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A Comparison of Luteinizing Hormone and Prostaglandin F_2 α Stimulation of Pseudo-Pregnant Rat Ovarian Steroidogenesis in Vitro

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A COMPARISON OF LUTEINIZING HORMONE AND PROSTAGLANDIN $F_2\alpha$ STIMULATION OF PSEUDO-PREGNANT RAT OVARIAN STEROIDOGENESIS IN VITRO

by
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INTRODUCTION

The functional mammalian corpus luteum, unlike other endocrine tissues, has only a brief life span during which it actively secretes its hormone. With each sex cycle, new corpora lutea develop only to undergo rapid regression. The duration of active steroidogenesis and secretion by the corpus luteum varies from species to species, but is rarely longer than thirty days (Asdell, 1964; Nalbandov, Cook, Kalterbach, and Keyes, 1966). During the secretory period, luteal tissue is involved with the synthesis of progesterone from metabolic precursors such as acetate and cholesterol. Progesterone in turn is the steroid responsible for maintaining a uterus receptive for nidation and for maintenance of early pregnancy. If for any reason the luteal system malfunctions, implantation and thus pregnancy cannot result.

The life cycle of a corpus luteum can be divided into four stages. First, is the stage of formation. This is the period following rupture of the follicle and filling of the follicular cavity with blood and lymph (Nalbandov, 1964). The second stage is that of maturation during which the granulosa cells (and in some cases thecal cells) become enlarged and lipid filled. Steroidogenesis is just beginning at this time. Third, is the stage of active secretion. This period is characterized by maximum size

and steroidogenic activity of the corpus luteum. The fourth, and final stage, is that of regression. At this time, steroid production is leveling off and will eventually cease. Concurrent with this is the invasion of more connective tissue, fat and hyaline-like substances among the luteal cells.

Opinions on the mechanisms which direct the activity of the corpus luteum remain controversial. This thesis attempted to relate the roles of luteinizing hormone (LH), prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and adenosine 3',5'-cyclic mononucleotide (cyclic AMP) in ovarian steroidogenesis.

The actual investigation undertaken involved a comparative study of the effects of LH, $PGF_2\alpha$, theophylline (a methyl xanthine which blocks phosphodiesterase and thus allows accumulation of cyclic AMP) and mixtures of each on luteinized rat ovarian tissue in an in vitro system. Included in the experiment was a quantitative measurement of the de novo synthesis of progesterone and 20α-hydroxy-Δ^4-pregnen-3-one by luteal tissue incubated with the above compounds. Besides measuring total steroid production, that which was synthesized from acetate-1-^{14}C was also determined.

LITERATURE REVIEW

The somewhat misleading term "prostaglandin" was coined by von Euler (1935) to describe an active principle which he found in human seminal plasma and sheep vesicular glands. The compound was both water soluble and ether soluble, lowered blood pressure of rabbits and stimulated various smooth muscles.

The name prostaglandin is now used for any of fourteen or more well defined natural substances which are extractable from several different mammalian organs, and whose biological effects range well beyond those known in 1935. It is also applied to some of the metabolites and to many semi-synthetic and totally synthetic congeners (Bergström, Carlson, and Weeks, 1968; Pickles, 1967).

Prostaglandins are all described as derivatives of prostanoic acid, a name given to a hypothetical molecule by Bergström, Ryhage, Samuelsson, and Sjövall (1963). The structure of prostanoic acid is given in FIGURE 1.

The stereochemistry of substituents on the five membered cyclopentane ring is designated alpha or beta; alpha substituents are oriented on the same side of the ring as the carboxy side-chain attached to C-8, and beta substituents are oriented in the opposite plane or on the same side as the alkyl (C-13 to C-20) side-chain.

There are four main families of prostaglandins.

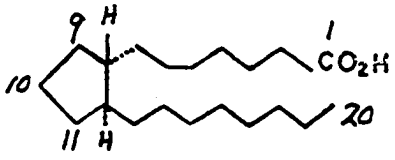
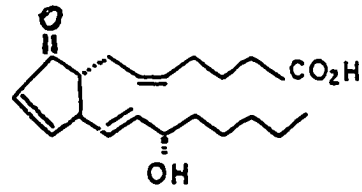


FIGURE 1. Prostanoic Acid: Hydrogen Atoms are Shown only Where Necessary to Indicate the Configuration; that on C-8 is in the Beta Position, that on C-12 in the Alpha Position.

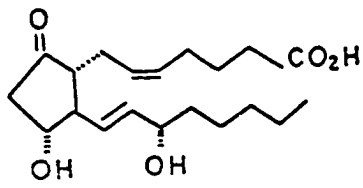
These are designated as E, F, B and A. The structural differences among each of these members is given in FIGURE 2. Besides the four main groups, there are also 19 hydroxyl derivatives which at the moment appear somewhat inactive biologically (Pickles, 1967a).

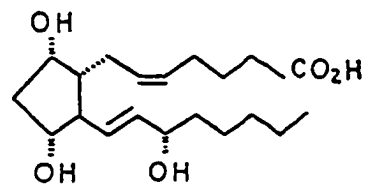
The accepted abbreviation for prostaglandins is PG. Therefore, a prostaglandin belonging to the F family can be shortened to PGF. The degree of unsaturation of the side-chains is indicated by a subscript. A subscript of 1 indicates the presence of a trans double bond at carbon thirteen; a subscript of 2 indicates a cis double bond at carbon five in addition to the above trans bond; if 3 is the subscript, a third cis double bond at carbon seventeen is indicated. FIGURE 3 shows location of these double bonds in the F family of prostaglandins. All naturally occurring PG's contain an alpha-OH group at C-15 and a trans double bond in the C-13,14 position (Pickles, 1967a).

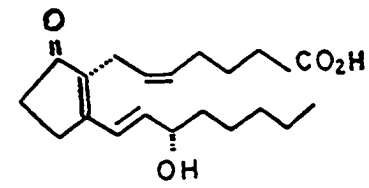
Biosynthesis of prostaglandins is effected by an enzyme system associated with the microsome fraction and a heat stable factor present in the supernatant (van Dorp, 1967; van Dorp, Beerthius, Nugteren, and Vonkeman, 1964; Samuelsson, 1967). The prostaglandins are synthesized from fatty acids such as dihomo-γ-linolenic and arachidonic acid (Bergström, Danielsson, Klenberg, and Samuelsson, 1964; Bergström, Danielsson, and Samuelsson, 1964; van Dorp, et al., 1964; Samuelsson, 1967). The three oxygen atoms



A

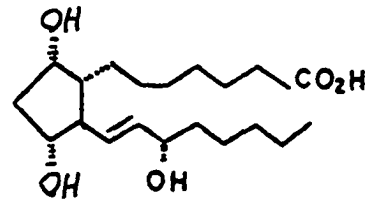




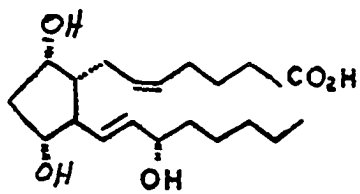


E F B

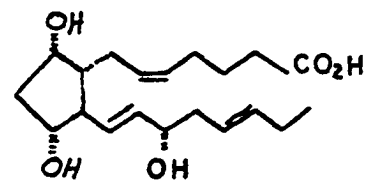
FIGURE 2. Structural Differences Among Prostaglandins E, F, B and A. The Differences are Found in the Cyclopentane Ring with the Side Chains at C-8 and C-12 the Same in all Families.



$PGF_1\alpha$



$PGF_2\alpha$



$PGF_3\alpha$

FIGURE 3. Configuration of $PGF_1\alpha$, $PGF_2\alpha$ and $PGF_3\alpha$. $PGF_1\alpha$ has the trans Double Bond at C-13, $PGF_2\alpha$ has in Addition a cis Double Bond at C-5 and $PGF_3\alpha$ has a Third trans Double Bond at C-17.

present at carbon atoms 9, 11 and 15 are derived from molecular oxygen and it is of special interest that the two oxygen atoms of C-9 and C-11 are derived from the same oxygen molecule (Pickles, 1967a). A cyclic peroxide thus appears to be the direct precursor of both the E and F prostaglandins (Speroff and Ramwell, 1970a).

In nature prostaglandins have been found in a wide variety of tissue, including decidua, uterus, semen, amniotic fluid and many others (Pickles, Hall, Best, and Smith, 1965; Bygdeman and Samuelsson, 1966; Pickles, 1967). The tissue content of prostaglandins is measured in nanograms and concentrations are easily varied by pH, temperature and oxidative conditions (Speroff and Ramwell, 1970).

Some of the experiments run with prostaglandins in vivo have tried to relate prostaglandin activities with male infertility (Bygdeman and Samuelsson, 1966; Hamberg and Samuelsson, 1966) absorption through the vagina as related to female fertility (Asplund, 1947) inhibition or stimulation of spontaneous motility of isolated non-pregnant human myometrium (Bygdeman and Eliasson, 1963; Bygdeman, 1964; Bygdeman and Hamberg, 1967) and an active role in the physiology of labor (Karim, 1968).

With regards to the ovary, very little work involving prostaglandins has been done. A prostaglandin-like material was reported in the ovaries of cows and sheep by von Euler and Hammarström (1937). Interest has now been

awakened due to the search for the uterine "luteolytic" agent of many animals (Howe, 1968; Pharriss, 1970). Hysterectomy during pseudopregnancy results in a prolonged life span of the corpus luteum in the rat, hamster and rabbit and extends the normal estrus cycle to the length of gestation in the guinea pig, sheep, pig and cow (Howe, 1968; Butcher, Barley, and Inskeep, 1969).

One of the initial works using chemically synthesized prostaglandins demonstrated a shift in progesterone production to 20α-hydroxy-Δ^4-pregnen-3-one in pseudopregnant rats following infusions of $PGF_2\alpha$ (Pharriss and Wyngarden, 1969). This shift in steroid production following 1 mg/kg infusions of $PGF_2\alpha$ indicates that the prostaglandin is exerting a luteolytic effect on the corpora lutea.

A hypothesis that the life span of the corpus luteum is influenced by ovarian venous drainage has been proposed (Pharriss and Wyngarden, 1969; Pharriss, 1970). The basis of this theory is built on the fact that many animals have a common utero-ovarian venous pathway which appears to be the only connection between the ovary and uterus and the fact that the uterus is known to cause luteolysis by some means (Butcher, et al., 1969; Pharriss, 1970).

If it were possible to find an agent in the uterus which might constrict the utero-ovarian vein, it could account for the luteolytic effect of the uterus. $PGF_2\alpha$ has been indicated as a possible candidate for the uterine

"luteolysin" (Pharriss, 1970). Two reasons for the choice are; 1) $PGF_2\alpha$ is found in the uterus, and more importantly, 2) $PGF_2\alpha$ has been shown to be a powerful venoconstrictor (DuCharme, Weeks, and Montgomery, 1968).

It has been demonstrated that incubations of pseudopregnant rat ovarian tissue with $PGF_2\alpha$ *in vitro* results in increased progesterone synthesis (Pharriss and Wyngarden, 1967). This increase in progesterone production strongly indicates a luteotrophic effect of $PGF_2\alpha$ *in vitro*.

From the above *in vivo* and *in vitro* studies one finds a paradox in the effects of $PGF_2\alpha$ on the rat ovary. A conclusion which can be drawn from this paradox is that the luteolytic effect of $PGF_2\alpha$ *in vivo* is in all likelihood not due to direct luteal toxicity of the material.

In an attempt to resolve the dilemma as to the luteal effect of $PGF_2\alpha$, the following observations were made by Pharriss and Hunter (1970): $PGF_2\alpha$ does not mimic the effect of gonadotropins on ovarian weight gain or ovulation; animals receiving PMS with 500 µg $PGF_2\alpha$ had significantly increased uterine weights over animals treated with the corresponding doses of PMS alone; in both PMS primed and unprimed animals, $PGF_2\alpha$ markedly retarded ovarian growth induced by HCG. Speroff and Ramwell (1970) found PGE_2, PGE_1, PGA_1 and $PGF_2\alpha$ to stimulate steroidogenesis in bovine luteal tissue. Prostaglandin E_2 was observed to have the greatest stimulatory effect, being half as effec-

tive as LH on a molar basis.

Bovine luteal tissue was incubated with either PGE_2 or LH and cyclic AMP production measured (Marsh, 1970). In every experiment, it was observed that PGE_2 and LH increased the amount of cyclic AMP produced. When measuring steroidogenesis, it was further found that the extent of stimulation of steroid production correlates well with the increase in cyclic AMP formed in the same tissue.

Adenosine 3',5'-cyclic monophosphate has been demonstrated to be present in many tissues (Sutherland and Rall, 1960) and considerable evidence has accumulated indicating this nucleotide as an important regulator of a number of enzymes and cellular processes (Sutherland, Rall, and Menon, 1962). Studies on the mechanism of hormone action have suggested that several hormones effect their respective target tissues by regulating the intracellular concentration of cyclic AMP (Creange and Roberts, 1965; Robison, 1968).

Cyclic AMP was implicated as a possible intermediate in the action of ACTH on the adrenal gland (Grahame-Smith, Butcher, Ney, and Sutherland, 1967). They showed that cell-free preparations of adenyl cyclase from rat adrenals and beef adrenal cortex responded to the addition of ACTH with increased cyclic AMP accumulation. They found also that cyclic AMP concentration increased temporarily in response to ACTH before any increase in the rate of steroidogenesis

could be detected.

In the bovine corpus luteum and the rabbit ovary there is evidence to suggest that 3',5'-cyclic AMP is an intermediate in the action of LH on steroidogenesis (Marsh, Butcher, Savard, and Sutherland, 1966; Dorrington and Kilpatrick, 1967). The addition of LH to slices of bovine corpus luteum causes a rapid increase in the level of cyclic AMP which precedes the increase in the rate of steroidogenesis (Marsh, et al., 1966).

The intracellular level of cyclic AMP in the ovary may be controlled in one of three possible ways (Dorrington and Baggett, 1969); 1) LH may directly affect the activity of adenyl cyclase, 2) LH may indirectly affect adenyl cyclase i.e. LH may interact with a molecule which is distinct from adenyl cyclase, but this interation may result in activation of adenyl cyclase, and 3) LH may increase the level of cyclic AMP by inhibiting phosphodiesterase.

LH has been shown to have an effect on the activity of adenyl cyclase in the rabbit ovary in vitro (Dorrington and Baggett, 1969). Further, it was observed that this effect is consistent and reproducible. Stimulation by LH on the synthesis of cyclic AMP is rapid; a significant effect is observed after two minutes of incubation, and the level of cyclic AMP remains elevated in the LH-containing homogenates throughout the incubation period.

The addition of LH and PGE_2 to incubating slices of a bovine corpus luteum causes a rapid accumulation of 3',5'-cyclic AMP which precedes the increase in progesterone synthesis (Marsh, et al., 1966; Marsh, 1970). Puromycin does not inhibit the action of LH on cyclic AMP accumulation thus eliminating the concept of obligatory protein synthesis for this stimulation.

The addition of adenosine 3',5'-cyclic monophosphate to incubating slices of bovine corpus luteum significantly stimulates steroid synthesis (Marsh and Savard, 1966). The optimal concentration of cyclic AMP for maximum stimulation of steroidogenesis in this tissue was determined as 100 µm/5ml of medium or 0.02M. In this same study specificity of the response to cyclic AMP was tested. Other nucleotides compared were 3'-AMP, 5'-AMP and ATP. The results showed that none of these nucleotides were effective in stimulating progesterone synthesis in corpora lutea which readily responded to LH and cyclic AMP.

The addition of LH to incubations maximally stimulated by cyclic AMP does not lead to a further increase in steroidogenesis (Marsh, et al., 1966). In regard to the utilization of acetate-$1^{14}C$ and cholesterol-$7^{3}H$ in progesterone synthesis, 3',5'-cyclic AMP closely resembles luteinizing hormone. Specifically, cyclic AMP increases both parameters of progesterone synthesis i.e. total steroid synthesized and that synthesized from acetate-^{14}C (Marsh and

Savard, 1966). The increase in acetate-^{14}C incorporation by LH however, is usually greater than the amount of steroid synthesized, while the extent of the increase in acetate-^{14}C incorporation by 3',5-AMP is about equal to the increase in the quantity of progesterone produced. These results are in accord with the proposal that 3',5'-AMP is mediator of the action of LH on steroidogenesis in the bovine corpus luteum. Attempts to stimulate steroidogenesis by cyclic AMP in homogenates of luteal tissue have been unsuccessful to date.

Dorrington and Kilpatrick (1967) found that 3',5'-AMP mimics the action of LH on progesterone synthesis by rabbit ovarian tissue in vitro. Stimulation of the tissue by luteinizing hormone or cyclic AMP greatly enhances the synthesis of 20α-hydroxy-Δ^4-pregnen-3-one, the progesterone content being increased to a smaller extent.

Thus, from the literature it has been shown that LH is capable of stimulating steroidogenesis in luteal tissue and in all probability this stimulation is mediated by 3', 5'-cyclic AMP. It has also been demonstrated that in some tissues prostaglandins are capable of stimulating steroid synthesis. Evidence of the role of cyclic AMP in this instance however, is very scant. A logical question arising out of the literature thus becomes: are gonadotropins and prostaglandins both involved with the same pathways in steroidogenesis, and if so, is the ultimate effect of gonadotropins mediated by prostaglandins?

METHODS AND MATERIALS

Ovaries were obtained from immature Holtzman rats (28-32 days old) in which pseudopregnancy had been induced via subcutaneous injection of 50 I.U. Pregnant Mare's Serum (Equinex, Ayrest) followed 56 hours later with 25 I.U. Human Chorionic Gonadotropin (Upjohn). The injections of both substances were given in a saline vehicle at a volume of 0.25 ml.

Animals were sacrificed by cervical dislocation on either day six or day nine following HCG injection. The ovaries were immediately excised, trimmed of oviduct and bursa and finely minced. Mincing was accomplished by use of a scalpel and the bottom of a 500 ml beaker which had been filled with ice and inverted. By this technique, the tissue was kept cold until time for incubation. The period from the removal of the ovaries until the start of the incubation was approximately two hours.

Krebs-Ringer bicarbonate buffer containing glucose (1mg/ml) and acetate-1^{14}C (New England Nuclear) 50 mC/mM (2.5 uC/vial) served as the basic incubation medium. In experiments in which the effects of luteinizing hormone (NIH-LH-S16 Ovine) prostaglandin $F_2\alpha$ (Upjohn) theophylline (Upjohn) or a mixture of these were investigated, these compounds were added directly to the incubation media. The acetate-1^{14}C was added in order to quantitate the

amount of this material incorporated into steroid. Each incubation vial contained a final volume of 4 ml.

Incubations were carried out in a Dubnoff Metabolic Shaker at 37°C and were gassed continuously with 95%O_2-5% CO_2. Incubations ranging from five minutes to four hours were used. Enzymatic activity of the tissue was stopped by the addition of 5 ml 2.5% NaOH. The vials were then stored in a freezer at -10°C to await extraction.

For extraction, tissues and media were decanted into Broeck hand homogenizers and were homogenized 50 strokes at 1000 RPM using a Vari Speed Stirrer (Scientific Products). The homogenates were poured into 125 ml separatory funnels. Homogenizers and incubation vials were then rinsed with 15 ml 2.5% NaOH and 25 ml anhydrous ether and the rinses added to the funnels. Finally, to this mixture, 100 µl progesterone-1,2^{3}H (40 C/mM) and 100 µl 20α-hydroxy-Δ^4-pregnen-3-one-1,2^{3}H (40 C/mM) both from New England Nuclear, were added for future determination of the per cent recovery of these steroids.

The total contents of the funnel were then mildly mixed and allowed to settle for ten minutes. At this time, the ether layer was pipetted off and placed in a 250 ml separatory funnel. Three more ether washes of the homogenate were made in the following manner; add 20 ml ether, shake for 30 seconds, let settle and pipet off; add 20 ml ether, shake 60 seconds, let settle and pipet off; add 20

ml ether, shake 90 seconds, let settle and pipet off. The final combined ether volume was thus 85 ml. This extract was washed twice with 10 ml water per wash. Following the second wash, the samples were left overnight to complete partition. The next day the water layer was discarded and the ether poured into 250 ml round bottom boiling flasks and evaporated to dryness over a steam bath. Flasks were then stored in a freezer at -5°C.

Thin-layer chromatography was employed to isolate the steroids of interest. The flasks were rinsed with small volumes (1 ml) of chloroform and these rinses were spotted onto a thin-layer chromatography plate (Uniplate® precoated thin-layer chromatography plates; precoated with Silica Gel GF, 250 microns thick, Analteck Inc., Wilmington, Del.). Plates were run twice in the first dimension with an ethyl acetate:cyclohexane (1:1) solvent system, and once in a second dimension in methylene chloride:diethyl ether (5:2). Between each run, the plates were allowed to air dry for five minutes.

Following final development of the chromatograms, the plates were placed under a U.V. light source for location of the progesterone and 20α-hydroxy-Δ^4-pregnen-3-one spots. These two areas were then scraped from the thin-layer plates using small spatulas and the silica was quantitatively transferred into 2 ml coarse sintered glass filters. The filters in turn were placed on 12 ml graduated centri-

fuge tubes. A total of 5 ml of absolute ethanol was added to each of the filters for elution of the steroids from the gel. The filtered solutions were mixed on a Vortex Jr. Mixer and a one tenth volume aliquot was removed. This aliquot was put into a scintillation vial, 15 ml diatol added, then counted to quantitate both the amount of ^{14}C and ^{3}H present. The ^{14}C count would represent the amount of acetate-1^{14}C incorporated and the ^{3}H count would give the per cent recovery of the steroid.

The apparatus used for counting samples was a Packard Tri-Carb Liquid Scintillation Spectrometer, model 574 (Downers Grove, Ill.). The instrument was set to count ^{14}C and ^{3}H simultaneously with a 17.1% efficiency for tritium and a 27.2% efficiency for ^{14}C.

The remaining alcoholic solution was evaporated to dryness under reduced pressure in water at 60°C on a Rinco rotary evaporator. Chloroform was used to wash down the sides of the sample tube and was subsequently evaporated under pure nitrogen.

Final quantitation of the steroids was made on a flame ionization gas chromatograph (F&M Scientific Corporation, Model 500 with Model 1609 flame ionization attachment). The chromatograph was equipped with a modified glass coil column (conversion 609/1609 Applied Science) which was six feet in length, with a 1/4 inch outside diameter and a 3.5 mm inside diameter. Packing for the

column was 1% Silicone Rubber SE-30 on Diatoport S, 80-100 mesh (Hewlett-Packard). Helium was the carrier gas and was delivered at a pressure of 50 psi and a flow rate of 145 ml/min. The temperature of the column oven was 225°C and that of the injection port and detector 260°C. Chart speed was set at 3.52 cm/min. 5α-cholestan-3β-ol hydrate (Upjohn) at a concentration of 0.2 μg/μl in chloroform was used as the internal standard against which the steroid unknowns were quantitated.

Statistical analysis involved determination of means $\pm$ standard errors. Significance was obtained by the one tailed Student t-test for the difference between two means. A difference was considered significantly greater when the P value $< .05$. P values have been listed to indicate degrees of significance.

RESULTS

Sodium acetate-1^{14}C was added to the first six experiments, but such low incorporations were observed its addition to further incubations was omitted. Likewise, further development of the results of the radioisotopic study will not be considered until the discussion section.

The first experiment involved a kinetic determination of the effects of luteinizing hormone on six day pseudopregnant rat ovarian tissue. Each vial contained 4 ml Krebs Ringer bicarbonate buffer. Incubation times investigated were zero minutes, five minutes, ten minutes and twenty minutes. The two steroids measured were progesterone and 20α-hydroxy-Δ^4-pregnen-3-one. Means $\pm$ standard errors for this experiment are listed in TABLE 1. The results indicate that within the first twenty minutes of incubation no significant increase in steroidogenesis exists between controls and LH treated ovaries.

It should be noted that there was a difference between zero minute and twenty minute values indicating that steroid synthesis was occurring.

A second kinetic study using luteinizing hormone on six day pseudopregnant rat ovaries is reported in TABLE 2. In this experiment, it should be noted that incubation times were zero minutes, fifteen minutes, thirty minutes and sixty minutes. No statistically significant increase

TABLE 1

Effects of LH on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	9.5 ± 0.95	8.1 ± 0.56
0 Min LH	9.3 ± 1.08	14.1 ± 0.42
5 Min Control	13.2 ± 0.55	9.1 ± 0.28
5 Min LH	15.9 ± 1.94	9.8 ± 1.76
10 Min Control	19.8 ± 0.16	10.2 ± 2.99
10 Min LH	19.9 ± 0.53	11.1 ± 3.25
20 Min Control	23.2 ± 1.28	11.5 ± 0.53
20 Min LH	23.4 ± 1.40	11.4 ± 0.50

* 20α-Hydroxy-Δ^4-pregnen-3-one

** In all cases, two incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 2

Effects of LH on Progesterone and 20α-Dihydroprogesterone* Synthesis *In Vitro* in Six Day Pseudopregnant Rat Ovaries

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	6.5 ± 0.54	11.5 ± 1.00
0 Min LH	6.7 ± 0.20	13.2 ± 1.43
15 Min Control	15.8 ± 1.30	13.5 ± 0.03
15 Min LH	15.8 ± 0.47	15.3 ± 2.48
30 Min Control	21.1 ± 6.38	13.6 ± 1.93
30 Min LH	17.5 ± 2.69	17.1 ± 1.73
60 Min Control	18.6 ± 1.19	17.2 ± 4.93
60 Min LH	22.4 ± 0.65 $P < .05$	17.8 ± 4.93

* 20α-Hydroxy-Δ^4-pregnen-3-one

** In all cases, two incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

was found during the first thirty minutes of incubation, however, after sixty minutes there was an increase ($P < .05$) in synthesis of progesterone due to luteinizing hormone. There was no statistical significance in any incubation period in the 20α-hydroxy-Δ^4-pregnen-3-one levels. From this study, the indication was incubation of one hour with LH stimulated ovarian progesterone synthesis in vitro.

Having demonstrated a time when LH significantly stimulated progesterone production, the next step was to see if $PGF_2\alpha$ would respond similarly. TABLE 3 lists the results of an experiment using $PGF_2\alpha$ at a concentration of 10 µg/ml. Ovarian tissue from six day pseudopregnant rats was incubated for zero minutes, fifteen minutes, thirty minutes and sixty minutes either with plain buffer or buffer plus $PGF_2\alpha$. This study failed to demonstrate any significant increase from the controls by the treated groups. Again, there is a statistical difference between zero minute values and sixty minute values indicating that the system was supporting synthesis.

Incubation times were then extended to two hours in the presence of $PGF_2\alpha$. From the results, TABLE 4, indications are that $PGF_2\alpha$ in a two hour incubation still failed to stimulate production of progesterone or 20α-hydroxy-Δ^4-pregnen-3-one.

The decision was then made to go to four hour incubations and to compare the actions of LH and $PGF_2\alpha$ when com-

TABLE 3

Effects of $PGF_2\alpha$ on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	8.8 ± 1.75	9.5 (1)
0 Min $PGF_2\alpha$	9.0 ± 0.90	10.6 ± 0.64
15 Min Control	18.9 ± 0.44	10.5 ± 0.27
15 Min $PGF_2\alpha$	17.0 ± 6.38	8.6 ± 2.09
30 Min Control	25.1 ± 0.03	11.0 ± 1.39
30 Min $PGF_2\alpha$	24.0 ± 6.38	11.7 ± 0.23
60 Min Control	30.5 ± 4.34	15.7 ± 0.88
60 Min $PGF_2\alpha$	26.4 ± 4.17	14.5 ± 1.51

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis following the mean value, two incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 4

Effects of $PGF_2\alpha$ on Progesterone and 20α-Dihydroprogesterone* Synthesis *In Vitro* in Six Day Pseudopregnant Rat Ovaries During One and Two Hour Incubations

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	7.8 ± 0.47 (2)	6.4 (1)
0 Min $PGF_2\alpha$	6.5 ± 1.26 (2)	8.4 ± 0.21 (2)
1 Hour Control	31.3 ± 2.63	18.0 ± 2.06
1 Hour $PGF_2\alpha$	31.0 ± 1.35	17.3 ± 1.61
2 Hour Control	38.0 ± 0.99	21.9 ± 1.76
2 Hour $PGF_2\alpha$	33.6 ± 5.26	19.6 ± 0.82 (2)

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis following the standard error value, three incubations were averaged for each treatment and are reported in µg steroid per gram of wet tissue weight as means ± standard errors.

bined with theophylline.

TABLE 5 lists the results of the first four hour study. In this experiment, LH, theophylline or a combination of the two were added to the incubation fluid and then placed in vials with six day pseudopregnant rat ovaries. When each of the treatments were compared to the four hour control the following was noted: addition of theophylline yielded non-significant values, LH and LH plus theophylline were significantly greater ($P < .05$ and $P < .025$ respectively). There were no differences between any combination of the other treatments. All of the 20α-hydroxy-Δ^4-pregnen-3-one values were unchanged from the four hour control.

In TABLE 6 are the results of a four hour study using $PGF_2\alpha$, theophylline or a combination of the two. The tissue in this experiment, as in the previous ones, was six day pseudopregnant rat ovarian tissue. There was no statistically significant difference between the control value and those of $PGF_2\alpha$ or theophylline. However, a significant increase in progesterone synthesis above the controls ($P < .025$) was found in the $PGF_2\alpha$ plus theophylline vials. With the 20α-hydroxy-Δ^4-pregnen-3-one values, there was significance of all three treatments from the control (all $P < .025$).

Two more studies were performed in order to see if the results represented in TABLES 5 and 6 could be repeated. TABLE 7 lists the results when LH was used as the

TABLE 5

Effects of LH and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis *In Vitro* in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Progesterone

Treatment**	Total Steroid	De Novo Synthesis	
0 Min Control	11.5 ± 0.09 (3)		
0 Min LH + Theo	8.3 ± 0.71 (2)		
4 Hour Control	35.6 ± 3.86 (3)	23.2 ± 3.86	
4 Hour Theo	41.5 ± 4.07 (3)	33.2 ± 4.07	
4 Hour LH	43.0 ± 3.32 (4)	34.7 ± 3.32	$P < .05$
4 Hour LH + Theo	48.4 ± 4.17 (3)	40.1 ± 4.17	$P < .025$

* 20α-Hydroxy-Δ^4-pregnen-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 5 (cont.)

Effects of LH and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Treatment**	20α-Dihydroprogesterone*	
	Total Steroid	De Novo Synthesis
0 Min Control	14.3 ± 5.12 (3)	
0 Min LH + Theo	15.6 ± 1.02 (3)	
4 Hour Control	28.6 ± 2.98 (3)	14.3 ± 2.98
4 Hour Theo	26.7 ± 5.86 (3)	11.2 ± 5.86
4 Hour LH	36.4 ± 5.72 (4)	20.8 ± 5.72
4 Hour LH + Theo	29.7 ± 1.91 (4)	14.1 ± 1.91

* 20α-Hydroxy-Δ^4-pregnen-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 6

Effects of $PGF_2\alpha$ and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis *In Vitro* in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Progesterone

Treatment**	Total Steroid	De Novo Synthesis
0 Min Control	8.0 (1)	
0 Min $PGF_2\alpha$ + Theo	9.1 ± 0.45 (2)	
4 Hour Control	27.1 ± 0.79 (3)	19.1 ± 0.79
4 Hour Theo	30.8 ± 2.19 (3)	21.7 ± 2.19
4 Hour $PGF_2\alpha$	29.8 ± 2.67 (4)	20.7 ± 2.67
4 Hour $PGF_2\alpha$ + Theo	39.4 ± 3.88 (4)	30.3 ± 3.88 $P < .025$

* 20α-Hydroxy-Δ^4-pregnen-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 6 (cont.)

Effects of $PGF_2\alpha$ and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Treatment**	20α-Dihydroprogesterone* Steroid Present	De Novo Synthesis
0 Min Control	15.2 (1)	
0 Min $PGF_2\alpha$ + Theo	9.5 ± 2.08 (2)	
4 Hour Control	23.1 ± 1.55 (3)	11.9 ± 1.55
4 Hour Theo	31.2 ± 3.21 (2)	21.7 ± 3.21 $P < .025$
4 Hour $PGF_2\alpha$	32.3 ± 1.73 (4)	22.8 ± 1.73 $P < .025$
4 Hour $PGF_2\alpha$ + Theo	28.0 ± 1.64 (3)	18.6 ± 1.64 $P < .025$

* 20α-Hydroxy-Δ^4-pregnen-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 7

Effects of LH and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis *In Vitro* in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Progesterone

Treatment**	Total Steroid	De Novo Synthesis			
			C	T	LH***
0 Min Control	7.8 ± 1.27 (2)				
0 Min LH + Theo	LOST				
4 Hour Control	15.2 ± 0.69	7.4 ± 0.69			
4 Hour Theo	17.3 ± 0.82	9.6 ± 0.82	$P < .05$		
4 Hour LH	30.0 ± 1.23	22.2 ± 1.23	$P < .001$	$P < .001$	
4 Hour LH + Theo	41.9 ± 3.91	34.1 ± 3.91	$P < .001$	$P < .001$	$P < .025$

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis, four incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

*** C = Compared to Control - T = Compared to Theo - LH = Compared to LH

TABLE 7 (cont.)

Effects of LH and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Treatment**	Total Steroid	20α-Dihydroprogesterone* De Novo Synthesis
0 Min Control	11.1 ± 0.99 (2)	
0 Min LH + Theo	11.3 ± 0.46 (2)	
4 Hour Control	27.1 ± 0.95 (3)	16.1 ± 0.95
4 Hour Theo	16.5 ± 1.97	5.2 ± 1.97
4 Hour LH	17.7 ± 1.19	6.9 ± 1.19
4 Hour LH + Theo	16.7 ± 3.63 (2)	5.4 ± 3.63

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis, four incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

stimulating gonadotropin. The ovarian tissue utilized was from six day pseudopregnant rats. These results show that progesterone synthesis with theophylline ($P < .05$) LH ($P < .001$) and LH plus theophylline ($P < .001$) was greater than the control. Also shown in this study was that progesterone synthesis with LH ($P < .001$) and LH plus theophylline ($P < .001$) was greater than that with theophylline alone and LH plus theophylline ($P < .025$) was greater than that of LH alone. Thus, a potentiation of steroidogenesis was observed with LH plus theophylline over LH alone or theophylline alone indicating that LH is stimulating the adenyl cyclase system.

Results reported in TABLE 8 were from a study using six day pseudopregnant rat ovaries and $PGF_2\alpha$. The effects of both theophylline alone and $PGF_2\alpha$ alone were non-significant when compared to the control values. With the combination of the two however, significant increases were observed ($P < .025$). Progesterone synthesis with $PGF_2\alpha$ ($P < .025$) and $PGF_2\alpha$ plus theophylline ($P < .001$) was greater than that with theophylline alone. The difference between $PGF_2\alpha$ and $PGF_2\alpha$ plus theophylline was found not to be statistically significant.

In order to test the effect of different pseudopregnant lengths, two studies were undertaken utilizing nine day pseudopregnant ovaries. TABLE 9 contains the results using the gonadotropin LH, theophylline or a combination

TABLE 8

Effects of $PGF_2\alpha$ and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Progesterone

Treatment**	Steroid Present	De Novo Synthesis		
		C	T	PG***
0 Min Control	4.9 ± 0.72 (3)			
0 Min $PGF_2\alpha$ + Theo	6.1 ± 0.71 (3)			
4 Hour Control	31.0 ± 2.94	26.1 ± 2.94		
4 Hour Theo	31.7 ± 2.15	25.6 ± 2.15		
4 Hour $PGF_2\alpha$	37.9 ± 2.19	31.8 ± 2.19	P < .05	
4 Hour $PGF_2\alpha$ + Theo	42.8 ± 1.97	36.7 ± 1.97	P < .025	P < .005

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis, four incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

*** C = Compared to Control - T = Compared to Theo - PG = Compared to $PGF_2\alpha$

TABLE 8 (cont.)

Effects of $PGF_2\alpha$ and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Six Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

20α-Dihydroprogesterone*

Treatment**	Steroid Present	De Novo Synthesis
0 Min Control	13.2 ± 1.60 (3)	
0 Min $PGF_2\alpha$ + Theo	12.9 ± 0.31 (3)	
4 Hour Control	27.0 ± 3.53 (3)	13.8 ± 3.53
4 Hour Theo	23.0 ± 2.30	10.1 ± 2.30
4 Hour $PGF_2\alpha$	22.4 ± 3.27	9.5 ± 3.27
4 Hour $PGF_2\alpha$ + Theo	19.8 ± 3.63	6.9 ± 3.63

* 20α-Hydroxy-Δ^4-pregnen-3-one

** Unless indicated by a number in parenthesis, four incubations were averaged for each treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

TABLE 9

Effects of LH and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Nine Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	3.7 ± 3.18 (2)	33.7 ± 5.12 (3)
0 Min LH + Theo	3.9 ± 0.82 (2)	44.2 ± 2.70 (2)
4 Hour Control	29.3 ± 1.01 (4)	38.7 ± 5.56 (4)
4 Hour Theo	26.8 ± 0.56 (3)	28.4 ± 1.04 (3)
4 Hour LH	28.8 ± 2.96 (2)	38.2 ± 1.12 (3)
4 Hour LH + Theo	28.4 ± 2.18 (4)	31.0 ± 2.55 (4)

* 20α-Hydroxy-Δ^4-pregnen-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

of the two. In all cases, there were no significant differences between treated and control or between different treatments.

TABLE 10 has the results from the study in which $PGF_2\alpha$ and theophylline were used on nine day old luteal tissue. As with the LH experiment, there were no differences between any of the values.

TABLE 10

Effects of $PGF_2\alpha$ and Theophylline (Theo) on Progesterone and 20α-Dihydroprogesterone* Synthesis In Vitro in Nine Day Pseudopregnant Rat Ovaries During a Four Hour Incubation

Treatment**	Progesterone	20α-Dihydroprogesterone
0 Min Control	7.0 ± 0.35 (2)	14.8 ± 1.82 (2)
0 Min $PGF_2\alpha$ + Theo	6.9 ± 0.12 (3)	15.9 ± 2.66 (3)
4 Hour Control	19.0 ± 3.39 (3)	21.7 ± 1.27 (3)
4 Hour Theo	17.5 ± 1.83 (3)	23.0 ± 2.61 (3)
4 Hour $PGF_2\alpha$	24.3 ± 1.34 (3)	22.2 ± 3.04 (3)
4 Hour $PGF_2\alpha$ + Theo	18.7 ± 1.90 (3)	21.2 ± 3.76 (3)

* 20α-Hydroxy-Δ^4-pregnene-3-one

** The numbers in parentheses indicate the number of incubations averaged for that treatment and are reported in μg steroid per gram of wet tissue weight as means ± standard errors.

DISCUSSION

Pharriss, et al. 1968, demonstrated that prostaglandin $F_2\alpha$ would stimulate steroidogenesis in vitro in pseudopregnant rat ovarian tissue. The animals employed in their experiment were Sprague-Dawley (Upjohn) females in which pseudopregnancy was induced via vaginal stimulation. The study reported in this paper utilized immature Holtzman rats which were superovulated by PMS followed by HCG. It should thus be noted that in both cases natural physiological conditions did not exist. Natural here being considered as mated females. Still, it was felt that ovaries would respond through similar biochemical pathways when exposed to gonadotropins or prostaglandins.

Originally two concepts were to be tested in this study. The first was to construct kinetic curves for the effects of luteinizing hormone and prostaglandin $F_2\alpha$ on ovarian steroidogenesis. This would involve a dynamic study in which various incubation times would be employed to give information on the time pattern of steroid synthesis. The second and most important concept was to demonstrate that both LH and $PGF_2\alpha$ were working through a common mediator: cyclic adenosine 3',5'-monophosphate.

There was no direct measurement made of cyclic AMP levels. Instead, an indirect method was employed to indicate stimulation of adenyl cyclase and thus cyclic AMP

production. This indirect method involved incubating tissue with LH or $PGF_2\alpha$ in the presence of theophylline, and measuring resulting steroid production.

Two known ways of regulating levels of cyclic AMP which lend themselves to measurement are through its synthesis from ATP via adenyl cyclase and through its catabolism to 5'-AMP via phosphodiesterase. The methyl xanthines, such as caffeine and theophylline, are known inhibitors of the 3',5'-adenocyclo-phosphodiesterase enzyme responsible for the 3'-desterification of cyclic AMP to 5'-AMP. Assuming cyclic AMP as an intracellular mediator of hormone action, if one observed an increase in steroidogenesis with hormone plus theophylline over hormone alone or theophylline alone, it would not be illogical to conclude that the hormone was acting through the adenyl cyclase system to stimulate cyclic AMP production. Measurement of increased steroidogenesis with hormone plus theophylline, therefore, was employed as our indirect indicator that cyclic AMP production was occurring.

Two parameters of steroid production were investigated. The first of these was measurement of the amount of progesterone and 20α-hydroxy-Δ^4-pregnen-3-one produced, and the second, quantitation of acetate-1-^{14}C incorporated into these steroids.

In none of the studies was there any detectable incorporation of the radioactive acetate into steroids, even

when incubation times were extended to four hours. Possible explanations for the failure of the ovaries to incorporate acetate-1^{14}C are: there might have been high enough concentrations of other precursors, such as cholesterol, so that acetate was not required; there may have been adequate intracellular stores of acetate with no mixing with radioactive material; a third possibility is that the radioactive acetate was diluted out by large intracellular cold acetate pools; finally, it is possible that 20α-hydroxy-Δ^4-pregnen-3-one was being oxidized back to progesterone. In experiments seven and eight, a decrease in 20α-hydroxy-Δ^4-pregnen-3-one with an increase in progesterone was observed. Because this was noted in only two of the experiments, no conclusions regarding this as the reason for failure of acetate-1^{14}C incorporation could be made.

From experiment one, the difference between zero minute values and ten minute values gives evidence that steroidogenesis was occurring. However, these data show that within twenty minutes there was no difference in steroid production between control incubations and LH treated ones. It should be emphasized that these ovaries were superovulated and were at a peak of steroidogenic activity, therefore, longer incubation times might be required before stimulation by LH would be observed. Another explanation is that perhaps a series of enzymatic steps on

steroidogenesis and an increase in steroid production is noted only after a longer incubation time.

A second experiment was performed in which the length of incubation was increased to one hour. Here a statistical increase ($P < .05$) was found in progesterone levels from LH treated incubations above the control incubations. In this particular system, therefore, a one hour incubation with LH is required before significant stimulation over the controls is observed.

Because of the inability to demonstrate steroidogenic differences during shorter incubation periods, it was decided to abandon the kinetic aspect of the experiment and concentrate on static comparisons of LH and $PGF_2\alpha$.

Having demonstrated a stimulatory effect by LH in one hour's time, a similar incubation using $PGF_2\alpha$ was tried. Results in TABLE 3 suggest the failure of $PGF_2\alpha$ to stimulate steroidogenesis. The reason for $PGF_2\alpha$'s lack of activity is not known. It could be that in order to stimulate steroid production, $PGF_2\alpha$ has to enter the corpora luteal cells themselves. Since $PGF_2\alpha$ is easily oxidized, much of the compound might be metabolized before entrance into the cell. Thus, a longer incubation would be required to attain a high enough intracellular titer to effect stimulation. Another possibility for which there is no evidence is that there may be specific sites on the membrane through which $PGF_2\alpha$ enters the cell at a controlled rate,

i.e. not limited by the nucleotide's concentration. This again would require longer incubations to attain adequate intracellular titers necessary for stimulation.

Incubation times were then extended to two hours. Results in TABLE 4 show that even two hours was not enough for $PGF_2\alpha$ to stimulate steroidogenesis.

It has been demonstrated with three hour incubations that PGE_1, PGE_2, $PGF_2\alpha$ and PGF_1 all increased steroidogenesis in the bovine corpus luteum (Speroff and Ramwell, 1970). Furthermore, in regard to acetate-$1^{14}C$ incorporation, it was found prostaglandins ascending order of potency to be: $PGA_1 < PGF_2\alpha < PGE_1 < PGE_2$.

Because of these findings, as well as our two hour results, four hour incubations were run. In these studies, the effects of LH and $PGF_2\alpha$ were compared. Theophylline was utilized in order to test whether both compounds might be working through the common intracellular mediator, cyclic AMP. Besides the work by (Pharriss et al., 1969) in rats, Marsh (1970) demonstrated a stimulatory effect by PGE_2 on adenyl cyclase in the bovine corpus luteum. In an earlier study it was demonstrated that cyclic AMP could indeed stimulate steroidogenesis in the bovine corpus luteum (Marsh and Savard, 1966). Furthermore, they found that the addition of LH to incubations maximally stimulated by 3',5'-AMP did not lead to further increase in steroidogenesis. It thus appears in the bovine corpus luteum that

both PGE_2 and LH are mediated via cyclic AMP.

The first four hour incubation employed luteinizing hormone as the stimulatory agent. It was not surprising to observe a significant increase in progesterone synthesis ($P < .05$) in treated incubations over controls, as this had already been shown with one hour incubations. There were no statistical differences in 20α-hydroxy-Δ^4-pregnen-3-one values. This argues against the idea of reconversion of the reduced steroid to progesterone.

Because many of the values in TABLE 5 were nonsignificant due to high standard errors, the experiment was repeated to see if these errors could be lowered. TABLE 7 lists the results of the repeated experiment. Significant increases in progesterone production by theophylline ($P < .05$), LH ($P < .001$) and LH plus theophylline ($P < .001$) over controls were found. There was a demonstration of a statistical increase ($P < .001$) and LH plus theophylline ($P < .001$) as compared to theophylline alone; and LH plus theophylline ($P < .025$) as compared to LH alone. This final result may be interpretated that LH is working through cyclic AMP via stimulating adenyl cyclase.

The first four hour experiment utilizing $PGF_2\alpha$ yielded statistical differences in progesterone production only between the controls and $PGF_2\alpha$ plus theophylline ($P < .025$). This was a curious result since theophylline alone and $PGF_2\alpha$ alone demonstrated no statistical difference. Thus,

there appeared to be a synergism between the two compounds. This was also the first experiment in which there was a difference in 20α-hydroxy-Δ^4-pregnen-3-one values. Theophylline, $PGF_2\alpha$ and $PGF_2\alpha$ plus theophylline all demonstrated increases in progesterone synthesis ($P < .025$) as compared to the control.

Because of the high standard errors of experiment six, it was repeated to see if results might become significant with lower errors. TABLE 8 lists the results of this second experiment. Again, the only treatment in which progesterone values were statistically greater from the controls was the $PGF_2\alpha$ plus theophylline ($P < .025$).

Because steroidogenic stimulation above controls in these studies were not as great as others reported in the literature (Marsh and Savard, 1966; Pharriss et al., 1968), a different approach was made. Previously, all ovarian tissue investigated was six days pseudopregnant. The choice of six days was based on the fact that rat corpora lutea are at peak functional activity sometime around day five or six of pseudopregnancy. It was felt that perhaps in our case, involving superovulation, that at six days pseudopregnancy, tissue would be maximally active and no increase could be attained through further stimulation of any sort. For this reason, a study was undertaken using nine day old pseudopregnant ovaries. By this age, the luteal tissue should be well beyond its steroidogenic peak

and declining.

The results from these studies are given in TABLES 9 and 10. They show a complete lack of significance regardless of the treatment employed or the steroid measured.

One observation with the nine day pseudopregnant ovaries utilized in the LH study was the high concentration of 20α-hydroxy-Δ^4-pregnen-3-one in the unincubated controls. One characteristic of a regressing corpus luteum is the increase in production of 20α-hydroxy-Δ^4-pregnen-3-one (Lidner and Shelesnyak, 1967).

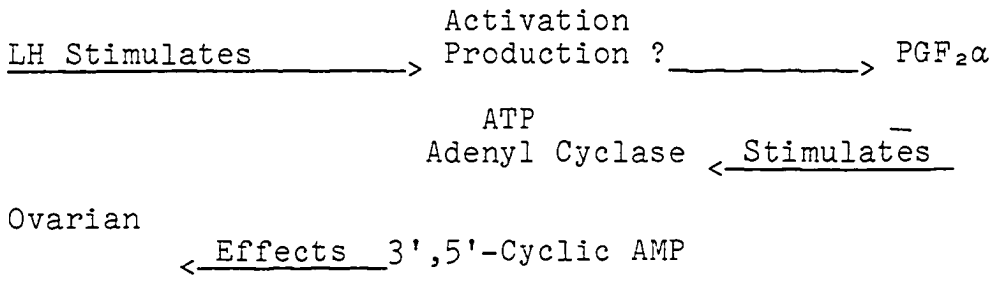
The conclusion from experiments nine and ten was that the ovaries had regressed to the point of unresponsiveness to stimulation by LH or $PGF_2\alpha$.

Several interpretations can be drawn from the total investigation. LH alone is capable of stimulating significant increases in rat ovarian steroidogenesis and can accomplish this with a one hour incubation. Although $PGF_2\alpha$ alone was shown by others to increase rat ovarian steroidogenesis (Pharriss, et al., 1968), no supporting values were obtained in this study. It should be reminded, however, that a different system was employed in the two investigations. Both LH and $PGF_2\alpha$ appear to be working through adenyl cyclase. This indication is based on their potentiation in effect when combined with theophylline. The increase in progesterone synthesis was in some cases paralleled by a decrease in 20α-hydroxy-Δ^4-pregnen-3-one

but as it was not consistent, no conclusion can be drawn implicating this reconversion as a major pathway.

Because both LH and $PGF_2\alpha$ appear to work through cyclic AMP, the possibility exists that LH exerts its effects on the ovary through $PGF_2\alpha$. This effect could be through increased synthesis of $PGF_2\alpha$ or activation of some inactive form of $PGF_2\alpha$.

One proposed biochemical pathway for rat ovarian steroidogenesis based on this study and others can be schematically represented as follows:



The only point of stimulation in the above schematic which was indicated in our study was $PGF_2\alpha$'s effect on the adenyl cyclase system. Exactly how and where each of the other stimulations take place is not postulated. It should be emphasized that other possible pathways besides the given example exist. The reasons for choice of the above schematic were that it was both logical and should lend itself to experimental evaluation.

More elegant experiments of the above need to be made. Incubations with $PGF_2\alpha$ and LH should be run in which

amounts of cyclic AMP are directly quantitated. As additional evidence that these compounds are influencing the adenyl cyclase system and not the phosphodiesterase enzyme, these experiments should be performed in the presence and absence of a methyl xanthine. Another more involved experiment, would be the development of a suitable assay system i.e. antibody assay, for $PGF_2\alpha$, and then incubate ovaries with LH and measure $PGF_2\alpha$ titers. If LH were working by increasing production of $PGF_2\alpha$, this would show up in the assay. If, however, LH affected activation of pre-existing $PGF_2\alpha$, the assay might not detect this. Another approach would be to incubate with LH and then run kinetic determinations on $PGF_2\alpha$ production, cyclic AMP production and finally steroidogenesis. If the pathway in this paper is correct, one should find a rise in $PGF_2\alpha$ followed by a rise in cyclic AMP and finally an increase in steroidogenesis.

The purpose of a three messenger system in ovarian steroidogenesis is difficult to explain. One possible theory would involve feedbacks. A three messenger system might allow for direct feedbacks (positive or negative) within the ovarian cells themselves and thus not involve the pituitary ovarian axis. Direct local control such as this would be more sensitive, localized and rapid than the gross feedback occurring through the pituitary. This form of feedback would thus allow direct control of single

cells, whereas feedback on the pituitary effects the whole ovary.

To date, all attempts to disclose the mechanisms controlling ovarian biochemical pathways have failed. With the development of better techniques, as well as the concentrated effort being put forth, these pathways should soon be determined. Once these pathways have been disclosed, the process of developing the "perfect" contraceptive will be greatly facilitated and an answer to effective population control given.

SUMMARY

Minced pseudopregnant rat ovaries were incubated for four hours with LH, LH plus theophylline, $PGF_2\alpha$ or $PGF_2\alpha$ plus theophylline and the steroids progesterone and 20α-hydroxy-Δ^4-pregnen-3-one quantitated. Results demonstrated that LH alone was capable of stimulating steroidogenesis and LH plus theophylline resulted in further stimulation of progesterone synthesis. $PGF_2\alpha$ alone failed to enhance progesterone production over controls, but $PGF_2\alpha$ in the presence of theophylline resulted in statistically greater steroid production. Results indicate that both LH and $PGF_2\alpha$ are mediated by adenyl cyclase (cyclic AMP production) in stimulating rat ovarian steroid synthesis.

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