EFFECT OF COFACTORS AND LUTEOTROPIC HORMONE ON THE BIOSYNTHESIS OF PROGESTERONE BY PORCINE LUTEAL TISSUE

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CHAPTER I

INTRODUCTION

The ovaries of many domestic species have been studied extensively and all have demonstrated steroidogenic capacity. The sow is unique among the farm animals being the only polytocous animal in this group.

The pig is well suited for studies in reproductive physiology because it has a recurring estrous cycle which lasts 21 days, a luteal phase which lasts 10 to 14 days and a sufficient number of large corpora lutea to prepare an _in vitro_ model. Porcine corpora lutea are available in all seasons of the year and collection of large quantities of luteal tissue at any time is not a problem.

Duncan _et al._ (57) first demonstrated that porcine corpora lutea slices, though synthesizing progesterone _in vitro_, did not respond to gonadotropins by increased synthesis as do slices from other species. Cook _et al._ (41) after investigating steroidogenesis in porcine luteal tissue _in vitro_, observed that steroidogenesis in luteal tissue from the cow and ewe was much greater. They suggested that this may have been due to low cholesterol stores in porcine luteal tissue. Furthermore, they reported a significant _in vitro_ response to gonadotropins only in tissues collected within a few minutes after the death of the animal, even though there was a wide range of variation. Savard _et al._ (162) and Armstrong _et al._ (12) after investigating steroidogenesis in bovine luteal slices, concluded that degenerative changes occurred in the

luteal tissues during their preparation because of the time elapsing between cessation of the blood supply to the organ and its removal from the donor animal. This, they suggested, accounted for the lowered rates of progesterone synthesis observed *in vitro*. Mason *et al.* (127) and Savard *et al.* (163) demonstrated that addition of cofactors to incubating slices of bovine luteal tissue stimulated progesterone synthesis. Bjersing *et al.* (24) also reported an increase in progesterone synthesis in porcine granulosa cell aggregates when incubated with several cofactors.

On the basis of the foregoing reports, it can be postulated that cofactors and precursor pool levels in swine luteal tissue are lower than in other species and that during the collection of tissues, the levels of these labile metabolites may fall below the concentrations necessary for a steroidogenic response to gonadotropins. One can postulate further, that addition of the metabolites most likely involved (Bjersing *et al.* (24), Marsh and Savard (126), and Savard *et al.* (163)) would reconstitute the system, making it responsive to tropic hormones. Because porcine luteal tissues are more available in large quantities, such a reconstituted system could provide a readily available model for extensive research in steroidogenic mechanisms.

To test the postulations, a series of *in vitro* experiments were conducted in which porcine luteal minces were incubated according to the technique of Duncan *et al.* (57).

CHAPTER II

LITERATURE REVIEW

The Corpus Luteum

The reproductive system of the female is composed of the ovaries and an accessory system. The ovaries are paired in all mammals. One important component of the ovary is the corpus luteum which is formed following ovulation. Histologically, it is composed primarily of granulosa cells, however, thecal cells may contribute to its formation. Hypertrophy and hyperplasia of these two cell types account for the increase in the size of the corpus luteum. Corpus luteum (yellow body) is therefore a term applied to the endocrine gland, which develops in the ruptured ovarian follicle after maturation and discharge of the ovum. The corpus luteum differs from the other endocrine glands in that it is a transitory structure with a short, but variable life span.

The first recorded records describing the corpus luteum date back to the sixteenth century, when Volcherus Coiter, in 1573 and Fallopius, in 1584 described the ovaries as "cavities" filled with fluid and sometimes with yellow solid. There however, exists some evidence that approximately 30 years prior to these dates, Vesaluis observed a corpus luteum in the ovary of a young girl. It was during the sixteenth century that man began his inquiry into the anatomical nature of the internal organs of man and other animal species.

In 1672, Regnier de Graaf recognized a relationship between the corpus luteum and the presence of a fetus in the uterus. De Graaf also observed that the corpus luteum would disappear after parturition and that the corpus luteum developed as a result of ovulation. Malpighi, in 1689, with the use of the microscope, accurately described the corpus luteum and he is credited with applying to it the name "corpus luteum" (16,45).

In 1827, von Baer, working with the bitch, concluded that luteal tissue is of thecal origin and this work is acknowledged as the first attempt to pinpoint the source of the corpus luteum. However, in 1842, Bischoff, working with the rabbit, suggested that the source of lutein tissue was the granulosa cells lining the follicle. The beginning of a firm basis for the origin of the corpus luteum began with Sabotta in 1895 to 1897. Sabotta carefully observed the formation of the corpus luteum in the mouse and rabbit and assumed that granulosa cells gave rise to the lutein cells (16). During this early period interest came to focus upon the luteal structures and many species were investigated, when for example, in 1893, a corpus luteum was shown to be present in reptiles. Interested readers may refer to review articles by Asdell (16) and Corner (45) concerning luteal-like structures in other vertebrates.

Loeb (110), in 1906, published the results of his study of the formation of the corpus luteum in the guinea pig. He strongly affirmed that granulosa cells invade the ruptured follicle and take part in the formation of the corpus luteum. He reported that thecal cells were observed to protrude into the granulosa. He also noted that the theca interna and granulosa cells assumed a similar appearance in the ruptured follicle. This corroborated the earlier observations and suggestions of

Schon whom in 1863, did similar studies in cats. He found that lutein cells may arise from both the granulosa and theca interna cells (16). In 1919, Corner (46) published an excellent review on the origin of the corpus luteum in the sow. He studied the formation of the corpus luteum and demonstrated that the granulosa cells do become the "lutein" cells. In 1937, Mossman's (135) study of the ovary of the pocket gopher indicated that the glandular cells of the corpus luteum are derived from the follicular epithelium, but not from the theca interna. In 1948, Harrison (88) reviewed the literature up to that time and surmised that the majority of the lutein cells are derived from the granulosa. Nalbandov (136) reported that it is the granulosa cells of the ovulated follicle which, by hypertrophy and hyperplasia, are formed into the corpus luteum; but that the lutein cells can arise either from the theca interna, or the granulosa, or both, depending upon the species. Greep (79) notes that after cytomorphosis is complete, the two cell types are easily identified. It is therefore generally accepted that in eutherian mammals the granulosa cells become transformed into the luteal cells of the corpus luteum (196).

Corner (46), while studying the formation of the corpus luteum in the sow, also observed that there is a ramification of an extensive vascular complex from the theca interna throughout this newly formed structure. Again, 35 years later, Corner (44) carefully followed the development, organization and breakdown of the corpus luteum in the monkey, observing that on the second day following ovulation, blood vessels invaded the granulosa of the corpus luteum and this vascular network also was derived from the theca interna. Corner (43) and Elder (62) are in agreement that the capillary bed throughout the luteal tissue is so

complex that every cell in the gland is in contact with a blood vessel. These observations are corroborated by Harrison (88), whose review of the literature suggests that in most species studied the blood vessels of the corpus luteum are well vascularized by the time implantation of the fetuses occur.

Not only is there an extensive vascular network throughout the corpus luteum, but there also exists a reticular network which appears to vary with each species. Loeb's (110) observations suggested that connective tissue permeated the ruptured follicle of the guinea pig ovary. Corner (43) has also observed in the sow, that the capillary endothelial lining is the source of the reticular connective tissue which forms a dense network about all the lutein cells, thereby holding them together. In addition, Corner's (44) studies of the monkey ovary suggest that the fibroblast-like cells are apparently derived from the endothelium (as are the blood vessels) and enter the luteal cavity about the fourth day after ovulation. It is further suggested that these fibroblastic cells produce the connective tissue of the luteal cavity and apparently arise from the invading vascular network. Harrison (88) and Greep (79) concur that the connective tissue proliferates from the theca interna cells and permeates between the luteal cells to form the reticular network. It should be noted that when Cook et al. (41) studied luteal progesterone synthesis in the cow, ewe and pig, they observed that swine corpora lutea were very soft and permeated by what these workers termed "rafts of connective tissue". In 1951, another distinctive cell was demonstrated in the human corpus luteum. White et al. (190) observed what they termed "K cells" appearing in the granulosa lutein layer. These cells were thought to invade the corpus luteum by

some special means, unlike the theca interna and accompanying blood vessels which invaginate into the collapsed membrana granulosa.

While studying the corpus luteum of pregnancy in the sow, Corner (45) also noted that the luteal cells arising from the granulosa cells became laden with lipoid materials, underwent mitotic division and thereby increased in number. Corner further proposed that these cells lose most of their fatty inclusions, pass into the corpus luteum and lodge among the granulosa cells throughout the whole structure. Corner (46) also suggested that some of the thecal cells persist for the duration of pregnancy as elements of the corpus luteum, but he also noted that as gestation progressed, the lutein cells became less elaborate; however, they did not change in form. It was also observed in the cow, by Elder (62), that the lutein cells of early pregnancy were rich in protoplasmic material, while containing a well defined granular nucleus. Enders and Lyons (63) observed that lutein cells formed a functioning corpus luteum characterized by an abundant tortuous, tubular, agranular endoplasmic reticulum and mitochondria. The endoplasmic reticulum is more abundant in the lutein cells of pregnant animals. These characteristics are similar to the characteristics of cells in other tissues associated with secretory functions. These characteristics influenced White et al. (190) to suggest that the K cells of the human corpus luteum may be the site of intense localization of ketogenic lipids, if not the site of production or utilization of steroids. It should be noted that the glandular nature of the corpus luteum was pointed out very early by Prenant in 1898, at which time, he suggested that its function was secretory. Mossman (135), as a result of his studies with the pocket gopher, noted that there are two other glandular elements in the ovary besides the

corpus luteum tissue. They are the thecal gland tissue and the interstitual tissue.

The corpus luteum, although a transient structure, plays a very important role in the reproductive process of the female. The secretory activity of the corpus luteum, which is essential for pregnancy, has been well established (6,47,73,102,114). It has also been shown that the length of time that the corpus luteum is essential during pregnancy varies among the species.

Steroidogenesis

The biosynthesis of ovarian hormones takes place in a heterogeneous cell population regulated by the pituitary gonadotropins and is therefore under the influence of the neuroendocrine control of the hypothalamus. Within this framework, the recurring phenomena of follicular development, ovulation and corpus luteum formation takes place and the steroid hormones are secreted at each stage of the cycle which regulates reciprocally the very gonadotropins which influence their formation. The ovary produces two general classes of hormones - first the steroids, typified by estrogens and progestins and second, a protein or polypeptide hormone, relaxin (76).

Among the first workers studying the female sex hormones were Frank and Gustavson (69) who obtained a potent lipid-soluble extract from the corpora lutea of cows and swine which was able to induce hyperemia and hyperplasia of Müller's Tract and mammae in rats. They showed these extracts to contain carbon, hydrogen and oxygen. Allen et al. (7), in similar studies, demonstrated the presence of hormones in human ovaries. They injected either liquor folliculi or tissue implants into

ovariectomized rats and observed changes in the vaginal epithelium. The highest hormone contents were observed in ovaries in the first half of pregnancy. Corner (48) also investigated the sites of formation of female sex hormones and assumed that the most probable site of estrogenic hormone synthesis to be in the theca interna cells of the ovary. In 1943, Dempsey and Bassett (51) investigated sex hormone influence in the rat ovary by using histochemical techniques. They were able to establish the presence of sterol hormones in the corpus luteum and also noted that the corpus luteum contained the least steroid during the period of this gland's greatest secretory activity. Shortly thereafter, Everett (65) demonstrated microscopically the presence of lipids in the corpus luteum of the rat. He suggested that cholesterol was the important lipid. In 1947, Claesson and Hillard (37), using polarization, optical and histochemical methods, demonstrated the presence of cholesterol in the theca interna of the rabbit ovary and this sterol was shown to vary in concentration with the sexual phase. Claesson _et al_. (36) later reported that cholesterol was the main lipid constituent in the ovary. In 1951, by means of cytochemical techniques, Barker (20) described the presence of lipid droplets in the granulosa-lutein cells and assumed that the presence of lipid filled cells represented the site of production of ovarian steroid hormones.

The first successful attempt to demonstrate _in vitro_ synthesis of steroid hormones came in 1940 when Dansby (49) perfused isolated bull testes within one hour after slaughter and observed comb growth in chickens injected with the perfusate. In 1951, an enzyme was shown to be present in the corpus luteum by Samuels _et al_. (158). This enzyme was shown to be capable of oxidizing the Δ^5-3-ol steroid structure to a

conjugate form. In 1953, Werthessen et al. (189) reported that the isolated, perfused sow ovary was capable of synthesizing cholesterol, estrogen and β-estradiol from acetate-1-^{14}C as a precursor. A year later, Hayano et al. (89) prepared and incubated bovine corpus luteum homogenates and demonstrated steroid formation. Rabinowitz and Dowben (146) showed that homogenates and tissue slices of canine ovaries incorporated acetate-2-^{14}C into cholesterol, estradiol and estrone. Soloman et al. (175) in 1956, incubated bovine ovarian tissue and reported the conversion of progesterone to androstenedione. In 1958, Wattenberg (187), using histochemical techniques, demonstrated the presence of a steroid hydrogenase in bovine luteal tissue and suggested that it was essential to progesterone formation.

These investigations of the 1930's, 1940's and 1950's have established that one of the principle sites of steroid metabolism in the ovary is the corpus luteum. There has been an attempt to divide steroidogenesis in the corpus luteum into two pathways. The first, synthesizing only progestins and the second, capable of formation of numerous steroids including estrogens (163).

Human corpus luteum preparations have been shown to yield great quantities of estradiol. Inguilla et al. (100) reported the synthesis of eight steroids in slices of human luteal tissue. Their study revealed that this ovarian component synthesizes a pattern of steroids different from that of the follicle. Hammerstein (87) also using the corpus luteum of the human ovary, incubated the gland in vitro in the presence of acetate-1-^{14}C. He obtained seven radioactive steroids, including progesterone. In a related experiment, Griffiths et al. (80) showed that human tissue consisting almost exclusively of granulosa cells, when

incubated in Kreb's-Ringer bicarbonate solution also possessed steroidogenic capability. In other experiments, Ryan and Short (156) demonstrated that preparations of equine granulosa and thecal cells possessed steroidogenic activity. In 1961, Taylor (184) utilized histochemical techniques and demonstrated that rat luteal and thecal tissues are capable of progesterone synthesis. Over the years, isotope incorporation studies have shown that progesterone is a prominent corpus luteum product in several species (9,10,33,125,161,183). In general, *in vitro* findings agree with *in vivo* studies of human ovarian venous blood from ovaries containing a corpus lutuem (132).

Biochemical investigations have shown that the ovary has the capacity to convert acetate and cholesterol to other steroid compounds. Werbin *et al.* (188) have demonstrated that *in vivo*, cholesterol is also a precursor of estrone. Sweat *et al.* (181) using bovine and human ovaries, Huang and Pearlman (98) using human ovaries, Huang and Pearlman (97) using rat ovaries and Mason *et al.* (127) using bovine ovaries demonstrated the capacity of luteal tissue to synthesize labeled progesterone from radioactive acetate, proving the corpus luteum to be an important site not only for elaboration of estrogen but progesterone as well. It was observed by Telegdy and Savard (186) in the rabbit, that the corpus luteum incorporated approximately 20-times more radioactivity into the eight steroids measured on a per gram basis, than the interstitium. Pregnenolone and progesterone were two of the most highly radioactive steroids identified. O'Donnell and McCaig (139) using acetate-1-^{14}C as a precursor, demonstrated that ovarian slices from cases of Stein-Leventhal's syndrome (a clinical entity in which there is hyperplasia of the theca interna cells) were capable of synthesizing labeled estrone,

17β-estradiol and oestriol. Steroidogenic activity was improved with the addition of fumarate, glucose and nicotinamide in Kreb's-Ringer phosphate medium.

With the accumulation of data on hormone synthesis in the ovary and other tissues the past several years it appears that there is a common pathway existing for the biosynthesis of all steroid hormones. The granulosa and theca interna appear to be the cellular sites of ovarian steroidogenesis. The theories which have been presented to explain the regulation of steroid hormone formation are still open to investigation. Short (171) has proposed a two-cell theory in which the granulosa cells produce progesterone and the theca interna cells produce estrogens. This theory implies that both cell types are necessary for complete steroid formation. Another theory proposed (53) is that there are alternate pathways for the synthesis of specific steroid hormones. Estrogen biosynthesis is thought to occur by one pathway in the follicle and by another in the corpus luteum. It has been proposed too that the synthesis of a specific hormone may be dependent upon the presence of a specific cofactor. For example, it has been shown that most of the major steps in steroid hormone formation require reduced triphosphopyridine nucleotide (TPNH), also designated as reduced nicotinamide adenine dinucleotide phosphate (NADPH). Another possible mechanism regulating specific steroid hormone synthesis is the inhibition or activation of ovarian enzymes by the synthesized ovarian steroids. Still another regulatory mechanism that has been proposed is that gonadotropins either activate or inhibit key enzymes in the steroidogenic pathway.

Steroidogenesis and Pyridine Nucleotides

In 1951, Samuels et al. (158) using preparations of placental, luteal, testicular or adrenal cortical tissue demonstrated that these tissues were capable of converting cholesterol and Δ^5-3-ol intermediates to active hormones. They found that addition of diphosphopyridine nucleotide (DPN), also designated as nicotinamide adenine dinucleotide (NAD), augmented these reactions. Hayano et al. (90) observed that the addition of adenosine triphosphate (ATP), Mg^{++} and NAD augmented steroidogenesis in bovine adrenal and luteal tissue incubations, thereby increasing the yield of 6β-hydroxy-11-deoxy-corticosterone. They also noted that NADP in the presence of fumarate, oxygen and a pH of 7.4 could replace ATP, Mg^{++} and NAD. A year later, Lynn et al. (112) successfully prepared an aqueous particle free extract of adrenal, testicular and ovarian tissues and found that the aqueous phase required augmentation with ATP and NAD for activity. These extracts, when incubated with cholesterol-4-^{14}C, yielded several radioactive steroids. Glock and McLean (71) found that NADP was present in large concentrations in the ovaries of rabbits and guinea pigs and in 1959, Wiest (191), using rat ovarian tissue, observed that NADPH was important for steroidogenesis. In 1960, Duncan et al. (57) incubated porcine corpora lutea slices in Kreb's-Ringer bicarbonate buffer medium and glucose under an oxygen atmosphere. They were able to show for the first time that progesterone was synthesized by porcine luteal tissue. The addition of NAD to the incubation media resulted in an increase in progesterone synthesis. In a later study, Duncan et al. (58) also observed that the addition of NAD and pregnenolone to incubating porcine luteal tissue increased the

synthesis of progesterone. Mason et al. (127) in 1961, using the bovine corpus luteum as a model, demonstrated that NADP, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase caused a marked increase in progesterone synthesis (6 to 8 times that of control tissues), however, the use of NAD or NADH in comparable concentrations to that of NADP did not appreciably stimulate progesterone formation. Mahajan and Samuels (117) demonstrated that equine corpus luteum homogenates, with a NADPH generating system, were steroidogenically active. In 1964, Hall and Koritz (83) used a mitochondrial preparation from bovine corpora lutea and demonstrated that cholesterol is converted to pregnenolone in the presence of NADP. In 1966, Dorrington and Kilpatrick (54) and Solod et al. (173), both using rabbit luteal tissue, Armstrong and Black (12), using bovine luteal tissue, and Channing and Villee (33), using rat luteal tissue, demonstrated that the addition of NADP to the incubating tissues augmented steroidogenesis. In 1967, Jackanicz and Armstrong (101) observed similar effects using a rabbit ovarian tissue homogenate augmented with NADP, as did Shima et al. (170), using the luteinized rat ovary and Bjersing and Carstensen (24), using porcine granulosa cells. More recently, similar work (81,85,169) has confirmed the aforementioned reports.

Steroidogenesis and Enzymes

It has been reported that the presence of certain regulatory enzymes are related to variations in steroidogenesis. In 1944, Corner (42) found that the granulosa cells of the ovary from the sow, guinea pig and bitch were deficient in the enzyme, alkaline phosphatase, whereas the theca interna had large deposits present. In the rabbit ovary

the enzyme concentration was shown to be opposite, whereas the rhesus monkey had equal concentrations in both cell layers. Goode et al. (74) in 1965 reported similar results in the sow. Akins (5) found that alkaline phosphatase progressively declined as the porcine corpus luteum matured and this activity declined after day 15. Beyler and Szego (23) found that a decrease in β-glucuronidase activity (another of the hydrolytic enzymes) occurred in the rat corpus luteum as progesterone synthesis increased.

In 1946, Buddulp et al. (30) studied ATP-ase activity in rat corpora lutea. They observed this enzyme to be decreased during the first half of prenancy when the output of progesterone was greatest.

In 1947, Mayer et al. (131) observed that succinic dehydrogenase activity increased in the rat corpus luteum of pregnancy and reached a maximum at day 11 and remained constant to day 20, then decreased. Ahrén et al. (3) studied succinoxidase activity and observed a correlation with luteinizing hormone (LH) concentrations. This enzyme was observed to increase significantly when LH was added to incubating granulosa cells of the rat ovary. An earlier experiment by Eckstein (60) showed an increase in ovarian weight and succinic dehydrogenase activity when rats were treated with pregnant mare's serum (PMS). Dehydrogenases are enzymes which catalyze reactions in electron transfer and is accompanied by transfer of hydrogen ions. Dehydrogenases catalyze a number of reactions which involve NAD and NADP.

In 1951, Samuels et al. (158) observed an unnamed, but active enzyme in adrenal, ovarian, placental and testicular tissue, which was thought to be important in the synthesis of steroid hormones. In 1953, Hayano et al. (90) reported the presence of 6β-hydroxylase in bovine

corpora lutea. In 1956, Soloman et al. (175) demonstrated the presence of 17α-hydroxylase in bovine ovarian tissue and in 1962, Short (171) described the presence of a 17α-hydroxylase in equine ovaries, but little activity was observed in the corpus luteum. A year later, Mahajan and Samuels (117) obtained similar results. In 1967, Axlerod and Goldzieher (17) produced evidence of 17α-hydroxylating, 3β-ol dehydrogenase, 17β-hydroxysteroid dehydrogenase and desmolase activities in ovarian tissue from a human pseudohermaphrodite.

In 1963, Ichii et al. (99) observed a relationship between LH and 20α-hydroxylase in bovine luteal tissue. Later, Chatterton and Greep (35) observed a similar relationship in rat ovaries, in vitro. This enzyme was thought to effect progesterone synthesis. Davenport and Mallette (50) found that rabbit ovarian homogenates contained 20α-hydroxysteroid dehydrogenase which required NADP. Popkin et al. (145) obtained similar results in rat ovarian tissue. Balogh et al. (19) demonstrated that in the rat ovary the enzyme 20α-hydroxysteroid dehydrogenase appears only after LH stimulation. Wiest et al. (192,193) and Kidwell et al. (105) also showed that LH produced an increase in 20α-hydroxysteroid dehydrogenase activity in rats. They observed an increase in estrogen secretion, ovulation and corpus luteum formation. Balogh (18) demonstrated histochemically that the corpus luteum of the rat was the richest source of this enzyme and also suggested that this further indicated the site of progesterone synthesis.

Goldberg et al. (72) using histochemical techniques demonstrated the presence of steroid-3β-ol-dehydrogenase in the human ovarian, placental and adrenal tissues. This enzyme activity was shown to be closely correlated with progesterone production. Rubin et al. (154) using

quantitative biochemical assays and histochemical techniques, studied Δ^5-3β-hydroxysteroid dehydrogenase activity in adrenals and ovaries of various mammals. They noted that this enzyme had a role in the conversion of prenenolone to progesterone. Later, Rubin and Deane (153) using rat luteal tissues demonstrated that administered gonadotropins resulted in an increase in ovarian Δ^5-3β-hydroxysteroid dehydrogenase activity. Davenport and Mallette (50) studying rabbit ovary homogenates, found that the 3β-hydroxysteroid dehydrogenase utilized NAD. Popkin et al. (145), using the rat corpus luteum, obtained similar results.

Ichii et al. (99) observed the presence of a cholesterol side-chain cleaving enzyme in bovine corpora lutea. They noted that LH stimulated the activity of the enzyme. Bjersing and Carstensen (24) found that the cholesterol side-chain cleaving enzyme activity was low in porcine granulosa cells.

In 1958, Wattenburg (187) found a dehydrogenase in adrenal, luteal, placental and testicular tissue which was involved in progesterone synthesis. Marks and Banks (122), in 1960, noted that glucose-6-phosphate dehydrogenase also had a role in the formation of steroid hormones and in 1961, Mason et al. (127) observed that G-6-P and its dehydrogenase was able to markedly increase progesterone synthesis. In 1962, Studzinske et al. (180) noted four NADPH-linked dehydrogenases in the human adrenal cortex, a) isocitric dehydrogenase, b) glucose-6-phosphate dehydrogenase, c) glucose-6-phosphogluconic dehydrogenase and d) malate dehydrogenase. A year later, Savard et al. (162), while studying the role of NADPH in bovine and human corpora lutea, found that the four dehydrogenases described by Studzinski et al. (180) were present in large amounts. It was noted by Savard and co-workers, that the four

dehydrogenases appeared to be capable of generating NADPH needed for the synthesis of progesterone in luteal slice preparations. Glucose-6-gluconate and glucose-6-phosphate dehydrogenases were found to be most effective. They also found that the addition of exogenous G-6-P and NADP further increased the formation of progesterone. The addition of exogenous NADP stimulated steroidogenesis only to a small extent as this cofactor must first penetrate into the cell. Wiest et al. (192) found that G-6-P dehydrogenase was high in rat luteal tissue and that LH administration induced an increase in this enzyme. Eckstein and Landsberg (61) showed that PMS enhances the activity of succinic, malic and isocitric dehydrogenases of the tricarboxylic acid (TCA) cycle in the ovaries of mature rats. Nielson and Warren (138) studied NADPH generation in the human ovary and observed that the luteal tissue contained high levels of the four dehydrogenases which were described by Savard et al. (162) and Studzinski et al. (180). Betz and Warren (22) studied G-6-P dehydrogenase activity in the human placenta, a steroidogenic organ. This enzyme is important in the production of NADPH. The administration of bovine LH by McKerns (115) to young female rats was shown to augment G-6-P dehydrogenase activity, therefore increasing the rates of formation of NADP and NADPH in the ovaries. He showed NADPH to be necessary for the hydroxylation reactions in the conversion of cholesterol to progesterone. Hirshfield and Koritz (95) obtained an increase in progesterone synthesis from large particle preparations of the bovine adrenal cortex and the bovine corpus luteum when augmented with G-6-P and its dehydrogenase. Similar results were obtained by Kidwell et al. (105) using superovulated rat ovaries.

Steroidogenesis and TCA Cycle Intermediates

In 1953, Hayano et al. (90) prepared washed beef adrenal homogenates and observed 11β-hydroxylation of deoxycorticosterone to corticosterone when supplemented with Mg^{++} and fumarate. If either component was absent, no synthesis occurred. Malate and succinate were shown to effectively replace fumarate, but cis-asconitate, citrate, α-ketogluterate, ascorbic acid, acetate, oxalacetate, lactate, pyruvate, hexose diphosphate, triose phosphate, glucose or asparate caused no measurable effect. Similar results were obtained by Grant and Brownie (78) using bovine adrenal mitochondrial preparations. They also showed that isocitrate could replace fumarate. Sweat and Lipscomb (182) found fumarate and malate to be highly effective in steroidogenesis and seemed to exert their effect in systems which generate NADPH. Their apparent role then is to supply NADPH. These workers observed that when NADP was added to NADH in the presence of fumarate or malate, increased activity resulted. Grant (77) also reported fumarate to have an important role in NADPH generation.

In 1960, Behrman and Duboff (21) studied the metabolic role of the TCA cycle in the human ovary. They quantitatively recovered all the members of the TCA cycle. They suggested that this is one of the important sources of energy within the ovary. They also observed that the metabolic pathway is mainly via the TCA cycle. Duncan et al. (57) augmented porcine luteal slice incubations with glucose and Lynn et al. (111) reported that addition of glucose in vitro or in vivo prior to the experiment, enhanced progesterone synthesis in bovine luteal slices.

These findings lend support to the theory that a pathway for

steroid synthesis, common to the adrenal gland and ovary, exists and that this pathway may be the same in other steroidogenic organs. Ichii et al. (99), Savard et al. (161) and Wattenberg (187) suggest that the steroidogenic pathway is the same in adrenal tissues as in corpus luteum tissues.

The following review on progesterone synthesis is presented in chronological order; however, the reader may refer to Figure 1 to better understand the development of the current concept of progesterone synthesis.

Progesterone Biosynthetic Pathways

An important accomplisment of biochemistry has been the elucidation of the biosynthetic pathway of cholesterol and therefore progesterone. The primary pathway is thought to involve acetic acid, mevalonic acid and squalene. The main biosynthetic steps from acetate to cholesterol to progesterone have been shown in many species and the pathways are thought to be common to the species that have been studied. With this concept in mind then, the adrenal cortex, ovary, placenta and testes which synthesize steroid hormones and secrete them into the blood-apparently possess the same enzyme systems.

In the 1940's, through the use of isotope techniques, it was demonstrated that all the carbon atoms of the cholesterol molecule were directly derived from acetyl coenzyme A (acetyl co A). Progesterone, in turn, was shown to be synthesized from cholesterol. The steroid molecule, cholesterol, is a very versatile compound in the cell, being converted to a variety of compounds such as bile salts, androgens, estrogens and corticoids - to name a few. Progesterone derived from

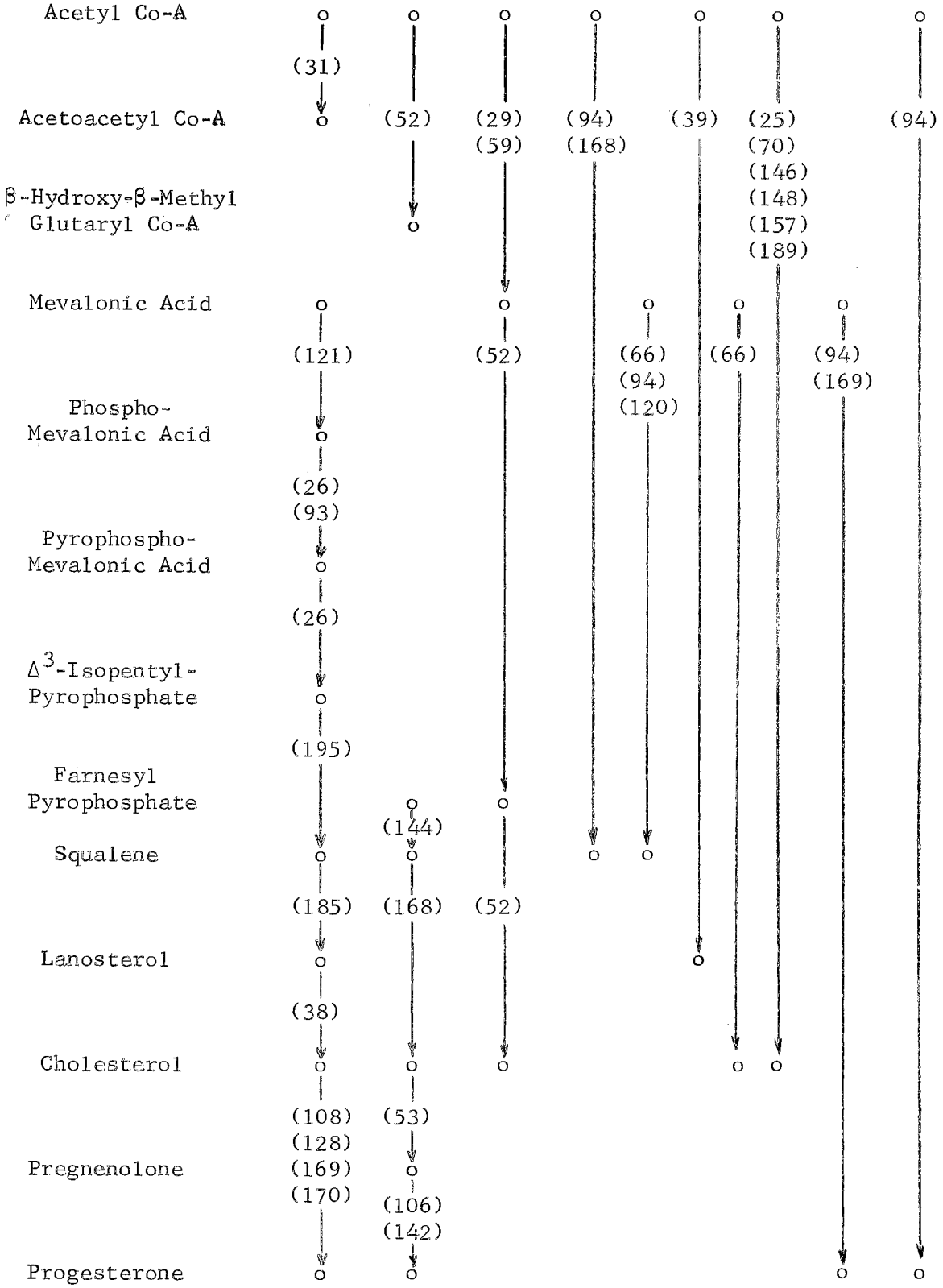

Figure 1. Documented Flow Diagram Proposing a Probable Progesterone Biosynthetic Pathway

cholesterol not only has an important role in female reproductive physiology as a circulating hormone, but also serves as the central steroid and a ubiquitous intermediate in the production of the many steroid hormones in the adrenals, ovaries, placenta and testes.

In 1946, the synthesis of cholesterol from acetate precursor was first observed by Block et al. (25) using a rat liver slice preparation. In this experiment, the need for oxygen was demonstrated, as no synthesis was obtained in anerobic conditions. Brady (27) in 1957, used a Warburg Apparatus and showed that testosterone was synthesized from acetate in preparations of human, porcine and rabbit testes. Werthessen et al. (189) perfused porcine ovaries with a perfusate containing acetate-1-^{14}C and labeled cholesterol was shown to be synthesized.

Steroid research continued and in 1954, Frantz and Bucher (70), using rat liver homogenates augmented with nicotinamide, Mg^{++} and NAD, also observed that acetate-1-^{14}C was incorporated into cholesterol. During that year, Schwenk et al. (168) isolated squalene from porcine liver for the first time. Liver, perfused with ^{14}C-carboxyl labeled acetate, was observed to synthesize squalene-^{14}C and the recovered radioactive squalene when fed to, or injected intraperitoneally into rats, was converted to cholesterol-^{14}C. Also in 1954, Pearlman et al. (142) using homogenates of human placenta in Kreb's-Ringer phosphate buffer, observed the conversion of pregnenolone to progesterone. In this experiment, it was found that NAD was an essential cofactor.

In 1955, Rabinowitz and Dowben (146), using viable tissue slices and homogenates of canine ovaries, were able to demonstrate acetate-2-^{14}C incorporation into labeled cholesterol, estradiol and estrone. Reich and Lehninger (148) in the same year, added ATP, NAD and fumarate

to both bovine and canine adrenal cortex homogenates. They concluded that the adrenal preparations were capable of converting acetate to cholesterol and the recovered cholesterol was shown to be converted to corticosteroids in the adrenal homogenates. A year later Clayton and Bloch (39) demonstrated that rat liver homogenates, when augmented with NAD, Mg^{++} and nicotinamide, were capable of synthesizing lanosterol from acetate precursors. In addition, Clayton and Bloch (38), experimenting further with rat liver homogenate preparations, also showed that lanosterol could be converted to cholesterol. Soloman et al. (174) used homogenates of bovine adrenal glands incubated in the presence of ATP, NAD, fumarate, Mg^{++} and nicotinamide and obtained data by which they postulated that 20α-hydroxysteroid was an intermediate between cholesterol and progesterone and this linked cholesterol to progesterone.

In 1957, Dituri et al. (52), using heart muscle, liver or yeast preparations, were able to show that labeled acetate was converted to β-hydroxy-β-methyl glutaric acid (HMG). They also demonstrated, by using rat liver homogenates, that mevalonic acid (MVA) was incorporated into fornesenic acid which was in turn converted into cholesterol. Squalene was shown to be an intermediate. During the same period, Tchen and Bloch (185) were able to show that NAD, NADP or nicotinamide would augment the conversion of squalene to lanosterol in rat and swine liver homogenates. It was also shown that when the homogenates were washed, the steroidogenic activity was lost, but could be restored by the addition of NADP, G-6-P and its dehydrogenase. Oxygen was shown to be ineffective in increasing this conversion. Concurrently, Ryan and Engel (155) found that the addition of ATP, NAD and nicotinamide, to bovine adrenal microsomal preparations was necessary for maximum hydroxylating

activity. This reaction is thought to be specific for the synthesis of progesterone and its derivatives. They found that a NADPH generating system and atmospheric oxygen could augment this reaction.

Two years later, Ferguson et al. (66) using yeast preparations, and Markley and Gurin (120), using rat liver homogenates, provided data that MVA is somehow eventually converted to squalene. Also, Ferguson et al. (66) provided additional data that MVA is a precursor to cholesterol. They also showed that the addition of a NADPH generating system or exogenous NADPH was required for activity and that NADH was only partially so. Markley and Gurin (120) were able to establish in their rat liver homogenate system that ATP, Mg^{++}, NADP and G-6-P were required as cofactors in the conversion of MVA to squalene. Block et al. (26) using a similar system, were able to show that ATP and Mg^{++} were required cofactors in the conversion of phosphomevalonic acid to pyrophosphomevalonic acid and the conversion of pyrophosphomevalonic acid to Δ^3-isopentyl pyrophosphate. In addition, Yuan and Block (195) used yeast preparations to show that Δ^3-isopentyl pyrophosphate is converted to squalene. Agranoff et al. (2), through the use of yeast preparations, demonstrated that the isomerization of Δ^3-isopentyl pyrophosphate was dependent on Mg^{++}. Durr and Rudney (59) in 1960, demonstrated that acetate was converted to MVA, that NADPH was a necessary cofactor and that two moles of NADP were reduced for each mole of coenzyme A released from HMG-Co A.

In 1961, Markley and Smallman (121) made a rabbit liver preparation and showed that the first phosphorylation step for MVA required the cofactors ATP and Mg^{++}. They also showed this cofactor requirement to be the same in yeast. Hellig and Popják (93), also using liver preparations, demonstrated that 5-phosphomevalonate was converted to

5-pyrophosphate mevalonate at an optimum rate in the presence of ATP and Mg^{++} at a pH of 7.3. Popjak et al. (144), also using liver incubation techniques, demonstrated the requirement for NADPH in the conversion of farnesyl pyrophosphate to squalene. Ryan and Smith (157) used a human ovary preparation and showed that cholesterol was synthesized from acetate in good yield.

Staple et al. (178) demonstrated the requirement of ATP and NAD for the enzymatic cleavage of the cholesterol side-chain in the adrenals, liver, ovary and testes. It was observed that coenzyme A, Mg^{++}, ADP, AMP and nicotinamide had little, if any effect. However, it was noted by Constantopoulos and Tchen (40) that ATP, coenzyme A and fumarate could be used as a NADPH generator and was effective in cholesterol side-chain cleavage. Halkerson et al. (82), using bovine adrenal cortex mitochondrial fractions, were able to show that G-6-P and its dehydrogenase was effective as a NADPH generator for cholesterol side-chain cleavage. Fumarate and Mg^{++} were also shown to be effective for cholesterol side-chain cleavage. Schimizu et al. (164) used rat testes and human placenta to establish that ATP, NAD and Mg^{++} were cofactors and were required for the conversion of 20α-hydroxycholesterol to pregnenolone and progesterone. They suggested that this data linked cholesterol to progesterone.

In 1963, Caldwell and Drummond (31) used bovine liver preparations and reported that acetoacetyl coenzyme A was synthesized from acetate precursors and in turn was converted to HMG-Co A. Brodie et al. (29) used a purified pigeon liver system and were able to suggest a pathway from acetyl Co A and Malonyl Co A to MVA. It was shown that NADPH was an essential cofactor.

During 1964, it was shown by Hirshfield and Koritz (96) that calicum ions have some stimulatory effect in the synthesis of pregnenolone from endogenous precursors in large particle preparations of rat adrenals. Also in 1964, Koritz (106) showed that NAD was required for the conversion of pregnenolone to progesterone and that NADP was less effective than NAD. Mason and Savard (128), using bovine corpus luteum slices, were able to show that NADPH increased progesterone synthesis primarily through its action on cholesterol transformation.

In 1965, Hellig and Savard (94) also used bovine corpus luteum slices and showed that mevalonate-2-^{14}C, as well as acetate, can be converted to squalene, progesterone and other steroids. Dorfman (53) reported that NAD was required for the conversion of pregnenolone from endogenous precursors in rat adrenal studies and the addition of nicotinamide to the system increased synthesis. They reported that the biosynthetic steps from cholesterol to pregnenolone required NADPH. They also reported that this cofactor was essential for cholesterol side-chain cleavage in bovine adrenal cortex preparations. It is thought the NADPH concentration in the system may be maintained by metabolism of TCA intermediates or by the presence of G-6-P and its dehydrogenase along with exogenous NADP. Dorrington and Kilpatrick (54), using rabbit ovarian tissue and Shima et al. (170), using luteinized rat ovaries, observed that NADPH stimulated the conversion of cholesterol-^{14}C to progesterone-^{14}C.

In 1968, Hall and Young (85) incubated slices of bovine adrenal cortex, bovine corpus luteum and rabbit testes, and showed that cholesterol-7α-^{3}H was converted to progesterone-7α-^{3}H. Similar results were obtained by Koritz et al. (108) using rat adrenal homogenates.

Seifert and Hansel (169) incubated slices of bovine corpus luteum and reported that progesterone was synthesized from both cholesterol and MVA. Nicotinamide caused higher rates of progesterone synthesis, possibly by maintaining the integrity of the pyridine nucleotides. Progesterone synthesis was also shown to be consistently enhanced by NADPH.

When one evaluates the foregoing chronological review, a distinct sequence of biochemical events unfolds and lends confidence to the proposed progesterone biosynthetic pathway depicted on page 21.

Luteinizing Hormone

The role of LH is presented in a chronological sequence except when it is necessary to clarify some point.

In 1926, Smith (172) demonstrated in the rat that a pituitary extract was capable of exerting an influence upon the gonads. Later in 1931, Fevold *et al.* (67) obtained a commercial, desiccated anterior pituitary lobe powder and were able to demonstrate the presence of two hormones designated as follicle stimulating hormone (FSH) and luteinizing hormone (LH). They used the rat to test the potency of the two hormones. Eight years later, McShan and Meyer (116) demonstrated the carbohydrate property of pituitary luteinizing preparations and that LH was therefore a glycoprotein. The carbohydrate moiety did not seem necessary for physiological activity, however. Luteinizing hormone fractions from sheep and swine have been isolated in pure forms, but physiochemical studies have shown that these two preparations are different. The stability of the biological activities of bovine, ovine and porcine LH to a range of conditions and agents was studied by Adams-Mayne and Ward (1) in 1964. For these three species LH was shown to resist changes of pH,

but was inactivated by heating to 100° C for six minutes, performic acid oxidation, urea denaturation or treatment with trypsin.

In 1962, Schwartz and Bartosik (166) demonstrated changes in pituitary LH content during the rat estrous cycle and observed a minimal content at estrus. Data obtained by Ramirez and McCann (147) on the LH activity at various stages of the estrous cycle in the rat suggested that a surge of LH secretion occurred prior to ovulation. Luteinizing hormone was assumed to be necessary for the final stages of follicular growth and ovulation and capable of causing ovulation and maintenance of the corpus luteum of the estrous cycle (28). Schwartz and Caldarelli (167) in 1965, observed in the rat, that the plasma LH concentration prior to, and at ovulation, increased. A year later, Naller et al. (137), using blood samples from the rat, and Anderson and McShan (8), using blood samples from cattle, rats and swine, obtained data similar to that of Schwartz and Caldarelli.

Luteinizing hormone is thought to act upon the follicle by inducing a proteolysis of the follicle wall, thus producing a stigma through which the follicular fluids and the ovum escape. Following ovulation, the granulosa and theca cells of the follicle undergo proliferation (luteinization) and the mass of new cells become the corpus luteum or yellow body. This change in follicular cells into lutein cells is somehow dependent upon LH and the newly formed corpus luteum is a highly active organ, synthesizing and secreting large amounts of progesterone and estrogens.

In 1965, Gorske et al. (75) obtained data suggesting that in vitro effects of LH on rabbit luteal tissue showed only a small and variable response. Their data differed with that obtained by Pool and Lipner

(143) in that the latter group observed that LH readily induced the incorporation of tritiated uridine and valine into rabbit ovaries *in vivo* implying induction of protein (enzyme) synthesis. Rennels (149) studied the ultra structure of the luteal cells of rats treated with PMS or HCG or HCG and PMS; they demonstrated the presence of reduced lipids, a high order of mitochondrial development, smooth and granular endoplasmic reticulum and golgi complexes. The luteal cells of the HCG-PMS treated animals also had numerous membrane granules. They proposed that the presence of the granules indicated a secretory function of the luteal cells. Sammelwitz *et al.* (159), using pigs and rats in a number of experiments, presumed that LH was involved in the formation and maintenance of the corpus luteum.

In 1947, Everett (64) injected ovine LH into rats and observed cholesterol storage in the corpus luteum within 18 hours. Although he did not observe cholesterol depletion, he assumed that it did occur. In 1961, Parlow (141), using the pseudopregnant rat, showed that LH stimulates secretion of luteal estrogen as well as cholesterol depletion. In 1963, Savard and Casey (160) noted that LH added to bovine corpus luteum slices containing acetate-1-^{14}C increases 3- to 5-fold, the incorporation of ^{14}C into progesterone. A year later, Mason and Savard (129) found that amounts of LH as low as 0.01 to 0.02 μg/g of luteal tissue were sufficient to stimulate progesterone biosynthesis in bovine luteal tissue. Rice *et al.* (150) also found that LH augmented steroidogenesis in human corpora lutea taken at the luteal phase of the menstrual cycle and from women with etopic pregnancy. Armstrong *et al.* (15) studied the effects of LH on the luteinized rat ovary and recognized that LH stimulated progesterone synthesis.

In 1965, Hellig and Savard (94), using bovine corpus luteum slices, demonstrated that addition of LH brings about an increased conversion of acetate-1-^{14}C and mevalonate-2-^{14}C to progesterone. In a similar experiment, Koritz and Hall (107) obtained data and suggested that LH increases steroidogenesis in bovine corpus luteum by accelerating the conversion of cholesterol to progesterone. Channing and Villee (34) reported that LH administered to rats *in vivo*, doubled subsequent conversion of cholesterol to pregnenolone when the ovarian tissues were later minced and incubated *in vitro*.

In a later experiment, Channing and Villee (32) administered LH to rats three hours prior to sacrificing, then studied the effects of LH on the incubated minced ovaries. They observed that acetate-1-^{14}C incorporation in LH pretreated ovaries doubled that of non-pretreated preparations. They postulated that LH caused three effects in the corpus luteum: a) increased progesterone, b) increased glucose uptake and c) increased glycogen phosphorylase. Solod *et al.* (173) injected cholesterol-7α-^{3}H or -4-^{14}C intravenously into pseudopregnant rabbits prior to the administration of LH. A large increase in the secretion of 20α-hydroxy-pregn-4-en-3-one, accompanied by a comparable increase in radioactivity of this steroid, was observed in the venous plasma. The addition of LH to ovary slices from rabbits previously injected intravenously with labeled precursors prior to sacrifice, resulted in increased steroid production. Major and Armstrong (118), Rice and Segaloff (152) and McDonald *et al.* (113) demonstrated that LH increases steroid production in rat ovarian tissue and Dorrington and Kilpatrick (55) observed a similar response when measuring progestational steroids in rabbit ovarian venous blood. Similar, but later experiments (103,170,

171,194) confirmed these results.

The studies of LH in steroidogenesis have shown an increased formation of progesterone. This increase must result from some point in the steroidogenic pathway which is sensitive and responsive to the LH effect. In 1965 Koritz and Hall (107), using bovine corpus luteum preparations, obtained data whereby they suggested that LH acted at a point between acetate and cholesterol to stimulate steroidogenesis. In the same year, Savard et al. (163) suggested that LH acted at a variety of points in the pathway of progesterone synthesis; that being between acetate and squalene, squalene and cholesterol or cholesterol and progesterone. Also in 1965, Hall and Koritz (84) incubated slices of bovine adrenal cortex, bovine corpus luteum and rabbit testes in the presence of LH, and reported that the stimulation of steroidogenesis seemed to occur at a point between cholesterol and pregnenolone. In 1966, Armstrong (9), in an experiment using rabbit ovarian tissues, obtained data from which he was able to suggest that LH increased progesterone synthesis at a point subsequent to the biosynthesis of cholesterol. A year later Major et al. (119), using rat luteal tissues, suggested that LH acts at a point between cholesterol and progesterone. The probable sites of LH action are demonstrated in Figure 2.

LH has been shown to exert other effects in addition to that of steroidogenesis. In 1958, Parlow (140), and also in later experiments (141) observed that LH administered to PMS-primed rats resulted in a linear reduction of ascorbic acid when plotted semi-logrithmically. He suggested that ascorbic acid is involved in steroidogenesis. Mills and Schwartz (133) and Foreman (68) in later experiments obtained similar results. Armstrong (11), Armstrong and Greep (13) and Armstrong et al.

(14) were able to demonstrate that LH caused an increased in vitro glucose uptake in rat ovarian tissue slices which was shown to be related to the rate of ascorbic acid reduction, suggesting a correlation between glucose and ascorbic acid levels. They also showed that lactic acid production was increased. The experiments of Channing and Villee (34) and Hamberger and Ahrén (86) confirmed these results. Ahrén and Kostyo (4) also observed that LH stimulated glucose uptake and oxygen utilization in rat ovary preparations.

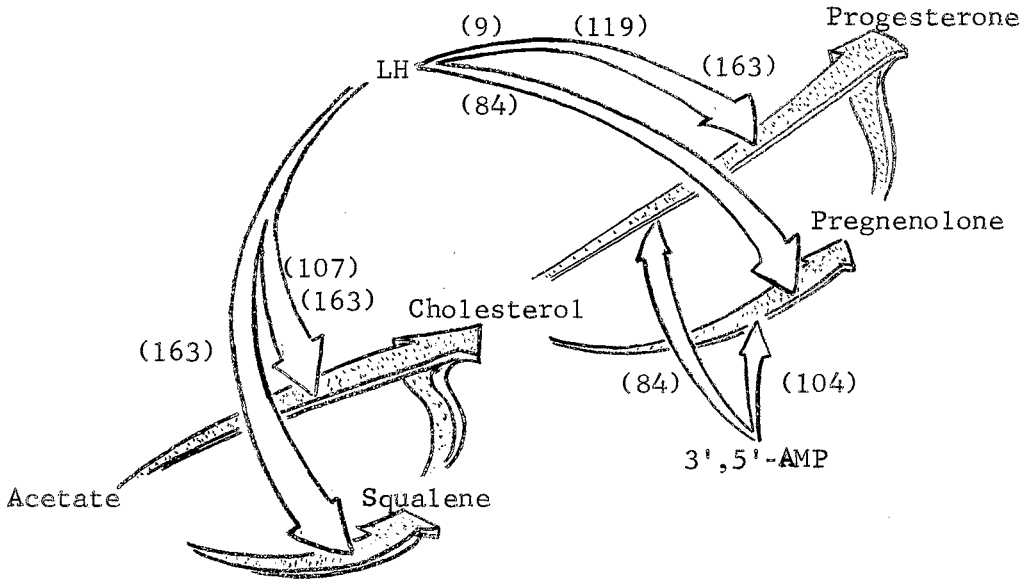

Figure 2. Documented Illustration Proposing Probable Sites of LH and 3',5'-AMP Action

Luteinizing Hormone and 3',5'-AMP Effects

In 1958, Haynes (91) added cyclic adenosine-3',5'-monophosphate (3',5'-AMP) to incubated slices of beef adrenal cortex and observed an

increase in phosphorylase activity. He thought that the increased phosphorylase activity permitted glycogen to be broken down at an increased rate to glucose-1-phosphate and then converted rapidly to G-6-P. Glucose-6-phosphate was reported to be utilized in the dehydrogenase system to provide NADPH, which in turn provided energy for a number of steps in steroidogenesis. In a later experiment, Haynes et al. (92) observed that 3',5'-AMP, when added to incubating rat adrenal glands, caused an increased steroid production. They also noted that substances closely similar in structure to 3',5'-AMP had no effect.

In 1965, Stanifield and Robinson et al. (177) demonstrated the presence of glycogen and phosphorylase in the corpus luteum of the cow and rat. They observed that LH increased the activity of luteal phosphorylase along with a slight decrease in total glycogen. They suggested that LH may act at a diversity of sites in the corpus luteum, including glycogen breakdown, sugar transport and directly upon steroidogenic enzymes. In 1966, Marsh et al. (123) reported that LH caused an accumulation of 3',5'-AMP in bovine corpus luteum slices and that inactivated LH was without effect. They noted that progesterone would increase following 3',5'-AMP accumulation. In an earlier experiment, Marsh and Savard (125) added 3',5'-AMP to incubating slices of bovine corpus luteum and observed a significant increase in the rate of progesterone synthesis without an increase in phosphorylase activity. A year later, Marsh and Savard (124) found that ovine LH increased phosphorylase activity when added to incubating slices of bovine corpora lutea, but 3',5'-AMP was without effect. They also noted that 3',5'-AMP did not stimulate corpora lutea phosphorylase. In 1966, Marsh et al. (123) demonstrated the presence of 3',5'-AMP in bovine luteal tissue. They observed that addition

of LH to incubating slices of luteal tissue caused a rapid accumulation of this cyclic nucleotide. However in 1967, Jackanicz and Armstrong (101) failed to obtain steroidogenesis in rabbit ovarian mitochondria preparations when 3',5'-AMP was added to the media.

In 1965, Hall and Koritz (84), using slices of bovine corpus luteum incubated with tritiated cholesterol, observed that LH and 3',5'-AMP stimulated steroidogenesis at some step between cholesterol and progesterone. In the same year, Karaboyas and Koritz (104) found 3',5'-AMP to stimulate the transformation of acetate-2-^{14}C and cholesterol-7α-^{3}H to corticosterone in rat adrenal slices and to cortisol in bovine adrenal cortex slices. The results of this experiment suggested that 3',5'-AMP acts at a point between cholesterol and pregnenolone. The sites of 3',5'-AMP action are illustrated in Figure 2.

In 1965, Levine and Vogel (109) investigated 3',5'-AMP effects on heart muscle tissue in the rat and suggested that this cofactor penetrates intact cell membranes of all types in sufficient quantities to regulate intracellular metabolic reactions such as steroidogenesis.

It is generally accepted that the exact mechanism by which 3',5'-AMP acts upon luteal steroidogenesis is not understood although Marsh and Savard (126) do suggest that exogenous 3',5'-AMP has an effect very similar to that of LH. Luteinizing hormone seems to be the only pituitary factor which is capable of stimulating steroidogenesis. Other hormones used have not been entirely free of LH as a contaminant.

From the literature presented in the review, it has been shown that steroidogenically, porcine luteal tissue preparations are not significantly enhanced by gonadotropins when compared to other species. Because porcine tissues in different stages of the estrous cycle and pregnancy

are more readily available, the porcine model needs to be improved. Therefore this experiment was limited to a single species - Suis domesticus - and was designed to determine whether porcine luteal tissues incubated in vitro, augmented and reconstituted with a group of selected metabolites would be more responsive to gonadotropic hormones.

CHAPTER III

MATERIALS AND METHODS

Porcine ovaries were collected at an abattoir and the corpora lutea were extirpated, minced and incubated to establish routine *in vitro* techniques. The corpora lutea minces were extracted and chemically assayed for progesterone to establish the analysis techniques. No data from these preliminary investigations were analyzed.

Experimental Design

Three experiments were designed and replicated four times to determine whether porcine luteal tissue, incubated *in vitro*, and augmented with cofactors[1], would provide a reconstituted system that would provide a model for the study of the effects of gonadotropic hormones on steroidogenesis.

The luteal tissues were incubated in Kreb's-Ringer bicarbonate buffer medium with 200 mgm glucose added for each 100 ml of solution. Five hundred mgm of luteal tissue were added to each incubation flask. Ovine luteinizing hormone (NIH-LH-S11-Ovine)[2] was used as the gonadotropic hormone in this study. From the data based on the studies of

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[1]Sigma Chemical Company, St. Louis, Missouri.

[2]Endocrinology Study Section, National Institute of Health, Bethesda, Maryland.

Akins (5), 0.05 μg/ml were used to test the gonadotropic response of the luteal preparation. The following group of cofactors were used together to augment the system in those flasks supplemented with cofactors: fumarate, 0.0043 mg/ml; nicotinamide, 0.0042 mg/ml; adenosine-3',5'-monophosphate, 0.0002 mg/ml; adenosine-5'-monophosphate, 0.0011 mg/ml; adenosine-5'-triphosphate, 0.0021 mg/ml; β-diphosphopyridine nucleotide (NAD), 0.0050 mg/ml; β-diphosphopyridine nucleotide, reduced (NADH), 0.0100 mg/ml; triphosphopyridine nucleotide (NADP), 0.0110 mg/ml; triphosphopyridine nucleotide, reduced (NADPH), 0.0039 mg/ml; and glucose-6-phosphate, 0.0062 mg/ml.

The arrangement of treatments in each of the three experiments is described below.

In the first experiment, the minces were not washed and biologically active LH was used.

Treatment No.

1 Non-incubated control

2 Incubated control

3 Each flask augmented with cofactors

4 Each flask augmented with cofactors and LH

5 Each flask augmented with LH

In the second experiment, the luteal minces were washed twice with Kreb's-Ringer bicarbonate buffer solution after setting aside a small portion of the luteal mince tissue for treatment No. 2 and then dividing them into the following groups:

Treatment No.

1 Non-incubated control

2 Incubated controls (Not washed)

3 Incubated controls

4 Each flask augmented with cofactors

5 Each flask augmented with cofactors and LH

6 Each flask augmented with LH

In the third experiment, the luteal tissue minces were not washed, but were divided into the following treatment groups and heat inactivated LH was used:

Treatment No.

1 Non-incubated control

2 Incubated control

3 Each flask was augmented with cofactors

4 Each flask was augmented with cofactors and heat inactivated LH

5 Each flask was augmented with heat inactivated LH

Glassware and Instruments

Wide mouth, screw cap collection jars with a capacity of 450 ml were used. Twenty-five ml Erlenmeyer incubation flasks were used. A 6.0 x 6.0 inch glass plate was used for the mincing of the tissues. All glassware was soaked in a strong detergent solution for 12 hours, washed in warm soapy water, and thoroughly rinsed in tap water followed by a distilled water rinse. The pipettes used in the experiment were washed first in a chromic acid bath, followed by a tap water rinse and a distilled water rinse. All glassware were dried in a hot air oven at 150° C until dry.

Single-edge razor blades were used to mince the tissues on the 6.0 x 6.0 glass plate and a small stainless-steel spatula was used to move

and weigh out the tissue for incubation. The razor blades were cleaned in a detergent solution, followed by a tap water, distilled water and acetone rinse. New corks were used to stopper the incubation flasks.

Solvents

The organic chemicals used in these experiments were dichloromethane and diethyl-ether of reagent grade quality. Benzene and ethyl acetate used in the thin-layer chromatography system were of nano-grade quality. Spectrograde toluene was used to prepare the scintillation cocktail. Reagent grade tetrahydrofuran (THF) was used to resuspend residues prior to the thin-layer steps, in the preparation of the scintillation vials and in gas-liquid chromatography (GLC) procedures.

Incubation Medium

Kreb's-Ringer bicarbonate buffer medium, with glucose, (see Appendix) was prepared the evening prior to the day of the experiment and refrigerated overnight. The sodium bicarbonate was added on the morning of the experiment and the pH of the medium was adjusted to pH 7.4. The Kreb's-Ringer bicarbonate buffer medium was divided between the collection jars, incubation flasks and "wash" for the luteal tissue. The cofactors and/or LH were added to a prescribed volume of incubation medium and then added to the incubation flasks by means of an automatic syringe[3]. All preparations were carried out in a cold room.

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[3]B-D Cornwall Continuous Pipetting Outfit, #3056, Becton and Company, Rutherford, New Jersey.

Collection of Ovaries

Porcine ovaries of early pregnancy, as determined by embryo length (less than 40 mm, crown to rump), were collected at an abattoir[4] in the morning on the day of the experiment. The ovaries of gilts and sows with unknown reproductive histories were observed as they passed through the processing line approximately 28 minutes after electrocution. Ovaries, which appeared normal and in early pregnancy, were removed from the uterine tract and each corpus lutuem was incised to facilitate chilling. One ovary was removed from the tract and retained for the experiment. The opposite ovary was discarded. A maximum of eight ovaries were collected for each experiment. The ovaries were placed in collection jars containing chilled Kreb's-Ringer bicarbonate buffer. The jars were packed in an ice chest and transported to the laboratory cold room.

Preparation of Luteal Tissue

The corpus luteum of the porcine ovary was removed from its connective tissue capsule and the extraneous tissue discarded. The luteal tissues from the ovaries were combined, placed on a glass plate and minced together, using a cleaned single-edge razor blade. During the mincing procedure, the tissue was repeatedly mixed by means of a stainless steel spatula to ensure a random, homogenous pooled mixture. Following mincing, 500 mg of the tissue were weighed[5] and transferred into the incubation flasks.

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[4]Wilson and Company, Oklahoma City, Oklahoma.

[5]Model LG Precision Balance, Federal Pacific Electric Company, Newark, New Jersey.

Incubation

The incubation was started within two to three hours following the collection of the ovaries. The tissues were incubated for two hours with constant agitation in an atmosphere of 95% oxygen and 5% carbon dioxide at 37° C. The water bath shaker[6] was draped with a plastic tent to maintain the atmosphere. The gas flow rate was 4 cubic feet per hour. Following incubation, the air in each flask was replaced with nitrogen gas to reduce oxidation of products, stoppered with new corks and stored at -20° C until extraction.

Extraction of Progesterone

The contents of each flask were thawed and the tissue homogenized in the incubation medium, using a 15 ml Potter-Elvehjem homogenizer[7]. The homogenate was transferred into an 85 ml conical glass stoppered centrifuge tube. The flask, as well as the homogenizer, was washed with a volume of dichloromethane and the wash added to the homogenate in the conical tube. Four millimicrocuries (mμc) of progesterone-4-^{14}C[8] were added to each homogenate in the conical tube. The extraction steps were carried out using a procedure described by Stabenfeldt (176) and modified by Mills (134). A volume of dichloromethane equal to two and one-half volumes of the homogenate was added to the conical tube. The contents

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[6]Arthur H. Thomas Company, Philadelphia, Pennsylvania.

[7]W. H. Curtin and Company, Germany.

[8]New England Nuclear Corporation, Boston, Massachusetts.

of the tube were thoroughly mixed on a Vortex[9] shaker and then centrifuged[10] at 1500 rpm for ten minutes. The lower layer of dichloromethane was pipetted off and transferred to a 250 ml round bottom flask. This extraction step, using dichloromethane, was repeated with a two and one-half volume aliquot and a two volume aliquot. Following extraction by dichloromethane, this solvent was evaporated, using a Rotovapor[11] flask evaporator at 45 to 48° C, under reduced pressure, leaving a residue in the bottom of the flask.

The dry residue was extracted three times with 5.0 ml portions of diethyl-ether and each ether wash was transferred to a 35 ml conical glass stoppered centrifuge tube. Five ml of 1.0 N sodium hydroxide were added to each of the samples which were first resuspended in ether to saponify the saponifiable lipids. The saponification process required that the contents of the stoppered tube be shaken by hand 200 times, releasing the pressure every 50 shakes. After shaking, the emulsion formed was broken by centrifuging at 1500 rpm for ten minutes. The lower aqueous layer was pipetted off and discarded and the ether was then washed two times with 5.0 ml of glass distilled water. Centrifugation was required after each wash to break the emulsion. After removal of the second water wash, the sample was centrifuged again to remove the last drop of water. The ether was evaporated to dryness in a water bath at about 45° C under a nitrogen atmosphere. The inside of each tube was

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[9]W. H. Curtin and Company, Germany.

[10]International High-Speed Centrifuge, Model HR-1, International Equipment Company, Needham Heights, Massachussetts.

[11]Rotovapor Model VE-50, Rinco Instruments Company, Inc., Greenville, Illinois.

washed with 5.0, 2.0 and 1.0 ml of ether respectively, beginning at the tip and advancing down toward the bottom with each wash. Following each wash, the ether was evaporated as previously described.

The residue in the bottom of the tube was resuspended in a small amount of THF and transferred, using a capillary pipette, to a 20 x 20 cm silica gel plate. The silica gel[12] was layered onto the glass plate at 0.25 mm thickness using a commercial spreading apparatus[13], then allowed to dry and activated by heating at 110° C for one hour. The tube was washed down two additional times with smaller volumes of THF and each volume also spotted onto the plate.

The spotted plates were developed in glass chromatography jars containing 100 ml of a 4:1 ratio of benzene to ethyl acetate. The plates were allowed to develop until the solvent progressed to within 2.0 cm of the upper edge, and were then removed. When thoroughly dry, the plates were observed under ultraviolet light and the fluorescing band, with a retention time corresponding to the progesterone marker, was marked. This band was scraped from the plate and eluted with 3.0 to 4.0 ml of ether by using a suction device with a porous glass filter. The ether was evaporated to dryness under a nitrogen atmosphere as previously described.

Preparation of Scintillation Vials and Standards

At the beginning of the extraction procedure, a standard vial was

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[12]MN-Silica Gel G-HR/UV 250, Macherey, Nagel and Company, Duren, Germany.

[13]Brinkman Instruments, Westbury, New York.

prepared by adding a quantity of progesterone-4-^{14}C to a scintillation vial, equal to the quantity that was added to each sample. Prior to GLC, each sample was resuspended in 50.0 µl of THF and 5.0 µl of the reconstituted sample were transferred into a scintillation vial. Ten ml of scintillation fluid were added to each vial and each vial counted for ten minutes, in a Packard Tri-Carb Liquid Scintillation Spectrometer (Series 314-E)[14]. Corrections were made for background, machine efficiency and quenching.

Gas-Liquid Chromatography and Analysis

Following the transfer of 5.0 µl of a sample to a scintillation vial, a specific volume (usually 2.0 to 5.0 µl) of the sample was chromatographed on a three-foot glass column (4.0 mm I. D. X 6.0 mm O. D.) packed with 1% QF-1 on 100/60 mesh Gas Chrom Q[15], using a Barber-Coleman Series 5000[16] instrument equipped with a Model 5121 hydrogen flame detector. Nitrogen was used as the carrier gas with a flow of 48 ml/min from the column. The injection port, column bath and detector temperatures were 250°, 220° and 230° C respectively.

Peak areas were measured by planimetry[17] and quantitation was accomplished by comparing the peak areas of the unknowns to a standard curve derived by plotting the areas of progesterone standards injected in known quantities. Progesterone (µg/g luteal tissue) was then

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[14]Packard Instruments, La Grange, Illinois.

[15]Applied Science Laboratories, College Station, Pennsylvania.

[16]Barber-Coleman Company, Rockford, Illinois.

[17]Ott-Planimeter, W. H. Curtin and Company, Germany.

calculated by multiplying the predetermined dilution factors, and the percent recovery factor. Percent recovery was determined by counting the quantity of labeled standard remaining in the final residue in a scintillation counter.

Statistics

Statistical analysis included an Analysis of Variance and Duncan's New Multiple-Range Test performed according to the methods outlined by Steele and Torrie (179).

CHAPTER IV

RESULTS AND DISCUSSION

In order to evaluate the steroidogenic potential of porcine luteal tissue minces of early pregnancy augmented with cofactors, a study was conducted in three experiments as described in Chapter III. The criterion for steroidogenic activity was progesterone synthesis. The method of extraction and quantitation of the steroid was identical to that used by Mills (134), modified after Stabenfeldt (176). Mills reported that after purification by thin-layer chromatography and quantitation by gas-liquid chromatography, it could be verified by mass spectroscopy, that the steroid isolated by this method was indeed progesterone.

Experiment I was performed using unwashed, pooled luteal tissues. Data from this experiment along with a review of the design can be found in Table I. The order in which the treatments are listed in this and other tables was determined by the results of Duncan's New Multiple-Range Test.

Analysis of the data (Table II) revealed that the increase in progesterone synthesis in the treatment groups was very highly significant ($P < 0.005$). The addition of cofactors or cofactors plus LH or LH alone augmented steroidogenesis. It was also observed, using the Duncan's New Multiple-Range Test (Table III), that there were no significant differences between treatment groups 3 to 5 at the 1% level. Increases in progesterone synthesis, over the controls, with the addition of

TABLE I

PROGESTERONE SYNTHESIS* IN EXPERIMENT I

Replica No.	Ovaries in Pool**	TREATMENTS									
		Non-incubated Control (1)		Incubated Control (2)		LH (3)		Cofactors and LH (4)		Cofactors (5)	
		Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.
1	8	78.43		99.13	9.07	124.31	7.04	128.56	2.63	150.63	11.12
2	7	62.23	2.82	85.13	5.81	88.04	5.90	93.09	16.36	87.13	1.86
3	7	90.29	2.23	111.75	1.97	107.79	2.49	119.51	10.94	130.17	3.75
4	6	75.36	5.74	97.98	3.17	104.01	4.68	120.41	10.31	104.17	1.69
5	6	72.96	3.75	90.15	3.11	113.01	5.77	106.75	5.30	102.77	13.61
Mean (all replicas)		75.85		96.83		107.43		113.66		114.95	
±S.E.		6.74		4.54		5.98		6.30		11.26	

*µg/g luteal tissue

**One ovary from each animal

TABLE II

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATION IN EXPERIMENT I

Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F
Total	24	10,049.63	418.73	
Replicas	4	3,408.93	852.23	9.44*
Treatments	4	5,196.56	1,299.14	14.40*
Error	16	1,444.07	90.25	

*P < 0.005.

TABLE III

DUNCAN'S NEW MULTIPLE-RANGE TEST APPLIED TO PROGESTERONE CONCENTRATION* IN EXPERIMENT I

Treatment	1	2	3	4	5
Mean	75.85	96.83	107.43	113.66	114.95
S_x = 4.25		————	————	····	····
		····			

Value of P d. f. = 16	5% 2	3	4	5	1% 2	3	4	5
SSR	3.00	3.15	3.23	3.30	4.13	4.34	4.45	4.54
LSR	12.75	13.39	13.73	14.03	17.51	18.45	18.91	19.30

*μg/g luteal tissue

5% level ————

1% level ····

cofactors were approximately 15%, cofactors plus LH, 14% and LH alone, 9%.

Experiment II was performed using twice washed, pooled luteal tissues. An unwashed portion from the same pool was set aside as an additional control. Data from this experiment, along with a review of the design, are shown in Table IV. A critical examination of Table IV, showed that progesterone synthesis was decreased approximately 25% in all treatment groups when compared with treatment 7 (unwashed, incubated control) and treatment 2 of Experiments I and III (unwashed, incubated control). An analysis of variance (Table V) showed that increases in progesterone synthesis in the treatment groups was very highly significant ($P < 0.005$). Duncan's New Multiple-Range Test indicated that there are no significant differences (at the 1% level) between treatment groups 3 to 6. Increased synthesis of progesterone over that of treatment 4 (washed, incubated control) upon the addition of cofactors, was approximately 8% and for cofactors plus LH, 8%. The addition of either active or heat inactivated LH did not produce a change in steroid synthesis when compared with treatment 4 (washed, incubated control). Treatment group 7 (unwashed, incubated control) showed a steroidogenic response similar to treatment group 2 of Experiments I and III (unwashed, incubated control).

In Experiment III, nonwashed, pooled luteal tissue and heat inactivated LH was added as a treatment to verify observed LH effects on steroidogenesis in the treated groups. Progesterone synthesis was observed to increase over that of the control groups in a manner similar to that observed in Experiment I. Data recorded in Table VIII showed that increases in progesterone synthesis were very highly significant

TABLE IV

PROGESTERONE SYNTHESIS* IN EXPERIMENT II

Replica No.	Ovaries in Pool**	TREATMENT***													
		Non-Incubated Control (1)		Heat Inactivated LH (2)		LH (3)		Incubated Control (4)		Cofactors (5)		Cofactors and LH (6)		Non-washed, Incubated Control (7)	
		Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.
1	8	52.12	2.90	57.69	3.50	68.13	1.84	68.19	1.53	68.42	4.00	62.81	1.84	76.31	1.99
2	7	48.88	3.09	34.20	2.19	36.38	1.08	56.45	3.38	60.01	1.35	60.13	2.13	81.50	2.31
3	6	76.10	4.83	91.63	0.02	99.06	5.28	92.32	7.76	98.69	6.81	103.32	6.80	119.97	2.97
4	7	49.90	1.65	66.47	8.69	65.42	1.45	68.17	7.98	71.67	7.29	73.65	5.32	92.17	2.60
Mean (all replicas)		56.60		62.50		67.25		71.28		77.20		77.75		92.44	
±S.E.		7.18		11.86		12.81		7.54		8.40		9.89		9.76	

*μg/g luteal tissue.

**One ovary from each donor animal.

***All minces twice washed except treatment 7.

TABLE V

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATION IN EXPERIMENT II

Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F
Total	27	11,118.51	411.80	
Replicas	3	7,169.64	2,398.88	51.74*
Treatments	6	3,117.51	519.59	11.25*
Error	18	831.36	46.19	

*P < 0.005

TABLE VI

DUNCAN'S NEW MULTIPLE-RANGE TEST APPLIED TO PROGESTERONE CONCENTRATION* IN EXPERIMENT II

Treatment	1	2	3	4	5	6	7
Mean	56.50	62.50	67.25	71.28	77.20	77.75	92.44

S_x = 3.4

Value of P d. f. = 18	5% 2	3	4	5	6	7
SSR	2.97	3.12	3.21	3.27	3.32	3.35
LSR	10.10	10.61	10.91	11.12	11.29	11.39

Value of P d. f. = 18	1% 2	3	4	5	6	7
SSR	4.07	4.27	4.38	4.46	4.53	4.59
LSR	13.84	14.52	14.84	15.16	15.40	15.61

*µg/g luteal tissue

5% level ————

1% level ·····

TABLE VII

PROGESTERONE SYNTHESIS* IN EXPERIMENT III

Replica No.	Ovaries in Pool**	TREATMENTS									
		Non-incubated Control (1)		Incubated Control (2)		Heat Inactivated LH (3)		Cofactors & Heat Inactivated LH (4)		Cofactors (5)	
		Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.	Mean	±S.E.
1	4	111.92	1.84	130.99	4.08	112.50	0.51	112.50	2.60	158.50	10.71
2	5	81.84	5.40	115.65	2.46	136.83	4.48	141.90	4.98	149.50	10.81
3	4	58.38	2.19	115.80	14.12	117.25	15.42	136.33	4.43	130.25	8.94
4	4	72.38	2.23	96.20	3.47	109.25	0.25	108.35	4.69	99.17	5.96
Mean (all replicas)		81.13		114.66		118.96		124.77		134.35	
±S.E.		11.35		7.13		6.18		14.62		13.13	

*µg/g luteal tissue.

**One ovary from each donor animal.

TABLE VIII

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATION IN EXPERIMENT III

Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F
Total	19	12,056.14	634.53	
Replicas	3	3,707.23	1,235.74	8.16*
Treatments	4	6,531.07	1,632.77	10.78*
Error	12	1,817.84	151.49	

*$P < 0.005$

TABLE IX

DUNCAN'S NEW MULTIPLE-RANGE TEST APPLIED TO PROGESTERONE CONCENTRATION* IN EXPERIMENT III

Treatment	1	2	3	4	5
Mean	81.13	114.66	118.96	124.77	134.35
$S_{\bar{x}} = 5.51$		————	————		
		.....	.....	.....	

Value of P	5%				1%			
d. f. = 12	2	3	4	5	2	3	4	5
SSR	3.08	3.23	3.33	3.36	4.32	4.55	4.68	4.76
LSR	16.97	17.80	18.35	18.51	23.31	25.07	25.79	26.23

*µg/g luteal tissue.

5% level ————

1% level .....

($P < 0.005$). Duncan's New Multiple-Range Test of the data (Table IX) did not reveal significant differences between treatment groups 3 to 5, but did indicate that groups 3 to 5, were significantly different than the incubated control groups at the 5% level. Increases in progesterone synthesis (over that of the controls) with the addition of cofactors were approximately 14%, cofactors plus heat inactivated LH, 9% and heat inactivated LH alone, 4%.

It was noted in these three experiments that the incubated controls responded with at least a 20% increase in progesterone synthesis over that of the unincubated control groups.

In these three experiments, none of the replicas failed to synthesize progesterone, either with or without the addition of cofactors and/or LH. These results demonstrated that all porcine luteal tissue preparations retained steroidogenic capacity _in vitro_. These observations are consistent with those of Cook _et al_. (41).

The ovaries in this experiment were collected at slaughter. This step in the experimental procedure is similar to that of Duncan _et al_. (56,57) and Bjersing and Carstensen (24), but differs from that of Cook _et al_. (41) who obtained their ovarian specimens by laparotomy, while the gilts were anesthetized, and started incubations immediately. Armstrong and Black (12) in their report strongly supported the concept that there was a need for prompt removal of tissues after the death of the donor animal. They suggested that degenerative changes may have occurred in the tissues during the time elapsing between cessation of the blood supply to the ovary and its removal from the carcass. In the experiments reported herein, the porcine ovaries were collected 25 to 30 minutes following electrocution and the luteal mince preparations were

incubated approximately two hours after collection, yet progesterone synthesis was observed. Bjersing and Carstensen (24) reported steroid biosynthesis from granulosa cell aggregates that were not incubated until three hours after slaughter. Seifert and Hansel (169) established that two hours incubation time was sufficient to detect LH induced stimulation of progesterone synthesis.

In this study, it was observed that when exogenous LH was present in the incubating porcine minces, there was an increase in progesterone synthesis over that of the control groups. These observations are consistent with those of Cook et al. (41), who demonstrated that either bovine, ovine or porcine LH was capable of eliciting a steroidogenic response, when measuring progesterone. Luteinizing hormone also has been shown to result in increased progesterone synthesis in diverse mammalian ovarian tissues (15,18,32,35,41,54,118,161,165,174). However, there are reports to the contrary (56,81,130,151,172).

The addition of LH in Experiment I resulted in a 14% increase in progesterone synthesis. The level of progesterone in the incubation media in this experiment was 105.43 μg/g luteal tissue (wet weight) and was comparable to the percent increase reported by Cook et al. (41).

Examination of the data of LH effects in Experiment II (washed luteal tissue), showed no augmentation of progesterone synthesis. The use of heat inactivated LH did not cause any significant change in progesterone synthesis in 4 of 4 replicas. Heat activated LH was also shown to be without effect in Experiment III. These data are consistent with those reported by Adams-Mayne and Ward (1).

In this study, because cofactors (page 37) were used in combination when the system was augmented, it was not possible to ascribe a specific

role to a single cofactor. It should be noted that by reviewing the reports of other workers (see Review of Literature), it was assumed that all the cofactors in this study had some essential role in progesterone synthesis.

In Experiment II (washed luteal tissue) there was a decrease in steroidogenesis of approximately 25% in all treatment groups except the unwashed group (Treatment 7) which synthesized progesterone at the same rate as the unwashed, incubated controls (Treatment 2) in Experiments I and III. Careful examination of Table IV revealed that the washed minces retained their ability to synthesize progesterone, but at a lower level. It was noted that the greatest steroidogenic activity was in the treatment groups augmented with cofactors. There were however, no significant differences between treatment groups 3 to 6 (Table IV). It would appear from this part of the study, that cofactors are necessary for steroidogenesis as neither active or heat inactivated LH elicited steroidogenic responses greater than Treatment 4 (washed, incubated control) when cofactors were not present. From the data obtained in Experiment II, it can also be postulated that washing resulted in a loss of essential factors necessary for efficient steroidogenesis and the addition of cofactors resulted in a partial restoration of specific factors necessary for steroidogenesis and an increased synthesis. However it was noted that the decrease in steroidogenic activity upon washing, apparently resulted in the loss of an unknown factor essential to LH activity that was not restored when the cofactors were added.

In Experiments I and III, the cofactor treatment groups were observed to significantly increase progesterone synthesis ($P < 0.005$). There was approximately a 14% increase over Treatment 2 (unwashed,

incubated control) to which cofactors were not added. Bjersing and Carstensen (24) added the same cofactors to incubating granulosa cell preparations, but they did not report any effect of the combined cofactors upon steroidogenesis. Data obtained in Experiment II revealed a lower level of augmentation of steroidogenic activity by cofactors than in either Experiment I or III.

In all cases LH or cofactors increased progesterone synthesis, but LH and cofactors in combination did not appear to be additive.

CHAPTER V

SUMMARY

A study was designed to determine whether porcine luteal tissues incubated *in vitro*, augmented and reconstituted with a group of selected cofactors, would respond to LH and thereby provide a model to study steroidogenesis. Progesterone was used as a criterion of luteal function and LH activity.

Porcine ovaries, from gilts in early pregnancy, were collected at an abattoir. The corpora lutea were extirpated from the ovaries, pooled and minced. Five hundred mg of luteal tissue were incubated in Kreb's-Ringer bicarbonate buffer medium according to techniques described by Duncan *et al*. (57). Progesterone was extracted and measured according to methods described by Stabenfeldt (176) and modified by Mills (134). Precision in the analysis was maintained by measuring the quantity of previously added ^{14}C-labeled progesterone recorded by scintillation spectrometry.

Data collected revealed that porcine luteal tissues are viable and capable of steroidogenic activity two to three hours after collection. Evaluation of these data also indicated that all samples of the pooled tissues had similar steroidogenic activities and were therefore homogeneous. Washed minces retained their ability to synthesize progesterone and responded to the addition of cofactors by increased synthesis, but at a lower level when compared to unwashed preparations. When LH or

heat inactivated LH was added to unwashed incubating minces, no significant changes in steroidogenic activity were observed. The addition of LH to unwashed incubating minces resulted in increased steroidogenesis comparable to that reported by Cook et al. (41); however, heat inactivated LH was without effect. Although cofactors were shown to significantly augment steroidogenic activity in both washed and unwashed minces, there were no observed additive effects when LH was used in combination with cofactors.

The foregoing indicated that washing removed a factor or factors essential to LH action that was not replaced when the group of selected cofactors were added to the washed preparations.

It appears from this study that porcine luteal tissues, collected at an abattoir and pooled, minced and incubated unwashed up to three hours later, could be used for steroidogenic studies, with or without adding cofactors.

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APPENDIX A

TABLE X

KREB'S-RINGER BICARBONATE GLUCOSE BUFFER MEDIUM

Solutions		
0.90 NaCl	(0.154 M)	0.9001 gm/100 ml
1.15% KCl	(0.154 M)	1.1481 gm/100 ml
1.22% $CaCl_2$	(0.154 M)	1.2209 gm/100 ml
2.11% KH_2PO_4	(0.154 M)	2.0958 gm/100 ml
3.82% $MgSO_4 \cdot 7H_2O$	(0.154 M)	3.7950 gm/100 ml
1.30% $NaHCO_3$	(0.154 M)	1.2939 gm/100 ml

1. Make up five-times concentrated stock solutions.

2. Gas the $NaHCO_3$ solution with CO_2 for one hour.

3. Take from the concentrated stock solutions the following aliquots:

NaCl	10.0 ml
KCl	0.4 ml
$CaCl_2$	0.3 ml
KH_2PO_4	0.1 ml
$MgSO_4 \cdot 7H_2O$	0.1 ml

4. To these add 43.6 ml distilled water, making a total of 54.5 ml.

5. Take 42.0 ml of this solution and add to it 8.0 ml of concentrated $NaHCO_3$ solution. Adjust the pH to 7.4. Gas the solution for 10 minutes with a mixture of 95% oxygen and 5% carbon dioxide.

6. Add 200.0 mg glucose/100 ml of the solution.

VITA 3

Richard Whitney Williams

Candidate for the Degree of

Doctor of Philosophy

Thesis: EFFECT OF COFACTORS AND LUTEOTROPIC HORMONE ON THE BIOSYNTHESIS OF PROGESTERONE BY PORCINE LUTEAL TISSUE

Major Field: Physiology

Biographical:

Personal Data: Born in Childress County, Texas, June 5, 1935, the son of Raymond and Evelyn Williams.

Education: Attended grade school in Erick, Oklahoma; graduated from Erick High School in 1953; received the Bachelor of Science degree from the Southwestern State College, Weatherford, Oklahoma, with a major in Art and Biology, in May, 1957; received the Certificate of Medical Technology from the St. Paul's Hospital School of Medical Technology, Dallas, Texas, in April, 1962; received the Master of Science degree from the East Texas State University, Commerce, Texas, in May, 1966; completed the requirements for the Doctor of Philosophy degree in August, 1969.

Professional Experience: Employed as a Technical Illustrator for Chance-Vought, Inc., Dallas, Texas, from 1957-1960. Employed as a Medical Technologist for Sherman Community Hospital, Sherman, Texas, from 1962-1964. Employed as a graduate teaching assistant at East Texas State University, Commerce, Texas, from 1964-1966. Employed as a graduate research assistant, from 1966-1968 and as a graduate teaching assistant, from 1968-1969, at Oklahoma State University, Stillwater, Oklahoma.

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