The effect of ergocornine on the stimulation of progesterone biosynthesis in bovine corpus luteum tissue

by Charles Leslie Soliday

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY
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Abstract:
An in vitro incubation system was used to assess the effects of ergocornine (ECO) on the stimulation of progesterone synthesis in "bovine corpus luteum tissue. Luteal tissue slices were incubated in Krebs-Ringer bicarbonate buffer under various experimental conditions: 1) incu- bated controls; 2) incubations containing ECO; 3) incubations stimulated by LH, PGE2, or cAMP; 4) stimulated incubations containing ECO. Luteinizing hormone (200 ng/ml) increased ($p<0.001$) the total progesterone content of the incubated luteal tissue to 246 ±7 (ug progesterone/g luteal tissue) compared to the incubated control level of 169 ± 7 ug/g. ECO alone decreased ($p<0.01$) progesterone synthesis from an incubated control level of 169 ± 7 to 139 - 7, ug/g. "When ECO (10-4M) was included with LH in the incubation media, progesterone synthesis was reduced ($p<0.01$) from an LH-stimulated level of 246 ± 7 to 188 ± 7 ug/g. The inhibitory effect of ECO on IH stimulation of progesterone synthesis was verified by decreased acetate-1-14C incorporation into de novo progesterone. In a range of 10-7 to 5 x 10-4M ECO, the inhibitory effect of ECO on LH-stimulated progesterone synthesis was found to be concentration dependent with 10-4M ECO having maximal effect. When PGE2 (10 ug/ml) was added to incubated tissue, progesterone synthesis was significantly ($p<0.01$) stimulated to a level of 204 ± 9 ug/g compared to incubated controls which had a progesterone level of 148 ± 11 ug/g. With the addition of ECO (10-4M) to the PGE2 stimulated system the progesterone level was significantly repressed to 148 ± 11 ug/g. Cyclic - AMP also stimulated an increase in progesterone synthesis (215 ± 11 vs. 142 ± 12 ug/g for the control). However, the addition of ECO to this cAMP stimulated system does not lower progesterone (196 ± 11 ug/g) to a significantly ($p<0.01$) different level. In light of the fact that IH and PGE2 are known to stimulate adenylate cyclase in the production of cAMP it was apparent the ECO may be affecting the activity of this enzyme or cAMP-phosphodiesterase. In luteal tissue homogenates 10-4M ECO inhibited cAMP-phosphodiesterase activity slightly by 14%. However, ECO was found to have a larger inhibiting effect on overall adenylate cyclase activity.

In a tissue slice assay the accumulation of 14C - cAMP in 30 min. was stimulated to a 5143 ± 861 cpm/100g tissue level compared to a control level of 1030 ± 89. When 10-4 M ECO was included with LH the 14C - cAMP accumulation dropped to a 2335 - 466 cpm/lOOg level. In luteal tissue homogenates LH (20 ug/ml) stimulates the production of cAMP at a rate of 539 ± 4l pmoles/15 min./40 mg of homogenized tissue (e.u.) compared to a control rate of 246 ± 30 e.u. The addition of ECO to the LH stimulated system results in a significantly ($p<0.01$) decreased rate of cAMP production (276 ± 8 e.u.). Ergocornine also inhibited PGEg stimulation of adenylate cyclase from 399 ± 25 e.u. to 327 ± 21 e.u. This evidence suggests that the inhibition of LH and PGEg stimulation of progesterone synthesis by ECO is due to an inhibition of LH and PGE2 stimulation of adenylate cyclase activity by the alkaloid.

THE EFFECT OF ERGOCORNINE ON THE STIMULATION OF PROGESTERONE BIOSYNTHESIS IN BOVINE CORPUS LUTEUM TISSUE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

An in vitro incubation system was used to assess the effects of ergocornine (ECO) on the stimulation of progesterone synthesis in bovine corpus luteum tissue. Luteal tissue slices were incubated in Krebs-Ringer bicarbonate buffer under various experimental conditions: 1) incubated controls; 2) incubations containing ECO; 3) incubations stimulated by LH, PGE_2, or cAMP; 4) stimulated incubations containing ECO. Luteinizing hormone (200 ng/ml) increased ($p<0.01$) the total progesterone content of the incubated luteal tissue to 246 ± 7 (ug progesterone/g luteal tissue) compared to the incubated control level of 169 ± 7 ug/g. ECO alone decreased ($p<0.01$) progesterone synthesis from an incubated control level of 169 ± 7 to 139 ± 7 ug/g. When ECO (10^{-4}M) was included with LH in the incubation media, progesterone synthesis was reduced ($p<0.01$) from an LH-stimulated level of 246 ± 7 to 188 ± 7 ug/g. The inhibitory effect of ECO on LH stimulation of progesterone synthesis was verified by decreased acetate-1-^{14}C incorporation into de novo progesterone. In a range of 10^{-7} to 5 x 10^{-4}M ECO, the inhibitory effect of ECO on LH-stimulated progesterone synthesis was found to be concentration dependent with 10^{-4}M ECO having maximal effect. When PGE_2 (10 ug/ml) was added to incubated tissue, progesterone synthesis was significantly ($p<0.01$) stimulated to a level of 204 ± 9 ug/g compared to incubated controls which had a progesterone level of 148 ± 11 ug/g. With the addition of ECO (10^{-4}M) to the PGE_2 stimulated system the progesterone level was significantly repressed to 148 ± 11 ug/g. Cyclic - AMP also stimulated an increase in progesterone synthesis (215 ± 11 vs. 142 ± 12 ug/g for the control). However, the addition of ECO to this cAMP stimulated system does not lower progesterone (196 ± 11 ug/g) to a significantly ($p<0.01$) different level. In light of the fact that LH and PGE_2 are known to stimulate adenylate cyclase in the production of cAMP it was apparent the ECO may be affecting the activity of this enzyme or cAMP-phosphodiesterase. In luteal tissue homogenates 10^{-4}M ECO inhibited cAMP-phosphodiesterase activity slightly by 14%. However, ECO was found to have a larger inhibiting effect on overall adenylate cyclase activity. In a tissue slice assay the accumulation of ^{14}C - cAMP in 30 min. was stimulated to a 5143 ± 861 cpm/100g tissue level compared to a control level of 1030 ± 89. When 10^{-4}M ECO was included with LH the ^{14}C - cAMP accumulation dropped to a 2335 ± 466 cpm/100g level. In luteal tissue homogenates LH (20 ug/ml) stimulates the production of cAMP at a rate of 539 ± 41 pmoles/15 min./40 mg of homogenized tissue (e.u.) compared to a control rate of 246 ± 30 e.u. The addition of ECO to the LH stimulated system results in a significantly ($p<0.01$) decreased rate of cAMP production (276 ± 8 e.u.). Ergocornine also inhibited PGE_2 stimulation of adenylate cyclase from 399 ± 25 e.u. to 327 ± 21 e.u. This evidence suggests that the inhibition of LH and PGE_2 stimulation of progesterone synthesis by ECO is due to an inhibition of LH and PGE_2 stimulation of adenylate cyclase activity by the alkaloid.

INTRODUCTION

Ergot alkaloids have been reported to have a variety of physiological effects on mammalian species among them the disruption of the female reproductive system. Cattle consuming toxic levels of ergot have been observed to have poor breeding efficiency, as evidenced by poor conception rate or abortion. The mechanism of this ergot alkaloid action in cattle has not been well characterized. However, extensive experimental investigations have been conducted with rats to determine the mode of action of ergot alkaloids. Single injections of one of the ergot alkaloids, ergocornine, given to female rats at critical times during their reproductive cycle result in certain reproductive dysfunctions. The primary site of this ergot alkaloid effect is thought to be at the pituitary gland where the alkaloids have an inhibitory action on the release of hormones necessary to stimulate progesterone biosynthesis in the corpus luteum. Although the corpus luteum has remained a suspected site of a secondary ergot alkaloid action, the direct effect of ergot alkaloid on progesterone synthesis in luteal tissue has not been previously tested. It will be the purpose of this thesis to investigate a possible direct effect of an ergot alkaloid on corpus luteum tissue.

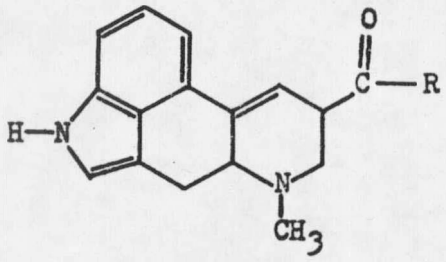
Ergot Alkaloids

The term "ergot" is a common name for species of fungi of the genus Claviceps which parasitize the grain heads of various cultivated grains

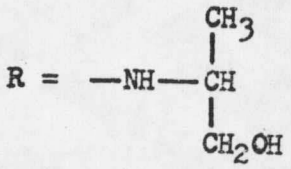
and wild grasses. Ergot occurs to some extent every year on cereal grasses such as wheat, barley, and rye and in pasture grasses. After infecting the open floret the fungus filaments ramify throughout the grass ovarial tissue eventually compacting into a dark purple, hard structure called the sclerotium which forms in place of the grain kernel. These sclerotia are composed of lipids, carbohydrates, inorganic constituents and alkaloids. Among the alkaloids are a group of amine, amino acid, and cyclopeptide derivatives of lysergic acid (Fig. 1) that have pronounced physiological effects on animals ingesting them.

Individual animals display a wide variation of reactions to the ingestion of ergot alkaloids in their feed grains and grasses. Some of the general symptoms of ergot poisoning of cattle, swine, sheep and poultry are: 1) gangrene of the extremities; 2) severe convulsions; 3) ulceration of the gastrointestinal tract; 4) poor breeding efficiency; and 5) decreased lactation (for a review see Kingsbury, 1964). Ingestion of comparatively larger amounts of ergot results in the convulsive type ergotism, in which contraction of arteriole masculature and smooth muscle of the gastrointestinal tract are important in producing the characteristic symptoms and lesions. Continuous ingestion of small amounts of ergot daily results in the gangrenous type ergotism. The stimulatory effect of ergot alkaloids on smooth muscle causes vasoconstriction resulting in a great restriction of blood supplied to the extremities. Ingestion of ergotized feed also seems to disrupt the

Figure 1. The Ergot Alkaloids

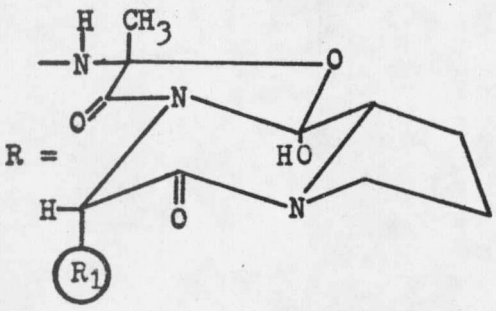


Lysergic acid nucleus

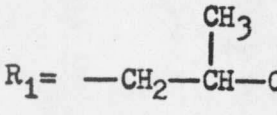


R = ergonovine

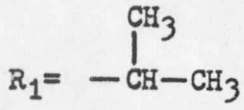
Ergotamine group



R_1= ergotamine

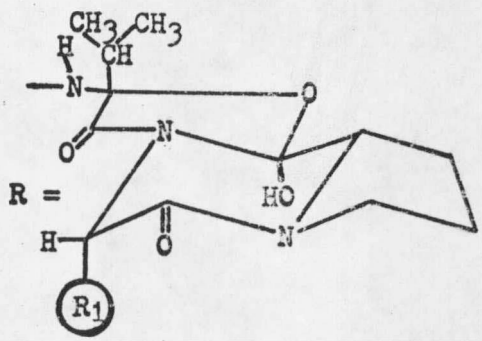


R_1= ergosine

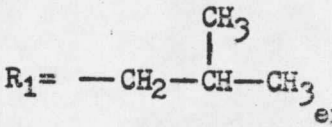


R_1= ergovaline

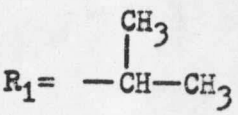
Ergotoxine group



R_1= ergocristine



R_1= ergokryptine



R_1= ergocornine

cyclic reproductive hormone levels in female animals resulting in poor conception rates (Dinnusson et al., 1971) and decreased lactation (Peace and Shaw, 1967). Although the mode of action of these alkaloids has not been fully characterized, great insights have been made in determining some of the mechanisms by which ergot alkaloids cause their effects. One area of active research has been the action of ergot alkaloids in the disruption of rat reproductive functions.

The Effect of Ergocornine on the Rat Reproductive System

An extensive investigation has been done on the mechanism of action of ergot alkaloids on the female reproductive system of the rat. A single injection of ergocornine methanesulfonate in the first six days after fertilization will prevent implantation of the blastocyst (Varavudhi et al., 1966) or cause reabsorption of an implanted fetus (Kisch and Shelesnyak, 1968). An injection of exogenous progesterone or prolactin into an ergocornine treated rat, will neutralize the effects of the alkaloid (Shelesnyak, 1958). Since it is progesterone that maintains the uterine wall for implantation and pregnancy, it would seem that a decreased level of progesterone is the end result of an ergocornine injection. In the ovary, the corpus luteum is the tissue responsible for the production of progesterone. In the rat, prolactin (Lamprecht et al., 1969) and luteinizing hormone (LH) (Loewit et al., 1969) are required to maintain the corpus luteum and stimulate

progesterone synthesis.

It has been found that ergocornine prevents any cyclic increases in serum prolactin levels and also significantly reduces the serum prolactin level. Pituitary prolactin and LH levels were found to be reduced after ergocornine treatment (Wuttke et al., 1971). Experimental results indicate that the ergocornine action is mediated through the hypothalamus by increasing prolactin inhibitory hormone and decreasing luteinizing releasing factor, and that ergocornine can directly act on pituitary cells to prevent prolactin release (Malven and Hoge, 1971; Lu et al., 1971). Therefore, the primary mechanism for the ergot alkaloids action on the hypothalamus and anterior pituitary resulting in low serum levels of prolactin and LH which are necessary for the maintenance of the corpus luteum and stimulation of progesterone synthesis from the corpus luteum to maintain the uterine wall.

Futher research with rats offered a possible explanation for the decreased progesterone activity due to ergocornine induced low prolactin levels. Progesterone is converted to a relatively inactive form, 20α-dihydroprogesterone, by the enzyme 20α-hydroxysteroid dehydrogenase in ovarian tissue (Wiest and Forbes, 1964). The activity of this enzyme fluctuates with the cyclic release of LH and prolactin from the pituitary gland (Lindner and Zmigrod, 1967). From additional experimental evidence, it appears that a dual control system exists where LH stimulates steroidogenesis toward progesterone synthesis by decreasing

20α- hydroxysteroid dehydrogenase activity (Wiest et al., 1968). Since ergocornine administration results in decreased prolactin levels, pseudopregnant rats show increased 20α-hydroxysteroid dehydrogenase activity (Lamprecht et al., 1969) and increased ovarian content of 20α-dihydroprogesterone relative to progesterone (Lindner and Shelesnyak, 1967) when injected with the alkaloid.

Throughout the investigation of decreased progesterone levels in rats due to ergocornine, a direct effect of the alkaloid on steroidogenesis in the corpus luteum remained a suspected site of action. The interpretation of the experimental results of Kraicer and Strauss (1970), who found that ergocornine administered at a critical time will block ovulation in the rat, is indicative of such an effect of the alkaloid on corpus luteum function. The timing of this ergocornine injection is not as critical as that of other ovulation blocking drugs -- atropine, dibenamine, barbiturates, and chlorpromazine which are know to inhibit neurosecretion of the ovulation inducing hormone releasing factors (Kraicer and Strauss, 1970). Since ergocornine does not exhibit the same temporal specificity, it does not operate in the same way as do these other drugs. Because it acts earlier than the other drugs, ergocornine may block some process which takes place well before the ovulatory peak of LH. The alkaloid action could be due to inhibition of the facilitatory action of progesterone in ovulation-induction. The alkaloid may directly inhibit a cyclic corpora lutea secretion of small amounts of

progesterone which have been indicated to influence the timing of the preovulatory pulse of gonadotropin secretion (Acker and Alloiteau, 1968).

A direct effect of ergocornine on the corpus luteum would be difficult to detect in the in vivo experimental designs used in the previously mentioned investigations because of the prior effect of ergocornine on the pituitary gland. By controling gonadotropic hormone levels, the pituitary controls corpus luteum function thus overshadowing the observation of any direct effect of ergocornine on the corpus luteum. The action of ergocornine in blocking increased progesterone levels may not be entirely at the pituitary level. The reproductive disfunctions caused by ergocornine may be due, at least in part, to the interference of normal steriodogenisis resulting in the loss of an optimal steroid environment essential for normal reproductive function.

To test the direct effect of ergocornine on the corpus luteum, the tissue needs to be placed in an isolated environment free of outside influences. The assessment of progesterone synthesis from an in vitro corpus luteum tissue slice incubation would provide the desired situation. Corpora lutea from cows would provide the quantity of specialized tissue needed for in vitro tissue slice incubations. Before suggesting possible experiments to test for a direct effect of ergocornine on the corpus luteum, the tissue and its function needs to be described.

Corpus Luteum Tissue Development

The bovine ovary is a heterogeneous tissue composed of several endocrinologically active structural subunits which undergo functional changes during the cow's 21 day estrous cycle. These structural subunits include the follicles, corpora lutea, and the specialized cells of the nongerminal elements. The normal endocrine function of the ovary is dependent on the cycle of follicular development, ovulation, corpus luteum formation, and corpus luteum regression which are all apparently under the control of gonadotropic substances derived predominantly from the anterior pituitary, placenta, and uterus (reviews: Savard et al., 1965; Lobel and Levy, 1968). The names of the pituitary gonadotropins are discriptive of the changes they bring about in the ovaries of experimental animals -- follicle-stimulating hormone (FSH), luteinizing hormone (LH), and luteotropic hormone (LTH).

At any given time, the ovary has a population of follicles in various stages and hormonal activity. The follicles consist of the ovum around which the two cell types, ganulosa and theca, are arranged in a concentric fashion. During its development, the theca layer becomes vascularized and the follicle becomes cystic and fills with fluid. A mature follicle ovulates upon a gonadotropin signal, and the remaining follicular tissue transforms to a corpus luteum through proliferation and vascularization of the granulosal and thecal cells. These two parenchymal cellular types of the pre-ovulatory follicle

are intermingled and the morphological distinction between them becomes blurred. However, the bulk of the luteal cells appear to be derived from the granulosa. Thecal cells can be identified along the edges of the trabeculea which contain the large blood vessels. This structural change is accompanied by a striking hormonal change from a follicle that predominately produces estrogens in microgram quantities to a corpus luteum that produces milligram quantities of progesterone.

By the nineth day post-ovulation, a full spectrum of as many as six different morphological cell types (Foley and Greenstein, 1958), are present in the corpus luteum. These luteal cells may exhibit a variety of different characteristic in histological preparations, but the histochemical preparations demonstrate the luteal cells to possess the same enzymatic profile. Therefore, the classification of luteal cells according to size and shape may be convenient for descriptive purposes, but appears to have little foundation with respect to cellular functional ability.

The first sign of cyclic corpora lutea degeneration is infiltration of lymphocytes which begin about day 14 and increase steadily to the end of the cycle. Until day 17, the enzymatic activities in the luteal cells appear strong, but there is an increase in lipid droplets. By day 18, the walls of the small blood vessels have thickened and a constriction of the vascular network has occured. Following the collapse of the vascular system within the corpus luteum tissue, the tissue shows a

decline in enzyme activity and an increase in free lipids. After the next ovulation, the luteal cells shrink rapidly until only large blood vessels are left surrounded by macrophages heavily laden with lipofuchsin.

As has been described, corpus luteum has a changing, limited functional life span, therefore the stage of development must be defined for metabolic studies. Measurements of the progesterone concentration of the peripheral venous blood of cows during the estrous cycle reveal a slow increase of progesterone to about day 9 post-ovulation where it peaks at some level before dramatically declining at about day 18 or 19 (Gomes et al., 1963). In addition to different corpus luteum tissue levels of progesterone (Gomes et al., 1963), there also appears to be marked differences in the in vitro stimulatory response of luteal tissue taken at different stages of the estrous cycle (Armstrong et al., 1964a; Armstrong and Black, 1966). The response to LH is least in tissue taken 2 to 7 days post-estrous when compared to tissue obtained 8 to 18 days post-estrous. The ability of the tissue to synthesize progesterone in vitro is maximal 4-13 days post-estrous and then gradually declines to day 19 where no detectable synthesis occurs (Armstrong et al., 1964a). Conversion of tritiated cholesterol to radioactive progesterone is greater at day 11-13. Histological studies show that from follicle rupture until day 7, the luteal cells are rapidly proliferating and increasing in size. These cells are capable of progestin synthesis during this time, but its not until after

active mitotic division that the fully formed luteal cells are capable of maximal progestin synthesis capacity and exogenous gonadotropin stimulation (Lobel and Levy, 1968).

When comparing the two parameters of LH stimulatory ability and progesterone synthesis ability, the influence of endogenous gonadotropin on the experimental tissue appears to be an important factor. Progesterone formation may be maximal from endogenous gonadotropin during the early development of the corpus luteum and not capable of further stimulation by addition of exogenous LH.

Steroid Products of the Corpus Luteum

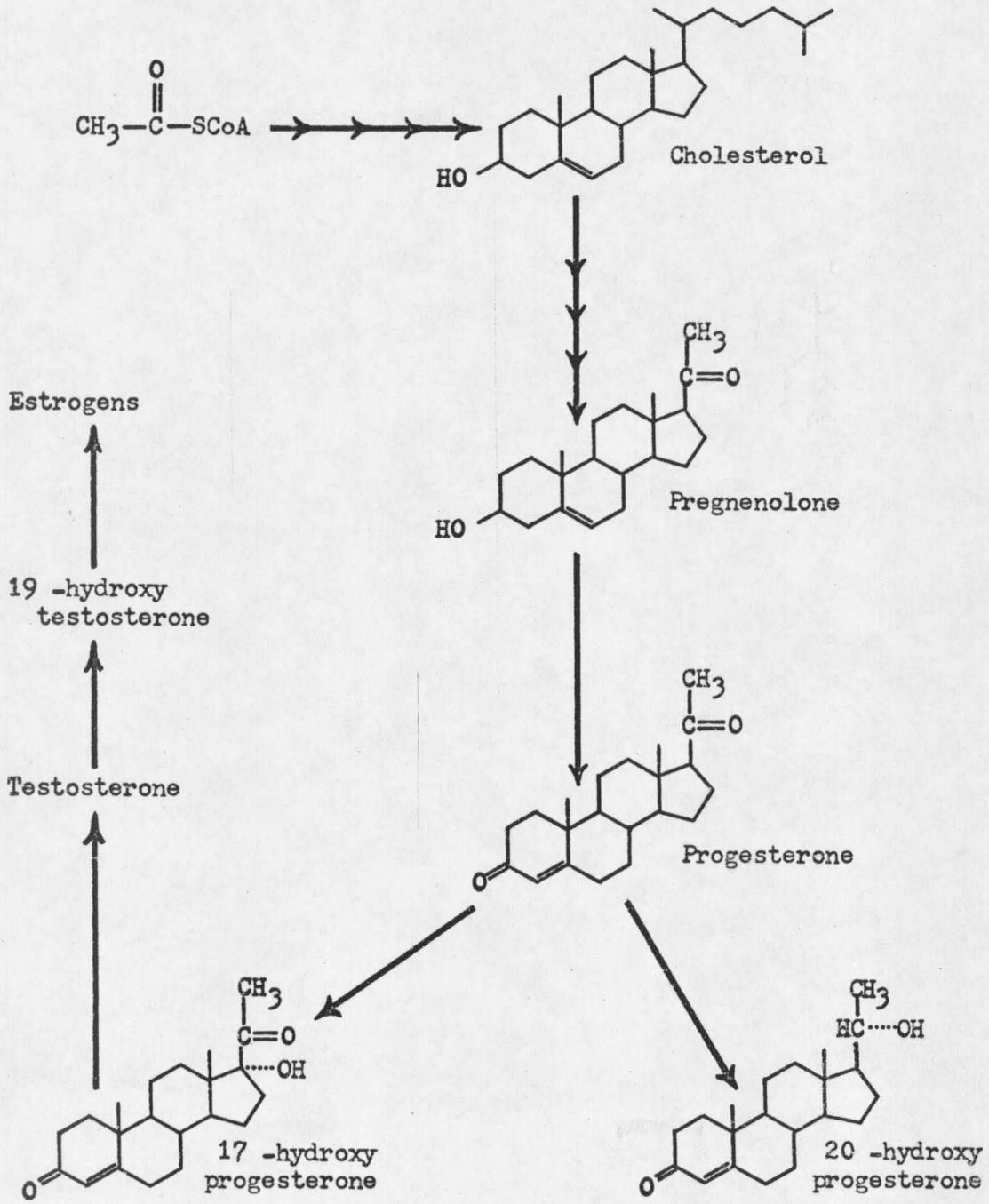
The steroid products of the individual tissue compartments of an ovary have been quantitatively measured through in vitro methods. The incorporation of radioactive steroidogenic precursors has provided a quantitive method to characterize the spectrum of steroids formed in the tissue and also indicated the relative proportion of each. The influence of gonadotropins on the quantitative formation of the steroid products has also been demonstrated. The in vitro system employed to study bovine corpora lutea function involves the incubation of tissue slices in Krebs-Ringer bicarbonate buffer at pH 7.4 for 2-3 hours as developed by Suarez, Sota and Demare (1960), and Armstrong (1964). To this system radioactive precursors and gonadotropic stimulators are

added and the resulting steroid products are extracted from the tissue, separated by chromatography, and analyzed.

According to Savard et al. (1965), the corpora lutea of various mammalian species may be divided into two groups: those which produce only progestins and those producing both progestins and estrogens. Although the bovine corpus luteum developes from a preovulatory follicle which produces estrogens before its rupture (Lobel and Levy, 1968), it falls into the group that synthesizes only progestins. When ^{14}C-1-acetate is included in bovine CL tissue incubations, only two radioactive steroids are recovered (Savard and Burdulis, 1961) both of which are progestins. These have been characterized as progesterone and 20β-hydroxy-Δ^4-pregnan-3-one (20β-dihydroprogesterone) (Fig. 2). The estrogen fractions are devoid of radioactivity. When ^{14}C-4-testosterone was used as substrate, again no radioactivity was recovered with estrogen carriers (Savard and Burdulis, 1961). Incubations with ^{14}C-4-progesterone result in only one radioactive product, 20β-dihydroprogesterone (Hayano et al., 1954, and Savard and Burdulis, 1961). There was no radioactivity in the chromatograms in the regions occupied by 17-hydroxyprogesterone and Δ^4-androstenedione.

Therefore, it appears that the bovine corpus luteum does not contain the enzymes, 17-hydroxylase and the aromatizing enzyme complex,

Figure 2. The steroidogenic pathway



to continue steroidogenesis from progesterone to androgen and estrogens as does the other ovarian tissues.

Effect of Gonadotropins on Corpus Luteum Tissue in vitro

The tissue slice incubation system has been utilized to test for stimulatory activity of substances on progesterone synthesis. The gonadotropin or other substance of suspected gonadotropic activity is added to tissue incubations and the tissue level of progesterone after incubation is compared to control incubation tissue levels.

Gonadotropins were first reported to cause a significant increase in progesterone synthesis in tissue slices by Mason et al. (1961). In a detailed study, Mason et al. (1962) went on to demonstrate that human chorionic gonadotropin (HCG), horse and bovine pituitary gonadotropin extract, and a highly purified sheep pituitary luteinizing hormone significantly enhanced progesterone synthesis. The stimulatory effect of these gonadotropins appears to be specific rather than due to any non-specific protein. Tissue that was responsive to LH was shown to be inactive to bovine serum albumin and adrenocorticotropic hormone (ACTH). Steroidogenesis in the adrenal gland is stimulated by ACTH.

Mason et al. (1962) continued to demonstrate the specificity of the gonadotropin protein by testing the activity of denatured LH. Sheep LH was exposed to hydrogen peroxide at room temperature and when added to the in vitro tissue incubation failed to stimulate progesterone

synthesis over control values. Although prolactin is an accepted tropic hormone for the corpus luteum of the rat (Astwood, 1964), it has no activity *in vitro* on progesterone synthesis in the bovine corpus luteum. Follicle-stimulating hormone (FSH) and growth hormone (GH), initially demonstrated progesterone synthesis stimulation, however, subsequent investigation by Mason *et al.* (1964a) concluded, as suspected, that these hormone preparations were contaminated by trace amounts of LH. Armstrong and co-workers (1964a) confirmed that highly purified bovine LH is active in stimulating progesterone synthesis *in vitro* in luteal tissue.

The incubating bovine slice preparation is extremely sensitive to small quantities of LH. The minimum effective dose of LH is between 0.01 and 0.02 ug per gram of tissue or 0.002 and 0.004 ug per milliliter of incubation media as reported by Mason and Savard (1964a). These low dose levels of LH causing stimulation in the *in vitro* system are considered to be in the physiological range.

Cyclic AMP Stimulation of Luteal Tissue

Guided by the work of Haynes *et al.* (1960), implicating cAMP as a mediating agent of ACTH stimulation of steroidogenesis in the adrenal cortex, Marsh and Savard (1964) investigated the possibility that this cyclic nucleotide might also act as a mediator of the stimulatory action of LH on steroidogenesis in incubating slices of bovine corpora lutea.

In this early study cAMP at a concentration of 0.002 M caused a slight increase in progesterone synthesis which was much smaller than the increase brought about by LH. A concentration study using graduated doses from 0.002 M to 0.04 M cAMP demonstrated that maximal stimulation of progesterone synthesis, as measured spectrophotometrically and by ^{14}C-acetate incorporation, occured at 0.02 M cAMP (Marsh and Savard, 1966). The level of progesterone produced by maximal cAMP stimulation is comparable to that caused by saturating amounts of LH. Hall and Koritz (1965b) also found that high concentrations of cAMP are required to increase the conversion of ^{3}H-7-cholesterol into progesterone in incubating luteal slices. Marsh and Savard (1966) demonstrated the specificity of the cAMP response by showing that the structurally related nucleotides, 3'-AMP, 5'-AMP or ATP at 0.02 M, do not effect progesterone synthesis in the in vitro system.

The concentration of exogenous cAMP needed for maximal stimulation of progesterone synthesis in tissue slices far exceeds endogenous levels of cAMP (8.5 nanomoles per gram) found in the corpus luteum (Marsh et al., 1966). High concentrations of exogenous cAMP have also been needed to stimulate liver and adrenal cells (Sutherland and Rall, 1960). Since homogenates of these later tissues require much smaller concentrations of cAMP to effect stimulation, it was concluded that the high concentrations of exogenous cAMP was needed due to the inability of the nucleotide to easily penetrate the cell membrane. In homogenates of corpora

lutea, Marsh and Savard (1966) were unable to demonstrate a stimulation of progesterone synthesis with either high or low concentrations of cAMP.

The Effect of Prostaglandins on Progesterone Synthesis

In a number of species it was found that the prostaglandin, $PGF_{2\alpha}$, when administered subcutaneously, resulted in a decrease of progestin blood levels along with a decrease in fertility (Pharriss et al., 1968, 1969; Blatchley and Donovan, 1969). Paradoxically, when prostaglandin was added to an in vitro luteal tissue incubation, a stimulation of progesterone synthesis over control incubations was observed (Bedwani and Horton, 1968; Pharriss et al., 1968). Speroff and Ramwell (1970) characterized the effects of prostaglandins on progesterone synthesis in bovine corpora lutea slices. All of the prostaglandins they examined (decending order of potency: $PGE_2 > PGE_1 > PGF_{2\alpha} > PGA_1$) increased progesterone synthesis compared to control incubations as determined by spectrophotometric assay of total tissue progesterone and by incorporation of ^{14}C-acetate into progesterone synthesized de novo. The peak of PGE_2 stimulation occured at a dose of approximately 1.0 ug/ml.

The in vitro stimulatory activity of $PGF_{2\alpha}$ is contradictory with the anti-fertility and luteolytic in vivo effects of this prostaglandin when large doses are given to rats (Pharriss and Wyngardon, 1969), guinea pigs (Blatchley and Donovan, 1969), rabbits (Pharriss, 1970),

sheep (McCracken et al., 1970), hamsters (Gutknecht et al., 1971), and cows (Louis et al., 1973, 1974). The evidence supporting the concept that PGF_2 is the uterine luteolytic agent responsible for the onset of corpus luteum regression is very convincing. In sheep, $PGF_{2\alpha}$ is released in relatively high concentrations from the uterus at the time of luteal regression and thought to be transferred from the utero-ovarian vein to the ovarian artery by a counter-current mechanism (McCracken et al., 1972). When an intrauterine device (IUD) is placed in the uterus of sheep, the corpus luteum does not develop normally (Hawk, 1968) and the endometrial $PGF_{2\alpha}$ level increased (Wilson et al., 1972). In a report by Spilman and Duby (1972) using the IUD model system, the following observations were made; 1) $PGF_{2\alpha}$ was increased in the endometrium at the site of the IUD and the uterine vein plasma, but 2) when an inhibitor of prostaglandin synthesis (indomethacin) was added, the increases in $PGF_{2\alpha}$ were abolished, 3) corpus luteum development was inhibited by an IUD, but 4) the inhibition of corpora lutea by the IUD was blocked by indomethacin. This evidence indicates, insofar as indomethacin is truly a specific inhibitor of prostaglandin synthesis, that the uterine luteolytic factor induced by an IUD is a prostaglandin.

Although $PGF_{2\alpha}$ has been shown to be luteolytic in vivo, various investigators have reported increased synthesis of progestins in rat, mouse, rabbit, cow, rhesus monkey, and human ovarian tissues incubated with $PGF_{2\alpha}$ (Lauderdale, 1974). Additional characterization of this

stimulatory effect strongly indicates this effect to also be of physiological significance. Stimulation by prostaglandins closely parallels the properties of LH stimulation as characterized by Speroff and Ramwell (1970) in the following experiments: (1) The increased ^{14}C-progesterone formed de novo from ^{14}C-acetate in the presence of prostaglandins has about the same specific activity as the ^{14}C-progesterone formed by gonadotropin stimulation; (2) The time-response curves for PGE_2 and LH are similar; (3) There is no additive effect when saturated doses of prostaglandin are added to incubations with saturating doses of either HCG or LH; (4) Cycloheximide equally blocks the steroidogenic response to either PGE_2 or LH.

The Steroidogenic Pathway in the Bovine Corpus Luteum

Much of our understanding of the pathway of progesterone synthesis and its tropic stimulation has resulted from studies of the incorporation of radioactive precursors such as ^{14}C-acetate, ^{14}C-mevalonate, ^{14}C-squalene, and ^{14}C- or ^{3}H-cholesterol in bovine tissue (Mason et al., 1962; Savard and Casey, 1964; Mason and Savard, 1964a; Savard et al., 1965; Hellig and Savard, 1965, 1966; Hall and Koritz, 1965a,b). Incorporation of ^{14}C-acetate has provided the best tool of establishing the pathway from acetate to cholesterol and progesterone and the gonadotropin stimulation of this pathway. Mevalonate-2-^{14}C was used to label squalene, cholesterol, and progesterone in bovine corpus luteum slices

(Hellig and Savard, 1965a), but the incorporation is less efficient than would have been expected from liver and yeast studies. LH added *in vitro* increases the incorporation of ^{14}C-mevalonate into progesterone but not into cholesterol (Hellig and Savard, 1965a). Attempts to introduce exogenous ^{14}C-squalene as precursor in luteal tissue failed (Mason and Savard, 1964; Savard *et al.*, 1965), likely due to the insolubility of the hydrocarbon to the aqueous incubation media. However, the precursor roles of squalene were demonstrated using an anaerobic incubation to accumulate ^{14}C-squalene from exogenous ^{14}C-acetate and then admitting oxygen to the system to allow for the disappearance of ^{14}C-squalene to form increased radioactive sterols (Hellig and Savard, 1966). Incorporation of radioactive cholesterol into progesterone has demonstrated its obligatory role in the biosynthetic pathway (Mason and Savard, 1964; Hall and Koritz, 1965a,b). However, certain experimental difficulties must be recognized when the incorporation of radioactive cholesterol into progesterone is used as a measure of gonadotropin stimulation of steroidogenesis. Initially, there is a problem of getting cholesterol, which is insoluble in incubation media, across cell membranes into the biosythetic precursor pool. Once across the cell membrane this radioactive exogenous cholesterol is substantially diluted by the vastly greater quantity of endogenous cholesterol already contained by the tissue. To further complicate labeling by cholesterol, there appear to be various "metabolic pools" (Savard *et al.*, 1965; Armstrong, 1966)

of cholesterol in the tissue into which the exogenous cholesterol may enter. While at least one of these "metabolic pools" actively supplies precursor for progesterone synthesis the other pools have other metabolic fates. The proportions of exgenous cholesterol that enter into the various "metabolic pools" is an unknown when the results of cholesterol labeling experiments are interpreted.

The time course labeling of ^{14}C-acetate into the individual components of the nonsaponifiable fraction suggest that the biosynthetic pathway in the corpus luteum is as follows: squalene → lanosterol C_{28} and C_{29} sterols → cholesterol → progesterone (Hellig and Savard, 1966). Figure 2 is an illustration of the sequencial appearance of ^{14}C label in these compounds in the pathway. The dynamic aspects of labeling in this time-course experiment is evident from surge-like appearances of radioactivity among the intermediates in stepwise fashion. There is a rapid and linear rate of radioactive incorporation of ^{14}C-acetate into squalene for the first hour of incubation and then levels off and decreases when monitored for 4 hours. After 15 minutes of incubation, ^{14}C labeled lanosterol appears, and not until after a 30 minute lag time does labeled cholesterol appear. The precursor relationships are evident by comparing the slope of the rate of labeling of each. The precursor reaches its maximum rate of ^{14}C incorporation just before the next compound in the pathway begins to increase its rate of incorporation. When the incubations were carried out to 4 hours,

the radioactivity of lanosterol and the C_{28} and C_{29} sterol fractions declined while cholesterol labeling continues increasing (Hellig and Savard, 1966). This most likely is due to the depletion of the labeled acetate pool before the cessation of turnover of the intermediary pools.

In comparing specific activities of squalene, cholesterol, and progesterone, squalene consistantly has a greater specific activity than progesterone indicating the precursor role of squalene (Hellig and Savard, 1966). These studies show the specific activity of the total cholesterol was consistantly less than either squalene or progesterone. This evidence supports the concept of "metabolic pools" of cholesterol. Since the specific activity of progesterone can not be greater than its precursor cholesterol, the cholesterol utilized in progesterone synthesis would have to come from a "steroidogenic pool" of relatively higher specific activity than progesterone. This implies that the distribution of radioactive cholesterol is not uniformly equilibrated with the preformed cholesterol of the tissue, therefore, newly synthesized cholesterol must remain temporarily in a compartmentalized state (Armstrong, 1967; Criol-Bosch and Romanoff, 1966).

Exogenous labeled cholesterol has been extensively used in elucidating the steroidogenic pathway: cholesterol → 20-hydroxycholesterol → 20, 22-dihydroxycholesterol → Δ^5-pregnenolone and isocaproic aldehyde (Tamaoki and Pincus, 1961; Ichii et al., 1963; Hall and Koritz, 1964). The transformations of ^{14}C-26-cholesterol and ^{14}C-4-cholesterol have

resulted in the demonstration of 20-hydroxycholesterol and 20, 20-dihydroxycholesterol as key intermediates in the cleavage of the cholesterol side chain. The 20-hydroxylation step has been studied in a cell-free system by Hall and Koritz (1964) who confirmed its sub-cellular localization and its NADP requirement. They suggested it to be the rate-limiting reaction in the steroidogenic pathway.

LH Stimulation of ^{14}C-acetate Incorporation

The incorporation of radioactive precursors has been utilized to study the site of LH action on the steroidogenic pathway. Bovine corpus luteum tissue stimulated by LH *in vitro* consistantly has both an increased incorporation of ^{14}C-acetate and increased formation of microgram quantities of progesterone (Savard and Casey, 1964; Savard *et al.*, 1965; Marsh and Savard, 1966). The specific activity of LH stimulated progesterone is higher but of the same order of magnitude when compared to the specific activity of the increased progesterone of control incubations. Since cholesterol is the obligatory precursor of progesterone, the increased specific activity of LH stimulated progesterone should be a direct reflection of the specific activity of the cholesterol from which it is formed. As proposed by Savard *et al.*, (1969), this implies that LH has two sites of action: 1) stimulation of the conversion of acetate and the other precursors into cholesterol, and 2) stimulation of cholesterol transformation to progesterone.

When Mason and Savard (1964a) and Marsh et al., (1966) examined the transformation of ^{3}H-7-cholesterol, they found a significant portion of the labeled precursor was incorporated into progesterone in tissue slice incubations. LH added to the system increased the incorporation of label into progesterone, however, the increase of microgram quantities of progesterone was greater. This resulting lower specific activity of LH stimulated progesterone indicates LH favors synthesis from precursors other than labeled cholesterol. This evidence supports the Savard et al., (1969) hypothesis that LH stimulatory action of progesterone biosynthesis is not confined to the transformation of cholesterol but also involves stimulated incorporation of pre-cholesterol precursors.

Other investigators, Armstrong and Hall in particular, argue that this second action of LH on pre-cholesterol precursors is unnecessary. Based on their experimental data (Armstrong, 1966; Hall, 1966; Armstrong et al., 1964a; Major et al., 1967), they contend that the depletion of the steroidogenic cholesterol pool under LH stimulation is sufficient to promote new synthesis of cholesterol from acetate due to the removal of a negative feed back action from the mass of cholesterol. In fact, Hall and Koritz (1965a) reported results from ^{3}H-cholesterol incorporation into LH stimulated progesterone contrary to those of Mason and Savard (1964b) and Marsh et al., (1966). Using the same in vitro bovine tissue slice incubation but a slightly different method of entering exogenous ^{3}H-cholesterol to the media, the specific activities of LH stimulated

progesterone in Hall and Koritz's experiments were never less than control values.

Marsh and Savard (1966) compared ^{14}C-acetate incorporation into progesterone synthesis stimulated by cAMP to LH stimulated and control incubation of bovine corpus luteum tissue. The cyclic nucleotide stimulated an increased formation of micrograms of progesterone in the same manner as LH. However, the specific activity of the progesterone from this cAMP stimulated incorporation of ^{14}C-acetate is about the same as the control tissue specific activity unlike the higher specific activity observed with LH stimulation. These results indicate that the action of cAMP is confined to transformation of cholesterol into progesterone, whereas, the increased specific activity from LH stimulation indicates an LH site of action before cholesterol in addition to the stimulation of cholesterol transformation into microgram quantities of progesterone. Therefore, LH appears to have an additional site of action earlier than cholesterol in the biosynthetic pathway as well as parallel action with cAMP. When ^{3}H-cholesterol was used as a radioactive precursor, LH and cAMP demonstrated the same increase in specific activity (Hall and Koritz, 1965a) compared to control incubations.

In their study of prostaglandin stimulation of progesterone synthesis in bovine luteal tissue, Speroff and Ramwell (1970) also compared ^{14}C-acetate incorporation under the conditions of prostaglandin stimulation, LH stimulation, and control synthesis of progesterone.

They found the specific activity of the newly formed progesterone under LH stimulation or prostaglandin stimulation to be of the same order of magnitude, thus demonstrating a possible parallel stimulation of progesterone biosynthesis by prostaglandin and LH.

Mechanism of Gonadotropin Action on Steroidogenesis

After the first demonstration of the *in vitro* stimulation of progesterone synthesis by gonadotropin (Mason *et al.*, 1962), it seemed natural for the investigators to continue to follow the example of ACTH stimulation of corticosteroidogenesis in the adrenal cortex. The concept of tropic hormone action of ACTH as advance by Haynes (1957, 1958, 1960) was that ACTH initially acts on adenylate cyclase to increase the cAMP level which in turn activates a phosphorylase enzyme of the adrenal cortex. The activated phosphorylase acts on glycogen to produce glucose-1-PO_4 and glucose-6-PO_4. Glucose-6-PO_4 is metabolized via the pentose phosphate shunt resulting in increased amounts of the nucleotide NADPH. This nucleotide stimulates corticosteroid synthesis through its action as a cofactor for many of the steps in the steroidogenic pathway.

In the initial studies of gonadotropin stimulation by Mason *et al.*, (1962) it was found that NADP + glucose-6-PO_4 caused increase progesterone synthesis. In a subsequent report, LH was shown to stimulate phosphorylase of the bovine corpus luteum (Marsh and Savard, 1964), thus remaining consistent with the Haynes hypothesis. However, experiments

where LH was added to incubations in the presence of saturating amounts of NADPH demonstrate an additive effect by these stimulators (Savard et al., 1963). This would not have occurred if NADPH were the limiting factor.

In ^{14}C-acetate incorporation studies, LH and NADPH were found to have differing effects on the incorporation of radioactivity (Savard and Casey, 1964). The LH stimulation resulted in the formation of de novo progesterone of roughly the same specific activity as incubated controls. On the other hand, exogenous NADPH resulted in formation of progesterone of much lower specific activity. It is obvious from these results that the progesterone synthesized in the presence of NADPH could not have come from the same pool of labeled precursors as the progesterone formed in the control or in the LH stimulated slices. When ^{3}H-7-cholesterol was added as a radioactive precursor, NADPH increased the conversion of this precursor to a far greater extent than did LH (Mason and Savard, 1964a). Again the progesterone synthesized by LH stimulation had to have come from a different precursor pool than the progesterone synthesized by NADPH stimulation. It was concluded (Savard and Casey, 1964; Mason and Savard, 1964b) from these experimental results that because of the distinct qualitiative differences in the precursor pools of progesterone synthesized in the presence of the two agents, the stimulator actions of LH and NADPH on steroidogenesis are independent.

Another broad concept concerning the mechanism of hormone action on its target tissue involves the synthesis of proteins and the associated genetically linked regulatory mechanism (Karlson, 1963). As applied to the action of gonadotropin on the corpus luteum, this implies that LH stimulates steroidogenisis through a stimulation of the synthesis of certain key enzymes in the steroidogenic pathway. To test this hypothesis Savard et al., (1965) introduced an inhibitor of protein synthesis, puromycin, to the tissue slice incubation system. When puromycin was added to the incubation media along with LH, the stimulatory action of LH was completely inhibited. Another known inhibitor, actinomycin D, had the same effect of suppressing LH stimulation of progesterone synthesis as puromycin. Actinomycin D indirectly inhibits protein synthesis through its inhibiting action on RNA synthesis (Breuer and Davis, 1964). The inhibitory effect of these two antibiotics on LH stimulation imply that LH may cause increased progesterone synthesis through a stimulation of the protein synthesis of key enzymes in the steroidogenic pathway.

The inhibitory effect of the protein synthesis inhibitors is also seen with cAMP and PGE_2 stimulation of progesterone synthesis. Puromycin inhibits the increased progesterone synthesis when incubated with tissue slices in the presence of 0.02 M cAMP in the same manner it inhibited LH stimulation (Marsh et al., 1966). When cycloheximide was included in

incubations containing LH or PGE_2, the stimulation of progesterone synthesis by both agents was equally blocked (Speroff and Ramwell, 1970).

Further similarities between the steroidogenic stimulating agents are seen in the results of incorporation studies of radioactive precursors. The incorporation of ^{14}C-acetate into de novo progesterone synthesis is stimulated by PGE_2 in the same manner as LH (Speroff and Ramwell, 1970). Cyclic AMP stimulated the incorporation of both ^{14}C-acetate and ^{3}H-7-cholesterol into progesterone resulting in similar specific activities to LH stimulation (Marsh et al., 1966). This also strongly indicates that the effect of cAMP, like that of LH cannot be mediated through NADPH.

Since these three agents, LH, cAMP, and prostaglandin, all seem to effect progesterone biosynthesis in the same manner, their actions must in some way be related to each other. Their effects on steroidogenic stimulation are not additive. When LH and cAMP (Marsh and Savard, 1966) or LH and PGE_2 (Speroff and Ramwell, 1970) are added collectively to tissue incubations, the progesterone level is not any greater from combined stimulators than from each stimulator alone. The possibility of a mediatory role for cAMP in the LH stimulatory response was investigated by measuring the changes in endogenous cAMP concentration due to the addition of LH to incubating tissue (Marsh et al., 1966). It was found that LH increased endogenous cAMP by 100 fold, and this response in bovine luteal tissue was specific for LH compared to other hormones.

Marsh and co-workers (1966) also found that endogenous cAMP reached maximum concentration 7.5 minutes after addition of LH, whereas, an increase in progesterone synthesis was not observed until 15-30 minutes into the stimulated incubation. This time-relationship is indicative of a cAMP mediator role in LH stimulatory action.

Further investigation by Marsh (1970a) into the relationship between cAMP and LH found that LH stimulates the enzyme, adenylate cyclase, which produces cAMP from ATP. It was found that LH had no effect on phosphodiesterase which could have accounted for increased endogenous cAMP through an inhibition of its activity.

This mediator role of cAMP is common for many other hormones in other tissues (reviews, Robison, 1971, 1972) and has been termed "the second messenger concept" (Fig. 3). The hormones released from their cells of origin are the first messengers which interact with the specific receptors of their target tissue. This interaction results in adenylate cyclase activation, thereby, increasing the intracellular level of cAMP. The cAMP is the second messenger which interacts with one or more systems within a cell to produce the effect associated with the particular hormone.

The possibility that prostaglandin stimulation of progesterone synthesis may also be mediated by cAMP, was investigated by Marsh (1970a, 1971). The prostaglandins stimulate adenylate cyclase following the same general order of activity observed in their stimulatory effect on steroidogenesis. PGE_2 and PGE_1 resulted in more than a 100% stimulation over

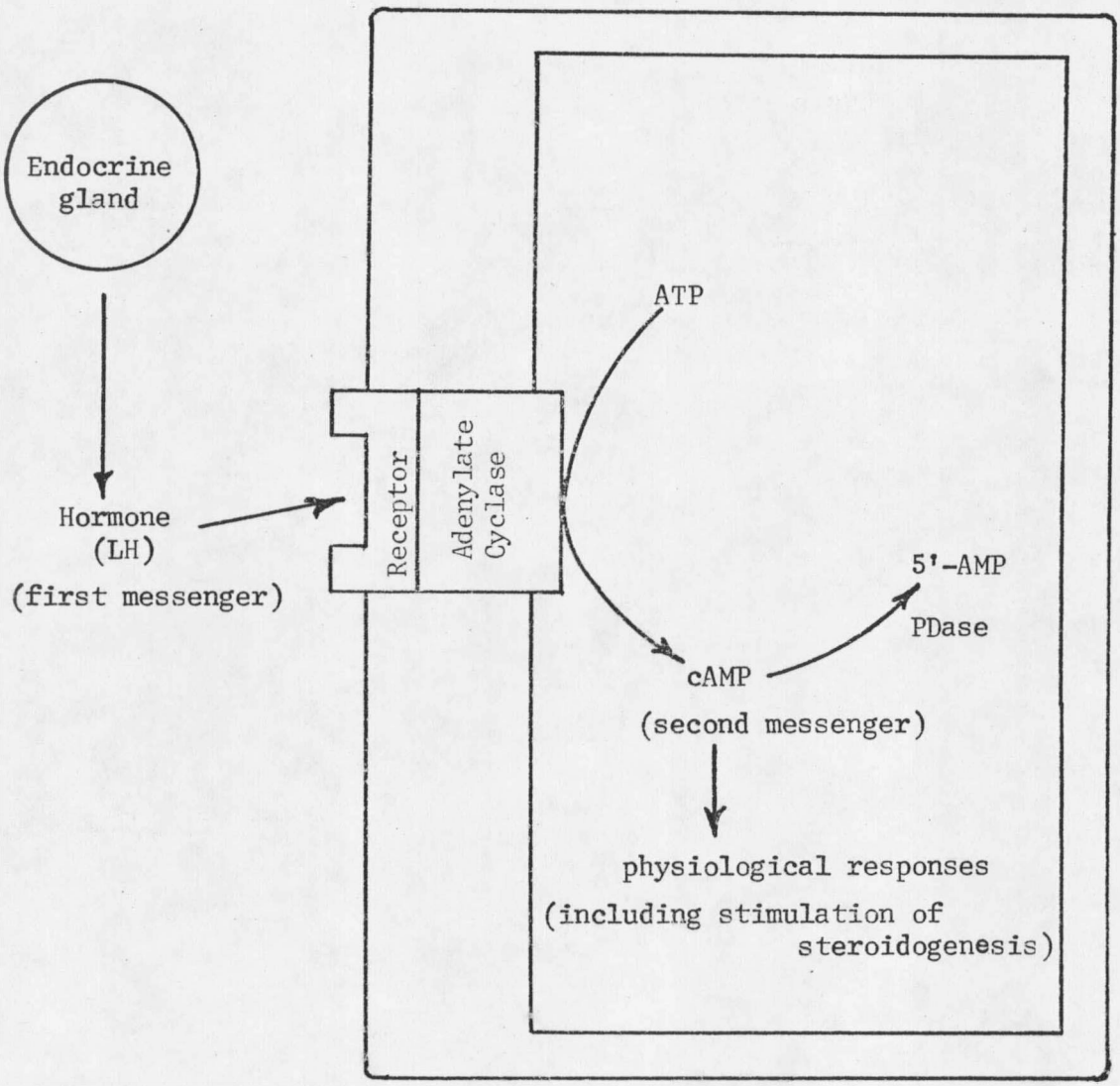


Figure 3. The second messenger concept.

control activity, whereas, $PGF_{2\alpha}$ and PGA_2 resulted in about a 50% stimulation. The functional significance of this effect of prostaglandins on luteal tissue is uncertain. Marsh (1971) demonstrated that LH and PGE_2 added together doubled the stimulation of adenylate cyclase compared to stimulation by either one of the agents added singly. This would indicate that the two effects are separate phenomena and that PGE_2 is not a mediator of the effect of LH on adenylate cyclase. There are individual receptor sites for gonadotropins and prostaglandin in bovine corpus luteum cell membranes (Rao, 1973) also indicative of two separate phenomena. However, Kuehl et al., (1970) concluded that the LH effect on adenylate cyclase is mediated by PGE_2 in their studies with mouse ovaries. They found that a prostaglandin antagonist, 7-oxa-13-prostynoic acid, not only blocks PGE_2 stimulation of adenylate cyclase, but also blocks LH stimulation of adenylate cyclase. Although this observation indicates a mediatory role of prostaglandin on LH stimulation, they observed additive effects of LH and prostaglandin on ^{14}C-cAMP accumulation.

The Effect of Ergot Alkaloids on Adenylate Cyclase

The stimulatory action of LH and PGE_2 on progesterone synthesis, has been shown to be mediated by their stimulatory effect on adenylate cyclase. Ergot alkaloids have a history of blocking stimulatory responses of adenylate cyclase by other agents in various tissues.

The early studies on epinephrine, Dale in 1906 reported that ergotoxine and ergotamine blocked the pressor effects of epinephrine on sympathetic nerve stimulation without blocking the vasodilator effect of the hormone. This began the work which separated catecholamine responses into the alpha or beta adrenergic receptor classifications. Dihydro-derivatives of ergot alkaloids were found to be even more potent alpha adrenergic blocking agents than their parent compounds (Rothlin, 1947).

Dihydroergotamine at high concentration has been found to have numerous physiological responses in addition to its action as an alpha adrenergic antagonist. Most of these additional responses seem to involve cAMP as a key intermediate which is a characteristic of a beta adrenergic effect (Sutherland, 1968). In rats, dihydroergotamine blocks epinephrine and cAMP induced hyperglycemia (Northrop and Parks, 1964). The blockage of the hyperglycemic effect of cAMP is interpreted as an indication that the site of action of the alkaloid is beyond adrenergic receptors and that dihydroergotamine may block the activation of phosphorylase by cAMP or increase the rate of inactivation of the cyclic nuceotide. In the dog, it was demonstrated that 0.1 mM dihydroergotamine blocked catecholamine activation of adenylate cyclase of the heart and liver (Murad et al., 1962). Ergotamine inhibits the adrenalin-potentiated production of cAMP in rat liver homogenates (Berthet et al., 1957). Glucagon, adrenalin, and noradrenalin increase cytoplasmic phosphopyruvate carboxylase activity

through the induction of cAMP which is inhibited by the ergot alkaloid (Yeung and Oliver, 1968). Dihydroergotamine has shown variable effects upon lipolysis. Scriabine and co-workers (1968) reported that dihydroergotamine prevents catecholamine stimulation of lipolysis in rat adipose tissue fragments (Booker and Calvert, 1967) and isolated fat cells (Fain, 1970). In an investigation using cells isolated from both brown and white rat adipose tissue, Ward and Fain (1971) found that dihydroergotamine influences the intracellular level of cAMP by its effect on two different enzymes. The ergot alkaloid inhibits phosphodiesterase resulting in increased levels of cAMP and it also inhibits the activation by catecholamines of adenylate cyclase resulting in decreased levels of cAMP.

STATEMENT OF THE PROBLEM

The action of LH on the corpus luteum has not been sufficiently characterized to the point where we fully understand the interaction of the hormone with its receptor and the following mediatory steps toward increased steroidogenesis. One approach to the elucidation of the mechanism behind LH stimulation of progesterone synthesis has been to break the system down into its component parts and attempt to fully characterize each part. Corpora lutea slices and homogenates have provided isolated model systems where the concentrations of endogenous controlling chemical components may be measured or changed by exogenous additions. By the addition of one chemical component and the measure of the response to this addition by quantitating the effect on other components, the inter-relationships between the various components of the system may be determined. There are indications that ergot alkaloids could directly influence the interactions between some of the important mediators in the LH stimulation of progesterone synthesis.

One of the chemical mediators in steroidogenesis in incubated corpora lutea slices and homogenates has been found to be cAMP. Investigators have shown many times that ergot alkaloids block hormonal stimulation of cAMP in several different types of tissue. One example is the ergotamine inhibition of the adrenalin-potentiated production of cAMP which would normally induce an increase in cytoplasmic phosphopyruvate carboxylase activity (Yeung and Oliver, 1968). It could be postulated that ergot

alkaloids may inhibit LH-potentiated production of cAMP in a similar fashion in the corpus luteum.

Although a primary effect of ergot alkaloids on animal reproductive dysfunctions appears to result from direct action on the pituitary gland, there is also some indication of a direct effect of the alkaloid on the corpus luteum. The purpose of this research has been to determine if the physiologically active ergot alkaloid, ergocornine, directly effects LH stimulation of progesterone biosynthesis in the corpus luteum. The incubated luteal slice system employed by previous investigators provides the necessary isolated conditions required to investigate any direct effects ergocornine may have on stimulation of steroidogenesis. The cow provides the quantity of luteal tissue needed for the repetitions of different experimental conditions required to define the system and demonstrate any effect of ergocornine on that system.

The first step in the investigation would be to establish a luteal tissue incubation system capable of showing increased progesterone synthesis with the addition of LH to the incubation media. Stimulation of progesterone synthesis may be measured by determining spectrophotometrically the quantity of progesterone extracted from the LH treated tissue and media and comparing it to the quantity of progesterone extracted from a control incubation. Progesterone synthesis may also be measured by the incorporation of ^{3}H-acetate into de novo progesterone. Once LH stimulation is

established, ergocornine may be added to the incubation to see if it has an effect on progesterone synthesis.

Steroidogenesis in luteal tissue has been shown to be stimulated by prostaglandins and cAMP. The effect of ergocornine on stimulation by these two agents should be tested. Since PGE_2 stimulates progesterone synthesis in the same manner as does LH, a similar effect of ergocornine on stimulation by both agents might be expected. However, it should be kept in mind that separate adenylate cyclase receptor sites for the gonadotropin and the prostaglandin have been demonstrated. Exogenous cAMP stimulates steroidogenesis mimicking the rise of endogenous cAMP due to LH or PGE_2 stimulation of adenylate cyclase. By determining the effect of ergocornine on exogenous cAMP stimulation of progesterone synthesis, the site of alkaloid action should be narrowed. If ergocornine effects the stimulatory action of cAMP in the same way it effects LH stimulation, the results would indicate the alkaloid action to be beyond LH stimulation of adenylate cyclase. However, if ergocornine does not effect exogenous cAMP stimulation in the same manner as its effect on LH stimulation, the results would indicate a possible effect of the alkaloid on the adenylate cyclase system.

To test a possible effect of ergocornine on adenylate cyclase, the alkaloid my be added to adenylate cyclase assay systems stimulated by LH. Adenylate cyclase activity may be measured in tissue homogenates, and the accumulation of cAMP from adenylate cyclase may be measured in tissue slices. Since ergot alkaloids have been implicated in an inhibitory

action on adenylate cyclase stimulation in other tissues, this would seem to be a possible site of ergocornine action in corpus luteum tissue. The ergot alkaloids have also been shown to effect the activity of cAMP-phosphodiesterase. A possible ergocornine action on phosphodiesterase activity should also be determined.

The results from the experiments outlined above should demonstrate the suspected direct effect of ergocornine on the stimulation of progesterone biosynthesis. The initial experiment will show if such an effect exists, and the following experiments will be used to characterize this possible direct effect of ergocornine on the stimulation of steroidogenesis.

EXPERIMENTAL MATERIALS AND METHODS

Corpus Luteum Tissue

Tissue for the *in vitro* incubation studies was prepared from bovine corpora lutea. The corpora lutea were extracted from cows through an incision in the anterior wall of the vagina dorsolateral to the cervix. Since the corpora lutea are at maximal stimulatory capacity between days 10-13 of the cow's estrus cycle (Armstrong and Black, 1966; Inskeep, *et al.*, 1967), careful records of the estrus cycles of the donating cows were kept, and corpora lutea were taken only during this three day period. After taking a corpus luteum, the cow was allowed at least one full, undisturbed cycle before another corpus luteum was taken. Each cow could provide up to four corpora lutea before accumulated scar tissue prevented the extraction of additional corpora lutea.

Immediately after extraction, the corpus luteum was placed in 0.9% NaCl solution (saline) on ice and rushed to the cold room ($4^{\circ}C$) for further preparation. Once in the cold room the extraneous connective tissue was peeled from the corpus luteum before cutting it into quarters. Each quarter was sliced carefully with a razor blade and the slices randomly placed in a petri dish containing cold saline over a circle of filter paper. Each slice was again cut into at least three pieces and randomly placed in a second petri dish of cold saline. Through this randomizing process it was hoped that relatively homogenious samples could be taken from the total tissue. Individual tissue samples were

blotted on filter paper before weighing them to 175 ± 5 mg samples for incubation.

Tissue Incubation

The techniques of in vitro corpus luteum tissue incubation as described by Suarez, Soto and Demare (1960) and further developed by Mason et al., (1964), and Seifart and Hansel (1968) provide a ready tool for testing the effects of various agents in vitro on corpus luteum function. The tissue slice samples were placed in 5 ml of Krebs-Ringer bicarbonate buffer (Umbreit et al., 1957) at pH 7.4 containing glucose (2 mg/ml). Progesterone synthesis stimulators (LH, 200 ng/ml, NIH-LH-B8; PGE_2, 10 ug/ml, provided by Dr. J. E. Pike, Upjohn, Inc.) were added where appropriate after a 10 minute pre-incubation of tissue in media containing the test substance, ergocornine (75 uM, ergocornine hydrogen maleate, provided by Sandoz Pharmaceuticals, Inc.), when called for. Stimulation of the luteal tissue with 0.02 M cAMP (Adenosine-3':5' cyclic-monophosphoric acid, sodium salt, Plenum Scient. Res., Inc.) did not lend itself to a pre-incubation due to the large quantity of cAMP required for stimulation. In some experiments 1 uCi ^3H-acetate (sodium salt, spec. act. 0.05 ci/mM, ICN Pharmaceuticals, Inc.) was added to the incubations to measure de novo progesterone synthesis. The incubations were carried out in 25 ml erlenmeyer flasks placed in a Dubnoff metabolic shaker at 37°C under 95% O_2 and 5% CO_2. After two hours, the incubations were terminated by freezing the incubation samples on dry ice and stored at -20°C.

Each corpus luteum provided enough tissue for three to four experimental groups consisting of four or five different incubation conditions per experimental group. The experimental groups contained an incubated control, incubated tissue with ergocornine, an incubation stimulated by LH or PGE_2 or cAMP, an incubation containing one of the stimulators plus ergocornine. The incubations were started sequentially through the experimental group before continuing on to the second experimental group, etc. This mode of initiating incubations tends to help eliminate any bias due to slight loss of tissue response with time. The time from when the corpus luteum was taken from the cow until the start of the last incubation was kept under two hours.

Progesterone Analysis

Progesterone analysis of the incubated tissue followed the procedures developed by Seifart and Hansel (1969). Before beginning the extraction and analysis of progesterone, a standard amount of ^{14}C-4-progesterone (approx. 10^4 cpm, spec. act. 52.8 mCi/mM, Calatomic, Inc.) was added to each incubation sample to provide a measure of chemical recovery after completion of the analysis. After thawing, the tissue and media were added to a round bottom flask and extracted in 70 ml of boiling ethanol for one hour, the supernatant was poured off and filtered, and the residue refluxed for an additional 30 minutes in 50 ml of ethanol. The combined filtrates were evaporated on a rotary evaporator under vacuum in a 60°C water bath. Five samples were spotted on each silica gel plate (thickness

0.5 mm) containing 2.5 mg fluorescent zinc silicate/g silica gel. The plates were first developed in a solvent system of hexane: ethylacetate (5:2) and then after drying, developed in a second solvent system of benzene: ethylacetate (3:1) in the same dimension. This resulted in the orange pigments migrating to the top of the plate while the progesterone, visulaized under a U.V. lamp, was at an R_f of about 0.4. The area of the plate containing the progesterone was scraped off into centrifuge tubes and the progesterone extracted from the silica gel by three, 2 ml washes with diethylether. After evaporation under a stream of N_2, the extracted progesterone was further purified on silica gel plates (Thickness 0.25 mm) developed in a solvent system of isopropylether: ethylacetate (5:2). The progesterone areas of the plates were scraped into sample vials to which 3 ml of methanol were added. One column on each plate was left blank and an equivalent progesterone area was scraped from this column to serve as a blank for that set of progesterone samples during spectrophotometric analysis. The sample vials were thoroughly mixed and centrifuged before spectrophotometric quantitative analysis on a Cary-14 spectrophotometer at a wavelength of 240 nm. Aliquots of each sample were evaporated and then dissolved in 0.4% PPO in toluene for assessment of radioactivity by a Beckman IS 100 liquid scintillation spectrometer. All samples were corrected to 100% recovery and progesterone was expressed per gram of luteal tissue.

A time effect on the tissue, measured from the time it was sliced until it was placed in the incubator, was corrected for by use of covariance analysis (Steel and Torrey, 1960).

Separation and Analysis of Progestins

A liquid-liquid chromatography system was developed to quantitate the relative percentages of progesterone and 20β-dihydroprogesterone which account for the total progestins of the corpus luteum. After trying different solvent systems and flow rates, it was found that progesterone and 20β-dihydroprogesterone can be separated on a 2 mm X 1 m Corasil C_{18} column eluted with Methanol: H_2O (66:34) at a 0.9 ml/min. flow rate employing the Waters Assoc., ALC 202 liquid chromatograph. The luteal tissue extract was injected on the column, and quantitation was determined by disc integration of the recorder trace.

Preparation of Homogenates

Homogenization of bovine corpora lutea for the assessment of adenylate cyclase activity and cAMP-phosphodiesterase activity followed the procedures of Sutherland, et al., (1962), and Marsh, (1970a,b). After obtaining a corpus luteum as previously described, it was taken to the cold room (4°C), cut into quarters, and the capsular tissue removed. Approximately 2.5 g of lutea tissue was sliced and minced with a razor blade before placing it in the glass mortar of a Potter-Elvehjem homogenization apparatus. After the addition of 10 ml of cold 0.02 M

glycylglycine (pH 7.4) containing 0.01 M $MgSO_4$, the homogenization was accomplished by no more than seven up and down strokes of the motor-driven Teflon pestle in the glass mortar. The mortar was surrounded by an ice water bath during the homogenization. The homogenate was strained through cheese cloth to remove connective tissue and kept on ice for immediate use.

Assessment of Adenylate Cyclase Activity

The measurement of adenylate cyclase activity in luteal tissue homogenates was carried out as described by Marsh (1970a,b). One tenth of a ml of homogenate suspension was added to 0.51 ml of a solution containing 25 umoles of tris (pH 7.4), 24.4 umoles theophylline, 1.2 umoles ATP, 10 uCi ^{3}H-ATP (^{3}H-2-Adenosine 5'-triphosophate tetrasodium, spec. act. 15 Ci/mm, Schwarz/Mann), 1.8 umoles Mg, 1.54 umoles NaCl, 0.12 mg bovine serum albumin, and the appropriate test substance. The test substances were added in the following concentrations; LH (10 ug/ml), PGE_2 (10 ug/ml), and ergocornine (100 uM). The reaction mixture was incubated with shaking in a Dubnoff metabolic incubator, in air, at 37°C for 10 minutes. The reaction was terminated by immersing the incubation tubes in a boiling water bath for three minutes. Blanks were made by boiling the reaction mixture before incubation. The formation of cAMP was assessed by measuring the conversion of ^{3}H-ATP into ^{3}H-cAMP.

The purification of cAMP was achieved through the combination of ion-exchange chromatography and precipitation with zinc sulfate and

barium hydroxide as first described by Krishna et al., (1968) and then by Forn and Krishna (1971). The cAMP was found to be 99% pure after this analytical procedure as checked by ion-exchange and paper chromatography and electrophoretic techniques, as well as by crystalization to constant specific activity.

The cAMP assay procedure starts with the addition of 0.1 ml of a solution (0.2 mg/ml) of carrier cAMP to the homogenate. Centrifugation of the homogenate produces a supernatant fluid which was chromatographed on a Dowex 50W-X8 (200-400 mesh) column (0.4 X 4.5 cm), prepared by pipetting 2 ml of a 1:1 slurry of resin and water into a glass wool pugged, disposable pipet. The column was eluted with water and 2 ml fractions were collected. The results of a trial run of the ion-exchange separation of ATP, ADP, cAMP are shown in Fig. 4. About 75% of the cAMP elutes out in the third fraction. After elution from the column, the cAMP fraction was treated with 0.2 ml each of 5% $ZnSO_4$ and 2.5% $Ba(OH)_2$. It has been observed by Krishna et al., (1968) that the addition of $ZnSO_4$ and $Ba(HO)_2$ quantitatively precipitates ATP, ADP, 5'-AMP, adenine, and inorganic phosphate while more that 99.9% of the cAMP remains in the supernatant. The resulting precipitant is centrifuged and the supernatant is transferred to another tube for a second $BaSO_4$ precipitation. After centrifugation, an aliquot of the resulting supernatant was measured for absorption at 260 nm to calculate the recovery of cAMP. A 1.0 ml aliquot of supernatant was added to 15 ml of cocktail D(4 g PPO

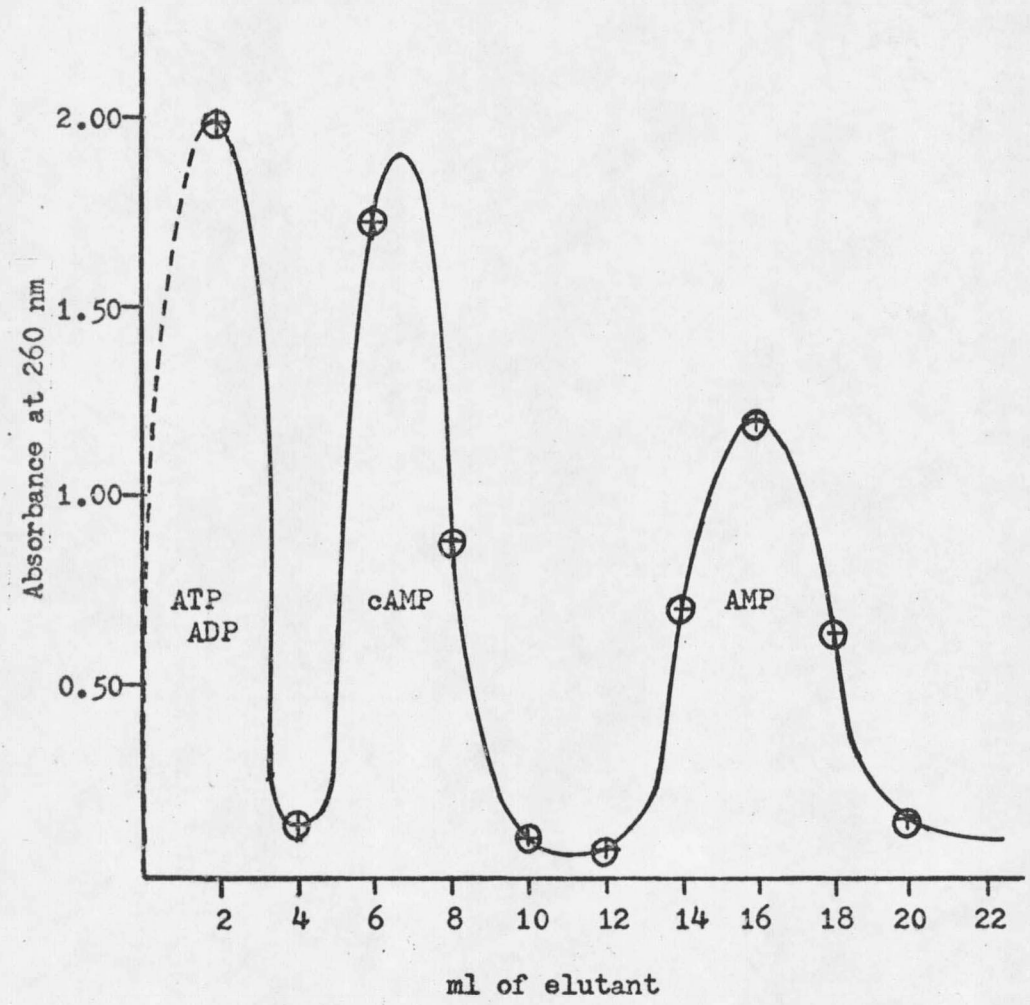


Figure 4. Separation of ATP, cAMP, and AMP on a Dowex 50W X8 column.

and 100 g naphthalene in 1 liter of dioxane) for liquid scintillation counting by a Beckman IS100 scintillation spectrometer.

Assessment of cAMP-Phosphodiesterase Activity

The assay for cAMP-phosphodiesterase consists of a two stage enzymatic isotopic procedure as developed by Thompson and Appleman (1971). Phosphodiesterase in the homogenate hydrolized ^{3}H-cAMP to ^{3}H-5'-AMP. A snake venom nucleotidase further hydrolized ^{3}H-5'-AMP to ^{3}H-adenosine. An ion-exchange resin was used to bind all of the

$$^{3}\text{H-cAMP} \xrightarrow[\text{homogenate phosphodiesterase}]{1} {}^{3}\text{H-5'-AMP} \xrightarrow[\text{nucleotidase}]{2} {}^{3}\text{H-Adenosine}$$

charged nucleotides leaving ^{3}H-adenosine as the only labeled compound to be counted.

The homogenate incubation for phosphodiesterase was the same as that described for adenylate cyclase except for a change in substrate and the elimination of theophylline. In place of ATP, 0.06 umoles of cAMP and 0.3 uCi of ^{3}H-cAMP (Adenosine-8-^{3}H-3', 5'-cyclic phosphate, spec. act. 21.8 Ci/mM, ICN, Inc.) were added per 0.51 ml of incubation media. The reaction was initiated by the addition of 0.1 ml of homogenate and incubated with shaking in a Dubnoff metabolic incubator, in air, at 37°C for 15 minutes before terminating by immersion in a boiling water bath for three minutes.

After the boiled incubations were cooled, they were put back into the metabolic incubator and 0.1 ml (1 mg/ml) of snake venom nucleotidase (Ophiophagus hannah, Sigma Chem.) added. This reaction was incubated for 30 minutes at 37°C before terminating by the addition of 1 ml of a 1:3 slurry of AG 1-X2 (200-400 mesh) in water. After centrifugation a 1.0 ml aliquot was added to 15 ml of cocktail D for assessment of radioactivity by liquid scintillation counting.

Measurement of Cyclic AMP Synthesis in Tissue Slices

Cyclic AMP formation was measured as described previously by Humes et al., (1969) and Kuo and DeRenzo (1969). This radiometric assay permits the in vitro measurement of adenylate cyclase activation in the intact cell by the determination of newly synthesized cAMP.

The bovine corpus luteum tissue was collected and sliced as previously described for the preparation of tissue slice incubation. Tissue samples of approximately 175 mg were initially incubated for one hour in 1.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with one-half the prescribed Ca^{++} concentration and containing 1 uCi of ^{14}C-8-adenine (52 mCi/mM, Schwarz/Mann). The 10 ml erlenmeyer incubation vessels were flushed with 95% O_2: 5% CO_2 and stoppered before incubation at 37°C in the Dubnoff metabolic incubator. The purpose of this one hour pre-incubation is to produce intracellular ^{14}C-ATP from the ^{14}C-adenine.

After the one hour pre-incubation, 0.5 ml of 20 mM theophylline and 1 mg/ml bovine serum albumin was added to each incubation vessel

along with the appropriate stimulating agents and ergocornine. Each incubation vessel was again flushed with 95% O_2: 5% CO_2 before further incubation for various time periods. The incubations were terminated by freezing the tissue and media on dry ice. The samples were then stored at -20°C until analysis for ^{14}C-cAMP.

Before analysis, ^{3}H-cAMP (3 X 10^4 cpm) was added to the incubation samples to measure recovery along with 0.5 ml of 0.05 M tris buffer (pH 7.5) containing 1.25 mM cAMP as carrier and 0.01 M theophylline. The incubation samples were then homogenized by the Potter-Elvehjem apparatus. After the homogenate was precipitated with 0.2 ml of 20% trichloralacetic acid and centrifuged, the supernatant was precipitated with 0.1 ml of 5% $ZnSO_4$ and 0.1 ml of 2.56% $Ba(OH)_2$, centrifuged, and then precipitated again with $BaSO_4$ and centrifuged. The supernatants were transferred to 10 ml erlenmeyer flasks and evaporated in a vacuum oven at 60°C. The samples were taken up in 0.5 ml of water and placed on a 0.4 X 4.5 cm, Dowex 50W-X8 (200-400 mesh) column which was eluted by water. The 2 ml fraction containing the cAMP was added to 15 ml of cocktail D for assessment of ^{14}C and ^{3}H activity by liquid scintillation counting. The ^{14}C-cAMP produced by tissue adenylate cyclase was corrected to 100% recovery from the recovery of ^{3}H-cAMP.

The incorporation of ^{14}C-adenine into ^{14}C-ATP was also assessed in this tissue slice system. The luteal tissue was prepared and incubated in the same manner as the pre-incubation step described above.

However, the pre-incubations were stopped at various time intervals by freezing on dry ice.

Adenine and ATP were extracted from the tissue as described above employing the homogenization procedure, and the resulting supernatant was lyophilized. Each residue was redissolved in 0.2 ml of a solution containing 1.25 mM ATP and 1.25 mM adenine. A 90 ul aliquot of each sample was spotted on cellulose (MN-cellulose powder 300G, 250 microns thick) thin-layer plates for separation of the adenine and ATP. After developing the cellulose plates using the solvent system of butanol-acetone-acetic acid-5% NH_4OH-water (4.5:1.5:1:1:2, by volume) (Marsh and LeMaire, 1974), the plate was viewed under a U.V. light and the appropriate areas corresponding to ATP and adenine standards were scraped into separate vials. Each sample was eluted with 1.5 ml of water and a 1.0 ml aliquot was added to 15 ml of cocktail D for liquid scintillation counting.

RESULTS AND DISCUSSION

The *in vitro* incubation system provides a means of investigating the direct effect of an agent such as ergot alkaloid on progesterone production in isolated corpus luteum tissue. The entire steroidogenic process is conserved in the tissue slice incubation which includes; 1) the endogenous precursors of progesterone, 2) the enzymatic pathway to convert those precursors to progesterone, and 3) the control apparatus by which hormones stimulate the enzymatic pathway to increase the production of progesterone. Tangible quantities of progesterone, which can be measured spectrophotometrically are produced by as little as 100 mg of incubated tissue. The progesterone biosynthetic pathway may be stimulated by the addition of LH (Mason *et al.*, 1962), prostaglandins (Speroff and Ramwell, 1970), or cAMP (Marsh and Savard, 1966) to the incubation media. The resulting production of measurable increases in progesterone tissue levels stimulated by these agents, provides one way of investigating the regulatory mechanism of steroidogenesis. The endogenous precursor pool may be radioactively labeled by exogenous ^{14}C-acetate or ^{3}H-cholesterol to provide the investigator another means of monitoring the steroidogenic process.

The Effect of Ergocornine on LH Stimulated Progesterone Synthesis

When ergocornine (ECO) was added to the incubation media of the *in vitro* incubation system, it had the effect of suppressing progesterone biosynthesis. The corpus luteum has been a previously suspected site

of ECO action (Draicer and Strauss, 1970; Shelesnyak, 1958; Zeilmaker and Carlson, 1962; Lobel et al., 1966), but the direct effect of ECO on luteal tissue had not been demonstrated. As can be seen in Figure 5, ECO results in decreased progesterone synthesis compared to control incubations as determined from the spectrophotometric measurement of the total progesterone content of the incubation samples. Unincubated luteal tissue samples were found to contain 35-50 ug of progesterone per g of luteal tissue. The addition of LH to the tissue incubation results in a stimulated level of progesterone synthesis comparable to that observed by other investigators (Seifart and Hansel, 1968; Marsh and Savard, 1966; Veenhuizen et al., 1972). When ECO was added along with LH, the stimulatory action of the hormone was suppressed.

The actual progesterone values and their statistical significance are expressed in Table I. There is a significant difference ($P<0.01$), as determined by the student's test, between the LH stimulated condition and the LH plus ECO condition no matter how the data is expressed. Ergocornine at 75 uM suppressed 80% of the progesterone biosynthesis produced by maximal LH stimulation (200 ug H/ml). The progesterone content is lowered by the addition of ECO to the LH incubated condition to a level that does not differ significantly ($p<0.01$) from the incubated control. When the data was treated by covariance analysis, a significant difference ($p<0.01$) is observed between incubated tissue and tissue incubated with ECO in the absence of exogenous LH. Covarience analysis

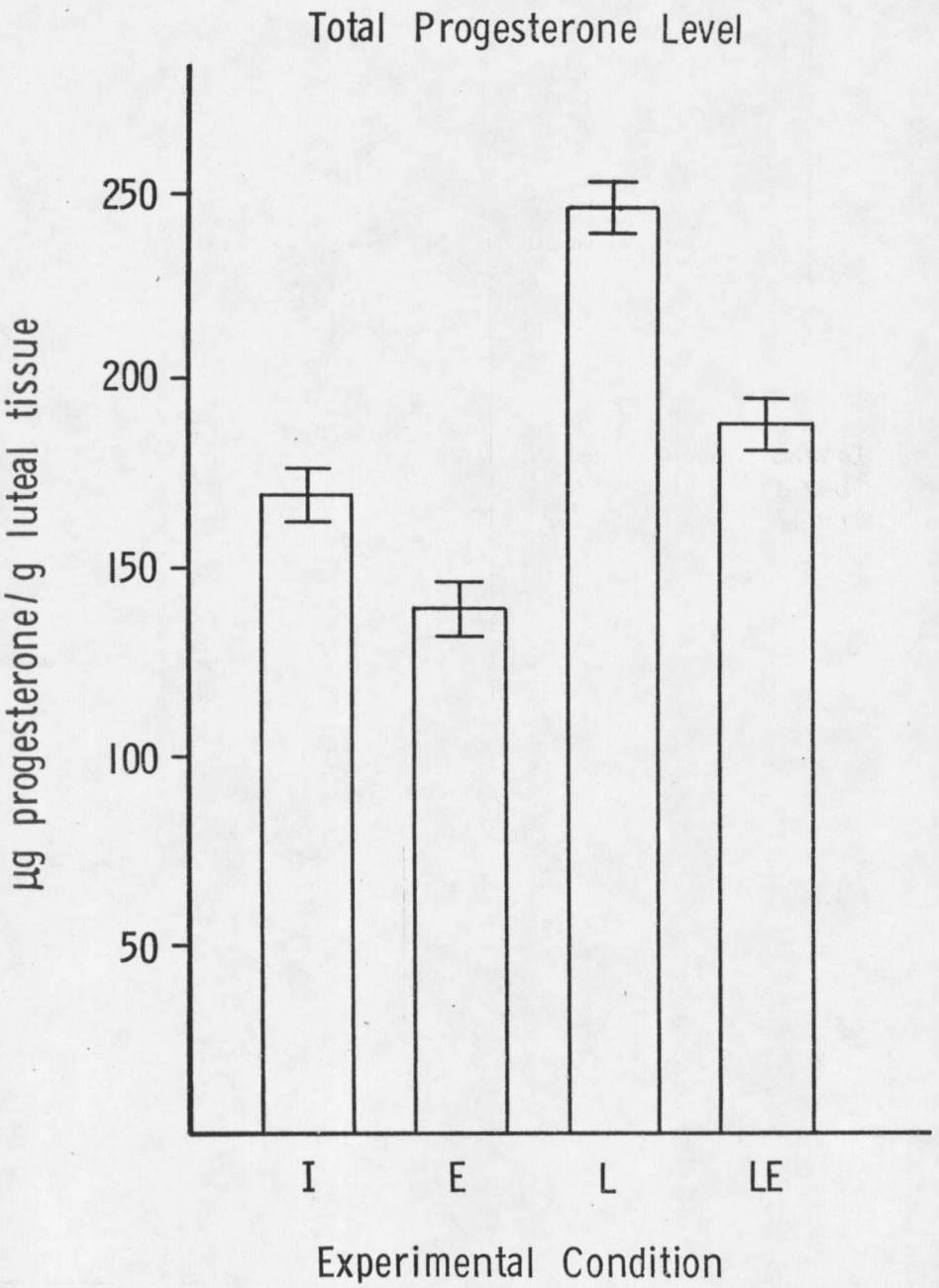


Figure 5. The effect of ECO on LH stimulated and control tissue steroidogenesis. (I) incubated control, (E) incubated with 75 uM ECO, (L) incubated with 200ng LH/ml, (LE) incubated with LH and ECO.

Table I. Effects of LH and ECO on progesterone biosynthesis in luteal tissue

			Total progesterone ug/g tissue*			
	no. of CL	no. of samples	Incubated control	ECO	LH	LH + ECO
Covariance corrected	3	10	169.2 ± 6.8^{a}	139.5 ± 6.8^{b}	246.3 ± 6.8^{c}	187.9 ± 6.8^{a}
Uncorrected	3	10	156.5 ± 8.0^{ab}	138.3 ± 8.0^{a}	220.3 ± 8.0^{c}	175.1 ± 8.0^{b}
Covariance corrected	7	21	158.6 ± 5.5^{a}		214.4 ± 5.5^{b}	172.1 ± 5.5^{a}
Uncorrected	7	21	142.2 ± 5.7^{a}		200.4 ± 5.7^{b}	160.1 ± 5.7^{a}

*All values are least square means ± SE

^{abc}Values with different superscripts differ significantly by $p<0.01$

attempts to correct the data for decreased progesterone biosynthesis due to the effect of time of tissue preparation before the start of incubation (Moody and Hansel, 1969). As can be seen in Table I the covariance analysis results in slightly higher progesterone values and decrease in the standard error of the mean.

Whether or not the slight depressive effect of ECO on progesterone synthesis in tissue incubations is due to the effect of ECO on endogenous hormone stimulation is a point of speculation. According to Niswender et al., (1969) the blood serum levels of LH in the cow are 12-60 ng/ml on the day of estrous and then fall off to a 1.5-2.5 ng/ml basal level during the luteal phase. Therefore, when the corpus luteum is taken from the cow, the tissue would contain some unknown level of endogenous LH. Considering the in vitro experiments show a minimum effective dose of 10 ng of LH/g of tissue or 2 ng of LH/ml of incubation media (Mason and Savard, 1964) for stimulation of progesterone synthesis over control incubation, it seems very possible that an endogenous LH stimulation is occurring in the incubated controls.

Another observation indicative of a possible endogenous gonadotropin effect may be seen in the results of adding puromycin to incubating luteal tissue. Puromycin effectively blocks LH stimulation of progesterone synthesis by its inhibitory action of protein synthesis. When Savard et al., (1965) were investigating this puromycin effect, they also observed a slight decrease in progesterone synthesis when puromycin was added to

incubating tissue in the absence of exogenous LH. This effect is very similar to the slight decrease in progesterone synthesis observed when ECO was added to incubating tissue.

When investigating the effect of a single injection of ECO on the progestin content of rat ovaries, Lindner and Shelesnyak (1967) found that ECO caused a sharp rise in ovarian 20α-dihydroprogesterone while the progesterone level decreases resulting in reduced phsiological activity. This observation necessitated the determination of 20β-dihydroprogesterone levels in the present in vitro system containing ECO in the incubation media.

The relative percentage of 20β-dihydroprogesterone of total progestins in the bovine corpus luteum tissue incubations agree with the normal 5 to 20% range observed by other investigators (Veehuizen et al., 1972; Gorski et al., 1958; Gomes and Erb, 1965).

In Table II are expressed the average percentages of progesterone and 20β-dihydroprogesterone for the four incubation conditions. The presence of ECO in the incubation media did not seem to influence the

Table II. The effect of ECO and LH on the ratio of 20β-dihydroprogesterone to progesterone.

Incubation Condition	Incubated Control	ECO	LH	LH + ECO
% progesterone	83.2 ± 1.2	83.3 ± 1.4	86.8 ± 0.4	86.3 ± 0.5
% 20β-dihydro-progesterone	16.8 ± 1.2	16.7 ± 1.4	13.2 ± 0.4	13.7 ± 0.5

progestin ratio. Unlike the *in vivo* effect observed in rats, the ratio of 20β-dihydroprogesterone to progesterone remained the same under the various *in vitro* treatment conditions of this study.

An experiment was conducted to establish the effective range of ECO on LH stimulated luteal tissue. The inhibitory action of ECO was found to be dependent upon ECO concentration. As can be seen in Fig. 6, ECO reaches its maximal suppression of LH stimulation at a concentration of about 100 uM. Incubations were conducted out to ECO levels equivalent to 500 uM without a further reduction in LH stimulation of progesterone biosynthesis. A precipitate of ECO appears in the incubation media before reaching the 100 uM level which may limit any concentration effect above this ECO level.

A series of experiments were performed using the incorporation of ^{3}H-acetate into progesterone as an indication of *de novo* progesterone biosynthesis in incubated luteal tissue. The three levels of LH did not seem to show real differences in stimulated incorporation (Table III) indicating the system to be maximally stimulated at even the lowest LH level employed. Therefore, the data was combined to show the average ^{3}H-acetate incorporations. In Table III the data indicates that incorporation into the progesterone of LH stimulated tissue is significantly ($p<0.01$) greater than incorporation into incubated controls. When ECO was added to the incubations along with LH, the incorporation level drops back to that of the incubated controls. Therefore, the incorporation of ^{3}H-acetate into progesterone during tissue incubations reflect the results of the

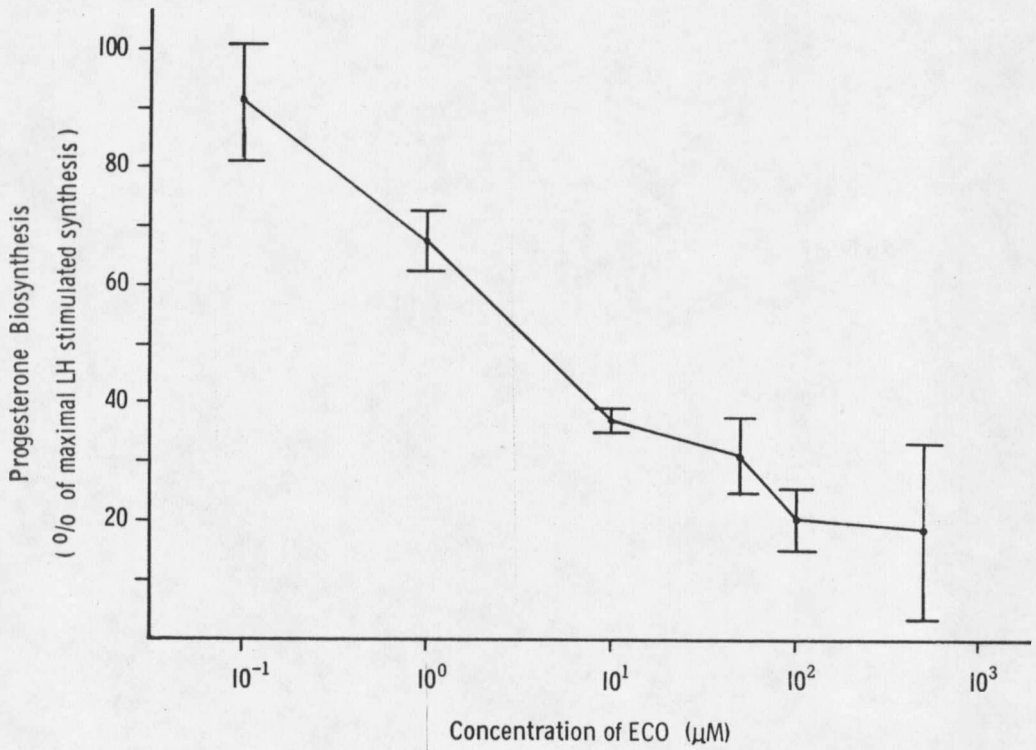


Figure 6. The effect of ECO concentration on LH stimulated steroidogenesis.

Table III. The effect of ECO on LH stimulated progesterone biosynthesis as measured by ^{3}H-acetate incorporation

	EXPERIMENTAL CONDITIONS		
LH concentration (ng/ml)	Incubated control	LH	LH + ECO
	(dpm/g tissue) x 10^{-3}		
0	29.3±2.3*[a]		
20		39.1±6.6[b]	27.5±2.3[a]
100		37.3±6.2[b]	28.9±2.4[a]
200		42.9±6.6[b]	29.7±3.4[a]
Average ^{3}H-acetate incorporation	29.3±2.3[c]	40.6±6.4[d]	28.7±2.6[c]
Specific activity (dpm/ug progesterone)	185±21	189±34	168±26

* Each experiment represents the mean ± standard error of determinations from four tissue incubations.

[ab] values with different superscripts differ significantly by $p < 0.05$

[cd] values with different superscripts differ significantly by $p < 0.01$

spectrophotometric determinations. The specific activities of the incubated control, LH stimulated, and LH and ECO incubation were about the same indicating that the extent of the ^{3}H-acetate incorporation is parallel for the different incubation conditions. The results in specific activity imply that LH increased progesterone synthesis without changing the precursor pools from those observed in control incubations. Savard and co-workers (1964,1965 and 1966) have shown a slight increase in specific activity of the LH stimulated tissue compared to controls. This implies that LH is stimulating incorporation of acetate over control incubations as well as stimulating progesterone synthesis. In looking at their data they show variable specific activity for different experiments and many of these values are the same as those specific activities seen in the control. It should also be noted that in their specific activity determinations 10-20 uCi of ^{14}C-acetate was used per incubation, whereas only 1 uCi of ^{3}H-acetate was used for obtaining the data in Table III. The important point made by the data in Table III is that ECO inhibits the LH stimulation of incorporation of acetate into progesterone. This supports the same conclusion drawn from the spectrophotometric determinations of total progesterone from the tissue incubations.

Effect of ECO on Prostaglandin Stimulation

Prostaglandins have the paradoxical effect of decreasing the circulating progestin level when administered subcutaneously to live animals and increasing the progesterone level of *in vitro* bovine corpus luteum tissue incubations (Speroff and Ramwell, 1970; Bedwani and Horton, 1968).

Since PGE_2 was reported to be the most active stimulator of the prostaglandins, it was utilized to stimulate progesterone synthesis in the study of the effect of ECO on stimulation of steroidogenesis. When PGE_2 (10 ug/ml) was added to the in vitro incubation system, it significantly ($p<0.01$) stimulated progesterone synthesis compared to the incubated control (Table IV). The progesterone level of the PGE_2 incubations was just under but comparable to the LH stimulated level (Fig. 7). When ECO was added along with PGE_2 to the incubation media, the progesterone level dropped significantly ($p<0.01$) below the PGE_2 stimulated level. The ECO suppressed PGE_2 stimulation of progesterone synthesis to a level that did not significantly differ from that of incubated controls. The statistical results were the same when the data was adjusted for a time effect by covariance analysis.

The effect of ECO on either LH stimulation or PGE_2 stimulation appears to be about the same. This is not surprising since the stimulator properties of prostaglandins closely parallel those of LH stimulation. Similarities are observed in specific activity of the increased incorporation of ^{14}C-acetate into progesterone, time response curves, and cycloheximide inhibition (Speroff and Ramwell, 1970). More important is the fact that both stimulate adenylate cyclase. The common inhibitory effect by ECO is indicative of some common intermediate between LH and PGE_2 stimulation of progesterone synthesis.

Table IV. Effect of ECO on PGE_2 stimulation of steroidogenisis in luteal tissue

Incubation condition	Incubated control	LH	PGE_2	PGE_2+ECO
No. Observations	9	9	15	15
Progesterone (ug/g) uncorrected	148.3 ± 11.5^a	215.3 ± 11.5^b	203.8 ± 9.0^b	147.6 ± 9.0^a
Progesterone (ug/g) covariance corrected	161.7 ± 8.6^a	234.8 ± 8.6^b	226.1 ± 6.8^b	179.9 ± 6.8^a

a,b Averages on the same line with different superscripts differ significantly ($p < .01$).

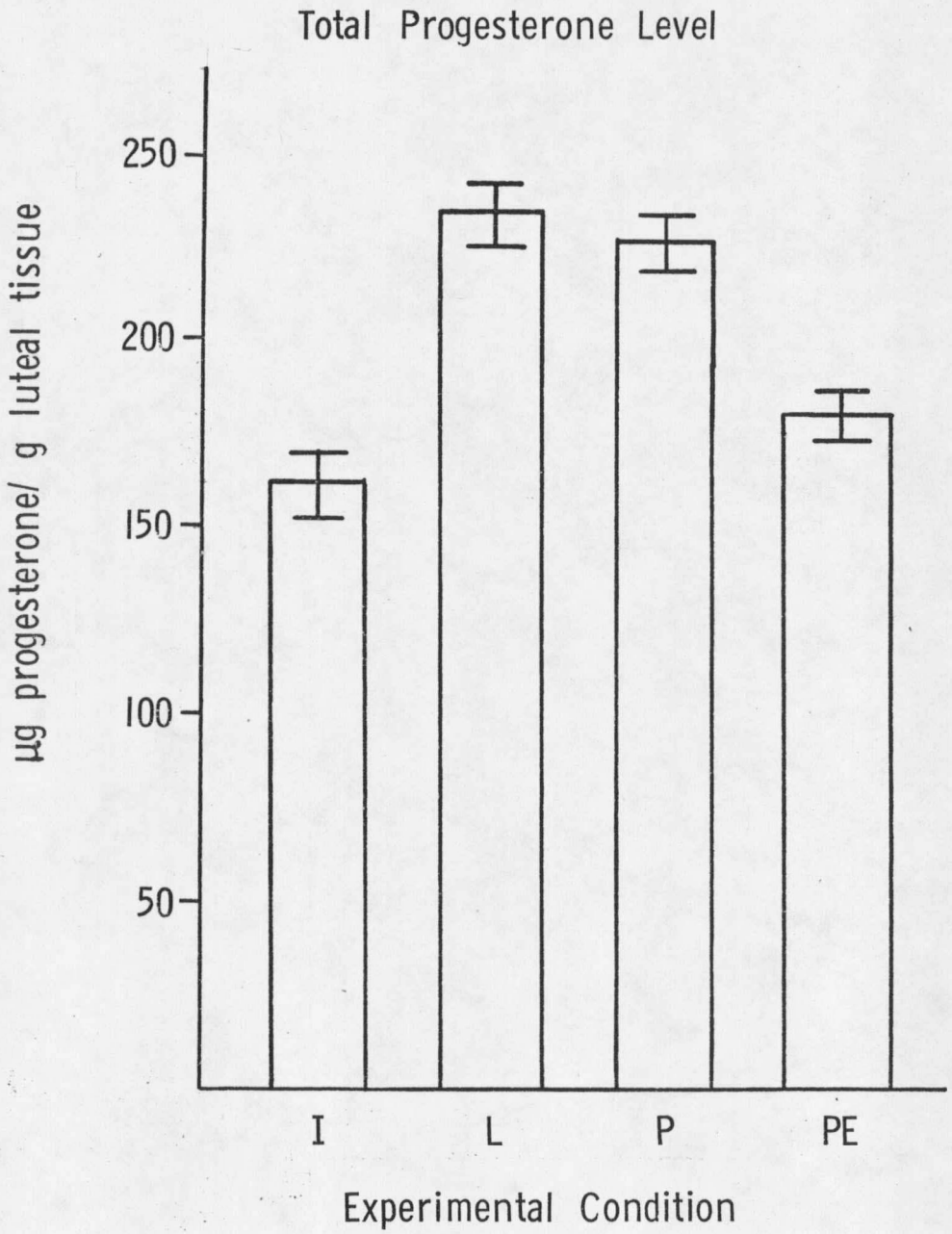


Figure 7. The effect of ECO on PGE_2 stimulation of steroidogenesis. (I) incubated control, (L) incubated with 200 ng LH/ml, (P) incubated with 10 ug PGE_2/ml, (PE) incubated with PGE_2 and 75 uM ECO.

When PGE_2 was added to homogenates of bovine corpora lutea, adenylate cyclase was stimulated to produce cAMP (Marsh, 1970b). This implies that PGE_2 stimulation of steroidogenesis is mediated by cAMP as is LH stimulation. In view of the vast structural dissimilarities between prostaglandins and gonadotropins it was not surprising when Rao (1973) demonstrated separate receptor sites for PGE, and human chorionic gonadotropin (HCG) in the cell membranes of bovine corpora lutea. The gonadotropin receptor was specific in binding HCG and LH, whereas the prostaglandin receptors were very specific for PGE_1 and PGE_2. This evidence suggests that prostaglandins and gonadotropins bind to different sites on the same receptor of adenylate cyclase or that each binds to different receptor molecules for adenylate cyclase.

The experimental evidence builds a strong case for both the luteolytic effect and the contrary steroidogenic stimulatory effect to be existing physiological phenomena. Perhaps these paradoxical effects may co-exist, considering that prostaglandin injected into an animal may have its effect on the corpus luteum as a whole organ sub-structure or its periphery, whereas the in vitro effects of the prostaglandins are confined to the tissue itself. When interpreting in vitro and in vivo experimental results, the difference in time an agent has to exert its action must be considered. The in vitro incubations demonstrate a short term effect of prostaglandins, whereas the in vivo experiments demonstrate a long term effect. Therefore, prostaglandin may stimulate progesterone synthesis when confined to luteal

tissue as seen in a short term in vitro experiment, but the long term effect on the organ substructure results in luteolysis and resulting decreased progesterone released from the ovary.

It has been proposed by Pharriss (1970) that $PGF_{2\alpha}$ is luteolytic due to a vasoconstrictive effect resulting in a local reduction of ovarian blood flow. Prostaglandin $F_{2\alpha}$ has been shown to reduce ovarian venous blood flow in the rat and rabbit (Pharriss et al., 1968). The fact that PGF_2 is the only potent venoconstrictor among the prostaglandins (Ramwell et al., 1968) would explain the luteolytic ineffectiveness of the other prostaglandins when given in vivo.

Certain in vitro studies indicate that prostaglandins mimic tropic agents in the adrenal gland (Flack et al., 1968) and thyroid gland (Onaya et al., 1969) as well as luteal tissue. Unlike tropic agents, prostaglandins are endogenous in the target tissue (Speroff and Ramwell, 1970) and may be synthesized from certain endogenous long chain fatty acid precursors (Claesson, 1954). It would not be difficult to invision a localized tissue or cellular effect by a short lived, endogenously produced prostaglandin. Since prostaglandins are rapidly degraded, a long-term effect on the corpus luteum would not be expected. The prostaglandins may serve as local regulators by modifying the effects of hormones at the tissue level. They could act as intercellular messengers of an LH response to the surrounding corpus luteum cells, or they may play a role in the cellular mechanism by which

hormones exert their effects. Perhaps the mobilization of cholesterol esters and fatty acids by LH (Armstrong et al., 1969) are intimately related to the biosynthesis of prostaglandins and their resulting stimulatory action.

Effect of ECO on cAMP Stimulation

Since LH and PGE_2 both stimulate adenylate cyclase to produce cAMP, the effect of ECO and cAMP stimulation was determined. When cAMP (0.02 M) was added to the incubation media, progesterone synthesis was stimulated to a level significantly greater ($p<0.01$) than the incubated control (Figure 8). The addition of ECO to the incubation media along with cAMP resulted in a progesterone level still significantly ($p<0.01$) greater than the control incubation. This was definitely in contrast to the results of ECO inhibition of both LH and PGE_2 stimulation. In fact, only a slight decrease in progesterone level is observed when ECO is included with cAMP in the incubation media to the cAMP stimulated condition.

The stimulated levels of progesterone expressed in Table V, are comparable to those values obtained by Marsh and Savard (1966) -- 207 ug progesterone/g luteal tissue for the 0.02 M cAMP condition and 233 ug/g for the saturated LH stimulatory condition. The concentration of exogenous cAMP needed for maximal stimulation of progesterone synthesis is relatively high compared to the endogenous, LH stimulated concentration

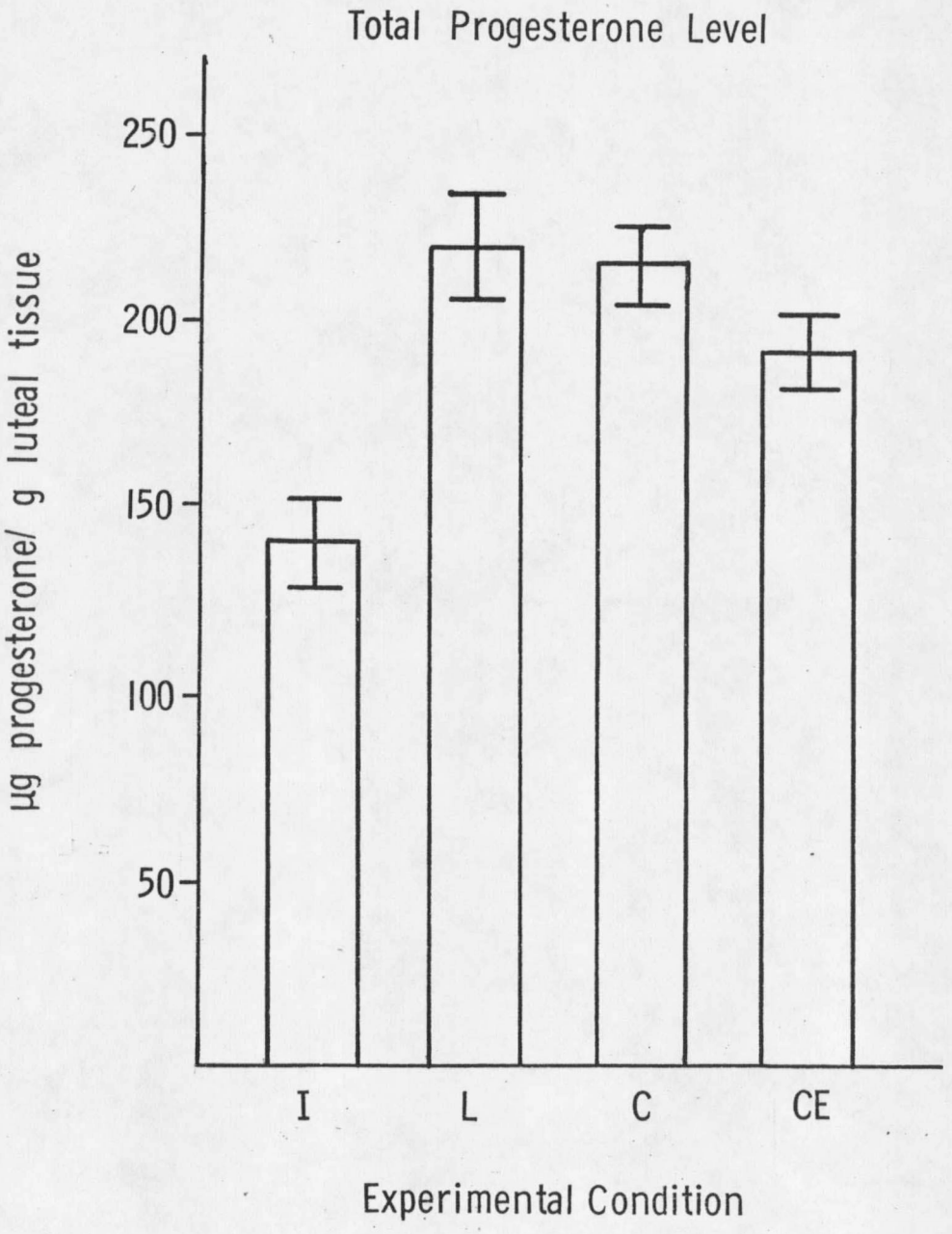


Figure 8. The effect of ECO on cAMP stimulation of steroidogenesis. (I) incubated control, (L) incubated with 200 ng LH/ml, (C) incubated with 0.02M cAMP, (CE) incubated with cAMP and 75 uM ECO.

Table V. Effect of ECO on cAMP stimulation of steroidogenisis in luteal tissue

Incubation condition	Incubated control	LH	cAMP	cAMP+ECO
No. Observations	7	5	9	9
Progesterone (ug/g)	141.8 ± 12.5^{a}	222.5 ± 15.2^{b}	215.7 ± 11.0^{b}	195.7 ± 11.0^{b}
No. Observations	9		13	13
Progesterone (ug/g)	140.8 ± 12.5^{a}		230.3 ± 10.3^{b}	210.9 ± 10.3^{b}

a, b Averages on the same line with different superscripts differ significantly ($p<0.01$).

of 8.5 ± 4.1 nanomoles/g (Marsh et al., 1966) found in the corpus luteum. This requirement for high concentrations of nucleotide has also been observed in studies on exogenous cAMP stimulation of intact liver and adrenal cells (Sutherland and Rall, 1960). Since much smaller concentrations of cAMP were needed to stimulate homogenates, they decided that perhaps the cyclic nucleotide does not effectively penetrate the cell membrane. This may be true in corpora lutea slices, but Marsh and Savard (1966) were unable to stimulate steroidogenesis by high or low levels of cAMP in homogenates of corpora lutea and the same attempts were unsuccessful in our lab. Another factor which may influence the effective concentration of exogenous cAMP is the enzyme, cAMP-phosphodiesterase, which may be inactivating the cAMP that does manage to get through the cell membranes. If the dibutyryl derivative N^6-2'-O-dibutyryl-3', 5'-AMP is used, the effective concentration can be reduced 100 fold (Marsh, 1969). The dibutyryl derivative is much more permeable to cell membranes and it is not hydrolized by phosphodiesterase.

By incubating corpus luteum tissue with the three known stimulators of steroidogenesis along with ECO, it has been determined that an inhibitory action of ECO on stimulation of progesterone synthesis appears to take place prior to cAMP stimulation. Considering that cAMP is the second messenger for LH and PGE_2 stimulation, the results indicated that the action of ECO may be to effectively prevent an

increase in the intracellular concentration of cAMP due to the first messengers. The intracellular level of cAMP is raised by LH and PGE_2 through their stimulatory action on the enzyme adenylate cyclase. This stimulatory action may be blocked by ECO thus preventing a rise in intracellular cAMP. Another possible way ECO may inhibit the increase in intracellular cAMP is through a stimulatory action on cAMP-phosphodiesterase. Further investigation of the effect of ECO on the stimulation of steroidogenesis proceeded with the determination of the effect of ECO on adenylate cyclase and phosphodiesterase activity.

The Effect of ECO on Phosphodiesterase

Cyclic AMP is hydrolized to physiologically inactive 5'-AMP under the influence of one or more phosphodiesterases. Most cells contain at least two phosphodiesterases, one with a low Km for cAMP (on the order of 10^{-6} M) and the other with a higher Km (about 10^{-4} M) (Thompson and Appleman, 1971), located in the soluble as well as in the particulate fractions. Homogenates of corpus luteum tissue contain phosphodiesterase activity as reported by Marsh (1970a). Since the inhibitory action of ECO on LH and PGE_2 stimulation could possibly be due to a stimulatory action of ECO on phosphodiesterase, the effect of ECO on phosphodiesterase activity was assessed.

Homogenates of luteal tissue had an enzyme activity of 40 nanomoles/15 min. X 20 mg luteal tissue for phosphodiesterase as measured by the

conversion of ^{3}H-cAMP to ^{3}H-5'-AMP. When LH or PGE_2 were added to the homogenates, the phosphodiesterase activity was unchanged by these steroidogenic stimulators as was also observed by Marsh (1970a,b). The classic phosphodiesterase inhibitor, theophylline, added at near saturated levels (0.04 M) reduced the catabolism of cAMP to a near negligible level. Dihydroergotamine as well as ECO was added to homogenate incubations at two concentrations, 10^{-3} M and 10^{-4} M. Neither alkaloid was completely soluble at the 10^{-3} M level. The two ergot alkaloids had a slight inhibitory effect on phosphodiesterase as expressed in Table VI. At the concentration of ECO commonly used in the previous experiments (10^{-4} M), the inhibition of phosphodiesterase was 13.7% of the control which is a small effect but significant ($p<0.01$).

Some of the ergot alkaloids and lysergic acid derivatives are known phosphodiesterase inhibitors in various tissues. Kukovetz and Poch (1969) demonstrated that bromo-LSD reduces myocardial phosphodiesterase in adipose tissue. Iwangoff and Enz (1972) reported the inhibition of phosphodiesterase in cerebral grey matter by dihydroergotamine and a large group of analogous compounds. Iwangoff and Enz (1973) also reported the inhibitory effect of dihydroergotamine and hydergine (an equimolar mixture of dihydroergotamine, dihydroergocristine, and dihydroergokryptine) on the phosphodiesterase activity in different organs (heart, kidney, liver, and brain) of the cat. A slight

Table VI. The effect of ergot alkaloids on phosphodiesterase activity.

Molar Conc. of Alkaloid	% Inhibition of Phosphodiesterase Activity*
10^{-4}M ECO	13.7 ± 2.0#
10^{-3}M ECO	14.7 ± 1.3
10^{-4}M DHE	15.5 ± 2.2
10^{-3}M DHE	19.8 ± 0.8

* Phosphodiesterase activity of the control was 40 n moles/15 min./20 mg tissue (homogenized).

Values differ significantly from control enzyme activity ($p<0.01$).

inhibitory effect of the alkaloids on phosphodiesterase was generally observed for all cases in the cat organ study comparable to the inhibitory effect of ECO and dihydroergotamine observed in bovine corpora lutea homogenates. The cat brain had the highest phosphodiesterase activity (39 u moles/30 min. X 100 mg tissue) and was inhibited 15.3% of the control by 10^{-4} M dihydroergotamine. This can be compared to an activity of 40 n mole/15 min X 20 mg tissue for bovine corpora lutea homogenates and a 13.7% inhibition of the control by 10^{-4} M ECO. Marsh (1970a) reported a 250 n mole/15 min. X 20 mg tissue for phosphodiesterase activity in bovine corpora lutea at a higher substrate level.

This slight inhibition of phosphodiesterase by ECO in bovine corpora lutea homogenates indicates that ECO would tend to increase the intracellular level of cAMP. An increase in cAMP results in an increase in progesterone synthesis which is contrary to the inhibitory action of ECO observed on stimulated steroidogenesis in incubated luteal tissue. The slight inhibiting effect of phosphodiesterase is not likely to have a dominating effect on intracellular levels of cAMP compared to an LH or PGE_2 stimulation of adenylate cyclase. Since phosphodiesterase is inhibited rather than stimulated by ECO, it cannot be the site of the ECO inhibition of stimulated progesterone synthesis. It is difficult to assess this effect of ECO inhibited phosphodiesterase on the overshadowing ECO inhibited levels of progesterone synthesis observed in

tissue incubations. Perhaps the inhibiting effect of ECO on stimulated steroidogenesis would be even greater in the absence of inhibitory effect on phosphodiesterase.

The Effect of ECO on Adenylate Cyclase Activity

The assessment of adenylate cyclase activity was made in corpora lutea homogenates and in luteal tissue slices through the measurement of accumulated newly formed cAMP from radioactive precursors added to the two systems. Tissue and cellular integrity are preserved in the tissue slice system providing the intact cellular system sometimes required for full expression of hormonal activation. The tissue integrity may be an important factor considering the nature of adenylate cyclase and its activation by more than one agent. One difficulty of the tissue adenylate cyclase assay is labeling the ATP substrate pool. Phosphorylated nucleotides are relatively impermeable to the cell. Homogenates provide a cell free system that will readily accept radioactive ATP into its substrate pool. Of course, cellular integrity is lost along with some adenylate cyclase activity. The homogenate does provide a system where any degradation of accumulating cAMP by phosphodiesterase can be ruled out by complete inactivation of the enzyme by an inhibitor such as theophylline.

The effect of ECO on LH stimulation of adenylate cyclase was determined by measuring the accumulation of ^{14}C-cAMP in tissue slices.

The intracellular ATP substrate pool was labeled by the conversion of extracellular ^{14}C-adenine to intracellular ^{14}C-ATP. Adenine was reported to penetrate lipocytes with relative ease and, in the presence of glucose as an energy source, was found to be almost exclusively converted to intracellular ^{14}C-ATP (Humes et al., 1969). This

$$^{14}\text{C-adenine} \longrightarrow {}^{14}\text{C-ATP} \xrightarrow[\text{cyclase}]{\text{adenylate}} {}^{14}\text{C-cAMP}$$

conversion was demonstrated to take place in bovine corpus luteum slices. When labeled adenine was added to incubating tissue slices, an increase in ^{14}C-ATP and the concurrent depletion of ^{14}C-adenine was measured with time. As can be seen in Figure 9, maximal conversion to ^{14}C-ATP plateaued after 60 minutes on incubation.

After a 60 minute pre-incubation, LH and ECO were added to the appropriate incubations to determine the effect of ECO on LH stimulation of adenylate cyclase activity. The measured amounts of ^{14}C-cAMP accumulated under the control incubation condition, LH stimulated condition, and LH plus ECO condition incubated for different time intervals are compared in Figure 10. The results are expressed as the mean ± the standard error of three experiments. The addition of LH (1.0 ug/ml) resulted in a marked increase in cAMP accumulation in the luteal tissue compared to incubated control tissue. When ECO (100 uM)

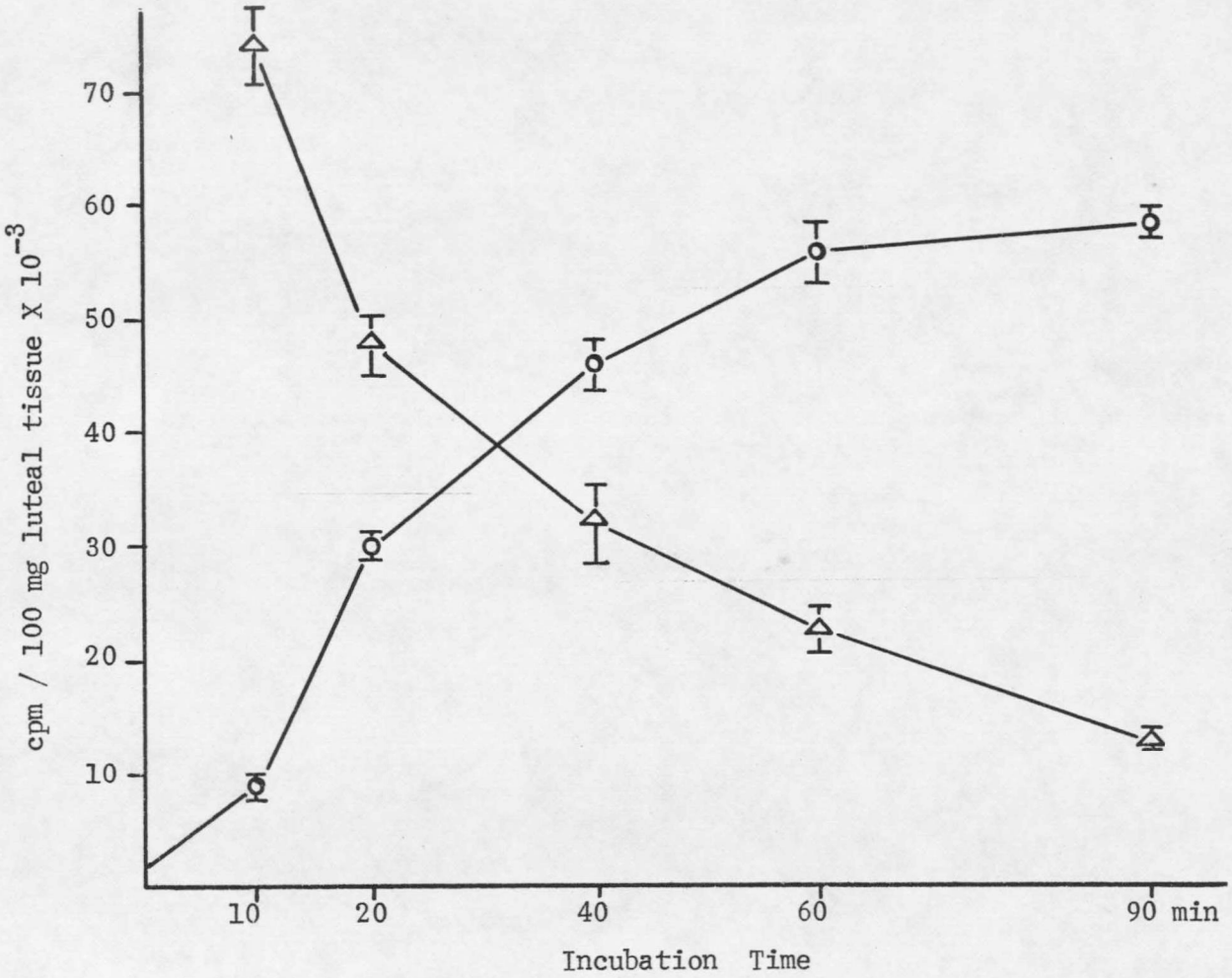


Figure 9. Conversion of ^{14}C-adenine to ^{14}C-ATP in luteal tissue. (○) ^{14}C-ATP, (△) ^{14}C-adenine.

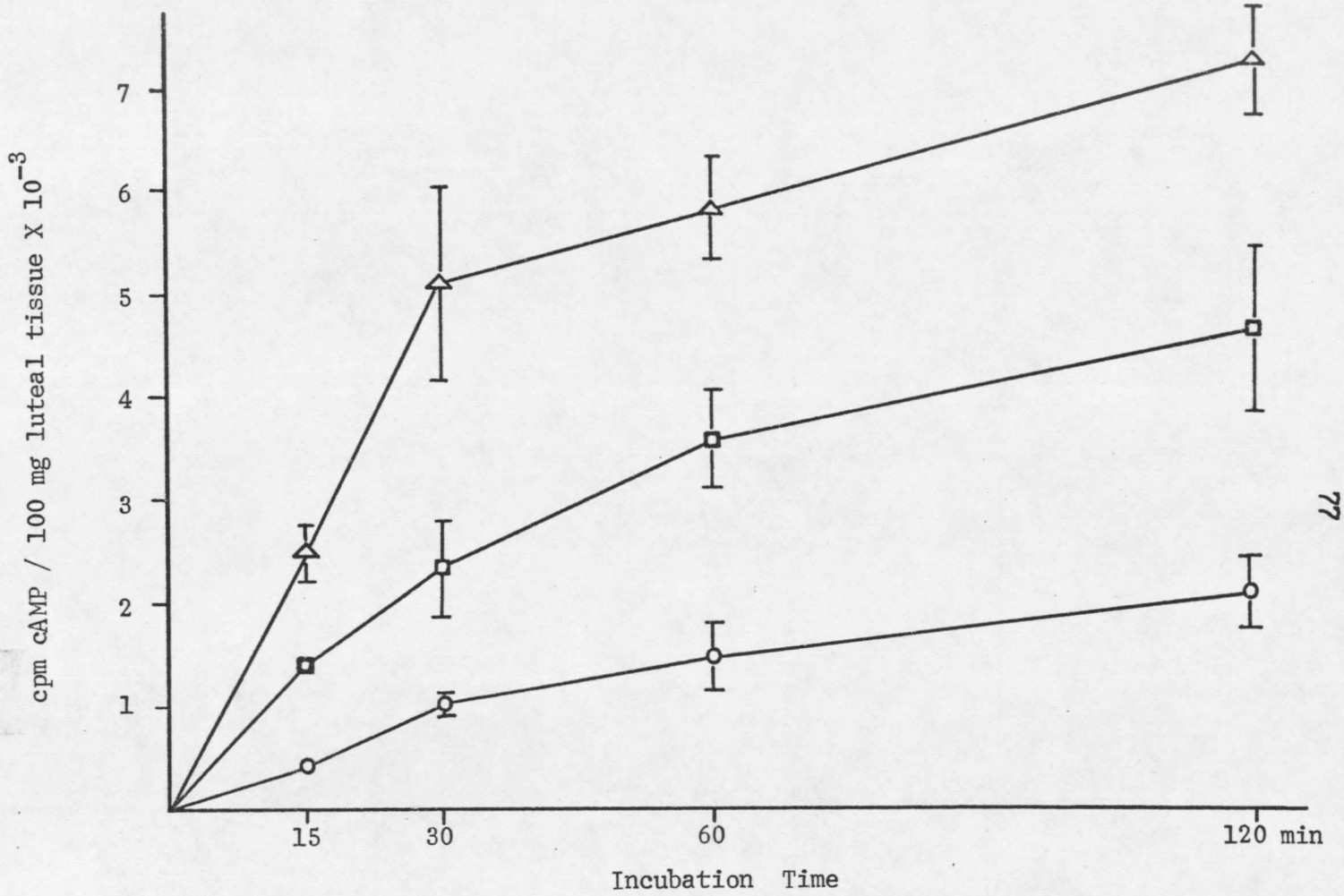


Figure 10. Cyclic AMP accumulation in luteal tissue. (o) incubated control, (□) LH plus ECO, (△) LH stimulated.

was added to the incubation media along with LH, the accumulation of cAMP was substantially decreased from the LH stimulated condition. These results suggest that ECO has an inhibitory effect on the LH stimulation of adenylate cyclase activity in intact corpus luteum tissue.

Luteal tissue homogenates were also used to assess the effect of ECO on the stimulation of adenylate cyclase in a cell free system. The ATP substrate pool was tagged with the addition of 10 uCi of ^{3}H-ATP to each incubation. Adenylate cyclase activity was determined from the amount of ^{3}H-ATP converted to ^{3}H-cAMP during the incubation. The inclusion of a high concentration of theophylline (0.04 M) in the assay incubation and the relatively short incubation time of 15 minutes assures an ineffectual phosphodiesterase activity in the homogenate.

The addition of LH (20 ug/ml) to the homogenate incubation, containing the equivalent of 40 mg of luteal issue, results in stimulated adenylate cyclase activity as previously demonstrated by Marsh (1970a). The mean activities ± standard error as determined by Marsh in bovine corpora luteal homogenates were 116 ± 25 p moles/20 min. X 40 mg for the control, 469 ± 95 for LH stimulated, and 4140 ± 720 for NaF stimulated adenylate cyclase. As can be seen in Figure 11, the enzyme activities determined under the same conditions are in the same range as those reported by Marsh. The addition of ECO (100 uM), once again, had the effect of inhibiting LH stimulation of adenylate cyclase. The results

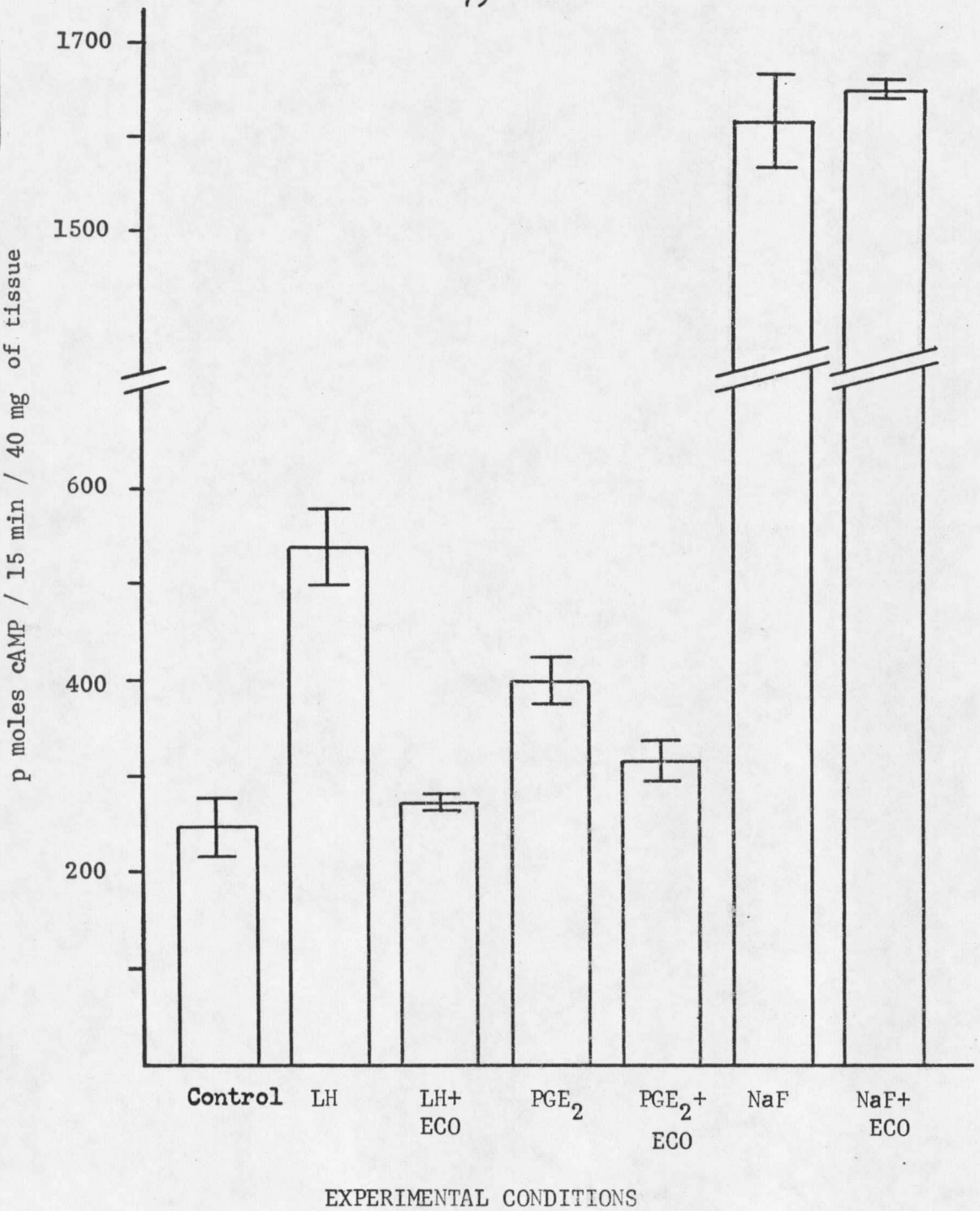


Figure 11. Adenylate cyclase activity in luteal tissue homogenates

expressed in Figure 11 are the means ± the standard error of three experiments for LH and LH plus ECO while the remaining values are the means ± the range for two experimental groups. Statistical analysis demonstrate the enzyme activities for the LH condition and the LH plus ECO condition to be significantly ($p<0.01$) different.

The addition of PGE_2 (10 ug/ml) to the homogenate also results in stimulated adenylate cyclase activity but not to the same extent as does LH (Figure 11). This same observation was made by Marsh (1970b). The addition of ECO appears to reduce the stimulatory effect of PGE_2 on adenylate cyclase in the same manner as it reduced LH stimulation.

An even greater stimulatory effect of adenylate cyclase was observed with the addition of NaF (0.01 M) to tissue homogenate. The stimulation of adenylate cyclase by NaF has been observed consistently in the study of the effect of other hormones on this mediatory enzyme in various target tissues (Sutherland et al., 1962). The magnitude of the NaF effect always seems to significantly exceed the effect of a stimulatory hormone. The addition of ECO did not effect the stimulatory action of NaF as it has suppressed LH and PGE_2 stimulation of adenylate cyclase. In a similar fashion, another ergot alkaloid, dihydroergotamine, inhibits the norepinephrine stimulation of adenylate cyclase but does not effect NaF stimulation of the enzyme (Ward and Fain, 1971). It seems likely that NaF interacts with some component of the adenylate cyclase system which is common to all tissues and that

this interaction is different from the effect of the hormone, which is very specific in most tissues studied.

The nature of the effect of ECO on adenylate cyclase in bovine corpora lutea tissue is difficult to assess. The ergot alkaloid inhibits the stimulatory action of both LH and PGE_2 on adenylate cyclase. Initially it seems logical to speculate that the alkaloid prevents the binding of LH and PGE_2 to an adenylate cyclase receptor site. However, one must keep in mind the specificity these sites have demonstrated. Separate receptor sites for gonadotropin and prostaglandin were shown to exist in bovine corpus luteum tissue (Rao, 1973). Considering the structural dissimilarities between LH, PGE_2, and ECO it is difficult to invision the alkaloid to be competing with LH and PGE_2 for their respective binding sites. Hormones such as adrenocorticotropin, glucagon, norepinephrine and epinephrine which stimulate adenylate cyclase in their respective target tissue, have no effect on adenylate cyclase in bovine luteal tissue (Marsh, 1970a). Yet ergot alkaloids have demonstrated inhibitory actions on norepinephrine stimulation of adenylate cyclase in lipocytes (Ward and Fain, 1971), catecholamine activation of adenylate cyclase in the heart and liver of dogs (Murad et al., 1962) and rat liver (Berthet et al., 1957), and glucagon and catecholamine increase of cAMP in the rat liver (Yeung and Oliver, 1968). The general inhibitory effect of ergot alkaloids on stimulation of adenylate cyclase must result from some common action on the enzyme to prevent its

activation. Recent experimental evidence (Schmidt, et al., 1974) indicates liver and platelet adenylate cyclase has activated dephospho-- and inhibited phospho--forms controlled by an adjoining phosphoprotein phosphatase which activates phospho--adenylate cyclase by dephosphorylation. Perhaps the ergot alkaloids are preventing hormone stimulation through a direct inhibitory action on the activation of adenylate cyclase by a common activation process such as the one just mentioned above.

SUMMARY

The Effect of ECO on the Stimulation of Steroidogenesis

The initial experimental evidence demonstrated a direct effect of ergocornine on the production of progesterone in the corpus luteum. The physiological importance of the ECO inhibited progesterone would be hard to determine in light of the action of ECO on pituitary hormone output. However, this direct effect in tissue progesterone biosynthesis must be taken into account when discussing the effects of ECO on mammal reproductive dysfunctions.

Although the decreased progesterone levels in rats following ECO injections has been traced to inhibition of pituitary release of prolactin and LH (Wuttke, et al., 1971), there may be other significant effects resulting from a direct effect of ECO on luteal tissue. Kraicer and Strauss (1970) pointed out that ECO may interupt a preovulatory surge of progestins responsible for setting off the gonadotropin ovulatory pulse. Ergocornine was also found to decrease progesterone levels when adminstered to the mouse (Carlsen et al., 1961), the ferret (Blatchley and Donovan, 1967), and man (Shelesnyak et al., 1963) although this later observation came into question following subsequent investigation (Lindner et al., 1967).

Hormone levels of cattle treated with ergot alkoids have not been measured. However, cattle consuming toxic levels of ergot have shown reproductive dysfunctioning. Since the bovine corpus luteum is a well characterized, specialized tissue and since it is relatively large in mass, it seemed to be the ideal tissue to use to investigate the effect of ECO on steroidogenesis.

The corpus luteum is characterized as a specialized tissue which functions to produce progesterone. This progesterone is released into the blood stream where is activates or maintains the female reproductive system. The production of progesterone in the corpus luteum is controlled by the serum level of LH released from the pituitary. Therefore, the pituitary, under the influence of the hypothallmus and reproductive hormone feedback, controls the cyclic female reproductive rhythm.

Investigators have shown that LH stimulates progesterone synthesis in the corpus luteum via the "second messenger" cAMP. The prostaglandins also stimulate steroidogenesis in luteal tissue via cAMP. However, neither the relationship between LH and prostaglandins nor the physiological significance of prostaglandin stimulation has been determined. The "second messenger system" (Fig. 3, p. 31) provides a means for: (1) the recognition of a hormone by specific target tissues, (2) the transmission of the

hormone message across the cell membrane, and (3) the amplification of the hormone message into the stimulated cellular processes.

Initial experiments demonstrated that ECO inhibited LH stimulation and PGE_2 stimulation of steroidogenesis (Fig. 12). However, the alkaloid did not result in the large reduction of steroidogenesis when the tissue incubations were stimulated by exogenous cAMP. These results place the effect of the ergot alkaloid at the center of the "secondary messenger system". The inhibitory effect of ECO is prior to cAMP mediation of LH and PGE_2 stimulation of progesterone synthesis (Fig. 13).

The intracellular level of cAMP is controlled by the activity of two enzyme systems (Fig. 13). Ergocornine could inhibit LH and PGE_2 stimulation of steroidogenesis by inhibiting adenylate cyclase or increasing phosphodiesterase both resulting in the break-down of the mediation of LH or PGE_2 stimulation. Ergocornine was found to have a slight inhibitory action on phosphodiesterase which would result in increased intracellular cAMP levels. However, ergocornine was found to block LH and PGE_2 stimulation of adenylate cyclase, which would result in decreased intracellular cAMP and subsequent break-down of mediation of LH and PGE_2 stimulation of steroidogenesis. The magnitude of ECO inhibition of adenylate cyclase should over-shadow the small inhibitory effect of ECO on phosphodiesterase. As a result of homogenization, phosphodiesterase does have a much higher enzymatic activity than adenylate cyclase in homogenates. However, Marsh (1970a) pointed out

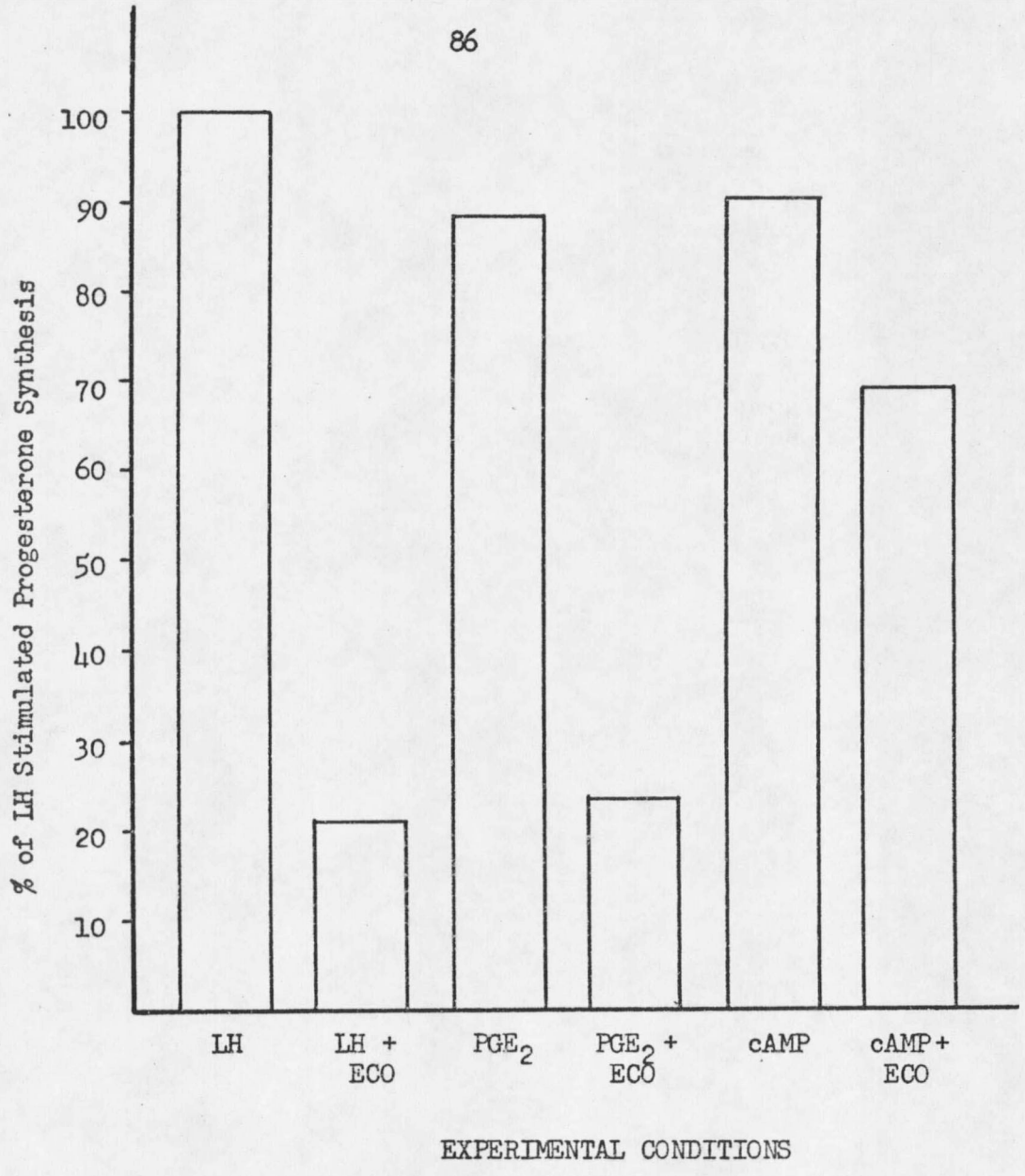
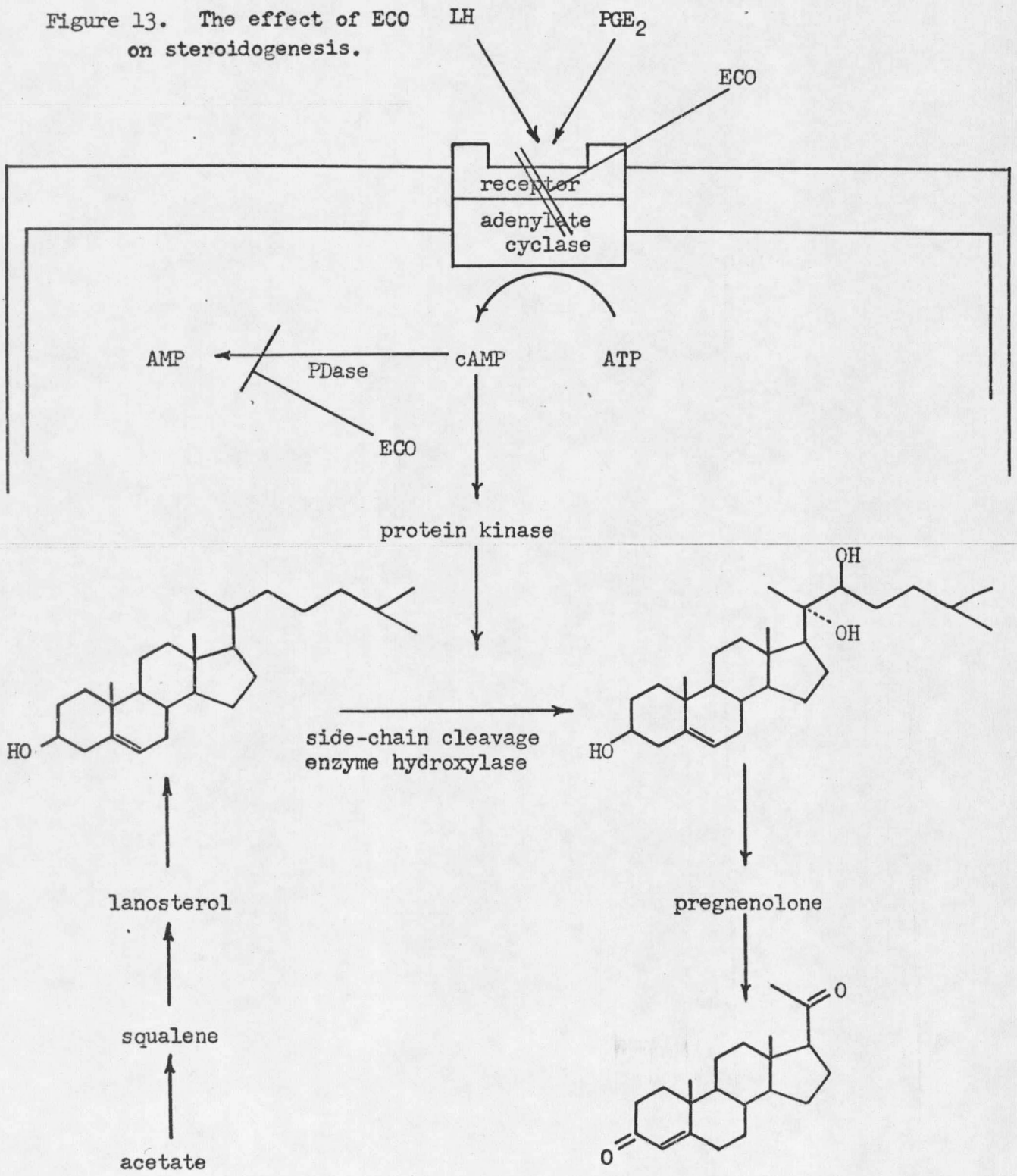


Figure 12. The effect of ECO on the stimulators of progesterone synthesis in luteal tissue. Incubation conditions as described in previous figures.

Figure 13. The effect of ECO on steroidogenesis.



that cAMP accumulates in the cell in the presence of this large potential phosphodiesterase activity indicating that either very little of the phosphodiesterase activity is expressed or that there is some compartmentalization which separates cAMP from this destructive enzyme. Of course homogenization releases the phosphodiesterase activity which must be inhibited by theophylline when assessing adenylate cyclase activity.

The primary effect of ergocornine inhibition of stimulated progesterone, therefore has been demonstrated to take place by an inhibitory action of the alkaloid on the activation of adenylate cyclase. From the experimental data presented, one may only speculate on the nature of this ECO inhibitory action on adenylate cyclase. Since the stimulatory action of both LH and PGE_2 are interrupted it appears that ECO interfers with an activation process of adenylate cyclase between the receptor sites and the enzyme. A general inhibitory action of ergot alkaloids on adenylate cyclase activation would also explain ergot alkaloid disruption of hormone stimulation in the other tissues mentioned -- adipose (Ward and Fain, 1971), heart (Murad et al., 1962), and liver (Berthet et al., 1957, Yeung and Oliver, 1968).

As a result of the ECO inhibitory action, LH or PGE_2 fail to stimulate an increase in the intracellular level of cAMP in bovine luteal tissue. Normally, LH or PGE_2 stimulated adenylate cyclase would increase the intracellular level of cAMP which activates certain protein kinases in bovine luteal tissue as shown by Menon (1973) (Fig. 13). The activated

protein kinases induce increased progesterone synthesis through certain phosphorylation reactions. Both LH and cAMP have been shown to directly activate a protein kinase that phosphorylates histones, whereas, only cAMP activates a different protein kinase to phosphorylate ribosomal proteins (Azhar and Menon, 1974). These phosphorylations could possibly increase the protein synthesis of enzymes in the steroidogenic pathway. Another phosphorylation reaction has been shown by Caron et al., (1974) to stimulate the activity of the rate-limiting enzyme of the steroidogenic pathway. The side-chain cleavage enzyme system in bovine luteal tissue is stimulated by a cAMP-dependent protein kinase phosphorylation of some protein in the cytochrome P-450 fraction of the enzyme complex.

From this discription it can be seen how the ergot alkaloid induced reproductive dysfunctions observed in cattle could possibly result from a direct effect of the alkoid on the progesterone synthesis in the corpus luteum.

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Soliday, Charles L
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