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Progestin metabolism in pregnant mares was investigated in five experiments. The following progestins were analyzed: [5α-pregnane-3,20-dione (5α-DHP), 4-pregnene-3,20-dione (P4), 3β-hydroxy-5-pregnen-20-one (P5), 3β-hydroxy-5α-pregnan-20-one (3β-5P), 20α-hydroxy-5α-pregnan-3-one (20α-5P), 5-pregnene-3β,20β-diol (P5-ββ), 5α-pregnane-3β,20β-diol (ββ-diol), and 5α-pregnane-3β,20α-diol (βα-diol)]. Gas chromatography/mass spectrometry (GC/MS) techniques allowed both definitive identification and quantification of steroids. Sample preparation involved the addition of internal standards, extraction, and derivatization (methoxime/t-butyldimethylsilyl).

In Experiment 1, plasma samples from both fetal and maternal vessels of six late-gestation mares indicated predominant progestins to be: uterine vein (20α-5P mean=767.6+/-S.E.=217.8 ng/ml, βα-diol 202.2+/-44.5 ng/ml), maternal artery (20α-5P 533.2+/-201.8 ng/ml, βα-diol 150.1+/-28.5 ng/ml), fetal vein (5α-DHP 216.4+/-24.4 ng/ml, P4 15.5+/-2.8 ng/ml), and fetal artery (P5 630.8+/-298.5 ng/ml, P5-ββ 131.4+/-51.8 ng/ml, 3β-5P 117.9+/-17.0 ng/ml, ββ-diol 32.3+/-12.8

ng/ml). The variation in levels of hormones in these vessels implies metabolism of progestins occurs in several locations. In Exp. 2, deuterium (D_4) P5 was infused into fetal arteries of two late-gestation mares. D_4-metabolites in plasma varied over time ($P < .05$), with D_4-3β-5P, D_4-P4, and D_4-5α-DHP higher ($P < .05$) in fetal vessels, and D_4-20α-5P and D_4-βα-diol higher ($P < .05$) in maternal vessels. The primary mare metabolite was D_4-20α-5P, while the major fetal metabolite was D_4-3β-5P. In Exp. 3, tissues from two late-gestation mares were incubated with D_4-P5. Highest production ($P < .05$) of D_4-P4 occurred in the placenta. D_4-5α-DHP was greater ($P < .05$) in the endometrium, placenta, and fetal ovary. D_4-3β-5P was higher ($P < .05$) in the fetal ovary, adrenal and liver, and mare endometrium. Some D_4-20α-5P was produced by endometrium and fetal tissues. More D_4-P5-ββ was produced by fetal liver ($P < .05$). D_4-βα-diol was produced primarily by endometrium ($P < .05$). In Exp. 4, estrogens, progestins, an androgen, and urokinase plasminogen activator were identified in nine embryos (d 14 to18). In Exp. 5, exogenous 5α-DHP and 3β-5P were not as effective as P4 in inhibiting estrus, ovulation, or equine luteinizing hormone .

The following metabolic model is suggested by this research. Placenta and endometrium metabolize P5 to P4 and 5α-DHP. Both 3β- and 20β-hydroxylations occur in the fetus, while 20α-hydroxylations occur in mare endometrium. Identification of steroids in early embryos indicates elaborate enzyme systems early in gestation. As noted in Exp. 5, higher concentrations of 5α-DHP and 3β-5P may be necessary to elicit the same biological activity as P4. This area of research could enable development of methods to determine feto-placental integrity in mares, and possibly other species.

METABOLISM OF PROGESTINS IN THE PREGNANT EQUINE

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INTRODUCTION

Over the past several decades, substantial technological advances have been made which facilitate analyzing the endocrinology of pregnancy in most species. Immunoassays with specific antibodies to the various hormones, coupled with advanced equipment in chromatography and ultrasound have allowed scientists and clinicians to make significant contributions to this area of research. The interrelationships of the maternal, fetal, and placental units are of functional importance to the success of the pregnancy, and thus are the focus of intensive investigation. Without harmony within and between these units, complications result which can culminate in a loss of pregnancy which in turn cause both economic and sentimental losses to the livestock producer.

Thus the focus of this research was on a critical time in the livestock production scheme; the gestation of young. The equine was investigated, which has some rather unique characteristics, yet still serves as a good model for endocrine investigations. In a series of five related studies, some of the complexities of steroid hormone metabolism in the equine pregnancy were studied. Particular attention was focussed on the numerous progestins of the equine fetus and pregnant mare. With expanded knowledge of the metabolic interrelationships of these progestins, such as their sources and biological actions, it may allow the development of methods to determine feto-placental integrity in the mare, and subsequently in other species as well.

REVIEW OF LITERATURE

PROGESTIN RESEARCH

1. Early Work on Progestins

Nearly sixty years ago, on April 11, 1934, results from an experiment in which 4-pregnene-3,20-dione (progesterone) was discovered, were presented to the German Society for Internal Medicine. Of the scientists involved in the four laboratories which were simultaneously working on the discovery, A. Butenandt and L. Rusicka shared the Nobel prize for their contribution to the study of female sex steroids. In a review article discussing this early work (Butenandt and Westphal, 1974), it was noted that the ovaries of 50,000 sows were required to isolate 20 mg of pure corpus luteum hormone.

At about the same time, some early studies on equine steroids in urine and tissues were underway. Haslewood et al. (1934) noted that pregnant mare urine contained a substance that appeared to have properties similar to the pregnane-diol previously identified in pregnant women's urine. Somewhat later, Brooks et al. (1952) identified two progestins of pregnant mare urine to be 5α-pregnane-3β,20α-diol and 5α-pregnane-3β,20β-diol, and isolated an additional seven progestins of undetermined structure. Chromatography and ultraviolet absorption spectrophotometry were methods used in these discoveries.

Short (1959) used paper chromatography and ultraviolet absorption spectrophotometry methods with a progesterone detection limit of 4 ng/ml (described in Short, 1958). He determined that the placental tissues of mares had high levels of progesterone, but was

unable to detect it in the uterine venous blood or in jugular blood of the mare in later gestation (120 to 310 d). Interestingly, Short (1959) also reported progesterone to be in the umbilical cord blood, and postulated that during later stages of gestation, pregnancy was maintained by a local effect of placental progesterone on the uterus. It was also concluded that the reduced progestins identified in the mare's urine in late-pregnancy may reflect the shift from ovarian to placental production of progesterone, and possibly the transfer of the progesterone metabolic site from the mare's liver, and other maternal organs, to the uterus.

It was not until the 1960's that more intense research on progesterone began. Progesterone had long been associated with pregnancy maintenance, due to its ability to decrease the excitability of the myometrium (Pearlman, 1948). In the 1950's and 1960's it was discovered that in addition to its "progestational" qualities, progesterone held inhibitory properties for the gonadotropins and was a feasible human contraceptive (Garcia and Pincus, 1964). Then much interest was given to steroid research in all species. In the early 1960's, a number of radioimmunoassays (RIA) and improved competitive protein binding assays (CPB) were developed, which were more sensitive than previous techniques, and allowed the routine measurement of progesterone, as well as other steroids, even at low levels. The scientific literature of the years that followed was filled with steroid hormone profiles throughout the estrous cycle and during gestation for horses and other livestock species.

2. General Reproductive Endocrinology of Mares

A complete discussion of reproduction in the mare, with supporting research cited, is in the recent edition of Reproductive Biology of the Mare (Ginther, 1992). In addition, a general account of the basic endocrine parameters in the cyclic and pregnant mare are discussed in a review by Hyland (1990). This section is a brief summary of general reproductive endocrinology of the mare, as noted from these two references.

The mean length of the estrous cycle in the mare is 21.7 d, with the mean lengths of diestrus being 14.9 d and estrus 6.5 d. Mean duration of gestation is 340 d, but can vary from 310 to 374 d. A number of hormones are associated with the estrous cycle and pregnancy.

Reproductive hormones in mares fall under one of the following structural categories: glycoproteins, proteins and peptides, fatty acids, or steroids. The glycoprotein gonadotropins in the mare are equine follicle stimulating hormone (eFSH), equine luteinizing hormone (eLH), and equine chorionic gonadotropin (eCG). These hormones consist of both an α and a β-subunit. The α-subunit is similar between each gonadotropin, while the β-subunit is hormone specific. The equine gonadotropins contain high concentrations of sialic acid, which is thought to prolong hormone half-life and prevent rapid breakdown in the liver. The decapeptide, gonadotropin releasing hormone (GnRH), is produced and secreted from the hypothalamus and transported to the anterior pituitary via the portal vessels, stimulating production and release of eLH and eFSH. It is known that GnRH is released in a pulsatile

fashion, and may therefore be responsible for the rythmic release of both eLH and eFSH observed in mares.

Concentrations of eLH in peripheral circulation are low during diestrus, and increase a few days prior to the onset of estrus. Maximum concentrations are reached 1 or 2 d after ovulation, decreasing over the next 4 to 5 d. Mean circulating eFSH levels are reciprocally related to the eLH levels during diestrus. Peak concentrations of eFSH are noted in diestrus. The eFSH stimulates the development of follicles, while eLH is responsible for the final growth of the follicle and ovulation.

Cells of trophoblastic origin which form the endometrial cups of the pregnant mare produce eCG from approximately 40 d gestation to a peak between 55 and 65 d, with undectable or very low levels by 100 to 150 d. The function of eCG is not completely understood. Due to its characteristics, which are similar to both eFSH and eLH, eCG may be responsible for the formation of secondary corpora lutea of pregnancy. Immunological protection of the fetus from the mother may be mediated with eCG. Murphy and Martinuk (1991) published a complete review on this unique hormone in the mare.

A variety of peptide and protein hormones, in addition to GnRH which was mentioned previously, have been identified in the mare (referenced from Ginther, 1992). Oxytocin, an octapeptide, is produced by the hypothalamus and stored in the posterior pituitary. In domestic ruminants, oxytocin is also produced in the corpus luteum. In the pregnant mare, oxytocin stimulates milk let-down and uterine contractions at the time of birth. Oxytocin may also be involved with prostaglandin release at the time of luteolysis in cyclic mares. Prolactin, a 199 amino acid hormone, is produced by the anterior

pituitary. Its functions in the pregnant mare appear to be primarily mammary gland development and milk secretion. Relaxin, a polypeptide produced in the uterus and placenta of pregnant mares, is important in the preparation for birth. Inhibin, which is produced by follicles, appears to selectively inhibit eFSH. Opioids are a family of peptides produced in the brain and have suppressive effects on gonadotropin secretion. Melatonin is known to be involved with seasonality regulation. This indole hormone is produced in the pineal gland.

Prostaglandins are 20-carbon fatty acids synthesized by a variety of tissues. The primary prostaglandin involved in reproductive processes is PGF2α. Due to its short half life, a metabolite of PGF2α, known as PGFM, is used in assay systems to monitor peripheral levels. In the cyclic mare, sharp peaks of PGFM are noted 14 to 17 d post-ovulation, coincidental with luteolysis. Prostaglandins also cause smooth muscle contractions.

The numerous steroids in the mare are all derived from the common precursor, 3β-hydroxy-5-cholestene (cholesterol). Steroidogenic pathways of steroids include enzymatic conversions of cholesterol to 3β-hydroxy-5-pregnen-20-one (pregnenolone), and then to progesterone. Several pathways may be possible from progesterone. Progesterone can be converted to 17α-hydroxy-4-pregnene-3,20-dione, which can then be metabolized to 4-androstene-3,17-dione (androstenedione) and the various estrogens (3-hydroxy-1,3,5(10)-estratrien-17-one or estrone, 1,3,5(10)-estratriene-3,17β-diol or estradiol, and 1,3,5(10)-estratriene-3,16α,17β-triol or estriol). An alternate pathway of metabolism may be from progesterone to 20α-hydroxy-4-pregnen-3-one or 5α-pregnane-3,20-dione, followed by

hydroxylations to form other progestin metabolites. The third pathway from progesterone is to the various corticosteroids. The progestins, estrogens, and androgens are the steroid groups of importance in reproduction in mares. Sources of these steroids are many, but include ovarian follicles, corpora lutea, adrenal cortex, placenta, and the feto-placental unit.

The ovarian follicles produce estrogens (primarily estradiol) commencing approximately 7 d prior to ovulation, with a peak estradiol concentration noted in the peripheral circulation at 2 d prior to ovulation. Peripheral concentrations are low during diestrus. The corpus luteum produces progesterone which increases in the peripheral circulation 1 d after ovulation. Concentrations reach a maximum within approximately 5 d after ovulation and are maintained until 13 or 14 d, when luteolysis occurs.

Large quantities of estrogens are produced in the pregnant mare and excreted in the urine. In the mare, unlike most other species, estrogen secretion decreases towards the end of pregnancy. Estrone sulphate is produced from approximately 37 to 200 d, with a rapid increase between 70 and 200 d. The source of early pregnancy estrogens (35 to 40 d) appears to be primarily the maternal ovary's corpus luteum (Daels et al., 1991). In later pregnancy, during the third month to the seventh or eighth month, the feto-placental unit is involved in the production of estrogens. From approximately 200 d to term a decrease is noted in both plasma and urinary estrogens (Ginther, 1992).

Progesterone, from the corpus luteum, increases after ovulation in both pregnant and non-pregnant mares. In the pregnant mare, these levels are maintained with a slight decrease to 30 d noted. The increase

to maximum levels between 60 and 120 d of gestation is due to secondary ovulations as well as progestin contributions from the feto-placental unit. Low levels are attained between 180 to 300 d gestation, after corpus luteum regression. The feto-placental unit contributes to the progestin pool from 60 or 70 d and throughout gestation with an increase observed during the last 30 d of pregnancy. The progestins of the pregnant mare will be discussed in greater detail in the following section.

3. Progestins in Mares

In a series of four projects, Bhavnani et al. (1969 to 1973) infused a number of isotopically labelled precursor and steroid compounds (7-^{3}H-dehydroisoandrosterone, 4-^{14}C-androstenedione, ^{14}C-acetate, ^{3}H-cholesterol, ^{14}C-squalene, and ^{14}C-mevalonic acid) into the umbilical vessels of pregnant mares, and monitored the radiolabelled steroid metabolites excreted in the mare's urine using paper and thin layer chromatography (TLC) methods. In the first two studies (Bhavnani et al., 1969 and 1971), attention was focussed on estrogen formation. In the last two studies (Bhavnani et al., 1973a,b), both estrogen and progestin metabolites were analyzed. Their reports indicated that 3β-hydroxy-5α-pregnan-20-one, 5α-pregnane-3β,20β-diol, and 5α-pregnane-3β,20α-diol, derived from ^{14}C-squalene (Bhavnani et al., 1973b) and from ^{14}C-mevalonic acid (Bhavnani et al., 1973a), were the predominant progestins in the urine of the pregnant mare. These studies verified portions of the metabolic pathways for both the estrogens and progestins.

In the 1970's, numerous reports of the mare's plasma progesterone profiles during the estrous cycle and pregnancy were determined using either CPB or RIA. Smith et al. (1970), Plotka et al. (1972), and Stabenfeldt et al. (1972), all indicated that in the non-pregnant, cycling mare, plasma progesterone concentrations determined with CPB, were at <1 ng/ml during estrus and increased to 5 to 8 ng/ml at 5 to 10 d after ovulation. These higher levels were maintained briefly, and decreased to <1 ng/ml after luteolysis. This basic profile of progesterone during the estrous cycle is still accepted today.

Levels of plasma progesterone during pregnancy were reported by several groups. Allen and Hadley (1973), and Van Niekerk et al. (1973), both utilizing CPB assays, examined early gestation and found progesterone increased initially (6 to 17 ng/ml), but a gradual decrease was then observed. Allen and Hadley (1973) observed the decrease from 20 to 30 d of gestation, while Van Niekerk et al. (1973) observed progesterone to decrease in concentration from 10 to 14 d (approximately 2 ng/ml), with an increase in concentration from 17 to 30 d (approximately 4 ng/ml).

The following year, Squires et al. (1974), using a RIA, reported that plasma progesterone concentrations during pregnancy decreased between 7 and 19 d (10 to 8 ng/ml), increased rapidly from 32 to 44 d with maximum concentrations at 90 d (15 ng/ml). A decrease to 10 ng/ml occurred between 150 to 180 d. Smith (1974) indicated that plasma progesterone, determined with a CPB assay, increased in concentration with some variations between animals, during the first 60 d of pregnancy, decreased to a minimum at 180 d, and then reached the maximum level of 11.5 ng/ml (significantly higher than the mean for

the whole gestation of 7.6 ng/ml) from 270 d to term. Using a RIA, Burns and Fleeger (1975) determined that during the last 90 d of gestation, plasma progestins increased from 25 ng/ml at 90 d prepartum, to 60 ng/ml at 10 d before foaling, with a slight decrease within days prior to delivery.

The general trend for early gestation progesterone levels appears to be an initial increase after ovulation, followed by a decrease at 30 d, with a subsequent increase. The variations reported in early gestation in the various studies were minor, and probably due to animal and assay variations. However, there appeared to be some major discrepencies in the literature regarding progesterone levels of later pregnancy. With Short's report (1959) indicating that no progesterone was in the systemic circulation of mares in late-gestation, it would seem that these more recent assays may be over-estimating the amounts of progesterone in later pregnancy.

Holtan et al. (1975b,c) purified the samples with Sephadex LH-20 column chromatography prior to analysis in CPB assays, and verified that high concentrations of progesterone existed in early pregnancy up to 150 d, as was previously reported. A maximum concentration of 15.2 ng/ml was attained at 64 d. However, in contrast to the other studies, at 180 to 300 d, only 2 ng/ml of progesterone was reported with an increase to 4 ng/ml in the last 30 d of gestation. In addition, these investigators found very low concentrations of 17α-hydroxy-4-pregnene-3,20-dione except between 40 and 120 d, and during the last 30 d of gestation. Two fractions were reported to first appear between 30 and 60 d of gestation and increase gradually to 300 d, followed by a

rapid gain in concentration to parturition. Both compounds were noted to cross-react with two different RIA's for progesterone.

Another group (Ganjam et al., 1975) determined with both RIA and CPB assay that there were two peaks of plasma progestins; one in the third month which coincided with eCG secretion and secondary corpus luteum formation, and the other in the eleventh month and probably from placental sources. After purification, the levels of progesterone reported by these CPB assays for later pregnancy were reduced, but still present. These researchers also speculated that unknown metabolites of progesterone may be contributing to the progesterone levels in late-pregnancy. In a study conducted by Barnes et al. (1975) using TLC and RIA, jugular vein samples were analyzed, and fetal samples were also collected via indwelling catheters. They indicated that progestins, which may include progesterone metabolites, were present in high concentrations in both fetal and maternal samples.

The unidentified progestins reported to be present in the later stages of gestation were identified with gas chromatography/mass spectrometry (GC/MS) by both Atkins et al. (1974) and Holtan et al. (1975a). Atkins et al. (1974) identified 5α-pregnane-3,20-dione, and indicated that progesterone declined in concentration during late-gestation. Additionally, they claimed 20α-hydroxy-4-pregnen-3-one was measurable with RIA. Holtan et al. (1975a) identified 5α-pregnane-3,20-dione, 3β-hydroxy-5α-pregnan-20-one, and 20α-hydroxy-5α-pregnan-20-one in pregnant mare circulation, from both intact and ovariectomized mares, as well as in umbilical vessel blood. High amounts of progesterone were found in placental tissues. Holtan et al. (1975a) postulated that since these three pregnanes identified were

much higher in the fetal vessels compared to the maternal circulation, the feto-placental unit was the most probable source. However, Atkins et al. (1976) using RIA and GC/MS found 5α-pregnane-3,20-dione in the peripheral plasma of cyclic and pregnant mares during the first 150 d of pregnancy. They therefore concluded that possibly the ovary in early pregnancy, as well as the feto-placental unit in later gestation produce 5α-pregnane-3,20-dione.

Seamans et al. (1979), using column chromatography and a RIA with a nonspecific antibody, found high levels (133 ng/ml) of 5α-pregnane-3,20-dione 80 h prior to parturition, with a decline following this peak. This group also reported a combined hydroxy-5α-pregnanone fraction at concentrations in excess of 1600 ng/ml up to 8 h before foaling. Progesterone levels were in the range of 4 to 8 ng/ml at the time of foaling according to their report. Moss et al. (1979) infused [1,2,6,7-^{3}H] progesterone into the uterine artery of pregnant mares or umbilical vein, and collected samples from indwelling catheters in uterine and fetal vessels. Using TLC to isolate the various progestins, more radioactive 5α-pregnane-3,20-dione was found in the fetal vein samples than the other vessels. Higher levels of radioactive 3β-hydroxy-5α-pregnan-20-one were found in the fetal artery, and more radioactive 20α-hydroxy-5α-pregnan-20-one was noted in the maternal vessels. They concluded that 5α-pregnane-3,20-dione was produced by the placenta, 20α-hydroxy-5α-pregnan-20-one was from maternal sources, and 3β-hydroxy-5α-pregnan-20-one from the fetus primarily.

Seren et al. (1981) investigated progesterone, 17α-hydroxy-4-pregnene-3,20-dione and 20α-hydroxy-4-pregnen-3-one levels in pregnant mare plasma, using rather non-specific antibodies in RIA

systems. The 17α-hydroxy-4-pregnene-3,20-dione and progesterone were higher in the second and third months of pregnancy which coincides with elevated eCG levels and secondary luteinizations. Concentrations of 20α-hydroxy-4-pregnen-3-one were noted to be low (2 ng/ml) during the first three months of pregnancy, with a rise beginning in the fifth month, and even higher levels (80 to 120 ng/ml) were attained in the last month of gestation suggesting a source other than the ovary. This study indicated that their antibody for 20α-hydroxy-4-pregnene-3-one cross-reacted with 20α-hydroxy-5α-pregnan-3-one, which was previously noted to be high near parturition. Therefore, the levels indicated may include a contribution by the 5α-pregnane metabolite.

Holtan et al. (1979) found that the ovaries are dispensable after 50 to 70 d of gestation, as determined by ovariectomy of pregnant mares. This work therefore further supported the varying sources of these additional progestins in later pregnancy. It was indicated from this study that the placenta was the most probable source of progestins to maintain pregnancy after 50 to 70 d. Prior to this time, ovarian progesterone was essential to pregnancy maintenance. Pashen and Allen (1979) found that removal of fetal gonads did not affect the concentrations of progestins, and pregnancy was maintained. However, they did notice estrogens decreased immediately after fetal gonadectomy, and PGF2α metabolite (PGFM) were depressed at the time of foaling. Gonadectomized foals were dysmature and died soon after birth. Mares carrying these foals had weak uterine contractions and required assistance in foaling. This report suggested that the fetal gonad supplies precursors for estrogen metabolism which occurs in the

placenta. Without these estrogens fetal development is impaired. Estrogens prime the uterus to produce prostaglandins and activate oxytocin receptors at birth. Therefore, the lack of estrogens probably caused the weaker uterine contractions in the mares with gonadectomized foals. Progestins by contrast were not affected, thus they must be produced and metabolized independent of the fetal gonads.

Several progesterone metabolites have been identified in late-gestation. Without doubt the high levels of progesterone noted in the later stages of the equine pregnancy by several researchers were due to non-specific cross-reactivity of the various progestins with progesterone antiserum in RIA and CPB assays. GC/MS techniques developed for steroid analysis (see discussion in the following section) were the solution to the cross-reactivity problems associated with immunoassays. Definitive identification of the various progestins is attained using molecular weights and specific ions indicating the predictable breakdown fragments.

Holtan et al. (1991) performed an extensive investigation utilizing GC/MS and deuterium labelled internal standards. Identification of three 5-pregnenes, two 4-pregnenes, and seven 5α-pregnanes were reported. Eight of these progestins were examined throughout pregnancy in the mare. The predominant progestins in pregnant mare jugular vein plasma near term were 20α-hydroxy-5α-pregnan-3-one (400 to 2100 ng/ml) and 5α-pregnane-3β,20α-diol (100 to 350 ng/ml). These two steroids, in addition to four more 5α-pregnanes (30 to 100 ng/ml), were initially detected between 30 and 60 d of gestation, and increased gradually throughout pregnancy with a rapid increase observed in the last 30 d prior to foaling. Progesterone

levels were similar to all previous reports for early gestation, but it was at undetectable levels (assay sensitivity was at least 0.5 ng/ml) during mid- to late-pregnancy. Samples obtained from indwelling catheters in the fetal and maternal vessels of late-gestation mares (250 to 300 d) were also analyzed in this study. Fetal artery steroids were: pregnenolone (422 ng/ml), 5-pregnene-3β,20β-diol (171 ng/ml), 3β-hydroxy-5α-pregnan-20-one (277 ng/ml), and 5α-pregnane-3β,20β-diol (221 ng/ml). The predominant steroid of the fetal vein was 5α-pregnane-3,20-dione (205 ng/ml), with low but detectable progesterone (12.7 ng/ml). The jugular vein concentrations, as described above, were similar to the uterine arterial levels, and the uterine vein had approximately double those amounts. These data suggested that the fetus produces pregnenolone which is metabolized to progesterone and 5α-pregnane-3,20-dione by the placenta, with 3β- and 20β-hydroxylations accomplished by the fetus, and 20α-hydroxylations occurring in the mare endometrium.

Progestin concentrations have been noted to change in abnormal pregnancies. Decreased progestins associated with complications during pregnancy were observed in studies using non-specific RIA systems (Ousey et al., 1987; and Santschi et al., 1991). It has also been reported by Holtan et al. (1991) using both GC/MS and RIA methods that mares delivering prematurely or aborting have decreasing pregnane levels, in particular 20α-hydroxy-5α-pregnan-3-one. In a study concerning progestins of normal and dysmature foals (Houghton et al., 1991), it was determined that pregnenolone and 5-pregnene-3β,20β-diol were higher in premature foals. A recent study (Rossdale et al., 1992) found increases in maternal plasma progestins following injections of adrenal

stimulants to the fetal circulation, indicating that the fetus may be involved with normal progestin production. It is probable that levels of certain progestins in the late-gestation mare may be found to reflect the fetal status, and be useful in identifying problems during pregnancy, or impending abortions. More research is needed to confirm concentrations of the various progestins in both maternal and fetal systems throughout gestation in both normal and abnormal pregnancies.

4. Development of GC/MS Methods for Steroid Analysis

Concerns regarding the use of both anabolic steroids and corticosteroids to enhance performance in race horses were apparent in the 1970's. Similar problems were of course being experienced in human athletic events. Methods to detect these hormones, or their metabolites, in urine and blood were developed to monitor this problem. The GC/MS analysis of pregnant women's urine was already used routinely. Swedish scientists had used GC/MS to identify numerous metabolites (5α and 5β-pregnane-diols, -triols, and -tetrols as well as pregnene-diols) in pregnant women's urine and fecal samples (Eriksson and Gustafsson, 1970; Eriksson et al., 1970; and Janne and Vihko, 1970). Eriksson and Gustafsson (1970) analyzed urine from a woman in her 37th wk of pregnancy, while Eriksson et al. (1970) analysed fecal samples from the same individual. The majority (88%) of pregnane-diols in urine were free and glucuronide fractions of the 5β configuration, whereas the remaining 12% of the pregnane-diols were di-sulphated 5α configurated compounds. Fecal pregnane-diols in the di-sulphated fractions were of the 5α configuration, whereas both 5α and 5β isomers were present in the free steroid fraction. Janne and Vihko (1970) identified three

hydroxylated progestins (3β,15α-dihydroxy-5α-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, and 5-pregnene-3β,15β,17α,20α-tetrol) in pregnant women's urine. They suggested that 15-hydroxylated progestins, which were suspected to be formed from progesterone in the feto-placental unit, may be useful in monitoring fetal well-being.

The methods for GC/MS identification of progestins have been improved over time with solid phase extraction protocols and purification techniques, and applied to plasma and tissue samples (Sjovall and Axelson, 1979; and Axelson and Sahlberg 1983). Incorporation of deuterium labelling procedures to analyze metabolism of the progesterone in humans has also been developed (Baillie et al., 1980; and Anderson et al., 1990). These two groups injected deuterium labelled precursor steroids intravenously to pregnant women, and collected blood periodically. Baillie et al. (1980) noted that the primary metabolic product in pregnant women was 5α-pregnane-3β,20α-diol. Anderson et al. (1990) determined 5α-pregnane-3α,20α-diol and 3α-hydroxy-5α-pregnan-20-one were the predominant products of progestin metabolism in pregnant women.

During the late 1970's and 1980's, GC/MS protocols were modified in order to detect a variety of steroids (androgen and corticosteroid metabolites) in horses, primarily in urine (Houghton et al., 1984, 1988; and Dumasia et al., 1986, 1989). These methods were subsequently applied in additional steroid studies in horses incorporating stable deuterium isotopes (Houghton et al., 1990). Holtan et al. (1991) included a detailed protocol which was successfully used in identifying and quantifying plasma progestins of the pregnant mare and fetus.

5. Tissue Incubation Studies

Tissue incubation studies are frequently useful in determining enzyme system locations. Although the data generated from such studies are limited since isolated systems are analyzed, some important findings have been published from such studies. Ainsworth and Ryan (1969) incubated placental tissue from a pony mare with either pregnenolone-7α-^{3}H or progesterone-4-^{14}C. The placental tissue converted radioactive pregnenolone to progesterone. In addition, radioactive pregnenolone and progesterone were metabolized to 20α-hydroxy-4-pregnen-3-one, 5α-pregnane-3,20-dione, 3β-hydroxy-5α-pregnan-20-one, 20α-hydroxy-5α-pregnan-3-one, 5α-pregnane-3β,20α-diol, and 5α-pregnane-3β,17α,20α-triol, as determined with chromatography.

Although useful in identifying enzymes in tissue, limited histochemical work has been done with equine tissues. A study published in 1975 (Flood and Marrable) addressed the distribution of several hydroxysteroid dehydrogenases (HSD), in a variety of equine placental and fetal tissues. They noted HSD to be present in placental and uterine tissues in all stages of pregnancy.

A recent study conducted by Hamon et al. (1991) determined from fetal and maternal tissue incubations that 5α-pregnane-3,20-dione production was derived primarily from endometrial metabolism of pregnenolone and progesterone. Placenta converted pregnenolone to progesterone. Negligible amounts of 5α-pregnane-3,20-dione were produced by fetal tissues (adrenal, liver, or testes). In this study, an

enzyme immunoassay (EIA) was developed with a specific antibody for 5α-pregnane-3,20-dione.

Marshall et al. (1991) determined from tissue incubations, that the source of extra-ovarian estrogens was the placenta. Incubations of fetal and maternal tissues with deuterium-enriched 17β-hydroxy-4-androsten-3-one (testosterone) and [4-^{14}C] testosterone allowed the identification of estrogen metabolism capabilities of the tissue. Chromatography and GC/MS methods were used in the identification of isotopically labelled estrogens in the placental tissues, indicating that this was the location of the majority of aromatase activity.

Tissue incubations have also been utilized to study steroid metabolism in other species. A study on the human placenta (Rabe et al., 1985), indicated that rates of synthesis of placental progesterone *in vitro* were autoregulated, and dependent on metabolic endproducts affecting the enzyme systems. In other studies (Powell and Challis, 1986; and Powell et al., 1986), human chorion tissues were used for incubation experiments. It was noted again in these two studies that various steroids can modulate the amount of progesterone produced in the chorion by affecting the enzymes involved. It was indicated that these steroids exert their control through the enzyme 3β-hydroxysteroid dehydrogenase, with 20α-hydroxy-4-pregnen-3-one being stimulatory to progesterone production, while testosterone, androstenedione, and 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone) were inhibitory. It was noted in these reports that the autoregulatory systems may be involved with the process of parturition in humans, by exerting local, paracrine effects on prostaglandin production. Possibly this type of control mechanism exists in other species as well.

Numerous studies have been completed using laboratory animals. Lerner and Eckstein (1976) determined that the immature rat ovary metabolized *in vitro* 5α-androstane-3α,17β-diol, 3α-hydroxy-5α-pregnan-20-one and 3α,17α-dihydroxy-5α-pregnan-20-one. This metabolism is characteristic only until puberty, when the metabolic capabilities change. It is possible that while still in utero, the late-gestation fetal gonad may be metabolically similar to the ovaries of these immature rats, and contribute to the progestins produced, as well as providing precursors for estrogen production in the placenta.

When rats were infused with radiolabelled progesterone, and subsequently the uterus removed and examined for progestins, it was noted that both the myometrium and endometrium take up and metabolize progesterone, with the amount dependent on the stage of the estrous cycle or pseudopregnancy (Redmond and Pepe, 1986). The endometrium metabolized more progesterone during pseudopregnancy than during the estrous cycle. They concluded that endometrial differentiation associated with pseudopregnancy appears to be associated with increased progesterone and its reduced or hydroxylated metabolites.

In a study using rabbits, animals of both sexes were infused i.v. or i.p. with radioactive progesterone and then killed 10 to 45 min later (Senciall and Roberts, 1989). Various tissues, blood, and urine were collected and tested for steroid content. They noted that the 21-hydroxy-steroid metabolites (21-hydroxy-4-pregnene-3,20-dione, 3α,21-dihydroxy-5β-pregnan-20-one, 3α,20β,21-trihydroxy-5β-pregnane, and 3α,6α,21-trihydroxy-5β-pregnan-20-one) derived from progesterone were found in the liver, kidney, and urine.

Uterine endometrium from sows in mid-luteal phase of the estrous cycle was found to metabolize radioactive progesterone *in vitro* to ten progestins, of which two were identified to be 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one (Hendricks and Tindall, 1971). These researchers thought that it was possible that the 5α-reduction of progesterone may be necessary for it to exert its biological effects on the endometrium. They indicated that this type of situation exists in males where testosterone is converted to 17α-hydroxy-5α-androstan-3-one in order to exert its effects on the prostate.

In another study, it was noted that porcine steroidogenesis in the placental incubations varied throughout gestation, but appeared to be local metabolism since the variation was not observable in the peripheral circulation of the sow (Kukoly et al., 1984). Fisher et al. (1985) demonstrated that in endometrial and conceptus tissues from sows at 10 to 25 d of gestation, there were temporal changes in the conversion of progesterone to estrogens, which was not observed in pseudopregnant gilt endometrium. The ability of both the endometrium and conceptus to convert progesterone to estrogens may be important to maintenance of pregnancy in pigs. This group also demonstrated that radioactive progesterone was converted to non-polar progestin metabolites, not estrogens, in endometrium of pseudopregnant gilts. The presence of the conceptus appears to be necessary for estrogen production at this stage of pregnancy in swine and possibly other species as well.

The sources of progestins vary between species and at various stages of gestation. It has been well documented that ovariectomy of rats, rabbits, pigs, and goats in any stage of pregnancy results in

abortion, due to dependency on the primary corpus luteum for progesterone. However, in primates, horses, cattle, and sheep, ovariectomy after early pregnancy does not cause abortion, since the placenta produces progestins (Short, 1969).

Sheldrick et al. (1981) incubated placental cotyledons from sheep and goats, and determined that goat placental tissue was three times more active in progesterone metabolism than sheep placental tissue. Peripheral plasma concentrations of 5β-pregnane-3α,20α-diol in goats were similar to progesterone concentrations in sheep. Wango et al. (1991) demonstrated that isolated fetal placental binucleate cells (specialized trophoblastic cells) in sheep produce progesterone, whereas goats produce 5β-pregnane-3α,20α-diol predominately. This metabolism of progesterone could account for the placenta of goats being unable to maintain pregnancy after ovariectomy. A study by Wango et al. (1992), indicated that the sheep placental binucleate cells utilize both the cyclooxygenase and lipoxygenase pathways of the arachidonic acid metabolism to regulate placental steroid production, whereas goat placental cells use only the lipoxygenase pathway. This observation indicates that the mode of steroid regulation may differ in the two species, resulting in different progestins predominating.

In cattle, Shemesh et al. (1983) determined that placentomes from cows at 100 to 150 d of gestation, comprised of both fetal and maternal tissue, could secrete a regulatory protein to control progesterone production in co-incubated granulosa and luteal cells. This inhibitory protein may also play a role in regulating placental steroidogenesis. In other research, bovine binucleate trophoblastic placental cells were noted to be capable of metabolizing both

progesterone and prostaglandins, an apparent autoregulation (Reimers et al., 1985).

In the tissue incubation studies that have been discussed here, it would appear that some important information relevant to progestin metabolism has been generated. However, systematic approaches to analyze each component to the overall scheme of steroid metabolism during pregnancy, in each species, have yet to be undertaken. In addition, the biological significance, if any, of the progestin metabolites has not been examined sufficiently.

6. Biological Activity of Progestins

Research in mares has concentrated on progesterone's role in controlling eLH secretion and estrous synchronization, and the importance of progesterone to pregnancy maintenance, with little concern to the functional significance of the other progestins found in the mare. If these progestins have any biological activity, it may be significant since some are found at concentrations approximately 100 to 1000 times greater than progesterone during late-pregnancy.

As noted previously in this review, during the 1950's and 1960's interest was focussed on the fact that progesterone was inhibitory to the gonadotropins. Determination of the negative feedback properties of progesterone on eLH in mares has been documented by several researchers. In ovariectomized mares, Garcia and Ginther (1978) determined that exogenous progesterone inhibited the secretion of eLH during the summer breeding season. It was also noted that estradiol, in conjunction with progesterone, had even greater inhibitory effects on eLH. However, estradiol alone was stimulatory to eLH. Thompson et al.

(1991) determined, from an extensive study examining the effects of several steroids on gonadotropins, that progesterone does indeed possess inhibitory regulation properties to eLH in the ovariectomized mare.

In ovarian intact mares, a study conducted by Evans et al. (1982), found that progesterone alone inhibited eLH in cyclic mares, and if progesterone and estradiol were administered together, even greater inhibition of eLH was observed. Silvia et al. (1987) found that exogenous estradiol with endogenous progesterone did not inhibit eLH more than in the animals with endogenous progesterone only. Therefore, in the mare, there is substantial evidence for progesterone's inhibitory control on eLH. The precise mechanism for this control, and the effectiveness of other progestins to act similarly needs further investigation.

During the 1970's and 1980's, great efforts were directed to more efficiently managing reproduction in mares. The application of progesterone's inhibitory properties on the gonadotropins, thus estrus and ovulation, was utilized in these management programs. It was Loy and Swan (1966), that originally noted the usefulness of progesterone in synchronizing ovulation in mares. Dosages of 100 mg/d of progesterone in oil, injected i.m., beginning in the mid-luteal phase of the estrous cycle and continuing to 19 or 20 d post-ovulation, in nonpregnant mares, inhibited estrus and ovulation in all mares. Four of the six treated mares ovulated 6 d after the withdrawl of treatments. In postpartum mares treated with the same dosage from 4 through 12 d postpartum, all six mares showed inhibition of estrus and ovulation until treatment was withdrawn. Additionally, lower dosages of progesterone, 6α-

methyl-17α-acetoxyprogesterone (MAP) injected i.m., and melengestrol acetate (MGA) or MAP fed orally were all shown to be ineffective in inhibiting estrus and ovulation. MGA and MAP are synthetic progestins that had been used successfully in other species to inhibit estrus and ovulation.

Webel (1975) determined that an orally administered synthetic progestin (17β-hydroxy-4,9,11-estratrien-3-one) was effective in controlling estrus. Several studies followed (Palmer, 1979; Squires et al., 1979; Webel and Squires, 1982; and Squires et al., 1983) which further validated this progestin's effectiveness in control of estrus. In 1984, this synthetic progestin (Altrenogest®) was approved in the United States for commercial use to control estrus and ovulation in mares.

The inclusion of a prostaglandin injection at the end of progestin therapy, to induce luteolysis, and an injection of human chorionic gonadotropin (hCG) in the presence of a large follicle in the estrus that ensues after the progestin and prostaglandin treatments, provides even greater control of estrus and ovulation. (Holtan et al., 1977; Taylor et al., 1982). Excessive use of hCG should however, be cautioned due to antibody development, which in some mares causes a refractory response to subsequent treatments with the hormone (Roser et al., 1979; and Wilson et al., 1990).

Similar programs for progestin treatment in postpartum mares to delay foal heat have also been reported (Loy et al., 1975, 1982; Bristol et al., 1983; and Bell and Bristol, 1987). A study by Parry-Weeks and Holtan (1987), found Altrenogest® to be useful in synchronizing equine

embryo recipients and maintaining pregnancy. Luteal progesterone is essential to the maintenance of early pregnancy, as was demonstrated by Holtan et al. (1979) and Kastelic et al. (1987). Consequently, it has become a common practice of breeding farms to supplement problem mares with an exogenous progestin source (Shideler et al., 1982; and Jackson et al., 1986). The effectiveness of providing exogenous progesterone to pregnant mares was scrutinized by Allen (1984). Allen questions whether the declining progesterone in early pregnancy causes the mare to abort, or if the malfunctioning fetus causes the progesterone to drop. It has been documented that in cases of surgical removal of the conceptus or in spontaneous abortions after 40 d, eCG is not capable of maintaining luteal function in all mares, suggesting the existance of a luteotropin from the conceptus (Allen, 1979). This is an area which needs further investigation.

More extensive research in the area of biological actions of progesterone and the various progestin metabolites have been completed using the female rat as the model. Biological actions of progestins, relative to pregnancy maintenance and gonadotropin regulation, have been investigated. Sanyal and Villee (1973), found 5α-pregnane-3,20-dione to have no progestational qualities in ovariectomized rats, but that it was capable of supporting the free floating blastocyst. Only progesterone was capable of initiating implantation and maintaining pregnancy in these rats.

Sridharan et al. (1974) observed in female rats that pregnenolone, progesterone, and 5α-pregnane-3,20-dione were facilitative to ovulation. Larger amounts of pregnenolone and 5α-pregnane-3,20-dione were needed to elicit the same response that progesterone caused.

However, 3α-hydroxy-5α-pregnan-20-one was ineffective at even high dosages.

Zanisi and Martini (1974) examined the effects of estrogen and progesterone, as well as 5α-pregnane-3,20-dione, 3α-hydroxy-5α-pregnan-20-one, and 3β-hydroxy-5α-pregnan-20-one on LH regulation in ovariectomized female rats. Using various combinations of exogenous hormones and estrogen implants in the hypothalamus and anterior pituitary, they determined that progesterone and 5α-pregnane-3,20-dione were more effective in modulating GnRH and subsequently LH, than either 3α-hydroxy-5α-pregnan-20-one or 3β-hydroxy-5α-pregnan-20-one.

Nuti and Karavolas (1977) found progesterone, 5α-pregnane-3,20-dione, and 3α-hydroxy-5α-pregnan-20-one all to affect LH systemically in rats. They suggested that since progesterone is converted to metabolites such as these in neuroendocrine tissues, that the metabolites may be involved with the positive and negative feedback mechanisms on gonadotropins.

Krause and Karavolas (1980, and 1981) examined 5α-reductase and 3α-hydroxy-steroid oxidoreductase enzymes in adult female rat hypothalamic tissues. These enzymes convert progesterone to 3α-hydroxy-5α-pregnan-20-one via 5α-pregnane-3,20-dione. It was reported that 3α-hydroxy-5α-pregnan-20-one has modulatory effects on the gonadotropins, but this may actually be through 5α-pregnane-3,20-dione via a back conversion from 3α-hydroxy-5α-pregnan-20-one. From studies such as these, it appears that progesterone metabolites may play an important role in the regulation of the hypothalamo-

hypophyseal-ovarian axis. More work will be necessary to further address the complete mechanism involved, and to determine whether similar mechanisms exist in other species.

Additional studies with rats have addressed the effects of progestins on progesterone receptors and gamma amino butyric acid ($GABA_A$) receptors. Since $GABA_A$ receptors are abundant in the brain, progestins binding to them may be directly controlling gonadotropins. Morrow et al. (1987) and others indicated progesterone metabolites interact with $GABA_A$ receptors of the brain. Brann et al. (1990) then provided evidence that 3α-hydroxy-5α-pregnan-20-one induced gonadotropin secretion was mediated by $GABA_A$ receptors in the brain. Putnam et al. (1991) analyzed contraction control by progestins on uterine tissue from rats, and found that both 3β-hydroxy-5β-pregnan-20-one and pregnane-diol are mediated through the $GABA_A$ system to the uterus, but 5β-pregnane-3,20-dione and progesterone use progesterone receptors. The evidence that progestins use receptors other than the progesterone receptor, may help explain the actions of the progesterone metabolites. These mechanisms of action, at the receptor level, have not been adequately addressed.

EMBRYO RESEARCH

1. Maternal Recognition of Pregnancy; Steroids and Proteins of the Early Embryo

In the mare, 4 to 6 d after fertilization the zygote reaches the uterus, while the numerous unfertilized eggs stay in the oviduct for months, where they slowly degenerate (Short, 1969). The prolongation of corpus luteum function, or maternal recognition of pregnancy, is of

primary importance to the survival of this zygote in the uterus to allow its further development throughout gestation. The mechanism of pregnancy recognition in mares, and other species, appears to be rather complex. This has been the topic of extensive research in the past, and with many aspects of the mechanism still unclear, further investigations are needed.

Maternal recognition of pregnancy in the mare is thought to occur between 14 and 16 d post-ovulation (Hersham and Douglas, 1979). Other studies have shown it may be as early as 11 to 13 d (Goff et al, 1987). Steroids, in particular estrogens, have been analyzed in the early stages of gestation in mares. Although estrogen levels in the first month of gestation in mares resemble diestrus levels, the embryo is capable of estrogen production as early as 7 d, with an actual increase between 12 and 20 d (Zavy et al., 1979 and 1984; Heap et al., 1982; and Marsan et al., 1987). Goff et al. (1983) noted that estrogen production was enhanced by the addition of blastocoel fluids to dispersed embryos *in vitro*. Heap et al. (1991) noted that chorionic girdle, yolk sac, and allantochorion tissue contained varying amounts of aromatase activity in horse embryos. The donkey conceptus, by contrast, has similar aromatase activity in all tissues. The biological significance of high aromatase activity, for the formation of estrogens in the horse and donkey embryos, remains elusive.

Since the production of these estrogens (estrone, estradiol, as well as androgens) occurs at the crucial time for the maintenance of pregnancy, it has been proposed that they may be involved in maternal recognition of pregnancy in the mare, as they are in the pig (Flint et al. 1979; Heap et al., 1981). However, it has been established that

mechanisms in each species must differ since exogenous estrogens are not luteotrophic in the mare (Allen, 1979), but are in the sow (Flint et al., 1979). In addition to the secretion of estrone and estradiol from pig blastocysts, several secretory proteins are also produced. These proteins, when intralumenally infused in the sow, do not prolong the corpus luteum lifespan (Bazer et al., 1986, 1989). Therefore, it would seem the estrogens are indeed important in the sow's recognition of pregnancy.

In the ewe and the cow, an interferon-like trophoblastic protein is produced by the conceptus which reduces the release of prostaglandins from the uterus (Imakawa et al., 1987; Stewart et al., 1987, 1989; and Anthony et al., 1988). Wilson et al. (1992) have also identified and quantified estrone, estradiol, and progesterone in bovine embryos at the time of pregnancy recognition. Studies with mice (Wu, 1987; and Wu and Lui, 1990) have shown that pre-implantation mouse embryos can metabolize progesterone to its various metabolites, indicating an extensive enzyme system. Similar systems may also exist in livestock species.

The precise mechanism of maternal recognition of pregnancy in mares is not certain. Undoubtedly, the steroid production of the equine conceptus plays some role in the process. Marsan et al. (1987) noted that after dissociation with collagenase, the horse conceptus at 12 to 15 d is composed of high density cells (trophectoderm) which secrete primarily estrogen, and low density cells (endoderm) secreting progesterone. The interactions of these two cell types are probably crucial for steroid metabolism in equine embryos. This study used non-

specific RIA systems which did not allow the definite identification of the intermediate metabolites or the steroids measured.

Pregnancy signalling in the mare may involve several mechanisms acting together to reduce prostaglandin release. When the conceptus is in the uterus, lower prostaglandin concentrations are noted in the uterine vein (Douglas and Ginther, 1976), uterine lumen (Zavy et al., 1979, 1984), and the periphery (Kindahl et al., 1982). Since the co-incubation of conceptus membranes and endometrium result in decreased prostaglandin production from the endometrium, it is suggested the mobility and contact of the embryo with the maternal endometrium is important to pregnancy maintenance (Bergland et al.,1982; and Sharp et al., 1984). Sharp et al. (1989) partially identified a protein they called prostaglandin inhibitory factor which is transient, and its production required conceptus mobility. Watson (1991) noted that mares possess an intracellular inhibitor to prostaglandin in the endometrium which may be produced in pregnancy.

In addition to questions surrounding the sustenance of luteal function for pregnancy maintenance, there is a lack of evidence indicating precisely how the early embryo is protected from immunological rejection in most species. The cytotoxic antibody response in the mother is directed towards paternal fetal antigens of the major histocompatability complex (MHC). Antezak and Allen (1989) noted that the source of fetal MHC in the equine pregnancy is the trophoblast cells of the chorionic girdle which form the endometrial cups. The progenitor girdle cells express high levels of paternal MHC, while the non-invasive trophoblast cells of the allantochorion and the cells of the mature endometrial cups do not express MHC. Additionally,

Roth et al. (1990) identified an immunosuppressor factor from the equine conceptus which is undoubtledly involved in this immuno-protection of the fetus. The literature concerning mechanisms for pregnancy recognition and maintenance in all species indicates that many facts have been established, but much is yet unknown.

2. Plasminogen Activator Research

The early embryo in all species undergoes extensive cellular remodeling, tissue proliferation, hatching, and implantation or placentation processes which all require enzymatic reactions. Plasminogen activator is a protease produced by the blastocyst which converts the zymogen plasminogen to the trypsin-like enzyme, plasmin (Sherman et al., 1976). Plasminogen, which has been identified in the uterine lumen of swine and murine species, is the source of protease that the developing embryo controls by altering plasminogen activator availability (Fazleabas et al., 1983; and Finley et al., 1983).

Mouse blastocysts have been shown to produce plasminogen activator from trophoblast cells primarily (Sherman et al., 1976; Strickland et al., 1976). These reports indicated that plasminogen activator may participate in the invasive implantation of mice. Mullins et al. (1980) identified plasminogen activator in swine conceptuses. This group also identified a progesterone induced inhibitor to plasminogen activator, which they thought may explain the non-invasive implantation observed in swine. Fazleabas et al. (1983) found that estrogens stimulate the swine blastocyst to elongate, which enhances the release of plasmin inhibitors by the endometrium to control the proteolytic reactions of plasminogen activator that potentially could

damage the endometrium. This study indicated that the plasminogen activator was involved in tissue reorganization and proliferation.

Menino and Williams (1987) determined that plasminogen activator enhanced hatching of the bovine blastocyst. Two other reports, (Domon et al., 1973; Menino and O'Claray, 1986) also indicated that possibly plasminogen activator was involved with blastocyst hatching in the murine species. Plasminogen activator has been characterized in the bovine species (Coates and Menino, 1991) and the ovine species (Bartlett and Menino, 1991) to be an urokinase-type.

Plasminogen activator has been identified in the murine, ovine, porcine, and bovine blastocyst. However, its precise mechanism of action and purpose is still not completely understood. No research has been published regarding plasminogen activator identification in the equine embryo.

CURRENT OBJECTIVES:

In conclusion to this entire literature review, it would seem a number of areas need further investigation. The research objectives of this current research will focus primarily on the progestin metabolism of the pregnant mare and fetus during later gestation. In addition, the early equine embryo will be examined regarding its steroid production, as well as presence of the plasminogen activator. With GC/MS techniques, definitive identification and specific monitoring of the steroids is possible. Finally, the biological actions of some of the progestins will be monitored in cycling mares.

MATERIALS AND METHODS

EXPERIMENT 1: PROGESTINS OF THE LATE-GESTATION MARE AND FETUS

1. Purpose

As indicated in the literature review, a number of progestin compounds have been identified in the pregnant mare during the later stages of gestation. Only one study (Holtan et al., 1991) indicated actual concentrations and identification of these progestins. Therefore, in this experiment, cannulae were placed into fetal and maternal vessels of late-gestation mares in order to confirm the identity and amounts of the following plasma progestins: 5α-pregnane-3,20-dione (5α-DHP), 4-pregnene-3,20-dione (P4), 3β-hydroxy-5-pregnen-20-one (P5), 3β-hydroxy-5α-pregnan-20-one (3β-5P), 20α-hydroxy-5α-pregnan-3-one (20α-5P), 5-pregnene-3β,20β-diol (P5-ββ), 5α-pregnane-3β,20β-diol (ββ-diol), and 5α-pregnane-3β,20α-diol (βα-diol). With cannulations of the vessels, collection of samples over time was attempted.

2. Animal Protocol

In this study, as well as all of the following experiments, the animals used were mature pony mares. All were maintained in the same outdoor environment on a standard grass hay and grain diet. From this research herd, six pregnant pony mares were chosen for the cannulation experiment. The gestational ages of the mares were: #46 (290 d), #48 (299 d), #59 (285 d), #75 (313 d), #76 (160 d) and #81 (313 d).

Prior to the surgeries, four cannulae were prepared for each of the six mares. For ease of insertion and flexibility, the cannulae were comprised of two types. The entire cannula was approximately 1.2 m in length, with .3 m of small diameter (.040 mm o.d. X .025 mm i.d.) Micro-Renathane (Braintree Scientific, Braintree, MA) tubing bonded with Silastic Medical Adhesive (Dow Corning Corporation, Midland, MI) to .9 m of larger diameter (.040 mm i.d. X .085 mm o.d.) Silastic tubing (Dow Corning Corporation). The four cannulae were bevelled on the end of insertion, and marked at measured intervals which allowed the surgeon to monitor the length inserted into each vessel. The opposite end was color coded such that each vessel could be identified externally. The cannulae were flushed three times with TDMAC-Heparinizing solution (Braintree Scientific), which coated the inside of the tubing to help prevent clot formation and subsequent blockage. The cannulae were then dried with N_2, packaged, and sterilized with Anprolene gas.

Mares were maintained under general anaesthesia with artificial ventilation for the cannulation procedure, which required approximately 3 to 6 h in a standard surgical setting (Comline et al., 1975; and Silver, 1980). An incision was made, and the cannulae were placed into four vessels: uterine or maternal artery, uterine vein, fetal artery, and fetal vein. In the first two mares, lateral abdominal incisions were made. However, due to problems with herniations at the suture line after surgery, mid-ventral abdominal incisions were used in subsequent surgeries.

Once all cannulae were situated and determined to be patent, they were extruded from the body via a puncture in the upper flank region. Since the fetal vessels, especially the fetal artery, were more prone to

blockage due to clot formation, a continuous flush device (Intraflo II with filter, Abbot-Sorenson, Santa Fe Springs, CA) was filled with dilute sterile 5 IU/ml heparinized saline (Kendall McCaw Laboratories, Irvine, CA), and attached externally to the cannula.

The mares recovered from surgical anaesthesia and were standing within a few hours. Banamine (200 mg, Scherring, Kenilworth, N.J.) and antibiotics (Genocin 400 mg and Penicillin $4X10^6$ IU, both from Pfizer, New York, N.Y.) were administered i.m. twice daily for six days following the surgery. Fetal well being was monitored via trans-abdominal ultrasound (Aloka 210-DX with 5-MHz probe, Corometrics Medical Systems Inc., Wallingford, CT) twice daily, as well as packed cell volume (PCV) on the fetal samples. Temperature, pulse, and respiration were closely monitored on the mares. Blood samples, obtained from each cannula, were collected into heparinized tubes or syringes twice daily, centrifuged, and the plasma kept at -20 °C until assayed. Each cannula was flushed with a dilute sterile 5 IU/ml heparinized saline solution, and left filled with a more concentrated 200 IU/ml heparinized saline solution.

3. Assay Protocol

a. Progestin Standards

All plasma samples were analyzed with GC/MS procedures, using a protocol similar to that described by Holtan et al. (1991). Progestin standards were: 5α-DHP, P4, P5, 3β-5P, 20α-5P, P5-ββ, ββ-diol, and βα-diol, which were all obtained from Sigma, St. Louis, MO, or Steraloids Inc., Wilton, N.H., and used without further purification (see structures in Figures 1 and 2).

Deuterium labelled progestins were utilized as internal standards in the analysis of samples. Deuterium labels were incorporated into four of the progestins, 5α-DHP, P5, 3β-5P, and ββ-diol, with a protocol similar to that noted by Dehennin et al. (1980). Both P5 and 3β-5P were deuterated by a proton for deuterium exchange to yield [17,21,21,21-^{2}H]-P5 (D_4-P5), and [17,21,21,21-^{2}H]-3β-5P (D_4-3β-5P). Then D_4-3β-5P was reduced to form D_4-ββ-diol, or oxidized to form D_4-5α-DHP.

Approximately 250 mg of 3β-5P, and 250 mg of P5 were placed in separate 150-ml round bottom flasks with 20 ml of deuterated methyl alcohol, 1 ml of sodium deuteroxide (both from Sigma), and a few boiling chips. These mixtures were refluxed for 5 h, then the solvent evaporated with N_2 at approximately 30 °C. Five milliliters of deuterated water and 1 ml of deuterated acetic acid (both from Sigma) were added. The solution was then extracted three times with 20 ml of anhydrous ethyl ether (Mallinckrodt Specialty Chemical Co., Paris, KY). The extract was collected into 15-ml centrifuge tubes and the ether evaporated with N_2. Before recrystallization, each progestin was checked for deuterium incorporation on the GC/MS using full-scan mode (increased molecular weight by four). Some of the D_4-3β-5P was then used to make D_4-5α-DHP (oxidation), and D_4-ββ-diol (reduction)

For the reduction reaction to form D_4-ββ-diol, approximately 75 mg of D_4-3β-5P were dissolved in 10 ml of deuterated methyl alcohol, and 200 mg of sodium borohydride (Sigma) added. This solution was incubated in a water bath at 50 °C for 45 min. The reaction was stopped with 1 ml deuterated acetic acid. Ten milliliters of deuterated water were then added, and the mixture transferred to a separatory funnel where it was extracted with 100 ml of ethyl ether. The extract was

collected into centrifuge tubes and the ether evaporated with N_2. A small amount was checked on the GC/MS using full scan mode prior to recrystallization.

Another 75 mg portion of the D_4-3β-5P was oxidized to form D_4-5α-DHP. The D_4-3β-5P was dissolved in 2 ml of deuterated acetic acid, and six drops of a 5% chromium oxide (Sigma) solution added. This reaction mixture was incubated 1 h at room temperature in the dark, and stopped by adding 5 ml of deuterated water. It was then extracted three times with 15 ml of ethyl ether, the extract collected in centrifuge tubes, and the ether evaporated with N_2. The product was checked on GC/MS full scan mode prior to recrystallization.

All four deuterium labelled progestins were then recrystallized. To each progestin, 2 ml of deuterated water were added and heated in a water bath to 80 °C. Deuterated methyl alcohol was added stepwise until the mixture went into solution. Each was then cooled at room temperature for 2 h, and placed in the freezer overnight. The crystallized yield was filtered over paper under vacuum. Since some went into the liquor portion, it was refiltered at least one additional time. The products were then transferred to aluminum foil, and dried in a warming oven overnight. At least 30 mg of each were recovered. All were derivatized and checked for purity on full-scan mode of the GC/MS.

A set of stock standards (1 mg/ml) were made by dissolving each of the eight non-deuterium progestins, and each of the deuterium labelled standards in methyl alcohol (spectro. grade, Mallinckrodt). From these 12 concentrated stock solutions, a mixture of the eight non-

deuterium progestins, and a mixture of the four deuterium labelled standards at the concentration of 10 µg/ml were prepared in methyl alcohol.

The two mixtures were used to prepare standard sets for each assay. For each of the eight progestins, standard regression response lines were developed by adding a constant amount (400 ng) of the deuterium mix, and varying amounts of the non-deuterium mix (100, 200, 400, and 800 ng) to 5 ml of plasma from an ovariectomized mare. While P5 , 5α-DHP, 3β-5P, and ββ-diol were all calibrated from their respective deuterium labelled internal standards, P4 used the D_4-5α-DHP internal standard, 20α-5P used the D_4-3β-5P, and P5-ββ and βα-diol both used the D_4-ββ-diol internal standard. These calculations were conveniently done by the GC/MS software program (MS Chem Station - HP G1034B, Hewlett Packard, Kennett Square, PA). The area of each progestin standard was divided by its appropriate deuterium internal standard resulting in the response ratios, which were graphed against the amount ratios. All unknown concentrations in samples were then calculated from these standard lines. In addition to the standards in plasma, standard sets were also prepared without plasma in order to determine extraction efficiencies.

To analyze plasma samples, from 4 to 5 ml of each sample were pipetted into 20-ml glass scintillation vials, and 400 ng of the deuterium mix internal standards were added. All samples and standards were extracted and derivatized using the protocols outlined in the following sections.

b. Extraction of Steroids

A solid phase extraction was used for the plasma samples and standards. C18 Sep-Pak Plus cartridges (Waters Associates, Milford, MA) were activated with 2 ml of methyl alcohol followed by 5 ml of water. Plasma samples and standards in plasma were placed into 10-ml plastic syringes and passed through the Sep-Pak dropwise. By slowly pushing samples through, extraction efficiency was greatly enhanced. The sample vial was then rinsed with 1 ml of water that was also passed through the Sep-Pak. Then 3 ml of water, and 2 ml of hexane (HPLC grade, Mallinckrodt) were passed through the Sep-Pak with glass syringes. The steroids were then eluded from the Sep-Pak with 4 ml of ethyl ether, which again were passed through gently with a glass syringe. The extract was collected in 3.7-ml glass screw cap vials with teflon lined caps (Fisher Scientific, Pittsburgh, PA), and the ether evaporated with N_2 at 45 °C in a Pierce Reacti-Vap Evaporating Unit (Pierce, Rockford, IL).

c. Derivatization

Derivatizing reagents were added to each sample and standard. Sixty microliters of a 2% methoxyamine-hydrochloride in pyridine (MOX reagent, Pierce) solution was pipetted into each vial and vortexed. The capped vials with MOX reagent were incubated at 60 °C for 30 min in the evaporating unit. Vials were then uncapped, and the pyridine evaporated with N_2. The MOX reagent derivatized the progestins with oxo-groups to methoxime.

In the second step of the procedure, TBDMS (or tert-butyldimethylsilyl) derivatized unhindered hydroxyl groups present on

progestins. Fifty microliters of a 5% solution of diethylamine in dimethylformamide (both from Pierce), plus 25 µl of MTBSTFA [N-(tert-butyldimethylsilyl) N-methyltrifluoracetamide] also from Pierce, were added to each vial, vortexed and incubated at 80 °C for 45 min. To stop the reaction, .5 ml of water were added and vortexed. Then the samples were extracted twice with 4 ml of ethyl ether, and the ether evaporated with N_2. It was important to rinse the walls of the vials with a few drops of ether to concentrate the steroids at the bottom of the vials. The samples were resuspended in 25 µl of n-undecane (Sigma), warmed slightly and vortexed. Each sample was transferred to capped autosampler vials (32 mm X 11 mm, Hewlett Packard) for GC/MS analysis.

d. GC/MS Program

The Hewlett Packard 5890 Series II gas chromatograph with an Alltech fused silica capillary column (30 m X .25 mm i.d., SE-30, Alltech, Deerfield, IL) had a temperature program of 20 °C per min from 150 °C to 310 °C, with total run time of 25 min per injection. The Hewlett Packard autosampler injected 1 µl of each sample in splitless mode. Interfaced to the gas chromatograph, was a Hewlett Packard 5971A Mass Selective Detector. The full-scan mode (FSM) was used for identification and qualitative runs, while the selected ion mode (SIM) was used for quantitative work. The system was controlled by the computer with the MS Chemstation software, which compared steroid peak areas to internal standards, and calculated ng/ml amounts from the response lines created by the standard ratios.

e. P4 EIA Cross-reactivity

Sample sets from three of the mares cannulated (#46, #48, and #59) were also analyzed with a commercial P4 EIA kit (Novo Biolabs Ltd., Cambridge, UK). Since most of the samples tested on this EIA resulted in maximum levels on the assay standard curve (10 ng/ml P4), it was suspected that progestins other than P4 were binding to the P4 antibody. Cross-reactivity experiments with the progestins known to be present in late-gestation mare and fetal samples (5α-DHP, P4, P5, 3β-5P, 20α-5P, P5-ββ, ββ-diol, and βα-diol) were then undertaken on the P4 kit assay. Each of the progestins were tested at 10, 100, and 1000 ng/ml concentrations in plasma from an estrous mare.

The assay is a competitive EIA technique. A detailed protocol for the procedure and all reagents needed are included with each 96-well microtiter plate kit. The microtiter plates are pre-coated with P4 antibody. Aliquots (20 µl) of the plasma samples and each standard (1, 2.5, 5, and 10 ng/ml) were added to duplicate wells, and 100 µl of conjugate added to each well. The plates were incubated at room temperature for 20 min. The plates were inverted to remove well contents, and washed three times with a washing solution. The substrate and amplifier were pipetted to each well (100 µl of each), and incubated at room temperature for 10 min. The reaction was stopped with a .3M sulphuric acid solution. Absorbance of standards and samples were read at 492 nm on a Titertek Multiscan Plus MKII microelisa automatic plate reader (Flow Laboratories Inc., Inglewood, CA).

4. Statistical Analysis

All additional calculations of data and assay statistics, such as coefficient of variation, recovery percentages (accuracy of added hormones), sensitivity, and extraction percentages, were done on Macintosh Excel software (Apple Computer Inc., Cupertino, CA).

Due to problems encountered in the long-term cannulations of the pregnant mares (herniations, abortions, and blockage of cannulae) over extended time, samplings were irregular. Consequently, only initial samplings were used for statistical analysis. A randomized block design analysis of variance (general linear model or GLM) on SAS (Cary, N.C.) was used to determine mare (random, block effect) and vessel (fixed, treatment effect) differences for the eight progestins (dependent response variables). A multiple comparison (least significant difference or LSD, with SAS) was also applied to the data set if significant differences were found to determine where variations occurred in the data. In addition, multiple paired t-tests were run to determine differences between each of the eight hormones within each vessel (SAS). The data was log 10 base transformed due to obvious unequal variances in hormone levels.

EXPERIMENT 2: PROGESTIN METABOLIC STUDIES UTILIZING DEUTERIUM PREGNENOLONE INFUSIONS TO FETAL VESSELS IN LATE-GESTATION MARES

1. Purpose

The differences in concentrations of the progestins between maternal and fetal vessels indicated in the literature suggest several metabolic sites. The objectives of this study were to determine, with

infusions of a labelled precursor steroid (deuterium P5) to the fetal circulation, and samplings over time, possible sources and metabolic locations of the various progestin metabolites. The non-ionizing deuterium label would be easily traced, with GC/MS analysis of plasma samples, to the subsequent progestins down the metabolic pathway from P5.

2. Animal Protocol

Two pregnant pony mares at 290 d (#46) and 313 d (#81) gestation were cannulated as noted in Experiment 1. After the cannulae were in place and patent, 10 mg of D_4-P5 (prepared as indicated in Experiment 1) were infused into the fetal arterial cannula. Samplings from each of the four cannulae were collected at timed intervals (0, 10, 20, 40, 60, 90, 120, and 160 min) after infusion. To prevent hemorrhagic shock in the fetus, only approximately 2 ml of plasma were collected at each fetal sampling which necessitated pooling of these samples prior to analysis. At least 5 ml samples of plasma were collected from the maternal vessels each time. All plasma samples were centrifuged and stored at -20 °C until assayed. At the end of the sampling period, the animals were euthanized.

3. Assay Protocol

a. Standards

In the samples collected in this experiment, the steroids of interest should be deuterium labelled progestins derived from the infused D_4-P5. Consequently, deuterium labelled progestins could not act as internal standards, and instead, 4-cholesten-3-one (Steraloids)

was used. A 10 µg/ml solution of 4-cholesten-3-one in methyl alcohol was prepared. Using the same principles outlined for Experiment 1, 4-cholesten-3-one (2000 ng) was added to all plasma samples and standards as the internal standard, prior to extraction and derivatization.

A standard regression response line was established for each progestin by adding varying amounts of the progestin mix (100, 200, 400, and 800 ng), and a constant amount (2000 ng) of 4-cholesten-3-one to 5 ml of ovariectomized mare plasma. All progestin concentrations were calculated from the 4-cholesten-3-one.

b. Extraction of Steroids

Due to poor extraction efficiency of 4-cholesten-3-one in plasma by Sep-Paks, ether extraction was used on all standards and samples in this experiment. Plasma samples and standards (4 to 5 ml) were pipetted into 15-ml glass vials with teflon lined screw caps, and the internal standard added. Each sample or standard was extracted twice with 8 ml of ethyl ether. The extract was transferred into 3.7-ml glass vials with teflon lined screw caps, the ether evaporated with N_2, derivatized, and analyzed by GC/MS as indicated in Experiment 1.

4. Statistical Analysis

As in the first experiment, Macintosh Excel software was used for assay statistics. Statistical analysis of the data was completed using SAS. In general, it appeared that the progestin concentrations were higher at the 10 min sampling. Therefore, a randomized block design

was used to compare the eight progestin concentration differences (dependent response variables), between mares (random, block effect) and vessels (fixed, treatment effect) at this sampling time, with an analysis of variance (GLM). A multiple comparison (LSD) was also applied when concentrations differed to determine the sources of the variations. Multiple paired t-tests determined where progestin concentration differences existed within each vessel. In addition, since samplings were collected over time, a split plot repeated measures analysis of variance using GLM on SAS was used. The model had the variable of over time effects, in addition to the 10 min sampling design noted above. The data in this experiment, as in the first experiment, obviously did not have homogeneous variance, which necessitated log transformations of the data in all analyses.

EXPERIMENT 3: METABOLIC CAPABILITIES OF FETAL MATERNAL TISSUES FROM LATE-GESTATION MARES

1. Purpose

Incubation studies have proven to be useful in identifying enzymes present within specific tissues as was noted in the literature review. The purpose of this experiment was to identify metabolic capabilities of the various fetal and maternal tissues from mares in the later stages of gestation. By co-incubating the tissues with a labelled progestin precursor steroid (D_4-P5), it would be possible to determine which progestins each tissue could produce by GC/MS analysis of the incubation medium for deuterium labelled steroids derived from D_4-P5.

2. Animal and Tissue Incubation Protocol

Two pregnant pony mares both at 313 d gestation (#75 and #81) were used in this experiment. Each mare was euthanized and the tissue samples promptly collected into sterile containers, placed on ice, and transported to the laboratory for incubations. Tissues collected included mare uterine endometrium, placenta, fetal gonads (ovaries, since both foals were fillies), fetal adrenals, fetal liver, and fetal kidneys.

All equipment used in the incubations was autoclaved. Incubation medium was Medium 199 (M199) with Hank's salts and sodium bicarbonate (Sigma). It was sterile filtered and endotoxin tested. The stock solution of M199 was gassed with 95% O_2 and 5% CO_2 for 20 min. Five milliliters were pipetted to each of the sterile 25 ml Erlenmeyer Flasks, and kept at 4 °C. Two 1 g samples of each tissue were minced with scissors, and added to the flasks as duplicates. The flasks were gassed again briefly, capped with rubber stoppers, and placed in a shaking water bath at 37 °C for an 1 h pre-incubation. Medium blanks served as control incubations.

After the 1 h pre-incubation, the medium was removed and replaced with new medium. The flasks were again gassed briefly. To one of the duplicates of each tissue type, 25 µg of D_4-P5 were added. All flasks were then incubated for 4 h at 37 °C in the shaking water bath. Medium and tissue were then kept at -20 °C until assayed.

3. Assay Protocol

a. Standards

As in Experiment 2, the medium samples obtained from the tissue incubations were being analyzed for deuterium labelled metabolites from the D_4-P5 in the medium. Therefore, 4-cholesten-3-one was used as the internal standard as indicated in Experiment 2.

b. Extraction of Steroids

The 4-cholesten-3-one in medium extracted efficiently (>90%) through Sep-Paks, therefore all samples in this experiment were extracted as indicated in Experiment 1. Derivatization and GC/MS programming was also as noted in Experiment 1.

4. Statistical Analysis

Assay statistics were done with Macintosh Excel. SAS was used for the data analysis. Randomized block design GLM analysis of variance determined mare (random, block effect) and tissue (fixed, treatment effect) differences for the progestin concentrations (dependent response variable). Multiples comparison tests (LSD) were applied when differences occurred. In addition, multiple paired t-tests were done to determine hormone differences within each tissue. All data was log transformed due to obvious heterogeneous variance.

EXPERIMENT 4: STEROID METABOLISM AND THE IDENTIFICATION OF PLASMINOGEN ACTIVATOR IN EARLY EQUINE EMBRYOS

1. Purpose

Metabolic intermediates between the estrogens and progesterone produced by equine embryos in early gestation have not been identified. One of the objectives of this study was to identify with GC/MS both progestins and estrogens produced by the equine embryo during the 14 to 18 d stage. Steroid metabolism may be associated with maternal recognition of pregnancy, which occurs at this stage of gestation in mares. In order to trace progestin metabolism by deuterium labelling with GC/MS, some of the embryos were incubated with varying amounts of D_4-P5. Additionally, since no work has been published regarding plasminogen activator in the equine embryo, identification of this protein in the incubation medium and embryonic tissues was included in the protocol.

2. Animal and Embryo Incubation Protocol

Nine pony mares (#35, #43, #44, #60, #72, #77, #78,#80, and #83) were bred to the same pony stallion via artificial insemination. All mares were teased, palpated rectally, and scanned transrectally with ultrasound daily to accurately determine readiness for breeding, day of ovulation, or pregnancy.

All embryos collected were 14 to 18 d after ovulation. After identifying normal embryos via ultrasound, the embryo was removed with a transcervical uterine flushing (Squires et al., 1985). The flushing buffer was sterilized by filtration (Falcon 7105 .22 µm filter,

Becton Dickinson, Lincoln Park, N.J.), and consisted of Dulbecco's Phosphate Buffered Saline or PBS (Sigma) at pH=7.2 and 37 °C. All equipment and tubing used in the embryo collections were sterilized with gas or steam.

Flushing medium was collected into glass graduated cylinders, the embryo removed, and examined on a low power microscope to be sure it was intact. Each embryo was then transferred to a warmed culture dish (60 X 15 mm, Corning Glassworks, Corning, N.Y.) with 5 ml of the culture medium (Minimum Essential Medium or MEM, with Hank's salts, L-glutamine, sodium bicarbonate, 100 U/ml penicillin G, and 100 µg/ml streptomyocin, all from Sigma). In five of the embryos cultured, 1% heat inactivated horse serum (Gibco Lab, Grand Island, N.Y.) was also added to the culture medium. The other four embryos were used in plasminogen activator assays which required serum free medium. Medium was placed in the incubator at 39 °C in a humidified atmosphere of air in 5% CO_2, for 24 h prior to adding the embryo.

Each embryo was rinsed three times in medium, and then pre-incubated for 2 h. After the pre-incubation, the embryo was transferred to a new dish with medium, and incubated 24 h. Control medium blanks were also included with each incubation. In the culture of three embryos, varying levels of D_4-P5 were added to the 24 h incubation medium (2500, 5000 and 20,000 ng). After the 24 h incubation, the embryo and medium were stored at -20 °C until assayed.

3. Assay Protocols and Statistical Analysis

a. Steroid Assay Protocol

GC/MS progestin analysis of the medium from the embryo incubations was identical to the protocol for tissue incubations noted for Experiment 3. In addition to the progestins, an androgen and estrogens (see structures in Figure 3) were studied in these embryo samples.

Stock standards of 1 mg/ml were prepared of 4-androstene-3,17-dione (androstenedione, AND), 3-hydroxy-1,3,5(10)-estratrien-17-one (estrone, E_1), 1,3,5(10)-estratriene-3,17β-diol (estradiol, E_2), and 1,3,5(10)-estratriene-3,16α,17β-triol (estriol, E_3). A mixture of these four steroids at a concentration of 10 µg/ml in methyl alcohol was then prepared. Standard regression lines against 4-cholesten-3-one were developed for each hormone as was outlined for the progestins in Experiment 1. These four steroids were identified and quantified in five of the embryos collected.

Extraction of medium was accomplished with Sep-Paks. Derivatization and GC/MS programs did not differ from techniques discussed in Experiment 1. Assay statistics were done on Macintosh Excel. Statistically, the deuterium progestin concentrations and the various treatment levels were analyzed in a regression using SAS to deterimine if there was a relationship between amount of D_4-P5, and the metabolites formed. The estrogen data was analyzed as a one way analysis of variance (GLM) on SAS, with a multiple comparison test (LSD) to determine where differences existed between hormones.

b. Plasminogen Activator Assay Protocol

The embryo medium samples were concentrated from 2 ml to 50 µl in Amicon Centricon Microconcentrators (Amicon, Danvers, MA) which were centrifuged for approximately 10 min. To each concentrated medium sample, 50 µl of sample buffer was added (5.0% SDS, 20% glycerol, .0025% bromophenol blue, all in .125 M Tris HCl buffer, all supplies from Sigma). The embryo tissues were placed in 500 µl of sample buffer overnight at room temperature. The following day, each was vortexed, centrifuged to settle the tissue, and the supernatent then drawn off for the assay.

A SDS polyacrylamide gel electrophoresis assay was used to characterize plasminogen activator in the medium and embryonic tissues of four embryos collected. A standard protocol for SDS PAGE was used for this experiment (Bio Rad., 1987). One lane on each gel contained the following Bio Rad protein standards: rabbit muscle phosphorylase b (97.4 KD), bovine serum albumin (66.2 KD), hen egg white ovalbumin (45.0 KD), bovine carbonic anhydrase (31.0 KD), soybean trypsin inhibitor (21.5 KD), and hen egg white lysozyme (14.4 KD). Two other lanes were used for tissue type plasminogen activator standard (tPA) at 10 ng/ml, and urokinase type plasminogen activator standard (uPA) at .1 IU/ml (Sigma). Sample or standard (100 µl) was added to each well in a 4% stacking gel with 12% separating gel. Electrophoresis was conducted through the stacking gel at 20 mA, and 30 mA through the separating gel over approximately 3 h. After electrophoresis was complete, the gels were placed in 2.5% Triton X-100 (Sigma) in water, and shaken gently for 30 min, followed by a rinse in PBS.

At this time, a casein-agar zymograph was prepared (Granelli-Piperno and Reich, 1978; Vassalli et al., 1984). Four grams of nonfat dry milk (Carnation Co., Los Angeles, CA) was dissolved in 100 ml of buffer containing .0013 M $CaCl_2$-$2H_20$, .1 M glycine, .038 M Tris, and .005 M sodium azide (all from Sigma). Seven milliliters of the milk mixture at 55 °C was added to 7 ml of a 2% agarose (Sigma) in water at 55 °C. Purified human plasminogen was added to the milk-agarose solution to make a 30 µg/ml solution. Ten milliliters of this mixture was transferred to a 13 X 8 cm glass plate, and allowed to gel for 10 to 15 min. In addition, zymographs without human plasminogen were prepared for the detection of any nonspecific proteolytic activity.

A gel "sandwich" was then prepared with the acrylamide gel and the casien-agar zymograph which was wrapped in plastic wrap and incubated at 39 °C for 24 h. The gels were separated, and the zymograph fixed with 3% acetic acid for 10 min, and rinsed with tap water. Zymographs were dried and stained overnight with .05% Coomassie Brilliant Blue (Bio Rad) in a ratio of water/isopropanol/acetic acid of 65/25/10.

An additional assay was conducted using amiloride zymographs (Erickson et al., 1984). To prepare this zymograph, a 50:50 solution of 4% nonfat dry milk in buffer with 2% agarose in water was prepared. Two 9 ml aliquots of this mixture were combined with either 1 ml of 100 mM amiloride (Sigma) dissolved in PBS or a control blank of 1 ml PBS. The plasminogen was added to the mixture as indicated in the previous protocol, and tranferred to a 13 X 8 cm glass plate.

Protein migration was deterimined by measuring the distance (cm) from the edge of the separating gel to the center of the lytic zone,

after fixing the gel. From the distances measured for the set of standard proteins, a linear regression line was constructed for each assay on Macintosh Cricket Graph. Each distance (cm) was divided by the total distance and plotted against the log of the molecular weights (MW). From the equation generated, the mass of the unknown could be calculated.

EXPERIMENT 5: THE BIOLOGICAL ACTIVITY OF EXOGENOUS PROGESTINS IN CYCLIC MARES

1. Purpose

The biological effects of the progestins, other than progesterone, have not been investigated in the mare. The objective of this experiment was to examine the biological action of two progestins (5α-DHP and 3β-5P) in the nonpregnant mare. Control of gonadotropin (eLH), behavioral estrus, and ovulation were the parameters studied.

2. Animal Protocol

Twenty-seven estrous cycles, during the natural physiological breeding season, in eleven nonpregnant pony mares (#44, #57 #59, #60, #70, #71, #72, #73, #74, #75, and #78) were analyzed in this study. The mares were teased daily with a stallion to detect estrous behavior. Each mare was rectally palpated and scanned with ultrasound transrectally to monitor ovarian changes daily during estrus and every other day during diestrus. Blood sampling was also done daily during estrus and every other day during dietrus. Jugular vein blood was collected into heparinized tubes, centrifuged, and kept at -20 °C until assayed.

The treatment regime included progestin injections (i.m.) beginning at 6 d postovulation, and continuing for 12 d or until the mare returned to estrus. At 8 d postovulation, 5 mg of PGF2α (Lutalyse, Upjohn Co., Kalamazoo, MI), was administered to regress the corpus luteum (CL). Lysis of the CL following PGF2α injection was verified with the P4 EIA (Novo Biolabs).

The progestins tested in this study (P4 , 5α-DHP, and 3β-5P, all from Sigma), were dissolved in safflower cooking oil at a concentration of 5 mg/ml, and injected i.m. Mares were randomly chosen for treatments, with some mares used for up to three cycles. However, at least one cycle elapsed between treated cycles. In preliminary work, three daily dosage levels of 5α-DHP were assessed (10, 25, and 50 mg). Two mares were injected at each level, and it was determined that 50 mg may be the effective dose. Therefore, in the progestin treatment group, an additional seven mare cycles were used to further test 5α-DHP at the dosage level of 50 mg/ml. In addition, four mares were injected with 50 mg of 3β-5P, and two mares with 50 mg of P4. Controls included two untreated mares, as well as six mares treated with PGF2α only.

3. Assay Protocol

A non-competitive "sandwich" EIA was developed to analyze eLH levels in the plasma samples (Wilson et al., 1990). Ninety-six-well polystyrene square-bottom Immulon-4 microtiter plates (Dynatech, Chantilly, VA) were coated with 200 µl/well of the capture antibody (Clone 518B7-0220, antibody produced against the β-subunit of bovine LH at Monoclonal Antibodies, Mountain View, CA) at a concentration of

10 µg/ml in .01 M PBS at pH=7.0. Plates were covered with plate sealers (Flow Laboratories Inc., Inglewood, CA), and incubated overnight at room temperature. The following day, the plates were inverted to remove the antibody, and washed three times with EIA wash buffer comprised of .04% polyoxyethylene-sorbital monolaurate (Tween 20) and .15 M sodium chloride (both from Sigma) in distilled water. Plates were inverted and dessicated in a vacuum overnight at room temperature, and the assay started the following morning.

Unknowns and standards were tested in duplicate wells (200 µl/well). The eLH standards (based on NIH standard LH-S1, obtained from Sigma) were at concentrations of 0, 2, 4, 8, 16, 32, 64, and 128 ng/ml. The standards for each assay were diluted in .01 M PBS with .04% Tween 20, pH=7.0. Standards diluted in plasma resulted in a similar standard curve (r^2=.99), consequently buffer diluent could be used for the assays. After all samples and unknowns were added to the wells, the plate was covered with a sealer and incubated at room temperature for 2 h. Appropriate controls were included on each plate to monitor non-specific binding.

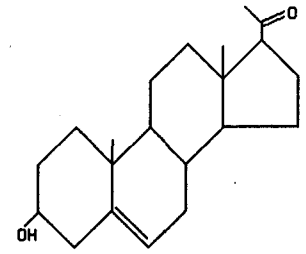
After incubation, plates were inverted and washed three times with EIA wash buffer. A second antibody conjugated to alkaline phosphatase (759G10-AP, antibody produced against the α-subunit of eCG and conjugated to alkaline phosphatase, Monoclonal Antibodies, Mountain View, CA), was added (200 µl) at the dilution of 1:300 in conjugate buffer (42 mM Trizma Base, 50 mM sodium chloride, 15 mM sucrose, .5 mM magnesium chloride, .1 mM zinc chloride, .01% Thimerosal, 1% bovine serum albumin, and .13% Tween 20, at pH=7.0; all

from Sigma). The plates were covered with sealers, and incubated 2 h at room temperature. After incubation, plates were inverted and washed three times with EIA wash buffer. Since the substrate (p-Nitrophenyl Phosphate, pNPP, Sigma) is light sensitive, it was prepared just prior to use. A 1 mg/ml solution of pNPP was prepared in a 1M diethanolamine buffer with .5 mM magnesium chloride and .01% sodium azide (all from Sigma) at pH=9.8. The substrate was added (150 µl/well), covered with a sealer, and incubated in the dark for 30 min. The reaction was stopped with 50 µl/well of 50 mM tetra-sodium ethylenediamine tetra-acetic acid (Sigma), pH=12.5.

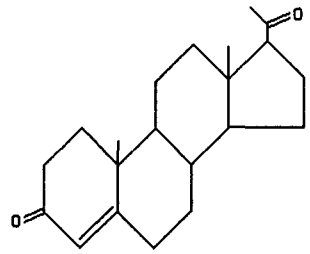
The optical densities were read at 405 nm on a Titertek Multiscan Plus MKII microelisa automatic plate reader (Flow Laboratories), with programming which calculated a standard curve from the eLH standards in each assay from which the concentrations (ng/ml) were calculated. A Titertek multichannel pipette (Flow Laboratories) was used for all pipetting across the plates.

4. Statistical Analysis

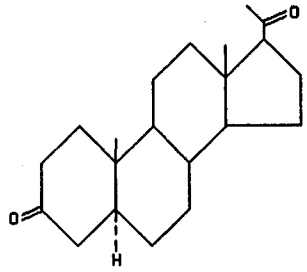
Several one way analyses of variance tests were conducted using Stat Graphics (STSC Inc., Rockville, MD) to determine differences between treatment groups (progestin treated, untreated mares, and PGF2α controls) for both the number of days from the initiation of treatment to ovulation, and the eLH concentrations on the day of ovulation subsequent to the treatment.



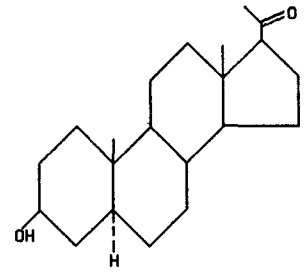
a) 3ß-hydroxy-5-pregnen-20-one (pregnenolone or P5)



b) 4-pregnene-3,20-dione (progesterone or P4)

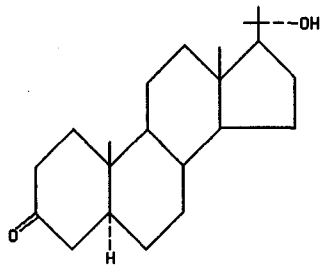


c) 5α-pregnane-3,20-dione (5α-DHP)

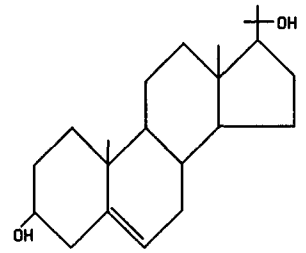


d) 3ß-hydroxy-5α-pregnan-20-one (3ß-5P)

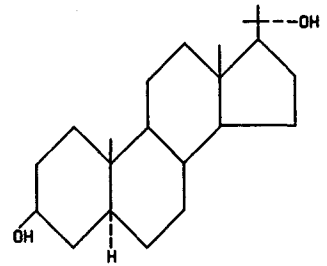
FIGURE 1: Progestin structures a) P5, b) P4, c) 5α -DHP, & d) 3ß-5P. Systematic names & some common names are indicated with each structure. Dashed lines in structures indicate α -configuration (below the plane). Solid lines indicate ß-configuration (above the plane)



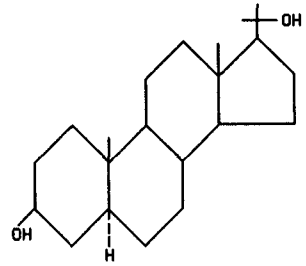
a) 20α-hydroxy-5α-pregnan-3-one (20α-5P)



b) 5-pregnene-3β,20β-diol (P5-ββ)

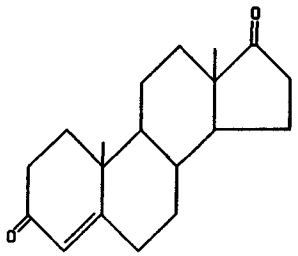


c) 5α-pregnane-3β,20α-diol (βα-diol)

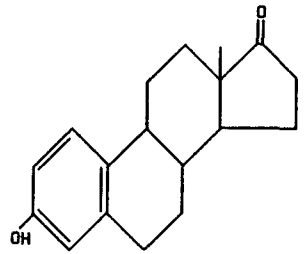


d) 5α-pregnane-3β,20β-diol (ββ-diol)

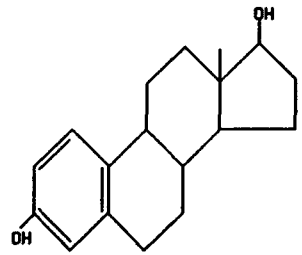
FIGURE 2: Progestin structures a) 20α -5P, b) P5-ββ, c) βα -diol, & d) ββ-diol.
Systematic names are indicated with each structure. Dashed lines in structures indicate α -configuration (below the plane). Solid lines indicate β-configuration (above the plane)



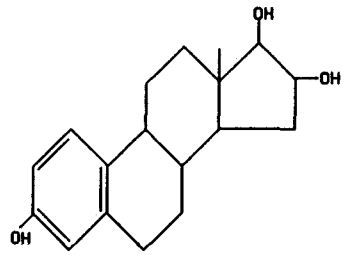
a) 4-androstene-3,17-dione (androstenedione or AND)



b) 3-hydroxy-1,3,5 (10)-estratrien-17-one (estrone or E1)



c) 1,3,5 (10)-estratriene-3,17β-diol (estradiol or E2)



d) 1,3,5 (10)-estratriene-3,16α,17β-triol (estriol or E3)

FIGURE 3: Estrogen and androgen structures a) AND, b) E1, c) E2, & d) E3. Systematic names & common names are indicated with each structure. Dashed lines in structures indicate α -configuration (below the plane). Solid lines indicate β-configuration (above the plane).

RESULTS

ASSAY RESULTS

1. GC/MS

Tables 1 and 2 contain the full systematic and abbreviated names, GC/MS retention times, and major ions monitored for the progestins and estrogens. Figure 4 is a total ion chromatogram on FSM of the progestin standards with deuterium labelled progestin internal standards, showing the individual peaks and retention times. These relative retention times, noted in Figure 4 (see caption), were consistent for all assays. The deuterium labelled progestins have retention times slightly before the unlabelled progestins. The doublet peaks observed for both 5α-DHP and 20α-5P are due to syn- and anti-isomers of methoxime at the carbonyl group of the third carbon.

Figures 5 through 8 show the individual full-scans, without background subtraction, of each progestin and deuterium labelled internal standard of the standard mix in the total ion chromatogram of Figure 4. Figure 5a shows the full-scan for D_4-5α-DHP, of which both 5α-DHP (Figure 5b) and P4 (Figure 5c) were quantified against. In Figure 6a, the full-scan of D_4-P5 is indicated. P5, which is quantified with D_4-P5, is in Figure 6b. D_4-3β-5P (Figure 7a) was used for the quantification of both 3β-5P (Figure 7b) and 20α-5P (Figure 7c). All three diols, P5-ββ (Figure 8b), ββ-diol (Figure 8c), and βα-diol (Figure 8d) were quantified with D4-ββ-diol (Figure 8a).

The method of quantification was described in the Materials and Methods section. The ions monitored for this quanitification on GC/MS

are noted in Table 1, and can be noted for each progestin on the full-scans. For progestins with deuterium labels, these ions have masses which are four units greater than unlabelled progestins. The major ion is the intact molecular ion (Figure 5c), or more commonly the molecular ion with a derivative group removed (all other progestins shown). With the MO/TBDMS derivatization, compounds with keto-groups have 29 added to their molecular weights for every keto-group present, while compounds with unhindered hydroxyl-groups have 114 added to their molecular weights for each hydroxyl group. Hybrid compounds with a keto- and a hydroxyl-group would have 114 and 29 added to their molecular weight. The keto-derivatives remove 31 from the total weight, while hydroxyl-derivatives remove 57 when fragmented.

In addition to the major ions monitored, other ions appear consistently, since each progestin breaks down in a predictable pattern of fragments. The progestins with 20-position keto-groups (Figures 5a,b,c, 6a,b, and 7a,b) have in addition to the major ion, a fragment with a mass of 100, or if deuterium labelled, a mass of 104. This fragment includes the 20-position derivative plus a portion of the D-ring of these compounds. When the compound has a hydroxyl-group at the 20-position (Figures 7c, and 8a,b,c,d), characteristic 103 and 159 mass ions, or 106 and 162 if deuterium labelled, appear on the scan. For identification purposes, these distinctive "fingerprints" allow definitive identification of unknown compounds.

The total ion chromatogram for the estrogen standards with 4-cholesten-3-one is shown in Figure 9a. Figure 9b is an individual full-scan of 4-cholesten-3-one, the internal standard used for the estrogen quantification since no deuterium labelling was feasible without the

21-carbon progestin structure. This internal standard was also used in the detection of deuterium labelled progestin metabolites. Figure 10a,b,c,d are individual scans of androstenedione and the estrogens (without background subtraction), showing the major ions monitored as well as the various fragments characteristic for each compound. The ion monitored was the intact molecular ion (Figure 10a), or the molecular ion with a single derivative group removed (Figure 10b and c). A major fragment was also used for the monitored ion (Figure 10d).

Representative standard curves are shown for a dione progestin (Figure 11a), a mono-hydroxy progestin (Figure 11b), a di-hydroxy progestin (Figure 12a), and an estrogen (Figure 12b). The standard curves varied due to differences in the response of the various progestins relative to their actual amounts in the GC/MS system.

Since internal standards were used in all assays, extraction efficiencies were not crucial. However, extraction efficiencies were calculated for each of the extraction techniques used and found to vary from 71% to 78% for plasma, and 94% to 97% for medium samples. Overall assay sensitivity, as determined by adding two standard deviation units to the lowest detectable reading, was usually at least .64 ng/ml.

All assay performance information from Experiment 1, for each progestin in plasma (Sep-Pak extracted), is listed in Table 3. Assay performance for Experiment 2, for plasma samples with ether extraction, is noted in Table 4. In both Experiments 3 and 4, medium samples were extracted with Sep-Pak. Assay performance for the progestins analyzed in these experiments are in Table 5. In Experiment 4, estrogens were also analyzed. Assay performance is noted in Table 6

for the estrogens studied in Experiment 4. The recovery % refers to the amount of a steroid measured with the assay compared to the actual amount added. In most cases, it was near 100%, but did range from 72.9% to 110.7%. The recoveries were determined with standards added to plasma at four concentrations and averaged over several assays (see notes on Tables 3 to 6). The coefficient of variation was also determined with standards added to plasma at four concentrations and tested within assays (intra assay %) as well as between (inter assay %) several assays (see notes on Tables 3 to 6). Intra assay % ranged from 4.0 to 15.3%, while inter assay % ranged from 4.1% to 23.3%.

Full-scans on GC/MS provide a definite identification of steroids as seen with the standards discussed previously in this section. Some examples of actual samples, which will be discussed in greater detail in the subsequent sections, are shown in Figures 13 to 17. In Figure 13a, a total ion chromatogram is shown for a fetal artery sample in Experiment 1. The large peak at 11.6 is P5 (full-scan in Figure 13b) and the small peak at 11.8 is 3β-5P (Figure 13c). Figure 14a,b,c,d are ion chromatograms from an uterine vein sample after infusion of D_4-P5 into the fetal artery. The GC/MS allows detection and identification of the deuterium labelled metabolites. In Figure 14a, the two compounds with the 404 ion are shown (3β-5P and 20α-5P), while their corresponding deuterium labelled metabolites with the 408 ion are seen in Figure 14b. Additionally, both labelled (ion 495 in Figure 14d) and unlabelled (ion 491 in Figure 14c) βα-diol can be detected.

Some of the deuterium labelled metabolites produced in the tissue incubations are shown in Figure 15. D_4-P4 was produced in endometrium (Figure 15a) and placenta (Figure 15b). D_4-P5-ββ was produced by fetal

liver (Figure 15c). Evidence of the production of deuterium labelled metabolites was also shown by embryos (Figure 16). A full-scan of the precursor added is shown (D_4-P5 in Figure 16c). Two metabolites are shown, D_4-P4 (Figure 16b) and D_4-3β-5P (Figure 16d). In Figure 17, evidence for the identification of P4 (Figure 17a), E1 (Figure 17b), and E2 (Figure 17c) in embryos is given.

2. SDS PAGE

A typical standard curve for the plasminogen activator SDS PAGE assay is depicted in Figure 18a. Molecular masses were determined from this curve as indicated in the Materials and Methods section.

3. Equine LH EIA

The intra-assay coefficient of variation was 8.3%, while the inter-assay coefficient of variation was 11.6%, which were determined from seven different concentration levels of eLH. Assay sensitivity, determined by adding two standard deviations to the 0 reading, was estimated to be .97 ng/ml. A typical standard curve for this assay is shown in Figure 18b.

In addition, the eLH EIA assay protocol was effectively used to determine equine chorionic gonadotropin (eCG) concentrations in plasma samples from pregnant mares in a sample set collected in an earlier study. Due to the structural similarities between eLH and eCG, both hormones will bind to the same antibodies.

EXPERIMENT 1: PROGESTINS OF THE LATE-GESTATION MARE AND FETUS

1. Progestin Concentrations Determined with GC/MS

The mean concentrations for each of the progestins (plus or minus standard error), in the uterine or maternal artery, uterine vein, fetal artery, and fetal vein were calculated for the six cannulated mares (Figures 19 and 20). There were no significant differences between the mares for all of the progestins except P4 and 20α-5P, which were different between mares ($P < .05$). Multiple comparison tests revealed that the mares differing from the others for P4 and 20α-5P concentrations were #75, #76, and #81.

Mare #76 had higher ($P < .05$) average P4 levels for the four vessels (12.8 ng/ml) than the other mares, due to her earlier stage of pregnancy (160 d). The differences for 20α-5P were due to both mares #75 and #81, who had average levels of 909.5 and 651.0 ng/ml respectively, which were higher ($P < .05$) than the other mares. This variation was due to #75 and #81 being at a slightly later stage of pregnancy (313 d). Mare #76, at a much earlier stage of pregnancy (160 d), had ($P < .05$) lower 20α-5P levels (83.5 ng/ml) than the other mares.

The progestin concentrations between the four vessels all differed ($P < .05$). The predominant progestin ($P < .05$) in the fetal vein (Figure 20b) was 5α-DHP (mean of 216.4 +/- S.E. of 24.4 ng/ml). P4 was also highest ($P < .05$) in the fetal vein (15.5 +/- 2.8 ng/ml), with lower levels in the fetal artery (7.8 +/- 5.1 ng/ml), and negligible amounts in the maternal vessels. In the fetal artery (Figure 20a), P5 (630.8 +/- 298.5 ng/ml), 3β-5P (117.9 +/- 17.0 ng/ml), P5-ββ (131.4 +/- 51.8 ng/ml), and ββ-diol (32.3 +/- 12.8 ng/ml) were all higher in

concentration ($P < .05$) than in the other vessels. The uterine vein (Figure 19b) had the highest concentration ($P < .05$) of both 20α-5P (767.6 +/- 217.9 ng/ml) and βα-diol (202.2 +/- 44.5 ng/ml), with the maternal artery (Figure 19a) having these two hormones predominating ($P < .05$), but at somewhat lower levels (20α-5P at 533.2 +/- 201.8 ng/ml and βα-diol at 150.1 +/- 28.5 ng/ml).

Within each vessel, some of the hormones differed significantly from the others. In the fetal artery (Figure 20a), P5 (630.8 +/- 298.5 ng/ml) was greater ($P < .05$) than both 3β-5P (117.9 +/- 17.0 ng/ml) and P5-ββ (131.4 +/- 51.8 ng/ml). The latter two hormones exceeded ββ-diol (32.3 +/- 12.8 ng/ml) in concentration ($P < .05$). All of these hormones were greater in concentration ($P < .05$) than the remaining four hormones which were all less than 18 ng/ml.

The mean level of 5α-DHP (216.4 +/- 24.4 ng/ml) in the fetal vein (Figure 20b) was greater than the other hormones at that vessel ($P < .05$). Levels of P5 (92.6 +/- 33.7 ng/ml), 20α-5P (57.2 +/- 36.5 ng/ml), and 3β-5P (41.5 +/- 4.7 ng/ml) did not differ significantly from each other, but did exceed the remaining four steroids ($P < .05$), which were all less than 15 ng/ml.

At the maternal artery (Figure 19a), 20α-5P (533.3 +/- 201.8 ng/ml) was greater than all other hormones ($P < .05$). The βα-diol (150.1 +/- 28.5 ng/ml) exceeded the remaining six hormones ($P < .05$). Both 5α-DHP (41.3 +/- 10.7 ng/ml) and 3β-5P (28.2 +/- 3.2 ng/ml) were not significantly different, but were greater ($P < .05$) than the other four hormones (which were all less than 3 ng/ml).

With the uterine vein samples (Figure 19b), 20α-5P (767.6 +/- 217.9 ng/ml) was the highest in concentration ($P < .05$), with βα-diol

(202.2 +/- 44.5 ng/ml) being the second in concentration level ($P < .05$). The 5α-DHP (66.4 +/- 12.4 ng/ml) exceeded the remaining five hormones ($P < .05$), with 3β-5P (20.3 +/- 2.8 ng/ml) being greater ($P < .05$) than the last four hormones which all were less than 3 ng/ml.

2. P4 EIA Cross-reactivity Studies

Samples from both maternal and fetal vessels in mares #59, #48, and #46 were tested on the commercially available P4 EIA kit, and found to produce a mean maximal reading of 10.84 +/- 0.34 ng/ml. Since some of the samples came from maternal vessels, which would normally have very low levels of P4 according to results published previously (Holtan et al., 1991), the eight steroids analyzed on GC/MS were tested on the EIA kit for cross reactivity with the P4 antibody. At the 1000 ng/ml level, all eight steroids recorded "positive" readings for P4, according to the kit manufacturer's biologically positive level of 2.5 ng/ml, which was also at 50% inhibition on the P4 standard curve. The 100 ng/ml level resulted in readings of over 2.5 ng/ml for 5α-DHP, P4, P5, and 3β-5P. At 10 ng/ml, only 5α-DHP and P4 were above 2.5 ng/ml. The concentrations at which each progestin reached the 50% inhibition capacity (2.5 ng/ml) or greater on the standard curve for the P4 assay are noted in Table 7. This implies that these hormones were able to competitively inhibit the binding of P4 to the P4 antibody in this assay.

EXPERIMENT 2: PROGESTIN METABOLIC STUDIES UTILIZING DEUTERIUM PREGNENOLONE INFUSIONS TO FETAL VESSELS IN LATE-GESTATION MARES

After the infusion of D_4-P5 into the fetal artery, plasma samples from both fetal and maternal vessels were collected over time in order to trace the deuterium labelled metabolites derived from D_4-P5. Metabolite identification is shown in Figure 14. The data accumulated in this experiment were multiple readings of the concentrations of eight progestins over a time period following the infusion. The sites included the fetal artery and vein, as well as a maternal artery and uterine vein from the two mares. In sampling from the fetal vessels, less blood volume was collected at each sampling to avoid hemorrhagic stress to the fetus. Consequently, for GC/MS analysis it was necessary to pool some of the samplings to attain adequate volumes resulting in fewer time readings in the fetal vessels compared to maternal vessels.

The D_4-P5 metabolite concentrations over the sampling time period for the two mares are presented in Figures 21 and 22. There were differences ($P < .05$) for all hormones in the various vessels, as well as over time differences ($P < .05$) for each hormone. In addition, there were differences ($P < .05$) between some vessels over time for D_4-P5 (infused precursor), D_4-5α-DHP, D_4-P4, D_4-3β-5P, D_4-20α-5P, and D_4-βα-diol. This variation was due to differences of the fetal vessels and maternal vessels, with D_4-3β-5P, D_4-P4, and D_4-5α-DHP being higher ($P < .05$) in fetal vessels, and D_4-20α-5P, and D_4-βα-diol higher in maternal circulation.

It was also observed from the data, that maximum concentrations of all hormones were noted at the ten minute sampling in the maternal

vessels and fetal artery (Figures 21 & 22). Even though the maximum reading for the fetal vein seemed to occur somewhat later than in the other vessels, data from the 10 min sampling were analyzed statistically. In statistical analysis of this data set, there were differences ($P < .05$) between vessels for D_4-P4, D_4-20α-5P, D_4-3β-5P, and D_4-βα-diol, with D_4-P4 and D_4-3β-5P higher in the fetal circulation, and D_4-20α-5P and D_4-βα-diol higher in maternal vessels (Figure 23). In comparing the levels of each deuterium labelled hormone within the four vessels it was revealed that the predominating progestins were D_4-20α-5P and D_4-3β-5P. Some hormones, in particular D_4-5α-DHP, D_4-P4, and the diols, were at very low levels.

EXPERIMENT 3: METABOLIC CAPABILITIES OF FETAL MATERNAL TISSUES FROM LATE-GESTATION MARES

The mean concentrations of D_4-P5 metabolites for each of the six tissues were determined (Figures 24 to 26). There were differences ($P < .05$) between the tissues for D_4-5α-DHP, D_4-P4, D_4-3β-5P, D_4-P5-ββ, and D_4-βα-diol.

Fetal ovary (Figure 24a), mare uterine endometrium (Figure 26b), and placental tissues (Figure 26a) produced more ($P < .05$) D_4-5α-DHP than the remaining tissues. Placental tissue (Figure 26a) produced more ($P < .05$) D_4-P4 than all other tissues. However, all other tissues incubated produced some D_4-P4. The concentration of D_4-3β-5P was higher ($P < .05$) for the fetal ovary (Figure 24a), adrenal (Figure 24b), liver (Figure 25a), and mare uterine endometrium (Figure 26b). Although D_4-20α-5P was made in the fetal ovaries (Figure 24a), adrenals (Figure 24b), liver (Figure 25a), and mare uterus (Figure 26b), the differences

were not significant. More ($P < .05$) D_4-P5-$\beta\beta$ was produced by the fetal liver (Figure 25a) than the other tissues. Maternal uterine tissue (Figure 26b) produced more of both D_4-$\beta\beta$-diol and D_4-$\beta\alpha$-diol ($P < .05$) than the other tissues. Metabolite identification is shown in Figure 15.

The order of hormones levels produced at each tissue was also determined. In each tissue, D_4-P5 and D_4-P4 predominated, with various other hormones being produced at lower amounts as mentioned in the preceding paragraphs. In the duplicate incubations of each tissue without D_4-P5, most of the progestins were identified in each tissue. It was not possible to ascertain whether they were actually metabolized or merely leaching out of the tissue.

EXPERIMENT 4: STEROID METABOLISM AND THE IDENTIFICATION OF PLASMINOGEN ACTIVATOR IN EARLY EQUINE EMBRYOS

1. Steroid Production

The predominant progestin ($P < .05$) in incubation medium of untreated embryos was P4 (Figure 27a). All other progestins tested for appeared in the incubation medium also, but at lower concentrations. In the incubation medium of embryos treated with D_4-P5 (Figures 27b and 28a,b), the metabolite from D_4-P5 produced in the highest amounts was D_4-P4. There was a direct relationship ($r=0.61$) between the amount of D_4-P5 added to the medium, and the level of D_4-P4 produced. The other deuterium labelled progestins were also produced by the embryos incubated with D_4-P5, but at lower levels than D_4-P4. They were identified with GC/MS full-scan (Figure16).

Five embryos were also analyzed for estrogens. There were no significant differences between the embryos for each hormone. The mean concentrations of the four hormones analyzed (Figure 29) were different ($P < .05$). Estradiol (420.9 +/- 95.1 ng/ml incubation medium) was greater ($P < .05$) than the other three hormones (estriol 192.9 +/- 57.9 ng/ml, estrone 97.3 +/- 28.7 ng/ml, and androstenedione 34.5 +/- 7.8 ng/ml). Positive identifications of these steroids were possible with GC/MS full-scans (Figure 17).

2. Plasminogen Activator

The SDS-PAGE assays allowed the identification of plasminogen activator in both the medium and embryonic tissue samples from four embryos. Figures 30 to 31 are photographs of some of the dried zymographs produced. In Figure 30a, lane 1 is a medium blank, lanes 2, 3 and 4 are embryo incubation medium samples, lanes 5 and 6 are embryo tissue samples, and lanes 7 and 8 have urokinase type plasminogen activator standards, with proteins standards in lane 9. Two weak bands were observed for the three medium samples at 72.4 KD and 51.3 KD. The two tissue samples produced four very strong bands at 91.2 KD, 83.2 KD, 72.4 KD, and 51.3 KD. Human urokinase plasminogen activator standards produced a band at 56.2 KD. Therefore the bands in actual samples at 51.3 KD are urokinase plasminogen activator. The band at 72.4 KD is tissue plasminogen activator, and the two bands at 83.2 KD and 91.2 KD are inhibitor complexed to the two plasminogen activators.

Figure 30b has in lane 1 a medium blank, lanes 2 and 3 embryo incubation medium samples, lanes 4 and 5 have diluted embryo tissue samples, lane 6 is a human urokinase plasminogen activator standard,

lane 7 is a human tissue type plasminogen activator standard, with protein standards in lane 8. Medium samples have two weak bands at 53.2 KD (urokinase plasminogen activator) and 75.4 KD (tissue plasminogen activator). Tissue samples have four stronger bands at 53.2 KD, 75.4 KD, 85.9 KD, and 93.7 KD. Again, the two higher KD bands indicate inhibitor bound to the two types of plasminogen activator. Human urokinase type plasminogen activator standard has a band at 59.3 KD, while human tissue type plasminogen activator standard has a band at 69.1 KD. No nonspecific proteolytic activity was noted in this zymograph.

Figure 31 illustrates the results of the amiloride (an inhibitor of urokinase type plasminogen activator) assay. Lanes 1 and 2 have the human tissue type plasminogen activator and the human urokinase type plasminogen activator, while lanes 3 and 4 have the two embryo tissue samples. The gel pictured in Figure 31a had no amiloride added, and therefore produced results similar to the previous gels. The embryo tissue samples had bands at 94.3, 87.9, 76.5, and 54.9 KD. Urokinase plasminogen activator standard was at 61.0 KD, and tissue type plasminogen activator standard at 67.8 KD. Figure 30b shows the results from the amiloride zymograph. No bands appeared for the lane 2 urokinase plasminogen activator standard, and the bands at 87.9 and 54.9 KD are absent in the embryo tissue samples, confirming the presence of urokinase plasminogen activator in these bands.

EXPERIMENT 5: THE BIOLOGICAL ACTIVITY OF EXOGENOUS PROGESTINS IN CYCLIC MARES

In the preliminary dosage determining trials, it was noted that both 10 and 25 mg per day of 5α-DHP were ineffective in inhibiting estrous behavior or ovulation. All four mares tested at these levels returned to estrus and ovulated, after receiving a PGF2α injection. The two mares that were treated with 50 mg of 5α-DHP remained in diestrus and did not ovulate until treatment was withdrawn. Consequently, the 50 mg level was chosen for further studies.

The subsequent testing involved injecting 50 mg of 5α-DHP during seven more mare cycles. These mares returned to estrus and ovulated after receiving a PGF2α injection. Four other mares were injected with 50 mg per day of 3β-5P, which did not inhibit estrus or ovulation either. Six mare cycles were used as PGF2α controls. These animals all returned to estrus within 2 to 4 d, and subsequently ovulated within 7 to 12 d. Two mares were injected with 50 mg P4 per day which inhibited estrus and ovulations until withdrawn. In addition two untreated control mares were included which had normal 21 d cycles.

In analyzing these data, the 5α-DHP and 3β-5P treated animals were compared to P4 or untreated mares, and PGF2α treated for eLH concentrations throughout the cycle (Figures 32a,b), eLH concentrations on the day of ovulation (Figure 33a), and days to ovulations (from d 6 postovulation, Figure 33b). The eLH concentrations were greater ($P < .01$) for the 5α-DHP (48.7 +/- 5.1 ng/ml), 3β-5P (68.1 +/- 7.5 ng/ml), and PGF2α (67.1 +/- 7.6 ng/ml) groups compared to the P4 and untreated controls (21.5 +/- 6.5 ng/ml). The eLH for the 5α-DHP, 3β-5P, and PGF2α groups were not significantly different.

Days to ovulation (Figure 33b), from the onset of treatment or d 6 postovulation, in the 5α-DHP group (14.8 +/- 1.2 days) and 3β-5P group (14.2 +/- 0.2 days) were not significantly greater than the PGF2α control mares (13.3 +/- .4 days). However, these three treatment groups had fewer days to ovulation ($P < .01$) than the P4 and untreated mares (20.8 +/- 1.1 days).

TABLE 1: Systematic and abbreviated names, retention times and ions monitored on GC/MS for each of the progestins.

Hormone Systematic Name (Abbreviated name)	Retention Time (min)	Ion Monitored
5α-pregnane-3,20-dione (5α-DHP)	11.03	343
	11.12	343
4-pregnene-3,20-dione (P4)	11.30	372
3β-hydroxy-5-pregnen-20-one (P5)	12.84	402
3β-hydroxy-5α-pregnan-20-one (3β-5P)	12.98	404
20α-hydroxy-5α-pregnan-3-one (20α-5P)	13.83	404
	13.99	404
5-pregnene-3β,20β-diol (P5-ββ)	16.46	489
5α-pregnane-3β,20β-diol (ββ-diol)	16.67	491
5α-pregnane-3β,20α-diol (βα-diol)	17.08	491

NOTES:
GC retention times for MO/TBDMS derivatives (30m X .25mm i.d. SE-30 column)
Most prominent ion - used for quantitation with selected ion mode
Doublet peaks are noted for 5α-DHP and 20α-5P

TABLE 2: Systematic and abbreviated names, retention times and ions monitored on GC/MS for each of the estrogens and an androgen.

Hormone Systematic Name (Abbreviated name)	Retention Time (min)	Ion Monitored
4-androstene-3,17-dione (AND)	8.95	344
3-hydroxy-1,3,5(10)-estratrien-17-one (E1)	10.41	356
1,3,5(10)-estratriene-3,17β-diol (E2)	13.52	443
1,3,5(10)-estratriene-3,16α,17β-triol (E3)	22.40	367

NOTES:
GC retention times for MO/TBDMS derivatives (30m X .25mm i.d. SE-30 column)
Most prominent ion - used for quantitation with selected ion mode

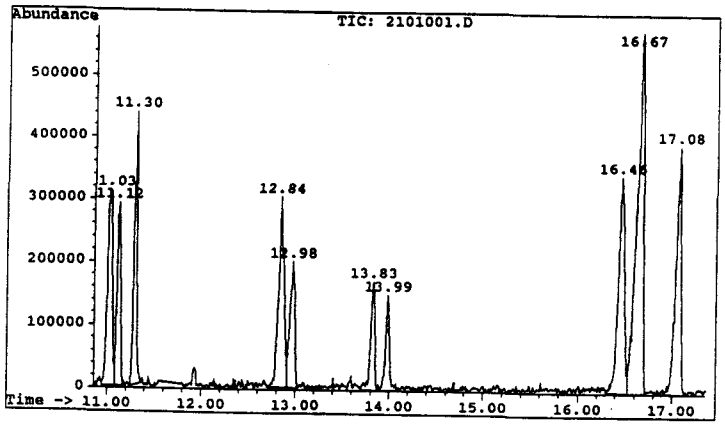
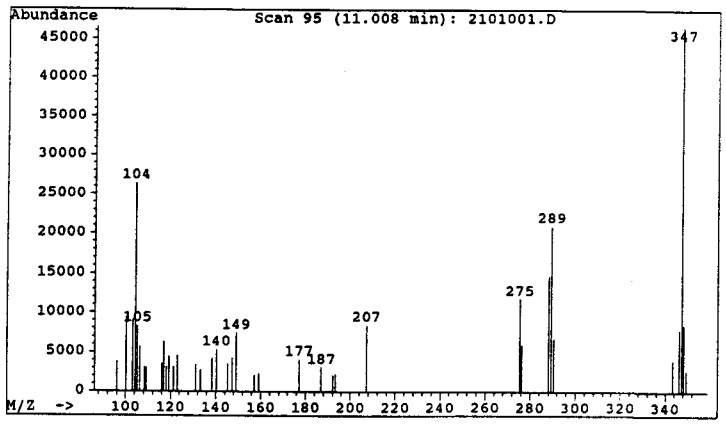
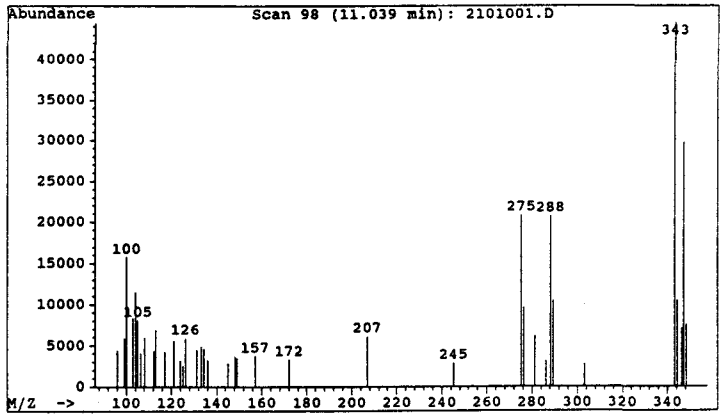


FIGURE 4: Total ion chromatogram of progestin standards on GC/MS full scan mode. Retention time is on the horizontal axis and abundance on the vertical axis. Major peaks indicated are: doublet 5α -DHP (11.03 & 11.12 min), P4 (11.30 min), P5 (12.84 min), 3β -5P (12.98 min), doublet 20α -5P (13.83 & 13.99 min), P5-β β (16.49 min), β β -diol (16.67 min), & β α -diol (17.08 min). See Table 1 for full systematic names.

a)



b)



c)

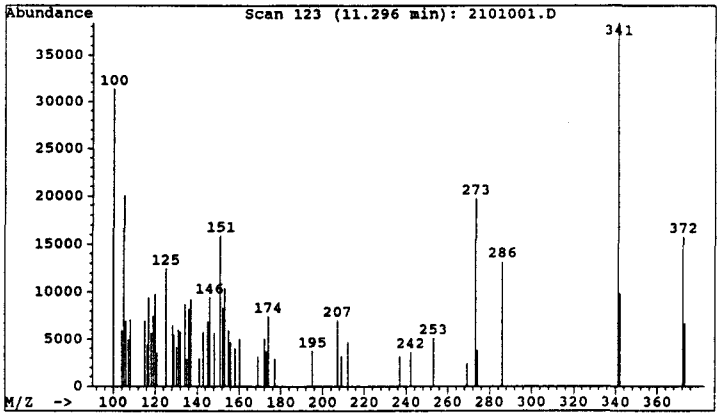
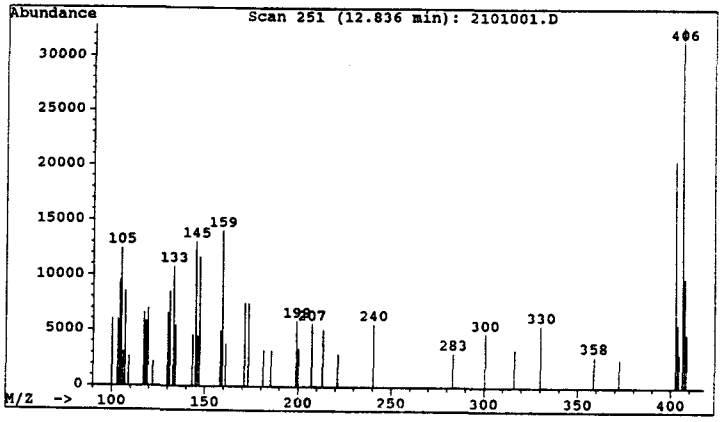


FIGURE 5: Full scans of a) D_4-5α -DHP, b) 5α -DHP, and c) P4. Major ions monitored are 347 for D_4-5α -DHP, 343 for 5α -DHP, and 372 for P4. Both 5α -DHP and P4 are quantified against D_4-5α -DHP. Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

a)



b)

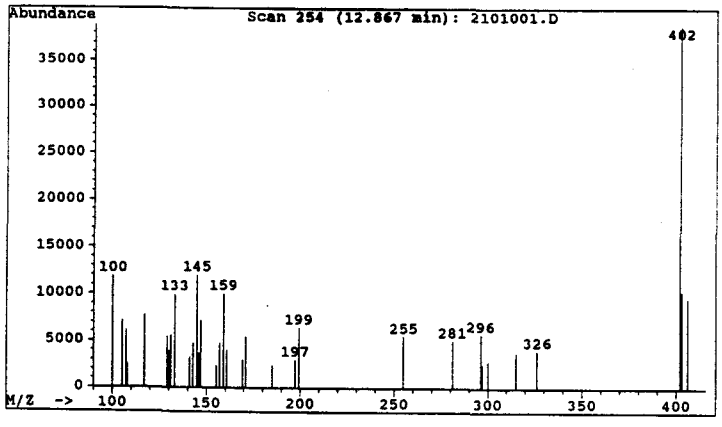
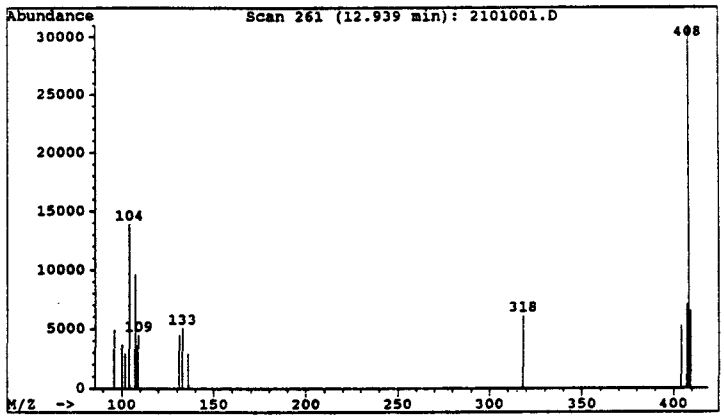
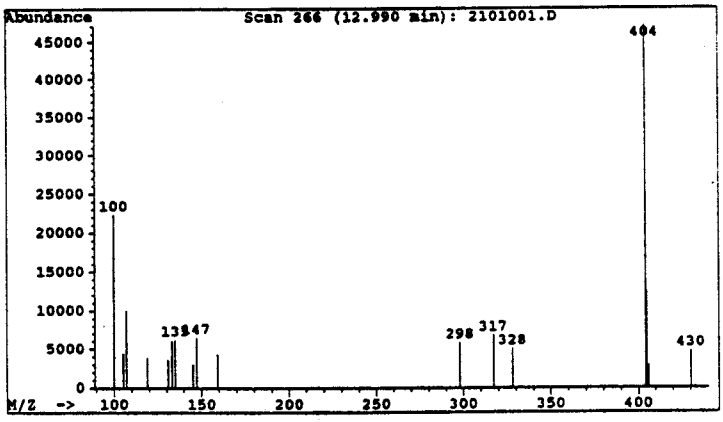


FIGURE 6: Full scans of a) D_4-P5 and b) P5. Major ions monitored are 406 for D_4-P5 and 402 for P5. P5 is quantified against D_4-P5. Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

a)



b)



c)

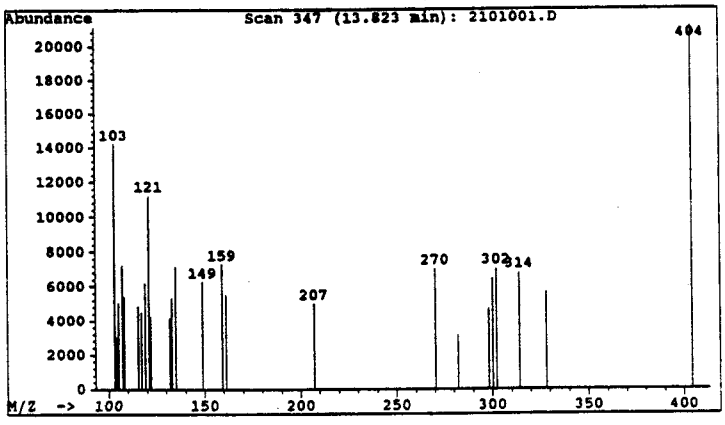


FIGURE 7: Full scans of a) D_4-3β -5P, b) 3β -5P, and c) 20α -5P. Major ions monitored are 408 for D_4-3β -5P, 404 for 3β -5P, and 404 for 20α -5P. Both 3β -5P and 20α -5P are quantified against D_4-3β -5P. Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

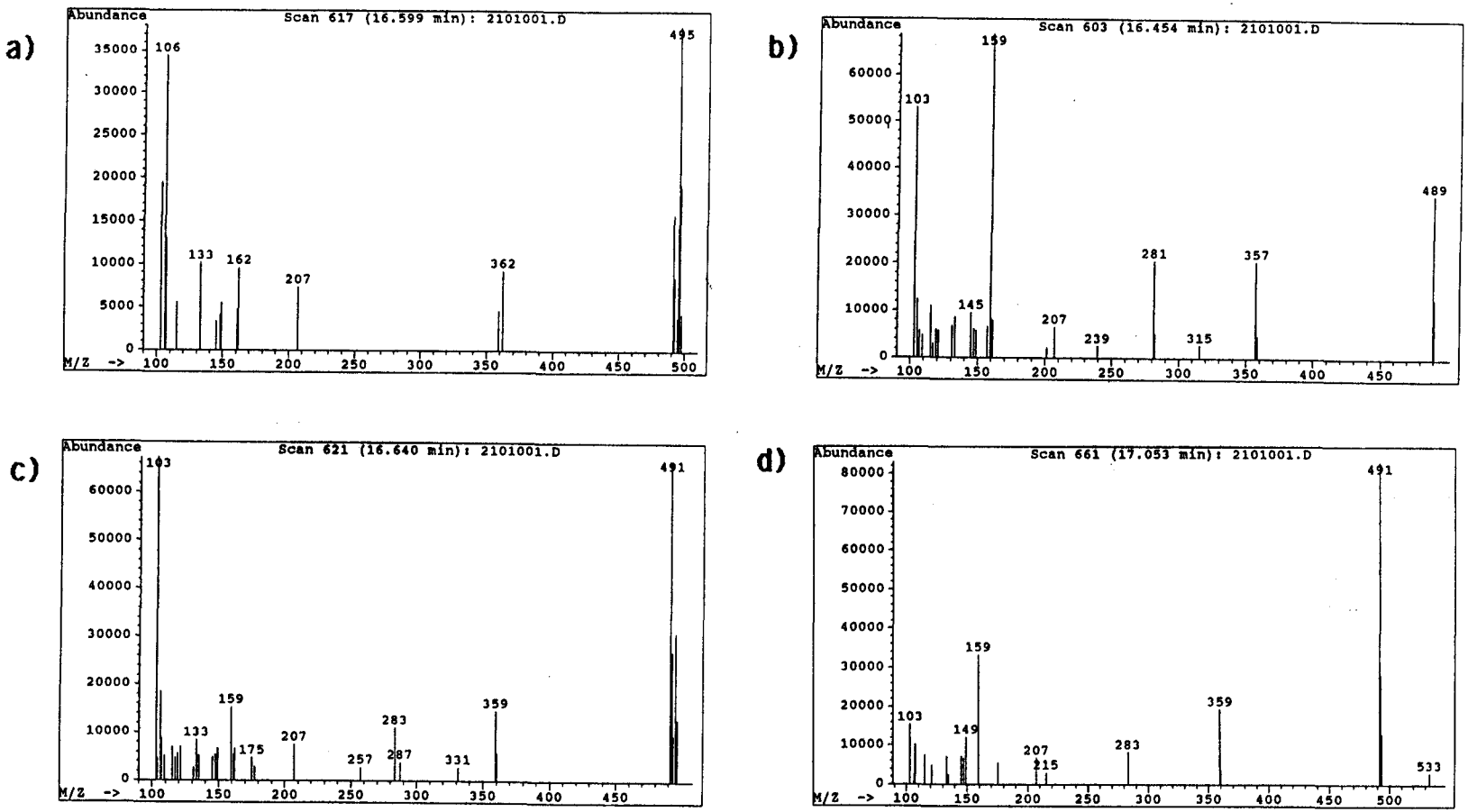
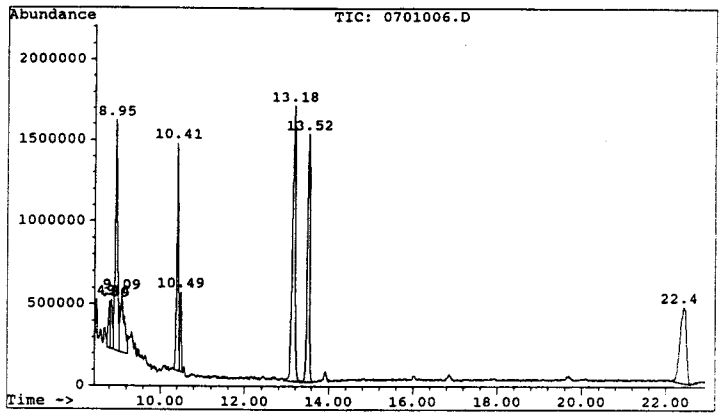


FIGURE 8: Full scans of a) D_4-β β -diol, b) P5-β β , c) β β -diol, & d) β α -diol. Major ions monitored are 495 for D_4-β β -diol, 489 for P5-β β , & 491 for both β β -diol and β α -diol. All three diols are quantified with D_4-β β -diol. Mass to charge ratio (m/z) is on the horizontal axis & intensity (abundance of ions) is on the vertical axis. See Table 1 for full names.

a)



b)

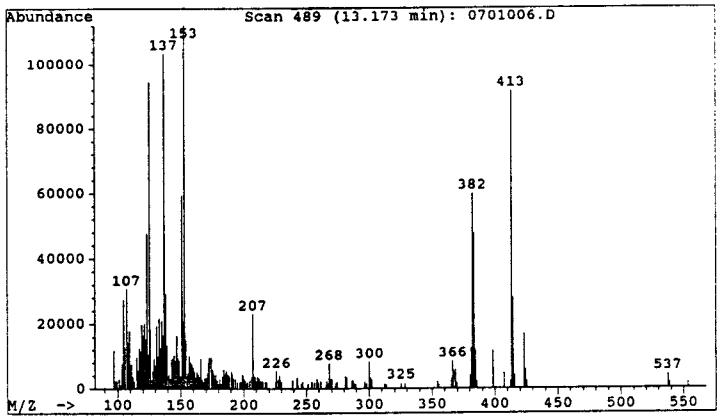


FIGURE 9: a) Total ion chromatogram of estrogen and androgen standards on GC/MS full scan mode and b) full scan of 4-cholesten-3-one. In a) retention time is on the horizontal axis and abundance on the vertical axis. Major peaks indicated are: AND (8.95 min), E1 (10.41 min), E2 (13.52 min), & E3 (22.40 min). The internal standard 4-cholesten-3-one is shown at 13.18 min. In b) a full scan of 4-cholesten-3-one is shown. The major ion monitored is 413. Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 2 for full systematic names.

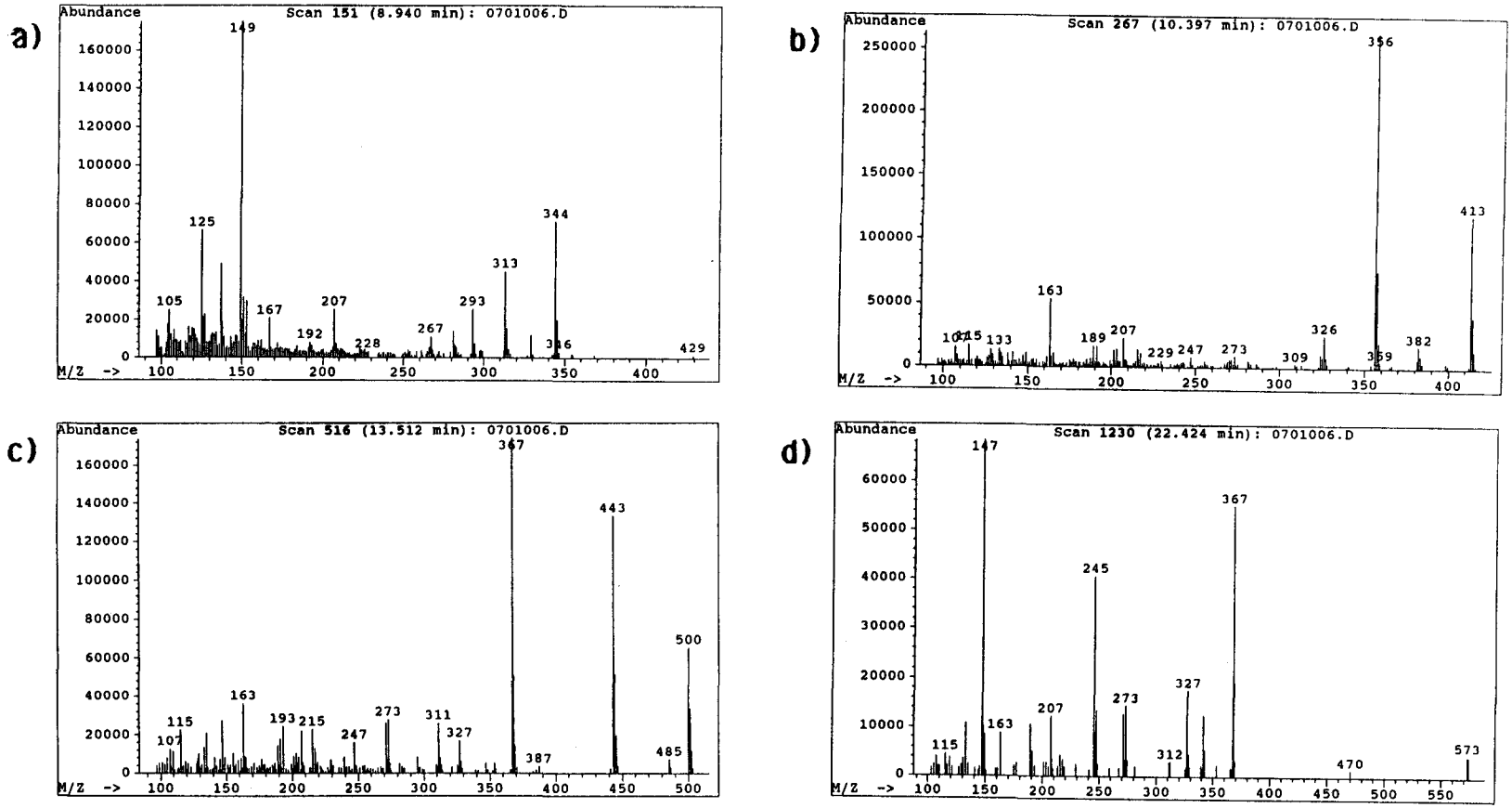


FIGURE 10: Full scans of a) AND, b) E1, c) E2, and d) E3. Major ions monitored are 344 for And, 356 for E1, 443 for E2, and 367 for E3. All four compounds are quantified against 4-cholesten-3-one. Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 2 for full systematic names.

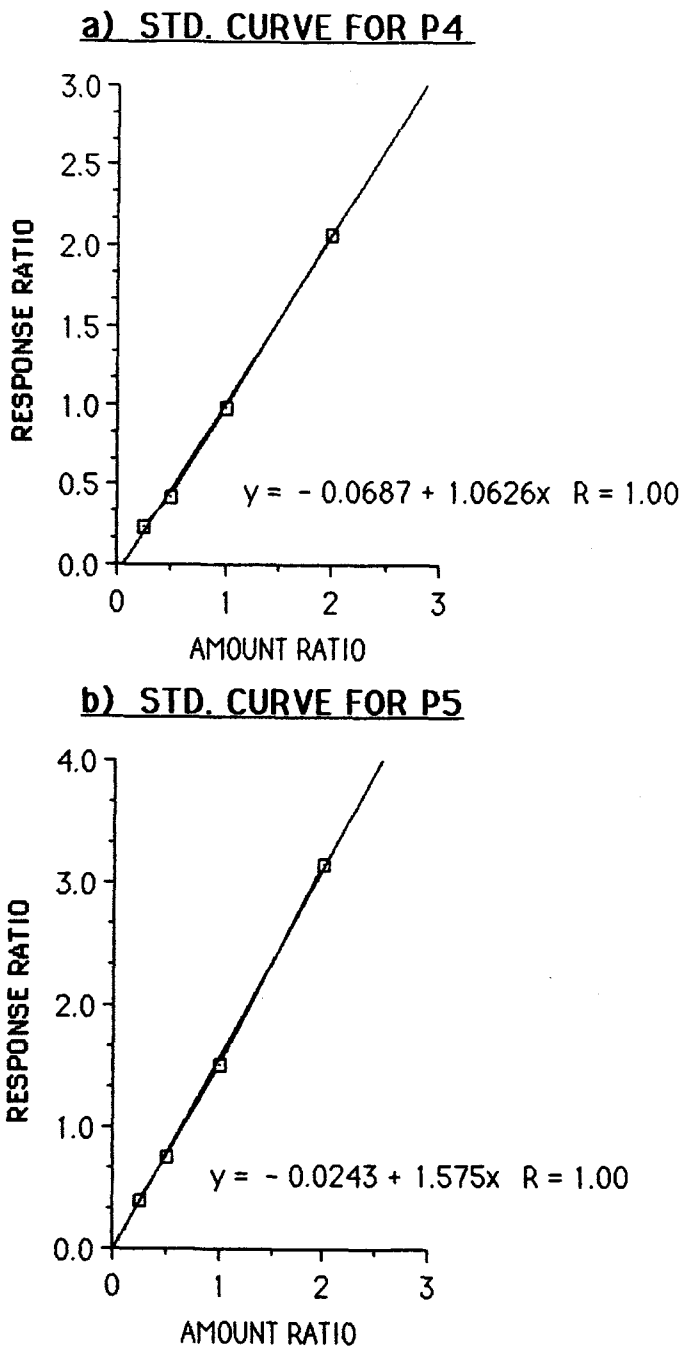
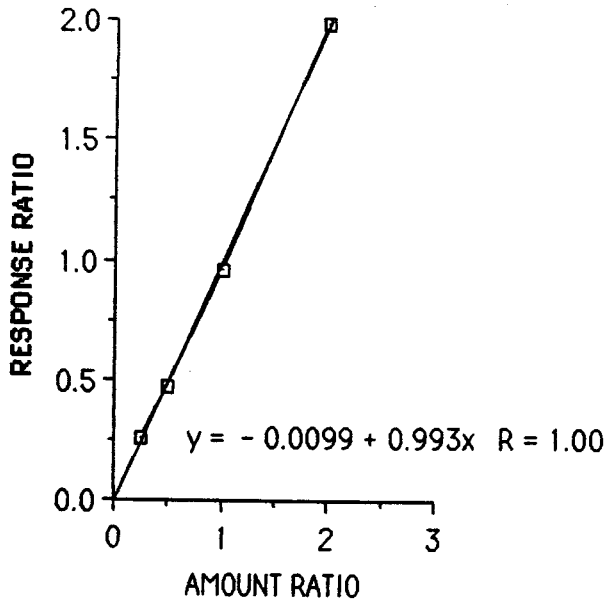


FIGURE 11: GC/MS standard curves for a) progestin-dione (P4) & b) mono-hydroxy-progestin (P5). Amount ratio (standard/ internal standard) is on the horizontal axis. Response ratio (standard/internal standard) is on the vertical axis. The slope, which varies for each compound, is indicated in the equation for the line. See Table 1 for full systematic names.

a) STD. CURVE FOR BB-DIOL



b) STD. CURVE FOR E2

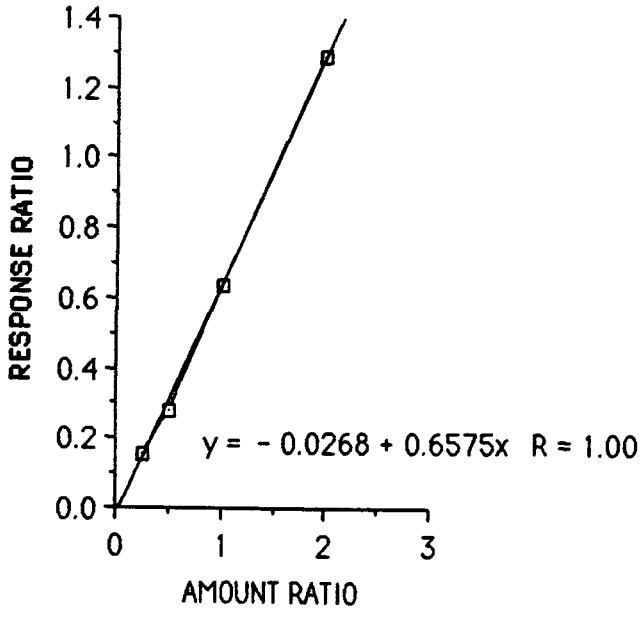


FIGURE 12: GC/MS standard curves for a) dihydroxy-progestin (β β -diol) & b) estrogen (E2). Amount ratio (standard/ internal standard) is on the horizontal axis. Response ratio (standard/internal standard) is on the vertical axis. The slope, which varies for each compound, is indicated in the equation for the line. See Table 1 & 2 for full systematic names.

TABLE 3: Experiment 1 GC/MS assay performance
Plasma samples with D_4-progestin internal standards extracted with Sep-Pak

Abbreviated Hormone Name (see Table 1 for full names)	**%Recovery (+/-SE)**	**Coefficient of Variation** Intra assay %	Inter assay %
5α-DHP	98.2 (3.9)	8.9	11.6
P4	93.2 (4.0)	9.4	11.7
P5	97.1 (4.0)	9.1	12.4
3β-5P	97.7 (4.0)	9.2	12.5
20α-5P	81.7 (4.3)	11.8	19.8
P5-ββ	99.5 (3.8)	8.5	13.0
ββ-diol	97.2 (3.8)	8.7	13.7
βα-diol	91.7 (3.9)	9.4	13.5

NOTES:
Mean % recovery (+/- standard error) of standards added to plasma, determined at 4 concentrations (25 to 200ng/ml) n=5 assays.
Coefficient of variation % (standards added to plasma at 4 concentrations over 9 assays).

TABLE 4: Experiment 2 GC/MS assay performance
Plasma samples with 4-cholesten-3-one internal standard ether extracted

Abbreviated Hormone Name (see Table 1 for full names)	**%Recovery (+/- SE)**	**Coefficient of Variation** Intra assay %	Inter assay %
5α-DHP	84.2 (6.4)	15.3	18.3
P4	87.7 (6.5)	14.7	23.3
P5	87.1 (6.5)	14.8	18.1
3β-5P	106.4 (6.9)	12.9	16.9
20α-5P	115.2 (6.9)	11.9	21.4
P5-ββ	109.9 (7.1)	12.9	20.5
ββ-diol	108.0 (7.5)	13.9	19.1
βα-diol	99.2 (7.5)	15.1	20.8

NOTES:
Mean % recovery (+/- standard error)of standards added to plasma, determined at 4 concentrations (25 to 200ng/ml) n=5 assays.
Coefficient of variation % (standards added to plasma at 4 concentrations over 5 assays).

TABLE 5: Experiments 3 & 4 GC/MS assay performance

Medium samples with 4-cholesten-3-one internal standard extracted with Sep-Pak

Abbreviated Hormone Name (see Table 1 for full names)	**%Recovery (+/- SE)**	**Coefficient of Variation** Intra assay %	Inter assay %
5α-DHP	102.4 (3.8)	7.4	16.1
P4	98.3 (3.8)	7.7	17.1
P5	105.0 (4.0)	7.7	13.4
3β-5P	102.7 (4.1)	8.1	16.4
20α-5P	99.3 (4.2)	8.5	22.4
P5-ββ	110.7 (4.3)	7.9	18.6
ββ-diol	105.6 (4.2)	8.0	20.1
βα-diol	106.8 (4.2)	7.8	17.4

NOTES:
Mean % recovery (+/- standard error)of standards added to medium, determined at 4 concentrations (25 to 200ng/ml) n=5 assays.
Coefficient of variation % (standards added to medium at 5 concentrations over 7 assays).

TABLE 6: Experiment 4 GC/MS Assay Performance

Medium samples with 4-cholesten-3-one internal standard, extracted with Sep-Pak.

Abbreviated Hormone Name (see Table 2 for full names)	**% Recovery**	**Coefficient of Variation** Intra assay %	Inter assay %
AND	72.9	9.6	10.6
E_1	86.4	7.9	8.6
E_2	92.5	7.4	7.6
E_3	109.9	4.0	4.1

NOTES:
Mean % recovery of standards added to medium, determined at 4 concentrations from 25 to 200ng/ml, n=2 assays.
Coefficients of variation % (standards added to medium at 4 concentrations over 2 assays).

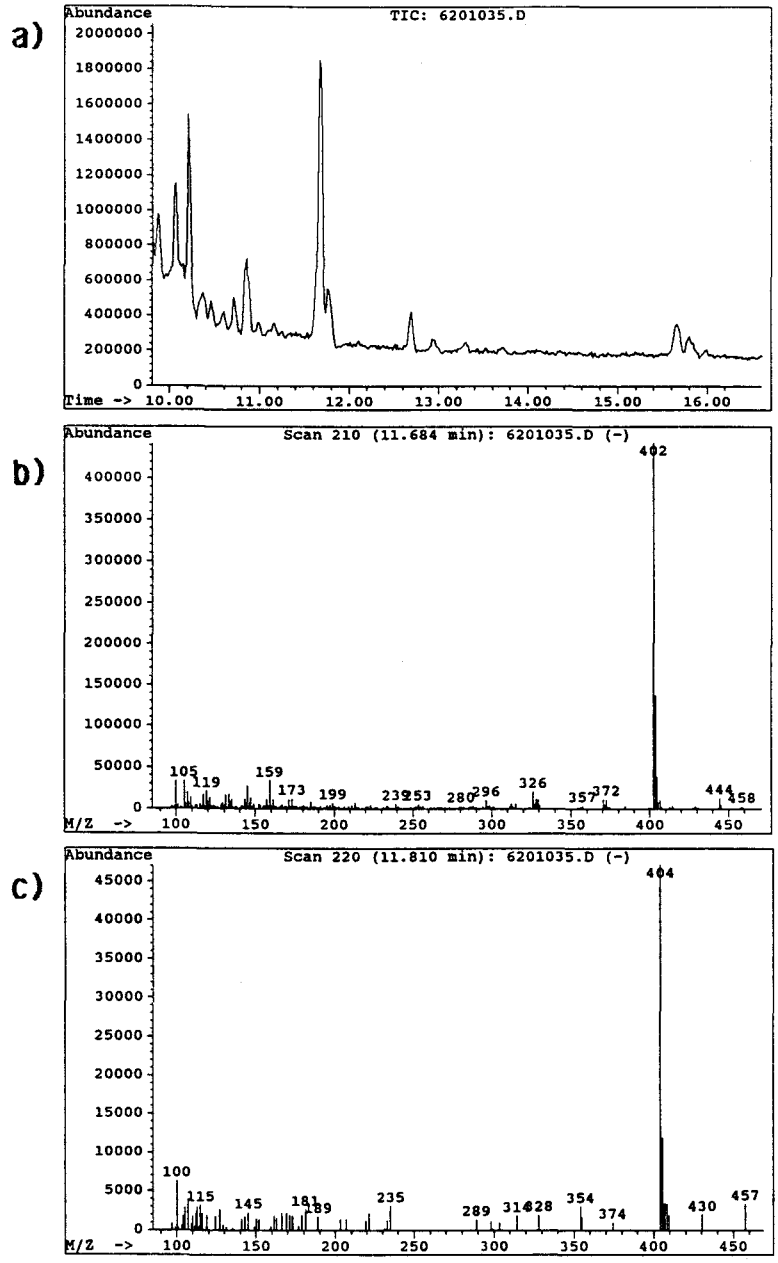


FIGURE 13: a) Total ion chromatogram & two full scans b) P5 and c) 3β -5P from a fetal artery plasma sample. In a) retention time is on the horizontal axis and abundance on the vertical axis. The P5 peak is at 11.6 min and the 3β -5P peak is at 11.8 min. In b) & c) mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

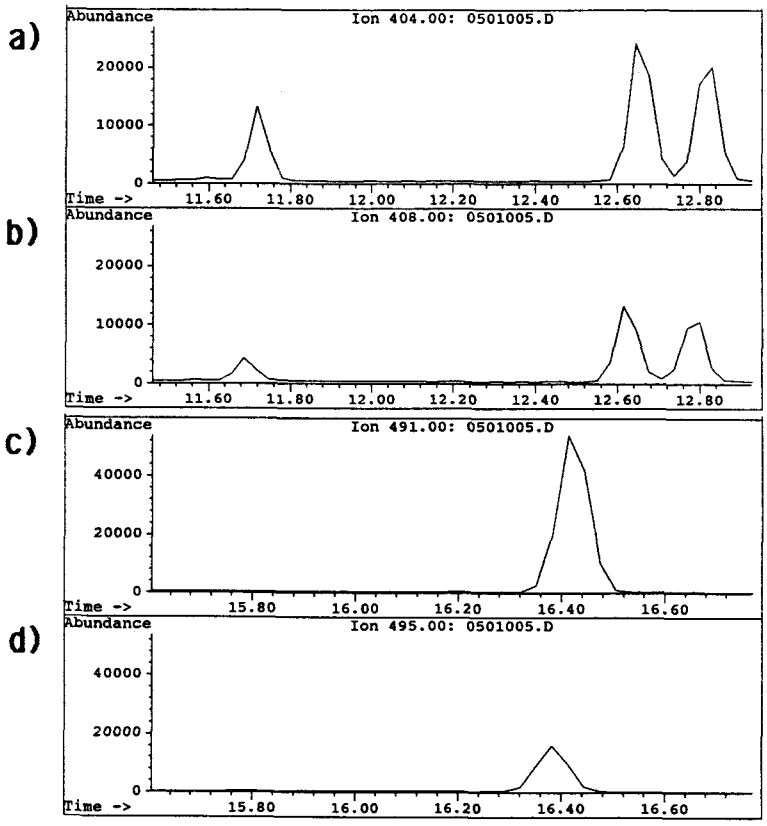


FIGURE 14: Ion chromatograms from an uterine vein sample after D_4-P5 infusion to the fetal artery. In a) both a single 3β -5P peak and doublet 20α -5P peaks have the 404 ion. In b) the corresponding D_4-labelled 3β -5P peak and doublet 20α -5P peaks with the 408 ion have retention times slightly ahead of the unlabelled compounds in a). In c) the single β α -diol peak is shown with the 491 ion. In d) D4-β α -diol is shown with the 495 ion. See Table 1 for full systematic names.

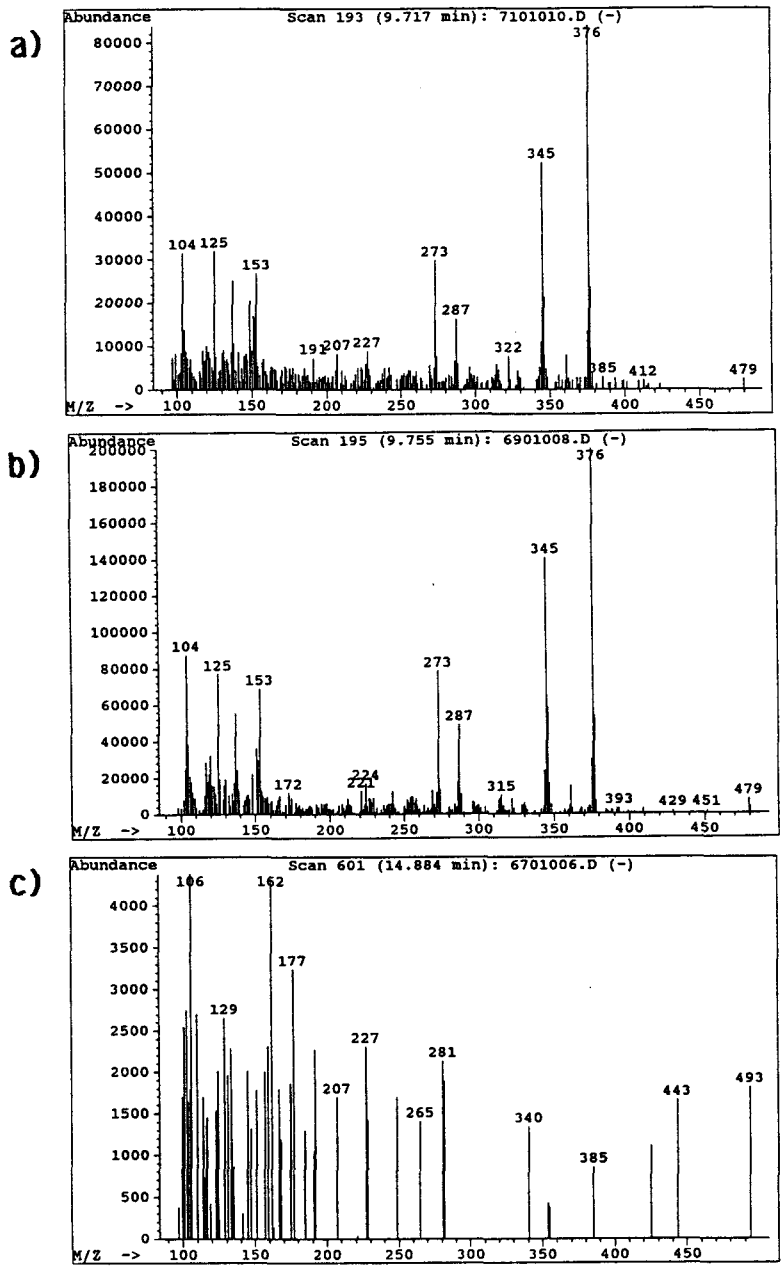


FIGURE 15: Full scans of incubation medium from a) D_4-P4 in uterine endometrium, b) D_4-P4 in placenta, and c) D_4-P5-β β in fetal liver. Note the differences in major ions compared to the regular standards (see Figures 5c & 8b). Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

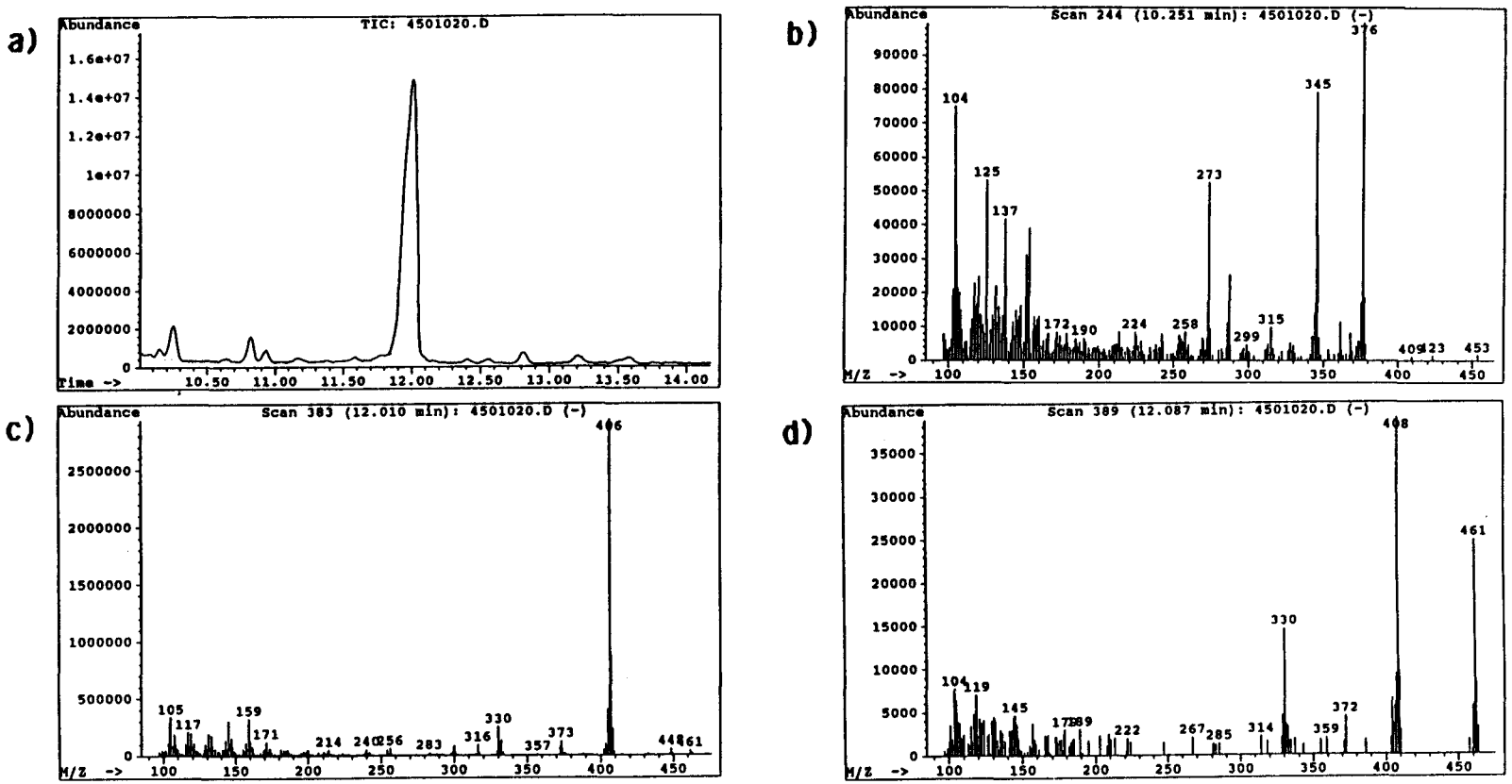


FIGURE 16: a) Total ion chromatogram & three full scans of b) D_4-P4, c) D_4-P5, & d) D_4-3β -5P from an embryo incubated with D_4-P5. In a) retention time is on the horizontal axis and abundance is on the vertical axis. The precursor D_4-P5 peak is at 12.01 min, the D_4-P4 peak is at 10.2 min, and the D_4-3β -5P peak is at 12.08 min. In b), c), & d) mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 for full systematic names.

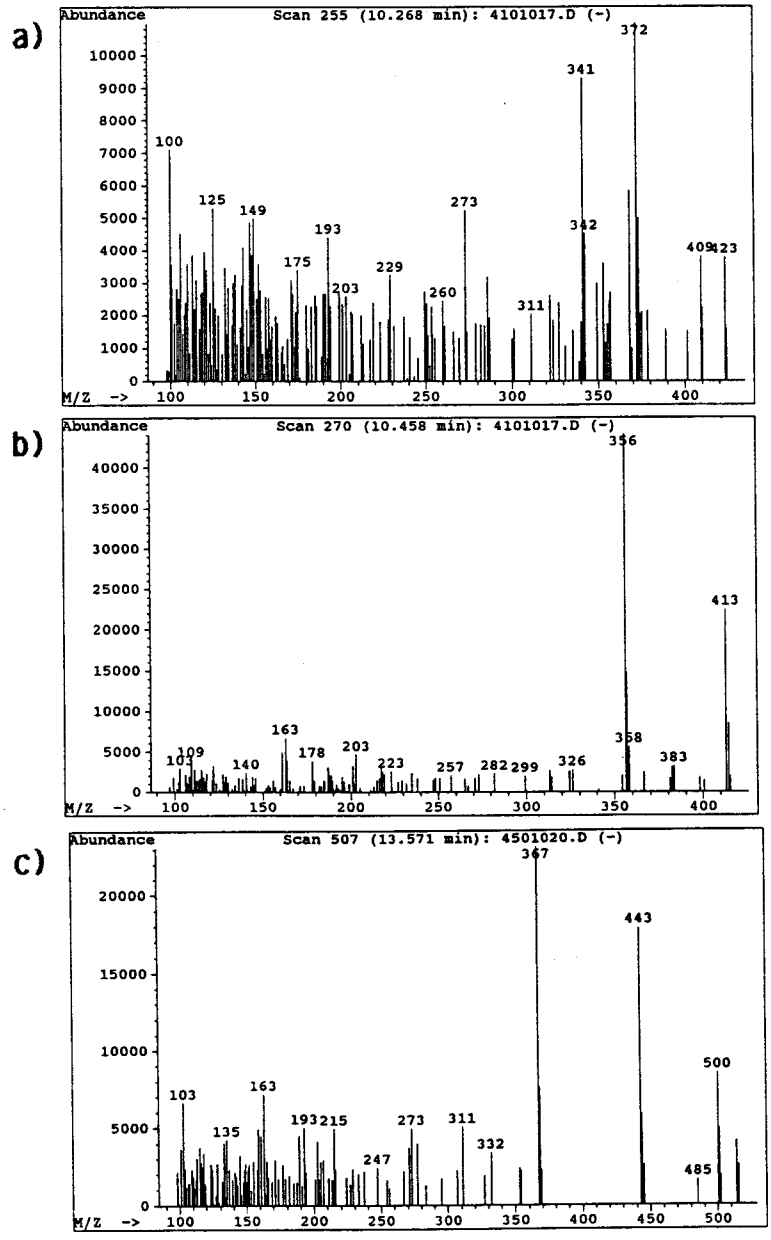
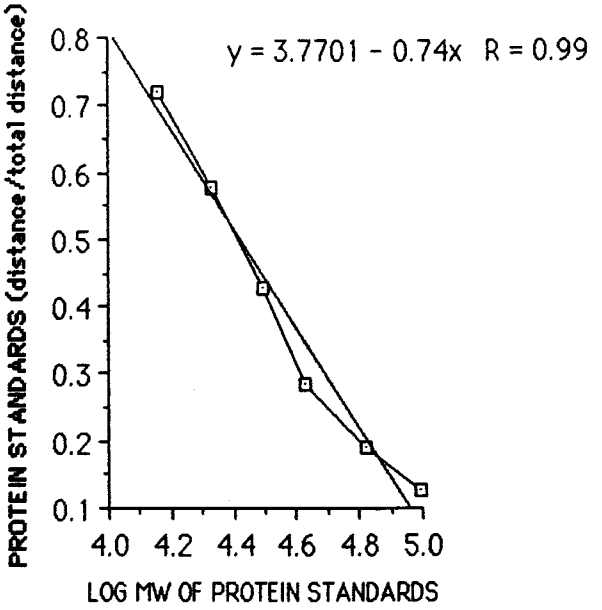


FIGURE 17: Full scans of steroids in embryo incubation medium a) P4, b) E1, and c) E2. Note the major ions are similar to regular standards (see Figures 5c & 10b,c). Mass to charge ratio (m/z) is on the horizontal axis and intensity (abundance of ions) is on the vertical axis. See Table 1 & 2 for full systematic names.

a) STD. CURVE FOR SDS PAGE



b) STD. CURVE FOR eLH EIA

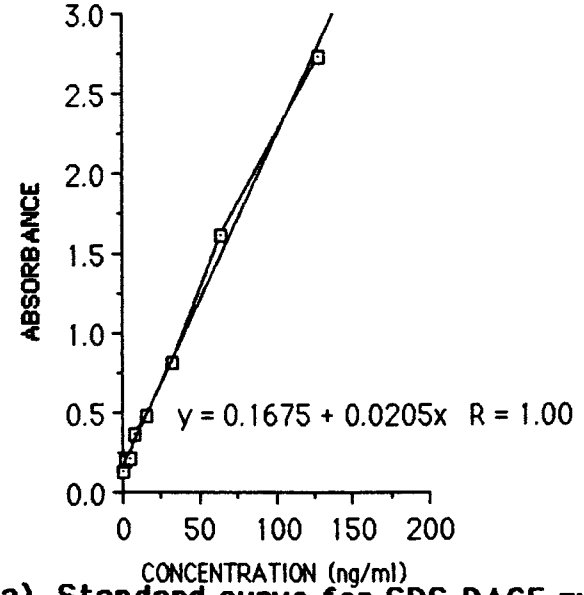


FIGURE 18: a) Standard curve for SDS PAGE zymograph & b) standard curve for eLH EIA. In a) LOG of molecular weight (MW) of protein standards is on the horizontal axis. Distance (cm) each protein migrated/total distance is on the vertical axis. Unknown protein mass was calculated by solving "x" in the equation of the line. In b) concentration of standards (ng/ml) is on the horizontal axis and absorbance at 405 nm is on the vertical axis. Unknown concentrations were determined by solving "x" in the equation of the line.

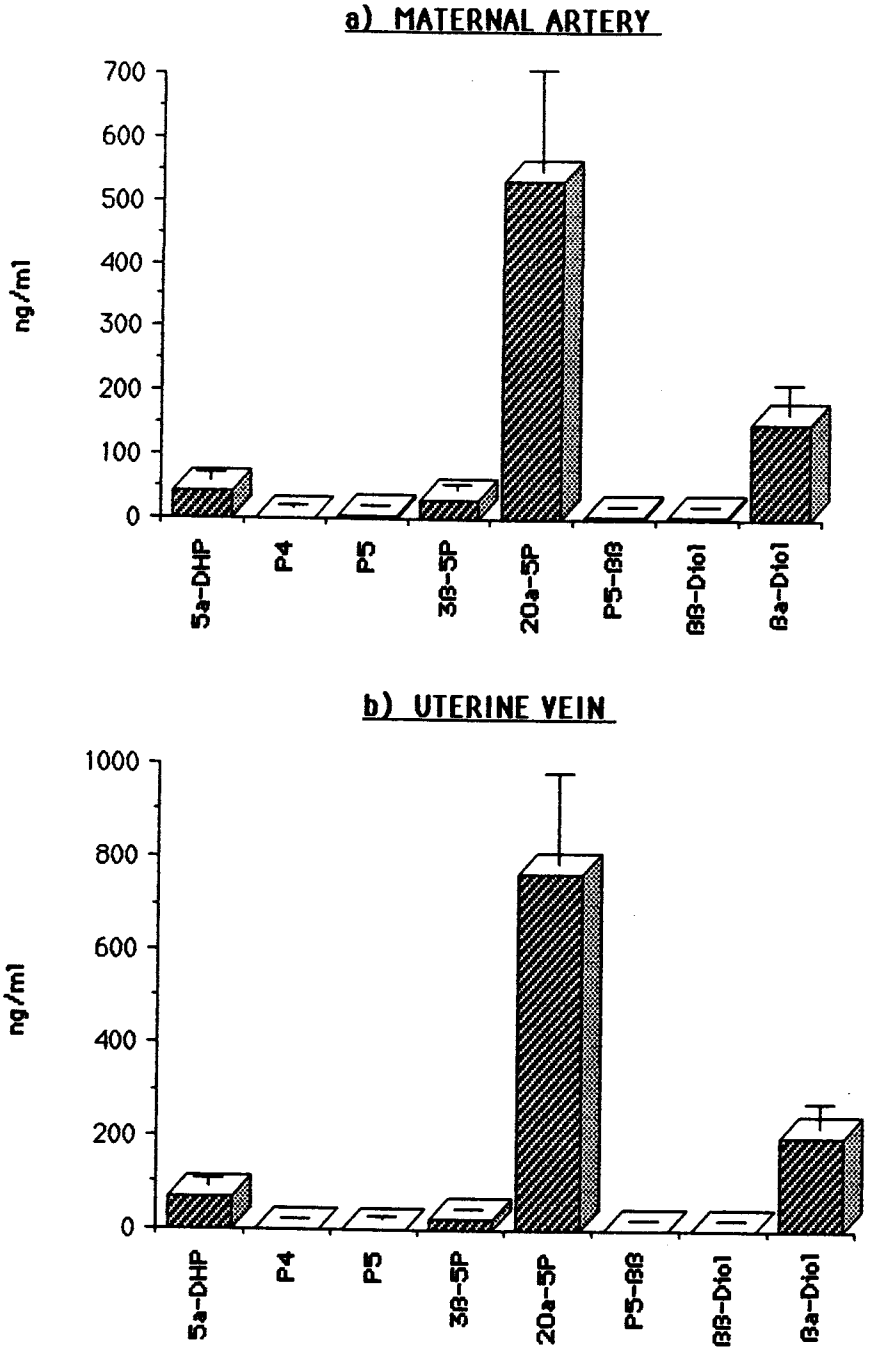


FIGURE 19: Mean concentrations of progestins determined by GC/MS for maternal vessels in the six pregnant mares studied in Experiment 1. Figure a) is maternal artery concentrations and figure b) is maternal vein levels. Each progestin is indicated on the horizontal axis and concentrations (ng/ml) are shown on the vertical axis. Standard error bars are included. See Table 1 for full systematic names.

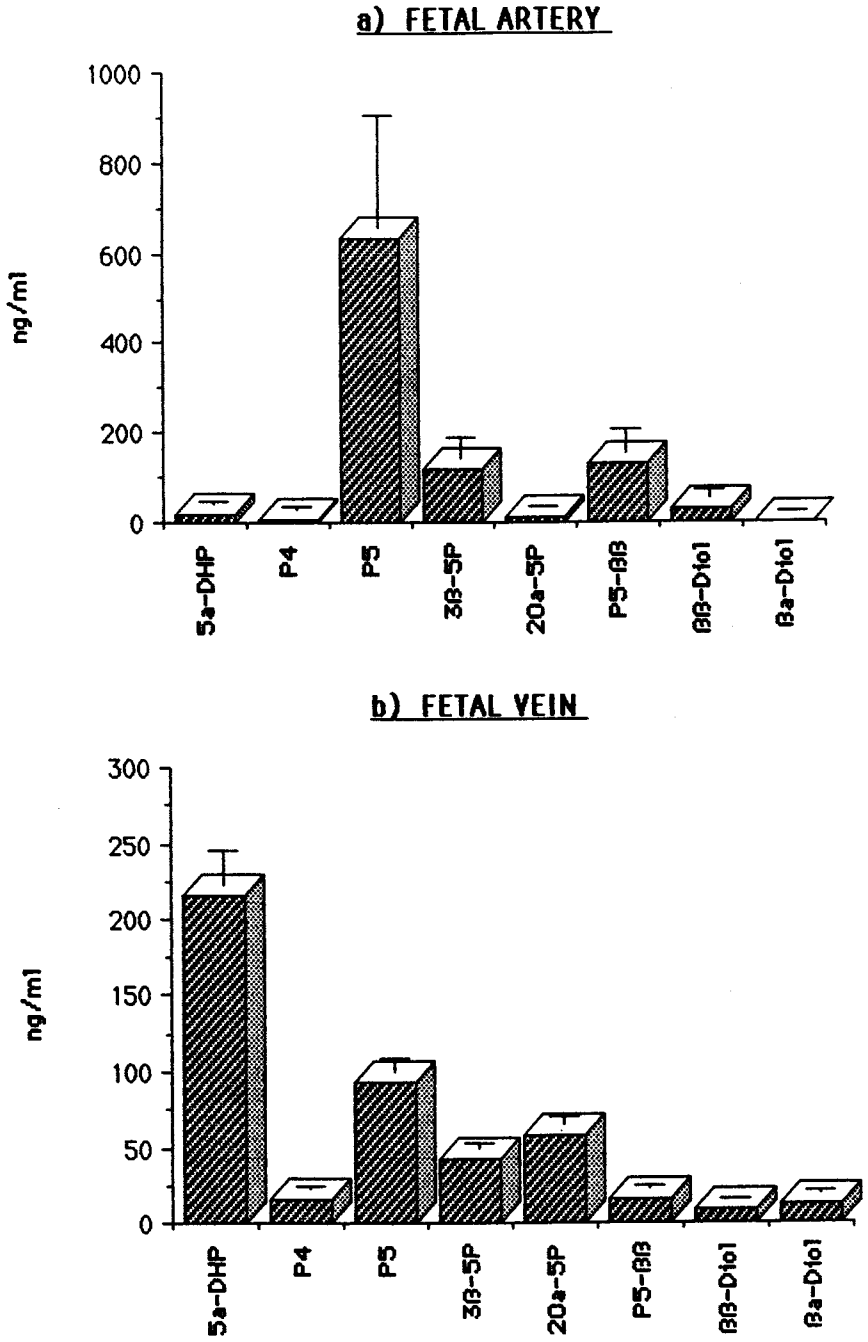


FIGURE 20: Mean concentrations of progestins determined by GC/MS for fetal vessels in the six pregnant mares studied in Experiment 1. Figure a) is fetal artery concentrations and figure b) is fetal vein levels. Each progestin is indicated on the horizontal axis and concentrations (ng/ml) are shown on the vertical axis. Standard error bars are included. See Table 1 for full systematic names.

TABLE 7: Progestins at 50% inhibition (2.5 ng/ml) or greater in the P4 EIA at each concentration level. Each progestin listed under the three actual concentrations inhibited the binding of P4 to the P4 antibody by at least 50%. This was equivalent to 2.5 ng/ml of P4 in this competitive EIA.

Concentration of Hormone		
1000 ng/ml	100 ng/ml	10 ng/ml
5α -DHP	5α -DHP	5α -DHP
P4	P4	P4
P5	P5	
3β - 5P	3β - 5P	
20α - 5P		

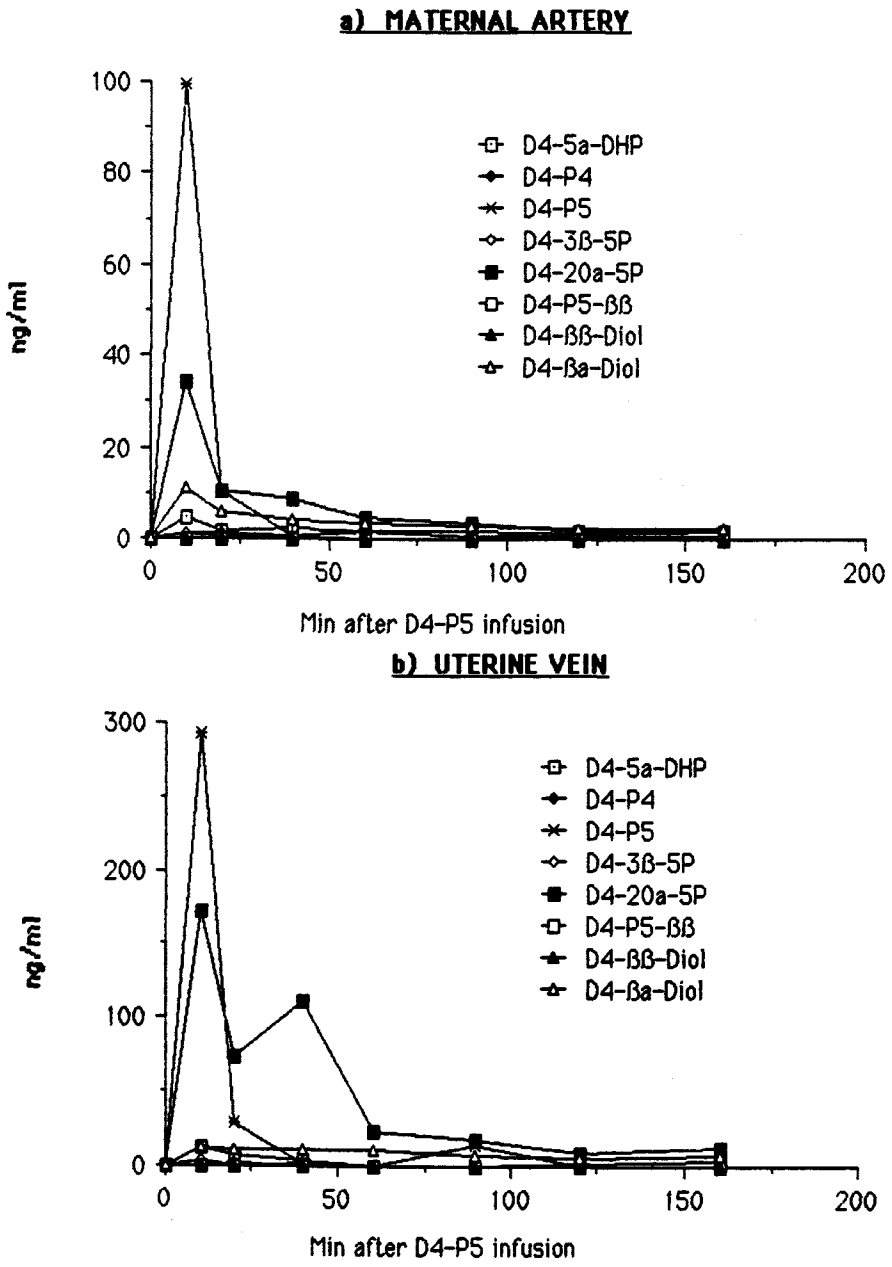


FIGURE 21: Mean concentrations of D_4-progestins over time determined by GC/MS for maternal vessels in the two pregnant mares studied in Experiment 2. Figure a) is maternal artery concentrations & figure b) is maternal vein levels. Each progestin is indicated in the legend. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis and time in min on the horizontal axis.

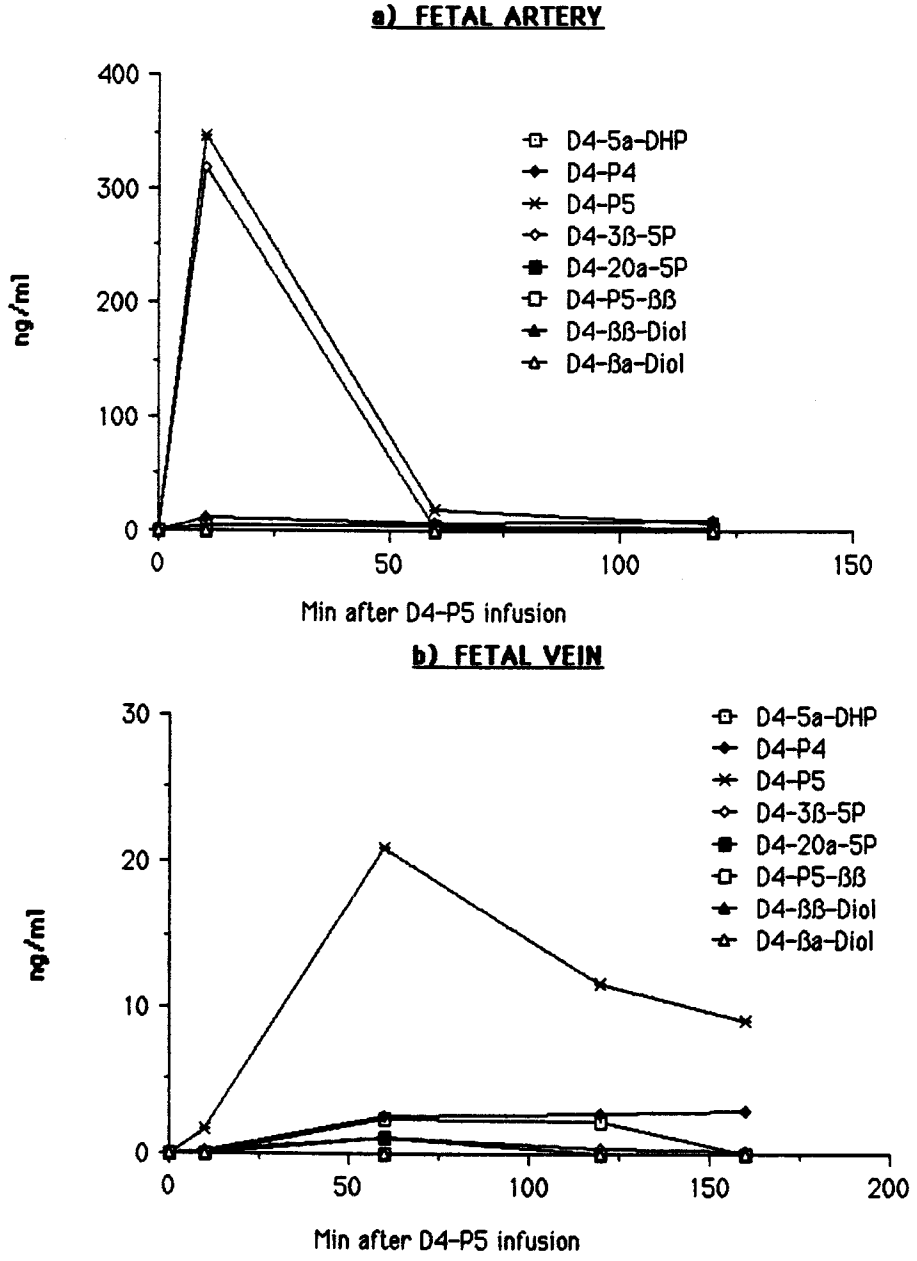


FIGURE 22: Mean concentrations of D_4-progestins over time determined by GC/MS for fetal vessels in the two pregnant mares studied in Experiment 2. Figure a) is fetal artery concentrations & figure b) is fetal vein levels. Each progestin is indicated in the legend. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis and time in min on the horizontal axis.

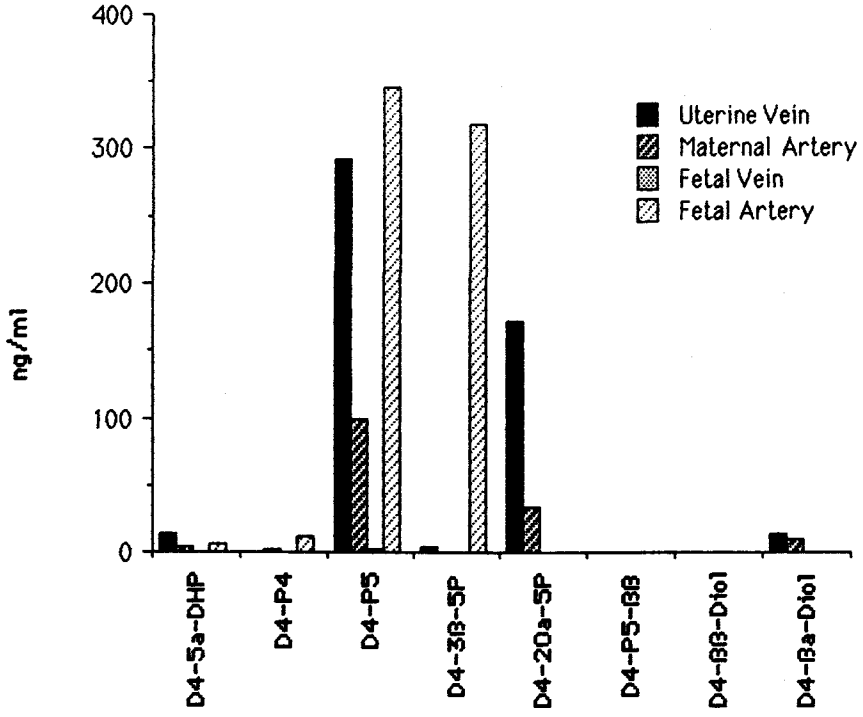


FIGURE 23: Mean concentrations of D_4-progestins determined by GC/MS for the 10 min samplings from the two pregnant mares infused with D_4-P5 in Experiment 2. Each hormone is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis. The sources of samples are indicated in the legend.

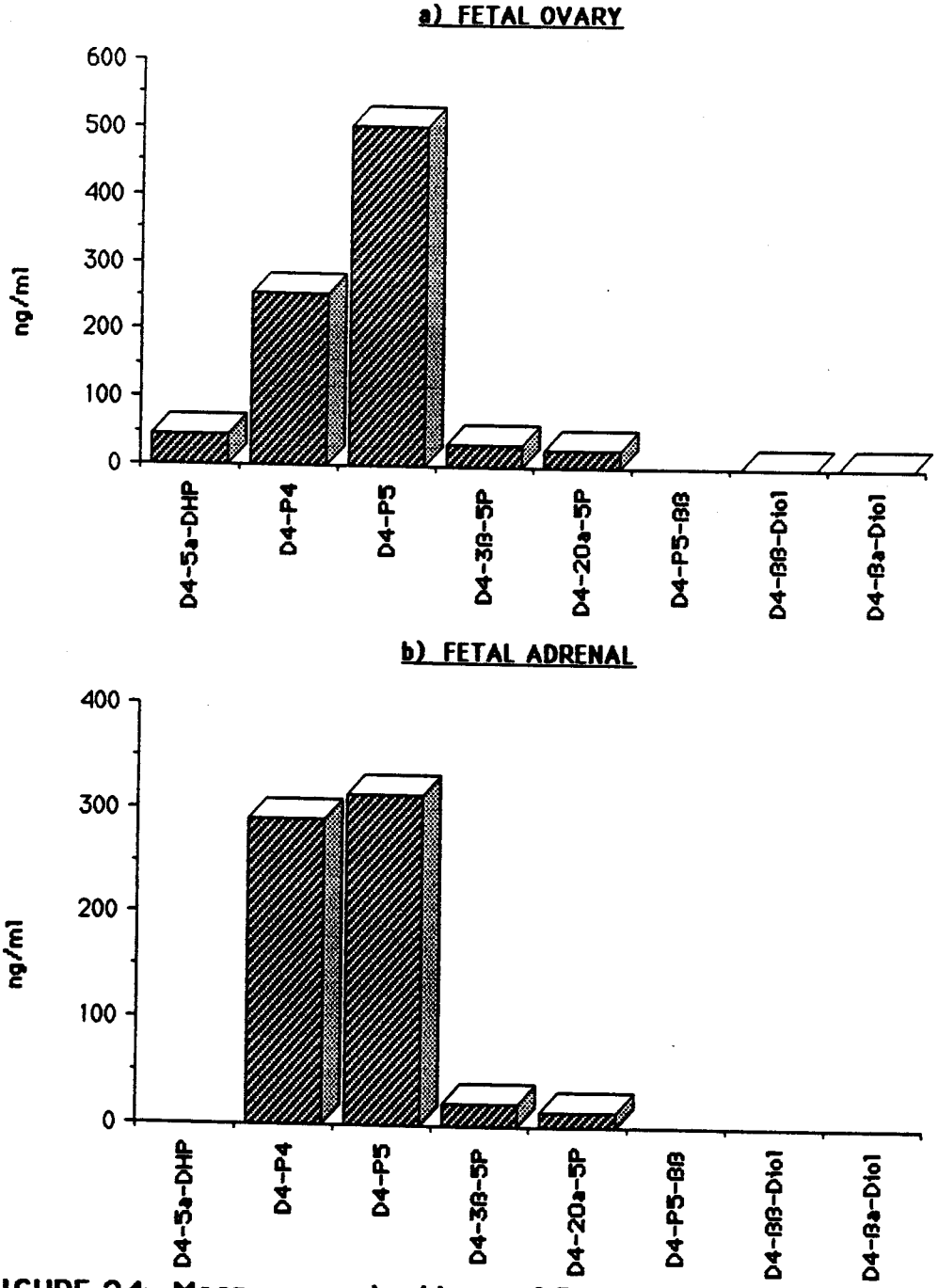


FIGURE 24: Mean concentrations of D_4-progestins determined by GC/MS for a) fetal ovary & b) fetal adrenal in Experiment 3. Each progestin is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis. (n=2)

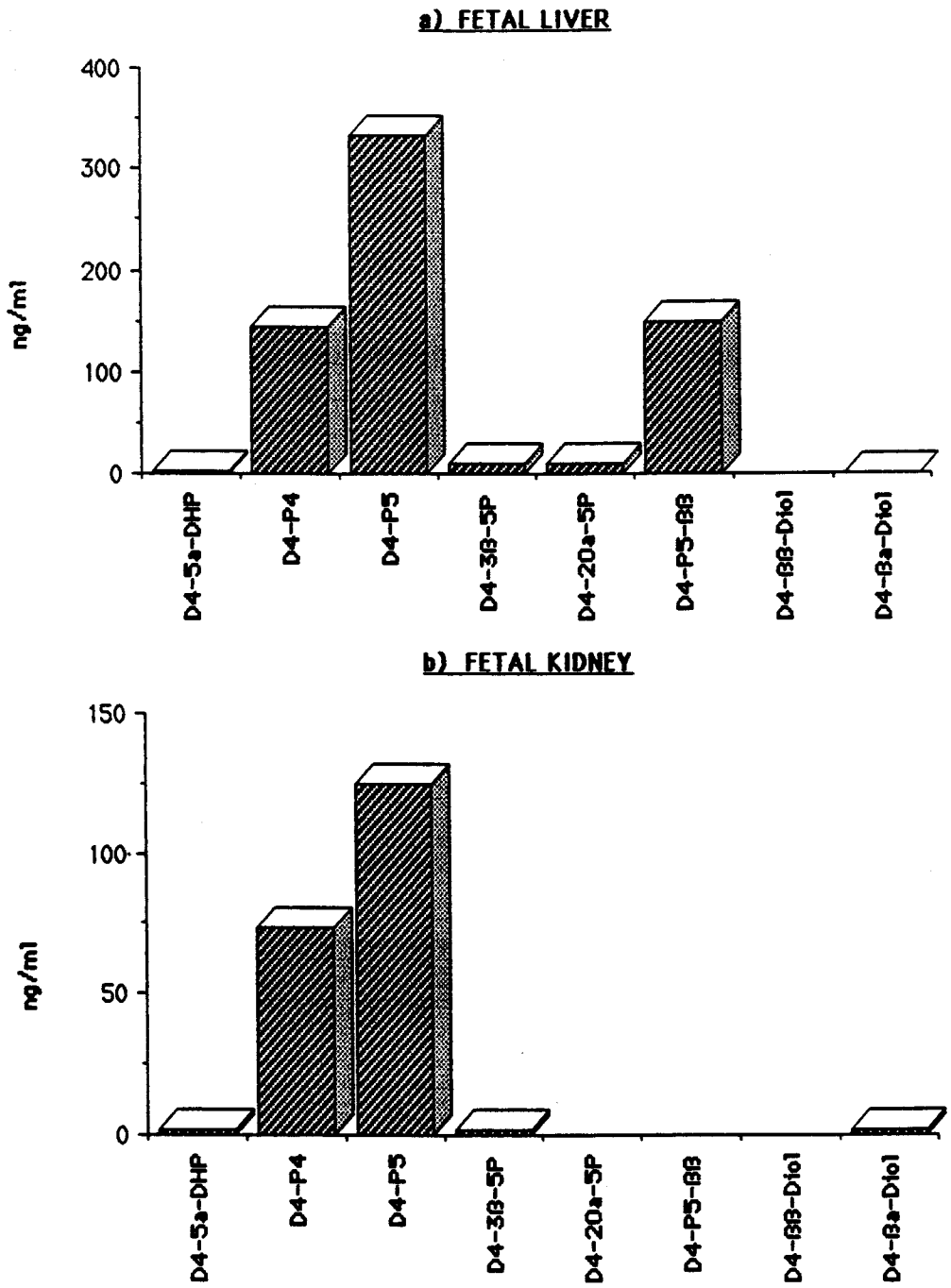


FIGURE 25: Mean concentrations of D_4-progestins determined by GC/MS for a) fetal liver & b) fetal kidney in Experiment 3. Each progestin is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis. (n=2)

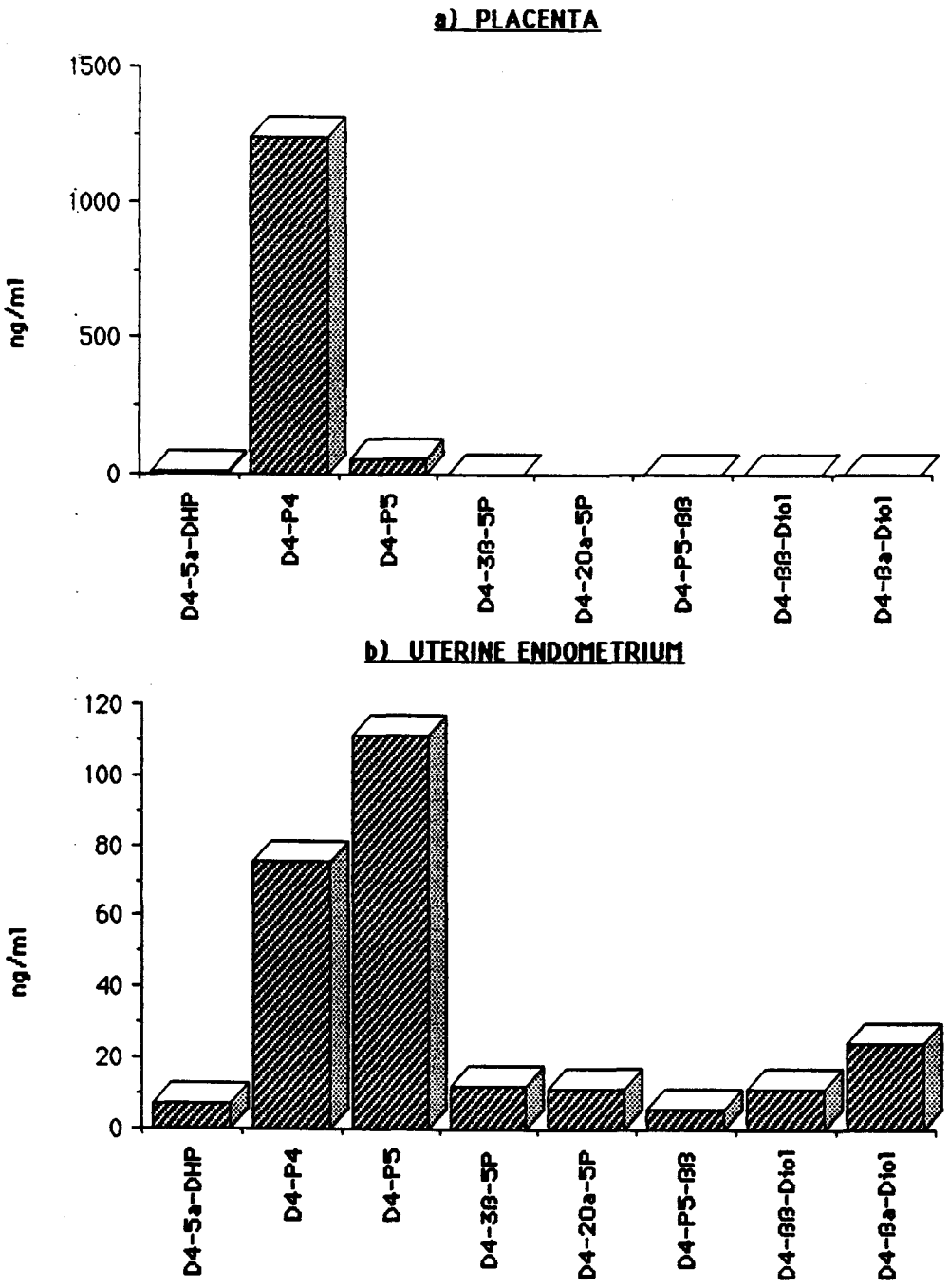


FIGURE 26: Mean concentrations of D_4-progestins determined by GC/MS for a) placenta & b) uterine endometrium in Experiment 3. Each progestin is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis. (n=2)

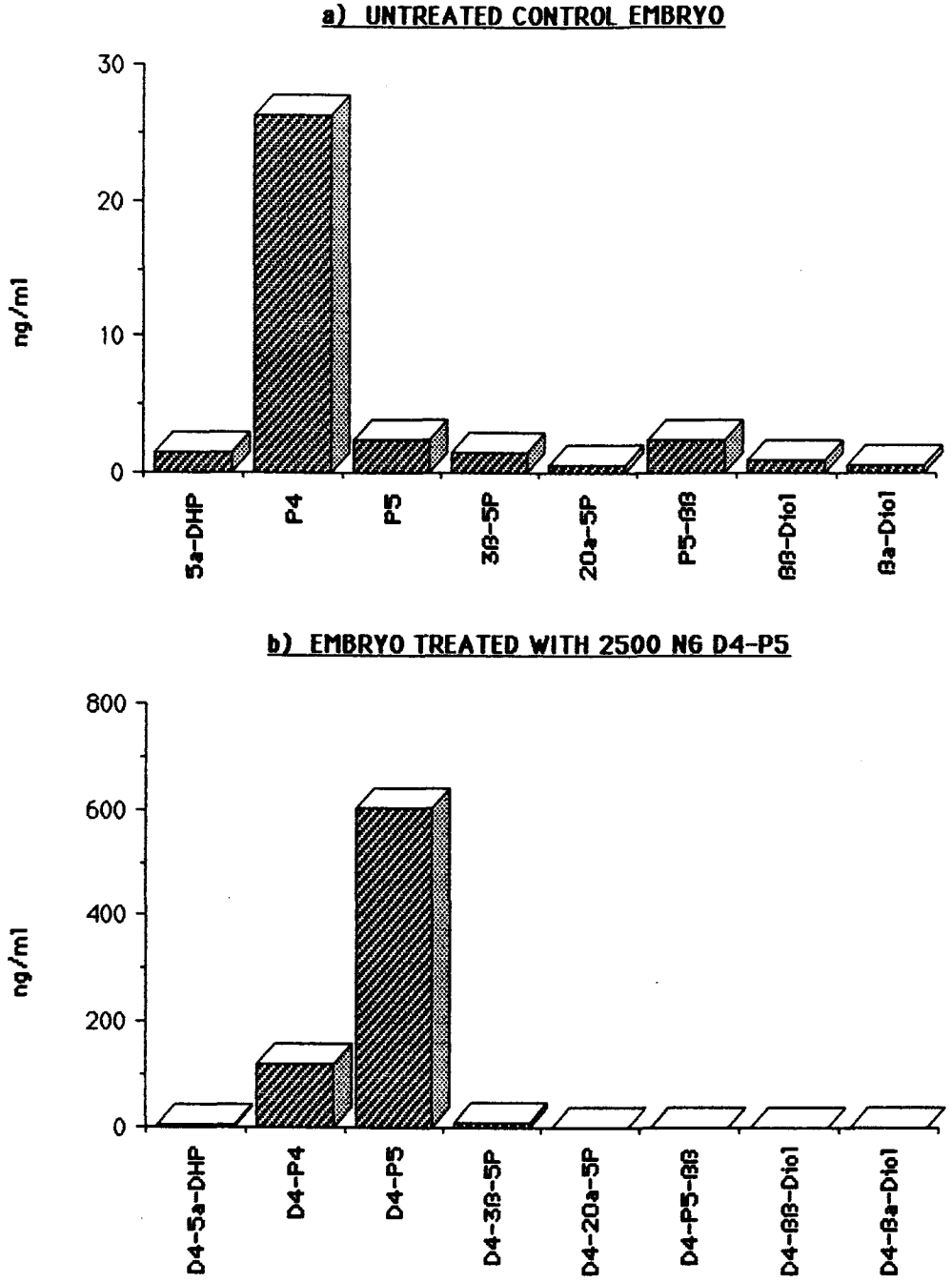


FIGURE 27: Concentrations of progestins determined by GC/MS for a) an untreated embryo & b) an embryo incubated with 2500 ng of D_4-P5 in Experiment 4. Each progestin is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis.

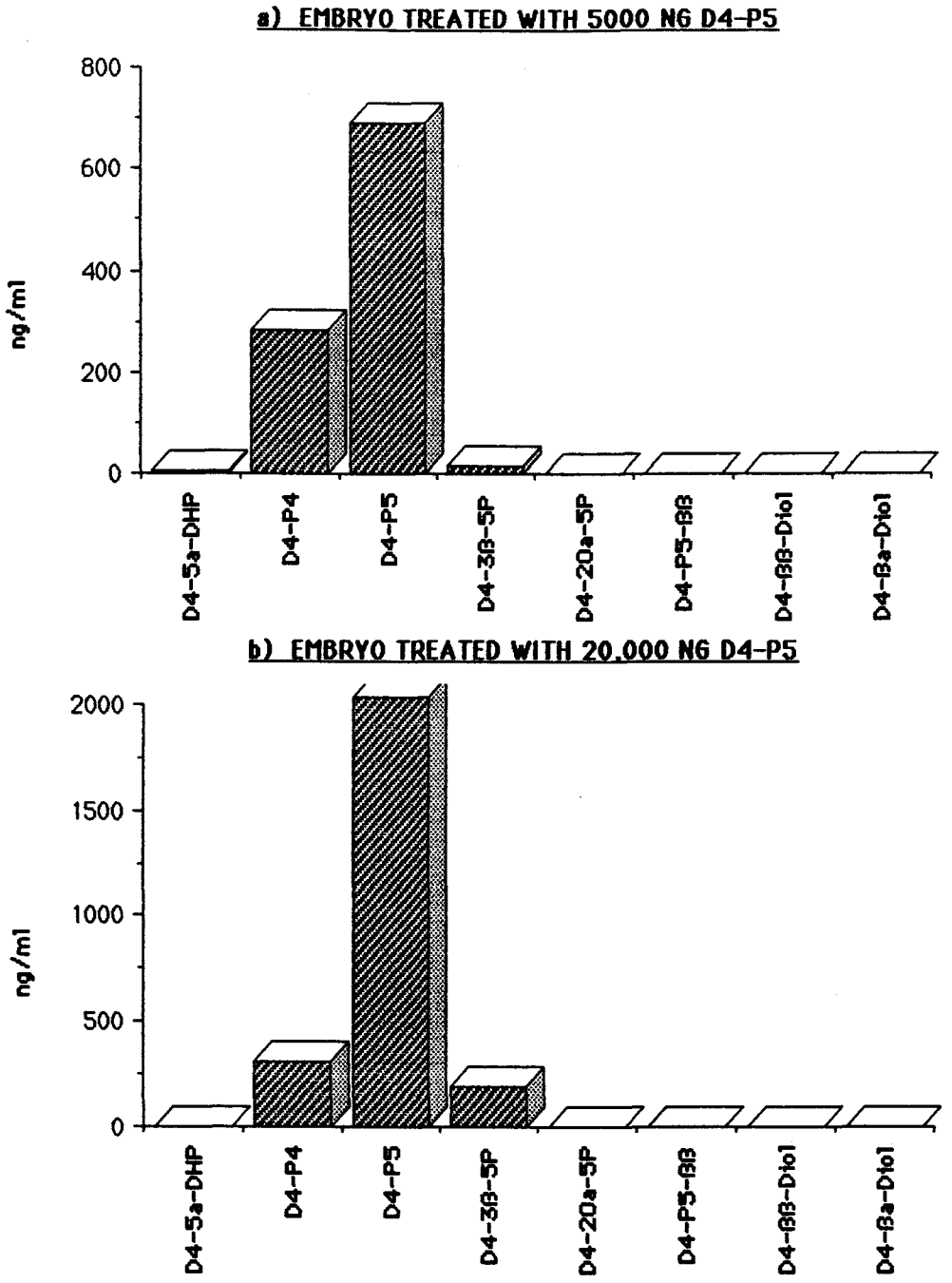


FIGURE 28: Concentrations of progestins determined by GC/MS for a) an embryo incubated with 5000 ng D_4-P5 & b) an embryo incubated with 20000 ng of D_4-P5 in Experiment 4. Each progestin is indicated on the horizontal axis. See Table 1 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis.

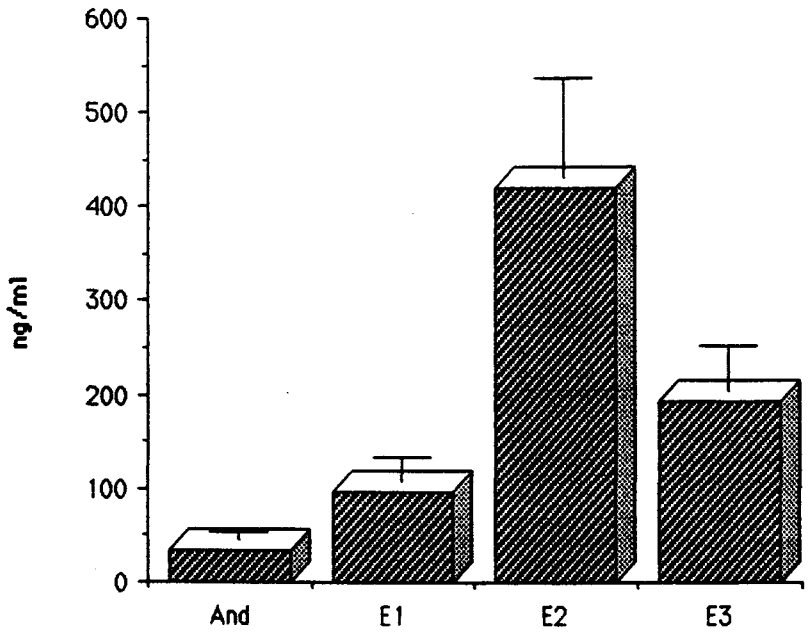


FIGURE 29: Mean concentrations of estrogens and an androgen determined by GC/MS for five embryos in Experiment 4. Each hormone is indicated on the horizontal axis. See Table 2 for full systematic names. Concentrations (ng/ml) are shown on the vertical axis. Standard error bars are included.

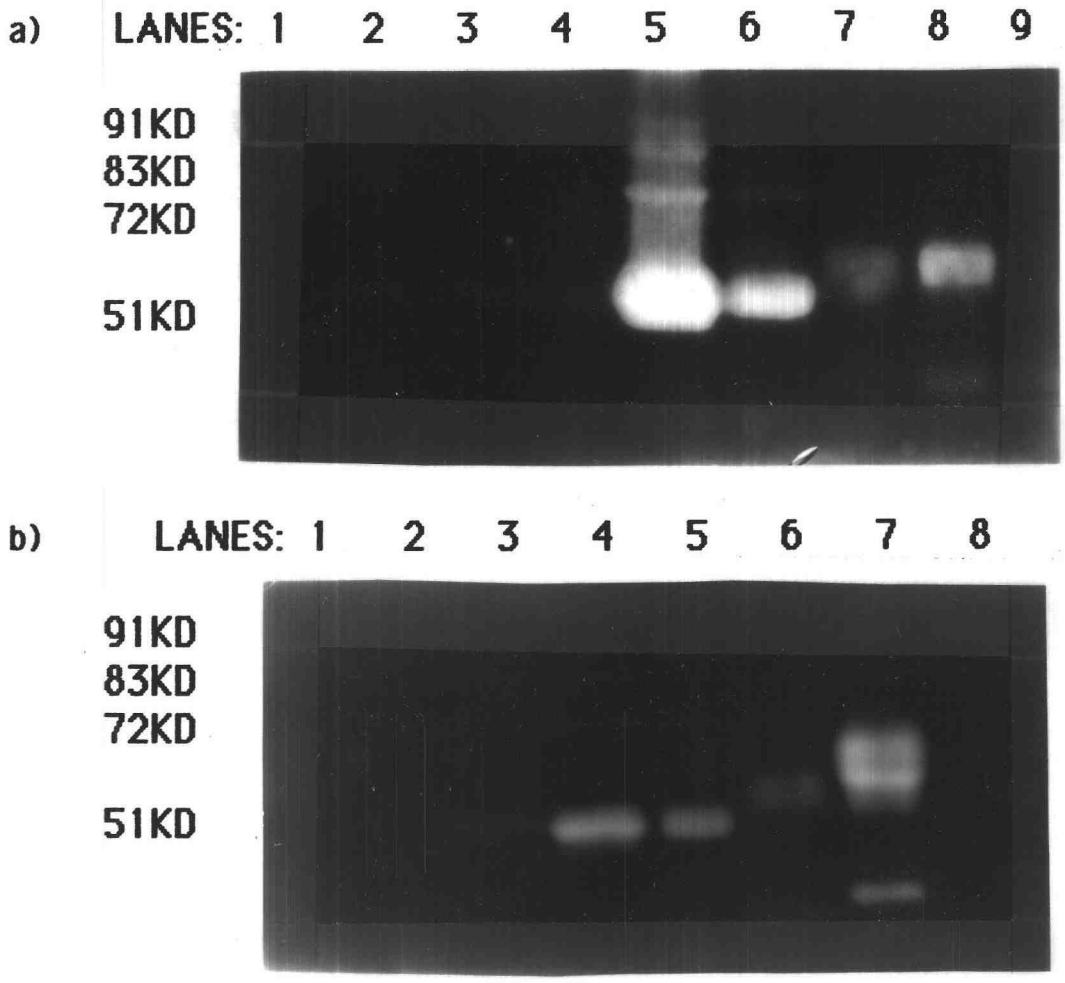


FIGURE 30: SDS PAGE Zymographs of equine embryo medium and tissue samples from Experiment 4. Lanes are indicated across the top and molecular mass (Kilo-daltons) of the protein bands are shown on the left of the photograph. In a) lane 1 is a medium blank, lanes 2 to 4 are medium samples, lanes 5 & 6 are embryo tissue samples, lanes 7 & 8 are plasminogen activator standards, and lane 9 has the protein standards. In b) lane 1 is a medium blank, lanes 2 & 3 are medium samples, lanes 4 & 5 are embryo tissue samples, lanes 6 & 7 are plasminogen activator standards, and lane 8 has the protein standards.

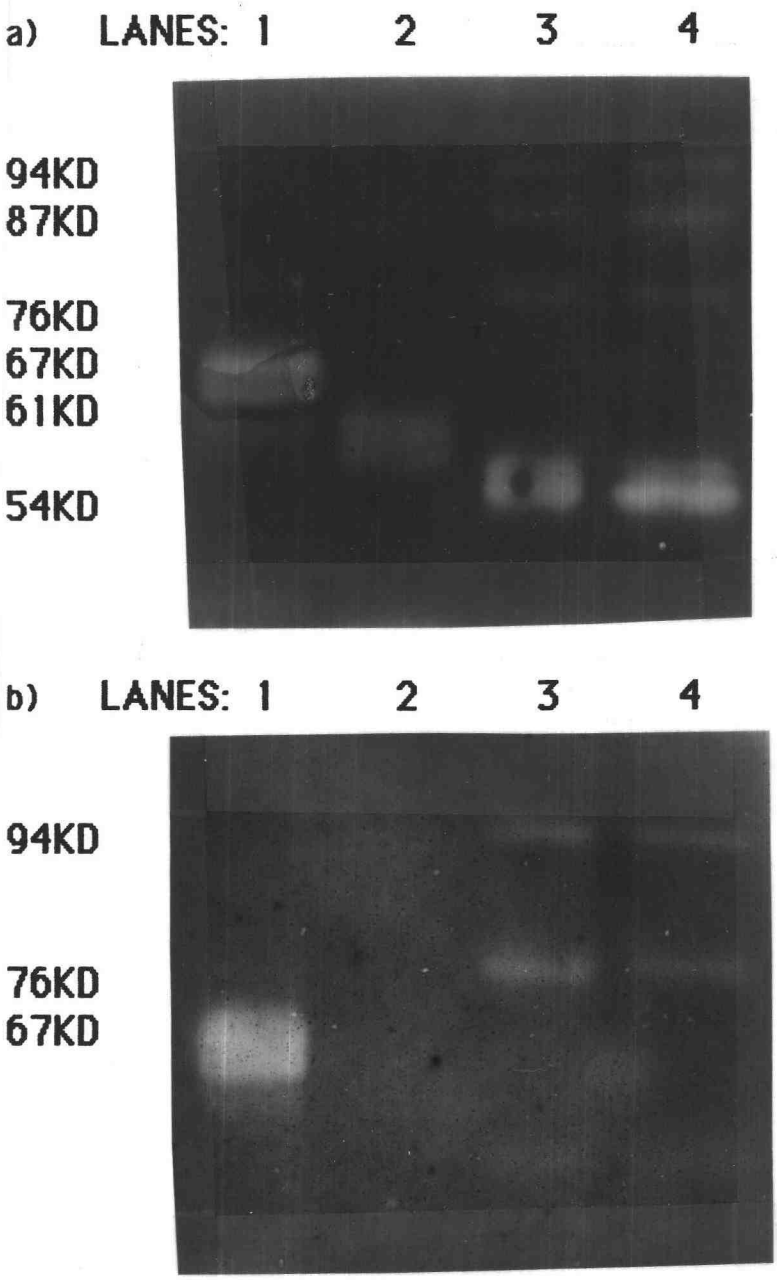


FIGURE 31: SDS PAGE Zymographs of equine embryo tissue samples a) without amiloride & b) with amiloride in Experiment 4. Lanes are indicated across the top and molecular mass (Kilo-daltons) of the protein bands are shown on the left of the photograph. Lanes 1 and 2 have plasminogen activator standards, and lanes 3 and 4 have embryo tissue samples.

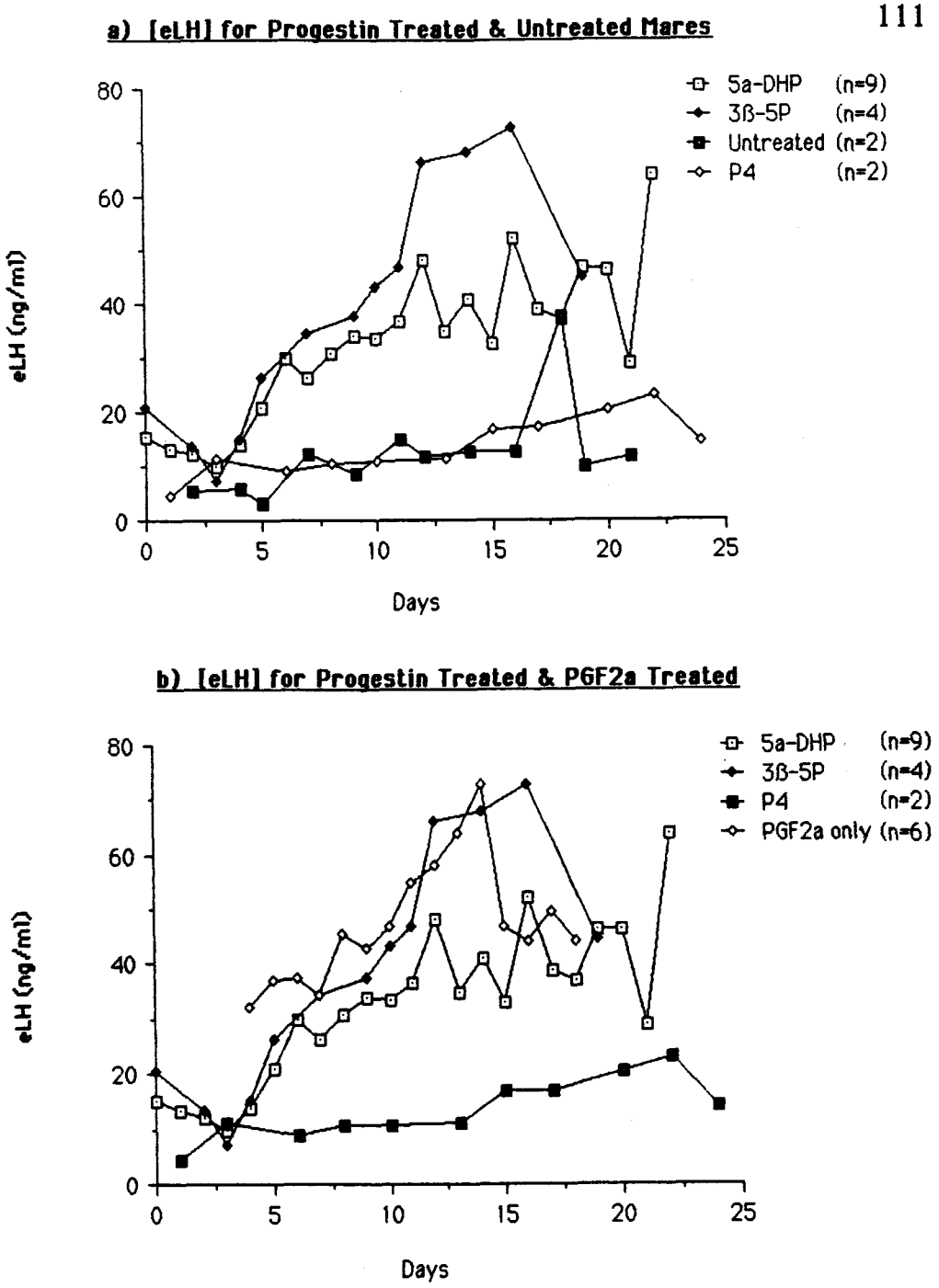
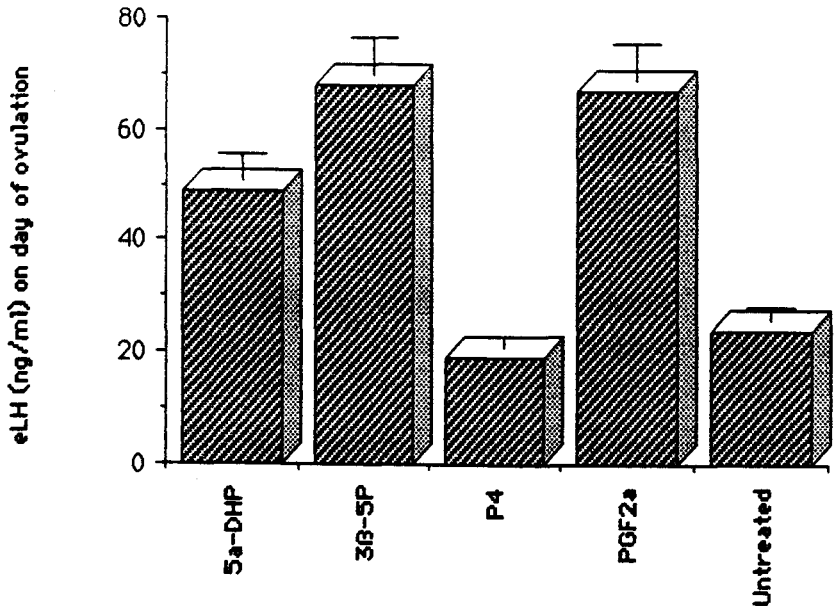


FIGURE 32: Mean concentrations of eLH determined by EIA in the non-pregnant mares treated with progestins in Experiment 5. Time (days) after d 6 postovulation is indicated on the horizontal axis and [eLH] in ng/ml is shown on the vertical axis. Figure a) shows the three progestin treated groups and the untreated controls. Figure b) shows the three progestin treated groups and the PGF2α controls.

a) [eLH] on the Day of Ovulation for all Treatment Groups



b) Days to Ovulation for all Treatment Groups

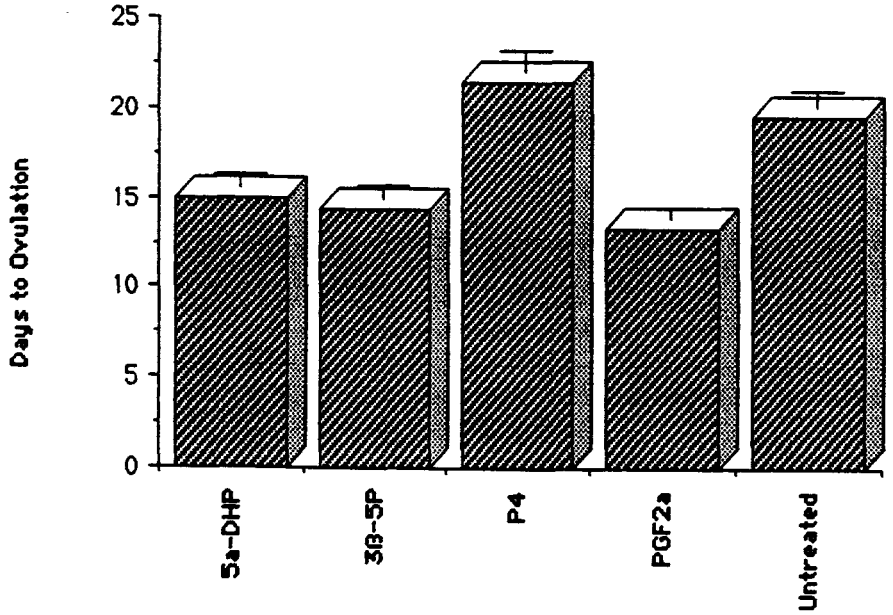


FIGURE 33: a) Mean concentrations of eLH determined by EIA & b) days to ovulation (after d 6 postovulation) in the non-pregnant mares in all treatment groups of Experiment 5. Each treatment group is indicated on the horizontal axis and in a) [eLH] in ng/ml on the day of ovulation is shown on the vertical axis or b) days are indicated on the vertical axis. Standard error bars are included.

DISCUSSION AND CONCLUSIONS

The GC/MS techniques were effective in determining steroid content in both plasma and medium samples in this research. With minor variations, from previously published techniques (Holtan et al., 1991), progestins and estrogens were identified and quantified with definitive specificity. The full-scans provided indisputable identification of the hormones, while the selected ion mode enabled quantification.

It is the specificity that gives GC/MS techniques a distinct advantage over immunoassays, as was determined in studies in the 1970's (Atkins et al., 1974 and 1976; Holtan et al., 1975a). The non-specific binding of progesterone antisera to the various progestins suspected in these immunoassay systems, was demonstrated with the cross-reactivity studies using the commercial P4 EIA in Experiment 1. Of the eight progestins, 5α-DHP, P5, 3β-5P and 20α-5P, reacted significantly at high concentrations (1000 ng/ml) in the P4 assay. At 100 ng/ml concentrations, 5α-DHP, P5 and 3β-5P reacted in the P4 assay. Since these hormones are near such concentrations physiologically, this finding is significant. It is highly probable that many immunoassay systems used today and in the past, react with other hormones of similar structure. Cross-reactivity studies should be included with results of immunoassays to prove their specificity for the hormone analyzed.

In addition to specificity, GC/MS offers range, precision, and accuracy which are comparable to, or surpass that of most immunoassays. However, even though GC/MS has proven to be a valuable

tool in research for identifying and quantifying these numerous progestins of similar structure, immunoassays still have their place in the routine analysis of "progestin" profiles in diagnostic work. As more knowledge is accumulated regarding the progestins of the pregnant mare, specific antibodies for certain progestins will inevitably be produced. Hamon et al. (1991) has already developed an EIA system with a specific antibody for 5α-DHP.

The use of deuterium labelled internal standards in the GC/MS assay system, as was previously published (Holtan et al., 1991), was used in the analysis of plasma samples in Experiment 1. In Experiments 2, 3 and 4, deuterium labelled precursor steroids (D_4-P5) were used in the experimental procedures to confirm the identity of metabolic products. This therefore necessitated the use of alternate internal standards (4-cholesten-3-one) for quantification of the deuterium labelled metabolites produced. The quantification of the eight progestins was against one steroid (4-cholesten-3-one) rather than four, as was the case with the deuterium labelled progestin internal standards. This resulted in slightly greater assay coefficients of variation for Experiments 2 through 4.

Extraction efficiencies in the experiments varied depending on the type of sample extracted (medium or plasma). Plasma extracted at approximately the same efficiency with Sep-Pak and ether extraction (about 70%). Medium samples however, extracted better (about 90%) than plasma through Sep-Pak. However, since internal standards were used in all samples, extraction efficiencies were not crucial. High recoveries of the steroids were noted throughout the various assays.

This recovery, or the ability of the assay to reliably report values comparable to actual amounts added, is important to GC/MS analysis.

The use of radiolabelled precursors has been used extensively in the study of equine hormones by Bhavnani et al. (1969, 1971, and 1973a,b). Moss et al. (1979) also utilized radiolabelled steroids in metabolic studies. Although their research provided data which enhanced the understanding of metabolic pathways, the use of radioactive labels resulted in problems in the disposal of the animals after the experiments. In equine tissue incubation studies, radiolabelled steroids have also been used in the past (Ainsworth and Ryan, 1969; Hamon et al., 1991). The use of radioactive labels in such studies does provide a reliable method to trace metabolism, but does have the added expense and precautions associated with the use of radioisotopes.

Deuterium labelling has been used in place of radiolabels in metabolic studies. These non-ionizing isotopes reduced the health threat to the research subjects. In addition, GC/MS can easily detect the difference of mass in analyzing samples with deuterium metabolites. Baillie et al. (1980) and Anderson et al. (1990) even used deuterium labelled precursor steroids intravenously in pregnant women. In Experiment 2, deuterium infusions (D_4-P5) to fetal arterial vessels provided a progestin metabolite label which was identified in the samples collected. The use of deuterium labelled precursor steroids (D_4-P5) has also proven to be a useful technique for tissue (Hamon et al., 1991; and Experiment 3) and embryo (Experiment 4) incubation metabolic studies.

The GC/MS and associated techniques were applied in Experiments 1 through 4. In Experiment 1, the identity and concentration of

progestins in fetal and maternal vessels of late gestation mares were confirmed and found to compare favorably with the only previously published study of this kind (Holtan et al., 1991). Experiments 2 and 3, again utilizing GC/MS, provided new and important information on the metabolism of progestins during pregnancy. In Experiment 2, primary metabolites were determined for the maternal circulation (20α-5P) and fetal circulation (3β-5P). Experiment 3 indicated specific sites of metabolism for some progestins (placenta produced P4, placenta and endometrium produced 5α-DHP, fetal tissues produced 3β-5P, fetel liver produced P5-ββ, and endometrium produced βα-diol). The GC/MS identification of the steroids (progestins, estrogens, and an androgen) in the early equine embryos in Experiment 4, has not been documented in the literature previously. In the embryo work, plasminogen activator was identified and characterized which is also new information. Experiment 5 provided evidence that at least two of the progestins found in pregnant mares (5α-DHP and 3β-5P) are not as physiologically active as P4 at comparable concentrations in the cyclic mare. The biological actions of the progestins (excluding P4) have not been investigated previously in horses.

In Experiment 1, all six mares appeared to have similar levels of each individual progestin, with the exception of the earlier gestation (160 d) mare having higher P4 levels ($P < .05$) indicating that the corpus luteum was still contributing P4. The two mares at 313 d had higher 20α-5P levels ($P < .05$), which was due to advanced gestational age. In previous work (Holtan et al., 1991), a rapid increase in progestins during the last 30 d pre-partum was observed.

Tremendous variations ($P < .05$) were noted between the four vessels for the eight hormones. It was noted that P4 was highest ($P < .05$) in the fetal vein (15.5 ng/ml), with the fetal artery lower (7.8 ng/ml), and negligible amounts in the maternal circulation. This finding is consistent with the early work published by Short (1959) which indicated P4 was found only in the placenta and umbilical cord blood. Holtan et al. (1991) also found similar concentrations of P4 in cannulations of the fetal vessels. The explanation for the differential from fetal vein to artery is evident from tissue incubation studies. Hamon et al. (1991) noted that placental tissue was capable of the metabolism of P5 to P4, with low conversion of P4 to 5α-DHP, but found mare endometrium produced significant amounts of 5α-DHP from P4. Ainsworth and Ryan (1969), also using tissue incubations, found P5 and P4 were metabolized by the placenta, with some P4 converted to 5α-DHP, 3β-5P, 20α-5P and βα-Diol. Therefore, the concentration of P4 would increase from fetal artery to fetal vein, and since the endometrium metabolizes P4 to other metabolites, P4 is unlikely to appear in maternal vessels.

The predominant fetal vein progestin in Experiment 1 was 5α-DHP at 216.4 ng/ml. Holtan et al. (1991) also noted that 5α-DHP was highest in the fetal vein. Previous studies help explain this finding. With radiolabelled infusions, Moss et al. (1979) determined that P4 was converted to 5α-DHP in the placenta. This was also noted in Ainsworth and Ryan's work (1969). However, Hamon et al. (1991) indicated the endometrium produces 5α-DHP, which may pass back to the fetus.

The fetus is the probable source of P5 (630.8 ng/ml) since it is the predominant ($P < .05$) steroid in the fetal artery as noted in

Experiment 1, and previously by Holtan et al. (1991). This P5 from the fetus (via the fetal artery) is converted to P4 in the placenta, which is subsequently either metabolized further by the placenta and endometrium (and therefore P4 is not seen in the uterine vein or maternal artery circulation), or passed to the fetus (via the fetal vein) where it is converted to other metabolites.

The fetal artery also had the highest ($P < .05$) levels of 3β-5P (117.9 ng/ml), P5-ββ (131.4 ng/ml) and ββ-diol (32.3 ng/ml). The uterine vein had the highest ($P < .05$) levels of 20α-5P (767.6 ng/ml) and βα-diol (202.2 ng/ml). These relative relationships were also noted by Holtan et al. (1991). From these differences in levels it could be proposed that 3β- and 20β-hydroxylations occur on the fetal system, while 20α-hydroxylations occur on the maternal side. Holtan et al. (1991) noted a 50% decrease in concentration of 20α-5P from the uterine vein to the maternal artery, and therefore speculated that the endometrium produced 20α-5P. The maternal vessel concentration differences of 20α-5P noted in Experiment 1 (767.6 ng/ml in the uterine vein and 533.3 ng/ml in the maternal artery), also indicate that the endometrium produces 20α-5P.

With the infusions of D_4-P5 to the fetal artery (Experiment 2), it was noted that the major metabolism of P5 seemed to occur within the first 10 min after infusion. After 10 min, levels of all D_4-progestins declined in all vessels except in the fetal vein which decreased after the 60 min sampling. There were no samplings between 10 and 60 min analyzed from the fetal vessels since low sample volumes were collected to avoid hemorrhagic insult to the fetus. Subsequently, pooling of fetal samples was necessary to attain sufficient volumes for

GC/MS analysis. Therefore, the fetal vein decrease probably occurred just after 10 min. Since larger volumes were collected from the maternal vessels, without risking a serious decrease in blood volume, there were several samples of sufficient volume for analysis between 10 and 60 min. Additionally, it appears that an approximate 10-fold decrease in magnitude of the progestins occurred from the fetal artery to vein, indicating that the amount of D_4-P5 may not have been adequate to supply precursors for both maternal and fetal systems. The maternal system was at levels comparable to fetal artery concentrations. These findings will be helpful in the design of future protocols. More frequent, earlier samplings and increased concentration of infused precursor may help to determine progestin metabolism completely.

The results from Experiment 2 did indicate some of the major metabolites in the fetal and maternal systems. Deuterium labelled progestins varied between vessels and over time ($P < .05$), due to D_4-3β-5P, D_4-P4 and D_4-5α-DHP being greater in magnitude in the fetal vessels, and D_4-20α-5P and D_4-βα-diol predominating in the maternal circulation. The maternal artery had lower levels ($P < .05$) of the two predominant maternal D_4-progestins than the uterine vein. This supports the idea proposed by Holtan et al. (1991) that the 20α-hydroxylations do indeed occur in the maternal system.

However, the situation remains somewhat unclear regarding the fetal side of the metabolic scheme. The higher D_4-3β-5P in the fetal artery indicates 3β-hydroxylation in the fetus. Since very low levels of the remaining hormones were detected, it is possible that either in the 10 min sampling, only the last portion of the metabolism of the D_4-P5 was detected, or that the level infused was not physiologically high

enough to accurately trace metabolism completely, at least on the fetal side. It is highly probable that the metabolism is indeed very rapid in the placenta and endometrium since only low levels of D_4-P4 and D_4-5α-DHP were detected compared to other metabolites. This may indicate that they were merely transient metabolic intermediates since high levels of D_4-3β-5P were found in the fetal vessels, and high levels of D_4-20α-5P were seen in the mare's circulation. However, very low levels of the 20β-hydroxylated compounds (P5-ββ and ββ-diol) were noted in the fetal circulation, which may indicate that there were not enough precursor progestins available to form these metabolites in their usual magnitudes. The important findings of Experiment 2 were however, that the major metabolite of the mare is 20α-5P, while the major metabolite produced by the fetus is 3β-5P.

In Experiment 3, enzymatic capabilities of tissues suspected to be involved with the production of the various progestins were evaluated. Although all the tissues tested produced D_4-P4, placental tissue produced the most ($P < .05$). This agrees favorably with previous work (Short, 1959; Ainsworth and Ryan, 1969; Hamon et al., 1991). In a histochemical study (Flood and Marrable, 1975), it was noted that hydroxysteroid dehydrogenase enzymes are located in placental and uterine structures in all stages of pregnancy. This is the enzyme required for the conversion of P5 to P4.

The major sources ($P < .05$) of D_4-5α-DHP included the placenta, uterine endometrium and fetal ovary. These findings are consistent with the levels observed in the vessel cannulations of Experiment 1, and by Holtan et al. (1991), as well as tissue studies of Hamon et al. (1991).

The highest ($P < .05$) production of D_4-3β-5P occurred in the fetal ovary, adrenal, liver, and mare endometrial tissues. This production of D_4-3β-5P in the fetal tissues is in agreement with the model suggested previously regarding 3β-hydroxylations in the fetus (Holtan et al., 1991). Although uterine endometrial tissues produced some D_4-3β-5P, D_4-P5-ββ and D_4-ββ-diol *in vitro,* in the intact system with other precursors present, alternate enzyme systems may be activated. Several tissues (uterine endometrium, fetal ovaries, adrenals and liver) produced D_4-20α-5P, but none were significantly higher than the others. It is possible that since this metabolite is suspected to be metabolized in the mare's endometrium, *in vivo* other metabolites may be available for its production. The uterine endometrium produced more D_4-βα-diol than the other tissues which supports the idea that 20α-hydroxylation occurs on the maternal side. A significant ($P < .05$) amount of D_4-P5-ββ was produced by the fetal liver compared to other tissues. This fetal source may be activated in distressed foals since they produce elevated levels of this hormone and other 20β-hydroxylated compounds (Houghton et al., 1991; Rossdale et al., 1992).

It must be taken into consideration when analyzing data from tissue incubations that only single, isolated systems are examined and different metabolites may predominate *in vivo* Various additional precursors and feedback systems are present in the whole system. The impact of feedback of the progestins was examined and found to be significant in human studies (Powell and Challis, 1986; and Powell et al., 1986).

However, Experiment 3 provided a more extensive analysis of progestin metabolism in feto-placental tissues than any results published previously. The primary conclusions from this study indicate that the placenta and endometrium metabolize P5 to P4 and 5α-DHP. The fetal liver is the location of the production of P5-ββ, which is a hormone elevated by fetal stress. Additionally, fetal tissues produced 3β-5P.

Cummulatively, these first three Experiments would imply a complicated system of metabolism in the late-gestation mare. Additional studies such as these will be necessary to completely determine the entire metabolic scheme. However, at this time with these studies and previous work, it would appear that the fetus is the primary source of P5. This P5 is transported to the placenta and endometrium where it is enzymatically altered to form P4 and 5α-DHP. The 5α-DHP and P4 are transported to the fetus where 3β and 20β-hydroxylations occur to form 3β-5P and ββ-diol in the fetal liver, adrenals and gonads. Some of the P5 is also hydroxylated (probably mostly in the fetal liver) to form P5-ββ. The 5α-DHP formed in the mare's uterus is probably the primary precursor for the 20α-5P and βα-diol found in the maternal vessels. Some 5α-DHP obviously crosses back to the fetal vein due to the high levels detected in this vessel. A steroid metabolic flow diagram (Figure 34) and a diagram of the feto-placental unit with progestin sources indicated (Figure 35) help to visualize this metabolic model.

It is unknown why the pregnant equine produces this wide variety of progestin compounds. Research regarding the biological activity of these substances, with the exception of P4, has received little

attention. The feedback inhibition of the gonadotropins by P4 has been known for years, and has been extensively examined in mares (see Literature Review for details). However, no studies have been published analyzing biological activity of any other progestins in pregnant or cycling equines.

In Experiment 5, the biological activity of two of the progestins (5α-DHP and 3β-5P) were tested in non-pregnant, cyclic mares and compared to P4. At the same treatment levels, it was determined that neither 5α-DHP nor 3β-5P consistently block estrus and ovulation. By contrast, P4 treated control mares showed the expected prolonged diestrus and delayed ovulation until after treatment was withdrawn. It was also noted that the mares treated with these two reduced progestins, or PFG2α alone, showed somewhat elevated eLH concentrations compared to the P4 treated and the untreated control mares. Therefore, it seems that the negative feedback exerted to inhibit eLH release from the pituitary by P4 treated mares, and by endogenous P4 in the untreated control mares, was not elicited by 5α-DHP, 3β-5P, or in mares treated with only PGF2α.

From the studies in pregnant mares, it was noted that the reduced progestins are present at levels much higher than P4. Therefore, it may be that the levels of treatment in Experiment 5 were inadequate to demonstrate the actual physiological activity of these progestins. Work with cyclic rats (Sridharan et al., 1974) has indicated that larger amounts of P5 and 5α-DHP were necessary to elicit the same response as P4, and that 3α-5P was ineffective at any dosage level. Others have reported differential effectiveness of the progestins on inhibition of LH

in rats (Zanisi and Martinin, 1974; Nuti and Karavolas, 1977). Similar situations may exist in the equine.

The work in Experiment 4 examined the metabolism of the progestins from D_4-P5, as well as the determining the content of progestins and estrogens in the early equine embryo. Previous work (Marsan et al., 1987) indicated different cell types are responsible for the production of progesterone and estrogen. Several studies (see Literature Review for details) have indicated the presence of estrogens in the early embryo. The intermediate metabolites between the progestins and estrogens have never been addressed. Additionally, in the previous studies, non-specific RIA systems were used, consequently definite identifications of the hormones were not possible. With full-scan GC/MS, positive identifications of the estrogens and progestins were attained.

The results from the incubations of 14 d to 18 d embryos indicate that the metabolic capability is present for the production of the eight progestins which have been identified in late-gestation mares. All seven D_4-progestins derived from D_4-P5 were present in the incubation medium, with D_4-P4 being the predominant product. When the amount of D_4-P5 was increased, there was an increase in D_4-P4 produced. Large amounts of E2 were noted in the embryo medium as well as E3, E1 and AND. Since the embryos studied in this experiment were near the time of maternal recognition of pregnancy, it is probable that these steroids, in particular P4 and the estrogens, are involved with this mechanism. Therefore, steroid production by the embryo may be important to pregnancy maintenance in the early stages of gestation.

The identification and characterization of the plasminogen activator in the equine embryo has not been documented previously. The plasminogen activator appears to be primarily a 50 to 55 KD urokinase type, since it was inhibited by amiloride. This information indicates that the major plasminogen activator present in equine embryos is similar to that found in bovine embryos (Coates and Menino, 1991) and ovine embryos (Bartlett and Menino, 1991). In addition, however, there appears to be some tissue type plasminogen activator of about 70 to 75 KD. Inhibitor complexed to both types of plasminogen activator are suspected to have resulted in the two additional bands at approximately 88 and 95 KD.

The biological function of the plasminogen activator is speculative at this time in the equine as well as the other species. A number of ideas are discussed in the Literature Review section. Its presence in the equine embryos at this early stage may indicate a role in tissue reorganization. Since it is suspected that there are inhibitors present also, the peak activity of this enzyme may actually occur at some other time, such as at the time of placentation. The progestins and estrogens also found to be present in these embryos, may be involved with the expression of plasminogen activator as was noted by Mullins et al. (1980) and Fazleabas et al. (1983).

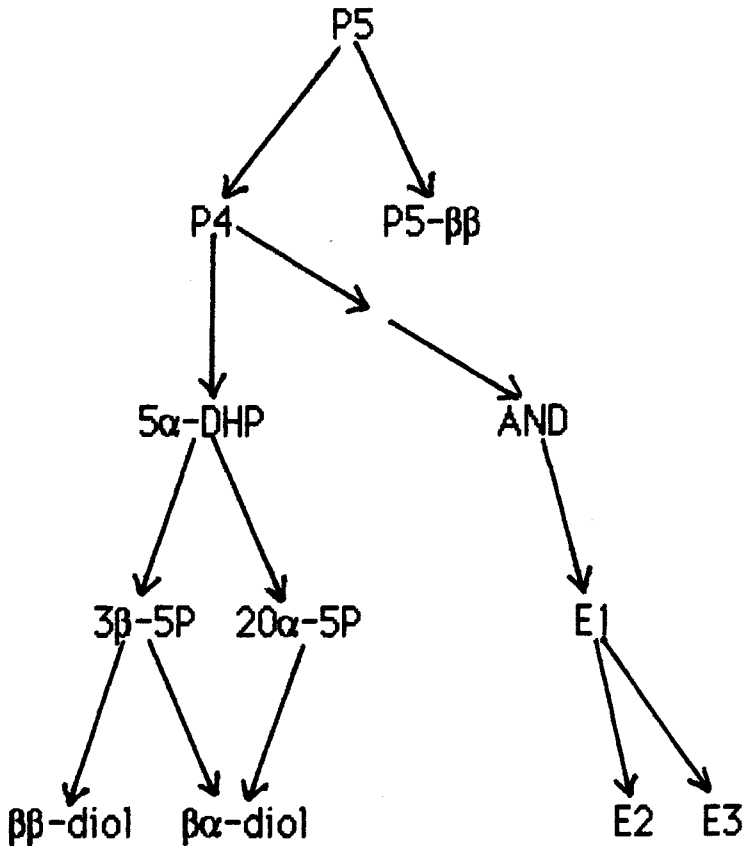


FIGURE 34: Steroid metabolic flow diagram. See Table 1 and 2 for full systematic names. See Figures 1, 2 and 3 for structures. Beginning with P5, the progestin metabolites are shown on the left side of the diagram. The androgen and estrogens found in the embryo work are shown on the right side of the diagram.

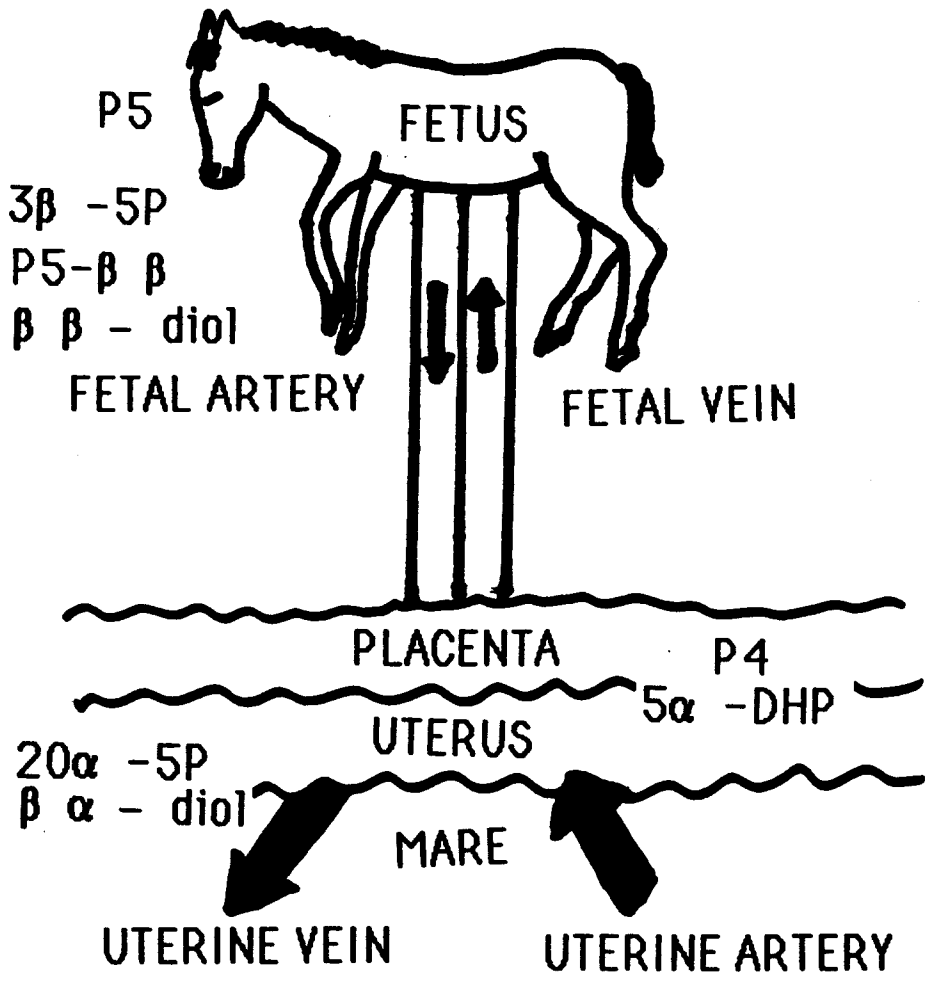


FIGURE 35: The feto-placental unit with progestin sources indicated. See Table 1 for full systematic names. P5 from the fetus (via fetal artery) is metabolized to P4 and 5α -DHP in the placenta and uterus. Some 5α -DHP goes to the fetus (via fetal vein) and is hydroxylated to 3β -5P and β β -diol (both high in fetal artery). The 5α -DHP in the uterus forms 20α -5P and β α -diol (both high in maternal vessels). Some P5 is hydroxylated in the fetal liver to P5-β β (high in fetal artery).

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