IN VITRO PROGESTERONE AND ESTRONE SYNTHESIS BY THE PORCINE PLACENTA AND ENDOMETRIUM AT 30, 60 AND 90 DAYS OF GESTATION

by

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(ABSTRACT)

The present studies were conducted to gain a better understanding of the effects of pregnenolone (P_5), human chorionic gonadotropin (hCG) and 3' 5', cyclic adenosine monophosphate (cAMP) on porcine placental and endometrial production of progesterone (P_4), testosterone (T) and estrone (E_1) at 30, 60 and 90 days of gestation. Duplicate 300 mg samples of placenta, endometrium or both (co-incubation) were incubated in medium199 containig either no P_5, P_5, P_5 + hCG or P_5 + cAMP for either zero (control), .5, 1 or 2 h.

The first study compared P_4 and E_1 production with or without addition of P_5. At d 30, 60 and 90, respectively, P_4 production (ng/g) increased significantly in the presence (vs absence) of P_5 in the incubation medium of placental (13.2 vs 7.5, 73.9 vs 42.7, 137.4 vs 113.5, respectively) coincubation (14.5 vs 10.0, 33.6 vs 22.3, 77.9 vs 49.4,

respectively) and endometrial (16.0 vs 13.3, 23.0 vs 16.0, 17.1 vs 6.7, respectively) tissue. Presence of P_5 increased E_1 production in d 60 (1.3 vs .7 ng/g) and d 90 (51.7 vs 34.6 ng/g) placental tissue and d 90 endometrial tissue (9.8 vs 8.0 ng/g).

In a second study, P_5 + cAMP increased (vs P_5 alone) P_4 in placental tissue at d 30 (11.6 vs 8.7 ng/g) and coincubation tissue at d 90 (103.7 vs 75.3 ng/g). Cyclic AMP stimulated increased P_4 synthesis (vs P_5 alone), throughout the incubation period in d 60 and d 90 tissue. E_1 production by endometrial tissue at d 30 (4.1 vs 2.9 ng/g), and placental tissue at d 60 (1.2 vs .9 ng/g). Presence of hCG in the incubation medium had no overall effect on either P_4 or E_1 accumulation. Only trace amounts of T were detected in either study, suggesting rapid aromatization of C_{19} steroids to estrogens.

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INTRODUCTION

Understanding of the mechanisms regulating prenatal mortality in swine is of economic importantance because litter size is an important component of profitable swine production. On a national average, 14 to 16 viable embryos per sow are present at the onset of pregnancy. However, only 10 to 11 of these embryos are born alive and only seven to eight of these piglets are weaned. Prenatal mortality in swine has been proposed to occur in two phases: 1) prior to d 25 and particularly between d 12 and 17 during the time of blastocyst expansion and maternal recognition of pregnancy (this embryonic loss occurs regardless of the number of potential pigs presents); and 2) between d 40 and term, particularly after d 70 due to placental insufficiency. The latter loss is the true limiting factor controlling litter size in potentially large litters. At present, our knowledge of the causes of prenatal mortality is very limited. Likewise, basic knowledge of the controlling mechanisms regulating the physiological and endocrinological interrelationships among the placenta, fetus and maternal system is limited. A potential key to understanding the dynamics of

fetal-placental-maternal function during gestation is an enhanced understanding of the steroidogenic capabilities of the conceptus unit and an elucidation of their controlling mechanisms.

The overall goal of this study is to gain a better understanding of porcine placental function, especially placental steroidogenesis and its relationship to conceptus development.

The specific objectives of this study were as follows: 1) to determine the capabilities of the porcine placenta and endometrium to produce progesterone (P_4), testosterone (T), and estrone (E_1) at 30, 60, and 90 days of gestation; and 2) to determine the effect on steroidogenesis of adding the precursor pregnenolone (P_5) and the potential steroidogenic stimulants 3', 5'-cyclic adenosine monophosphate (cAMP) and human chorionic gonadotropin (hCG).

REVIEW OF LITERATURE

The Porcine Estrous Cycle

Sexually mature female pigs are polyestrous throughout the year. They have estrous cycles which recur approximately every 21 days, with a normal range of 19 to 23 days (Anderson, 1980). These cycles commence at puberty and continue throughout the female's life, interrupted only by pregnancy, lactation or endocrine dysfunction (Hughes and Varley, 1980). Behavioral estrus lasts for 24 to 72 h and ovulation occurs 36 to 42 h after the onset of estrus. The duration of the ovulatory process requires approximately 3.8 h (du Mesnil du Buisson et al., 1970), with the range in ovulation rate of 10 to 25 ova. Following ovulation, the walls of the mature Graafian follicle of each ruptured follicle collapse. The remaining granulosa cells of the ruptured follicle mix with the theca cells which had been previously located outside the basement membrane and this mixture of cells hypertrophies to become the lutein cells of the corpus luteum (CL). Thus formed, the CL rapidly increases in size, such that it has a diameter of 8 to 9 mm by d 7 of the cycle. Progesterone secretion increases from

d 7 to maximum production between d 12 and 14 of the estrous cycle (Guthrie et al., 1972). Little morphological change then occurs until d 14 or 15 of the cycle. At this time, in non-pregnant females, luteal regression begins and plasma P_4 concentrations decline rapidly to basal levels (1 ng/ml or less) by d 17 to 18 (Anderson, 1980), leading to a recurrent estrous cycle unless interrupted by pregnancy or events leading to endocrine dysfunction.

Endocrine Control of the Estrous Cycle

Morphological, behavioral and endocrine changes throughout the porcine estrous cycle depend upon a complex interrelationship among hypothalamic, pituitary, uterine and ovarian hormones. The estrous cycle consists of an alternation between a follicular phase, which begins when the CL regresses and ends with ovulation of the mature Graafian follicle, and a luteal phase, which extends from formation of the CL after ovulation until the CL regresses.

During the follicular phase, the reproductive system is under the influence of estrogens secreted mainly by the granulosa cells of the follicle. Estradiol (E_2) concentrations in peripheral plasma begin to increase from 10-30 pg/ml to 60-90 pg/ml over a 6 d period during which follicles mature (Henricks et al., 1972; Shearer et al., 1972).

After estrus, E_2 levels decline to 10 to 20 pg/ml throughout the rest of the luteal phase of the cycle.

The anterior pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), both secreted in response to the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, are primarily responsible for stimulation of follicular growth and maturation (Anderson et al., 1967). Adenohypophyseal LH and FSH concentrations peak at estrus and are low during the luteal phase (Parlow et al., 1964). The surge release of FSH and LH at estrus is triggered by a positive feedback effect of E_2 from the preovulatory follicle. Recent evidence suggests that androgens are synthesized in the theca cells under the influence of LH and aromatized to E_2 in the granulosa cells of the follicle under the influence of FSH (Hansel and Convey, 1983). As ovulatory follicles grow in size, the number of LH receptors in the theca and granulosa cells increase. As a result, these follicles become more responsive to LH and acquire an increased ability to secrete E_2. The increase in E_2 concentrations trigger a pre-ovulatory LH surge which reaches a peripheral serum concentration of 4 to 5 ng/ml (Niswender et al., 1970). During the immediate post-estrus period, on d 2 and 3 following onset of behavioral estrus, FSH concentrations in peripheral serum reach

peak levels of 9.5 ng/ml and 9.0 ng/ml, respectively. The increase in FSH may play a role in recruitment of pre-antral follicles. Thus, estrogen from new follicles, plus P_4 from the newly formed CL feedback negatively on the hypothalamus and anterior pituitary to inhibit ptoduction and release of FSH and LH.

During the luteal phase of the cycle, the reproductive system is under the influence of P_4 secreted by the CL. The concentration of P_4 increases from preovulatory values of < 1.0 ng/ml (Henricks et al., 1972) to mid-luteal phase values of 20to 48 ng/ml (Edquist and Lamm, 1971; Henricks et al., 1972) before commencing a rapid decline to basal levels by d 17 and 18 of the cycle.

The uterus exerts control over the functional lifespan of the porcine CL. Spies et al. (1958), du Mesnil du Buisson and Dauzier (1959) and Anderson et al. (1961) found that bilateral hysterectomy in the early-to-mid-luteal phase of the estrous cycle resulted in CL maintenance for periods equal to or longer than the 114 d of a normal pregnancy. It has now been established that the pig uterine endometrium is the source of the uterine luteolysin prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and, under the influence of P_4, increased amounts of $PGF_{2\alpha}$ are secreted causing morphological regression of CL and cessation of P_4 secretion (Moeljono et al., 1977).

Bazer et al. (1982) demonstrated that $PGF_{2\alpha}$ acts as a luteolytic agent when injected into the uterine lumen or administered intramuscularly in swine on d 12 or later in the estrous cycle. Henderson and McNatty (1975) proposed that pig CL may remain refractory to $PGF_{2\alpha}$ until LH begins to dissociate from the luteal cell membrane receptors. About d 12, conformational changes within the luteal cell membrane may facilitate $PGF_{2\alpha}$ binding. The $PGF_{2\alpha}$ in turn, is proposed to inactivate the adenyl cyclase system to inhibit P_4 secretion and activate lysosomal enzymes to cause morphological regression of the CL.

Establishment of Pregnancy

Fertilization of ova shed at ovulation marks the beginning of gestation. In pregnant gilts, embryos move from the oviducts, site of fertilization, and into the uterine horns at about the 4-cell stage or about 60 to 72 h after onset of estrus. The embryo advances to the morula stage, a solid clump of cells, and by d 5 the embryos reach the blastocyst stage(Hafez, 1980). The resulting blastocyst consists of an outer layer of trophoblast cells, an inner cell mass and a central cavity or blastocoele surrounded by a transparent, and tough mucopolysaccharide coat, the zona pellucida. The trophoblast establishes and main-

tains physical and chemical contact with the mother. At later stages it is referred to as ectodermal chorion. The inner cell mass gives rise to the embryo, amnion, allantois and the mesodermal lining of the chorion (Steven, 1975). Between d 6 and 7 the blastocysts emerge from the zona pellucida (hatching). Blastocysts then undergo marked morphological changes and rapid growth beginning at d 10 of gestation. Blastocysts change from a spherical (3 to 10 mm diameter) to a tubular (10 to 50 mm long) and then to a thread-like filamentous form (> 100 mm long), attaining 700 to 1000mm in length by d 14 to 16 of pregnancy (Perry and Rowlands, 1962; Anderson, 1978; Geisert et al., 1982b). Initial blastocyst elongation occurs at a rate of 30-45 mm/h and apparently results from cellular hypertrophy and remodeling and not cellular hyperplasia (Geisert, 1982b). Then, during the final stage of elongation mitotic activity is high, as reflected by substantial increases in total RNA and DNA per conceptus (Geisert et al., 1982b).

Prior to blastocyst elongation, blastocysts unndergo a migratory activity both within and between the uterine horns (Polge and Dziuk, 1970; Geisert et al., 1982b). This intrauterine migration and distribution is influenced by E_2. Pope et al. (1982) recently found that when beads the size of embryos and impregnated with cholesterol are substituted

for embryos, they stay in the top of the horns; however, when impregnated with E_2, they are distributed along the uterine horns like embryos. After intrauterine migration, the long filamentous blastocysts lie end-to-end in a fixed position and follow the contour of the endometrial folds along the entire length of the uterine horns without overlapping (Anderson, 1978). Failure to establish this contact in both uterine horns leads to pregnancy loss (du Mesnil du Buisson, 1961; Anderson et al., 1966; Niswender et al., 1970). Furthermore, fewer than five blastocysts present within the uterus results in termination of pregnancy (du Mesnil du Buisson and Rombauts, 1963).

The trophoblast then establishes contact with the mesometrial surface of the endometrium and by d 18 an intimate contact between fetal and maternal epithelia by interlocking villi is established (Perry et al., 1976).

Several critical events are associated with blastocyst elongation between d 10 and 12 of pregnancy. First, estrogen production begins at the 10 mm tubular stage (d 12) (Geisert et al., 1982a). These estrogens appear to be the blastocyst signal for maternal recognition of pregnancy, resulting in maintenance of luteal function and a continuous supply of P_4 (Bazer and Thatcher, 1977; Frank et al., 1977; Patek and Watson, 1983). In order for luteal function to be

maintained in early pregnancy, the effects of the endometrial luteolysin $PGF_{2\alpha}$ must be negated. In nonpregnant gilts, P_4 enhances and/or induces $PGF_{2\alpha}$ synthesis by the uterine endometrium and secretion is associated with increased E_2 production by the ovarian follicles between d 12 and 18 of the estrous cycle (Zavy et al., 1980). Secretion of $PGF_{2\alpha}$ during the late luteal phase of the estrous cycle is in an endocrine direction, that is, toward the uterine venous drainage. However, estrogens produced by pig blastocysts, alter the direction of movement of $PGF_{2\alpha}$ so that its' secretion remains in an exocrine direction, toward the uterine lumen (Bazer and Thatcher, 1977; Zavy et al., 1980). Thus, sequestering $PGF_{2\alpha}$ in the uterine lumen and preventing it from entering the uterine venous drainage prevents CL regression. Furthermore, Frank et al. (1977) demonstrated that injections of estradiol valerate (E_2V) caused a re-direction of uterine prostaglandin ($PGF_{2\alpha}$) secretion so that the amount secreted into the utero-ovarian vein was decreased and that which was released into the uterine lumen was increased.

Another event associated with blastocyst elongation is the stimulation and sequestering of uterine gland secretions in an exocrine direction to provide nourishment for the developing blastocyst. Geisert et al. (1982a) reported the

accumulation of secretory vesicles in endometrial gland epithelium between d 10.5 and 12 of gestation. Concurrently with the onset of estrogen production by tubular and filamentous pig blastocysts between d 11 and 12 of gestation, there was an increase in total recoverable calcium, protein, acid phosphatase (index of uteroferrin) activity, $PGF_{2\alpha}$ and PGE_2 in uterine flushings (Geisert et al., 1982a; Fazleabas et al., 1982). Furthermore, administration of 5 mg of E_2V to nonpregnant gilts on d 11 resulted in the increase of those components by 24 h post-injection (Geisert et al., 1982c).

Geisert et al. (1982a) reported that uterine fluids obtained from pigs between d 10 and 14 contained proteins which serve as nutrients. The major protein present was uteroferrin, a P_4-induced protein (Knight et al., 1973) which during pregnancy transports iron from the endometrial epithelium to the developing conceptus (Roberts and Bazer, 1980; Buhi et al., 1982; Renegar et al., 1982). In nonpregnant gilts, between d 9 and 13 of the estrous cycle, uteroferrin is secreted into the lumen of the uterine gland. Beginning on d 14, however, uteroferrin begins to become localized within the uterine endometrial stroma where it possibly enters the interstitial fluid and/or vascular system for transport to the spleen or liver for degradation

(Bazer et al., 1982). In contrast to the changing pattern of localization of uteroferrin in nonpregnant gilts, uteroferrin is localized within the epithelial cells and lumen of uterine glands of pregnant gilts and continues to be secreted into the uterine lumen where it is available to provide a nutrient source to the developing conceptus for a major portion of gestation.

Furthermore, retinol and retinoic acid binding proteins have also been identified and are assumed to transport these two forms of vitamin A to conceptuses (Adams et al., 1981).

The Fetal Membranes of the Pig

Initially the porcine blastocyst develops independent of its uterine environment. However as the blastocyst increases in size, it becomes less autonomous and development increasingly depends on the local uterine environment. Initial attachment of the blastocyst to the uterine wall begins at about d 12 and is complete by d 24 of gestation (Crombie, 1970).

After elongation, the first mesodermal cells appear budded from the ectoderm of the embryonic disc to form a thin sheet of tissue between it and the underlying endoderm. This sheet of tissue increases in area and extends beyond the margins of the embryonic disc. The central portion of

this sheet of tissue, within the embryonic area, will be incorporated into the structure of the developing embryo, while the extra-embryonic portion will form part of the fetal membranes. Soon after the outgrowth of the extra-embryonic mesoderm, a split appears in the extra-embryonic membrane so that the extra-embryonic membrane becomes two-layered, one layer closely overlying the endoderm, the other underlying the ectoderm near the developing embryo. The cavity formed is the extra-embryonic coelom. The outer layer of mesoderm is raised, together with the covering ectoderm to form amniotic folds. By d 18 the amnion is formed from these amniotic folds which rise up on all sides of the embryo and fuse above the dorsal surface of the embryonic disc (Steven and Morriss, 1975). The trophoblast retains its continuity as the outermost membrane or chorion. The allantois, which first appears at about d 14, arises as an outgrowth of the hindgut of the embryo and carries extra-embryonic blood vessels to the chorion. Fluid accumulation within the cavity of the allantois distends the entire vesicle to intensify contact and initiate the spread of adhesion. By d 17, the allantois is as large as the embryo itself and the allantois' cresent-shaped horns extend toward opposite ends of the conceptus (Steven and Morriss, 1975). By d 19 the vascular mesodermal covering of the

allantois makes contact with a small area of the internal surface of the chorion, and this contact is progressively increased as the allantois expands to fill the extra-embryonic coelom. By d 26 the chorion is extensively vascularised by allantoic blood vessels (Heuser, 1927). The allantois, however, does not usually enter the extremities of the chorion, and in most instances the tips of the early conceptus degenerate and become necrotic.

The trophoblast consists of a single layer of cells resting upon a well-defined basement membrane. The relationship between trophoblast and uterine epithelium is predominantly one of simple apposition with interdigitation of fetal and maternal microvilli at all points where the two epithelia come into contact (Dantzer et al., 1981). However, in those areas of the chorion which lie opposite the mouths of the uterine glands, the trophoblast is invaginated to form prominent dome-like structures known as regular areolae (Brambel, 1933). The areolae which are distributed over the entire chorioallantoic surface, first appear by d 17 (King et al., 1982) and serve as specialized areas for absorption of the secretions of uterine glands (Amoroso, 1952; Chen et al., 1975; Friess, 1981). Knight et al. (1977) reported that the number of areolae on the surface of the placenta increased until d 50 of gestation.

By d 30 the chorion is extensively vascularised and the chorion and endometrial surface interlock with numerous low ridges or rugae and corresponding troughs or fossae (Friess, 1980). By d 40 the chorion can be divided into three areas: 1) the large placental zone which occupies the central region of the sac, 2) the laterally adjacent paraplacental zone and 3) the avascular extremeties (Steven and Morris, 1975) (figure 1).

Thus, the pig's placenta is diffuse in shape due to the fact the chorioallantois shows numerous small folds which interlock with corresponding endometrial folds and according to the classification of Grosser (1909), the placenta of the pig is epitheliochorial in structure consisting of six apposed tissue layers (three maternal, three fetal).

Steroidogenis During Early Pregnancy

Perry et al. (1973) first demonstrated conversion of tritium-labeled dehydroepiandrosterone (DHEA), androstenedione (A), P_4 and estrone-sulphate (E_1SO_4) to unconjugated E_2 and estrone (E_1) in preimplantation pig blastocysts at d 12 of gestation. This is a time at which the blastocyst undergoes rapid elongation from a spherical, flaccid sac of 5 mm in diameter to a fluid-filled filamentous bilaminar structure which measures about 1000 mm in length. Thus,

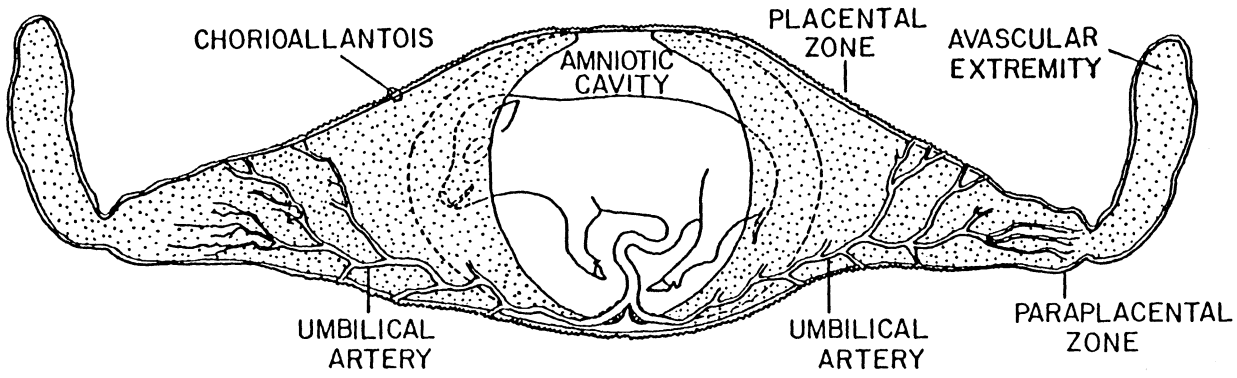

Figure 1. Fetal membranes of the pig

preimplantation pig blastocysts demonstrate Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and aromatase activity. Subsequent studies have confirmed and extended these findings using tritium labeled A, DHEA, testosterone (T), 17α-hydroxyprogesterone, P_4 and prenenolone (P_5) which were metabolized to E_1 and E_2 (Gadsby et al., 1976; Perry et al., 1976; Flint et al., 1979; Gadsby et al., 1980; Heap et al., 1981a). Other enzymes of estrogen synthesis, 17α-hydroxylase and C-17,20 desmolase have been found to be present in the blastocyst on d 16 and 20 (Heap, 1979). Furthermore, trophoblast explants produce estrogens, A and P_4 when cultured in defined medium containing acetate and cholesterol (Gadsby and Heap, 1979; Heap et al., 1981a).

Biochemical evidence for estrogen synthesis by pig blastocysts is supported by histochemical findings that hydroxysteroid dehydrogenases are present in trophoblast (Flood, 1974). Dehydrogenases have been identified that catalyse the oxidation of Δ^5-3β-hydroxysteroids and 17β-hydroxysteroids and these enzymes first appear at d 12 of gestation. The activity of Δ^5-3β-HSD increases sharply at d 15, whereas that of 17β-HSD disappears by d 16. The major pathways of steroid synthesis that have been identified are illustrated in figure 2.

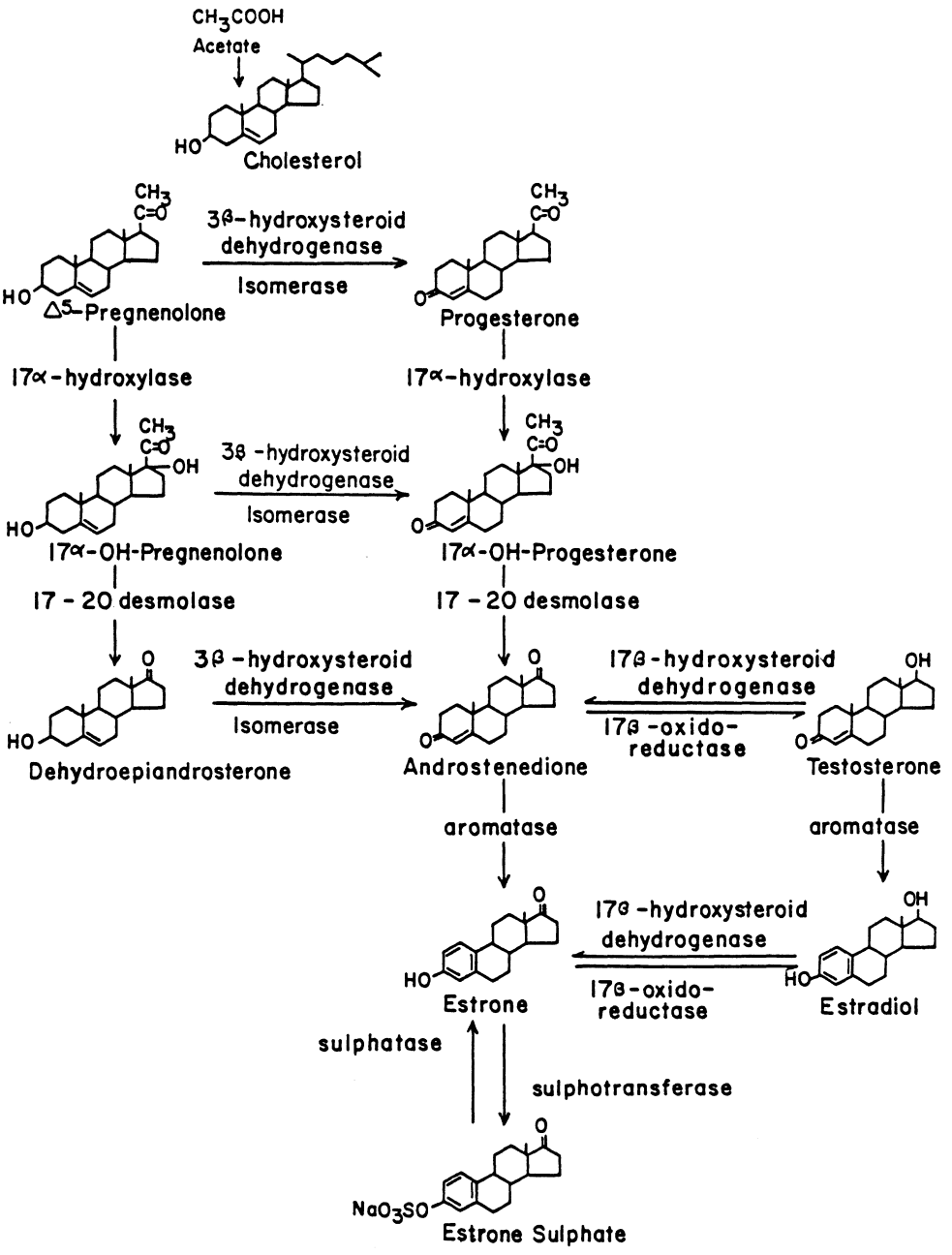

Figure 2. Steroid synthesis in the pig blastocyst

Since all the enzymes of estrogen synthesis from P_5 are present in the elongated blastocyst, it seems likely that the local presence of the E_2 essential for placentation can thus be ensured by the blastocyst itself. Evidence to support the theory that trophectoderm estrogens are synthesized de novo rather than from maternal P_4 has been obtained in gilts ovariectomized within 10 days of mating and treated with a synthetic, non-aromatizable progestin, medroxyprogesterone acetate (MPA) to maintain pregnancy (Heap et al., 1981b). Peripheral circulating E_1SO_4 concentrations were normal in ovariectomized animals, rising to levels identical to those in intact controls. Thus, ovarian P_4 is not essential for trophoblast estrogen synthesis. Furthermore, preimplantation pig blastocysts are capable of de novo synthesis of steroids in vitro when precursors such as acetate and cholesterol are supplied (Heap et al., 1980). Moreover, the concentration of E_1 and E_2 in uterine vein plasma exceeds that in arterial plasma between d 11 and 17 of gestation (Moeljono et al., 1977; Ford et al., 1982), suggesting hormone synthesis by the uterus.

Steroidogenesis of the Gravid Uterus

Evidence for the in vitro synthesis of E_1 and E_2 by elongated pig blastocyst from labeled steroid precursors, DHEA, A, T, P_4 and P_5 is well established (Perry et al., 1973; Gadsby and Heap, 1978; Flint et al., 1979; Heap et al., 1981b; Geisert et al., 1982a). These precursors may be derived in part from the maternal circulation; however, conversion of P_4 to androgens and conjugated estrogens by the uterine endometrium may also serve to provide pig blastocysts with precursors for conversion to free estrogens during early pregnancy. Thus, it would appear that the blastocyst and endometrium cooperate to produce steroids, particularly estrogens, in order to regulate their local concentrations.

Christie (1968) and Dufour and Raeside (1969) obtained histochemical evidence that endometrial tissue possesses both Δ^5-3β-HSD and 17β-HSD activity. The enzyme Δ^5-3β-HSD occupies a key position in the synthesis of steroid hormones. Its presence in endometrial tissue gives rise to the possibility that the endometrium of pregnant gilts may be able to synthesize P_4 from its immediate precursor, P_5. This possibility is suggested by several lines of evidence. First, Knight et al. (1977) observed that uterine artery-uterine vein (A-V) differences in plasma P_4 concentrations

were positive at all 11 stages of gestation studied between d 20 and 100. Conversely, A-V differences in E_1 and E_2 were negative at all stages of gestation. These data were interpreted to suggest that P_4 (of luteal origin) was being taken up and/or metabolized by the pregnant uterus and/or its contents.

Dueben et al. (1977, 1979) demonstrated the conversion of ^3H-P_4 to androstenedione, T, E_1, E_2 and conjugated E_1 and E_2 by the endometrium of pregnant gilts. In addition, Henricks and Tindall (1971) reported that P_4 ^{14}C was metabolized in vitro by the porcine endometrial tissue to at least ten metabolities, of which two have been identified as 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one. Thus, their results indicated that porcine endometrial tissue contained 3β- and 20α-HSD and 5α- and 5β-reductase enzyme activity. Craig (1982) determined that 3β-HSD is present in the endometrium on d 17 of gestation, and at every stage of pregnancy examined thereafter.

A third line of evidence to suggest that the endometrium is capable of P_4 synthesis is from a series of studies in which gilts were bilaterally ovariectomized on d 4 of pregnancy and treated with either 20, 50, 100 or 200 mg P_4/d to maintain pregnancy until either d 40 (Knight et al., 1974) or d 60 (Bazer et al., 1979). Gilts which were preg-

nant had plasma P_4 concentrations ranging from 7 to 26.5 ng/ml. However, nonpregnant gilts had P_4 concentrations of 163 to 428 ng/ml. Thus, events associated with establishment of pregnancy have a marked effect on P_4 metabolism by the uterus and/or conceptuses.

Dalton and Knight (1983) reported a positive UA-UV difference in P_4 concentration at d 50 of gestation following exogenous P_4 and E_1 supplementation for six different time intervals during gestation and in three control groups, again suggesting metabolism of P_4 within the uterus thereby providing steroid precursors for steroidogenesis.

Finally, the steroidogenic capabilities of the endometrium in late pregnancy were examined by Craig (1982). Endometrial tissue samples obtained from d 100 pregnant gilts were incubated with tritium labeled precursors, P_5, P_4 and 17α-hydroxyprogesterone. Craig (1982) reported that the major products of P_4 metabolism by the endometrium were 5β-reduced pregnanediols and pregnanetriols, together with 5β-P_5. Furthermore, the major products of A metabolism by the endometrium were 5β-reduced androstanolones and 5β-androstanediols.

Therefore, the endometrium and the blastocyst possess the steroidogenic capabilities to synthesize androgens from P_5 or P_4, and estrogens from androgens. As these 'free'

estrogens move towards the maternal circulation, they are acted upon by 17β-HSD and sulphotransferase in the uterine endometrium and converted to E_1SO_4. Therefore, E_1SO_4 is the primary estrogen in maternal plasma (Robertson and King, 1974) and allantoic fluid (Dueben et al., 1979). This concept explains the high unconjugated estrogen concentration acting locally at the interface between blastocyst and endometrium with no systemic effects due to high concentrations of 'free' estrogens (Bazer et al., 1982). Therefore, after exerting their local effect, but before leaving the uterus, the unconjugated estrogens are conjugated in the endometrium and myometrium, and secreted into maternal circulation as estrogen sulphoconjugates (George and Wilson, 1978).

Based on concentrations of E_1SO_4 in maternal plasma, estrogen secretion by pig conceptuses is triphasic with major detectable increases between d 10 and 12 (Stoner et al., 1981), d 16 and 30 (Robertson and King, 1974; Stoner et al., 1981) and d 60 to term (Robertson and King, 1974; Knight et al., 1977). Robertson and King (1974) reported that conjugated estrogens in the peripheral maternal plasma of pregnant animals increased from 60 pg/ml on d 16 to 3 ng/ml between d 23 and 30. This concentration then declined, but began a third rise about d 60 to peak values

just before parturition (Knight et al., 1977). During the second peak of E_1SO_4 production, unconjugated E_1 reached a value of only 15 pg/ml compared with a concentration of E_1SO_4 of 3 ng/ml. During the third phase of estrogen production, the concentration of E_1 was very high and similar to that of E_1SO_4. Stoner et al. (1981) reported a significant increase in E_1SO_4 concentrations in utero-ovarian vein plasma between d 11 and 12 of pregnancy followed by a decline until d 16. Furthermore, Geisert et al. (1982a) detected an increase in total recoverable E_2, E_1, estriol (E_3) and E_1SO_4 in uterine flushings of pregnant gilts as blastocysts increased in size from 5 to 8 mm in diameter to d 12 filamentous conceptuses, and then these estrogens decreased by d 14.

Porcine Placental Steroid Metabolism

It is clearly established that the maintenance of gestation in domestic farm animals and women depends on the continuous secretion of P_4, as well as estrogen, to maintain the uterus in the correct physiological condition to allow the conceptus to remain and develop in it normally (Allen, 1975). Placental production of P_4 in ewes (Denamur and Martinet, 1955), mares (Amoroso and Finn, 1962) and women (Tulsky and Koff, 1957) is sufficient to maintain gestation

after bilateral ovariectomy if the operation is carried out after d 50 in ewes, d 170 in mares and d 24 in the women. Gestation lengths in the above mentioned species are 145 , 350 and 280 d, respectively.

In the pig, maintenance of gestation depends on the continuous secretion of P_4 of luteal origin since bilateral ovariectomy or reduction of CL number below five in one step during any stage of gestation results in abortion within 2 to 3 d (du Mesnil du Buisson and Dauzier, 1957; 1959). Recently, Thomford et al. (1984) reported the maintenance of pregnancy until term in gilts following a stepwise reduction of the number of CL to 1 to 3. Progesterone was decreased 24 h after reduction of CL but returned to an intermediate level between pre- and post-reduction. They suggested several reasons for the resurgence in the level of P_4, one reason being P_4 was produced by an extraovarian source such as the fetus or placenta.

Christie (1968) and Dufour and Raeside (1969) provide histochemical evidence for the presence of Δ^5-3β- and 17β-HSD in placental tissue from the fourth week of gestation until term. Bloch and Newman (1966) in an earlier study, demonstrated the conversion of DHEA to A by the porcine placenta, suggesting the presence of an active Δ^5-3β-HSD system.

Ainsworth and Ryan (1966) demonstrated the in vitro conversion of ^{3}H-DHEA and ^{14}C-A to estrogens by porcine placental tissue obtained at d 112 of gestation, suggesting the presence of an active aromatase enzyme system. Furthermore, Choong and Raeside (1974) reported the presence of high concentrations of unconjugated and conjugated estrogens in placental tissue.

Knight et al. (1977) and Kukoly (1984) showed high concentrations of unconjugated estrogens in allantoic and amniotic fluid. Knight et al. (1977) reported that from d 60 to 100 of gestation, the concentration of estrone present in allantoic fluid increased from .9 ng/ml to 537.7 ng/ml. Kukoly and Knight (1984) reported an increase from 2.65 ng/ml to 2077 ng/ml during the same time period. In both studies, the E_1 concentration in allantoic fluid greatly exceeded the concentration of E_1 in maternal circulation.

Craig (1982) studied the synthesis and metabolism of P_4 and E_1 from tritium-labeled P_5, P_4, 17α-hydroxyprogesterone and A by the porcine placental tissue taken late in gestation. He reported that the activity of placental 17α-hydroxalase, C-17,20-lyase and aromatase increased between d 100 of gestation and term. Thus, the placenta possesses all enzymes necessary for the synthesis of estrogens from P_5 by the Δ^4 pathway.

Steroidogenic Effect of Human Chorionic Gonadotrophin

Human chorionic gonadotrophin (hCG) is a glycoprotein hormone produced by the syncytial trophoblast of the human placenta (Josimovich, 1973). It is comprised of two nonidentical peptide chains, designated alpha (α) and beta (β), which are attached noncovalently in the active hormone, but are biologically inactive as individual free subunits (Swaminathan and Bahl, 1970). The amino acid sequence of the α subunit of hCG is similar to the α subunit of human luteinizing hormone (hLH), porcine LH (pLH), ovine LH (oLH) and bovine LH (bLH) (Hafez, 1980). However, hormone specificity of each is determined by its distinctive β subunit. Human chorionic gonadotrophin is primarily LH-like in biological activity and it will invariably induce hypertrophy and luteinization of the interstitial and thecal cells in the ovaries of rats and mice (Albert and Derner, 1960). The precise physiologic role of hCG in human pregnancy is not fully understood. During the initial 6 to 8 weeks of pregnancy, hCG acts as a luteotropin, maintaining P_4 secretion by the CL. Progesterone produced by the CL promotes morphological and biochemical changes in the endometrium in preparation for implantation (Klopper and Fuchs, 1977). Placental hCG secretion insures P_4 production by the CL until the fetus and placenta assume steroid production at a later

stage in pregnancy (Siiter et al., 1974). Human chorionic gonadotrophin promotes several stages of steroidogenesis in the fetus and placenta. First, the conversion of cholesterol to P_4 is stimulated by hCG. Second, Lauritzen and Lehman (1965) have proposed that hCG stimulates the synthesis of DHEA by the fetal adrenal. Finally, hCG appears to stimulate the conversion of androgens to estrogens in the placenta via aromatization.

In a series of in vitro perfusion studies using human placental tissue, Troen and Gordon (1958) and Troen (1961) demonstrated that hCG stimulates general placental metabolism as measured by citrate utilization and aromatization of neutral and phenolic steroids. In addition, hCG promotes the conversion of E_2 to E_3.

However, Bedwani and Marley (1972), using guinea-pig placenta reported no significant effect of hCG on P_4 production by placental slices from intact or ovariectomized guinea pigs.

In a recent study, Onthank (1983) incubated ovine placenta tissue obtained from d 80 and d 115 of gestation with hCG in the presence or absence of P_5. Onthank (1983) reported that hCG had no effect on P_4 accumulation in the incubation medium.

Effect of Cyclic 3',5 Adenosine Monophosphate on Steroidogenesis

Cyclic 3',5' adenosine monophosphate (cAMP) has been called the second messenger due to its role as an intracellular mediator of the actions of a variety of amine and polypeptide hormones (Sutherland and Rall, 1960). The sequence of events leading to steroidogenesis in the porcine CL (Andersen et al., 1974) involves the binding of LH to specific cell membrane receptors and that interaction activates the enzyme adenylate cyclase, which leads to the production of cyclic AMP. Cyclic AMP, in turn, activates cAMP-dependent protein kinase, which catalyzes phosphorylation of a number of proteins in the cytoplasm and nucleus. When phosphorylated, the enzyme cholesterol esterase converts cholesterol esters into free cholesterol (Behrman and Armstrong, 1969). The entry of this increased amount of cytoplasmic cholesterol into mitochondria is a rate-limiting step (Mahefee et al., 1974; Robinson et al., 1975). A carrier protein facilitates the entry of cholesterol into mitochondria and a group of enzymes in the mitochondria cleave the side chain of the cholesterol molecule and converts the steroid to Δ^5-prenenolone (Gill, 1972). Pregnenolone formed in the processes of steroidogenesis passively diffuses from the mitochondria and is converted to P_4 or 17-hydroxypregnenolone in the microsomes. Additional trans-

formations occur to the extent that preformed enzyme systems are present in the cell.

As previously mentioned, the limiting step in the steroidogenic pathway and the site of LH and cyclic AMP action is between cholesterol and P_5 (Ichii et al., 1963; Halland Koritz, 1965; Hall and Young, 1968). There are, however, several ways by which cyclic AMP could carry this out. It could bring about (a) an increase in the amount of a cofactor such as nicotinamide adenine dinucleotide phosphate (NADPH), (b) an increase in the concentration of substrate cholesterol, (c) an increase in the availability of this cholesterol to the side chain cleavage enzyme by promoting its transport into the mitochondrion, (d) an activation of the cleavage systems, or (e) a decrease in a restraining influence on this enzyme system, perhaps by enhancing transport of an end product inhibitor, such as P_5, out of the mitochondrion (Marsh, 1976).

Marsh and Savard (1964) reported that the addition of exogenous cyclic AMP to incubating slices of bovine CL caused a significant stimulation of P_4 synthesis in terms of mass, acetate and cholesterol incorporation. Hall and Koritz (1965) conducted a similar experiment and demonstrated that cyclic AMP stimulated P_4 synthesis by accelerating a step between cholesterol and P_5.

LeMaire et al. (1971) reported a stimulation of P_4 synthesis in incubating slices of a human CL of ectopic pregnancy. Furthermore, the effect of the addition of exogenous cyclic AMP exceeded that of hCG. The greater effectiveness of cyclic AMP over hCG may be related to the fact that human CL of pregnancy have a diminished capacity to bind human LH or hCG (Cole et al., 1973; Rao et al., 1975) than CL of the menstrual cycle. Therefore, if hormone-induced ovarian refractoriness was at the receptor or cyclase level, then the addition of cyclic AMP would be able to overcome that block.

In addition to P_4 stimulation by cyclic AMP in luteal tissue, Cedard et al., (1970) observed that cyclic AMP stimulated the conversion of labeled testosterone to estrogens in perfused placenta. Dëmers et al. (1973) reported increased cyclic AMP concentrations when placental preparations were incubated with hCG, and Menon and Jaffe (1973) reported that hCG stimulated adenylate cyclase in a minced placental preparation.

However, Catt and Dufau, (1973) and Moyle and Ramachandran (1973) reported that low concentrations of LH can elicit a stimulation of steroidogenesis in other tissues without a detectable change in cyclic AMP. This observation might have been due to the fact that cyclic AMP was confined

to a small compartment of the cell, such as the regulatory subunit of protein kinase.

Onthank (1983) observed an increase in P_4 production by ovine placental tissue collected at d 115 of gestation and incubated with both P_5 and cAMP. However, in the absence of P_5, cAMP-treated d 115 ovine placental tissue did not accumulate greater concentrations of P_4 relative to ovine placental samples incubated in the absence of both P_5 and cAMP.

MATERIALS AND METHODS

A total of 24 sexually mature crossbred gilts of similar age (10 to 14 mo), weight (108 to 136 kg), and genetic background (Hampshire x Yorkshire x Duroc) were utilized in two studies. Gilts were assigned to these studies after they had exhibited at least two estrous cycles of normal duration (18 to 22 days). Gilts were bred 12, 24 and 36 h after the detected onset of estrus by either natural service or artificial insemination. At least two boars were used during all mating periods. The first day of estrus was designated as d 0. After breeding, each gilt was randomly assigned to be bilaterally hysterectomized at either d 30, 60 or 90 of gestation. Eight gilts were utilized at each of the three stages of gestation. Days 30, 60 and 90 of gestation were chosen as the periods of examination based upon the results of a previous study in our laboratory (Kukoly, 1984) in which the dynamic changes in progesterone (P_4) and estrone (E_1) production by the placenta were characterized at 12 stages of gestation between 20 and 110 d of gestation. Kukoly (1984) reported that placental production of P_4 rose in a linear manner from d 25 (4.65 ng/g of tissue) to d 40

(39.31 ng/g), plateaued between d 40 and d 50 and continued to rise from d 60 until peak levels at d 100 of gestation (231 ng/g).

Kukoly (1984) demonstrated that placental E_1 production exhibited a biphasic pattern which mimiced the pattern seen in fetal fluids and maternal blood pools. An early peak of E_1 at d 30 (3.47 ng/g of tissue) was followed by a decrease to .45 ng/g at d 35. A second E_1 increase began at d 70 and continued through d 110 of gestation (127.2 ng/g). Thus, d 30, 60 and 90 were chosen as the timepoints for further examination based upon the substantial and dynamic changes in P_4 and E_1 production by the porcine placenta previously observed during these periods.

Surgical and Data Collection Procedures

Gilts were not fed for 24 h prior to surgery. At surgery, initial anesthesia was induced with 1 to 1.5g of a 5% sodium thiopental solution (Abbott Labs, North Chicago, IL) injected into the ear vein using a 20 gauge needle attached to polyethylene tubing. A surgical plane of anesthesia was maintained with halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) in combination with a mixture of nitrous oxide and oxygen. During surgery, a blood sample was obtained from the anterior vena cava utilizing a 15 gauge

needle and a syringe containing 13.8% disodium ethylenediamine tetraacetate (EDTA) as an anticoagulant. Additional blood samples were collected from a uterine vein, a uterine artery and from the umbilical vein at d 90. All blood samples were centrifuged at 2000 xg for 10 min, decanted and stored at -20 C until analyzed for steroid concentrations via radioimmunoassay (RIA).

The reproductive tract was exposed following a mid-ventral laparatomy. The ovaries, uterine horns, uterine body and a portion of the cervix were surgically removed from the peritoneal cavity as previously described by Knight et al. (1977) and Dalton and Knight (1983). Following hysterectomy, the ovaries, oviducts and connective tissue were dissected from the uterus. The uterus was dissected along the mesometrial border and at least two conceptuses were carefully removed. An uncontaminated 15 ml sample of allantoic and amniotic fluid (except in the 30 d conceptus) was aspirated from each of the conceptuses with an 18 gauge needle attached to a 20 ml syringe. Fluid samples were labeled for each fetus and stored at -20 C until time of assay.

The placental membranes were carefully detached from the uterus as previously described by Dalton and Knight (1983) and Kukoly (1984). The chorioallantoic membrane was separated from the amnion and random sections from the

entire chorioallantois were immediately placed into a sterile beaker containing Medium 199 with Earlɔs Salts (M199, 37 C, Appendix Table 1, Gibco Laboratories; Grand Island, NY). Placental tissue samples obtained from one male and one female fetus were utilized at d 60 and 90. Kukoly (1984) demonstrated that sex of fetus did not significantly affect placental production of either P_4 or E_1. At d 30, due to the inability to determine the sex of the fetus by visual appraisal and to insure that a sufficient amount of tissue was present for incubation, three placental membranes were collected.

Endometrial tissue was carefully stripped from the uterus adjacent to the sites from which placental tissue was taken. The endometrial tissue was immediately placed into another sterile beaker containing M199 at 37 C. Placental and endometrial tissue samples were immediately transported to the lab for incubation.

In Vitro Incubations

Placental and endometrial tissue samples were then transferred to fresh M199 and minced into 1 to 2 mm^3 pieces with a sterile razor blade within 1 h after collection. The tissue samples were incubated in duplicate 300 mg samples of either a) placenta, b) endometrium or c) a combination of

placenta (150 mg) and endometrium (150 mg). Each tissue sample was weighed on a torsion balance, transferred to a culture tube containing 3 ml M199, placed in a Dubnoff water bath-shaker at 37 C and incubated in an atmosphere of 95% O_2:5% CO_2 for either 0 (control), .5, 1 or 2 h. At the end of the incubation period, the tubes were tightly capped and immediately placed in an icebath and subsequently frozen (-20 C) until assayed.

In experiment 1, the tissue samples were incubated in M199 either with or without the steroid precursor pregnenolone (P_5, 56 ng/ml M199). A total of 576 samples were generated (3 days of gestation x 4 gilts x 4 incubation periods x 3 types of tissue x 2 types of medium x 2 replicates). The M199 with P_5 will be referred to as M199-P_5 (Appendix Table 2).

In experiment 2, all of the tissue samples were incubated in a medium containing P_5 plus either human Chorionic Gonadotropin (hCG, 56 ng/ml + 100 I.U./ml) or P_5 plus N^6, O^2-Dibutyryladenosine 3', 5'-Cyclic Adenosine Monophosphate (cAMP, 56 ng/ml + 1.26 mg/ml, Sigma Chemical Company, St. Louis, MO). The M199-P_5 with either hCG or cAMP will be referred to as M199-P_5-hCG or M199-P_5-cAMP, respectively (Appendix Tables 3 and 4). In addition, in one gilt at d 30, 60 and 90 of gestation, an extra set of 2 h tissue sam-

ples were incubated (3 types of medium x 3 types of tissue x 2 replicates). At the end of the 2 h incubation period, 1.5 ml of 100% ethanol was added to each tube of the extra set of tissue samples. The extra set was included to compare the steroid production of the tissue samples in which the reactions were stopped by freezing with the steroid production of the tissue samples immediately inactivated by ethanol. Including the extra sets of 2 h tissue samples, a total of 918 samples were generated.

Protein Determination

As described by Kukoly (1984), the total protein content of the incubated tissue samples was determined due to the fact that it was not always possible to fully isolate the allantochorion from the nonvascularized amnion. Hence, it was suspected that wet tissue weight might not be an accurate measure of the quantity of tissue present which was capable of hormone production.

The total protein was determined by the Bio-Rad microassay, a dye binding technique described by Bradford (1976). The protein determination assay involved the binding of Coomassie Brilliant Blue-G-250 (Sigma Chemical Company, St. Louis, MO) to protein to form a protein-dye complex. The binding of the dye to protein occurs within

approximately 2 min and the protein-dye complex remains dispersed in solution for approximately 1 h.

Incubated tissue samples were thawed at 4 C and centrifuged (1200 xg) for 20 min to separate the tissue sample from the incubation medium. The medium was poured into a 13 x 100 polyethylene culture tube, capped tightly and restored at -20 C until time of assay. Five milliliters of saline were added to each tissue sample. Placental tissue samples were homogenized for two 10s pulses and endometrial and coincubation tissue samples were homogenized for two 15s pulses with a Brinkmann Polytron Homogenizer to insure cell disruption.

Bovine serum albumin (Sigma Chemical Company, St. Louis, MO) solution containing 10 to 100 µg of protein served as the protein standard. A volume of .1 ml of standard or homogenized tissue sample (in duplicate) was pipetted into 12 x 100 disposable culture tubes. Five ml of the protein reagent was added to the test tubes and the contents were mixed by vortexing. The absorbence was measured using a Gilford Spectrophotometer 250 at a wavelength of 595 and a linear regression line was calculated to estimate tissue protein.

Radioimmunoassays Procedures

Plasma, fetal fluid and incubated medium hormone concentrations were quantified utilizing a slight modification of radioimmunoassay procedures described by Abraham et al. (1971) for progesterone, Chung-Hsiu Wu et al. (1971) for estrone and Falvo et al. (1974) for testosterone. All assays were validated in our laboratory.

Preliminary assays of d 30, 60 and 90 samples were run to determine the approximate concentrations of P_4, E_1 and testosterone (T) in plasma and incubation medium samples. The established concentrations served as guidelines to determine the volume of incubation sample needed for extraction and the appropriate size aliquot necessary to ensure values which read on the standard curve. Data from these initial assays indicated that only trace amounts of T could be measured (table 1); therefore, the measurement of T in remaining samples was deleted. In addition, RIA for estradiol-17β in incubated medium was not conducted due to the large number of samples generated and based upon several lines of evidence indicating that while the pattern of E_2-17β mimics that of E_1, it is found in significantly lower concentrations throughout gestation than is E_1 (Robertson and King, 1974; Knight et al., 1977).

TABLE 1 LEAST-SQUARES MEANS OF TESTOSTERONE (ng/g ± S.E.) PRODUCTION FOLLOWING THE INCUBATION OF FETO-MATERNAL TISSUE IN EITHER PREGNENOLONE, PREGNENOLONE + hCG OR PREGNENOLONE + cAMP AT 30, 60 AND 90 DAYS OF GESTATION

Day of gestation	Treatment	Tissue[a]					
		N	Placenta	N	Coincubation[b]	N	Endometrium
30	Pregnenolone	8	.27 ± .03	8	.20 ± .06	8	.22 ± .08
	Pregnenolone + hCG	8	.24 ± .06	8	.16 ± .04	8	.23 ± .04
	Pregnenolone + cAMP	8	.25 ± .03	8	.19 ± .07	8	.18 ± .06
60	Pregnenolone	8	.10 ± .02	8	.06 ± .04	8	.09 ± .05
	Pregnenolone + hCG	8	.06 ± .03	8	.09 ± .03	8	.10 ± .03
	Pregnenolone + cAMP	8	.08 ± .04	8	.07 ± .04	8	.09 ± .03
90	Pregnenolone	8	.23 ± .1	8	.30 ± .05	8	.27 ± .06
	Pregnenolone + hCG	8	.25 ± .03	8	.32 ± .09	8	.21 ± .08
	Pregnenolone + cAMP	8	.30 ± .05	8	.25 ± .06	8	.25 ± .03

[a]Significant ($P < .001$) day of gestation effects.
[b]Combination of 150 mg of placenta and 150 mg of endometrium.

Progesterone Radioimmunoassay

Samples were thawed at 4 C and following addition of approximately 1000 cpm of tritiated progesterone (1, 2, 6,7-^{3}H(N)-P_4; Amersham, Arlington Heights, IL) to compensate for procedural losses, they were extracted twice with 3 ml certified petroleum ether (Fisher Scientific, Raleigh, NC). The extraction tubes were quick frozen (-70 C) and the petroleum ether decanted into a clear 13 x 100 mm tube and evaporated to dryness under air. The extracted samples were redissolved with methylene chloride:methanol (9:1, Fisher Scientific, Raleigh, NC) for aliquoting. Antibody, supplied by W. Hansel (Cornell University, Ithaca, NY) and tested at a dilution of 1:6000 (table 2), was added along with ^{3}H-P_4 to assay tubes in volumes of 100 µl. All tubes were incubated for 18 h at 4 C. A dextran-coated charcoal suspension was used to separate free and bound P_4. Intra- and inter-assay coefficients of variation were 9.39% and 7.16%, respectively. Mean extraction efficiencies determined by recovery of labelled P_4 were 88.3%. The concentration of P_4 in each sample was adjusted from ng/ml of medium to ng/gm of incubated tissue.

Table 2

CROSSREACTIVITY OF PROGESTERONE ANTIBODY WITH VARIOUS STERIODS [a]

Steroid	Percent Cross Reaction[b]
Progesterone	100
5α-dihydroprogesterone	1.97
5β-dihydroprogesterone	11.64
20α-OH-progesterone	1.90
20β-OH-progesterone	2.64
Pregnenolone	0.09
17α-OH-progesterone	3.22
Corticosterone	0.96
Cortisol	0.01
Estrone	<0.01
17β-estradiol	<0.01
Estriol	<0.01
17α-estradiol	<0.01
Androstenedione	0.01
5α-dihydrotestosterone	<0.01
dehydroepiandrostenedione	<0.01
Testosterone	0.03
Cholesterol	<0.01

[a] Beal et al., 1980

[b] Mass of progesterone required to inhibit 50% binding divided by mass of steriod required to inhibit 50% binding x 100.

Antibody dilution 1:6000.

Estrone Radioimmunoassay

The antibody used to measure E_1 in this study (kindly provided by R. B. Staigmiller, USDA Agricultural Experimental Station, Miles City, MT) was subsequently validated in our laboratory for use with porcine serum and M199. The antibody, tested at a dilution of 1:8500 was found to be highly specific with only a slight (11.2%) crossreactivity with estrone sulphate (table 3).

The extraction procedure was similar to the P_4 extraction procedure. Approximately 1000 cpm of tritiated estrone (E_1 (2, 4, 6, 7-^{3}H(N)), New England Nuclear, Boston, MA) were added to correct for procedural losses. Samples were extracted twice with 5 ml hexane:ethylacetate (3:1; HPLC grade, Fisher Scientific, Raleigh, NC). The hexane:ethylacetate was quick frozen (-70 C) and decanted into a clean 13 x 100 tube and evaporate to dryness under air. The extracted samples were redissolved with methylene chloride:methanol (9:1). Antibody and ^{3}H-E_1 were added to assay tubes in volumes of 100 µl. A dextran-coated charcoal suspension was used to separate free and bound E_1. Intra- and inter-assay coefficients of variation were 9.61% and 18.92%, respectively. Mean extraction efficiencies determined by recovery of labelled E_1 were 86.2%. The concentration of E_1 in each sample was adjusted from ng/ml to ng/gm of incubated tissue.

Table 3

CROSSREACTIVITY OF ESTRONE ANTIBODY WITH VARIOUS STEROIDS

Compound	Percent Cross Reaction[a]
Estrone	100
Estrone-3-sulphate	11.2
16-Ketoestradiol	<.1
16-Epiestriol	<.1
Pregnanetriol	<.1
Reichstein's Substance	<.1
17α-Hydroxyprogesterone	<.1
5β-pregnan-3,20-dione	<.1
Cortisone	<.1
16-17, Epiestriol	<.1
Δ^4 Androsten-3, 17-dione	<.1
5-α-pregnane-3,20-dione	<.1
DHEA	<.1
17α-Ethynlestradiol	<.1
DHT	<.1
19-Nortestosterone	<.1
Estriol	<.1
17α-Estradiol	<.1
D-Equilenin	<.1

[a]Using an antibody dilution of 1:8500. Mass of estrone required to inhibit 50% binding divided by mass of steroid required to inhibit 50% binding x 100.

Statistical Analysis

The data for progesterone and estrone levels were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1982). The preliminary analyses, incorporating the protein content of the tissues as a continuous variable, indicated that progesterone concentrations at d 30, 60 and 90 and estrone concentrations at d 90 were significantly influenced by protein content of the tissues. Therefore, the hormone values at each day of gestation were preadjusted for differences in protein content using the formula previously described by Kukoly (1984):

Adjusted hormone (ng/g) = hormone (ng/g) - regression coefficient (protein (mg/g) - mean protein (mg/g)).

The following statistical model was used to analyze the data:

$$Y_{ijklmn} = \mu + (DOG)_i + [GILT(DOG)]_{ij} + (TIME)_k + (TISSUE)_l + (TRT)_m + e_{ijklmn}$$

where μ is the overall mean for the tissue hormone concentration; $(DOG)_i$ is the day of gestation where i = d 30, 60 or 90; $GILT(DOG)_{ij}$ is the observation for the j^{th} gilt within the i^{th} day of gestation; $(TIME)_k$ is the effect of the k^{th} time (k = 0, .5, 1, 2 h) of incubation; $(TISSUE)_l$ is

the effect of the l^{th} tissue (l = placenta, placenta + endometrium, endometrium); $(TRT)_m$ is the effect of the m^{th} treatment effect (without P_5, with P_5, P_5 + hCG, P_5 + cAMP) and e_{ijklmn} is the random error associated with the observation of the m^{th} treatment on the l^{th} tissue within k^{th} time of j^{th} gilt within i^{th} day of gestation. The effect of DOG was tested using mean squares for GILT(DOG) as its error term. Day of gestation , tissue, time and treatment were assumed to be fixed effects, whereas GILT(DOG) was considered as random.

The analysis revealed a large variability among TRT, TIME and TISSUE means. Thus, the data were re-analyzed within DOG and TISSUE subclasses using a reduced model having only the effect of time and treatment as fixed and gilt as random.

In order to reduce the scaling effect in hormone production by the incubated tissue samples at d 30, 60 and 90, logarithmic transformations were used to stabilize the variance. The means were positively correlated with the variances (greater means were accompanied by greater variances), and the logarithmic transformation made the variances independent of the means. Tests for significance were also performed using the logarithmic scale. However, the least square means reported are the original scale of measurement.

Dunnett's comparison test (Steel and Torrie, 1960) was used to test the differences in hormonal concentrations at each time period from their respective time zero (control).

Data for gilt plasma hormone concentrations and fetal fluids were also analyzed using a GLM procedure for day of gestation effects. The appropriate least squares means were obtained from this analysis.

RESULTS

Protein Content of Incubated Tissue Samples

Mean protein content for placental and endometrial tissue samples incubated in combination and separately at d 30, 60 and 90 of gestation is depicted in figure 3. Kukoly (1984) observed an overall increase in placental tissue protein content throughout gestation. Likewise, the protein content of placental tissue in this study increased from d 30 to 60 and 90 of gestation. Furthermore, mean protein content for coincubation tissue increased gradually between d 30 (2.00 mg/g) and d 60 (2.76 mg/g) and between d 60 and d 90 (3.03 mg/g). Endometrial protein content increased between d 30 (3.52 mg/g) and d 60 (4.57 mg/g), but decreased at d 90 (4.21 mg/g).

Tissue protein concentrations were determined in order to adjust for variation in tissue samples due to errors in tissue sampling. Preliminary analyses, after incorporating the protein content of the tissues as a continuous variable, revealed significant protein content effects for progesterone production by placental ($P < .0001$), coincubation ($P < .001$) and endometrial ($P < .0001$) tissue samples at d 30, 60

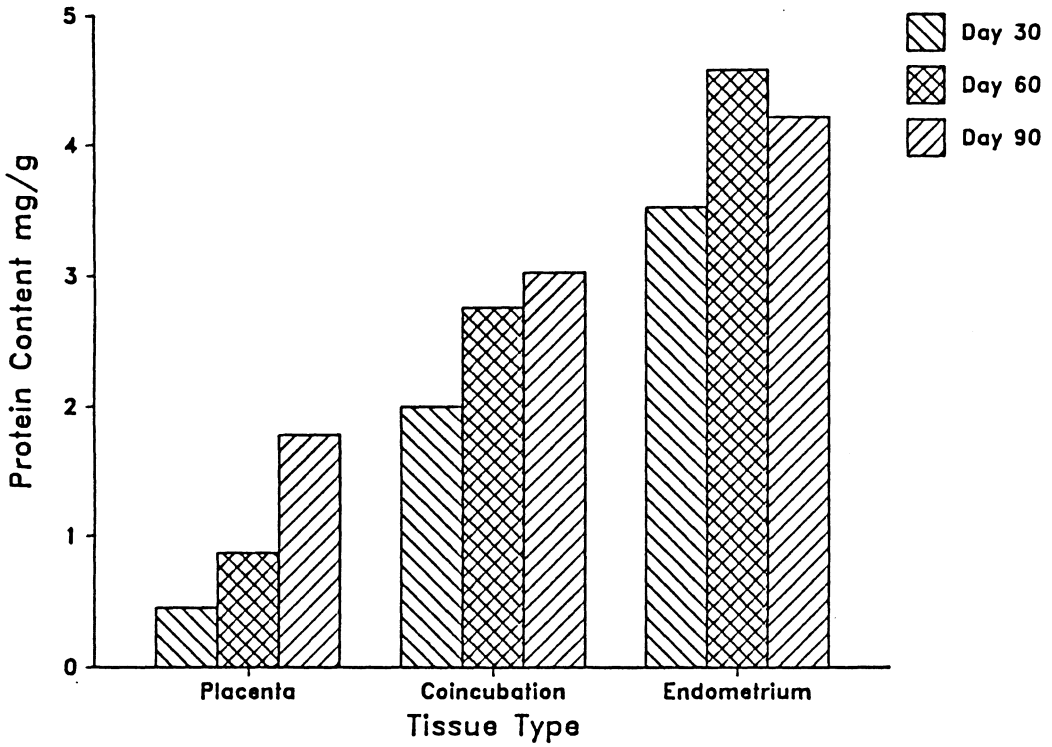

Figure 3. Protein content for porcine placental, coincubation and endometrial tissue at 30,60 and 90 days of gestation.

and 90 of gestation and estrone concentrations by placental ($P < .01$), coincubation ($P < .001$) and endometrial ($P < .001$) tissue samples at d 90 of gestation. Therefore, in order to decrease the error term, progesterone and estrone concentrations in the medium were adjusted to the mean protein content for each tissue within each day of gestation using individual coefficients of regression in the previously described formula.

Experiment One:

In Vitro Progesterone Synthesis by Feto-maternal Tissue in the Presence or Absence of Pregnenolone at 30, 60, and 90 Days of Gestation.

Results from least squares analyses of variance for P_4 concentration in placental, coincubation and endometrial tissue samples collected at d 30, 60 and 90 of gestation and incubated in the absence or presence of P_5 are presented in table 4. At d 30 of gestation, significant gilt effects were detected for placental ($P < .001$), coincubation ($P < .01$) and endometrial ($P < .001$) tissue samples incubated with or without P_5, indicating that P_4 production by feto-maternal tissue collected at d 30 of gestation varies among gilts. However, significant gilt effects were only observed for d 60 placental tissue samples ($P < .05$) and d 90 placental ($P < .001$) and coincubation tissue samples ($P < .001$).

TABLE 4 LEAST-SQUARES ANALYSES OF VARIANCE FOR THE EFFECTS OF INCUBATION TIME AND PREGNENOLONE TREATMENT ON PROGESTERONE PRODUCTION BY FETO-MATERNAL TISSUE OF GILTS AT 30, 60 AND 90 DAYS OF GESTATION

Day of gestation	Source of variation	df	Tissue: Mean squares: Placenta	Tissue: Mean squares: Coincubation[a]	Tissue: Mean squares: Endometrium
30	Gilt	3	288.88***	184.95**	395.84***
	Time	3	161.40***	.59	60.93
	Treatment	1	517.89***	319.09***	114.01*
	Time x treatment	3	49.35	3.56	4.65
	Error	53	18.73	42.50	56.23
60	Gilt	3	511.09*	173.75	199.72
	Time	3	22960.78***	1632.78***	581.93***
	Treatment	1	15551.50***	1859.28***	786.12**
	Time x treatment	3	1224.19*	485.16*	448.96**
	Error	53	220.62	138.41	91.08
90	Gilt	3	11874.67***	5152.83***	64.63
	Time	3	11190.53***	2444.31	15.33
	Treatment	1	9100.77**	12999.70***	1748.98***
	Time x treatment	3	2060.76	829.87	46.33
	Error	53	1613.79	575.42	46.57

[a]Combination of 150 mg of placenta and 150 mg of endometrium.
*P < .05.
**P < .01.
***P < .001.

Table 5 contains the least squares means for the effect of P_5 on P_4 synthesis by placental, coincubation and endometrial tissue samples at d 30, 60 and 90 of gestation.

Progesterone synthesis by placental tissue collected at d 30, 60 and 90 of gestation was significantly higher when P_5 was added to the incubation medium (table 5). The pooled effect of length of incubation on P_4 synthesis by P_5-treated and untreated placental tissue collected at d 30, 60 and 90 of gestation was significant ($P < .001$) (figure 4). At d 30 of gestation, during the first .5 h of incubation, P_4 concentrations in the medium rose slightly from 7.73 to 8.81 ng/g, continued to rise during the next .5 h to 9.98 ng/g and finally to 14.92 ng/g after 2 h. Placental tissue collected at d 60 of gestation was capable of synthesizing larger quantities of P_4 than tissue collected at d 30 over the duration of the incubation period. In d 60 samples, pooled P_4 concentrations rose from 18.05 (control) to 41.95 ng/g (.5 h), 66.50 ng/g (1 h) and 106.79 ng/g (2 h). In d 90 placental samples, the pattern of P_4 synthesis exhibited throughout the incubation period was not as dramatic as that of d 60 samples; however, there was a gradual increase in P_4 accumulation in the medium from 108.62 ng/g to 122.53 ng/g during the first 1 h of incubation and finally to 163.70 ng/g at the end of the incubation period (figure 4).

TABLE 5 LEAST-SQUARES MEANS OF PROGESTERONE (ng/g ± S.E.) PRODUCTION FOLLOWING INCUBATION OF FETO-MATERNAL TISSUE IN THE ABSENCE OR PRESENCE OF PREGNENOLONE AT 30, 60 AND 90 DAYS OF GESTATION[a]

Day of gestation	Treatment	Tissue[b]		
		Placenta	Coincubation[c]	Endometrium
30	Absence of pregnenolone	7.52 ± .77	10.01 ± 1.15	13.33 ± 1.33
	Presence of pregnenolone	13.21 ± .77***	14.47 ± 1.15***	16.03 ± 1.33*
60	Absence of pregnenolone	42.73 ± 2.62	22.31 ± 2.08	15.95 ± 1.69
	Presence of pregnenolone	73.91 ± 2.62***	33.59 ± 2.08***	22.96 ± 1.69**
90	Absence of pregnenolone	113.52 ± 7.10	49.43 ± 4.24	6.65 ± 1.20
	Presence of pregnenolone	137.37 ± 7.10**	77.94 ± 4.24***	17.12 ± 1.20***

[a]Treatment effects tested within each tissue at 30, 60 and 90 days of gestation.
[b]*P < .05; **P < .01; ***P < .001.
[c]Combination of 150 mg of placenta and 150 mg of endometrium.

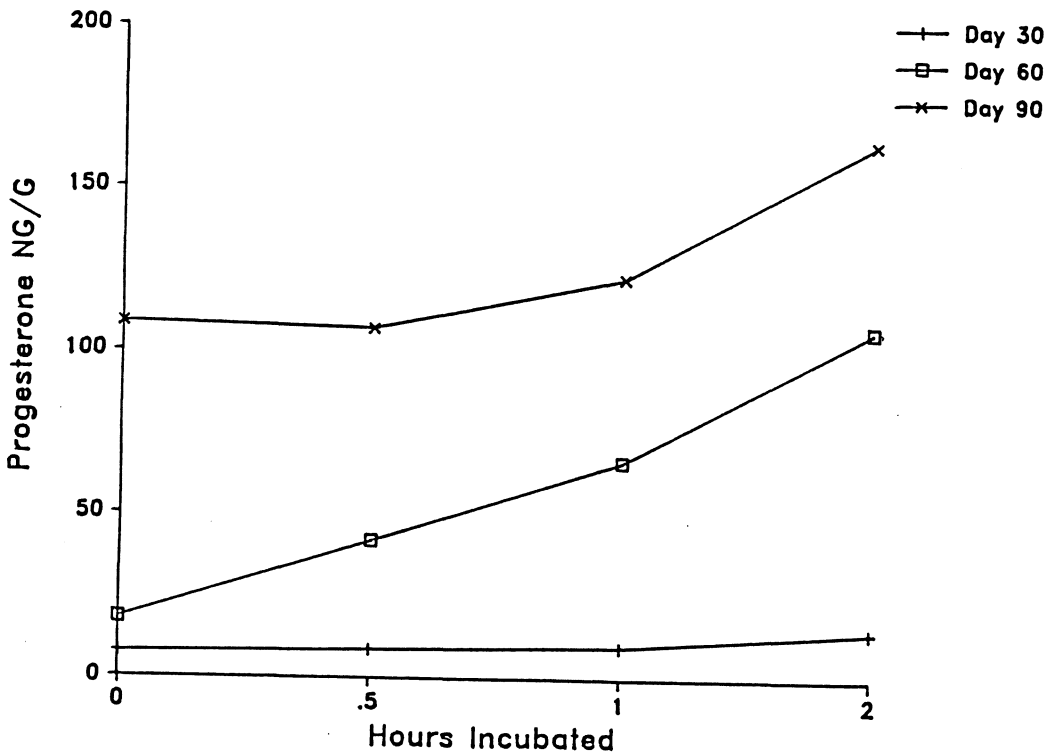

Figure 4. Combined mean progesterone concentrations in the medium of pregnenolone treated and untreated placental tissue collected at either day 30,60 or 90 of gestation.

The effect of P_5 treatment on P_4 production by coincubation tissue (a combination of 150 mg of placenta and 150 mg of endometrium) and endometrial tissue at d 30, 60 and 90 of gestation is also presented in table 5. In both tissue types, significant treatment effects were detected at each of the three stages of gestation examined.

In addition to significant differences attributable to the individual main effects tested within each tissue at d 30, 60 and 90 of gestation, the time by treatment interaction, tested within each tissue at d 60 of gestation was significant ($P < .05$) (table 6). In the absence of P_5, P_4 secretion by placental tissue samples increased two-fold during the first .5 h of incubation (13.72 to 27.51 ng/g). During the next .5 h and final 1 h, there was a further increase to 48.21 and 81.49 ng/g, respectively. Following the addition of P_5, P_4 concentration in the media of incubated placental tissue rose from 22.39 (control) to 56.39 ng/g (.5 h), 84.79 ng/g (1 h) and 132.08 ng/g (2 h). Thus, the presence of P_5 enhanced placental P_4 synthesis at each incubation period.

In d 60 coincubation tissue samples, P_4 synthesis in the absence of P_5 rose gradually from 14.05 (control) to 21.79 and 30.89 ng/g during the .5 and 1 h incubation periods, respectively. During the final hour, there was a

TABLE 6 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 60 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Absence of pregnenolone	13.72 ± 5.3[b]	27.51 ± 5.3[b]	48.21 ± 5.3[c]	81.49 ± 5.3[d]
	Presence of pregnenolone	22.39 ± 5.3[b]	56.39 ± 5.3[b]	84.79 ± 5.3[c]	132.08 ± 5.3[d]
Coincubation[e]	Absence of pregnenolone	14.05 ± 4.2[b]	21.79 ± 4.2[b]	30.89 ± 4.2[c]	22.51 ± 4.2[d]
	Presence of pregnenolone	13.64 ± 4.2[b]	30.36 ± 4.2[b]	43.61 ± 4.2[c]	46.75 ± 4.2[d]
Endometrium	Absence of pregnenolone	12.16 ± 3.4[b]	15.46 ± 3.4[b]	14.38 ± 3.4[b]	21.81 ± 3.4[b]
	Presence of pregnenolone	10.51 ± 3.4[b]	25.28 ± 3.4[b]	35.11 ± 3.4[c]	21.95 ± 3.4[d]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and time zero as control.
[bcd]Letters in same row with different superscripts differ from time zero ($P < .05$).
[e]Combination of 150 mg of placenta and 150 mg of endometrium.

decrease in P_4 synthesis (22.51 ng/g). In the presence of P_5, P_4 accumulation in the medium of coincubated tissue increased two-fold during the .5 h incubation time (13.64 vs 30.36 ng/g). During the next .5 h there was an increase to 43.61 ng/g and finally, at the end of the 2 h incubation period, P_4 levels of coincubated tissue in the presence of P_5 were 46.75 ng/g (table 6).

Finally, significant P_4 production by endometrial tissue incubated in the absence of P_5 was detectable at the end of the 2 h incubation period. In addition, mean P_4 production by endometrial tissue samples incubated in the presence of P_5 rose from 10.51 to 25.28 ng/g during the first .5 h. During the next .5 h there was a further increase to 35.11 ng/g, but by 2 h of incubation P_4 synthesis by endometrium had decreased to 21.95 ng/g.

Overall, P_4 secretion by placental tissue samples collected at d 30, 60 and 90 of gestation was higher ($P < .05$) following the addition of P_5 to the incubation medium. Similarly, P_4 production by coincubation tissue at each day of gestation examined was higher following the addition of P_5 ($P < .01$). However, the increase of P_4 synthesis by coincubation tissue was not as pronounced as the increase of P_4 synthesis by placental tissue. Likewise, the increase in endometrial P_4 synthesis in the presence of P_5 was smaller

than the increase in placental P_4 synthesis at d 30, 60 and 90 of gestation.

Although the time by treatment interaction tested within each tissue at d 30 and 90 of gestation was not significant, the interaction is presented in tables 7 and 8, respectively, in order to delineate the basic time trends.

TABLE 7 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 30 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Absence of pregnenolone	7.03 ± 1.5[b]	6.81 ± 1.5[b]	6.30 ± 1.5[b]	10.04 ± 1.5[b]
	Presence of pregnenolone	8.41 ± 1.5[b]	10.82 ± 1.5[b]	13.61 ± 1.5[b]	19.80 ± 1.5[c]
Coincubation[d]	Absence of pregnenolone	10.02 ± 2.3[b]	10.15 ± 2.3[b]	12.02 ± 2.3[b]	9.23 ± 2.3[b]
	Presence of pregnenolone	13.96 ± 2.3[b]	14.27 ± 2.3[b]	14.30 ± 2.3[b]	14.96 ± 2.3[b]
Endometrium	Absence of pregnenolone	10.75 ± 2.6[b]	13.49 ± 2.6[b]	14.49 ± 2.6[b]	14.01 ± 2.6[b]
	Presence of pregnenolone	12.58 ± 2.6[b]	17.09 ± 2.6[b]	16.86 ± 2.6[b]	17.69 ± 2.6[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.

[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

[d]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 8 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 90 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Absence of pregnenolone	105.19 ± 14.2[b]	83.7 ± 14.2[b]	111.35 ± 14.2[b]	146.96 ± 14.2[b]
	Presence of pregnenolone	112.03 ± 14.2[b]	130.1 ± 14.2[b]	133.69 ± 14.2[b]	180.44 ± 14.2[c]
Coincubation[d]	Absence of pregnenolone	49.81 ± 8.5[b]	56.58 ± 8.5[b]	49.96 ± 8.5[b]	47.36 ± 8.5[b]
	Presence of pregnenolone	57.95 ± 8.5[b]	79.59 ± 8.5[b]	104.91 ± 8.5[c]	63.28 ± 8.5[b]
Endometrium	Absence of pregnenolone	9.01 ± 2.4[b]	9.21 ± 2.4[b]	5.01 ± 2.4[b]	4.28 ± 2.4[b]
	Presence of pregnenolone	12.37 ± 2.4[b]	19.97 ± 2.4[b]	18.08 ± 2.4[b]	16.96 ± 2.4[b]

[a]Time x treatment effects tested within each tissue using Dunnetts test and respective time zero as control.
[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).
[d]Combination of 150 mg of placenta and 150 mg of endometrium.

In Vitro Estrone Synthesis by Feto-maternal Tissue in the Presence or Absence of Pregnenolone at 30, 60 and 90 Days of Gestation.

Least squares analyses of variance for estrone concentration in placental, coincubation and endometrial tissue samples collected at d 30, 60 and 90 of gestation in the absence or presence of P_5 are summarized in table 9. There were significant differences ($P < .05$) in placental, coincubation and endometrial estrone production among gilts at the same stage of gestation. Thus, E_1 production by embryonic and maternal tissues at d 30, 60 and 90 of gestation varies due to differences among gilts.

Estrone synthesis by placental tissue collected at d 60 and 90 of gestation was higher when P_5 was added to the placental samples (figure 5). Overall E_1 accumulation in the medium of d 60 and d 90 placental samples incubated in the presence of P_5 (vs absence) was 1.33 vs .74 ng/g and 51.72 vs 34.61 ng/g, respectively. Furthermore, only the time by treatment interaction for E_1 synthesis by d 60 placental tissue was significant ($P < .05$). In the absence of P_5, E_1 production by d 60 placental tissue did not significantly change throughout the incubation period (figure 6). However, in the presence of P_5, E_1 concentration rose from .83 to 2.42 ng/g during the first .5 h of incubation, then

TABLE 9 LEAST-SQUARES ANALYSES OF VARIANCE OF THE EFFECTS OF INCUBATION TIME AND PREGNENOLONE TREATMENT ON ESTRONE PRODUCTION BY FETO-MATERNAL TISSUE OF GILTS AT 30, 60 AND 90 DAYS OF GESTATION

Day of gestation	Source of variation	df	Tissue		
			Mean squares		
			Placenta	Coincubation[a]	Endometrium
30	Gilt	3	18.75***	12.24***	26.17*
	Time	3	.57	1.08	11.46
	Treatment	1	.74	4.05*	3.72
	Time x treatment	3	1.42	3.80	5.78
	Error	53	1.01	1.18	5.39
60	Gilt	3	6.21***	2.37***	2.54***
	Time	3	1.89	.21	.46*
	Treatment	1	5.51**	.11	.09
	Time x treatment	3	2.70*	.09	.14
	Error	53	.70	.11	.14
90	Gilt	3	11479.33***	7160.77***	279.29***
	Time	3	1502.09	1018.25*	10.28
	Treatment	1	4677.37*	135.42	46.47*
	Time x treatment	3	1814.28*	71.33	11.83
	Error	53	633.82	242.30	8.96

[a]Combination of 150 mg of placenta and 150 mg of endometrium.
*P < .05.
**P < .01.
***P < .001.

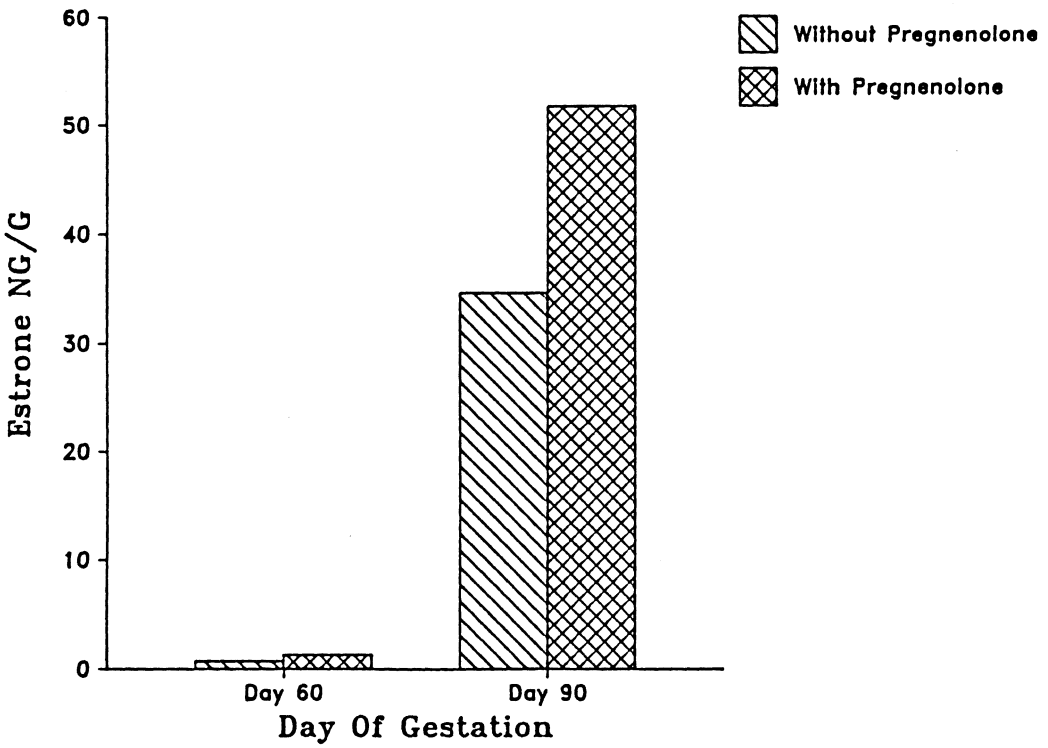

Figure 5. Mean estrone concentrations in the medium of incubated placental tissue collected at either day 60 or 90 of gestation in either the absence or presence of pregnenolone.

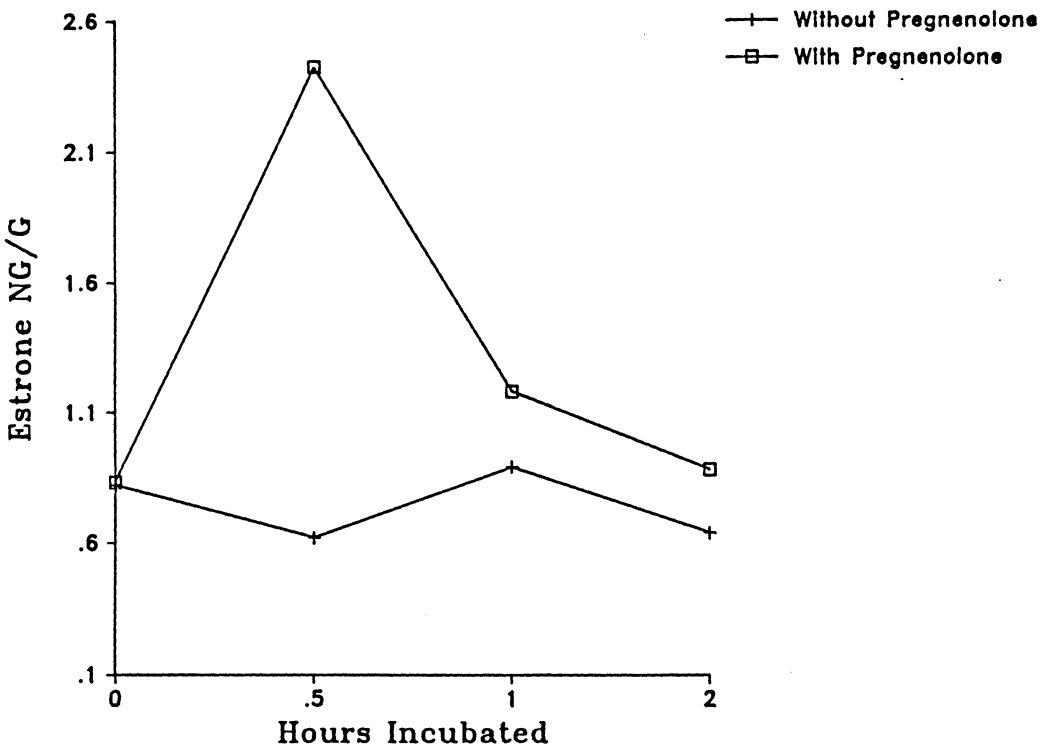

Figure 6. Mean estrone concentrations in the medium of either pregnenolone treated or untreated placental tissue collected at day 60 of gestation.

declined during the second .5 h (1.18 ng/g) and final 1 h (.88 ng/g). At the end of the 2 h incubation period, placental E_1 levels were similar to initial values.

The time by treatment interaction for E_1 synthesis by placental tissue collected at d 90 of gestation is shown in table 10. In the absence of P_5, placental E_1 synthesis increased slightly during the first .5 h of incubation and then declined during the next .5 and final 1 h (from 32.77 to 39.31 ng/g and then to 35.17 ng/g and 32.53 ng/g, respectively). Pregnenolone treated placental samples exhibited an increase throughout the incubation period from 32.82 (control) to 37.91 (.5 h), 56.83 (1 h) and 77.91 (2 h).

In addition to the detectable significant differences attributable to the main effects and their interactions for placental E_1 production at d 30 (Gilt), d 60 (Gilt, Treatment and Time by Treatment) and d 90 (Gilt, Treatment and Time by Treatment), overall placental E_1 production at the three stages of gestation examined exhibited a biphasic pattern similar to the one reported by Kukoly (1984). Figure 7 depicts the overall placental estrone production at d 30 (2.06 ng/g), d 60 (.82 ng/g) and d 90 (43.16 ng/g) of gestation.

Significant gilt effects were detected for E_1 synthesis by coincubation tissue at d 30, 60 and 90 of gestation ($P <$

TABLE 10 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 90 DAYS OF GESTATION

Tissue	Treatment	Time, h[a]			
		0	.5	1	2
Placenta	Absence of pregnenolone	32.77 ± 8.9[b]	39.31 ± 8.9[b]	35.17 ± 8.9[b]	32.53 ± 8.9[b]
	Presence of pregnenolone	32.82 ± 8.9[b]	37.91 ± 8.9[b]	56.83 ± 8.9[b]	77.91 ± 8.9[c]

[a]Time x treatment effects were tested using Dunnetts test and respective time zero as control.
[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

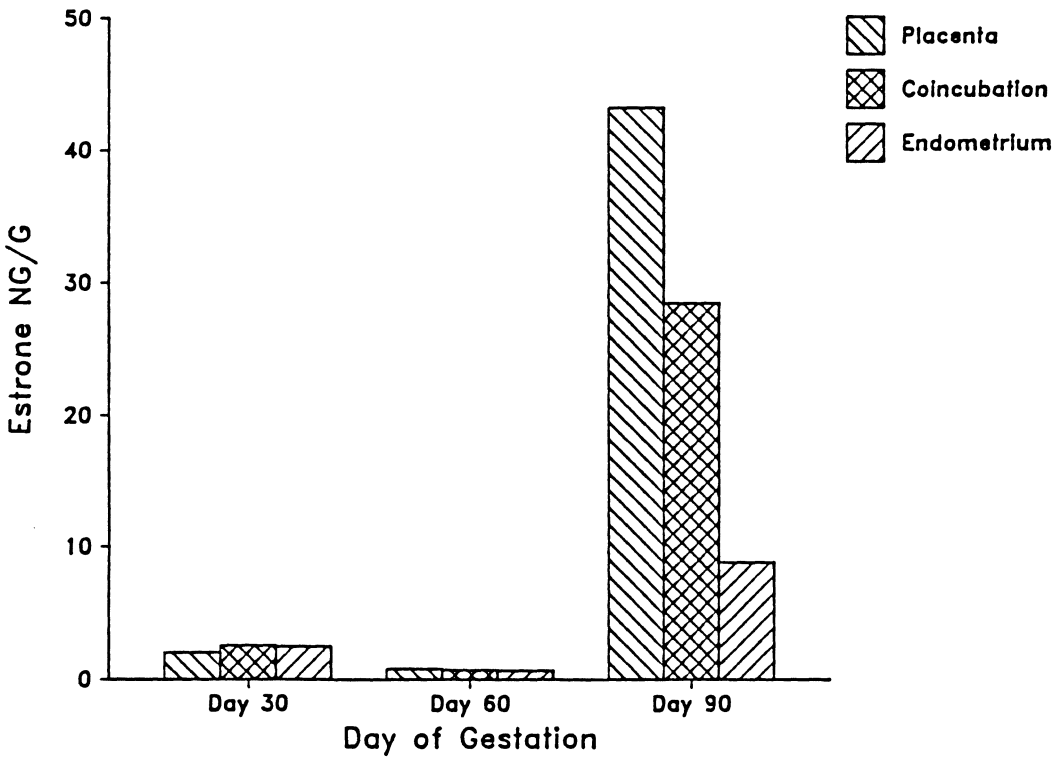

Figure 7. Combined mean estrone concentrations in the medium of pregnenolone treated and untreated placental, co-incubation and endometrial tissue collected at either day 30,60 or 90 of gestation.

.001). Furthermore, significant differences in E_1 production by coincubation tissue at d 30 and 90 were due to the main effects of treatment ($P < .05$) and time ($P < .05$), respectively (table 9). Overall E_1 production by coincubation tissue samples was greater in the presence of P_5 than in the absence of P_5 (3.41 vs 2.31 ng/g). The effect of length of incubation on overall E_1 synthesis by coincubation tissue collected at d 90 of gestation and incubated in the absence and presence of P_5 increased during the first h from 20.52 to 36.91 ng/g, then declined to 33.46 ng/g at the end of 2 h (figure 8).

The overall pattern of E_1 synthesis exhibited by coincubation tissue collected at d 30, 60 and 90 of gestation was similar to that of E_1 synthesis by placental tissue (figure 7). However, at d 90 of gestation, placental E_1 synthesis was almost two-fold greater than E_1 production by coincubation tissue.

At day 30, 60 and 90 of gestation significant gilt effects were detected for endometrial E_1 production, indicating that E_1 synthesis by endometrial tissue at each of the three days of gestation examined, varies among gilts (table 9).

At d 60 of gestation, length of incubation had an effect on the pooled values of E_1 synthesis by endometrial

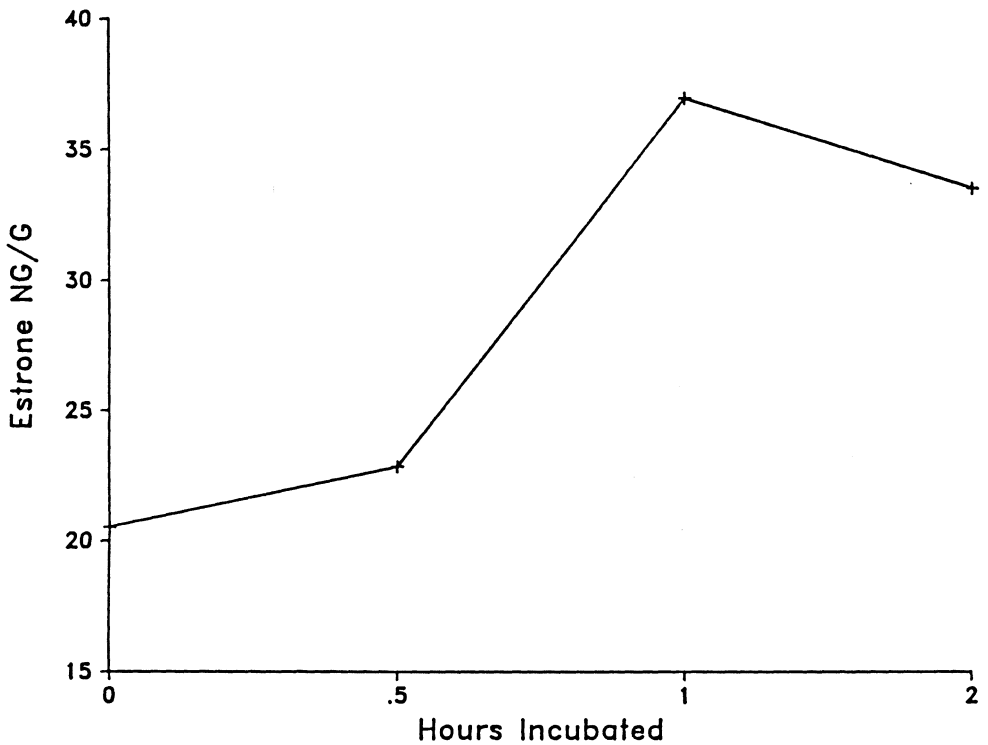

Figure 8. Combined mean estrone concentrations in the medium of pregnenolone treated and untreated coincubation tissue collected at day 90 of gestation.

tissue incubated in the absence and presence of P_5 ($P < .05$). Endometrial E_1 synthesis rose from .49 ng/g (control), to .64 ng/g (.5), .82 ng/g(1h), and .87 ng/g (2h). Overall E_1 synthesis by d 90 endometrial tissue was slightly greater when P_5 was present in the medium (9.80 ng/g) compared with its absence (7.97 ng/g).

A biphasic pattern for overall E_1 synthesis by endometrial tissue collected at d 30, 60 and 90 of gestation was observed (figure 7). However, E_1 production by endometrial tissue collected at d 90 of gestation was approximately five times less than placental E_1 synthesis by tissue collected at the same stage of gestation (8.85 vs 43.16 ng/g).

As mentioned previously, only the two-way interaction between the effects of time and treatment for E_1 synthesis by d 60 and 90 placental tissue was significant. However, the effect of length of incubation and treatment on E_1 synthesis by the remaining tissues at d 30 (table 11) 60 (table 12) and 90 (table 13) although not found to be significant (table 9) are presented in order to show data of E_1 secretion by P_5-treated and untreated feto-maternal tissues.

TABLE 11 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 30 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Absence of pregnenolone	1.99 ± .35[b]	1.98 ± .35[b]	2.71 ± .35[b]	2.04 ± .35[b]
	Presence of pregnenolone	2.09 ± .35[b]	2.51 ± .35[b]	2.15 ± .35[b]	2.84 ± .35[b]
Coincubation[c]	Absence of pregnenolone	2.24 ± .35[b]	2.64 ± .38[b]	1.40 ± .38[b]	3.01 ± .38[b]
	Presence of pregnenolone	3.73 ± .38[b]	3.42 ± .38[b]	3.01 ± .38[b]	3.03 ± .38[b]
Endometrium	Absence of pregnenolone	1.21 ± .82[b]	1.89 ± .82[b]	2.37 ± .82[b]	2.61 ± .82[b]
	Presence of pregnenolone	1.89 ± .82[b]	1.88 ± .82[b]	3.66 ± .82[b]	4.25 ± .82[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.
[b]Means did not differ from time zero ($P < .05$).
[c]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 12 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY COINCUBATION AND ENDOMETRIAL TISSUE AT 60 DAYS OF GESTATION

Tissue	Treatment	Time, h[a]			
		0	.5	1	2
Coincubation[c]	Absence of pregnenolone	.62 ± .11[b]	.74 ± .11[b]	.78 ± .11[b]	.79 ± .11[b]
	Presence of pregnenolone	.60 ± .11[b]	.61 ± .11[b]	.88 ± .11[b]	.86 ± .11[b]
Endometrium	Absence of pregnenolone	.48 ± .13[b]	.63 ± .13[b]	.71 ± .13[b]	.82 ± .13[b]
	Presence of pregnenolone	.51 ± .13[b]	.66 ± .13[b]	.92 ± .13[b]	.92 ± .13[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts and respective time zero as control.

[b]Means did not differ from time zero ($P < .05$).

[c]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 13. TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 90 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Coincubation[d]	Absence of pregnenolone	19.19 ± 5.5[b]	23.30 ± 5.5[b]	34.20 ± 5.5[b]	33.10 ± 5.5[b]
	Presence of pregnenolone	21.84 ± 5.5[b]	22.31 ± 5.5[b]	38.1 ± 5.5[b]	33.71 ± 5.5[b]
Endometrium	Absence of pregnenolone	7.51 ± 1.06[b]	8.22 ± 1.06[b]	7.68 ± 1.06[b]	8.40 ± 1.06[b]
	Presence of pregnenolone	8.81 ± 1.06[b]	8.99 ± 1.06[b]	10.90 ± 1.06[b]	10.41 ± 1.06[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.

[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

[d]Combination of 150 mg of placenta and 150 mg of endometrium.

Experiment Two:

Effects of Pregnenolone, Pregnenolone plus hCG or Pregnenolone Plus cAMP on in vitro Progesterone Synthesis by Feto-maternal Tissue at Day 30, 60 and 90 of Gestation.

Mean squares from the least squares analyses of variance for P_4 concentration in fetal and maternal tissue samples collected at d 30, 60 and 90 of gestation and incubated with P_5, P_5 plus hCG or P_5 plus cAMP are presented in table 14. Significant pooled time effects were detected for placental tissue incubated with P_5, P_5 plus hCG and P_5 plus cAMP at each of the three stages of gestation examined (table 15). At d 30 of gestation, P_4 concentrations rose only during the first .5 h from 7.39 to 12.21 ng/g, plateaued during the next .5 h, and then declined to 8.96 ng/g by the end of the 2 h incubation period. In d 60 placental samples, P_4 concentration rose sharply throughout the .5, 1 and 2 h time periods from 32.99 (control) to 50.80, 98.61 and 157.0 ng/g, respectively. Progesterone synthesis by placental tissue collected at d 90 of gestation rose from 116.31 (control) to 147.36, 166.91 and 209.86 ng/g at time .5, 1 and 2 h, respectively (table 15).

The overall pattern of P_4 accumulation in the medium of placental tissue at d 30, 60 and 90 in experiment two was similar to the pattern observed in experiment one (figure

TABLE 14 LEAST-SQUARES ANALYSES OF VARIANCE FOR THE EFFECTS OF TIME OF INCUBATION AND PREGNENOLONE, PREGNENOLONE + hCG OR PREGNENOLONE + cAMP TREATMENT ON PROGESTERONE PRODUCTION BY FETO-MATERNAL TISSUE OF GILTS AT 30, 60 AND 90 DAYS OF GESTATION

Day of gestation	Source of variation	df	Tissue Mean squares Placenta	Coincubation[a]	Endometrium
30	Gilt	3	305.13***	181.28***	182.96*
	Time	3	135.51***	91.41*	307.74**
	Treatment	2	67.01*	22.78	320.85
	Time x treatment	6	14.39	3.38	167.06
	Error	81	12.47	21.75	58.61
60	Gilt	3	18905.80***	9402.19***	10574.78***
	Time	3	92310.40***	10901.37***	1698.21***
	Treatment	2	297.45	429.36	1193.06***
	Time x treatment	6	930.33*	517.60	449.71**
	Error	81	783.82	433.90	152.11
90	Gilt	3	50265.87***	19078.78***	12424.51***
	Time	3	36815.70***	3820.67***	1897.56***
	Treatment	2	21605.40***	9239.98***	747.45
	Time x treatment	6	6733.37**	1177.01	360.60
	Error	81	2203.77	688.60	297.56

[a]Combination of 150 mg of placenta and 150 mg of endometrium.
*P < .05.
**P < .01.
***P < .001.

TABLE 15 LEAST-SQUARES MEANS FOR THE EFFECT OF TIME OF INCUBATION ON PROGESTERONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 30, 60 AND 90 DAYS OF GESTATION[a]

Day of gestation	Time	Tissue[b]		
		Placenta	Coincubation[c]	Endometrium
30	0	7.39 ± .72[b]	10.33 ± .95[b]	12.46 ± 1.56[b]
	.5	12.21 ± .72[c]	12.54 ± .95[b]	20.26 ± 1.56[c]
	1	12.07 ± .72[d]	14.86 ± .95[c]	14.48 ± 1.56[b]
	2	8.96 ± .72[b]	13.82 ± .95[d]	18.48 ± 1.56[d]
60	0	32.99 ± 5.1[b]	32.23 ± 3.80[b]	22.41 ± 2.25[b]
	.5	50.80 ± 5.1[b]	39.91 ± 3.80[b]	28.21 ± 2.25[b]
	1	98.61 ± 5.1[c]	58.47 ± 3.80[c]	35.27 ± 2.25[b]
	2	157.00 ± 5.1[d]	74.65 ± 3.80[d]	39.39 ± 2.25[c]
90	0	116.31 ± 9.58[b]	65.93 ± 5.35[b]	27.62 ± 3.52[b]
	.5	147.36 ± 9.58[b]	86.99 ± 5.35[b]	47.55 ± 3.52[c]
	1	166.91 ± 9.58[c]	88.48 ± 5.35[b]	35.79 ± 3.52[b]
	2	209.86 ± 9.58[d]	95.04 ± 5.35[c]	30.02 ± 3.52[b]

[a]Time effects were tested within each tissue at 30, 60 and 90 days of gestation using Dunnetts test and time zero as control.
[b]Letters with different superscripts differ ($P < .05$).
[c]Combination of 150 mg of placenta and 150 mg of endometrium.

9). In experiments one and two placental tissue collected at d 90 of gestation was capable of synthesizing greater quantities of P_4 than tissue collected at d 60 (125.40 and 160.11 ng/g vs 58.31 and 84.74 ng/g, respectively). Likewise, in experiment one and two, d 60 placental tissue was capable of producing greater quantities of P_4 than tissue collected at d 30 of gestation (58.31 and 84.74 ng/g vs 10.36 and 10.16 ng/g, respectively).

Significant treatment effects were observed for placental P_4 production by tissue collected at d 30 of gestation ($P < .05$). Addition of cAMP to P_5 treated d 30 placental tissue explants (vs d 30 placental tissue treated only with P_5) increased P_4 synthesis (11.57 vs 8.67 ng/g). In contrast to this, when hCG was added to P_5 treated d 30 placental tissue samples, P_4 levels similar to those found in the medium with only P_5 were detected (9.23 vs 8.67 ng/g).

An increase in placental P_4 production was also observed at d 60 and 90 of gestation. However, this increase was due to the interaction between the length of incubation and treatment (table 16). In d 60 placental tissue samples, addition of cAMP and hCG to P_5 treated placental tissue samples resulted in a five-fold increase in final P_4 accumulation in the medium between their respective time 0 concentrations.

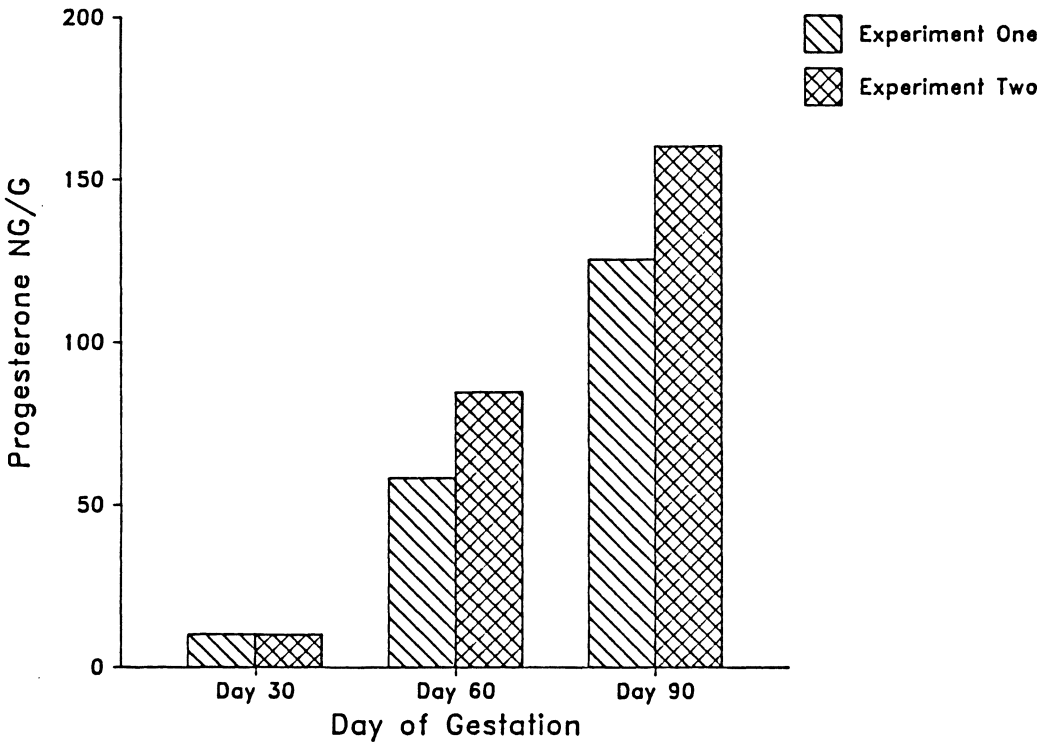

Figure 9. Mean progesterone concentrations in the medium of incubated placental tissue collected at either day 30, 60 or 90 of gestation in either experiment one or two.

TABLE 16 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE PRODUCTION (ng/g) BY PLACENTAL TISSUE COLLECTED AT DAY 60 AND 90 OF GESTATION

Day of gestation	Treatment	Time, h[a]			
		0	.5	1	2
60	Pregnenolone	34.45^{b}	58.96^{b}	93.19^{c}	144.16^{d}
	Pregnenolone + hCG	27.33^{b}	50.26^{b}	98.87^{c}	154.76^{d}
	Pregnenolone + cAMP	37.21^{b}	43.19^{b}	102.43^{c}	172.08^{d}
90	Pregnenolone	87.15^{b}	120.84^{b}	136.87^{b}	228.14^{c}
	Pregnenolone + hCG	129.16^{b}	129.54^{b}	170.36^{c}	155.66^{b}
	Pregnenolone + cAMP	132.61^{b}	191.69^{b}	193.49^{c}	245.78^{d}

[a]Time x treatment effects were tested within each tissue using Dunnetts test and time zero as control.

[bcd]Letters in same row with different superscripts differ from time zero ($P < .05$).

Moreover, in day 60 placental tissue explants treated only with P_5, a four-fold increase in final P_4 accumulation in the medium was observed. This increment is similar to the one previously detected by placental tissue incubated in the presence of P_5 in experiment one. Addition of cAMP to d 60 placental tissue samples incubated in the presence of P_5 resulted in a 27.92 ng/g increase in final P_4 concentration ($P < .05$) above that of P_4 synthesis by tissue samples incubated only in the presence of P_5.

In d 90 placental tissue samples, a two- and three-fold increase in final P_4 synthesis by samples incubated with P_5 (228.14 vs 87.15 ng/g) and P_5 plus cAMP (245.78 vs 132.61 ng/g) was observed (table 16). Furthermore, an increase in placental P_4 production in M199-P_5-hCG was detected during the first h from 129.16 to 170.36 ng/g. During the second h, placenta P_4 levels declined to 155.66 ng/g. Overall, at d 90 of gestation, placental progesterone accumulation in M199-P_5 and M199-P_5-cAMP was greater ($P < .05$) than P_4 accumulation in M199-P_5-hCG (table 16).

Significant overall time effects were detected for P_4 production by coincubation tissue samples incubated with P_5, P_5 plus hCG and P_5 plus cAMP at d 30 ($P < .05$), 60 ($P < .001$) and d 90 ($P < .001$) of gestation. In both d 30 and 60 of gestation, P_4 synthesis by coincubation tissue samples

rose significantly ($P < .05$) during the first h from 10.33 to 14.86 ng/g and from 32.23 to 58.47 ng/g, respectively (table 15). At the end of the second h of incubation, however, P_4 concentration in coincubation samples collected at d 30 of gestation decreased to 13.82 ng/g while P_4 concentration in d 60 samples increased to 74.64 ng/g. At d 90 of gestation, coincubation P_4 concentration rose from 65.93 to 86.99 ng/g during the first .5 h, then continued to gradually increase for the remainder of the incubation to 95.04 ng/g.

Addition of cAMP to P_5-treated d 90 coincubation tissue significantly increased ($P < .05$) P_4 accumulation compared with coincubation samples incubated in the presence of P_5 (103.69 vs 75.3 ng/g) (figure 10). However, hCG had no significant effect on P_4 synthesis by coincubation tissue in the presence of P_5 (figure 10).

Pooled P_4 synthesis by endometrial tissue incubated with P_5, P_5 plus hCG and P_5 plus cAMP and collected at d 30, 60 and 90 of gestation was significantly influenced by the length of the incubation period (table 14). However, d 60 endometrial P_4 production was affected ($P < .01$) by the two-way interaction between time and treatment.

In d 30 endometrial samples, P_4 rose from 12.46 to 20.26 ng/g during the first .5 h, then declined to 14.48

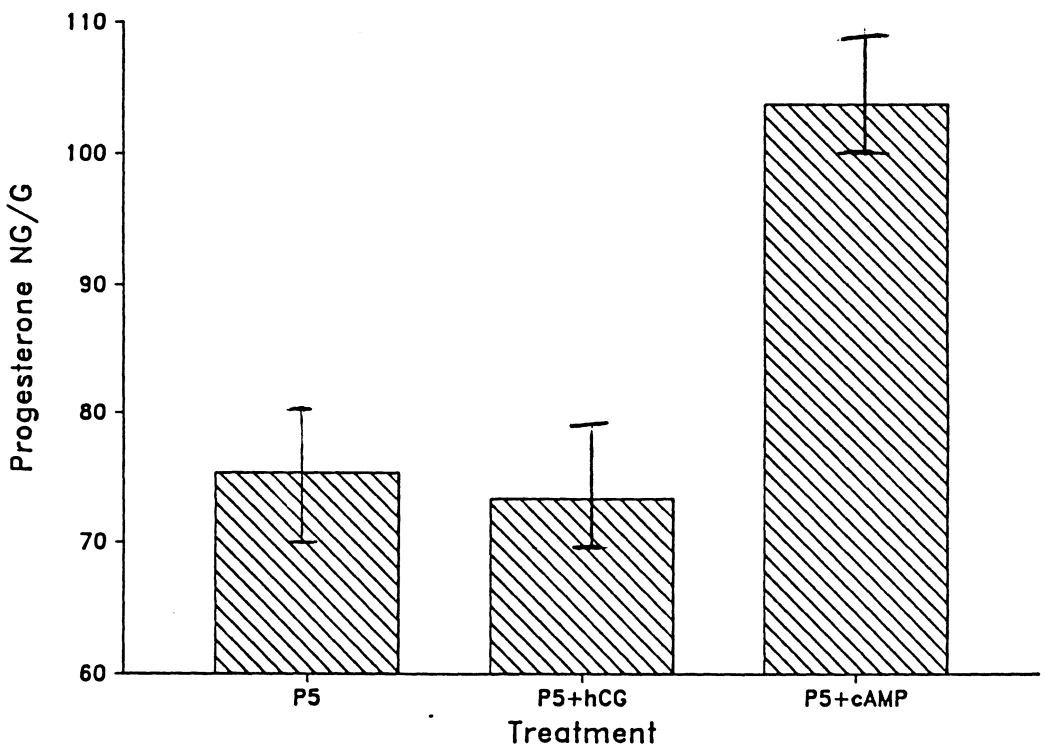

Figure 10. Mean progesterone concentrations in the medium of incubated coincubation tissue collected at day 90 of gestation in either the presence of pregnenolone pregnenolone+hCG or pregnenolone + cAMP.

ng/g during the second .5 h and then gradually increased to 18.48 ng/g by the end of the 2 h period (table 15). An increase in P_4 synthesis by endometrial tissue samples collected at d 90 of gestation occurred during the first .5 h from 27.62 to 47.55 ng/g, thereafter a gradual decrease was observed for the 1 and 2 h times (35.79 and 30.02 ng/g, respectively).

The increase in P_4 synthesis by endometrial tissue from gilts at 60 days of gestation was attributable to time by treatment effects ($P < .01$). During the first h, endometrial P_4 concentrations increased in medium with P_5 (from 28.35 to 36.58 ng/g), medium with P_5 plus hCG (from 16.17 to 25.6 ng/g) and medium with P_5 plus cAMP (from 22.71 to 43.63 ng/g) (figure 11). In endometrial tissue samples incubated in M199-P_5 and M199-P_5-hCG, P_4 accumulation increased by the end of the two hour period. However, in cAMP treated endometrial samples, P_4 concentration declined after the 2 h incubation time.

Although the length of incubation by treatment interactions for P_4 synthesis by d 30 placental, coincubation and endometrial tissue (table 17), d 60 coincubation tissue (table 18) and d 90 coincubation and endometrial tissue (table 19) were not significant, the data are presented on P_4 synthesis by feto-maternal tissues incubated with P_5, P_5 plus hCG or P_5 plus cAMP.

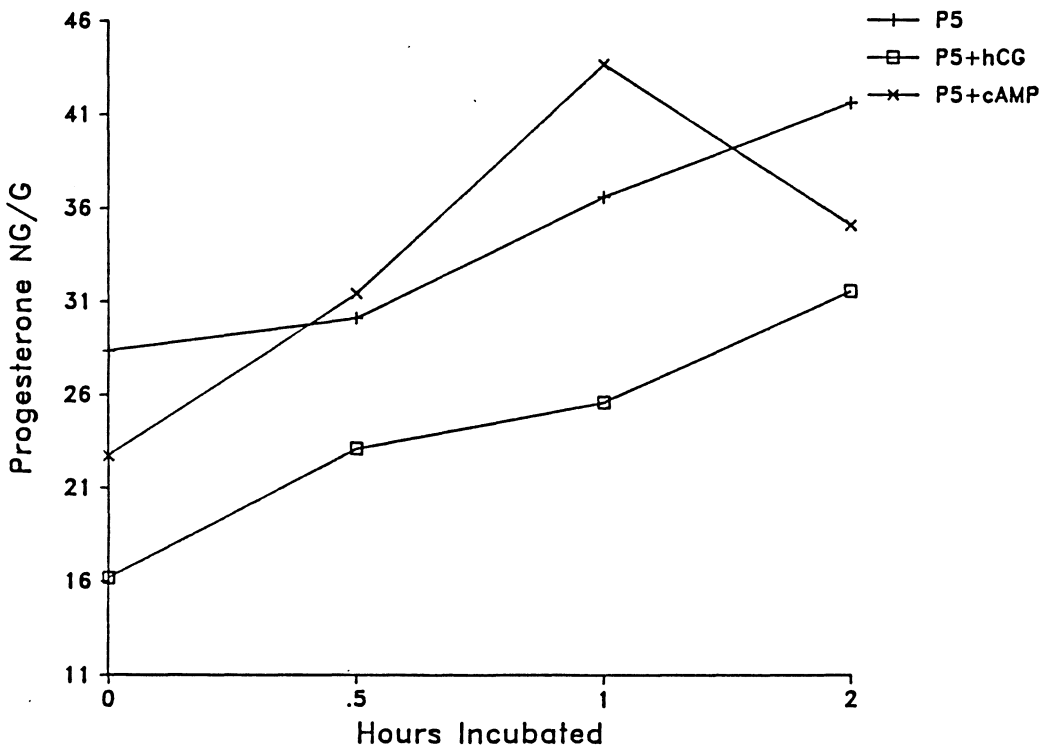

Figure 11. Mean progesterone concentrations in the medium of incubated endometrial tissue collected at day 60 of gestation in either the presence of pregnenolone, pregnenolone + hCG or pregnenolone + cAMP.

TABLE 17 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 30 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Pregnenolone	7.55 ± 1.3[b]	10.02 ± 1.3[b]	9.52 ± 1.3[b]	7.59 ± 1.3[b]
	Pregnenolone + hCG	6.48 ± 1.3[b]	13.58 ± 1.3[c]	11.66 ± 1.3[b]	8.07 ± 1.3[b]
	Pregnenolone + cAMP	8.15 ± 1.3[b]	12.91 ± 1.3[b]	15.02 ± 1.3[b]	11.20 ± 1.3[b]
Coincubation[d]	Pregnenolone	10.61 ± 1.65[b]	13.56 ± 1.65[b]	16.13 ± 1.65[b]	13.81 ± 1.65[b]
	Pregnenolone + hCG	10.06 ± 1.65[b]	11.39 ± 1.65[b]	13.47 ± 1.65[b]	12.78 ± 1.65[b]
	Pregnenolone + cAMP	10.31 ± 1.65[b]	12.65 ± 1.65[b]	14.96 ± 1.65[b]	14.87 ± 1.65[b]
Endometrium	Pregnenolone	12.04 ± 2.7[b]	16.37 ± 2.7[b]	12.95 ± 2.7[b]	17.16 ± 2.7[b]
	Pregnenolone + hCG	13.11 ± 2.7[b]	13.45 ± 2.7[b]	15.83 ± 2.7[b]	15.81 ± 2.7[b]
	Pregnenolone + cAMP	12.21 ± 2.7[b]	30.96 ± 2.7[c]	14.65 ± 2.7[b]	22.47 ± 2.7[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.

[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

[d]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 18 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY COINCUBATION TISSUE AT 60 DAYS OF GESTATION

Tissue	Treatment	Time, h[a]			
		0	.5	1	2
Coincubation[d]	Pregnenolone	32.17 ± 6.6[b]	40.59 ± 6.6[b]	60.50 ± 6.6[b]	76.67 ± 6.6[c]
	Pregnenolone + hCG	34.03 ± 6.6[b]	38.78 ± 6.6[b]	57.14 ± 6.6[b]	62.41 ± 6.6[c]
	Pregnenolone + cAMP	30.47 ± 6.6[b]	40.37 ± 6.6[b]	57.73 ± 6.6[b]	84.87 ± 6.6[c]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.
[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).
[d]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 19 TIME BY TREATMENT LEAST-SQUARES MEANS FOR PROGESTERONE (ng/g ± S.E.) PRODUCTION BY COINCUBATION AND ENDOMETRIAL TISSUE AT 90 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Coincubation[d]	Pregnenolone	57.88 ± 9.3[b]	68.97 ± 9.3[b]	83.11 ± 9.3[b]	91.23 ± 9.3[b]
	Pregnenolone + hCG	69.02 ± 9.3[b]	80.49 ± 9.3[b]	76.21 ± 9.3[b]	68.59 ± 9.3[b]
	Pregnenolone + cAMP	70.86 ± 9.3[b]	111.52 ± 9.3[b]	106.11 ± 9.3[b]	125.31 ± 9.3[c]
Endometrium	Pregnenolone	28.62 ± 6.1[b]	43.14 ± 6.1[b]	36.99 ± 6.1[b]	34.36 ± 6.1[b]
	Pregnenolone + hCG	30.36 ± 6.1[b]	40.60 ± 6.1[b]	25.80 ± 6.1[b]	23.91 ± 6.1[b]
	Pregnenolone + cAMP	23.89 ± 6.1[b]	58.92 ± 6.1[b]	44.59 ± 6.1[b]	31.75 ± 6.1[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.

[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

[d]Combination of 150 mg of placenta and 150 mg of endometrium.

In vitro Estrone Synthesis by Feto-maternal Tissue in the Presence of Pregnenolone, Pregnenolone Plus hCG or Pregnenolone Plus cAMP at 30, 60, and 90 Days of Gestation.

The results from the least squares analyses of variance for estrone concentrations in placental, coincubation and endometrial tissue at d 30, 60 and 90 of gestation and incubated with P_5, P_5 plus hCG or P_5 plus cAMP are presented in table 20. Significant gilt effects were detected for each tissue at each day of gestation. Thus, E_1 synthesis by feto-maternal tissue varied among gilts.

Other than significant gilt effects for E_1 production by placental tissue collected at d 30 of gestation, no other significant main effects were observed in d 30 placentas. However, at d 60 of gestation a time by treatment interaction ($P < .05$) was observed for placental E_1 synthesis. Figure 12 depicts the changes in placental E_1 synthesis over the length of incubation at each treatment. The greatest accumulation of placental E_1 concentration over time occurred in P_5 plus cAMP treated samples (from .73 to 1.14 ng/g).

At d 90 of gestation, addition of hCG to placental tissue samples treated with P_5 significantly ($P < .05$) decreased mean E_1 accumulation in the medium compared with tissue samples incubated only with P_5 (figure 13).

TABLE 20 LEAST-SQUARES ANALYSES OF VARIANCE FOR THE EFFECTS OF TIME OF INCUBATION AND PREGNENOLONE, PREGNENOLONE + hCG OR PREGNENOLONE + cAMP TREATMENT ON ESTRONE PRODUCTION BY FETO-MATERNAL TISSUE OF GILTS AT 30, 60 AND 90 DAYS OF GESTATION

Day of gestation	Source of variation	df	Tissue Mean squares Placenta	Coincubation[a]	Endometrium
30	Gilt	3	26.37***	27.77***	29.83**
	Time	3	.23	7.61*	59.94***
	Treatment	2	1.05	2.03	21.68*
	Time x treatment	6	.59	3.22	3.71
	Error	81	.56	1.61	5.14
60	Gilt	3	1.02***	2.37*	1.53***
	Time	3	.25	1.48	.11
	Treatment	2	.23	1.48	.40
	Time x treatment	6	.26*	1.01	.36
	Error	81	.10	.96	.22
90	Gilt	3	9622.34***	3965.13***	754.41***
	Time	3	1641.10*	51.62	35.12
	Treatment	2	1743.21*	52.75	36.29
	Time x treatment	6	390.93	170.93	63.24
	Error	81	524.39	85.28	25.89

[a]Combination of 150 mg placenta and 150 mg of endometrium.
*P < .05.
**P < .01.
***P < .001.

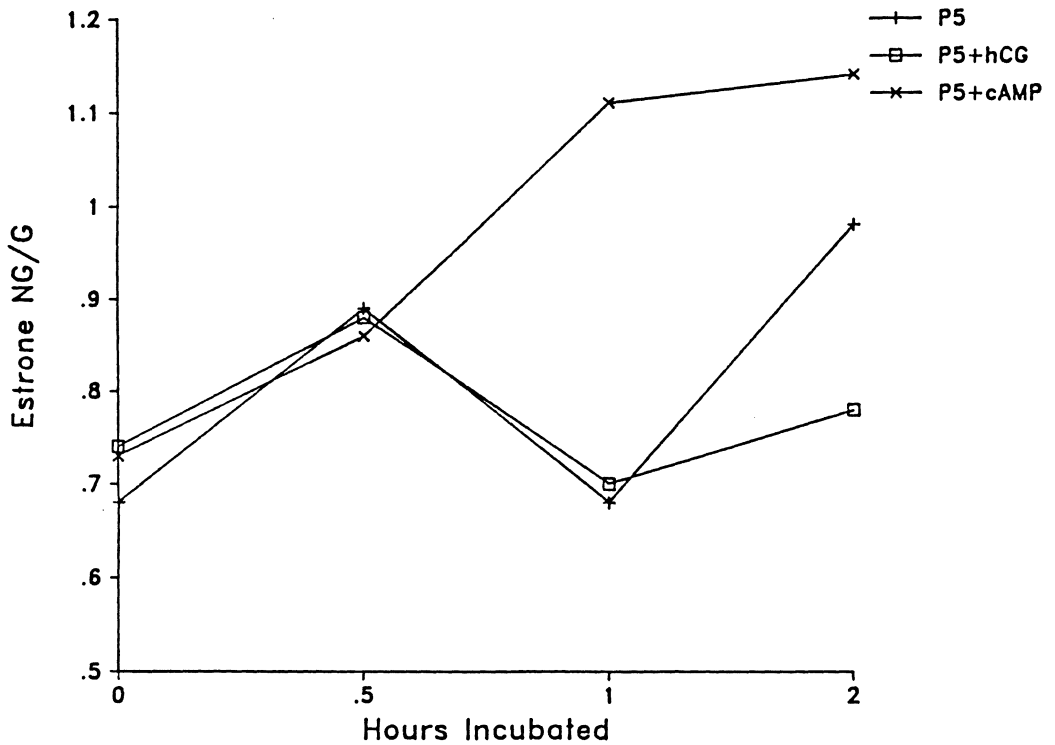

Figure 12. Mean estrone concentrations in the medium of incubated placental tissue collected at day 60 of gestation in either the presence of pregnenolone, pregnenolone + hCG and pregnenolone + cAMP.

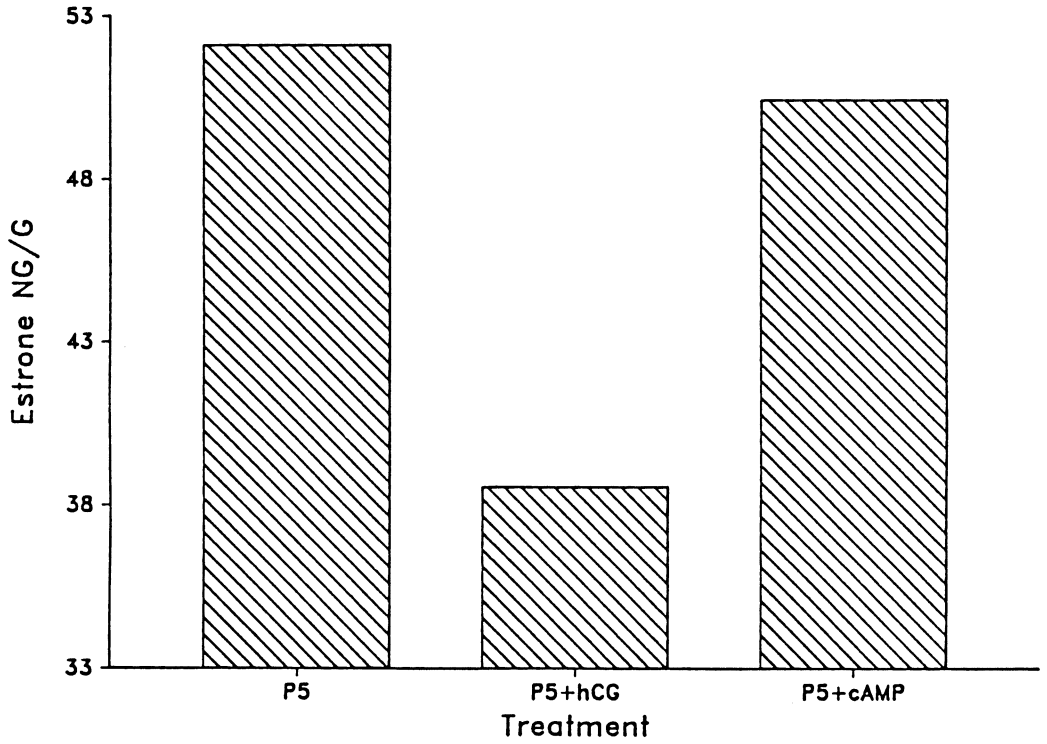

Figure 13. Mean estrone concentrations in the medium of incubated placental tissue collected at day 90 of gestation in either the presence of pregnenolone, pregnenolone + hCG or pregnenolone + cAMP.

Although few significant main interactions were observed for placental E_1 synthesis at d 30, 60 and 90 of gestation, the overall secretory pattern of E_1 production of the three stages of gestation examined was similar to the one seen in experiment one (figures 7 and 14). Likewise, the overall trend in E_1 synthesis by coincubation tissue samples at each day of gestation examined was similar to the trend observed in experiment one (figures 7 and 14).

Even though the time by treatment interaction for E_1 production by d 60 placental samples incubated in the presence of P_5, P_5 plus hCG or P_5 plus cAMP was the only significant interation between main effects ($P < .05$), the remaining data for feto-maternal tissues at d 30 (table 21), d 60 (table 22) and d 90 (table 23) of gestation are presented.

Differences in overall E_1 concentration among different treatments were observed for endometrial tissue collected at d 30 of gestation ($P < .05$). Addition of cAMP to P_5 treated endometrial tissue samples increased overall E_1 synthesis compared with tissue samples incubated only with P_5 (4.14 vs 2.86 ng/g). Human chorionic gonadotropin had no significant effect on overall E_1 accumulation in the medium of P_5 treated samples. Also in d 30 endometrial samples, final E_1 concentration was about three-fold higher than that of initial E_1 concentration (5.43 vs 1.84 ng/g).

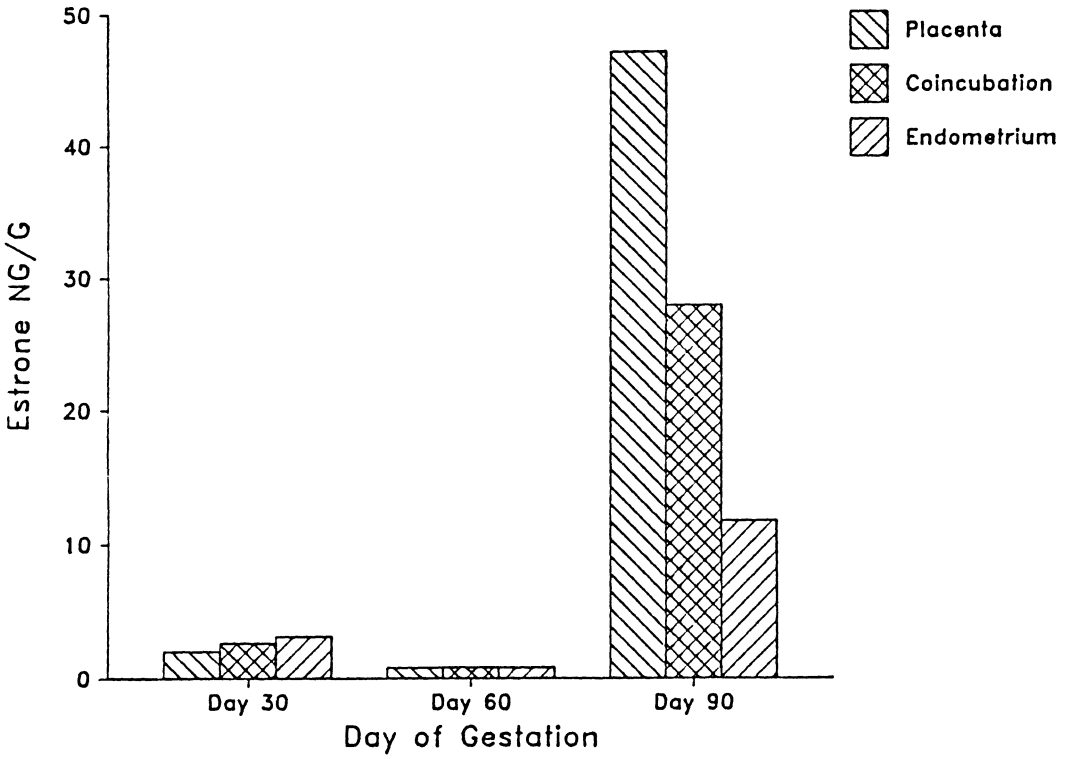

Figure 14. Combined mean estrone concentrations in the medium of pregnenolone, pregnenolone plus hCG and pregnenolone plus cAMP treated placental, coincubation and endometrial tissue collected at either day 30, 60 or 90 of gestation.

TABLE 21 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 30 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Pregnenolone	1.76 ± .26[b]	1.85 ± .26[b]	1.99 ± .26[b]	2.40 ± .26[b]
	Pregnenolone + hCG	2.57 ± .26[b]	2.28 ± .26[b]	2.04 ± .26[b]	2.11 ± .26[b]
	Pregnenolone + cAMP	2.02 ± .26[b]	2.05 ± .26[b]	1.72 ± .26[b]	1.81 ± .26[b]
Coincubation[d]	Pregnenolone	2.66 ± .45[b]	2.16 ± .45[b]	3.00 ± .45[b]	4.08 ± .45[b]
	Pregnenolone + hCG	1.96 ± .45[b]	1.88 ± .45[b]	3.62 ± .45[b]	2.72 ± .45[b]
	Pregnenolone + cAMP	2.59 ± .45[b]	2.30 ± .45[b]	1.87 ± .45[b]	3.39 ± .45[b]
Endometrium	Pregnenolone	1.88 ± .80[b]	1.76 ± .80[b]	3.68 ± .80[b]	3.95 ± .80[b]
	Pregnenolone + hCG	1.51 ± .80[b]	1.54 ± .80[b]	3.13 ± .80[b]	4.1 ± .80[b]
	Pregnenolone + cAMP	2.23 ± .80[b]	2.45 ± .80[b]	4.84 ± .80[b]	7.0 ± .80[c]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.

[bc]Letters in same row with different superscripts differ from time zero ($P < .05$).

[d]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 22 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY COINCUBATION AND ENDOMETRIAL TISSUE AT 60 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Coincubation[c]	Pregnenolone	.78 ± .31[b]	1.96 ± .31[b]	.79 ± .31[b]	.85 ± .31[b]
	Pregnenolone + hCG	.64 ± .31[b]	.85 ± .31[b]	.76 ± .31[b]	.75 ± .31[b]
	Pregnenolone + cAMP	.78 ± .31	.80 ± .31	.69 ± .31	.85 ± .31
Endometrial	Pregnenolone	.95 ± .15[b]	.83 ± .15[b]	.87 ± .15[b]	.87 ± .15[b]
	Pregnenolone + hCG	.67 ± .15[b]	1.09 ± .15[b]	.94 ± .15[b]	.95 ± .15[b]
	Pregnenolone + cAMP	.64 ± .15[b]	.77 ± .15[b]	.78 ± .15[b]	.72 ± .15[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.
[b]Means did not differ from time zero ($P < .05$).
[c]Combination of 150 mg of placenta and 150 mg of endometrium.

TABLE 23 TIME BY TREATMENT LEAST-SQUARES MEANS FOR ESTRONE (ng/g ± S.E.) PRODUCTION BY FETO-MATERNAL TISSUE AT 90 DAYS OF GESTATION

Tissue	Treatment	Time, h[a] 0	.5	1	2
Placenta	Pregnenolone	49.91 ± 8.1[b]	54.44 ± 8.1[b]	42.5 ± 8.1[b]	61.56 ± 8.1[b]
	Pregnenolone + hCG	37.70 ± 8.1[b]	32.91 ± 8.1[b]	36.0 ± 8.1[b]	47.65 ± 8.1[b]
	Pregnenolone + cAMP	38.66 ± 8.1[b]	62.83 ± 8.1[b]	37.8 ± 8.1[b]	62.40 ± 8.1[b]
Coincubation[c]	Pregnenolone	27.06 ± 3.26[b]	31.82 ± 3.26[b]	24.72 ± 3.26[b]	32.48 ± 3.26[b]
	Pregnenolone + hCG	23.06 ± 3.26[b]	23.75 ± 3.26[b]	32.96 ± 3.26[b]	26.22 ± 3.26[b]
	Pregnenolone + cAMP	28.90 ± 3.26[b]	29.72 ± 3.26[b]	31.40 ± 3.26[b]	22.63 ± 3.26[b]
Endometrium	Pregnenolone	13.08 ± 1.79[b]	9.6 ± 1.79[b]	11.69 ± 1.79[b]	13.1 ± 1.79[b]
	Pregnenolone + hCG	13.77 ± 1.79[b]	10.78 ± 1.79[b]	11.61 ± 1.79[b]	10.90 ± 1.79[b]
	Pregnenolone + cAMP	13.41 ± 1.79[b]	12.38 ± 1.79[b]	9.14 ± 1.79[b]	11.31 ± 1.79[b]

[a]Time x treatment effects were tested within each tissue using Dunnetts test and respective time zero as control.
[b]Means did not differ from time zero ($P < .05$).
[c]Combination of 150 mg of placenta and 150 mg of endometrium.

Although treatment and time effects were only found to be significant for endometrial tissue samples collected at d 30 of gestation, the overall trend of endometrial E_1 synthesis was similar to the trend seen in experiment one (figures 7 and 14).

Progesterone and Estrone Concentrations in Maternal Plasma

Anterior vena cava, uterine artery and uterine vein P_4 concentrations of gilts utilized in both experiments one and two are summarized in table 24. At each day of gestation examined, P_4 levels in the anterior vena cava were greater ($P < .001$) than those of the uterine vein or uterine artery. These results are similar to those previously reported by Knight et al. (1977) and Kukoly (1984). A comparison of uterine artery (UA) minus uterine vein (UV) P_4 concentrations (table 24) revealed a positive UA-UV difference at each of the three stages of gestation examined. However, only the UA-UV differences at d 30 ($P<.001$) and 60 ($P<.05$) of gestation were significant.

Conversely, UA-UV differences in E_1 concentrations were negative at d 30, 60 and 90 of gestation (table 25). However, only the UA-UV difference of E_1 concentration at d 90 was significant ($P < .001$). Both uterine artery and anterior vena cava concentrations of E_1 rose gradually between d

TABLE 24 PROGESTERONE CONCENTRATIONS (NG/ML ± S.E.) IN ANTERIOR VENA CAVA (AVC), UTERINE ARTERY (UA) AND UTERINE VEIN (UV) PLASMA

Day of gestation	AVC[a]	UA[bd]	UV[c]	UA-UV difference
30	49.50 ± 22.67	18.56 ± 2.00	6.61 ± 1.12	11.96
60	102.78 ± 22.67	10.45 ± 2.00	5.15 ± 1.12	5.30
90	79.76 ± 22.67	6.78 ± 2.00	3.36 ± 1.12	3.42

[abc]Columns with different superscripts are significantly different ($P < .001$).
[d]Significant ($P < .01$) day of gestation effects.

30 and d 60, then rose sharply by d 90 of gestation. Uterine vein E_1 concentration decreased slightly from d 30 (.086 ng/ml) to d 60 (.081 ng/ml) and then increased to 1.61 ng/ml by d 90 of gestation.

Changes in E_1 concentration in the uterine vein mimiced those of the uterine artery at each day of gestation examined (table 25). No differences were found in E_1 concentrations in the anterior vena cava and uterine artery.

Combined Progesterone and Estrone Concentrations in Fetal Fluids

Average P_4 concentrations in allantoic and amniotic fluid samples at d 30, 60 and 90 of gestation are reported in table 26. Significant day of gestation effects ($P < .001$) were detected for allantoic fluid samples at each day of gestation examined. Progesterone concentrations in allantoic fluid rose from .93 to at d 30 to 1.94 ng/ml at d 60, and continued to increase until d 90 of gestation (3.51 ng/ml). No significant changes in amniotic fluid P_4 concentration at either d 60 or d 90 of gestation were detected ($P > .5$). At d 90, mean umbilical vein P_4 concentration was 6.05 ng/ml.

Changes in allantoic and amniotic fluid E_1 concentrations at d 30, 60 and 90 of gestation are summarized in table 27. Significant day of gestation effects was for E_1

TABLE 25 ESTRONE CONCENTRATIONS (NG/ML ± S.E.) IN ANTERIOR VENA CAVA (AVC), UTERINE ARTERY (UA) AND UTERINE VEIN (UV) PLASMA

Day of gestation	AVC[a]	UA[a]	UV[a]	UA-UV difference[a]
30	.063 ± .06	.070 ± .06	.086 ± .08	-.016
60	.069 ± .06	.073 ± .06	.081 ± .08	-.008
90	.572 ± .06	.623 ± .06	1.61 ± .08	-.988

[a]Significant ($P < .01$) day of gestation effects.

TABLE 26 PROGESTERONE CONCENTRATIONS (NG/ML ± S.E.) IN ALLANTOIC (AL), AMNIOTIC FLUID AND UMBILICAL VEIN (UMV)

Day of gestation	AL[c]	AM	UMV
30	.93 ± .21	a	b
60	1.94 ± .21	.86 ± .18	b
90	3.51 ± .21	.70 ± .18	6.05 ± .81

[a]Amniotic fluid volume inadequate for hormone assay.
[b]Umbilical vein volume inadequate for hormone assay.
[c]Significant ($P < .001$) day of gestation effects.

TABLE 27 ESTRONE CONCENTRATIONS (NG/ML ± S.E.) IN ALLANTOIC (AL) AMNIOTIC FLUID AND UMBILICAL VEIN (UMV)

Day of gestation	AL[c]	AM[c]	UMV
30	5.01 ± 14.8	a	b
60	1.40 ± 14.8	.26 ± 1.6	b
90	136.90 ± 14.8	18.51 ± 1.6	14.16 ± 1.2

[a]Amniotic fluid volume inadequate for hormone assay.
[b]Umbilical vein volume inadequate for hormone assay.
[c]Significant ($P < .001$) day of gestation effects.

concentrations in allantoic and amniotic fluid ($P < .001$) were detected. Furthermore, a biphasic pattern in allantoic E_1 concentration was observed. Initial E_1 concentrations declined from 5.01 ng/ml at d 30 to 1.40 ng/ml at d 60. Thereafter, there was a sharp increase in E_1 concentrations until d 90 of gestation (136.90 ng/ml). Likewise, amniotic E_1 concentrations rose linearly from d 60 (.26 ng/ml) to d 90 (18.51 ng/ml). Mean E_1 concentration in umbilical vein samples at d 90 of gestation was 14.16 ng/ml.These results are similar to previous reports (Knight et al., 1977; Kukoly, 1984).

Progesterone and Estrone Differences in Frozen vs Ethanol Treated Feto-maternal Tissues

At the end of the 2 h incubation period, differences in P_4 and E_1 synthesis by placental, coincubation and endometrial tissue samples at d 30, 60 and 90 of gestation that were either frozen or killed with ethanol were statistically compared. Due to the variability in P_4 and E_1 synthesis by each tissue at each day of gestation, differences were changed to percentages and then compared. The overall mean difference in P_4 and E_1 concentration between frozen and ethanol treated tissue samples was 4.4% and 3.2%, respectively. For both hormones, no significant day of gestation, tissue or day of gestation by tissue effects were detected.

Thus, at the end of 2 h, no significant differences in P_4 or E_1 .synthesis were detected between frozen and ethanol treated tissue samples.This suggests that either method of halting steroid hormone synthesis by "killing" the tissue was equally effective.

DISCUSSION

Placental Steroidogenesis

Maintenance of gestation in domestic farm animals depends on high levels of P_4 in the maternal systemic circulation. That association depends on the continuous secretion of progesterone, the principal source being the corpus luteum (or corpora lutea). In the ewe, mare and cow, placental P_4 production is sufficient to maintain a gravid uterus after ovariectomy only if the operation is carried out after approximately d 50, 170 and 200 of gestation, respectively. In contrast to the ewe, mare and cow, removal of both ovaries from a sow during any stage of gestation results in abortion within 2-3 days (du mesnil du Buission and Dauzier, 1957).

Based on these results, it has long been assumed that the porcine placenta throughout gestation lacks the ability to synthesize significant amounts of P_4. Despite the fact that luteal P_4 is essential for maintenance of gestation in the pig, in a recently completed study in our laboratory (Kukoly, 1984) in vitro cultures of porcine placental explants collected between d 20 and 100 of gestation produced large quantities of P_4.

In this study, porcine placental tissue collected at d 30, 60 and 90 of gestation and incubated in the absence of P_5, synthesized a substantial amount of P_4. Production of P_4 increased linearly from d 30 to 90 of gestation. While the quantity of placental tissue used for incubation (300 mg) remained constant for the three stages of gestation examined, the capacity of the porcine placenta to produce significant quantities of P_4 differed depending upon the day of gestation. Placental tissue collected at 90 days of gestation was capable of synthesizing larger quantities of P_4 than tissue collected at d 60. Likewise, placental tissue collected at d 60 of gestation was capable of producing larger quantities of P_4 than tissue collected at d 30 of gestation. In addition, steady increases in allantoic fluid P_4 concentrations were detected at each of the three stages of gestation examined. Increases in P_4 synthesis by the porcine placenta as pregnancy proceeds could be the result of an increase or maturation of the enzyme systems located in the fetal membranes controlling the availability and utilization of the precursor P_5.

In the absence of P_5, P_4 was still being secreted into the medium after 2 h of incubation by placental samples collected at d 30, 60 and 90 of gestation. In contrast to these findings, under similar in vitro conditions, Kukoly

(1984) detected no further increase in P_4 production by placental tissue collected at either d 30, 60 or 90 of gestation between 1 and 2 h incubation periods suggesting that de novo synthesis of P_4 by the porcine placenta did not occur beyond 1 h.

In the present study, in the absence of added P_5, placental tissue apparently did not convert all of its endogenous source of P_5 to P_4, since increasing quantities of P_4 were still being produced at the end of the incubation period. This suggests that either some cholesterol is being converted to P_5 in order to replenish existing endogenous pools of P_5 or placental tissue has an adequate supply of endogenous P_5 for the in vitro production of P_4 until at least 2 h of incubation.

Addition of P_5 to the incubation medium of placental tissue collected at d 30, 60 and 90 of gestation resulted in a two-fold increase in final P_4 concentrations above that of P_4 in the medium of tissue incubated in the absence of P_5. Addition of P_5 also resulted in a linear increase in P_4 accumulation in the medium throughout the length of incubation. This suggests that although an endogenous source of P_5 is available for P_4 synthesis by placental tissue, the endogenous supply is not enough to saturate the enzymes 3β-HSD and/or 3-ketosteroid isomerase, which are responsible

for the conversion of P_5 to P_4, since addition of P_5 enhanced placental P_4 production. Although enzyme activity was not determined, the local regulation of enzyme supply by product inhibition due to an increase in P_4 biosynthesis did not appear to be taking place. Thus, the enzyme systems necessary for in vitro P_4 synthesis from P_5 in placental tissue collected at d 30, 60 and 90 of gestation do not appear to be lacking, inhibited or saturated.

Steroidogenic capabilities of the porcine placenta are supported by histochemical findings that $\Delta^5$3β- and 17-β-HSD are present in placental tissue from the fourth week of gestation until d 101 of gestation (Christie, 1968; Dufour and Raeside, 1969).

Overall P_4 production of placental samples incubated in the presence of P_5 plus hCG did not differ from P_4 production by placental samples incubated in only the presence of P_5 at any of the three stages of gestation examined.

In humans, after the fourth week of gestation, the function of the corpus luteum of pregnancy declines. The placenta then becomes the primary source of P_4 and, with the fetus, the placenta plays an increasing role in steroidogenesis. Human chorionic gonadotropin (hCG) promotes the conversion of cholesterol to P_5 (Klopper and Fuchs, 1977) by initially binding to specific cell membrane receptors and in

so doing, hCG stimulates the receptor coupled adenylate cyclase. The enzyme converts ATP to cAMP, the intracellular second messenger. The generated cAMP relays the message intracellularly by activating protein kinase to initiate two phosphorylation reactions which result in the stimulation of the enzyme cholesterol esterase which converts cholesterol esters into free cholesterol. Cholesterol is then carried into the mitochondria and converted to P_5 through a series of enzymatic reactions.

In light of the previous observations, data from this portion of the study would suggest that the overall hCG signal is not eliciting an increase in cAMP in order to stimulate steroidogenesis. This could possibly be due to: 1) a lack of specific hCG cell membrane receptors on the porcine placenta, 2) the supply of hCG to the porcine placenta was not enough to stimulate an increase in the concentration of cAMP in the target tissue, or 3) disappearance of binding sites due to an occupancy of the receptors during a period of down-regulation.

Although statistical comparisons were not made, it was apparent that the temporal patterns of P_4 accumulation in hCG plus P_5 treated placental samples collected at d 30 and 60 of gestation did not differ from those of P_4 accumulation by placental samples incubated only in the presence of P_5.

Although P_4 accumulation at each incubation period in both P_5 and P_5 plus hCG treated placental samples increased relative to their respective time zero interval, the observed increase in P_4 synthesis in both treatments was due to the addition of P_5.

At d 90 of gestation, although overall placental P_4 production did not differ in P_5 plus hCG (146.18 ng/g) and P_5-treated samples (143.25 ng/g) P_4 accumulation at 1 and 2 h incubation periods differed between the two treatments. Placental P_4 concentrations of P_5 plus hCG treated samples were higher and then lower than P_4 production by P_5-treated samples at 1 and 2 h of incubation, respectively (table 16). This suggests that at d 90 of gestation placental endocrine function allows the steroidogenic stimulus of hCG to occur due to either an increase in gonadotrophic cell-membrane receptor sites or maturation of the enzyme systems responsible for the increase in adenyl cyclase and cAMP steroidogenic activities. During the first h of incubation, hCG appeared to stimulate cAMP synthesis which in turn stimulated steroid synthesis. Once the hCG was bound, the hCG-receptor complex may have been internalized and degraded by lysosomal enzymes resulting in a decrease in the number of hCG receptors on the cell surface and consequently, desensitized to further biologic effects of additional hCG.

Addition of cAMP to P_5 treated placental tissue samples increased final P_4 accumulation at d 60 and 90 of gestation relative to final P_4 concentration of placental explants incubated in the presence of P_5 but without cAMP.

In a similar study conducted in our laboratory on the ovine placenta, Onthank (1983) reported that cAMP exerted an effect on P_4 accumulation when it was added to P_5 treated placenta tissue samples but not when added to tissue in the absence of P_5. Onthank (1983) suggested two possibilities: 1) cAMP did not exert a major effect in the steroidogenic pathway by acting through the protein kinases which increase the conversion of cholesterol and/or 2) that cAMP affected the steroidogenic pathway by exerting its effect on the 3β-HSD or isomerase enzyme systems responsible for the conversion of P_5 to P_4.

In the present study, these observations were not possible since the effect of cAMP on P_4 synthesis was only examined in the presence of P_5. However, since cAMP plus P_5 did exert a stimulatory role in placental P_4 production, then perhaps cAMP either enhances the enzyme systems necessary for the conversion of P_5 to P_4 or cAMP may decrease some restraining influence on the enzyme systems.

The overall effect of cAMP on d 90 placental P_4 (190.89 ng/g) exceeded that of hCG (146.16 ng/g). A possible

explanation is that the porcine placenta may have a diminished capacity to bind and to respond to hCG in terms of adenylate cyclase, cAMP accumulation and steroid synthesis, resulting in a block at the receptor or cyclase level. Addition of cAMP may be able to by-pass that block.

As previously mentioned, in the absence of precursor (P_5) or stimulants (hCG and cAMP) the capacity of the porcine placenta to produce significant quantities of P_4 increased linearly from d 30 to 90 of gestation. Maternal plasma P_4 levels of 10 to 25 ng/ml between d 25 and 100 of gestation have been previously reported (Guthrie et al., 1974; Robertson and King, 1974; Knight et al., 1977). Yet the increasing amounts of P_4 secreted by the placenta during gestation are not reflected in maternal systemic P_4 levels. There are two possible explanations for this: 1) P_4 synthesized by the porcine placenta may be converted to pregnane derivatives such as 5β-pregnanediol (Sheldrick et al., (1981) reported that in goats this steroid circulates in high concentrations during gestation and as an end result of its formation from P_4, the placenta fails to secrete P_4 in quantities sufficient to maintain gestation after ovariectomy); or 2) P_4 synthesized by the placenta is used locally and/or rapidly metabolized through androgens to estrogens.

In this study, in the absence of precursor (P_5) or stimulants (hCG or cAMP) an overall biphasic pattern of estrone production was observed at 30, 60 and 90 days of gestation with an exponential increase in E_1 at d 90 of gestation. Ainsworth and Ryan (1966) demonstrated the conversion of DHEA and androstenedione to estrogens with E_1 being the major estrogen formed by porcine placental tissue taken in late pregnancy (109 to 112 days post-coitum). Choong and Raeside (1974) reported the presence of high concentrations of unconjugated and conjugated estrogens in placental tissue and they suggested that the placenta was the site of synthesis.

In this study, as in previous investigations by Knight et al (1977) and Kukoly (1984), the concentration of estrone present in allantoic fluid (table 27) at d 60 and 90 of gestation greatly exceeded the concentration of estrone in the maternal circulation (table 25), providing additional evidence that the placenta is the site of synthesis.

Addition of P_5 increased E_1 accumulation by placental tissue collected at d 60 and 90 of gestation. Furthermore, an increase in E_1 synthesis during the first .5 h of incubation by P_5-treated placental samples collected at 60 (figure 6) and 90 days (table 10) of gestation was observed. This increase in E_1 synthesis may be a result of an increase in

available P_4 concentrations from P_5 treated placental tissue for the conversion to androgens and subsequent aromatization to estrogens. The enzyme systems necessary for the conversion of P_4 to androgens and then to estrogens do not appear to be lacking, inhibited or saturated since an increase in available P_4 results in a direct increase in E_1.

Although enzyme activity was not determined in this study, the porcine placenta at d 30, 60 and 90 of gestation appears capable of readily synthesizing estrone from C-21 steroids. Thus local levels of estrogens essential for embryonic development, distribution of the embryos along the uterine horns, increase of blood flow in the gravid uterus, and stimulation of cell division and RNA synthesis can be insured by the concepts itself. Moreover, estrogen synthesis in vivo is unaffected when the ovarian source of progesterone is removed surgically and pregnancy is maintained by treatment with medroxyprogesterone acetate, a non-aromatizable progestagen (Heap et al., 1981b) showing that ovarian progesterone is not essential for trophoblast estrogen synthesis. Thus, it would appear that the conceptus and endometrium interact to produce estrogens in order to regulate the local levels. This interaction will be discussed in subsequent sections.

In this study, hCG did not stimulate P_4 synthesis by P_5-treated placental tissue collected at d 30, 60 and 90 of gestation relative to P_5-treated placental samples incubated in the absence of hCG. Likewise, hCG did not exert a stimulatory effect on overall E_1 synthesis by P_5-treated placental explants. Thus, hCG had no apparent stimulatory effect on placental P_5 synthesis from cholesterol nor an effect on steroid synthesis beyond the point of P_5. Possible explanations for these results are the following: 1) there is a lack of receptor sites for hCG to bind to elicit an increase in cAMP or 2) there was an inadequate concentration of hCG to elicit a significant response since at d 30 of gestation placental E_1 concentrations were slightly higher in P_5 plus hCG treated samples than P_5-treated placental samples.

Placental tissue collected at d 60 of gestation and incubated in the presence of P_5 plus cAMP accumulated a greater amount of E_1 in the medium than samples incubated only in the presence of P_5. Thus, cAMP exerted a stimulatory effect on E_1. It was noted earlier that placental samples incubated in the presence of P_5 accumulated more E_1 at d 60 and 90 of gestation than samples incubated in the absence of P_5. Cyclic AMP had an additive effect above that produced by P_5, suggesting that 1) cAMP increases available P_4 for conversion to androgens and subsequent aromatization

to estrogens or 2) cAMP increases the amount of active aromatase.

Endometrial Steroidogenesis

Results from this study add to the increasing evidence to support the theory that the porcine placenta synthesizes steroids. The possibility that the endometrium also contributes to the production of steroids by the gravid uterus is also under investigation.

In this study, endometrial P_4 levels in the absence of P_5 were measured at each day of gestation examined. However, the magnitude of P_4 synthesized by endometrial tissue changed little between d 30 and 90 suggesting that the endometrium has a limited capacity for P_4 production from d 30 to 90 of gestation.

Overall P_4 synthesis by endometrial tissue samples was not nearly as dramatic as that of placental P_4 synthesis between d 30 and 90 of gestation. Although the enzymes 3β-HSD and isomerase which are responsible for the conversion of P_5 to P_4 are present, the endometrium appears to be less active than the placenta in converting C-21 steroids to P_4 between d 30 and 90 of gestation. These observations may be a result of 1) a limited supply of precursor since addition of P_5 did increase slightly P_4 synthesis, 2) the endom-

etrium regulates the local level of P_4 by metabolizing the hormone into less active derivatives like preganolones and pregnanediols (Henricks and Tindall, 1971) or 3) the steroid capabilities of the endometrium are dependent to some extent on the interaction or cooperation between the endometrium itself and the conceptus since the presence of an embryo in the uterus causes some enzymes involved in steroidogenesis to appear in the endometrium. For example, 3β-HSD appears in the uterine epithelium of pregnant ewes around d 12 of gestation, a 17β-HSD appears in mares and 3β- and 17β-HSD appear in the endometrium of sows once there is endometrium-conceptus contact (Christie, 1968; Dufour and Raeside, 1969; Flood 1974; Flood and Marrable, 1975; Flood and Ghazi, 1981).

In both experiments one and two, P_4 accumulation by P_5 treated endometrial samples throughout the length of incubation at d 30 and 60 of gestation was similar. However, at d 90 of gestation, P_4 accumulation throughout the incubation period of P_5 treated endometrial samples in experiment two was almost twice that of P_4 accumulation throughout the incubation time in experiment one. The reason for these results is unclear since endometrial tissues samples in both experiments were treated the same.

Addition of hCG to P_5-treated endometrial samples at d 30, 60 and 90 of gestation did not increase P_4 synthesis in endometrial tissue incubated only in the presence of P_5. Progesterone accumulation throughout the incubation period in both treatments was similar, suggesting that the observed increase in P_4 accumulation in both treatments above the accumulation observed by endometrial tissue incubated in the absence of P_5 was due to the stimulatory effect of the precursor P_5. This suggests that hCG does not elicit a stimulatory effect possibly due to lack of receptor sites or the response by the porcine endometrium to hCG stimulation is that of refractoriness probably due to depletion of the cholesterol available for steroidogenesis. Addition of P_5 to endometrial samples would restore the steroidogenic response.

Addition of cAMP to P_5-treated endometrial samples enhanced P_4 accumulation at d 60 of gestation beyond the accumulation of P_4 in the medium of P_5 and P_5 + hCG treated d 60 endometrial samples. If there is a block to hCG responsiveness at the level of adenylate cyclase activation then exogenous cAMP would be able to by pass that block and stimulate steroidogenesis.

Cyclic AMP exerted its greatest stimulatory effect during the first .5 h of incubation in d 30 endometrial sam-

ples. In contrast to this, in d 60 endometrial samples cAMP exerted a positive effect during the first and second .5 h of incubation. Thus, d 60 placental and endometrial tissue appear to be more responsive to cAMP plus P_5 and P_5 treatment than placental and endometrial tissue collected at d 30 or 90 of gestation. This may be related to the observation of Knight et al. (1977) that an adequate placenta and uterine environment during this period of gestation is critical in order to reduce secondary death losses that occur due to placental insufficiency.

Estrogens circulate in early pregnancy predominantly in the form of estrone sulphate (Robertson and King 1974). The endometrium regulates local estrogen levels through the activity of 17β-HSD permitting the interconversion of estradiol-17β and estrone; and by a sulphotransferase-sulphatase enzyme system allowing the interconversion of estrogen sulphate and estrone (Pack and Brooks, 1974; Dwyer and Robertson, 1980).

The sulphokinase responsible for this interconversion is a P_4 dependent enzyme and the activity of the enzyme system changes during the estrous cycle in parallel with circulating P_4 concentrations (Pack and Brooks, 1974). Furthermore, in vitro studies (Dueben et al., 1979) have demonstrated that endometrial tissue taken early in gestation (d

25) is capable of synthesizing estrogens from P_4 and thereby providing an aromatizable steroid to the blastocyst, that contributes toward estrogen synthesis in early pregnancy. In the present study, uterine artery minus uterine vein (UA-UV) differences in plasma P_4 concentrations were positive at d 30, 60 and 90 of gestation (table 24). Conversely, UA-UV differences in E_1 were negative at those same stages of gestation suggesting that P_4 (of luteal origin) was being taken up and/or metabolized by the endometrium and/or its contents.

In this study, measurable quantities of endometrial E_1 at 30, 60 and 90 days of gestation were detected. The overall concentration of endometrial E_1 at d 30 and 60 of gestation was similar to placental E_1 at 30 and 60 days of gestation, respectively. However, at d 90 of gestation, overall endometrial E_1 concentrations were one-fourth of the concentration of E_1 synthesized by d 90 placental tissue. Although enzyme activity was not measured, this suggests that d 30 and 60 of gestation placental and endometrial tissue have a similar capacity to synthesize E_1 from androgen precursors; however at d 90 of gestation, the capacity of the porcine placenta to synthesize E_1 increases possibly due to available substrate (C-19 steroids) or changes in the enzyme systems necessary for the supply of substrates.

If d 90 placental tissue is capable of providing adequate concentrations of P_4 and androgens for E_1 synthesis then either endometrial enzyme activity decreases or the endometrium converts estrone into estrone sulphate.

Addition of hCG to P_5 treated endometrial samples did not stimulate E_1 synthesis relative to E_1 synthesis by P_5-treated endometrial tissue. Furthermore, E_1 accumulation by endometrial tissue throughout the incubation period did not significantly differ from the temporal pattern of E_1 synthesis by P_5 treated samples. A dramatic increase in steroid production due to hCG has not been observed, which suggests either 1) a lack of hCG cell-membrane receptor sites or 2) if the tissue responds to the gonadotropin stimulus, after cAMP production a state of refractoriness to further stimulation causes a decrease in the number of specific binding sites or irreversible binding. The discrimination between true disappearance of receptors and irreversible occupancy of the binding sites by hCG has been investigated by immunocytochemistry (Van Straaten and Wensing, 1978). The hormone-receptor complex has been visualized through a double-antibody-peroxidase staining technique specific for hCG molecule. When down-regulation is induced in vitro in cell cultures from the testes of piglets a dissociation occurs between the amount of free and occupied

receptor. The free receptors decrease much faster than the occupied receptors (Haour et al., 1983). This finding suggests that irreversible occupancy of receptors occurs and that removal of the hormone-receptor complex is a slow process (up to 24 h) (Mather et al., 1982).

Addition of cAMP to P_5-treated endometrial samples slightly increased E_1 synthesis by d 30 endometrial samples throughout the incubation period relative to d 30 endometrial samples incubated only in the presence of P_5.

Coincubation Steroidogenesis

Placental and endometrial explants were co-cultured to evaluate placental and endometrial interactions at d 30, 60 and 90 of gestation. Overall P_4 synthesis by placental and endometrial explants collected at d 30, 60 and 90 of gestation and co-cultured in M199 without P_5 was approximately the average of P_4 values observed at d 30, 60 and 90 of gestation for placental and endometrial tissue incubated separately. Therefore, P_4 synthesis by coincubation tissue increased linearly between d 30 and 90 of gestation.

Although statistical comparisons were not made, it was apparent that the temporal patterns of P_4 concentration exhibited by the co-cultured tissue collected at d 30, 60 and 90 of gestation was approximately the average of the

temporal patterns of placental and endometrial tissue incubated separately. Placental tissue incubated separately in the absence of P_5 synthesized greater quantities of P_4 than endometrium incubated separately at each day of gestation examined. Since 150 mg of placenta and 150 mg of endometrium were incubated together for a total weight of 300 mg, which is the same amount of placenta and endometrial tissue incubated separately, and the values of P_4 for the coincubation tissue are approximately the mean values of the two tissue types incubated separately then the tissues do not seem to regulate each others steroidogeneic capabilities. Instead it acts like a mixture.

Addition of P_5 enhanced P_4 accumulation by coincubation tissue at each day of gestation examined. Thus, as was mentioned previously, the enzyme systems in each tissue do not appear to be lacking, inhibit or saturated; however, the mechanisms involved in hormonal regulation or cooperation between the two tissues is unclear.

Addition of hCG to d 60 P_5 treated coincubation tissue had a stimulatory effect on P_4 synthesis. However, no stimulatory effect was observed for placental and endometrial samples incubated separately in M199 with hCG plus P_5.

Addition of cAMP did not increase E_1 synthesis by coincubation tissue relative to coincubation tissue incubated in the presence of P_5.

Neither hCG nor cAMP exerted an overall stimulatory effect on E_1 synthesis by coincubation tissue incubated at 30, 60 and 90 d of gestation. However, cAMP stimulated E_1 synthesis in d 60 placental samples and d 30 endometrial samples incubated separately.

In summary, the present investigation revealed that 1) the porcine placenta is capable of synthesizing significant concentrations of P_4 and synthesis increases linearly between d 30 and 90 of gestation, 2) progesterone synthesis by the porcine placenta is enhanced by P_5 and cAMP, 3) the endometrium has a limited P_4 production capacity which changes little between d 30 and 90, 4) placental E_1 production increases significantly between d 30 and 90 and is enhanced by P_5 and cAMP at d 60 and 90 of gestation and 5) hCG had no overall effect on P_4 and E_1 accumulation in the incubation medium at 30, 60 and 90 days of gestation.

CONCLUSIONS

Basic information relative to the physiological and endocrinological interrelationships among the placenta, fetus and maternal system is extremely limited. Therefore, the present in vitro study was aimed at increasing our understanding of porcine placental and endometrial function, especially placental and endometrial steroidogenesis.

It must be emphasized that in vitro experiments may not exactly represent the conditions in vivo; nevertheless, the results from this study demonstrate that the porcine placenta is capable of synthesizing P_4 and E_1. Furthermore, P_4 synthesis by the porcine placenta increases linearly between d 30 and 90 of gestation and is enhanced by P_5 and cAMP. The placenta appears to possess all the enzyme systems necessary for the synthesis of P_4 and E_1 from P_5 by the Δ^4-pathway. Likewise, the endometrium synthesizes P_4 and E_1 in the absence and presence of P_5. However, the magnitude of P_4 synthesis by the endometrium appears to be limited since P_4 production changes little between d 30 and 90 of gestation.

Although no P_4 to T conversion was noted in vitro, an adequate source of androgens, which serve as precursors for estrogen production by the trophoblast/chorion of the conceptus is believed to exist.

Further research is underway to determine 1) the effect of the presence of the conceptus on endometrial steroid metabolism by comparing endometrial steroidogenesis in pregnant and pseudopregnant gilts; 2) the activities of the enzymes estrogen sulphatase, estrogen sulphotransferase, and aromatase in the endometrium and placenta during key periods of pregnancy and during pseudopregnancy and 3) the in vitro biotransformation by the placenta and endometrium of radioactively labelled P_4, A and E_1SO_4.

As our understanding of the steroidogenic capabilities of the fetal-placental-maternal unit progresses, so will our understanding and knowledge of the causes of prenatal mortality.

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APPENDIX

APPENDIX TABLE 1

Composition of Medium 199

Medium 199 powder mix (Gibco Laboratories, Grand Island, NY)	9.9 g/pkg
Sodium Bicarbonate ($NaHCO_3$)	2.2 g
Penicillin	100,000 I.U.
Streptomycin	100,000 µg
Amphotericin B (Fungizone)	250,000 µg
Deionized water	Total volume 1000 ml
pH	7.2

Medium was filtered through a pressurized millipore filter (.45 µm) and stored at 4 C until use.

APPENDIX TABLE 2

Composition of M199-P_5

Medium 199 (M199)	Total volume 150 ml
Pregnenolone (P_5)	.0084 mg/ml

APPENDIX TABLE 3

Composition of M199-P_5-cAMP

M199-P_5	Total volume 150 ml
N^6, O^2-Dibutyrylodenosine 3', 5'-cyclic Adenosine Monophosphate (cAMP)	188.7 mg

APPENDIX TABLE 4

Composition of M199-P_5-hCG

M199-P_5	Total volume 150 ml
Human Chorionic Gonadotropin (hCG)	15,000 I.U.

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