PROGESTERONE SYNTHESIS IN PORCINE LUTEAL TISSUE *IN VITRO*

By

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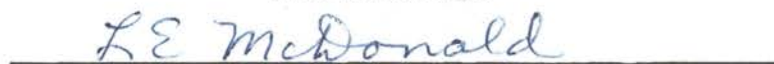

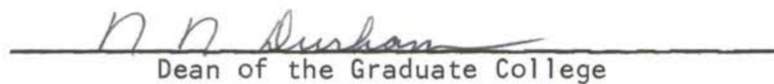

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CHAPTER I

INTRODUCTION

The finding by nineteenth century scientists that the death of the organism is not necessarily accompanied or followed by an immediate death of its cellular units provided the foundation for *in vitro* techniques. Bernard, in 1878, described the theoretical bases of maintaining organs in a functional state outside the neural and humoral influences of the whole organism and, in 1885, Roux successfully kept a chick medullary plate alive in a physiological solution. Tissue culture (intact cells incubated in artificial media under specified conditions of atmosphere and temperature) is considered to be the first of the *in vitro* models to be used in biological studies. Arnold, in 1887, started the work in this field by incubating frog leukocytes in a physiological solution to study their survival and migratory activity. These *in vitro* techniques, along with perfusion of isolated organs, incubation of tissue homogenates and slices, isolated subcellular fractions and enzyme systems are presently employed to study metabolic activities of health and diseased organs and tissues under different conditions and treatments. In the field of reproductive physiology, the foregoing methods have been used to investigate the hypothalamus-pituitary-ovary-uterus interrelationship as well as the functional aspects of each of these organs.

Incubation of tissues *in vitro*, in particular, is still being used as the principal technique to measure the single or multiple steroid

products, qualitatively and quantitatively, that are synthesized by the individual compartments of the ovaries of mammals, and to determine the required precursors, metabolic pathways and influences of several co-factors, gonadotropins and other substances on steroidogenesis.

Duncan *et al.* (1960) were the first workers to incubate slices of swine luteal tissue in Krebs-Ringer bicarbonate buffer medium to demonstrate that porcine corpora lutea cells are capable of retaining their ability to synthesize progesterone throughout an incubation period of six hours under fixed conditions of oxygen tension and temperature. This attempt was followed by another investigation (Duncan *et al.*, 1961) to study some of the factors that influence *in vitro* synthesis of progesterone by porcine luteal tissue slices.

Although Duncan's experiments indicated that porcine luteal tissues synthesized progesterone *in vitro*, he was unable to provide evidence that these tissues would respond to gonadotropins under the conditions of the experiment. Later reports by Cook *et al.* (1967) indicated that porcine luteal tissues respond to gonadotropins only if the tissues are collected and incubated within a few minutes after slaughter. Even though the tissues in their experiment responded to gonadotropin, there was only a 15% increase in luteal progesterone during a two hour incubation period. Increases of 100% or more have been reported for bovine luteal tissues treated in the same manner.

Because of the foregoing it was hypothesized that porcine luteal tissues need a more complete incubation medium or unidentified serum factors for efficient *in vitro* steroidogenesis. To test this hypothesis an experiment was designed, using the methods of Duncan *et al.* (1961) but with modifications of the incubation medium and additives.

CHAPTER II

LITERATURE REVIEW

Ultrastructures of the Lutein Cells

During the period of follicular growth, the cytoplasm of the granulosa cells is very rich in ribonucleoprotein particles and the cells undergo rapid multiplication. Their mitochondria are rod-shaped in appearance with parallel cristae of regular spacing. The endoplasmic reticulum is mainly the granular type. The granulosa cells synthesize protein actively (Bjorkman, 1962) and secrete follicular fluid which can be seen, by means of the electron microscope, in their interspaces (Hadeck, 1963). These cells can also synthesize mucopolysaccharides as indicated by their ability to incorporate ^{32}S. (Odeblad and Bostrum, 1953).

As the granulosa cells undergo luteinization, numerous large mitochondria, rounded in shape with tubular cristae such as those seen in adrenal cortical cells, make their appearance. This change is suggestive of a shift from protein synthesis (Odeblad and Bostrom, 1953). Further, the cytoplasm shows large numbers of vacuoles, the nucleus is vesicular and lacking visible chromatin, the nuclear envelop exhibits many small pores or annuli (Deane *et al.*, 1966) and the lysosome-like bodies and multivesicular bodies are increased in number. The cell in general seems to be particularly fragile (Bjorkman, 1962), and the tubular agranular endoplasmic reticulum is extensively developed along with marginal

foldings and protrusions on the perivascular spaces (Enders, 1962). Enders and Lyon (1964) reported that lutein cells from corpora lutea of pregnant rats and adult rats injected with prolactin are characterized by an abundant, tortuous tubular agranular endoplasmic reticulum such as that encountered in other tissues associated with triglyceride transport and synthesis, phospholipid synthesis and steroidogenesis, and with metabolism of acetoacetyl coenzyme A to mevalonic acid in the liver. They also reported that lutein cells from hypophysectomized rats have a less highly developed agranular endoplasmic reticulum, the mitochondria have irregular outlines with a relatively lucid matrix and the lipid droplets are small in size and less abundant. They suggested, too, that the luteotrophic hormone (LTH, prolactin) has a general effect on the metabolism of the lutein cell rather than solely affecting a specific subcellular structure.

McDonald and Goldfien (1965) studied the morphology of the Golgi apparatus of the rat granulosa cell during the estrous cycle. Before ovulation this subcellular structure is small. After ovulation, as the granulosa cells undergo luteinization, the Golgi apparatus shows enlargement reaching maximum size in late diestrus when it appears as a fine filamentous structure completely encircling the nucleus in many cells. They related this change in the morphology of the Golgi apparatus with the secretory activity of the corpus luteum.

Crisp (1965) studied the lutein cells of pregnant mice in midgestation. These cells were of two types, large and small, presumably derived from the granulosa and theca of the ruptured Graafian follicle, and there was a significant variation in the morphology and abundance of the agranular endoplasmic reticulum and mitochondria of each type of

cell. In general, the subcellular structures of the large cells were more abundant and better developed than in the small ones. Beside the vascular coloration and cellular hypertrophy induced by the luteotrophic hormone, there was a marked depletion of lipid vacuoles.

Wolthuis (1963) reported that in the immature female rat there was a correlation between the decrease in the number of luteal cell nuclei counted per surface unit, and the injection of increased amounts of prolactin. He suggested that the correlation could provide the basis for a prolactin assay.

Steroids of the Corpus Luteum

Progesterone and 20α-hydroxypregn-4-en-3-one have been recovered in the venous effluent of luteinized rat ovaries (Shima *et al*., 1967; Hashimoto *et al*., 1968). Corpora lutea of normal rats have not been incubated separately *in vitro* to study steroidogenesis. Most incubation studies have been performed with highly luteinized (pseudopregnancy) ovaries from rats previously injected with human chorionic gonadstropin (HCG) or luteinizing hormone (LH). The two hormones, progesterone and 20α-hydroxypregn-4-en-3-one, were isolated after incubation of slices with sodium acetate-^{3}H, without any evidence of estrogen or other hormone synthesis (Huang and Pearlman, 1962). Everett (1956a) reported that implantation does not occur in hypophysectomized rats, when the pituitary gland is transplanted beneath the kidney capsule one or two days after fertile mating, unless estrogen is administered. However, Macdonald and Armstrong (1965) found that injection of LH replaced estrogen injections in inducing implantation in such rats. They concluded that corpora lutea of normal rats are capable of secreting

estrogen concomitantly with progesterone secretion because uterine and vaginal changes usually induced by estrogen were not encountered when LH was injected in the absence of corpora lutea. Yuhara et al. (1963) incubated rat ovarian slices after 3 daily injections of HCG. Estrogen was synthesized in amounts detectable by bioassay.

The ovary of the rabbit is characterized by abundant, opaque, white "interstitial gland" tissue which stores substantial amounts of cholesterol and cholesterol esters (Claesson et al., 1953). The interstitial cells contain high concentrations of the enzyme Δ^5-3B-hydroxysteroid dehydrogenase (Rubin et al., 1963), whereas the luteal cell concentrations are very low (Deane and Rubin, 1965). The venous effluent of luteinized rabbit ovaries contains progesterone and 20α-hydroxypregn-4-en-3-one (Dorrington and Kilpatrick, 1966; Hilliard et al., 1968b; Mikhail et al., 1961; Simmer et al., 1963), and these two progestins were also isolated from the venous blood of ovaries lacking corpora lutea (Hilliard et al., 1961), and Graafian follicles. Therefore, rabbit interstitial tissues are capable of synthesizing these steroid hormones (Hilliard et al., 1963). According to Gorski et al. (1965) rabbit corpora lutea slices are capable of synthesizing labelled progesterone and 20α-hydroxypregn-4-en-3-one from acetate-2-^{14}C.

Progesterone, 20α-hydroxypregn-4-en-3-one, estrone and estradiol-17β were recovered from ovine ovarian venous blood during the mid and late luteal stages of the estrous cycle (Short et al., 1963). However, luteal tissue was found to contain only progesterone (Stormshak et al., 1963). This implied that the other steroids were synthesized by the ovine interstitial tissue.

Progesterone and 20β-hydroxpregn-4-en-3-one have been found in the

venous blood of pregnant and non-pregnant cow ovaries (Gorski et al., 1958). Kristoffersen (1960), and Zimbelman et al. (1961) also identified these two hormones in the luteal tissue of pregnant and non-pregnant cows. Further, it has been reported that bovine luteal tissue slices incubated with acetate-1-^{14}C synthesized labelled progesterone and 20β-hydroxypregn-4-en-3-one (Savard et al., 1965).

Mare luteal tissues are capable of synthesizing progesterone and 20α-hydroxypregn-4-en-3-one and trace amounts of 17α-hydroxyprogesterone. These tissues were also reported to have a weak 17-hydroxylase activity and little or no desmolase activity (Short, 1964). Mahajan and Samuels (1963) incubated mare luteal slices with progesterone, 17α-hydroxyprogesterone, androstendione and testosterone. Incubation with progesterone resulted in the synthesis of very small amounts of 17α-hydroxyprogesterone, androstendione and testosterone, but no estrogen. Incubation of mare luteal tissue slices with 17α-hydroxyprogesterone gave much smaller amounts of androstendione and testosterone without any indication of estrogen synthesis, whereas incubation with androstendione and testosterone produced some estrogen. This supports the finding of Short (1964) that mare luteal cells have little 17 -hydroxylase and desmolase activities, but suggests that these cells possess the enzymes involved in aromatization.

Huang and Pearlman (1963) incubated human corpora lutea slices with sodium acetate-^{3}H and reported the synthesis of labelled progesterone and 17α-hydroxyprogesterone. When progesterone-7-^{3}H was used as substrate there was synthesis of labelled 17α-hydroxyprogesterone, estrone and estradiol. Later reports by other workers indicated that, in vitro, human luteal tissue slices incorporate acetate-1-^{14}C into labelled

progesterone, 20α-hydroxypregn-4-4n-3-one, 17αhydroxprogesterone, androstendione, estradiol and estrone (Hammerstine et al., 1964; Rice et al., 1964; Savard et al., 1964). Human luteal cells appear to possess high Δ^5-3β-01 dehydrogenase activity, the enzymes involved in aromatization, and relatively low desmolase activity (Forleo et al., 1967).

Gomes et al. (1965) reported that isolation and identification of progesterone in ovarian venous blood of sows during the estrous cycle. However, 20α-hydroxypregn-4-en-3-one, 20β-hydroxypregn-4-en-3-one, 17α-hydroxyprogesterone, androstendione or pregnenolone were not found. Bjersing and Carstensen (1967) incubated porcine granulosa cells with testosterone as substrate and reported the synthesis of estrone and estradiol. Recently Cook et al. (1967) reported that porcine luteal tissue incubated with acetate-1-^{14}C synthesized labelled progesterone and 20α-hydroxypregn-4-en-3-one.

The Corpus Luteum and Pregnancy

The corpus luteum is indispensable for the maintenance of pregnancy throughout gestation in the rat (Johnson and Challans, 1930; Selye et al., 1935), rabbit (Corner, 1928; Firor, 1933) and goat (Meites et al., 1951). In the guinea pig, it is essential for less than full term (Herrick, 1928).

Presence of the corpus luteum is essential during the first half of gestation to avoid abortion in ewes (Casida and Warwick, 1945; Short, 1957).

Hart and Colege (1934) and Asdell (1964) reported that in the pregnant mare, ovariectomy could be performed on day 200 of gestation

without termination of pregnancy.

According to McDonald *et al*. (1953), the corpus luteum is essential for the maintenance of pregnancy in the cow until approximately the 200th day of gestation. Absence of the corpus luteum with progesterone deficiency prior to parturition leads to retained placenta (McDonald *et al*., 1954).

Ovariectomy or removal of the corpus luteum does not result in any abnormality in rhesus monkeys as far as the normal gestation period, parturition at term, postpartum involution of the uterus and lactation are concerned (Hartman, 1941; Hartman and Corner, 1947).

The corpus luteum of pregnancy in women can be removed a short time after implantation has occurred without terminating pregnancy (Tulsky and Koff, 1957).

Du Mesnil du Buisson and Dauzier (1957) reported that, in gilts, ovariectomy as early as four days post coitum, or as late as 106 days, resulted in abortion within three days. Progesterone is essential for the maintenance of pregnancy in such operated on animals (Day *et al*., 1959).

Luteotropic Substances

Factors controlling the activity of the corpus luteum have been attracting attention for several years. Historically, Dresel (1935) was the first to observe that prolactin can suspend estrus in the mature non-pregnant mouse. This observation was extended to the rat by Lahr and Riddle (1936). Astwood (1941) found that the injected prolactin could prolong the life span of the corpus luteum of the rat beyond the normal limit. He called prolactin the luteotrophic hormone

(LTH), or luteotrophin.

These findings in the rat and mouse inspired a long series of attempts to study the effect of prolactin on the corpus luteum of other species.

Prolactin cannot activate steroid release from the rabbit ovary containing functional corpora lutea of pregnancy or pseudopregnancy (Hillard et al., 1961). Rennie et al. (1964) reported that prolactin did not prevent atrophy of the corpus luteum following hypophysectomy in pseudo-pregnant or pregnant rabbits, nor did it produce changes in the corpora lutea of intact pseudopregnant or pregnant rabbits.

In guinea pigs, prolactin did not maintain the corpora lutea beyond the luteal phase (Aldred et al., 1961).

According to Kaltenbach et al. (1968b) prolactin did not maintain the corpus luteum in hypophysectomized pregnant and non-pregnant ewes.

Prolactin administered at selected times during the estrous cycle did not maintain the bovine corpus luteum beyond the luteal phase (Smith et al., 1957), and Simmons and Hansel (1964) reported that ovine prolactin did not prevent the anti-luteotropic effects of oxytocin in the cow. Bovine prolactin was used with similar results (Donaldson et al., 1965).

Hisaw (1944) found that prolactin could not maintain the corpus luteum beyond the luteal phase in rhesus monkeys. This was supported by the work of Bryans (1951). Further, prolactin will not maintain the corpora lutea of women (Bradbury et al., 1950; Holmstrom and Jones, 1949).

Prolactin did not prevent regression of the corpora lutea in pregnant gilts induced by daily injections of 400 mg of progesterone

(Sammelwitz and Nalbandov, 1958), and injection of ovine prolactin 3 days prior to slaughter on day 12 of the cycle does not alter the initial concentration of progesterone in swine luteal tissue (Duncan et al., 1961).

It could be concluded from the foregoing that prolactin is luteotropic in rats and mice only.

Other investigations have been made to determine the substances that have luteotropic activity based on whether each substance satisfies one or more of the following parameters:

1. Prolongs luteal activity in the cycling animal beyond the limits of the luteal phase.
2. Prevents luteal regression due to hypophysectomy or any action that induces regression.
3. Increases the rate of progesterone synthesis by luteal cells.

It has been reported that the estrous cycle of the rat was prolonged by HCG. Ovaries of the treated animals became heavily luteinized and their weight was increased significantly (Ketzman et al., 1931). Ender et al. (1953) reported similar results.

Injections of LH increased plasma progesterone levels (Major and Armstrong, 1966) and induced luteal regression in pseudopregnant rats (Rothchild and Schwartz, 1965). Rothchild and Schwartz (1965) observed, however, that administration of estradiol for 28 days reduced the incidence of luteal regression in intact pseudopregnant rats probably through depression of LH release from the anterior pituitary gland. Estradiol was ineffective in hypophysectomized rats. Administration of LH resulted in luteal regression in the intact rats which were treated

with estradiol. Bogdanove (1966) reported the initiation of pseudopregnancy and persistance of the corpora lutea in pseudopregnant rats for an additional 17 - 27 days after estrogen treatment.

Malven and Sawyer (1966), in a recent re-evaluation of prolactin effects on rat corpora lutea, observed a luteolytic response in hypophysectomized rats previously treated with doses of LH and follicle stimulating hormone (FSH) sufficient to induce ovulation. Prolactin injections started several weeks after hypophysectomy hastened the regression of the induced corpora lutea and reduced the ovarian weight. They related the regression to the loss of the capacity of the corpora lutea to be maintained in a functional state by prolactin due to hypophysectomy. This is in contrast to the previous findings that combined hypophysectomy and autotransplantation of the anterior pituitary (Everett, 1956b) and pituitary stalk section (Nickitovitch-Winer, 1965) to facilitate prolactin release resulted in persistant functional corpora lutea for months. The luteal regression reported by Malven and Sawyer (1966) could have been due to the LH and FSH injections. Malven et al. (1967) reported failure of prolactin to prolong corpora lutea life for more than 3 days in both hysterectomized and intact rats, but in hypophysectomized rats, prolactin maintained the corpora lutea for 20 days after ovulation.

It has been often reported that any substance with LH activity increased progesterone synthesis in the luteinized rat ovary slices (Huang and Pearlman, 1962; Armstrong et al., 1964; Major and Armstrong, 1966).

Kovacic's (1964) experiment supported the concept that prolactin has luteotropic activity in mice. Prolactin, when injected into intact

mice, suspended estrous cycle, and when injected into hypophysectomized mice persistence of corpora lutea occurred. He reported similar results with human LH and HCG in intact mice only (Kovacic, 1966). Ovine LH was not effective.

Injection of ovine LH into hypophysectomized pseudopregnant rabbits leads to persistance of corpora lutea, whereas prolactin fails to show any influence (Kilpatrick et al., 1964).

Administration of pregnant mare serum (PMS), HCG and LH induced increased progesterone release into the ovarian venous effluent of pregnant and pseudopregnant rabbits (Hillard et al., 1961). Okano et al. (1966) reported similar results with HCG when it was injected into pregnant rabbits. Dorrington and Kilpatrik (1966) found that small doses of ovine LH were required to induce increased output of progestins from psuedopregnant rabbit ovaries, whereas much larger amounts of ovine FSH and growth hormone were required to produce a similar effect. The action of these last two hormones could have been due to LH contaimination. Stormshak and Casida (1964, 1965) and Spies et al. (1966) have reported that administration of substances with LH activity induced regression of corpora lutea in pseudopregnant rabbits. Estrogen injections given con-commitantly were able to prevent such regression (Stormshak and Casida, 1965; Spies et al., 1966). Spies et al. (1966) suggested that LH might act on luteal tissue by interfering with endogenous estrogen through a direct effect on the ovary. Spies and Quadri (1967) investigated and supported the role of estrogens in the maintenance of rabbit corpora lutea. Key and Nalbandov (1967) found that destruction of the ovarian follicular tissue in pregnant rabbits by X-irradiation was followed by regressive changes in corpora lutea and cessation of synthesis and

release of progesterone leading to abortion. Luteinizing hormone and crude anterior pituitary powder were unable to maintain either the morphology of the corpora lutea or progesterone syntehsis in such animals. Daily injections of estradiol were effective in preventing regression of luteal tissue and in maintaining measurable quantities of progesterone in both the corpora lutea and the ovarian venous effluent. Whether estrogens are only luteotrophic or both luteotrophic and steroidogenic is not known.

Hilliard _et al_. (1968a) reported that administration of LH to pseudopregnant rabbits resulted in a lowered interstitium cholesterol store and undetectable release of 20α-hydroxypregn-4-en-3-one. Chronic prolactin treatment promoted cholesterol storage and restored the basal release of 20α-hydroxpregn-4-en-3-one. They noticed that hypophysectomy 24 hours after the LH injection, induced atrophic changes in the interstitial tissue and corpus luteum and cessation of progesterone release along with other changes. In such animals prolonged prolactin treatment led to hypertrophy of the interstitial tissue with elevation of cholesterol stores, but without any change in progesterone release. Treatment of these animals with estrogen maintained the corpora lutea and increased the release of progesterone over 20α-hydroxypregn-4-en-3-one. Prolactin and estrogen given simultaneously acted on both corpora lutea and interstitial tissue causing release of more 20α-hydroxypregn-4-en-3-one than progesterone. Thus, it can be postulated that, in the rabbit, estrogen acts primarily on the corpora lutea and prolactin acts primarily on the interstitial tissue to enhance steroidogenesis.

Incubation of rabbit luteal tissue (Gorski _et al_., 1965; Gospodarowicz, 1965) and luteinized or non-luteinized ovarian slices

(Dorrington and Kilpatrik, 1966; Gorski and Padnos, 1966) in the presence of LH results in increased synthesis of progesterone and 20α-hydroxypregn-4-en-3-one. Ovine prolactin is without effectiveness (Dorrington and Kilpatrik, 1966).

Rowlands (1961) observed that hysterectomy resulted in the persistance of functional corpora lutea in the guinea pig, with failure of ovulation and cessation of the estrus because of the anti-estrogenic action of high circulating levels of progesterone. Later he reported that exogenous estrogens did not prevent the onset of the luteal changes induced by hysterectomy (Rowlands, 1962). After hypophysectomy, the life span of corpora lutea were prolonged rather than shortened in hysterectomized guinea pigs (Rowlands, 1962) indicating that pituitary gonadotrophins in this species do not play an essential role in maintenance of the corpora lutea. Prolactin and LH in intact (Rowlands, 1962; Deanesly, 1966) and LH in hypophysectomized guinea pigs (Deanesly, 1966) did not show any luteotropic or luteolytic activity.

Hypophysectomy on days 2-5 of the estrous cycle of the ewe allows the corpus luteum to continue secreting normal amounts of progesterone up to 9 days (Denamur _et al_., 1966). Kaltenbach _et al_. (1968a) found that hypophysectomy on day 5 of the ovine estrous cycle was followed by luteal regression by day 12. Denamur _et al_. (1966) performed pituitary stalk section early in the cycle, to allow continuous prolactin secretion, with and without simultaneous hysterectomy and observed that in the first case the ovine corpus luteum was still secreting progesterone at a normal rate on day 18, whereas in the second case, stalk section only, the corpus luteum behaved as it would during a normal cycle. Short _et al_. (1963) failed to detect any increase in progesterone

in the ovarian venous blood of ewes following the injection of ovine FSH, LH, prolactin, HCG, PMS and an endometrial extract. Infusion of prolactin directly into the arteries of corpora lutea bearing ovine ovaries enhanced progesterone secretion which could be maintained only with continued prolactin infusion (Domanski and Dobrowlski, 1966). Kaltenbach et al. (1968b) reported that constant injection of crude luteinizing hormone into the sheep was enough to maintain the corpus luteum after the animal was hypophysectomized, whereas FSH and prolactin were ineffective. Denamur and Mauleon (1963) reported that estrogen prolonged the life span of the ovine corpus luteum beyond the luteal phase, but Kaltenbach (1968) found estrogen without effect on the functioning life of the corpus luteum in hypophysectomized ewes. Legault-Demare et al. (1960) incubated ovine luteal tissue slices in vitro and reported that PMS increased the amount of progesterone liberated into the incubation medium without any clear-cut evidence of an increase in progesterone synthesis. Kaltenbach et al. (1966, 1967) incubated ovine corpora lutea slices in vitro and added ovine LH, FSH and prolactin to the incubation medium, separately. Only LH caused a significant increase in progesterone synthesis.

Injections of HCG suspended the estrous cycle and caused maintenance of corpora lutea in cycling heifers (Wiltbank et al., 1961). Similar results were obtained with pituitary extracts and bovine LH (Donaldson and Hansel, 1965). Crude bovine anterior pituitary extracts, HCG, and LH, prevented the anti-luteotrophic effect of oxytocin injections in the cow, but bovine growth hormone, equine luteinizing hormone and ovine prolactin did not (Simmons and Hansel, 1964; Hansel, 1966). Human chorionic gonadotropin (Donaldson et al., 1965), as

well as LH injections (Brunner and Hansel, 1966), increased progesterone concentrations in bovine corpora lutea. Injection of estrogen caused regression of the corpus luteum in the cycling, pregnant and hypophysectomized heifers (Wiltbank, 1966).

Results of several experiments, in which bovine luteal slices were incubated in vitro, indicate that any substance with LH activity will increase the rate of progesterone synthesis (Mason et al., 1962; Marsh and Savard, 1964; Mason and Savard, 1964a; Savard and Casey, 1964; Hall and Koritz, 1965a; Lynn et al., 1965; Armstrong and Black, 1966; Hansel, 1966; Marsh and Savard, 1966; Hansel and Seifart, 1967; and Seifart et al., 1968). Bartosik et al. (1967) perfused luteal and follicular bovine ovaries in vitro and reported that both prolactin and LH increased the progesterone secretion rate of the corpora lutea bearing ovaries, whereas LH augmented the progesterone secretion rate of follicular ovaries also. Romanoff et al. (1966) perfused luteal bovine ovaries in vitro and observed that FSH, LH and prolactin significantly enhanced the rates of incorporation of 1-^{14}C-acetate into labelled cholesterol and progesterone.

According to Hisaw (1944) and Bryans (1951) HCG is able to prolong the functional life of the corpus luteum in the monkey. Human chorionic gonadotropin injections also prolong the life span of the functional human corpus luteum (Browne and Venning, 1938; Holstrum and Jones, 1949; Bradbury et al., 1950). In vitro, HCG and LH of human origin enhanced the synthesis of progesterone in human luteal tissue slices incubated with acetate-1-^{14}C, whereas ovine LH (Rice et al., 1964) and human prolactin (Savard et al., 1965) were ineffective. Le Maire et al. (1966) reported that ovine LH increased steroidogenesis significantly

when it was added to human corpus luteum slice preparations.

Duncan et al. (1961) incubated porcine luteal tissue slices in the presence of an unfractionated homogenate of gilt pituitary gland, ovine prolactin, HCG, PMS, oxytocin, relaxin and endometrial filtrates from different days of the estrous cycle. Endometrial filtrates from days 12 and 13 were the only additives causing increased progesterone synthesis. Others were without any influence, except the endometrial filtrates from days 16 and 18 which inhibited the synthesis of progesterone. Cook et al. (1967) reported that they were able to detect a significant increase in progesterone synthesis (15%) when swine corpora lutea slices were incubated with ovine, bovine and porcine LH. Follicle stimulating hormone and prolactin were ineffective. Nishikawa (1963) injected gilts with estrogen during the luteal phase of the estrous cycle. Follicle development was suppressed by the persisting corpora lutea resulting from the estrogen injections. Pituitary FSH concentrations were lowered whereas LH concentrations were raised. Regression of persisting corpora lutea allowed FSH and LH levels to return to normal. Gardner et al. (1963) also reported persistence of gilt corpora lutea by estrogen when treatment was started by day 11 of the estrous cycle. They observed a decrease in luteal tissue weight, an increase in progesterone concentration, and no change in the total progesterone content of the corpora lutea. Du Mesnil du Buisson (1966) hypophysectomized gilts during the luteal phase of the cycle and observed persistence of the corpora lutea until about day 14. According to du Mesnil du Buisson et al. (1964, 1965), application of hypophysectomy and hysterectomy in gilts during the luteal phase of the estrous cycle, followed by daily injections of HCG or LH, lead to further persistence

of the corpus luteum, whereas treatment with bovine or porcine prolactin was not effective.

Greenwald (1965) studied the action of exogenous estrogen on the corpora lutea of pregnancy in the hamster. Administration of estrogen on day 3 of pregnancy induced luteal regression unless FSH was also given. Greenwald (1967), in a more recent experiment, noticed that hypophysectomy after day 12 of pregnancy, when the placenta was established, did not cause luteal regression in the hamster, whereas regression occurred when hypophysectomy was performed on days 1, 4 and 8 of pregnancy. Daily inections of prolactin, but not LH or FSH, maintained the morphology of the corpus luteum but not its function. Injection of prolactin and FSH concomitantly maintained the histologic and functional aspects of the hamster corpus luteum. He suggested that the interaction of prolactin and FSH constituted the "minimal luteotropic complex" in the hamster. Perhaps such a "minimal luteotropic comples" exists in other species.

Steroidogenesis

Steroidogenesis occurs in adrenal cortical tissue, testicular interstitium, tissues of different ovarian compartments, and placenta. Steroidogenesis in adrenal tissue will be discussed briefly before reviewing steroid biosynthesis in luteal tissue.

Stone and Hechter (1954) perfused bovine adrenal glands with ^{14}C-labelled acetate, cholesterol and adrenocorticotrophic hormone (ACTH). The trophic hormone increased the incorporation of cholesterol into corticosteroids. Halkerston _et al_. (1961) and Koritz (1962) observed the need of reduced nicotinamide adenine dinucleotide phosphate (NADPH)

for cholesterol side-chain cleavage and synthesis of pregnenolone from cholesterol in the adrenal cortex. Conversion of cholesterol to pregnenolone includes the rate limiting step in steroid biosynthesis and that ACTH may act specifically at this point to stimulate 20α-hydroxylation of cholesterol (Koritz, 1962). It has been suggested, too, that high concentrations of pregnenolone in the mitochondria could inhibit conversion of cholesterol to 20α-hydroxycholesterol (Koritz and Hall, 1964). Another suggested mechanism by Koritz and Hall (1964) is that ACTH may stimulate conversion of cholesterol to pregnenolone by increasing the rate of removal of pregnenolone from mitochondria.

Haynes _et al_. (196) developed a hypothesis to explain the mode of action of ACTH on steroidogenesis in the adrenal cortex. The hypothesis suggested that 1) the initial action of the trophic hormone is to increase the conversion rate of adenosine triphosphate (ATP) to cyclic adenylic acid (3',5'-AMP), 2) intracellular accumulation of 3',5'-AMP induces a rapid rate of phosphorylase activation which in turn facilitates the breakdown of glycogen to glucose-1-phosphate and glucose-6-phosphate and 3) glucose-6-phosphate is then metabolized via the pentose phosphate pathway to produce large amounts of the co-factor NADPH which is essential for several reactions during corticosteroid biosynthesis.

The mode of action of the steroidogenic hormones on steroid biosynthesis in the corpus luteum was investigated by several workers. Tamaoki and Pincus (1961) reported the presence of the cholesterol side-chain cleaving enzyme desmolase in bovine ovarian tissues. They also reported the capability of the bovine luteal tissue homogenate to convert cholesterol-4-^{14}C to labelled progesterone. However, LH, FSH, PMS, HCG and prolactin failed to effect desmolase activity and

progesterone synthesis in the homogenate. Ichii *et al*. (1963) reported that LH stimulated side-chain cleavage in cholesterol using acetone-dried powder of mitochondria from bovine corpus luteum, whereas Jackanicz and Armstrong (1967), using intact mitochondria of rabbit ovarian interstitial cells, did not observe such stimulation when LH was added to their system. Hall and Koritz (1964) reported that LH did not produce a demonstrable effect on the conversion of cholesterol and 20α-hydroxycholesterol to pregnenolone and progesterone when incubated with an acetone-dried powder of mitochondria from bovine corpus luteum unless NADPH was added to the system. They also reported that 20α-hydroxycholesterol is an intermediate product. Hall and Young (1968) incubated intact bovine luteal cells and reported that LH increased the conversion of cholesterol to progesterone and suggested that this hormone stimulated steroidogenesis in intact lutein cells through 20α-hydroxylation of cholesterol. Other scientists are of the opinion that LH accelerates the conversion of preformed endogenous cholesterol to steroids (Armstrong *et al*., 1964; Major and Armstrong, 1966; Solod *et al*. 1966; Shima *et al*., 1967). Another report indicates that NADPH stimulates the conversion of both endogenous and exogenous cholesterol and its action is much faster than that of LH (Shima *et al*., 1967).

In addition to the foregoing, several attempts have been made to test the extent of application of the Haynes hypothesis (Haynes *et al*., 1960) on the action of the steroidogenic hormones in the corpus luteum.

It has been reported that phosphorylase activity was increased in bovine luteal tissue slices when ovine LH was added to the incubation medium (Marsh and Savard, 1964). A similar increase was reported for luteal phosphorylase in pseudopregnant rats following the *in vivo*

administration of LH (Stansfield and Robinson, 1965). Further, prior injection of LH or HCG increased glucose-6-phosphate dehydrogenase and 20α-hydroxysteroid dehydrogenase activity in the luteinized granulosa cells of superovulated rat ovaries (Kidwell _et al._, 1966).

Mason _et al._ (1962) observed that bovine and ovine LH and HCG increased the rate of synthesis of progesterone in bovine corpus luteum slices _in vitro_, and addition of an NADPH generating system to the incubation medium resulted in a greater rate of synthesis. The latter point was studied again by Marsh and Savard (1966a) who found that LH resulted in a further stimulation of progesterone synthesis in bovine corpora lutea slices preincubated _in vitro_ with high concentrations of NADPH. Other reports indicated that LH increased both progesterone synthesis and incorporation of acetate-1-^{14}C into progesterone, while exogenous NADPH increased progesterone synthesis only (Savard and Casey, 1964). The opposite was observed when cholesterol-7-^{3}H was used as a substrate (Mason and Savard, 1964b).

These observations on the differences in the actions of LH and NADPH gave a clear-cut evidence that the action of LH is not mediated via the formation of NADPH, a fact that is not consistent with the hypothesis of Haynes _et al._ (1960).

Cyclic 3',5'-AMP was not observed to activate luteal phosphorylase (Marsh and Savard, 1963) but was observed to stimulate increased synthesis of progesterone when added to the incubation media of bovine luteal tissue slices (Marsh and Savard,1966b). Thus, stimulation of phosphorylase is not part of the mechanism of LH action on the stimulation of steroidogenesis although LH results in accumulation of endogenous 3',5'-AMP in bovine luteal slices. Human chorionic gonadotropin

has similar effects in the human corpus luteum in vitro (Marsh _et al_., 1966).

Using acetate-1-^{14}C and cholesterol-7-^{3}H as substrates, Marsh and Savard (1966b) have shown that LH and exogenous 3',5'-AMP behave similarly but without any additive effect. Seven and a half minutes after the addition of LH to the incubation medium, a significant increase in the endogenous 3',5'-AMP was observed in bovine luteal slices, whereas 15-30 minutes elapsed until steroidogenesis started to show a significant increase (Marsh _et al_., 1966).

The observed results of LH and 3',5'-AMP actions suggest that LH brings about its action on steroidogenesis in luteal tissue through 3',5'-AMP which acts as a mediator (Marsh and Savard, 1966b; Marsh _et al_., 1966). By means of this mediator, LH can stimulate progesterone synthesis from endogenous "steroidogenic" cholesterol and by enhancing synthesis of such cholesterol from earlier precursors (Savard _et al_., 1965).

Addition of puromycin to the incubation medium, in recently reported experiments, did not affect the accumulation of endogenous 3',5'-AMP in bovine corpus luteum slices in the presence of LH (Marsh _et al_., 1966). However, Marsh and Savard (1966a) and Savard _et al_., (1965) have presented some unpublished data which indicated that puromycin could inhibit the action of LH on steroidogenesis at a step after the increase in the accumulation of 3',5'-AMP. Exogenous 3',5'-AMP was unable to increase steroidogenesis in the presence of this protein synthesis inhibitor. These observations suggested that LH might stimulate the synthesis of certain proteins which are essential for biosynthesis of progesterone, and such stimulation could be mediated through 3',5'-AMP also.

Gorski and Padnos (1966) also observed an inhibition of steroidogenesis by puromycin and cycloheximide. Actinomycin D, a potent inhibitor of ribonucleic acid synthesis, did not interfere with the action of LH on progesterone synthesis in luteinized rabbit ovary slices *in vitro*. They suggested that LH might affect a translational control at a step following synthesis of messenger ribonucleic acid, and this control in turn could regulate steroidogenesis.

From the foregoing extensive review of the literature, it was concluded that there is need of more research on steroidogenesis and that many studies can still be conducted using *in vitro* models. However, the literature review has suggested that porcine luteal tissues do not perform well *in vitro* and that the porcine model needs to be improved.

On this basis the present study was designed to determine whether porcine luteal tissues, incubated *in vitro*, need a more complete incubation medium or unidentified serum factors for more efficient biosynthesis of progesterone.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

An experiment, in three replicates, was designed in an attempt to improve *in vitro* steroidogenesis in porcine luteal tissue and to demonstrate a steroidogenic response to porcine serum, LH, LH plus serum and LH preincubated in serum. Incubation was performed in two different mediums in an attempt to find a medium in which porcine luteal tissues could synthesize progesterone at a better rate. Each replicate consisted of the incubation of forty samples of pooled chopped swine luteal tissue. Eight unincubated aliquots acted as controls.

The incubated samples were divided into two groups, twenty were incubated in Krebs-Ringer bicarbonate buffer medium (pH 7.4) and the other twenty were incubated in Waymouth's cell culture medium[1] (Waymouth, 1959). The twenty 20 ml incubation flasks, into which each group of tissue samples was transferred, were subdivided into five units of four flasks each:

First Unit - Each flask contained 7.5 ml incubation medium

Second Unit- Each flask contained 6.5 ml medium and 1 ml porcine (barrow) serum

Third Unit - Each flask contained 6.5 ml medium, 0.9 ml

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[1]Medium 752, without serum, Colorado Serum Company, Denver 16, Colorado.

porcine serum and 125 ug ovine luteinizing hormone (NIH-LH-S11-Ovine) dissolved in 0.1 ml normal saline solution.

Fourth Unit - Each flask contained 6.5 ml medium and 125 ug ovine luteinizing hormone dissolved in 0.1 ml normal saline solution and preincubated in 0.9 ml porcine serum at 37°C for three hours.

Fifth Unit - Each flask contained 6.5 ml medium and 125 ug ovine luteinizing hormone dissolved in 1 ml normal saline solution.

A preliminary incubation of a similar design was conducted mainly to test the capability of the swine luteal tissue to synthesize progesterone under the specified conditions.

Glassware and Instruments

The incubation flasks, as well as the glass plates, jars and pipettes which were used to prepare and conduct the experiment were placed in a concentrated solution of a detergent overnight. They were then rinsed with tap water, placed in a chromic acid bath for three hours and rinsed again with tap and distilled water. They were dried in an oven at 150°C for two hours, removed and refrigerated immediately. The incubation flasks were stoppered with cork stoppers, previously boiled in 5% sodium bicarbonate solution, and rinsed with distilled water.

The single-edged razor blades, scissors and small spatulas, used to prepare the tissue for incubation, were wiped with a swab soaked in carbon tetrachloride and then sterilized.

Incubation Media

Krebs-Ringer bicarbonate buffer medium (pH 7.4) was prepared in a cold room the night preceding the day of the experiment and was held

refrigerated, in a clean sterilized stoppered bottle until early the next morning when it was measured into the stoppered incubation flasks along with the cell culture medium. The porcine serum and physiological saline solution of ovine LH were added at that time. The ovine LH pre-incubated in porcine serum was the only additive added just prior to incubation time.

Solvents

Reagent-grade solvents were used for extraction without any further distillation. The solvents for the thin layer chromatography developing system were of nanograde quality. Spectrograde toluene was used in preparing the scintillation cocktail.

Collection of Ovaries

Ovaries of pregnant gilts, at about three to four weeks of pregnancy, based on crown-rump length (Morrissette, 1964) were collected at an abattoir in Oklahoma City, Oklahoma, early in the morning of the day of the experiment. The ovaries were collected within twenty-five minutes after killing the animals and put in glass jars containing prechilled Krebs Ringer bicarbonate buffer solution. The jars were packed in ice and transported to the laboratory cold room.

Preparation of the Luteal Tissue

The luteal tissues were removed from the ovaries and chopped, on a glass plate with a single-edged razor blade, with little loss of cellular integrity. The chopped tissue was carefully pooled and mixed to homogeneity. Five to seven hundred mg aliquots of the pooled tissue

were weighed and placed in the prepared labelled incubation flasks containing the assigned media and additives. The control aliquots were transferred into empty incubation flasks which were frozen and stored.

Incubation

Within two to two and a half hours after collection of the ovaries, the incubation was started. The tissues were incubated for two hours at 37°C. in a water bath shaker[2] at 52 cycles per minute in an atmosphere of 95% O_2 and 5% CO_2 with a gas flow rate of 5 cubic feet per hour. At the end of the incubation the flasks were stopped, after replacing the air with nitrogen gas, and stored at -30°C.

Progesterone Analysis

The content of each flask was thawed, within three days after incubation and homogenized in dichloromethane with a Potter-Elvehjem homogenizer.[3] The homgenate was transferred into a 70 ml conical stoppered tube. The flask as well as the homogenizer were washed with dichloromethane and the wash was added to the homogenate in the conical tube. An equal amount of ^{14}C-progesterone was added to each tube and mixed thoroughly. Extraction was carried out by following the procedure described by Yuhara et al. (1963) as modified by Stabenfeldt (1968). A volume of dichloromethane equal to two and a half times the volume of the homogenate was added. The tube was shaken for two minutes on a Vortex shaker[4] and then centrifuged at 9,000 R.P.M. for ten

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[2] Model 2156, Warner-Chilcott Laboratories, Richmond, California.

[3] Arthur H. Thomas Company, Philadelphia, Pa.

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

minutes at 10°C. The lower layer dichloromethane was pipetted into a 250 ml round-bottom flask. This extraction step was repeated three times. The dichloromethane was evaporated by means of a Rotavapor[5] at 48°C under negative pressure. The dried residue was washed with 5 ml of ether and transferred to a 40 ml conical stoppered tube. The washing step was repeated two more times and each wash was added to the conical tube. Saponification was accomplished by adding 5 ml of 1 N NaOH solution to the ether washes. The content of the tube was shaken by hand 200 times releasing pressure every 50 shakes. Five ml of glass distilled water was added to the tube and the mixture was shaken by hand 50 times. The tube was then centrifuged at 1,500 R.P.M. for ten minutes. The lower aquous layer was pipetted off and discarded. This step was repeated once more. The conical tube was centrifuged for a third time and the last drop of water was pipetted off. The ether was evaporated in a water bath at 48°C. under nitrogen. The inside of the tube was then washed down with 5, 2 and 1 ml of ether, respectively, beginning at the top and advancing toward the bottom with each wash. After each wash the ether was evaporated as before.

The residue at the bottom of the conical tube was dissolved in 200 λ tetrahydrofuran (THF) and the tube was shaken thoroughly on a Vortex shaker. Using a capillary pipette the contents of the tube was spotted on a 20 X 20 cm activated silica gel[6] thin layered glass plate. The tube was then washed down with 100 λ and 50 λ of THF, in that order, and each of these volumes was also spotted on the plate thus ensuring

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[5] Deluxe Model VE50, Rinco Instrument Co., Inc., Greenville, Illinois

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

negligible quantity of residue remained in the tube. Five of a standard progesterone[7] solution were spotted on the same plate. The dried spotted plate was developed in a glass chromatography jar containing 100 ml benzene: ethyl acetate (4:1 v/v). When the solvent front progressed to within 2 cm of the upper edge, the plate was removed from the chromatography jar and left to dry. The flourescent progesterone band was located in a dark room by means of an ultraviolet lamp and its relationship to the standard progesterone spot. The progesterone band was scraped off the plate and transferred into a 15 ml conical centrifuge tube.

Progesterone was extracted from the silica gel three times with 3 ml of ether. After each addition of ether the tube was shaken and centrifuged at 1,500 R.P.M. for three minutes and the ether solution was pipetted off and placed in another 15 ml conical centrifuge tube. After the third extraction, the ether was evaporated under nitrogen gas in a water bath at 48°C. The inside of the tube was washed down from the top three times with 1 ml of ether, each wash starting nearer to the bottom of the tube. The ether was evaporated after each washing in the manner previously described.

In a cold room, using precooled ether and pipettes, the content of the tube was reconstituted in 1 ml of ether. One tenth of the ether solution was pipetted into a prechilled scintillation vial containing 10 ml of scintillation fluid. The ether remaining in the test tube was again evaporated under nitrogen gas at 48°C in a water bath.

A blank scintillation vial was prepared, containing 10 ml of

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

scintillation fluid only to measure the background counts. A standard vial was prepared by adding to the 10 ml of scintillation fluid a quantity of ^{14}C-progesterone, equal to the quantity that had been added to the homogenate at the beginning of the extraction. The vials were counted for ten minutes by means of a liquid scintillation spectrometer[8] to determine the percent recovery of progesterone.

Gas-liquid chromatography[9] was used to determine the amount of progesterone left in the conical centrifuge tube. The dried residue of each tube was reconstituted with a known amount of THF and a specific volume of the solution was chromatographed on a 3-foot glass column packed with 1%QF on 100/120 mesh Gas-Chrom Q.[10] Nitrogen carrier gas was used at a flow rate of 48 ml/min from the column. The temperatures of the injection port, column bath and detector were 250°C., 225°C., and 230° C respectively.

By means of a planimeter[11] the peak areas of the injected quantities of the sample, as well as of the standards were measured. A standard curve was drawn by plotting the areas of the injected standards in order to calculate the amount of progesterone, in ug, in each chromatographed volume of the sample. Progesterone in ug/gm luteal tissue was then calculated by multiplying this quantity of progesterone by the several dilution factors, and loss factors due to experimental procedure and then dividing by the quantity of luteal tissue, in grams, extracted.

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[8] Packard Tricarb Model 314-B, Packard Instruments, La Grange, Ill.

[9] Gas-liquid Chromatograph, Barber-Coleman Series 5000, equipped with a Model 5121 hydrogen flame detector.

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

[11] Ott-Planimeter, W. H. Curtin and Co., Germany.

Statistical Analysis

Statistical analysis was performed under the supervision of the Department of Statistics, Oklahoma State University. Analysis of variance (Steel and Torrie, 1960) was conducted by means of IBM 7040 computer in the University Computer Center.

CHAPTER IV

RESULTS AND DISCUSSION

The preliminary trial, conducted prior to the experiment, indicated that the procine luteal tissues were capable of synthesizing progesterone under the collection, storage and incubation conditions of the experiment.

Table I presents the mean values of progesterone content of luteal tissue before and after incubation for a two-hour period in Krebs-Ringer bicarbonate buffer medium (pH 7.4) with 200 mg% glucose and Waymouth's cell culture medium, with and without treatments. Statistical analysis revealed that:

1. A significant amount of progesterone was synthesized in the procine luteal tissue during incubation in both Krebs-Ringer bicarbonate buffer and Waymouth's cell culture mediums (Appendix A, Table II.
2. Addition of porcine serum, LH, LH with serum, and LH preincubated in serum did not stimulate or inhibit progesterone synthesis _in vitro_ (Appendix A, Table III through X).
3. Preincubation of LH in porcine serum did not result in any change in the influence of LH on progesterone synthesis (Appendix A, Tables XI and XII).
4. Progesterone was synthesized at similar rates during

TABLE I

PROGESTERONE CONCENTRATION IN PORCINE LUTEAL TISSUE BEFORE AND AFTER INCUBATION IN VARIOUS MEDIUMS AND ADDITIVES

	Number of Samples	Concentration (ug/g)
Unincubated Control		85.6*** ± 15.62†
Krebs-Ringer Bicarbonate Buffer Medium	12	139.8 ± 0.25
Krebs-Ringer Medium Plus Serum	12	146.8 ± 3.92
Krebs-Ringer Medium Plus LH	12	143.9 ± 14.17
Krebs-Ringer Medium Plus LH and Serum	12	138.5 ± 5.67
Krebs-Ringer Medium Plus LH Preincubated in Serum	12	139.3 ± 5.28
Waymouth's Cell Culture Medium	12	137.0 ± 15.73
Waymouth's Medium Plus Serum	12	138.9 ± 6.18
Waymouth's Medium Plus LH	12	140.4 ± 6.10
Waymouth's Medium Plus LH and Serum	12	139.9 ± 12.35
Waymouth's Medium Plus LH Preincubated in Serum	12	135.3 ± 9.73

***$P < 0.001$

†S.E.

incubation in Krebs-Ringer bicarbonate buffer medium
and Waymouth's cell culture medium (Appendix A, Table XIII).

The results show that porcine luteal tissue retained the capability of synthesizing progesterone in vitro in both mediums under the conditions of the experiment. Duncan et al. (1960) reported the capability of porcine luteal tissue to synthesize progesterone throughout six hours of incubation in Krebs-Ringer bicarbonate buffer medium.

Failure of LH, in the present study, to inhibit or stimulate progesterone synthesis in the porcine is inconsistent with the findings of Duncan et al. (1961). They reported that addition of PMS, HCG and a homogenate of whole gilt pituitary gland to the incubation medium failed to inhibit or stimulate progesterone synthesis in porcine luteal tissues collected at different stages of estrous cycle and gestation and incubated in Krebs-Ringer bicarbonate buffer medium with 200 mg% glucose. However, the results of this experiment and the experiment of Duncan et al. (1961) are not in agreement with the report of Cook, et al. (1967) who presented data indicating increased rate of progesterone synthesis in porcine luteal tissues at different stages of estrous cycle and gestation by porcine, ovine and bovine LH added to Krebs-Ringer bicarbonate buffer medium (pH 7.4).

The major step in the experimental procedure of Cook et al. (1967) that is different from this experiment and that of Duncan et al. (1961) is the method of collecting the ovaries. In this work, as well as in that of Duncan et al. (1961), the ovaries were collected at slaughter, whereas Cook et al. (1967) obtained the ovaries by laparotomy while the gilts under anaesthesia. The lack of response to LH by steroidogenesis in porcine luteal tissues reported by Duncan et al. (1961) and in this

study can be attributed to some degenerative changes taking place in the luteal cells during the time elapsing between cessation of blood supply to the ovaries and their removal from the carcass prior to chilling. Such changes might take place in the cellular membranes, where the enzymes involved in the synthesis of 3',5'-AMP are located (Sutherland *et al*., 1965). As a result of such deterioration, endogenous 3',5'-AMP could not be accumulated and therefore LH would not bring about its action on steroidogenesis which is mediated through 3',5'-AMP (Marsh and Savard, 1966b; Marsh *et al*., 1966).

Armstrong and Black (1966), during their study of bovine luteal tissue, observed that keeping the ovaries under anaerobic conditions at body temperature, similar to the situation after the death of the animal, for about half an hour or more resulted in less progesterone synthesis in response to LH, but there was increased rate of steroidogenesis in the presence of an NADPH generating system. This is in comparison with biosynthesis of progesterone in bovine luteal tissue obtained surgically and incubated with LH and an NADPH generating system. They attributed the increased response in the anaerobically "aged" luteal tissue to the NADPH generating system to some breakdown of cell membranes which increased cell permeability as intact cells are poorly permeable to this system. Luft and Hechter (1957) have reported an increase in permeability in bovine adrenocortical cells due to anoxia.

In the experiment discussed herein the mean value of progesterone concentration after two hours of incubation in Krebs-Ringer bicarbonate buffer medium without additives was 157.7 ug/gm. The mean values reported by Duncan *et al*. (1960) and Duncan *et al*. (1961) were 145 and 153 ug/gm respectively in porcine luteal tissue collected on days 22 and

and 27 of gestation and incubated for the same period of time. The reduction in the mean values observed in this study as well as in those of Duncan et al. (1960, 1961) in comparison with those reported by Cook et al. (1967), could be attributed to the destruction of some essential factors such as NADPH and 3',5'-AMP due to the anaerobic conditions at body temperature which existed before the ovaries were removed from the carcass and chilled. Duncan et al. (1960) observed that addition of nicotinamide adenine dinucleotide (NAD) to the incubation medium stimulated further synthesis of progesterone.

Armstrong and Black (1966) have also reported that prolonged holding of the bovine luteal tissue near $0^{o}C$. did not cause any change in the ability to synthesize progesterone as well as in the response to LH. Thus it is not likely that significant changes in the luteal tissues occurred after chilling.

In previous studies on steroidogenesis in swine luteal tissue in vitro, porcine serum was overlooked as a source of some unidentified factors that could be essential for better synthesis of progesterone in the absence or presence of LH. Addition of 1 ml of porcine serum to the incubation medium, did not result in any changes in the rate of progesterone synthesis in the absence and presence of LH. Loss of essential factors during the collection and preparation of the serum cannot be excluded, however.

Maddock and Leach (1952) incubated HCG in plasma at $37^{o}C$. for 4 hours before it was assayed in rats. A two-fold increase in response was observed over that induced by unincubated HCG-plasma mixture. Maddock et al. (1953) suggested a mechanism by which HCG is protected by plasma against inactivation. Later, Parlow (1963) suggested the

possibility that some intermingling of the serum and LH might take place. In this study, LH was incubated in porcine serum for 3 hours at 37°C before it was added to the incubation media to explore the possibility that a serum-LH complex would influence steroidogenesis in vitro. Although negative results were obtained, the lack of response could have been due to cellular degenerative changes and destruction of some essential factors in the luteal tissue.

Cook et al. 1967 reported a 15% increase in progesterone concentration of porcine luteal tissue due to the addition of LH to the incubation medium, Krebs-Ringer bicarbonate buffer medium, while in ovine and bovine luteal tissues the increase was 30% and 40% respectively. Further, Seifart and Hansel (1968) reported a 200% increase in progesterone concentration in bovine luteal tissue after the addition of LH to Krebs-Ringer bicarbonate medium. These results have raised the question of whether a more complete incubation medium, along with better collection techniques (collection of tissues at laparotomy), are needed before porcine corpora lutea can synthesize progesterone at better rates in vitro and respond to LH.

Waymouth's cell culture medium was described by Waymouth (1959) as a chemically defined nutrient solution containing 40 components including amino acids, monosaccharides, vitamins and a buffer system. It was originally used for rapid proliferative growth of a specific strain of mouse cells. It is the most complete incubation medium available and theoretically should provide most of the essential nutrients and thereby facilitate steroidogenesis. However, progesterone synthesis in the presence of Waymouth's cell culture medium was no better than in Krebs-Ringer bicarbonate buffer medium.

It was concluded from this study that there is still a need for a better incubation medium or tissue collection method before effective *in vitro* studies involving porcine luteal tissue preparations are feasible. It is possible, however, that porcine luteal tissues are already maximally stimulated when collected and that additional stimulation of progesterone synthesis after addition of gonadotropins *in vitro* would occur only under the most ideal conditions and that improved techniques may not be the immediate answer to the problem.

CHAPTER V

SUMMARY AND CONCLUSIONS

An experiment, in three replicates, was conducted to study steroidogenesis by porcine luteal tissue incubated in Krebs-Ringer bicarbonate buffer medium and Waymouth's cell culture medium, and to investigate the effects of porcine serum, LH, LH plus serum and LH plus serum previously incubated when added to the incubation medium.

Ovaries from gilts at day 21-28 of pregnancy were collected at an abbatoir in Oklahoma City, Oklahoma, within 25 minutes after electrocution and bleeding, and chilled immediately. Luteal tissues from these ovaries were extirpated, chopped, pooled and incubated in Krebs-Ringer bicarbonate buffer medium (pH 7.4) and Waymouth's cell culture medium with and without additives consisting of porcine serum, ovine LH, serum plus LH, and LH preincubated in serum. The incubation was carried out at 37°C in a water bath shaker for two hours in an atmosphere of 95% O_2 and 5% CO_2 with a gas flow rate of 5 cubic feet per hour.

The results indicated that porcine corpora lutea were capable of synthesizing progesterone at similar rates in both mediums under the conditions of the experiment, and that none of the additives influenced the rate of progesterone synthesis.

This failure of different additives to stimulate further synthesis of progesterone was attributed to degenerative changes in the luteal cells and to the destruction of some essential factors in the luteal tissue

due to anaerobic conditions at body temperature which existed before the collection of the ovaries, and in the porcine serum during collection, preparation and storage.

From these findings it was concluded that effective *in vitro* studies on steroidogenesis in porcine luteal tissues may require even better incubation or tissue collection methods. However, the possibility of maximal stimulation of steroid biosynthesis by endogenous gonadotropins, at the time the luteal tissues were collected, should be taken into consideration. Consequently, additional stimulation by gonadotropins *in vitro* would occur only under the most ideal conditions and that improved techniques may not be the immediate answer to the problem.

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APPENDIX A

TABLE II

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATION BEFORE AND AFTER INCUBATION IN DIFFERENT MEDIUMS

Source	Degrees of Freedom	Sum of Squares	Mean of Squares	F
Total	143	87439.09		
Replicates	2	722.00	361.00	1.796*
Treatment	10	60386.68	6038.66	
Mediums	(1)	341.19	341.19	1.697*
Incubation in mediums with and without additives	(4)	554.57	138.64	0.689*
Mediums X incubation in mediums with and without additives	(4)	269.02	67.25	0.334*
No incubation versus incubation	(1)	59221.90	59221.90	294.650***
Error	131	26330.41	200.99	

*No significance
***$P<0.001$

TABLE III

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM AND IN KREBS-RINGER BICARBONATE MEDIUM PLUS SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium	11	139.8	9251.2
Krebs-Ringer Medium + Serum	11	146.8	6300.5

Calculated value of t (d.f. 22) = 0.645[1]

TABLE IV

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM AND IN KREBS-RINGER BICARBONATE MEDIUM PLUS LH

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium	11	139.8	9251.2
Krebs-Ringer Medium + LH	11	139.8	9251.2

Calculated value of t (d.f. 22) = 0.362[1]

[1]Tabulated value of t (d.f. 22, $P<0.5$) = 0.686

TABLE V

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM AND IN KREBS-RINGER BICARBONATE MEDIUM PLUS LH AND SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium	11	139.8	9251.2
Krebs-Ringer Medium + LH + Serum	11	138.5	6810.3

Calculated value of t (d.f. 22) = 0.117[1]

TABLE IV

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM AND in KREBS-RINGER BICARBONATE MEDIUM PLUS LH PREINCUBATED IN SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium	11	139.8	9251.2
Krebs-Ringer Medium + LH Preincubated in Serum	11	139.3	3919.2

Calculated value of t (d.f. 22) = 0.050[1]

[1] Tabulated value of t (d.f. 22, $P<0.5$) = 0.686

TABLE VII

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN WAYMOUTH'S MEDIUM AND WAYMOUTH'S MEDIUM PLUS SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Waymouth's Medium	11	137.0	2269.8
Waymouth's Medium + Serum	11	138.9	2474.5

Calculated value of t (d.f. 22) = 0.317[1]

TABLE VIII

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN WAYMOUTH'S MEDIUM AND WAYMOUTH'S MEDIUM PLUS LH

Treatment	Degrees of Freedom	Mean	Sum of Squares
Waymouth's Medium	11	137.0	2269.8
Waymouth's Medium + LH	11	140.4	3718.7

Calculated value of t (d.f. 22) = 0.505[1]

[1] Tabulated value of t (d.f. 22, $P<0.5$) = 0.686

TABLE IX

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN WAYMOUTH'S MEDIUM AND WAYMOUTH'S MEDIUM PLUS LH AND SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Waymouth's Medium	11	137.0	2269.8
Waymouth's Medium + LH + Serum	11	139.9	3688.3

Calculated value of t (d.f. 22) = 0.431[1]

TABLE X

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN WAYMOUTH'S MEDIUM AND WAYMOUTH'S MEDIUM PLUS LH PREINCUBATED IN SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Waymouth's Medium	11	137.0	2269.8
Waymouth's Medium + LH Preincubated in Serum	11	135.3	2263.9

Calculated value of t (d.f. 22) = 0.285[1]

[1]Tabulated value of t (d.f. 22, $P<0.5$) = 0.686

TABLE XI

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM AND WAYMOUTH'S MEDIUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium	11	139.8	9251.2
Waymouth's Medium	11	137.0	2269.8

Calculated value of t (d.f. 22) = 0.299[1]

TABLE XII

COMPARISON OF MEANS OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN KREBS-RINGER BICARBONATE MEDIUM PLUS LH AND SERUM AND KREBS-RINGER BICARBONATE PLUS LH PREINCUBATED IN SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Krebs-Ringer Medium + LH + Serum	11	138.5	6810.3
Krebs-Ringer Medium + LH Preincubated in Serum	11	139.3	3919.2

Calculated value of t (d.f. 22) = 0.088[1]

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[1]Tabulated value of t (d.f. 22, $P<0.5$) = 6.86

TABLE XIII

COMPARISON OF PROGESTERONE CONCENTRATION AFTER INCUBATION IN WAYMOUTH'S MEDIUM PLUS LH AND SERUM AND WAYMOUTH'S MEDIUM PLUS LH PREINCUBATED IN SERUM

Treatment	Degrees of Freedom	Mean	Sum of Squares
Waymouth Medium + LH + Serum	11	139.9	3688.3
Waymouth's Medium + LH Preincubated in Serum	11	135.3	2263.9

Calculated value of t (d.f.22) = 0.614[1]

[1]Tabulated value of t (d.f.22, P<0.51 = 6.86

TABLE XIV

REPLICATE NO. 1 PROGESTERONE CONCENTRATION IN PORCINE LUTEAL TISSUE (ug/g) BEFORE AND AFTER INCUBATION

Unincubated Control	Incubation in Krebs-Ringer Bicarbonate Buffer Medium					Incubation in Waymouth's Cell Culture Medium				
	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum
75.0	157.6	148.0	149.1	153.0	145.0	131.0	112.9	158.3	109.8	131.6
85.6	129.6	158.5	167.6	120.7	139.4	127.8	142.3	108.1	159.3	112.9
78.6	122.5	144.4	118.1	125.1	151.2	114.3	149.4	119.0	139.9	158.5
65.9	148.2	128.0	136.8	143.2	118.6	146.8	138.0	162.2	151.4	120.2
82.0										
73.2										
96.3										
79.5										

TABLE XV

REPLICATE NO. 2 PROGESTERONE CONCENTRATION IN PORCINE LUTEAL TISSUE (ug/g) BEFORE AND AFTER INCUBATION

Unincubated Control	Incubation in Krebs-Ringer Bicarbonate Buffer Medium					Incubation in Waymouth's Cell Culture Medium				
	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum
78.4	158.5	142.3	139.4	137.3	129.9	122.0	109.1	124.9	159.0	137.3
94.0	126.6	135.5	144.7	146.7	136.0	140.1	149.5	150.4	111.5	145.0
83.2	154.1	151.2	167.8	156.2	132.5	155.5	140.9	160.0	148.3	126.6
99.8	120.1	165.4	154.2	124.3	150.5	125.0	167.6	134.4	116.2	153.0
90.0										
95.4										
84.8										
97.2										

TABLE XVI

REPLICATE NO. 3 PROGESTERONE CONCENTRATION IN PORCINE LUTEAL TISSUE (ug/g) BEFORE AND AFTER INCUBATION

Unincubated Control	Incubation in Krebs-Ringer Bicarbonate Buffer Medium					Incubation in Waymouth's Cell Culture Medium				
	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum	Medium	Medium + Serum	Medium + LH	Medium + LH + Serum	Medium + LH Preincubated in Serum
94.7	145.4	154.5	134.2	146.7	154.5	138.4	150.8	161.1	159.6	122.0
89.8	137.4	162.7	148.3	158.1	138.5	131.7	128.0	126.9	143.0	137.3
92.9	158.1	129.2	139.4	121.7	130.3	150.3	141.3	134.8	134.5	151.5
91.9	120.2	142.6	128.0	129.3	146.3	161.7	137.6	144.8	147.3	128.1
96.5										
67.4										
83.5										
78.9										

APPENDIX B

KREBS-RINGER BICARBONATE BUFFER MEDIUM

SOLUTIONS:

0.90% NaCl	(0.154M)	0.9001g/100ml
1.15% KCl	(0.154M)	1.1481g/100ml
1.22% $CaCl_2$	(0.110M)	1.2209g/100ml
2.11% KH_2PO_4	(0.154M)	2.0958g/100ml
3.82% $MgSO_4.7H_2O$	(0.154M)	3.7950g/100ml
1.30% $NaHCO_3$	(0.154M)	1.2939g/100ml

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
$MgSO_4.7H_2O$	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 it to 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.

SCINTILLATION COCKTAIL

PPO[1]	4.0 g
POPOP[1]	0.3 g

Place in one liter spectro-quality toluene.[2]

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[1] Packard Instruments, La Grange, Illinois.

[2] Matheson Coleman and Bell, Norwood, Ohio.

VITA

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