ROLE OF RNA AND PROTEIN SYNTHESIS IN RABBIT OVARIAN FOLLICULAR TESTOSTERONE PRODUCTION

BY

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ABSTRACT

The role of RNA and protein synthesis in follicular testosterone production has been investigated in the rabbit ovarian follicle *in vitro*.

Isolated follicles were incubated (usually 2 hours) with labeled amino acids or uridine plus LH, cyclic AMP or cyclic GMP alone, or together with various metabolic inhibitors.

In dose response studies, testosterone production was stimulated at all concentrations of LH (0.1 to 10 μg/ml) tested ($p < .05$), with optimal stimulation occurring at 5 μg LH/ml ($p < .01$). However, a significant uptake of ^{3}H-leucine into follicular protein occurred only at $\geq$ 2.5 μg LH/ml ($p < .01$), with optimal stimulation occurring at 5 and 10 μg LH/ml. Cyclic AMP (5 and 10 mM) enhanced both testosterone production and the uptake of ^{3}H-leucine into follicular protein ($p < .01$). Lower cyclic AMP concentrations were ineffective. Neither LH nor cyclic AMP had any effect on the incorporation of labeled uridine into follicular RNA.

In time course studies, testosterone was stimulated within 15 minutes ($p < .01$), in the presence of LH (5 μg/ml) or cyclic AMP (5 mM). The incorporation of ^{3}H-leucine also increased with time in both LH and cyclic AMP treated follicles, compared to controls, but a significant difference was observed only after 90 and 60 minutes, respectively ($p < .01$). However, electrophoretic fractionation and radio-

autographic examination of total follicular proteins after exposure of LH and ^{35}S-methionine for 15, 60 and 120 minutes showed no apparent difference in the distribution of protein bands when compared to controls.

Actinomycin D (20, 80 and 160 μg/ml) together with LH (5 μg/ml) inhibited the incorporation of ^{3}H-uridine into follicular RNA by 79, 85 and 86%, respectively ($p < .01$). At these concentrations, no inhibitory effect on LH-induced testosterone production was observed. Paradoxically, Actinomycin D (1 μg/ml) enhanced LH-induced testosterone production above that elicited by LH alone.

Cycloheximide (20 and 10 μg/ml) inhibited LH-induced testosterone production by 64 and 57% ($p < .01$), as well as the uptake of ^{3}H-leucine into follicular protein by 94 and 93%, respectively. However, cycloheximide (1 μg/ml) did not inhibit LH-induced testosterone production, yet inhibited ^{3}H-leucine incorporation by 81.7%. Similarly, puromycin (40 μg/ml) inhibited LH-induced testosterone production by 66%, and the uptake of ^{3}H-leucine into protein by 74% ($p < .01$). However, puromycin (10, 1 or 0.1 μg/ml) did not inhibit LH-induced testosterone production, yet ^{3}H-leucine incorporation was inhibited by 58, 37 and 31%, respectively ($p < .01$).

The methylxanthines, theophylline (25, 10 and 1 mM) and MIX (5 and 0.5 mM) had no synergistic effects with cyclic AMP on follicular testosterone production. However, these methylxanthines inhibited the incorporation of ^{3}H-uridine (35 to 68%) and ^{3}H-amino acids (45 to 69%) into follicular RNA and protein.

Cyclic GMP (25, 10 and 1 mM) had no stimulatory effect on foll-

icular testosterone production or the uptake of ^{3}H-amino acids into protein. However, cyclic GMP (25 and 10 mM) significantly enhanced the uptake of ^{3}H-uridine into follicular RNA ($p < .01$).

These data collectively suggest that de novo RNA and protein synthesis are not required for acute LH-induced testosterone production in the rabbit follicle.

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ABBREVIATIONS AND TRIVIAL NAMES

androsterone	3α-hydroxy-5α-androstan-17-one
androstenedione	androst-4-ene-3,17-dione
cholesterol	cholest-5-en-3β-ol
dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
20α-dihydroprogesterone	20α-hydroxypregn-4-ene-3,20-dione
5α-dihydrotestosterone	17β-hydroxy-5α-androstan-3-one
estradiol-17β	estra-1,3,5(10)-trien-3,17β diol
estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
etiocholane	3α-hydroxy-5β-androstane
5α-pregnandiol	5α-pregnan-3α,20α-diol
pregnenolone	3β-hydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
17-hydroxyprogesterone	17α-hydroxypregn-4-ene-3,20-dione
3α,5β progesterone	3α-hydroxy-5β-pregnan-20-one
testosterone	17β-hydroxyandrost-4-en-3-one
A^{o}	angstrom unit (10^{-7} millimeters)
ACTH	adrenocorticotrophin
AIB	α-amino-isobutyrate
AMD	actinomycin D
ATP	adenosine triphosphate
C	carbon atom
^{o}C	degrees centigrade

Ci	Curie
cpm	counts per minute
cyx	cycloheximide
cyclic AMP	adenosine 3',5' cyclic monophosphate
cyclic CMP	cytidine 3',5' cyclic monophosphate
cyclic GMP	guanosine 3',5' cyclic monophosphate
cyclic IMP	inosine 3',5' cyclic monophosphate
cyclic UMP	uridine 3',5' cyclic monophosphate
$CHCl_3$	chloroform
dibutyryl cyclic AMP	N^6,O^2-dibutyryl adenosine 3',5' cyclic monophosphate
FSH	follicle stimulating hormone
g	gram
Gpp(NH)p	guanylylimidodiphosphate
GTP	guanosine triphosphate
h	hours
hCG	human chorionic gonadotrophin
KOH	potassium hydroxide
Kd	dissociation constant
l	liter
LH	luteinizing hormone
M	molarity (mole/liter)
mM	millimolar
m	meter
mm	millimeter
MEM	modified Eagles medium
Mg	magnesium

Mn	manganese
min	minute
MeOH	methanol
N	normality
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaF	sodium fluoride
NaOH	sodium hydroxide
PP	pyrophosphate
PCA	perchloric acid
PMSG	pregnant mares serum gonadotrophin
POPOP	1,4-bis-[-2(5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
pg	picograms (10^{-12} gram)
pur	puromycin
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
S	Svedberg unit
SDS	sodium dodecyl sulphate
S.E.M.	standard error from the mean
theo	theophylline
µCi	microCurie
µg	microgram

µl	microliters
UV	ultraviolet

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PREFACE

"Never measure the height of a
mountain until you have reached the top.
Then you will see how low it was."
(Dag Hammarskjold, Markings)

INTRODUCTION

The term "gonadotropic hormone" or "gonadotropin" refers to those hormones which stimulate the gonads[1]. With regard to the ovary, the primary gonadotropins are luteinizing hormone (LH) and follicle stimulating hormone (FSH).

During the estrous cycle, just before ovulation, LH and FSH are secreted in a surge-like fashion by the anterior pituitary in response to rising levels of estrogens[2]. The importance of LH and FSH is exemplified by the fact that hypophysectomy leads to the arrest of all ovarian functions[3]. Classically, LH was thought to regulate steroidogenesis, ovulation and luteinization, while FSH promoted follicular growth and development. Recent studies suggest a more complex interaction between LH and FSH in the regulation of ovarian functions[3].

The aim of the work reported here has been to elucidate the mechanism of action of LH on steroidogenesis in the rabbit ovarian follicle. Specifically, the role of ribonucleic acid (RNA) and protein synthesis in LH-induced steroidogenesis has been examined.

This introduction will attempt to summarize the current knowledge regarding the subcellular mechanism of LH (and FSH) action on follicular steroidogenesis.

FIGURE 1. Cross-section of a Graafian follicle. Letters correspond to, antrum (A), basement membrane (BM) cumulus oophorus (C), membrane granulosa (G), oocyte (O), theca externa (TE), theca interna (TI), vascular blood supply (V), and zona pellucida (Z).

Follicular Structure

The mammalian ovary contains follicles in all stages of development. The growing follicle may be assigned to one of three categories[4]: (i) primordial follicle - classified as having an oocyte surrounded by a single layer of spindle shaped (flat) granulosa cells; (ii) primary follicle - characterized by multi-layered cuboidal granulosa cells, a band of mucoidal substance (zona pellucida), well defined theca interna cells and an autonomous blood supply; (iii) secondary follicle - characterized by the formation of a fluid filled antrum, the proliferation of theca and granulosa cells, and the distinct formation of the cumulus oophorus and enlargement of the oocyte.

The mature or Graafian follicle appears as a translucent sphere on the surface of the ovary and has the following structural features (Figure 1): (i) antrum, (ii) basement membrane, (iii) cumulus oophorus, (iv) membrane granulosa, (v) oocyte, (vi) theca externa, (vii) theca interna, (viii) vascular blood supply, and (ix) zona pellucida.

Electron microscopic examination of the follicle has revealed a number of ultrastructural differences between the cells of the membrane granulosa and theca interna[5]. The theca interna cells are characterized by abundant mitochondria with tubular cristae, smooth tubular endoplasmic reticulum, a poorly developed Golgi complex and numerous lipid droplets.

In contrast, the granulosa cells possess sparse, oval shaped mitochondria with transverse cristae, rough endoplasmic reticulum, a well defined Golgi complex and lipid droplets which differ in size and number from those of the theca cells.

Follicular Steroidogenesis - Biosynthetic Pathways

The Graafian follicle has the capacity to synthesize the three major classes of sex steroids - androgens, estrogens and progestins - from acetate [6-11]. Our present knowledge of the steroid biosynthetic pathways is the cumulative efforts of scientists for two decades working with such steroid producing glands as the adrenal, testis and ovary. A brief description of the gonadal steroidogenic pathways (reviewed by Bhavnani and Woolever[12]) is shown in Figure 2.

Acetate (2C), in the form of acetyl coenzyme A, undergoes a series of condensation reactions in the cytoplasm to form cholesterol (27C). The cholesterol enters the mitochondria where the side chain is hydroxylated at positions C-20 and C-22 by the 20- and 22- hydroxylase enzymes. The 20-22 desmolase enzyme then cleaves the C-20,22 bond yielding the 21C steroid compound: pregnenolone. Molecular oxygen and NADPH are required for cholesterol side chain cleavage.

Once formed, pregnenolone enters the cytoplasm for further metabolism. The cytoplasmic enzymes, Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-isomerase, convert pregnenolone to progesterone. NAD is required as cofactor. Hydroxylation of pregnenolone and progesterone at the C-17 position by the microsomal enzyme 17 α-hydroxylase (NADPH required), followed by cleavage of the C-17 side chain by 17-20 desmolase, yields

two 19C keto androgens: dehydroepiandrosterone and androstenedione. The dehydroepiandrosterone can either be secreted directly or converted by Δ^5-3 β-hydroxysteroid dehydrogenase and Δ^5-isomerase to androstenedione. Reduction at the 17-keto position by 17β -dehydrogenase converts androstenedione to testosterone. In addition, both dehydroepiandrosterone and androstenedione can be aromatized to form estradiol-17 β and estrone.

Source of Follicular Steroidogenesis: Theca or Granulosa Cells

The source of follicular steroid production, particularly estrogens, has been disputed. One hypothesis is that the theca interna are the source of estrogens and androgens, while the granulosa cells secrete pregnenolone, progesterone and 20α-dihydroprogesterone [13-15]. A second hypothesis is that the granulosa and theca cells are both necessary for estrogen biosynthesis; the theca cells synthesize androgens while the granulosa cells aromatize these androgens to estradiol-17 β and estrone[16-25]. A third hypothesis is that both granulosa and theca cells produce estrogens[26].

Follicular Steroidogenesis: Effect of LH

Although the pattern of steroidogenesis during the estrous cycle is species specific, certain generalities can be made[27]. The Graafian follicle, of every species studied, is the primary source of preovulatory estrogens. During the follicular phase of the cycle, low levels of circulating gonadotropins (LH and FSH) stimulate the follicles to secrete small amounts of androgens, estrogens and progestins. The

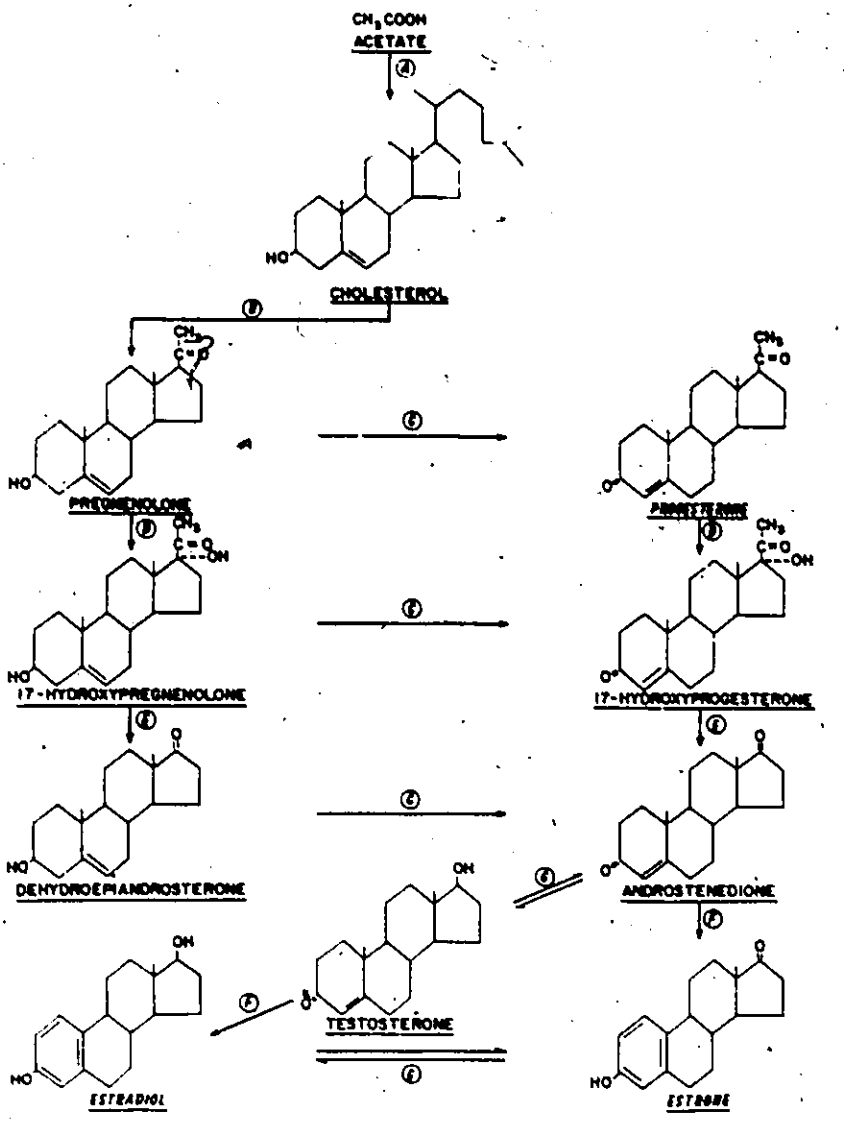

FIGURE 2. Steroid biosynthetic pathways. Key enzymes in the pathway are represented alphabetically:

(A) several enzymes; (B) 20, 22 desmolase; (C) 3β-hydroxysteroid dehydrogenase and Δ^5-isomerase; (D) 17α-hydroxylase; (E) 17-20 desmolase; (F) aromatase; and (G) 17β-hydroxysteroid dehydrogenase.

gonadotrophin surge stimulates increased production of androgens, estrogens and progestins which are then released into the ovarian vein and follicular fluid. Shortly after the gonadotropin surge, follicular steroidogenesis declines and remains at low levels until the next estrous cycle.

Two in vitro experimental approaches have been successfully used to study steroidogenesis in the ovarian follicle: (i) incubation of isolated intact follicles; and (ii) incubation of isolated theca or granulosa cells. Graafian follicles have been isolated by microdissection from the ovaries of the rabbit [9-11, 28, 29] and rat [30-32].

Isolated rabbit follicles incorporated ^{14}C-acetate into estrogens [6, 9-11]. Mills et al.[9] observed that both LH (0.0025-2.5 µg/ml) and FSH (1-100 µg/ml) stimulated approximately 5- to 15-fold increases in the incorporation of ^{14}C-acetate into estrone and estradiol-17 β in isolated rabbit follicles after 3 hours. The stimulatory effect of FSH was attributed to LH contamination since pre-treatment of FSH with either anti-LH serum or 6 M urea abolished the stimulatory effect.

Mills and Savard[10, 11] also studied the incorporation of ^{14}C-acetate into steroids from preovulatory rabbit follicles isolated before and after mating. They found that unstimulated follicles secreted small amounts of radioactive estrogens, androgens and progestins, into the incubation medium. Exposure of the follicles to LH (0.025 µg/ml) for 3 hours in vitro showed testosterone to be the major radioactive product, followed closely by 17α-hydroxyprogesterone. Follicles isolated 2 hours post-coitus synthesized predominantly

17α-hydroxyprogesterone, while ovulatory (12 hours post-coitus) follicles secreted progesterone. These findings were confirmed by YoungLai [29, 33, 34], using steroid radioimmunoassay procedures. YoungLai[35] also showed that a brief (less than 1 minute) in vitro exposure of the follicle to LH (5 μg/ml) was sufficient for later stimulation of steroidogenesis. A similar post-coital pattern of steroid secretion was observed in the plasma[36-39], follicular fluid[28,40] and follicular tissue[40] of the rabbit.

In rat follicles, isolated during proestrus Lieberman et al.[41] found estradiol-17β to be the major steroid secreted into hormone free medium during a 12-hour incubation period. Addition of LH (5 μg/ml) to these preovulatory rat follicles resulted in considerably more estradiol-17β than androstenedione and progesterone being released into the incubation medium after 4 hours. However, from 6 to 12 hours, progesterone was the major product observed (50-fold increase) with lesser amounts of estrogens (20-fold) and androgens (5-fold) being produced. These findings support the value of the intact Graafian follicle in vitro as an experimental model to study the preovulatory pattern of steroidogenesis.

Regulation of Estrogen Production: Role of FSH

FSH may regulate estrogen biosynthesis in the ovarian follicle. In the early 1940's, Fevold[42] and Greep[43], using uterine weight bioassay, first demonstrated that FSH and LH complemented one another in the stimulation of estrogen secretion in immature hypophysectomized rat ovaries in vivo. For the past thirty years, numerous in vivo

and *in vitro* studies have demonstrated the synergism between LH and FSH on ovarian estrogen biosynthesis, yet only a few studies have tried to define the nature in biochemical terms.

Moon *et al*[20] showed that ovaries from immature hypophysectomized rats, incubated *in vitro* for 72 hours with testosterone (10^{-7} M) responded to highly purified FSH (0.25 µg/ml) with significant increases (up to 900%) in estradiol-17β production. In subsequent studies, these authors[21] reported that monolayer cultures of rat granulosa cells, also synthesized estradiol-17β (163-fold increase) from exogenous testosterone in the presence of FSH, but not LH. Using an identical cell system, Erickson and Hseuh[24] found that in the presence of androstenedione (10^{-7}M), FSH (1-100 ng/ml), but not LH (200 ng/ml), stimulated a dose related (6-80 fold) increase in estrogen production. No estrogen secretion was observed in the absence of androstenedione.

Recently, Fortune and Armstrong[22, 23] proposed a "two cell -two gonadotropin" model for control of estrogen synthesis in the Graafian follicle. Their model was based on *in vitro* experimental evidence in which LH, but not FSH, stimulated testosterone production in cultured rat thecal cells. Monolayer cultures of granulosa cells did not synthesize testosterone, but under the influence of FSH exogenous testosterone or endogenous testosterone (from theca cells) was converted to estrogens. This indicated that LH-induced testosterone, synthesized by the theca cells, was transported to the granulosa cells and aromatized to estrogens under the influence of FSH. This theory is supported by similar observations

in ovarian follicles of the rat[24], hamster[18], sheep[44] and human[25, 45]. However, thecal cell preparations from rabbit[19, 28, 46], mare[47], hamster[18] and human[48] follicles have been found to synthesize androgen, estrogens and progestins.

It may be that both cell types (theca and granulosa) have specific aromatizing systems, the nature of which are dependent upon the species in question. The problem is pertinent and its solution may provide the cellular site of the action of gonadotropins on follicular steroidogenesis.

Luteinizing Hormone (LH)

LH (MW 28,000) is a glycoprotein composed of two dissimilar polypeptide subunits designated α and β. The carbohydrate moieties account for approximately 12-17% of the molecular weight of LH[49-51]. The primary amino acid sequences for the α and β subunits of LH for a number of species has been determined[50, 52]. The α (96 residues) and β (119 residues) subunits of bovine and porcine LH appear to be identical. However, interspecies variations also exist. For example, the extent of homology between the human and bovine LH α subunits vary by 22%, and the LH β subunits vary by 68%[50, 52].

The individual LH α and β subunits are biologically inactive[53, 54]. The LH receptor recognizes only the native hormone, not isolated subunits. Chemical modification of functional amino acid residues on both α and β subunits, followed by recombination studies, indicates the importance of the intact hormone for

biological activity[55].

Mechanism of Luteinizing Hormone Action

The current model depicting LH action on steroidogenesis is shown in Figure 3 (reviewed by Channing and Tsafriri[3]). Initially, LH is thought to bind to specific membrane receptors to stimulate adenylate cyclase activity and adenosine 3', 5' cyclic monophosphate (cyclic AMP) formation. The increased cyclic AMP activates a protein kinase which can act either by enhancing RNA synthesis or directly stimulating de novo protein synthesis. The activation of pre-existing regulatory enzyme has also been proposed[56]. Since the rate-limiting step is thought to occur in the mitochondria[57], a regulatory protein may act to: (i) increase cofactor (NADPH) availability; (ii) increase cholesterol availability; (iii) facilitate cholesterol transport into the mitochrondria; (iv) enhance cholesterol side chain cleavage activity; and (v) regulate the release of pregnenolone from the mitochondria. A more detailed description will be discussed later.

LH Receptor

Evidence that LH specifically binds to plasma membrane receptors of the granulosa and theca cells is supported by radioautographic and cell fractionation studies[58-62] in which ^{125}I-LH or ^{125}I-hCG bound preferentially to follicular theca and granulosa cell membranes. From LH receptor studies in the intact ovary, corpus luteum and testis,

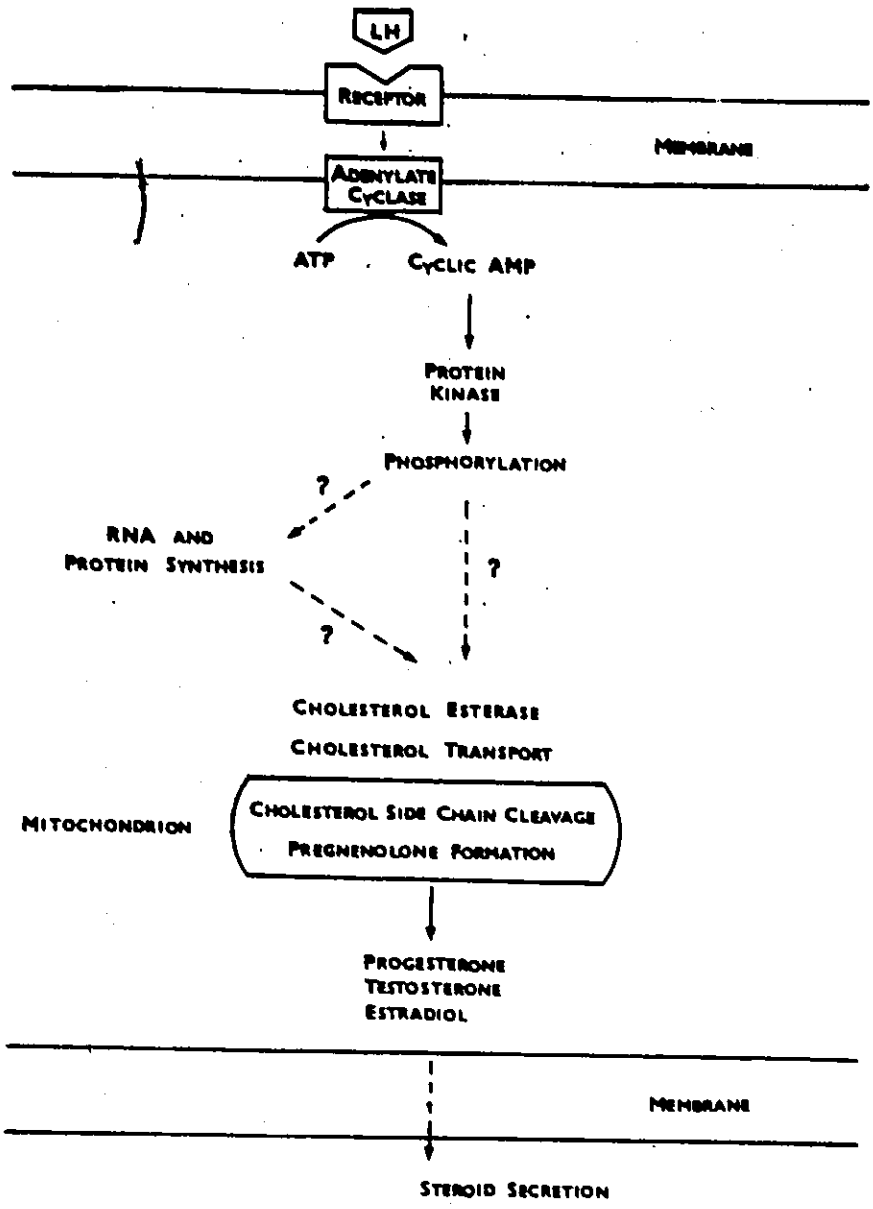

FIGURE 3. Schematic representation of the subcellular regulation of gonadal steroidogenesis by LH.

certain chemical and physical properties have been identified (reviewed by Ryan and Lee[63]).

The LH receptor appears to be a lipoprotein containing carbohydrate moities[64, 65] and exhibits distinct physical properties, such as, sedimentation coefficient (6.0-6.8 S), stokes radius (60-64Å) and a molecular weight of approximately 20000 daltons[66].

The LH receptor possesses distinct binding characteristics common to all LH sensitive tissues studied, namely, structural and steric specificity for LH, saturability, cellular specificity, high affinity for LH (Kd 1.4 to 1.7x10^{-10} M) and a relative degree of reversibility[63].

LH Receptor Regulation

The capacity of follicular tissue to bind LH increases with follicular maturation[67-71]. Follicles from immature or hypophysectomized female rats contain very few LH receptors, which are predominantly localized in the theca and interstitial tissue. In contrast, FSH receptors are exclusively found in the granulosa cells[58].

The regulation of the LH-receptor content, in the granulosa cells at least, appears to be under hormonal control. Sequential treatment of immature hypophysectomized female rats for 1 to 4 days with estradiol-17β, followed by 2 days with FSH, resulted in a 2-fold increase in ^{125}I-hCG binding in granulosa cells[72-74]. This ability of FSH to promote LH receptor affinity was directly proportional to estrogen pretreatment. In contrast, LH has been shown to act on

granulosa cells of Graafian follicles from these same animals to decrease the receptor affinity for LH and FSH[75]. The mechanism involved in modulation (increase or decrease) of these LH receptors remains unknown.

LH Stimulation of Adenylate Cyclase

The specific binding of LH to the plasma membrane of follicular cells has been correlated with activation of adenylate cyclase[31, 76-82]. Activation of follicular adenylate cyclase has been shown to be an extremely rapid, dose dependent process[77-79, 83], with intracellular cyclic AMP being significantly increased within 20 seconds after exposure of the follicle to LH[83]. This increase in cyclic AMP is maximal within 5 to 15 minutes and rapidly declines thereafter[81-83]. The follicle is thence refractory (desensitized) to further stimulation by LH (reviewed by Lamprecht et al.[84]).

Regulation of Adenylate Cyclase Activity: Desensitization

Desensitization, i.e. the inability of adenylate cyclase to be further stimulated by LH, may provide a regulatory control mechanism for LH action in the follicle. In vivo and in vitro exposure of Graafian follicles to high levels of LH results in rapid desensitization of adenylate cyclase responsiveness[76-79, 84-87] and loss in cyclic AMP production[77-79, 85] and steroidogenesis[11, 34]. In the isolated rabbit follicle, LH (0.1 µg/ml) stimulated a 10 to 12 fold increase in adenylate cyclase activity after 2 hours[78]. Higher LH concentrations

(0.8 or 8 µg/ml) had an almost complete inhibitory effect on adenylate cyclase activity. In vivo administration of an ovulatory dose of hCG to the female rabbit, one minute prior to sacrifice, resulted in 70% decrease in follicular adenylate cyclase activity.[78]

The mechanism responsible for refractoriness of adenylate cyclase may occur through any number of changes in the pathway from LH binding to generation of cyclic AMP. Several hypotheses have been advanced to explain this phenomenon (reviewed by Lamprecht et al.[84]; Liljekvist et al.[88]). These include: (i) a decrease in the number of LH binding sites; (ii) alterations in LH receptor-cyclase coupling; (iii) synthesis or induction of an inhibitory protein; and (iv) activation of phosphodiesterase.

Lamprecht et al.[84] tested these hypotheses in cultured rat Graafian follicles and presented evidence in favor of hypotheses (ii) and (iii), primarily by a process of elimination. Desensitization of adenylate cyclase to LH was not accompanied by a decrease in the total number of LH binding sites. Mills and McPherson[89] arrived at similar conclusions after rabbit Graafian follicles were rendered refractory by the endogenous LH surge of mating. The activation of phosphodiesterase resulting in enhanced rate of cyclic AMP degradation, also seemed unlikely since follicles were desensitized to LH in the presence of methylxanthines[84]. Moreover, LH had previously been shown to have no effect on phosphodiesterase activity in rat[90] and rabbit[91] ovaries. Nevertheless, follicles desensitized to LH responded normally to other stimulators of cyclic AMP (e.g. NaF, GTP

or its imido analog Gpp (N H)p, suggesting that impairment of LH receptor-cyclase coupling may be involved. The possibility of a putative protein inhibitor which could interfere with LH receptor-cyclase coupling was proposed when desensitization was prevented by transcriptional and translational inhibitors, Actinomycin D and cycloheximide[84].

The physiological significance of LH induced refractoriness of the adenylate cyclase system in the follicle is not obvious. It is possible that desensitization of the follicle may play an important role in the final stages of follicular development, steroidogenesis and ovulation, since the phenomenon is observed in vivo only after the preovulatory LH surge[11, 34, 78].

Adenosine 3', 5' Cyclic Monophosphate (Cyclic AMP)

Cyclic AMP is considered a "second messenger" for many polypeptide hormones in their respective target tissues[92]. In the mammalian ovary, as in most other tissues, the endogenous cyclic AMP concentration is in the order of 10^{-7} to 10^{-8} moles/kg wet weight[93]. The cyclic AMP concentration of any tissue depends on the rates of biosynthesis and degradation, catalyzed by the enzymes adenylate cyclase (E.C.4.6.1.1) and phosphodiesterase (E.C.3.1.4.1), respectively.

A. Formation

Cyclic AMP is formed from adenosine triphosphate (ATP) by a reaction catalyzed by membrane associated adenylate cyclase. The

divalent cations, magnesium or manganese, are required and an inorganic pyrophosphate (PP) is formed[92].

$$\text{ATP} \xrightarrow[\text{Mg++ or Mn++}]{\text{Adenylate Cyclase}} \text{Cyclic AMP} + \text{PP}$$

In isolated Graafian follicles of rabbits or rats, measurement of the effect of LH on the rate of conversion of either ^{3}H-adenosine / via ATP to cyclic ^{3}H-AMP[31, 76, 85, 94]; or conversion of ^{32}P-ATP to cyclic-^{32}P-AMP, resulted in increased cyclic AMP formation[77, 79, 86]. This suggested that cyclic AMP formation was due to the stimulation of adenylate cyclase by LH.

B. Degradation

It is currently accepted that the major pathway for the biochemical degradation of cyclic AMP, in all tissues, involves hydrolysis of the 3' bond of cyclic AMP yielding the noncyclic 5' adenosine monophosphate, 5'-AMP. The reaction is catalyzed by phosphodiesterase enzymes, isolated in multiple forms from the cytoplasm and membrane fractions of various tissues (reviewed by Amer and Kreighbaum[95]). The reaction is non-reversible and requires a variety of divalent cations, including magnesium, and possibly the presence of protein activators to express maximal activity[95].

In ovarian tissues, very little is known about the role of phosphodiesterases. Electrophoretic isolation of phosphodiesterases from the rat ovary[96] has revealed two forms of phosphodiesterase with apparent molecular weights of 135000 and 150000. Recently,

FSH, but not LH, was shown to activate phosphodiesterase *in vitro* in the rat ovary[90], suggesting another control site for FSH action.

Criteria for Establishing Cyclic AMP Involvement

Although cyclic AMP was originally discovered as a "second messenger" for glucagon and epinephrine stimulation of hepatic gluconeogenesis, it is now accepted as an intracellular mediator for a number of hormones, including LH. Robinson *et al.*[92] proposed several criteria to establish the involvement of cyclic AMP in a particular hormone action. With regard to LH-induced steroidogenesis in the gonads, these criteria may be summarized as follows: (i) LH should modulate adenylate cyclase activity; (ii) LH should induce a measurable change in the concentration of endogenous cyclic AMP levels; (iii) exogenous cyclic AMP should mimic the effects of LH on the subcellular events leading to steroidogenesis; and (iv) inhibitors of phosphodiesterases, such as methylxanthines, should mimic (when used alone) or potentiate (when used with) the effects of LH and cyclic AMP on steroidogenesis.

In many respects, each of these criteria have been successfully met. The first two criteria have already been discussed. The remainder of this introduction will concentrate on the role of cyclic AMP in steroidogenesis (criteria iii and iv), emphasizing possible subcellular sites of action.

Cyclic AMP and Follicular Steroidogenesis

Cyclic AMP has been shown to mimic the effects of LH on steroidogenesis in a number of ovarian tissue components including the corpus luteum[97-100], interstitial tissue[98, 101, 102] and Graafian follicle[103-105].

Mills[104] compared the effects of various concentrations of LH (0.005-50 µg/ml) and cyclic AMP (0.2-40 mM) alone, or together, on the steroid synthesizing capacity of the intact rabbit ovarian follicle after 3 hours in vitro. Both LH and cyclic AMP stimulated a dose dependent increase in all steroids measured in the incubation medium (progesterone, 17α-hydroxyprogesterone, testosterone, and estradiol-17β), with testosterone and 17α-hydroxyprogesterone being the major secretory products. The minimum threshold of steroidogenic response was produced by 0.005-0.05 µg LH/ml and 0.2-2mM cyclic AMP, while maximal responses were elicited at 5 µg LH/ml and 20 mM cyclic AMP. The finding that the combined steroidogenic effects produced by maximal concentrations of LH plus cyclic AMP were not additive, implied that both agents stimulated steroidogenesis by identical mechanisms.

YoungLai[105], also using isolated rabbit ovarian follicles, compared the effects of a variety of 3', 5' cyclic nucleotides on follicular steroidogenesis. Cyclic AMP (7.5 mM), dibutyryl cyclic AMP (2.5 mM) and cyclic CMP (10 mM), but not cyclic IMP (2.5 mM), cyclic GMP (3.5 mM) or cyclic UMP (10 mM), enhanced follicular steroidogenesis in vitro: androgens increased from 0.1 ng/ml to 10 ng/ml and progestins from 0.5 ng/ml to 3 ng/ml. In all cases,

follicular steroidogenesis was stimulated within 15-30 minutes, becoming maximal by 45-60 minutes, and declining only after the 3', 5' cyclic nucleotides were removed from the incubation medium. Theophylline (5mM), an inhibitor of phosphodiesterase, also stimulated follicular androgen and progestin production when used alone and synergized the steroidogenic responses of cyclic AMP. These results are consistent with the idea that cyclic AMP and perhaps other cyclic nucleotides, mediate the action of LH on follicular steroidogenesis.

Guanosine 3', 5' cyclic monophosphate (Cyclic GMP)

Guanosine 3', 5' cyclic monophosphate (cyclic GMP) has been also implicated as a second messenger in ACTH-enhanced steroidogenesis in the rat adrenal cortex[106]. A positive correlation between cyclic GMP accumulation, protein kinase activity and corticosteroidogenesis was reported[107].

In the isolated hamster follicle, LH has been shown to enhance cyclic GMP accumulation within 5 minutes[82]. In the isolated rabbit follicle, cyclic GMP was without stimulatory effect on steroidogenesis[105]. However, in the rat Graafian follicle, cyclic GMP was shown to stimulate prostaglandin synthesis[108], which in turn has been implicated in ovulation[109]. These findings collectively suggest a role for cyclic GMP in ovarian function.

Role of Protein Kinase

Since cyclic AMP-dependent protein kinase (ATP protein

phosphotransferase; E.C.2.7.1.37) was originally discovered by Walsh, et al.[110] to catalyze the phosphorylation of phosphorylase b kinase, two isoenzyme forms have been isolated from most tissues studied (reviewed by Nimmo and Cohen[111]). These have been referred to as 'type 1' and 'type 11' with respect to the order in which they are eluted on DEAE-cellulose chromatography and differ primarily in the size of their respective receptor subunits. In the rabbit Graafian follicle, type 1 and type 11 cyclic AMP-dependent protein kinase isoenzymes are regulated independently of one another[112-114]. Type 11 isoenzyme, when stimulated by LH, exhibits maximum activity during estrus and the preovulatory phase, whereas, type 1 activity predominates after corpus luteum formation. The physiological significance of these protein kinases or their role in the follicle is not known.

Increasing evidence indicates that the activation of cyclic AMP-dependent protein kinase may be an important step in the regulation of steroidogenesis. Parallel increases in LH-induced steroidogenesis and protein kinase activation have been demonstrated in the testis[115], ovary[116], and corpus luteum[117]. In Leydig cell suspensions, LH via cyclic AMP-dependent protein kinase enhanced the phosphorylation of three phosphoproteins which complemented the appearance of testosterone[118]. The role of these phosphoproteins in steroidogenesis is not known.

In ovarian tissues, cyclic AMP-dependent protein kinases have been shown to enhance RNA synthesis[119], phosphorylate nuclear and cytoplasmic phosphoproteins[112, 118, 119] and activate cholesterol side chain cleavage enzymes[120]. In reticulocytes, cyclic AMP-dependent

protein kinase phosphorylated the initiation factors (IF-E2 and IF-E3) known to be essential for eucaryotic protein synthesis[121]. In light of these multiple effects of the enzyme, it is a distinct possibility that gonadal steroidogenesis is controlled by the activation of cyclic AMP-dependent protein kinase.

Follicular RNA and Protein Synthesis

In the Graafian follicle, RNA and protein synthesis have been implicated in LH-induced steroidogenesis[32, 41, 122], oocyte maturation[32, 41, 123], ovulation[124-126], and luteinization[127]. Current thinking would suggest that these LH-induced events are a reflection of the genetic code transcribed to specific RNA species which in turn translate specific protein-enzymes to complete the message. However, these essential RNA and protein species have not been isolated in the Graafian follicle.

A. General Incorporation Studies

Given and colleagues[128] studied the *in vitro* incorporation of labeled precursors into RNA and protein in isolated rabbit ovarian follicles after pretreatment *in vivo* with gonadotropins. Injection of either LH or FSH for 1.5 hours *in vivo* resulted in a 60% increase in the incorporation of ^{14}C-uridine and ^{3}H-valine into follicular RNA and protein after 1 hour *in vitro*.

Mills[126] found that LH (5 µg/ml), added directly to the incubation medium, significantly stimulated the incorporation of

labeled methionine into protein (28% increase), but not uridine incorporation into RNA, in the rabbit follicle after 2 hours. FSH (5 µg/ml) and cyclic AMP (20 mM) stimulated both follicular RNA (~34%) and protein synthesis (~50%) during this same 2 hour period in vitro. In a later study, Mills and Felt[129] demonstrated that LH (5 µg/ml), but not FSH (5 µg/ml) or cyclic AMP (20 mM), caused a 35% increase in the transport of the non-metabolizable amino acid, ^{14}C-α-amino isobutylate (AIB), in the isolated rabbit follicle. This implied a role for LH on amino acid transport.

In pregnant mares serum gonadotrophin (PMSG) primed 30 day old rats, Nilsson[130] found that in vivo injection of LH (10 µg/rat), prior to in vitro incubation with ^{3}H-leucine, stimulated the incorporation of leucine into follicular protein by 22%. When these PMSG-primed follicles were incubated in vitro with LH, FSH or dibutyryl cyclic AMP, the incorporation of leucine into follicular protein was not seen. The difference between the in vitro incorporation of labeled precursors into RNA and protein in the rabbit and rat Graafian follicle has not been reconciled.

B. Role in Follicular Steroidogenesis:

Only two studies have attempted to directly correlate LH-induced steroidogenesis with de novo RNA and protein synthesis in the Graafian follicle and both have been unsuccessful[32, 41]. In cultured rat follicles, isolated during proestrus, Tsafriri et al.[32] were unable to demonstrate a direct stimulatory effect of LH (5 µg/ml) on the

incorporation of labeled precursors into follicular RNA and protein. Nevertheless, LH-induced progesterone synthesis was prevented by cycloheximide (5 μg/ml) and puromycin (80 μg/ml), concentrations which inhibited protein synthesis by 60 and 90%, respectively. Actinomycin D (8 μg/ml) reduced follicular RNA synthesis by more than 80% and completely abolished the LH-induced progesterone synthesis. Lower doses of Actinomycin D (0.8 and 0.08 μg/ml) inhibited both RNA and progestin synthesis in a dose dependent fashion. In later studies, Lieberman et al.[41] showed that puromycin (80 μg/ml), added 1, 2, or 3 hours after LH addition, suppressed follicular progesterone synthesis throughout a 6 hour incubation period. In contrast, when Actinomycin D (8 μg/ml) was added after 2 hours, LH-induced steroidogenesis continued undisturbed for the remainder of the incubation. From these studies, Lieberman et al.[41] proposed that LH-induced steroidogenesis was under transcriptional control in the rat follicle. Furthermore, a stable Actinomycin D-sensitive nucleic acid (presumably messenger RNA) was synthesized during the first 2 hours of LH stimulation, which was necessary for the synthesis of a labile regulatory protein. This regulatory protein was essential for steroidogenesis.

In the rabbit ovarian follicle, YoungLai[122] found that LH-induced androgen production was also prevented by puromycin (80 μg/ml) and cycloheximide (50μg/ml), but not by high concentrations of Actinomycin D (160 μg/ml) in vitro. These findings confirmed the work of Gorski and Padnos[131] in rabbit ovarian homogenates. Therefore,

it was concluded that LH-induced steroidogenesis, in rabbit ovarian tissue at least, was controlled at the translational level, possibly by the activation of a pre-formed messenger RNA species or the stimulation of de novo protein synthesis[122]. The different effects of Actinomycin D on LH-induced steroidogenesis in the rabbit and rat follicles may reflect different control mechanisms for steroidogenesis.

Hypothetical Models for the Role of Protein Synthesis in the Regulation of Steroidogenesis by LH

The regulation of steroidogenesis by a protein with a short half life has been suggested for the testis[1, 56, 132-135], adrenal cortex[136-144], ovary[131, 145, 146], Graafian follicle[32, 41] and corpus luteum[99, 147-149]. Three models are illustrated in Figure 4 which have been put forth to describe the role of protein synthesis in LH-enhanced steroidogenesis (reviewed by Janszen et al.[56])

(i) *Model 1* - the regulation of steroidogenesis by the de novo synthesis of a protein with a short half life;

(ii) *Model 2* - indirect mediation of steroidogenesis by a trophic hormone independent (regulatory) protein with a short half life;

(iii) *Model 3* - activation of pre-existing protein essential for steroidogenesis.

Evidence for each model will follow in the discussion.

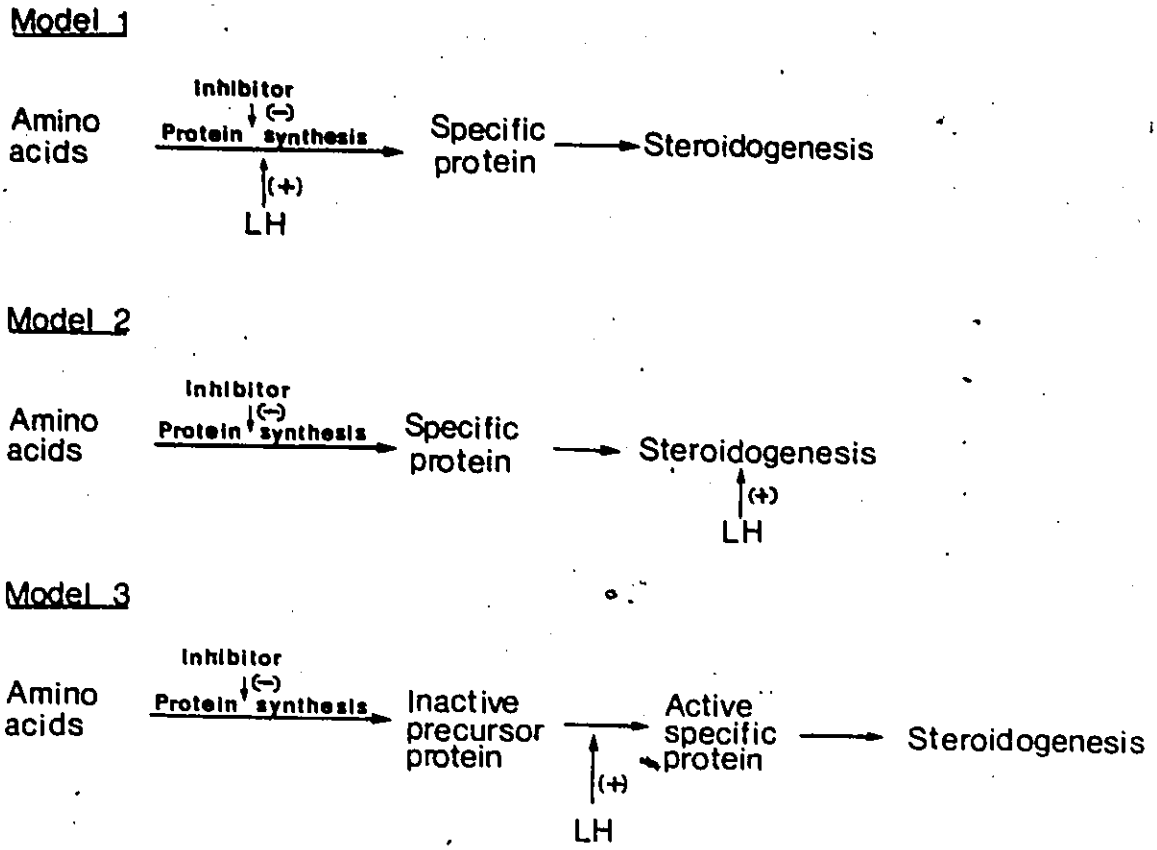

FIGURE 4. Hypothetical models depicting role of protein synthesis in the regulation of steroidogenesis by LH.

Possible Loci of Action of a Regulatory Protein

Since the rate-limiting step in the steroidogenic pathway is believed to be in the mitochondria, between cholesterol and pregnenolone formation[57], a labile regulator protein might act to increase: (i) cofactor (NADPH) availability; (ii) cytoplasmic cholesterol availability; (iii) cholesterol translocation into the mitochondria; (iv) cholesterol side chain cleavage activity; and (v) efflux of pregnenolone out of mitochondria.

A. Via increased cofactor: NADPH

The cholesterol side chain cleavage enzymes reside in the mitochondria[57]. This mitochondrial enzyme complex is composed of an NADPH-specific flavoprotein dehydrogenase, an iron sulfur protein (ovarian ferredox) and cytochrome P-450 as terminal oxidase[150]. NADPH is generated in the cytoplasm primarily as the result of glucose oxidation via the pentose phosphate pathway. Intra-mitochondrial NADPH may then be increased by way of the malate shuttle [151, 152].

Haynes and Berthet[153] and Haynes et al.[154] originally proposed (for the adrenal) that the availability of extra-mitochondrial NADPH was rate limiting in steroidogenesis. In ovarian tissues, a number of theories have evolved using the Haynes and Berthet postulate as a basis for LH control of steroidogenesis. These theories suggest a number of ways by which cytoplasmic NADPH may be increased (reviewed by Marsh[155]; Dorrington[156]): (i) via increased transport of glucose across the cell membrane[157-159]; (ii) via stimulation of

glycogen phosphorylase activity thereby increasing glucose 1-phosphate and eventually glucose 6-phosphate formation[160-162]; and (iii) via increased glucose 6-phosphate dehydrogenase activity which catalyzes the dehydrogenation of glucose 6-phosphate yielding NADPH[163-165]. These theories will not be discussed further, except to note that the evidence is inconclusive in support of a rate limiting role for NADPH in LH-induced steroidogenesis.

B. Via Increased Substrate: Cholesterol

LH may regulate follicular steroidogenesis by enhancing the availability of free, unesterfied cholesterol at the expense of cholesterol esters. Conceivably, this could be done in two ways: (i) by direct stimulation of cholesterol esterase (sterol ester hydroxylase, E.C.3.1.1.13) which catalyzes the hydrolysis of cholesterol esters; or (ii) by inhibition of cholesterol ester synthetase (sterol ester synthetase, E.C.6.1.6.11) which catalyzes the conversion of cholesterol into acyl esters. In ovarian tissues, the evidence for LH activation of either of these two enzymes is equivocal (reviewed by Marsh[155]). At present, the control mechanisms have not been worked out.

In the adrenal cortex, cyclic AMP-dependent protein kinase phosphorylation of purified cholesterol esterase increased cholesterol ester hydrolysis by 130%[143]. Cycloheximide had no effect on ACTH-induced cholesterol esterase activity, indicating that the activation of the enzyme rather than its de novo synthesis was essential[166]. These studies implied that the enzyme existed prior to phosphorylation

in an inactive dephosphorylate form.

C. Via Increased Transport of Cholesterol

Hermier et al.[99], using intact luteinized rat ovaries in vitro, suggested that an LH inducible-cycloheximide sensitive protein factor was involved in the translocation of cholesterol into the mitochondria. However, Leaver and Boyd[149] have clearly demonstrated that ^{14}C-cholesterol uptake, by mitochondria isolated from luteinized rat ovaries, was not affected by the in vivo administration of LH or cycloheximide 15 to 60 minutes prior to sacrifice. In the same experiment, however, cycloheximide did inhibit LH-induced cholesterol side chain cleavage by 40-50%. It was concluded that cholesterol transport into the mitochondria is primarily a physical partition whereas intra-mitochondria cholesterol side chain cleavage requires a cycloheximide sensitive (protein) factor. These results are supported by similar observations in the adrenal cortex[142, 144, 167].

D. Via Increased Cholesterol Side Chain Cleavage

Evidence for a cycloheximide or puromycin sensitive site in the mitochondria has been presented for the adrenal cortex[142, 144, 167, 168] and corpus luteum[148, 149]. These studies suggested that this putative protein factor is synthesized in the cytosol and acts inside the mitochondria on the binding of cholesterol to the side chain cleavage enzyme, cytochrome P-450. Nevertheless, Arthur and

Boyd[148] also demonstrated that puromycin can interact directly with luteal mitochondria cytochrome P-450 to inhibit cholesterol side chain cleavage in vitro. Such adverse effects of these translational inhibitors have not been adequately documented.

E. Via Increased Efflux of Pregnenolone from Mitochondria

Koritz and Hall[169] originally proposed that ACTH-regulated adrenal steroidogenesis by stimulating the efflux of pregnenolone out of the mitochondria, thereby releasing end product inhibition on cholesterol side chain cleavage. To date, there has been no supportive evidence for such a hypothesis in gonadal tissues (reviewed by Marsh[155]).

The Rabbit as an Experimental Model

The female rabbit, being a reflex ovulator, normally remains in continuous estrus until it is mated[170]. Ovulation occurs 10 to 12 hours post-coitus or after administration of an ovulatory dose of LH[171]. Coitus stimulates a rapid rise in serum LH, which is evident within 30 minutes, reaching a peak by 2 hours and declining by 4 to 6 hours[39, 172]. During this 12 hour preovulatory period, a number of biochemical and morphological changes in the follicle appears to be initiated by LH. Such changes may be summarized as follows:

(i) Ovarian steroids (particularly estradiol-17β, testosterone and 20α-hydroxyprogesterone) are secreted into the ovarian

vein[36-39] and follicular fluid[28, 40] and follow a pattern parallel to LH secretion;

(ii) Cyclic AMP levels rapidly increase within minutes and gradually decrease after 2 to 4 hours post coitus[85];

(iii) Protein kinase activity maximally increases within 10 minutes and remains elevated until ovulation[112];

(iv) De novo RNA and protein synthesis becomes evident after 2 hours post-coitus and is maintained until ovulation[125, 126];

(v) Prostaglandin synthesis increases only during the final 4 hours before ovulation[173, 174];

(vi) Morphological examination of follicles destined to ovulate showed connective tissue gradually decomposing and the granulosa cells beginning to luteinize[175].

At present, very little is known about the interrelationship of each of these subcellular events with one another or their function.

Purpose of this Research

The purpose of the research discussed in this thesis was to examine the correlation (and role) of RNA and protein synthesis with LH-stimulated steroidogenesis by the intact rabbit ovarian follicle *in vitro*.

In the rabbit follicle, since the *in vivo* synthesis of LH-induced RNA and protein[125, 126] are mimiced *in vitro*[126], the isolated follicle provided a means for investigating the role of RNA and protein synthesis in the control of follicular steroidogenesis. The

intact rabbit follicle *in vitro* offers an attractive experimental model whereby the concentration of LH and various test substances can be carefully controlled and their effects on steroidogenic processes measured. However, one major difficulty lies in the fact that the rabbit follicle consists of two principal cell types and effects that are seen with the whole follicle may not necessarily occur at the single cell level. Despite such difficulties, attempts were made to elucidate the role of RNA and protein synthesis in rabbit follicular steroidogenesis. It was of interest to determine:

(i) Whether LH could induce *de novo* RNA and protein synthesis in the rabbit ovarian follicle *in vitro*;

(ii) Whether cyclic AMP could mimic the effects of LH on *de novo* RNA and protein synthesis;

(iii) Whether a correlation existed between LH or cyclic AMP-induced steroidogenesis with *de novo* RNA and protein synthesis;

(iv) Whether a similar correlation existed between LH or cyclic AMP-induced steroidogenesis with RNA and protein synthesis, in the presence of inhibitors of transcriptional and translational prccesses;

(v) Whether a role (stimulatory or inhibitory) for cyclic GMP could be observed;

(vi) Whether inhibitors of phosphodiesterases could elicit effects similar to those of cyclic AMP on protein and RNA synthesis.

MATERIALS

ANTISERA: preparation, dilution, specificity

Antisera to androstenedione (S-1557#2), dehydroepiandrosterone (S-1507#7), estradiol-17β (S-1554#6) and progesterone (S-49#6) were kindly provided by Dr. G.E. Abraham, Department of Obstetrics and Gynecology, University of Southern California, Torrance, Ca., U.S.A.

Antiserum to testosterone was kindly provided by Robert W. Armstrong, Department of Medical Sciences, McMaster University, Hamilton, Ontario.

Specific details of these antisera are as follows:

Androstenedione antiserum

Antiserum (S-1557#2) was prepared by Abraham and Chakmakjian[176] by immunizing ewes against androstenedione-3-oxime-human serum albumin. A recommended dilution of 1/5,000 was used for radioimmunoassay (RIA). The antiserum cross-reacted with dehydroepiandrosterone (25%), androsterone (7.0%), ethiocholane (3%), testosterone (3%), 5α-dihydrotestosterone (1%), estrone (1%), and less than 0.01% with all other steroids tested.

Dehydroepiandrosterone antiserum

Antiserum (S-1507#7) was prepared by Buster and Abraham[177] by immunizing ewes against dehydroepiandrosterone-3β-monohemisuccinate-human serum albumin. A recommended dilution of 1/5,000 was used for RIA. The antiserum cross-reacted with androstenedione (12.5%) and less than

0.01% with all other steroids tested.

Estradiol-17β antiserum

Antiserum (S-1554#6) was prepared by Abraham et al.[178] by immunizing ewes against 6-keto-estradiol-17β-6 oxime-human serum albumin. A recommended dilution of 1/100,000 was used for RIA. The antiserum cross-reacted with estrone (0.6%) and less than 0.03% with all other steroids tested.

Progesterone antiserum

Antiserum (S-49#6) was prepared by Abraham et al.[178] by immunizing ewes against 11-desoxycortisol-21-monohemisuccinate-human serum albumin. A recommended dilution of 1/15,000 was used for RIA. The antiserum cross-reacted with 17α-hydroxyprogesterone (90%), 5α-dihydrotestosterone (2.3%), testosterone (1.2%), 20α-dihydroxyprogesterone (1.2%), 3α,5β-progesterone (0.5%), 5α-pregnandiol (0.3%) and less than 0.1% with all other gonadal steroids tested.

Testosterone antiserum

Antiserum was prepared by Armstrong[179] by immunizing female rabbits with testosterone-3-oxime-bovine serum albumin. A dilution of 1/3,000 was used for RIA. This antiserum cross-reacted with 5α-dihydrotestosterone (55%), androstenedione (0.6%), dihydroepiandrosterone (<0.1%), estradiol-17β (<0.1%) and progesterone (<0.1%.

Assay Standards

Standards for electrophoresis - cytochrome C, myoglobin, ovalbumin and albumin - were purchased as dry chemicals from Sigma Chemical Co., St. Louis, Missouri, U.S.A. These standards were made up in Dissolving

Buffer (0.5 mg/ml) and stored at -80°C.

RNA (yeast ribonucleic acid, type XI) and protein (bovine serum albumin, fraction V powder) assay standards were also purchased from Sigma Chemical Co. These RNA and protein standards were made up fresh in 0.3N KOH and 0.3 N NaOH, respectively, at a concentration of 1 mg/ml.

Steroid radioimmunoassay standards - androstenedione, dehydroepiandrosterone, estradiol-17β, progesterone and testosterone - were purchased from Steraloids Inc., Pawling, New York. These standards were prepared from a stock solution of 95% ethanol (100 mg/ml), and stored at 4°C.

Buffers

Tris-HCl

Tris-HCl was prepared by dissolving tris- (hydroxymethyl) aminomethane in distilled water to give 0.5M and 1.5M solutions; the pH was adjusted to 6.8 and 8.8 with concentrated HCl: These buffers were used as stacking and separating gel buffers, respectively, in polyacrilamide gel electrophoresis.

Dissolving Buffer

Dissolving Buffer (double strength) consisted of 0.5M tris-HCl, 2% ($\frac{W}{V}$) sodium dodecyl sulphate, 2% ($\frac{V}{V}$) mercaptoethanol and 20% ($\frac{V}{V}$) glycerol; the pH was adjusted to 6.0 using concentrated HCl.

Buffer A

Buffer A, used in steroid RIA, consisted of 0.015 M NaN_3, 0.15 M NaCl, 0.06 M $Na_2HPO_4.7H_2O$ and 0.03M $NaH_2PO_4.H_2O$. Buffer A was made

up in distilled water and adjusted to pH 6.8 using 1N HCl.

Hepes

HEPES was prepared by dissolving N-2-hydroxyethylpiperazine-N-2-ethanesuphoric acid in distilled water to give a concentration of 1mM. This buffer was added to the culture medium.

Culture Medium

Prepared modified Eagles Medium (MEM-F11) and methionine free MEM-199 were purchased from Grand Island Biological Co. (GIBCO), Grand Island, New York. Medium was fortified with 5% normal rabbit serum and L-glutamine (292 mg/liter); both purchased from GIBCO.

Cyclic Nucleotides

Adenosine $3^1, 5^1$ cyclic monophosphate (cyclic AMP, lot 33C-2150) and guanosine $3^1, 5^1$ cyclic monophosphoric acid (cyclic GMP; lot 114C-7320) were purchased as dry chemicals from Sigma Chemical Co., St. Louis, Missouri, U.S.A. These $3^1, 5^1$ cyclic nucleotides were dissolved directly in incubation medium, pH adjusted to 7.4 and made up fresh as required.

Dextran-coated Charcoal

Dextran coated charcoal consisted of 2.5 gm of charcoal and 0.25 gm of Dextran T-70 per liter of Buffer A. This solution was kept at 4°C and allowed to equilibrate for at least 3 hours before use.

Luteinizing Hormone (LH)

Ovine luteinizing hormone (NIH-LH-S18) was a gift of the Pituitary Hormone Distribution Program, National Institute of Arthritis and

Metabolic Disease, Bethesda, Md., U.S.A. LH-S18 had less than 5% FSH activity. LH was dissolved directly in the incubation medium and made up fresh as required.

Metabolic Inhibitors

The following inhibitors were purchased: Actinomycin D (lot C-45014/3) from Calbiochem, La Jolla, California; cycloheximide (lot C-6255), puromycin dihydrocholoride (lot 102C-2610) and theophylline (lot 82C-3070) from Sigma Chemical Co., St. Louis, Missouri, U.S.A.; 1,3-isobutyl methylxanthine (MIX; lot 85,845) from Aldrich Chemical Co., New York. Inhibitors were dissolved directly in the incubation medium, pH adjusted to 7.4 with 1N NaOH and made up fresh as required.

Radioisotopes

The following radioisotopes were purchased from New England Nuclear (Canada) Co., Dorval, Quebec: 1,2-^{3}H-androstenedione (49 Ci/mM), 7-^{3}H-dihydroepiandrosterone (20 Ci/mM), 6,7-^{3}H-estradiol-17β (48 Ci/mM), 1,2-^{3}H-progesterone (50.3 Ci/mM), 5,6-^{3}H-uridine (39.3Ci/mM), 2-^{14}C uridine (60mCi/mM), L-^{35}S-methionine (380 Ci/mM) L-4,5-^{3}H-leucine (50 Ci/mM) and L-^{3}H-amino acid mixture of various specific activities. Radioisotopic steroids were stored in 95% ethanol (5 μCi/ml) and prior to RIA, aliquots were dried down and redissolved in Buffer A. Radioactive precursors for RNA and protein were dried down and dissolved directly in the incubation medium.

Reagents (miscellaneous)

Other reagents were purchased: acrilamide from Bio Rad Laboratories, Richmond, California; NCS^{R} tissue solubilizer, PPO (2,5-diphenyloxazole) and POPOP (1,4-bis{2-(5-phenyloxazolyl)}-benzene from Amersham/Searle Corp., Don Mills, Ontario; sodium pentobarbitol, U.S.P. from Haver-Lockhart Laboratories, Calgary, Alberta; and Dextran T_{70} from Pharmacia (Canada) Ltd., Montreal, Quebec.

All other chemicals were reagent grade; solvents were redistilled prior to use.

METHODS

Animals and follicular preparations

Sexually mature New Zealand white rabbits weighing 2 - 3 kg were used. Ovaries, removed from live rabbits anesthesized with 2 ml of sodium pentobarbitol (65 mg/ml), were immediately placed in chilled normal saline solution. Follicles were dissected out under a Bausch and Laumb dissecting scope using Irex^{R} surgical miscroscissors and microtweezers. Each ovary usually contained 1 or 2 large follicles (1.5 - 2.0 mm diameter) and 6 to 8 smaller follicles (1 - 1.4 mm diameter). A representative picture of some isolated follicles is shown in Figure 5.

Experimental protocols

Protocol I

In the first protocol, duration 4 hours (Figure 6A), follicles were pooled and selected at random. Each experimental group contained 1, 3 or 6 follicles, depending on size. The isolated follicles were placed in disposable glass culture tubes (12 X 75 mm) and *preincubated* for 2 hours in 200 µl of Eagle's minimal essential medium (MEM). The medium was supplemented with 5% normal rabbit serum, L-glutamine (292 mg/l) and buffered to pH 7.4 with 10 ml/l of 1 mM HEPES and 20 ml/l of 7.5% sodium bicarbonate solution. A Forma Scientific CO_2 incubator was used and maintained at 37 ± 0.5°C in a humid atmosphere of 95% air and 5% CO_2. This was followed by an *incubation* period lasting up to 2

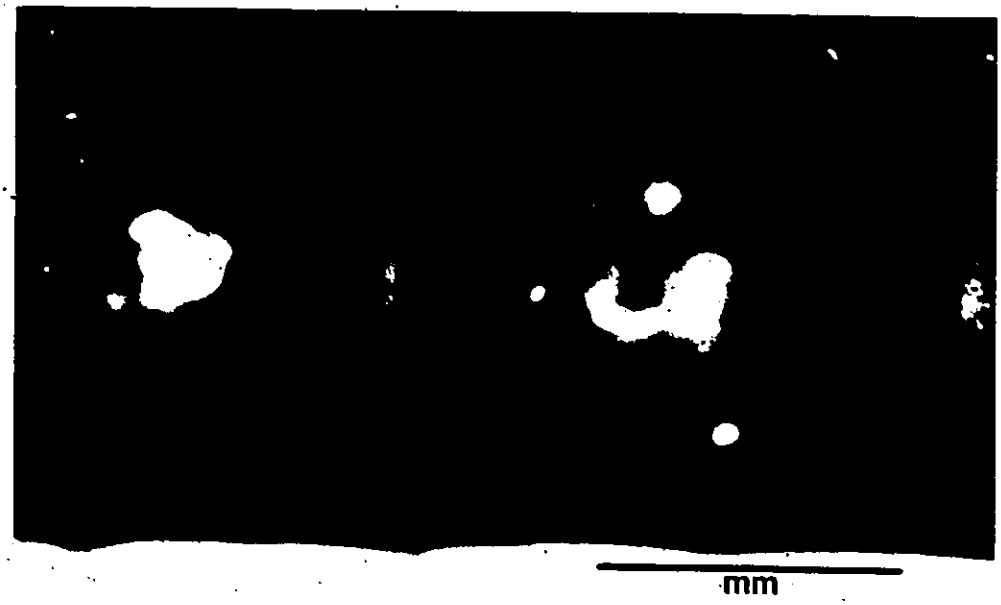

FIGURE 5. Representative picture of isolated rabbit ovarian follicles (1.0 - 1.5 mm diameter)

hours in which the medium was replaced with one containing LH, cyclic AMP, cyclic GMP, Actinomycin D, cycloheximide, puromycin, theophylline or 1,3-isobutyl methylxanthine (MIX) or a combination of LH or cyclic AMP plus these metabolic inhibitors. The radioactive precursors ^{3}H-uridine (0.5 µCi), ^{14}C-uridine (0.5 µCi), ^{3}H-amino acid mixture (0.5 µCi) and ^{3}H-leucine (0.5 µCi) were added separately in 10 µl medium. After each incubation, follicles and medium were frozen until RNA, protein and steroid determinations could be carried out.

Essentially the same 4 hour experimental protocol (Figure 6A) was used for electrophoretic studies, with the exception that methionine-free MEM and a Dubnoff Metabolic CO_2 incubator were used. Following the 2-hour preincubation period in MEM, single follicles (1.8 - 2.0 mm diameter) were incubated with ^{35}S-methionine (25 µCi/500 µl) together with LH or LH plus cycloheximide for different time periods (up to 2 hours) as indicated in the text.

Protocol II

In the second experimental protocol, duration 8 hours (Figure 6), isolated follicles (1 - 1.4 mm diameter) from individual rabbits were pooled and divided into an experimental and control group containing 6 follicles each. After the 2 hour preincubation in MEM, follicles were incubated in medium containing LH plus cycloheximide for 2 hours. The medium was then removed and follicles washed (six changes of medium) and reincubated in MEM alone for an additional 4 hours. During the incubation, a mixture of ^{3}H-amino acids was present from either 2 - 4 hours, 4 - 8 hours or 2 - 8 hours.

In a similar series of 8 hour experiments, pooled follices (3

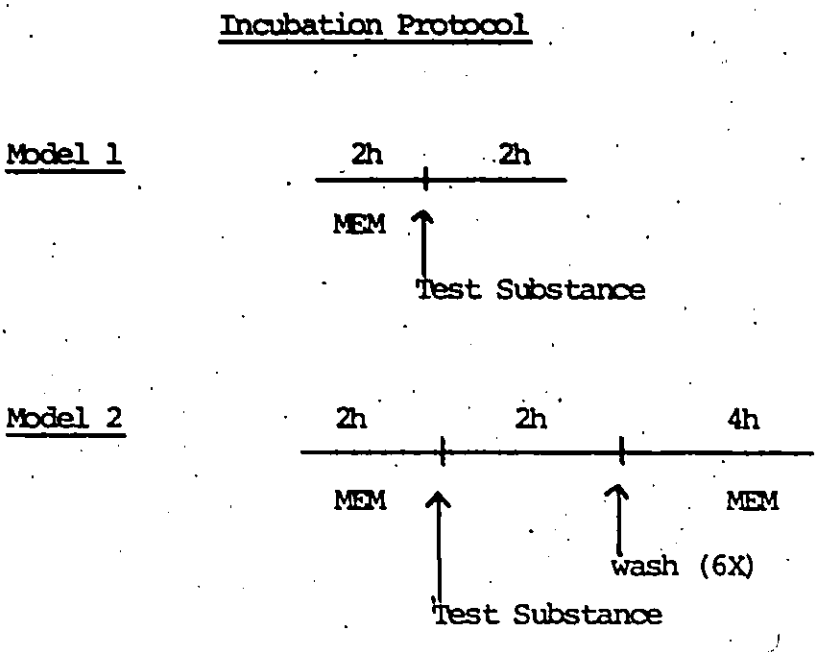

FIGURE 6. Models for incubation protocol.

per group) were incubated during the 2 - 4 hour period with either LH, LH plus cycloheximide, cycloheximide or MEM alone. After washing thoroughly in MEM the follicles were reincubated for the remaining 4 hours in MEM containing ^{3}H-amino acids.

Determination of Ribonucleic Acid (RNA) and protein

RNA and protein were determined using a modified procedure of Monro and Fleck[180]. A flowchart of the isolation procedure is illustrated in Figure 7. The follicles were washed in MEM and homogenized in 50 µl distilled H_2O (4°C) using custom made glass homogenizers (30 X 5 mm). The homogenate was transferred by Pasteur pipet to a 1.5 ml microtest tube (Brinkman) and kept on ice. The homogenizer and plunger were washed (3 times) with 150 µl H_2O (4°C) and the washings were also transferred to the microtest tubes. The homogenates were extracted (3 times) with diethylether (1 ml) and assayed for steroids.

RNA and protein were precipitated from the aqueous homogenate by adding 1 ml of 0.6N perchloric acid (4°C) and centrifuging at 12,000 x g in a Brinkman centrifuge (model 5412). The pellet was washed (4 times) with 0.2 N perchloric acid and an aliquot of the last wash counted to determine if any unincorporated label was still present. The pellet was dissolved in 615 µl of 0.3 N potassium hydroxide and incubated for 60 minutes in a shaking water bath 37°C. Yeast RNA standards were also made up in this same volume and treated in a similar manner. The protein fraction was precipitated by adding 315 µl of 1.2 N perchloric acid and letting the mixture stand on ice for 20 minutes. RNA was measured by UV spectrophotometry at 260 mµ and a 50 µl aliquot taken for determination

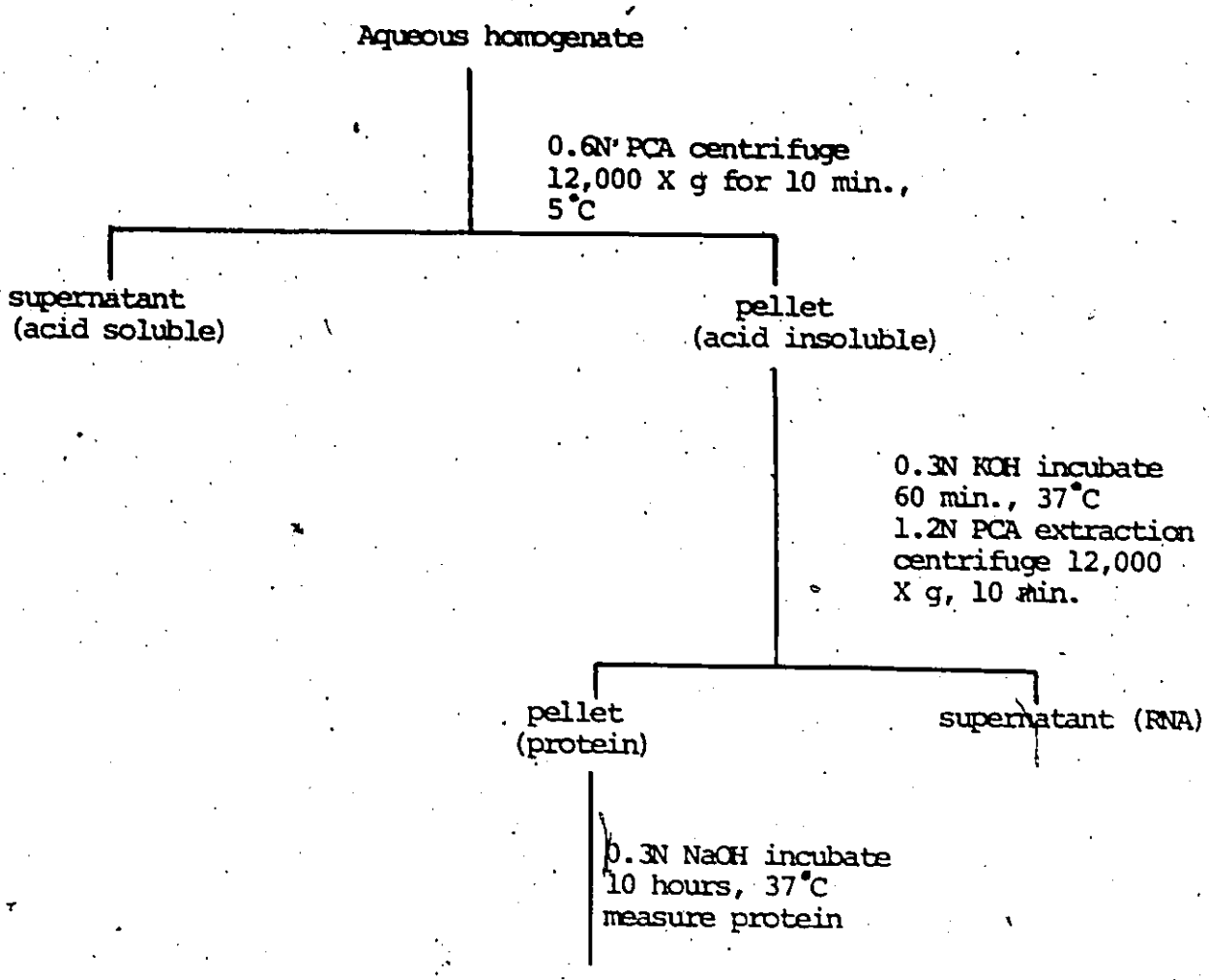

FIGURE 7. Scheme for extraction of RNA and protein from isolated rabbit ovarian follicles.

of radioactive incorporation. The pellet was again washed in 500 µl of 0.2 N perchloric acid and an aliquot counted to determine if any labeled RNA remained behind.

The acid insoluble precipitate containing protein was then dissolved in 600 µl of 0.3 N sodium hydroxide for 10 hours at 37°C in a shaking water bath. Protein was measured at 750 mµ by method of Lowry et al.[181], using bovine serum albumin as standard. In experiments where the uptake of tritiated amino acids into follicular protein was being determined, a 50 µl aliquot was counted for radioactive incorporation. By this method, the efficiency of extracting quality control samples of RNA and protein was 90 and 92%, respectively.

A toluene scintillation cocktail countaining 4 gm/liter PPO (2, 5-diphenyloxazole) and 0.1 gm/liter POPOP (1,4 bis-{2-(5-phenyloxazolyl)} benzene was used. To a 10 ml portion of this scintillator, 500 µl of NCS^{R} tissue solubilizer was added to absorb the aqueous radioactive sample. Radioactivity was counted in a Beckman liquid scintillation spectrometer (model LH 233) with automatic external standardization. The counting efficiency of ^{3}H and ^{14}C was approximately 43 and 93%, respectively.

Sodium dodecyl sulphate/polyacrilamide gel electrophoresis

Following the incubation in ^{35}S-methionine and LH or LH plus cycloheximide, the follicles were washed (2 times) in MEM, homogenized in 200 µl double strength Dissolving Buffer and heated at 100°C for 2 minutes. Samples to be analyzed were standardized by applying either equal counts (^{35}S-label incorporated into protein) or equal amounts of

protein to the gels. Protein determination was done by method of Lowry et al.[181], with bovine serum albumin as standard. Electrophoresis was carried out in linear 7.5 to 15% ($\frac{W}{V}$) sodium dodecyl sulphate/polyacrilamide slab gels containing 1% glycerol and run at a constant current of 30 Amperes as described by Laemili[182]. The gels were stained for 30 minutes with 0.1% ($\frac{W}{V}$) Coomassie Brilliant Blue, in a solution containing 25% ($\frac{V}{V}$) isopropanol and 10% ($\frac{V}{V}$) acetic acid. The gels were destained by several isopropanol/acetic acid washings and finally rinsed with water. The gels were dried under vacuum in a Bio-Rad gel slab dryer (model 224). Radioautograms of the ^{35}S-labeled follicular proteins were then obtained after the gels were exposed to Kodak RP Royal X-omat film which had been presensitized with red light.[183] The molecular weights of the proteins were determined by comparison with the migration of known standards: cytochrome C (12,500), myoglobin (17,800), ovalbumin (45,000) and albumin (67,000).

Radioimmunoassay (RIA) of steroid hormones

The steroid concentration in the follicular homogenates and culture medium was measured by RIA. RIA determinations for androstenedione, dehydroepiandrosterone, estradiol-17β, and progesterone were carried out on aliquots of incubation medium (10 or 20 µl) as well as ether extracts of follicular homogenates which had been dried under air and redissolved in Buffer A. For testosterone RIA, the medium was first extracted with 4 ml diethyl ether, dried down and redissolved in Buffer A. All samples and standards were then made up to

a constant volume of 100 µl with Buffer A before incubation with specific antibody and labeled steroid. Incubation procedures are as follows:

Androstenedione RIA

Antiserum to androstenedione (S-1557#2) and ^{3}H-androstenedione (10,000 cpm), each in 100 µl of Buffer A, were added consecutively to a glass test tube (12 X 75 mm) containing the sample. The sample was then vortexed and incubated for 16 to 20 hours at 4°C. The separation of free from bound androstenedione was accomplished by adding 1 ml of dextran coated charcoal solution, vortexing, incubating for 10 minutes (4°C), and finally centrifugation at 2600 RPM for 10 minutes. The supernatant was decanted into counting vials and mixed with 10 ml of a toluene scintillation cocktail containing 0.5% ($\frac{w}{v}$) of 2, 5 diphenyl-oxazole (PPO) and 10% ($\frac{v}{v}$) glacial acetic acid.

The results were expressed as total percent ^{3}H-androstenedione bound to the antibody, calculated as:

$$\% \text{ bound} = B/B_o \times 100$$

where B - cpm bound in unknown tubes and

B_o - cpm bound in tubes containing no added standards

The concentration of androstenedione was then calculated from known androstenedione standards (10 - 1,000 pg/100 µl Buffer A), run with each RIA. The immunoreactivity of interfering substances in the culture medium (2 pg) was subtracted from each sample. Since no significant difference in total follicular protein content was observed between individual follicles or groups of follicles in each experiment, the RIA results were standardized and expressed as ng steroid per mg protein for

both the incubation medium and follicular homogenate.

A typical androstenedione standard curve is plotted in Figure 8. The percentage of bound radioactivity is plotted against the logarithm of the dose of androstenedione. The assay had a sensitivity of 10 to 25 pg.

Dehydroepiandrosterone, estradiol, progesterone and testosterone RIA's -

The RIA protocol for each of these four steroids is essentially identical and therefore will be considered as a group. Antiserum to either dehydroepiandrosterone, estradiol-17β, progesterone or testosterone, in 100 µl Buffer A, was added to the sample, vortexed and incubated for 30 minutes at 20°C. This was followed by a 2 hour incubation at 4°C with either ^{3}H-dehydroepiandrosterone, ^{3}H-estradiol, ^{3}H-progesterone or ^{3}H-testosterone (10,000 cpm each) in 100 µl Buffer A. Separation of free from bound steroid was accomplished by adding 1 ml of a dextran-coated charcoal suspension, followed by centrifugation. The supernatant was decanted into scintillation vials, mixed with cocktail and counted. Calculation of steroid concentrations was carried out as described for androstenedione. Immunoreactivity of interfering substances in the medium was subtracted from all samples; dehydroepiandrosterone (1.5 - 3 pg), estradiol-17 (1.5 - 3 pg), progesterone (1.5 - 3 pg) and testosterone (15 - 25 pg). All RIA's had a sensitivity of 10 - 25 pg with the exception of testosterone which had a sensitivity of 35 - 50 pg. The minimal detection in each sample was 75 - 100 pg. The coefficient of variation between assays was less than 10% for all steroid RIA's.

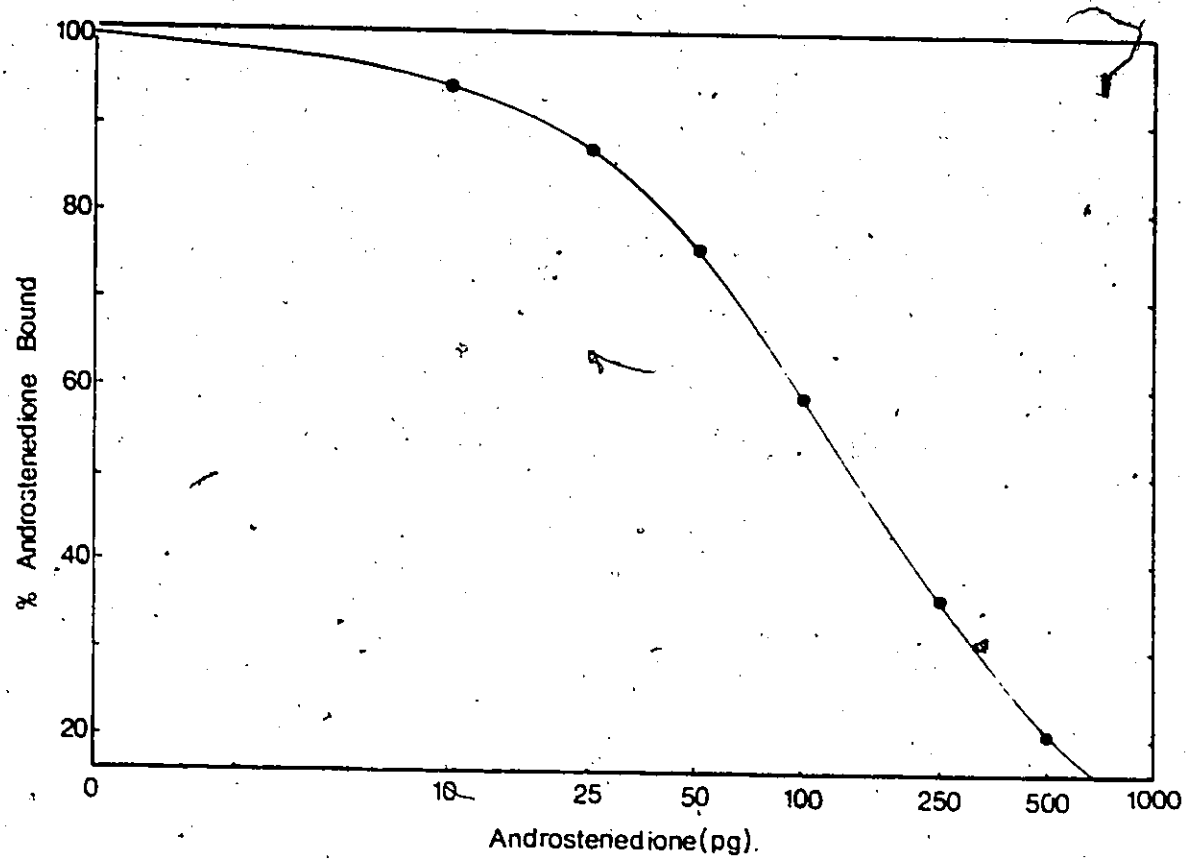

FIGURE 8. Representative standard curve for androstenedione radioimmunoassay.

Determination of ^{3}H-testosterone metabolism

After a 2 hour incubation with ^{3}H-testosterone (50,000 cpm) and various test substances, ^{14}C-testosterone (20,000 cpm) was added for recovery purposes to the incubation medium. The amount of ^{3}H and ^{14}C recovered after extraction was 69 and 83%, respectively.

The extraction procedure can be summarized in the flow chart, Figure 9. The intact follicles were homogenized in 50 µl H_2O and the homogenate was combined with the incubation medium. The homogenizer and plunger were then washed (3 times) with 150 µl H_2O and the washings also combined with the incubation medium. Steroids were then extracted (3 times) from the combined follicular homogenate and incubation medium with 6 ml $CHCl_3$:MeOH (2:1) and the extracts evaporated to dryness and redissolved in 25 ml benzene. The steroids were then separated into neutral and phenolic fractions using a benzene-sodium hydroxide partition[184]. The phenolic (estrogen) fraction was discarded due to insufficient amount of counts. The neutral benzene fraction containing radiolabeled androgens was dried under a stream of air and dissolved in 1 ml iso-octane saturated in ethylene glycol and chromatographed on microCelite columns according to methods described by Abraham et al.[185].

Chromatography

The columns were first prepared by mixing Celite with 0.5 ml/g of the stationary phase (iso-octane) in a plastic bag until homogenous[186]. The Celite was then dry packed in disposable 5 ml pipets. The extracts, dissolved in 1 ml iso-octane/ethylene glycol, were then placed on the microCelite columns. The columns were then developed with 3 ml of 5%

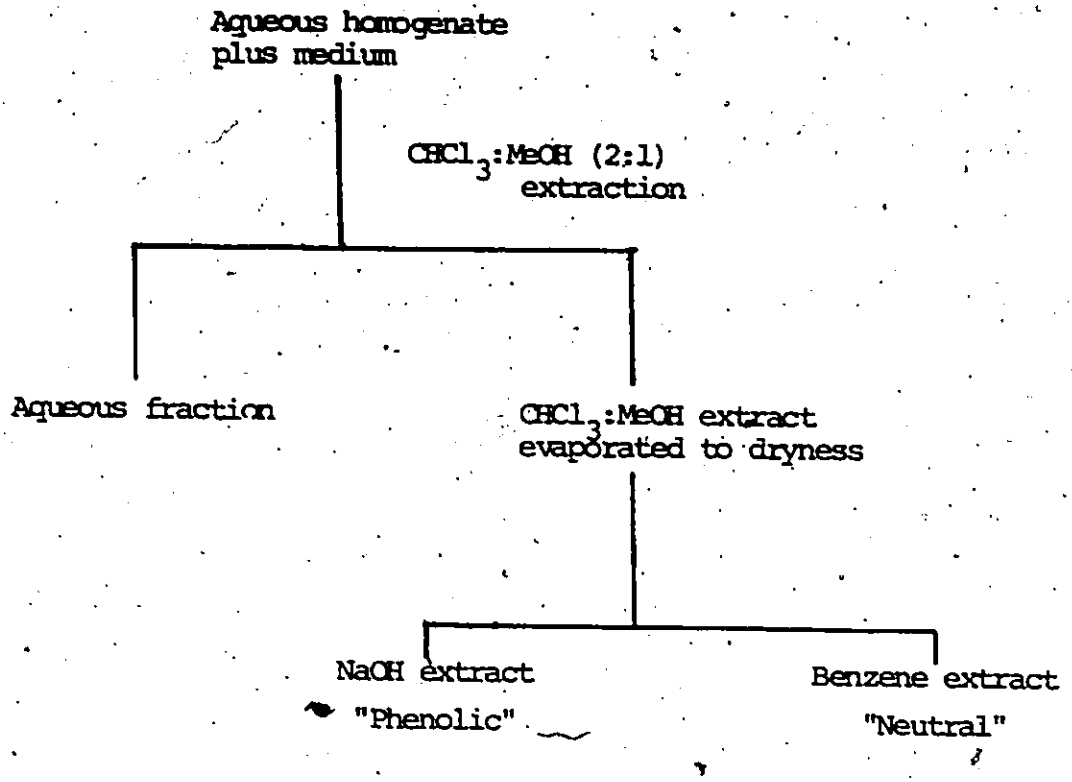

FIGURE 9. Scheme for extraction of steroids from rabbit follicular homogenate and incubation medium.

ethylacetate in iso-octane (3 times) under forced air pressure. After dihydrotestosterone had been eluted, the columns were then developed with 3 ml of 15% ethylacetate in iso-octane (3 times). These latter fractions contained radiolabeled testosterone.

Counting

Samples to be counted were dried down and dissolved in 10 ml of toluene containing 0.5% ($\frac{W}{V}$) of 2, 5 diphenyl-oxazole (PPO) and 10% ($\frac{V}{V}$) glacial acetic acid. The amount of ^{3}H- and ^{14}C- in samples containing both radioisotopes was estimated by the discriminatory ratio method[187], using the following equations:

$$^{3}H = N_1 - \frac{N_2}{b} \quad ; \quad ^{14}C = N_2 - N_{1a}$$

where N_1 = cpm in ^{3}H-channel

N_2 = cpm in ^{14}C-channel

$$a = \frac{^{3}H \text{ in } ^{14}C\text{-channel}}{^{3}H \text{ in } ^{3}H\text{-channel}}$$

$$b = \frac{^{14}C \text{ in } ^{14}C\text{-channel}}{^{14}C \text{ in } ^{3}H\text{-channel}}$$

The "a" and "b" ratios were determined from standards containing known amounts of ^{3}H or ^{14}C. The value of "a" was small (<0.1) and disregarded while the value of "b" was 5.0 - 5.5. Each sample was corrected for the amount of ^{14}C spilled into the ^{3}H channel. The counting efficiency of ^{3}H and ^{14}C were 42 and 90% , respectively.

Statistics

Statistical treatment of data was done either by: (i) one way analysis of variance followed by Duncan's New Multiple Range Test[188]; or (ii) by Students paired and unpaired "t" tests. Duncan's New Multiple Range Test provided a method for testing significant differences between individual means when more than two groups were compared. A p value of 0.05 or less was considered significant in all cases.

RESULTS

Testosterone Production

The production rate of testosterone may be defined as the total amount of testosterone synthesized de novo per unit time. The unstimulated rabbit Graafian follicle synthesizes very small amounts of androgens, estrogens and progestins from ^{14}C-acetate.[9-11] In addition, the unstimulated rabbit follicle (1.8 - 1.9 mm diameter) contains small amounts of testosterone (n=3; 10.2 ± 3.1 ng/mg protein), progesterone (n=3; 3.2 ± 1.8 ng/mg protein) and estradiol-17β (n=3; 1.9 ± 0.8 ng/mg protein), as measured by radioimmunoassay. However, upon LH (5 µg/ml) stimulation, testosterone is the major steroid produced by the isolated rabbit follicle[29,104] (Figure 10). Therefore, throughout this thesis, testosterone production was used as a marker for measuring the steroidogenic responsiveness of the follicle to LH and other test substances.

In order to rule out the possibility that the increased testosterone production was due to decreased metabolism, it was necessary to determine the extent of testosterone metabolism. Isolated follicles (1 per incubation) were incubated for 2 hours with ^{3}H-testosterone (50,000 cpm) and either LH (5 µg/ml), Actinomycin D (1 µg/ml) or LH (5 µg/ml) plus Actinomycin D (1 µg/ml). After extraction of steroids and partition into neutral and phenolic, the neutral fraction was chromatographed on microCelite columns and the testosterone fraction analyzed for radioactivity.[183] As shown in Table I, neither LH nor

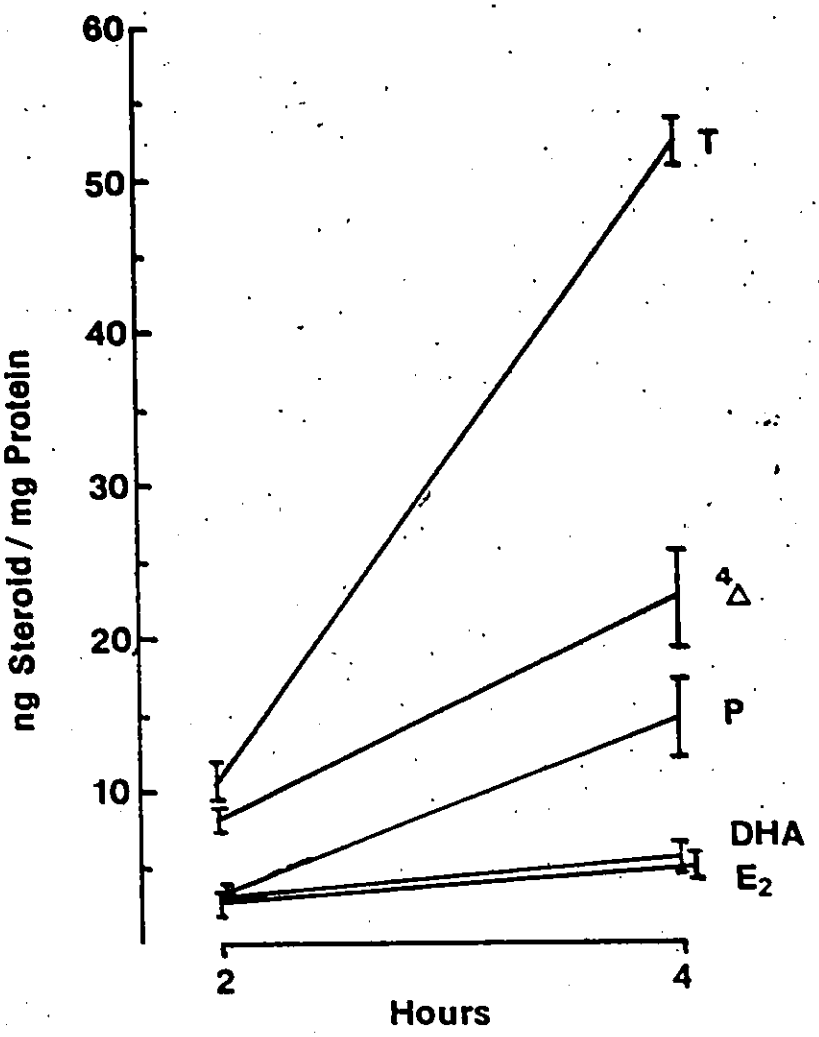

FIGURE 10. Effect of LH(5µg/ml) on androstenedione($^4\Delta$), dehydroepiandrosterone(DHA), estradiol-17β(E_2), progesterone(P), and testosterone(T) production by the rabbit follicle after 4 hours in vitro. Results are expressed as the mean±SEM(n=4; 1 follicle per incubation. Average follicular protein content was 135±17 µg.

TABLE 1

Effects of LH (5 µg/ml), LH (5 µg/ml) plus Actinomycin (1 µg/ml) and Actinomycin (1 µg/ml) alone on the metabolism of ^{3}H-testosterone in isolated rabbit follicles after a 4 hour incubation.

Treatment (µg/ml)	Unchanged ^{3}H-testosterone (%)
LH (5)	92.5 ± 0.40
LH (5) + AMD (1)	92.6 ± 0.13
AMD (1)	92.5 ± 0.24
Control	92.3 ± 0.40

Results are expressed as mean ± S.E.M. (n = 5, 1 follicle/ incubation. No statistical difference was observed between the percentage unchanged of testosterone after various treatments.

Actinomycin D, alone or together, had an effect on testosterone metabolism. More than 90% of the incubated ^{3}H-testosterone was recovered unchanged, regardless of treatment.

Effect of LH on follicular RNA, protein and testosterone production

Preliminary attempts to delineate the roles of RNA and protein synthesis in LH-induced steroidogenesis were unsuccessful. Following a 2 hour pre-incubation in MEM, follicles (6 per incubation) were incubated for 2 hours with LH (5 µg/ml) plus a mixture of ^{3}H-amino acids (Figure 11A) or ^{3}H-uridine (Figure 11B). LH significantly enhanced a 1- and 3- fold increase in testosterone production ($p < .01$), yet had no observable stimulatory effect on the incorporation of either ^{3}H-amino acids or ^{3}H-uridine into follicular protein and RNA. Similar protein results were observed when the follicles were incubated with the ^{3}H-amino acid mixture in amino acid free medium.

When follicles (6 per incubation) were incubated with ^{3}H-leucine in MEM, LH (5 µg/ml) significantly enhanced [illegible]ine incorporation into follicular protein ($p < .05$) from 2- to 4- hours of incubation (Figure 12). During the same 2 hour period, LH enhanced a 2.5-fold increase in testosterone production, measured in the incubation medium ($p < .01$).

Attempts were therefore made to examine the correlation between LH-induced ^{3}H-leucine incorporation into follicular protein with testosterone production. LH dose and time course studies were carried out.

Dose Response

Following the usual 2 hour pre-incubation in MEM, follicles (1 per incubation) were incubated with various doses of LH together with

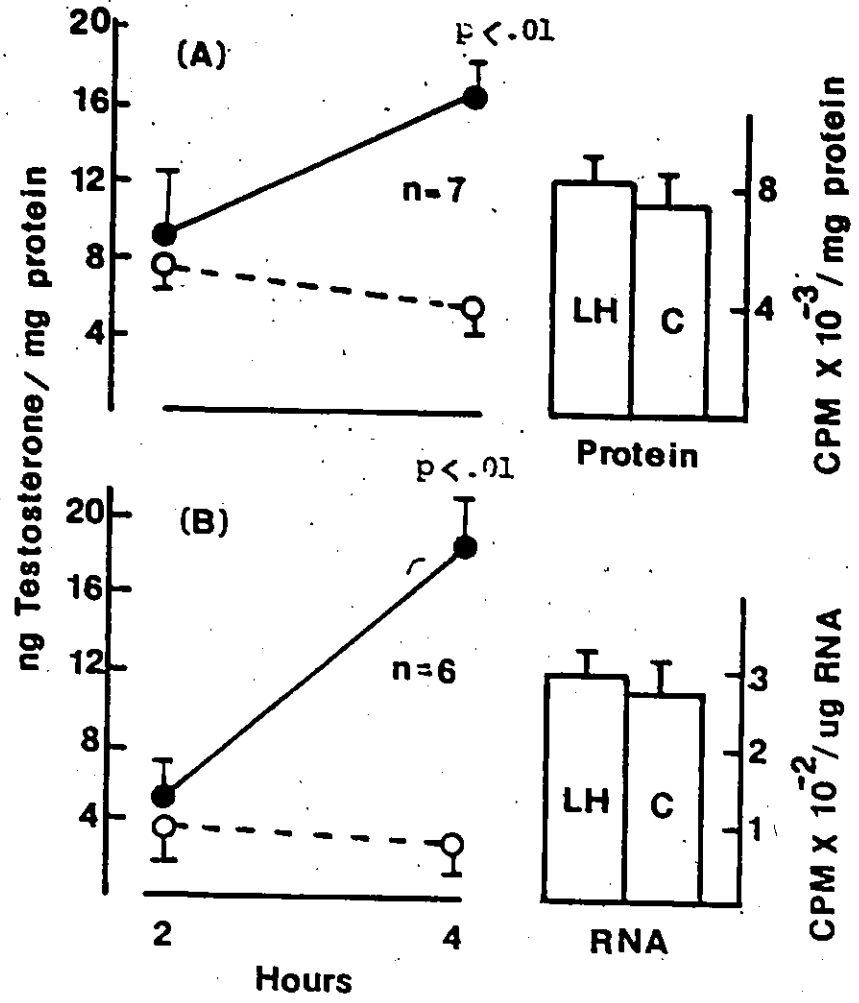

FIGURE 11. Effect of LH (5 µg/ml) on testosterone production and the uptake of a mixture of ^{3}H-amino acids into follicular protein (Figure 11A), and the uptake of ^{3}H-uridine into follicular RNA (Figure 11B) after 4 hours in vitro. Separate incubations were carried for protein and RNA determinations. Lines on left represent mean ± SEM (n=6 or 7; 6 follicles/ incubation) of testosterone production (medium) in the absence (o-----) or presence (●——) of LH. Histograms on right represent uptake of labeled precursors into follicular RNA and protein in the absence (C) or presence of LH. Statistical analysis was determined by Paired Student's T Test.

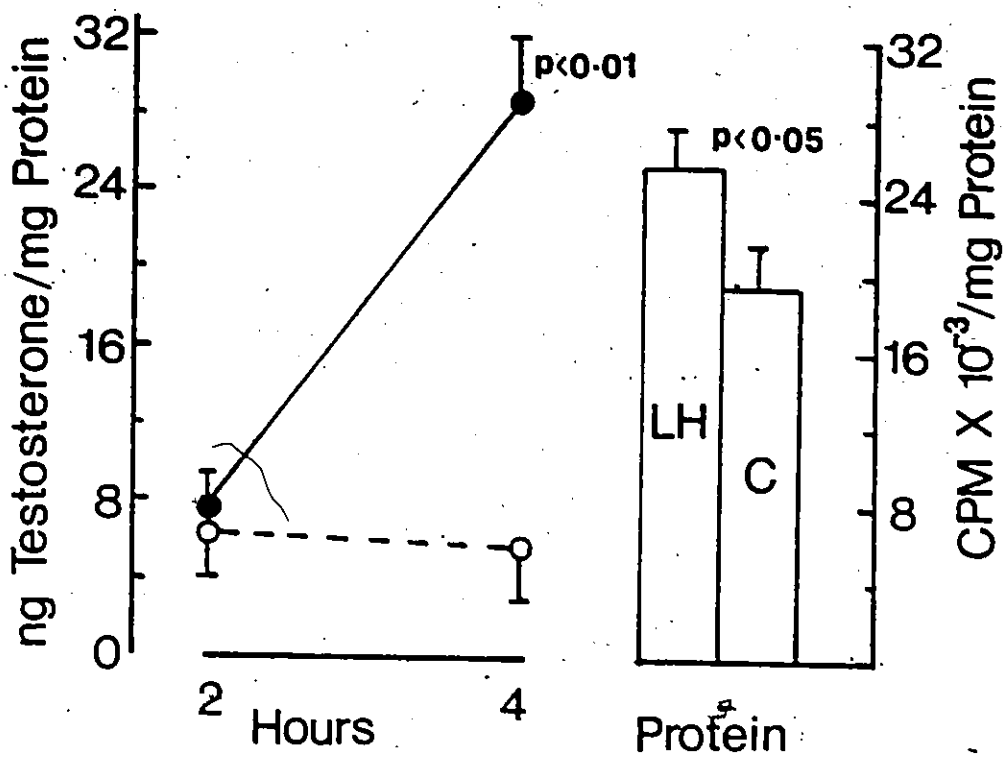

FIGURE 12. Effect of LH (5 µg/ml) on testosterone production and the uptake of ^{3}H-leucine into follicular protein after 4 hours in vitro. Lines on left represent mean ± SEM (n=5; 3 follicles/incubation) of testosterone, measured in the medium, in the absence (o- - - -) and presence (●——) of LH. Histograms on right represent uptake of ^{3}H-leucine into follicular protein. No statistical difference in follicular protein content (242 ± 36 µg/3 follicles) was observed between treatment groups. Statistical analysis was determined by Paired Student's T Test.

^{3}H-leucine for an additional 2 hours. As shown in Figure 13, total testosterone production (medium plus homogenate) was enhanced at all concentrations of LH (0.1, 0.5, 1.0, 2.5, 5.0 and 10 µg/ml), with optimal stimulation occurring at 5 µg LH/ml ($p < .01$). This pattern of testosterone was consistent when measured in both the follicular homogenates and incubation medium. In contrast, a significant uptake of ^{3}H-leucine into follicular protein was observed only at LH concentrations of 2.5 µg/ml or greater ($p < .01$), with optimal stimulation occurring at 5 and 10 µg LH/ml ($p < .01$).

Time Course Study

Using 5 µg LH/ml as an optimal stimulatory dose for all further studies, the time-course relationship between LH-induced testosterone and protein synthesis was examined.

Isolated follicles (1 per incubation) were incubated with LH (5 µg/ml) for 15, 30, 60 and 90 minutes, following a 2 hour pre-incubation in MEM (Table 2). In the presence of LH, testosterone production (measured in medium) was enhanced within 15 minutes ($p < .001$) and continued to rise with time. The uptake of ^{3}H-leucine also increased with time in both LH treated follicles and controls, but a significant difference was only observed at 90 minutes of incubation ($p < .01$). When the uptake of ^{3}H-leucine was compared only among LH treated groups, the incorporation was significantly increased with time: LH (90 minutes) $\simeq$ LH (60 minutes) > LH (30 minutes) > LH (15 minutes) ($p < .01$). Similar findings were observed among control groups (C) with: C (90 minutes) $\simeq$ C (60 minutes) > C (30 minutes) > C (15 minutes) ($p < .01$).

The effect of LH (5 µg/ml) on the electrophoretic pattern of

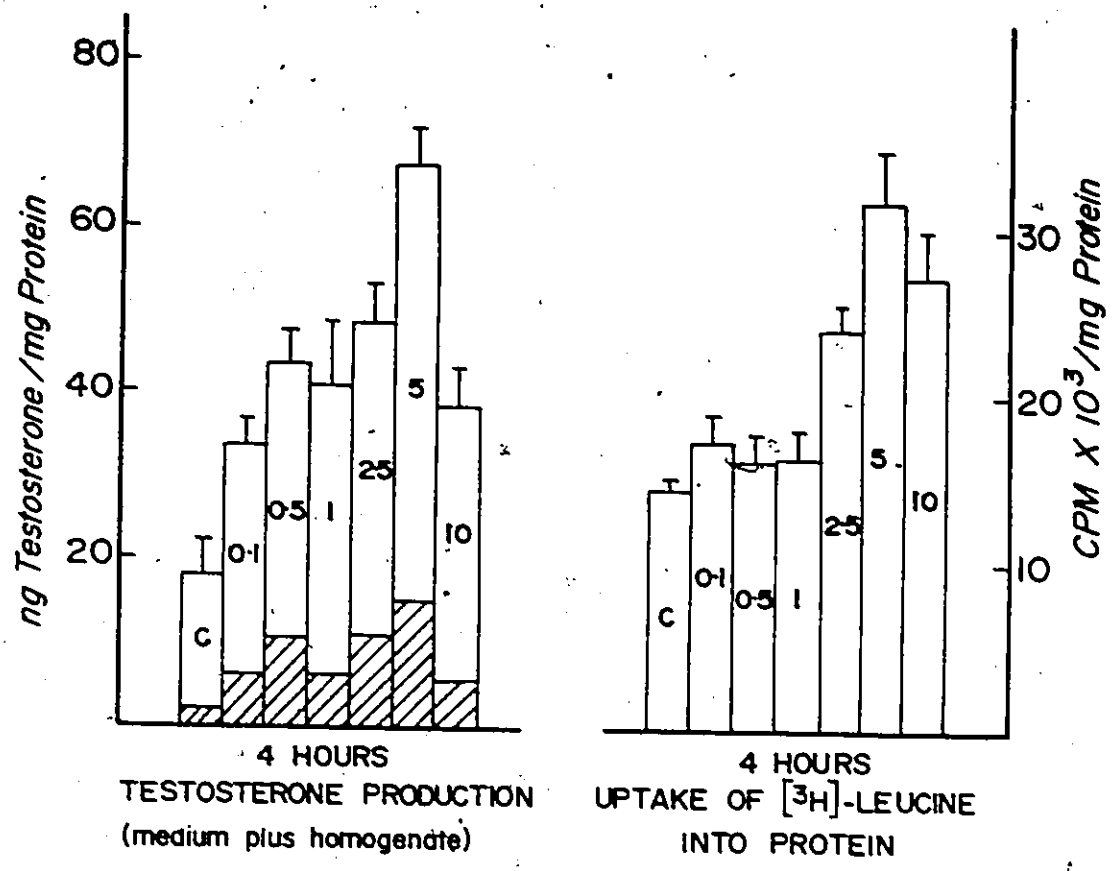

FIGURE 13. Effects of LH (0.1, 0.5, 1.0, 2.5, 5.0 and 10 µg/ml) on testosterone production and uptake of ^{3}H-leucine into follicular protein after 4 hours in vitro. Histograms on left represent total testosterone in medium (clear area) plus follicular homogenates (slashed area). Histograms on right represent uptake of ^{3}H-leucine into protein. Results are expressed as mean ± SEM (n=7; 1 follicle/incubation). No statistical difference in follicular size (1.9 ± 0.2 mm diameter) or protein content (128 ± 3.3 µg/follicle) was observed. Symbol "C" and "numerical inset" correspond to control and LH (µg/ml) treatments, respectively. Statistical analysis was determined by Duncan's New Multiple Range Test.

TABLE 2

Time course of LH (5 µg/ml) action on testosterone production and uptake of ^{3}H-leucine into protein *in vitro*.

Incubation time (minutes)			Testosterone Medium (ng/mg protein)	^{3}H-leucine incorporation (cpm x 10^{-3}/mg protein)
15	LH	(n=8)	13.4 ± 1.1 **	12.1 ± 0.5
	Control		6.4 ± 1.1	11.9 ± 0.6
30	LH	(n=4)	12.6 ± 2.4 **	17.6 ± 1.1
	Control		1.5 ± 0.4	15.1 ± 0.3
60	LH	(n=4)	22.2 ± 3.8 **	18.0 ± 1.8
	Control		5.2 ± 1.2	18.4 ± 0.6
90	LH	(n=4)	36.7 ± 10.6*	20.4 ± 0.4 *
	Control		7.0 ± 1.8	17.8 ± 0.9

* $p < 0.05$ vs control using Unpaired Student's "T" Test

** $p < 0.01$ vs control using Unpaired Student's "T" Test

Statistical analysis for uptake of ^{3}H-leucine with time using Duncan's New Multiple Range Test.

- Among LH treated groups only: $LH_{90} \simeq LH_{60} > LH_{30} > LH_{15}$
- Among control groups only: $C_{90} \simeq C_{60} > C_{30} > C_{15}$

Results are expressed as mean ± SEM. No difference in follicular size (1.6 ± 0.2 mm/follicle) or protein content (97.3 ± 4 µg/follicle) was observed between groups.

^{35}S-methionine incorporation into individual protein bands, after 15-, 60-, and 120-minute incubations, is shown in Figure 14. Electrophoretic fractionation of total cellular proteins did not reveal any new protein bands in sufficient quantities to be detected by radioautography.

Effect of cyclic AMP on follicular RNA, protein and testosterone production

If the hypothetical model (Figure 3) is correct, that is, LH exerts its action via cyclic AMP, then LH and cyclic AMP should have identical effects on testosterone production by the follicles. Experiments were therefore performed as for LH.

Dose response

The effects of cyclic AMP on testosterone production and uptake of labeled leucine and uridine into follicular protein and RNA are shown in Table 3. Following a 2 hour pre-incubation in MEM, follicles (1 per incubation) were incubated for 2 hours with cyclic AMP (0.1, 0.5, 1.0, 2.5, 5.0 and 10 mM) together with ^{3}H-leucine and ^{14}C-uridine. A positive dose dependent correlation was found between cyclic AMP-induced testosterone production and de novo protein synthesis. At cyclic AMP concentrations of 5 and 10 mM, both testosterone production and the uptake of ^{3}H-leucine into follicular protein were significantly enhanced ($p < .01$). Lower cyclic AMP concentrations ($\leq$ 2.5 mM) were ineffective. No effect on the incorporation of ^{14}C-uridine into follicular RNA was observed at any cyclic AMP concentration tested.

Time Course Study

Using 5 mM cyclic AMP as an optimal stimulatory dose for all

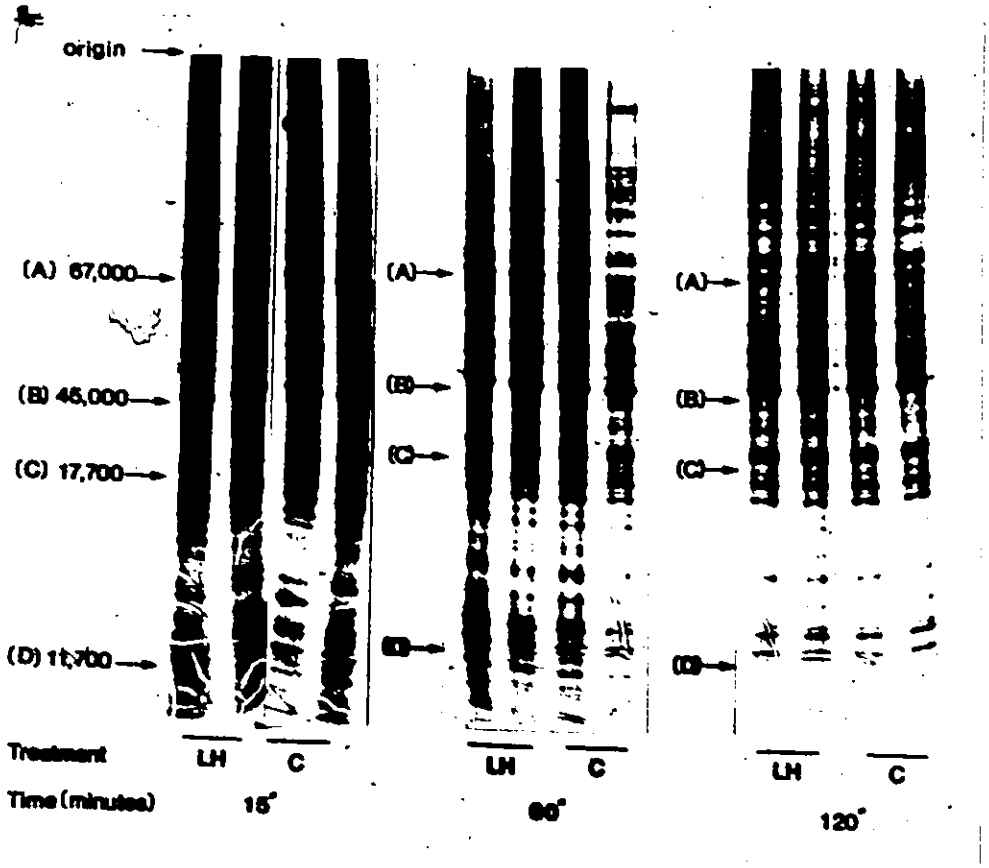

FIGURE 14. Time course study of the effect of LH on the incorporation of ^{35}S-methionine into follicular protein synthesis. Following a 2 hour preincubation in methionine free MEM, isolated follicles (3 per tube) were incubated with ^{35}S-methionine for 15, 60, or 120 minutes, in the absence or presence of LH (5 µg/ml). After extraction, total cellular proteins were fractionated on a 7.5 - 15% discontinuous sodium dodecyl sulphate/polyacrilamide gel. Radioautographic profile of fractionated proteins are shown in the figure. Standards used as molecular weight markers were (A) albumin MW 67000; (B) ovalbumin, MW 45000; (C) myoglobin, MW 17800; (D) cytochrome C, MW 12500.

TABLE 3

Effect of cyclic AMP (10, 5.0, 2.5, 1.0, 0.5, 0.1 mM) on testosterone production and incorporation of ^{3}H-leucine and ^{14}C-uridine in protein and RNA in the isolated rabbit ovarian follicle.

Treatment (mM)	Testosterone ng/mg protein	Uptake of ^{3}H-leucine into protein cpm x 10^{-3}/mg protein	Uptake of ^{14}C-uridine into RNA cpm/µg RNA
10	42.5 ± 6.4*	23.8 ± 0.8*	576 ± 38.6
5	37.0 ± 4.2*	25.1 ± 1.1*	486 ± 38.2
2.5	7.2 ± 1.4	20.1 ± 0.7	587 ± 22.7
1	4.5 ± 0.9	18.9 ± 1.1	521 ± 33.1
0.5	5.4 ± 1.9	17.8 ± 0.4	492 ± 34.5
0.1	2.9 ± 0.7	17.8 ± 0.7	536 ± 43.5
Control	3.2 ± 0.7	18.9 ± 0.7	581 ± 19.7

* $p < .01$ vs control using Duncan's New Multiple Range Test.

Results are expressed as mean ± SEM (n=10; 1 follicle/incubation). No difference in follicular size (1.8 ± 0.2 mm/follicle), protein (127 ± 7 µg/follicle) or RNA (13.2 ± 0.5 µg/follicle) content was observed between groups.

further studies, the time relationship between cyclic AMP-induced testosterone production and protein synthesis was examined.

Table 4 shows the effects of incubating isolated follicles (1 per incubation) with cyclic AMP (5 mM) and ^{3}H-leucine for 0.5, 15, 30, 60 and 90 minutes, following a 2 hour pre-incubation in MEM alone. In the presence of cyclic AMP, testosterone was stimulated within 15 minutes and continued to rise with time: cyclic AMP (90 minutes) > cyclic AMP (60 minutes) = cyclic AMP (30 minutes) > cyclic AMP (15 minutes) > cyclic AMP (0.5 minutes) ($p < .05$). No significant difference in testosterone production was observed among control groups with time.

The incorporation of ^{3}H-leucine also increased with time in both cyclic AMP treated and control follicles, but a significant difference was only observed after 60 minutes ($p < .01$). When the uptake of ^{3}H-leucine was compared only among cyclic AMP treated groups, there was significant incorporation with time: cyclic AMP (90 minutes) > cyclic AMP (60 minutes) = cyclic AMP (30 minutes) > cyclic AMP (15 minutes) > cyclic AMP (0.5 minutes) ($p < .05$). Similar findings were observed among control groups (C) with C (90 minutes) > C (60 minutes) = C (30 minutes) > C (15 minutes) > C (0.5 minutes) ($p < .01$).

Inhibitors of protein synthesis: puromycin and cycloheximide

Since the previous studies indicated that the steroidogenic response to LH and cyclic AMP may not be dependent upon macromolecular synthesis, inhibitor studies using puromycin and cycloheximide were undertaken to further elucidate this relationship. Both antibiotics inhibit protein synthesis at the translational level: puromycin causes

TABLE 4

Time course of cyclic AMP (5 mM) on testosterone production and uptake of ^{3}H-leucine into follicular protein after 4 hours *in vitro*.

Incubation time (min.)		Testosterone Medium (ng/mg protein)	^{3}H-leucine incorporation (cpm x 10^{-3}/mg protein)
0.5	cAMP (n=4)	-	0.9 ± 0.2
	Control	-	0.8 ± 0.2
15	cAMP (n=8)	3.4 ± 0.60*	2.3 ± 0.15
	Control	1.1 ± 0.20	2.1 ± 0.15
30	cAMP (n=8)	11.7 ± 1.20*	9.4 ± 1.3
	Control	2.3 ± 0.93	11.2 ± 1.4
60	cAMP (n=8)	14.1 ± 1.62*	18.9 ± 0.8*
	Control	2.4 ± 0.45	11.0 ± 0.5
90	cAMP (n=8)	18.4 ± 1.19*	21.6 ± 1.2*
	Control	1.44 ± 0.29	14.8 ± 0.4

*$p < .01$ vs control using Paired Student's "T" Test.

Results are expressed as mean ± SEM: No difference in follicular size (1.8 ± 0.2 mm/follicle) or protein content (129 ± 2.9 µg/follicle) was observed between groups.

premature termination of peptide synthesis, while cycloheximide prevents translocation.[189]

Effect of cycloheximide on LH-induced testosterone and protein synthesis

Preliminary experiments suggested that a discrepancy may exist between the cycloheximide-inhibitions of protein synthesis and LH-induced testosterone production. Cycloheximide inhibited by 50% the incorporation of ^{3}H-amino acids into follicular proteins at concentrations between 0.1 and 0.01 µg/ml (Figure 15). In the presence of LH (5 µg/ml), cycloheximide (50, 10 and 1 µg/ml) inhibited the incorporation of ^{3}H-amino acids into follicular protein by 85, 83 and 55%, respectively ($p < .01$) (Figure 16). However, testosterone production was inhibited to control values by cycloheximide concentrations of 50 and 10 µg/ml and not by 1 µg/ml.

To further investigate this discrepancy, using a single labeled essential amino acid, the inhibitory effects of cycloheximide (20, 10 and 1 µg/ml) on LH-induced testosterone production and the incorporation of ^{3}H-leucine into follicular protein were examined (Figure 17). Following a 2 hour pre-incubation in MEM, LH (5 µg/ml) alone significantly stimulated testosterone production above control values ($p< .01$) and the incorporation of ^{3}H-leucine into follicular protein ($p < .05$). Cycloheximide (10 µg/ml), Figure 17A, inhibited LH-induced testosterone production by 54.7% and the uptake of ^{3}H-leucine into follicular protein by 93% ($p < .01$). On the other hand, cycloheximide (1 µg/ml) did not inhibit LH-induced testosterone production, yet ^{3}H-leucine incorporation into protein was significantly inhibited by 71% ($p < .01$).

In the presence of cycloheximide (20, 10 and 1 µg/ml) alone, Figure

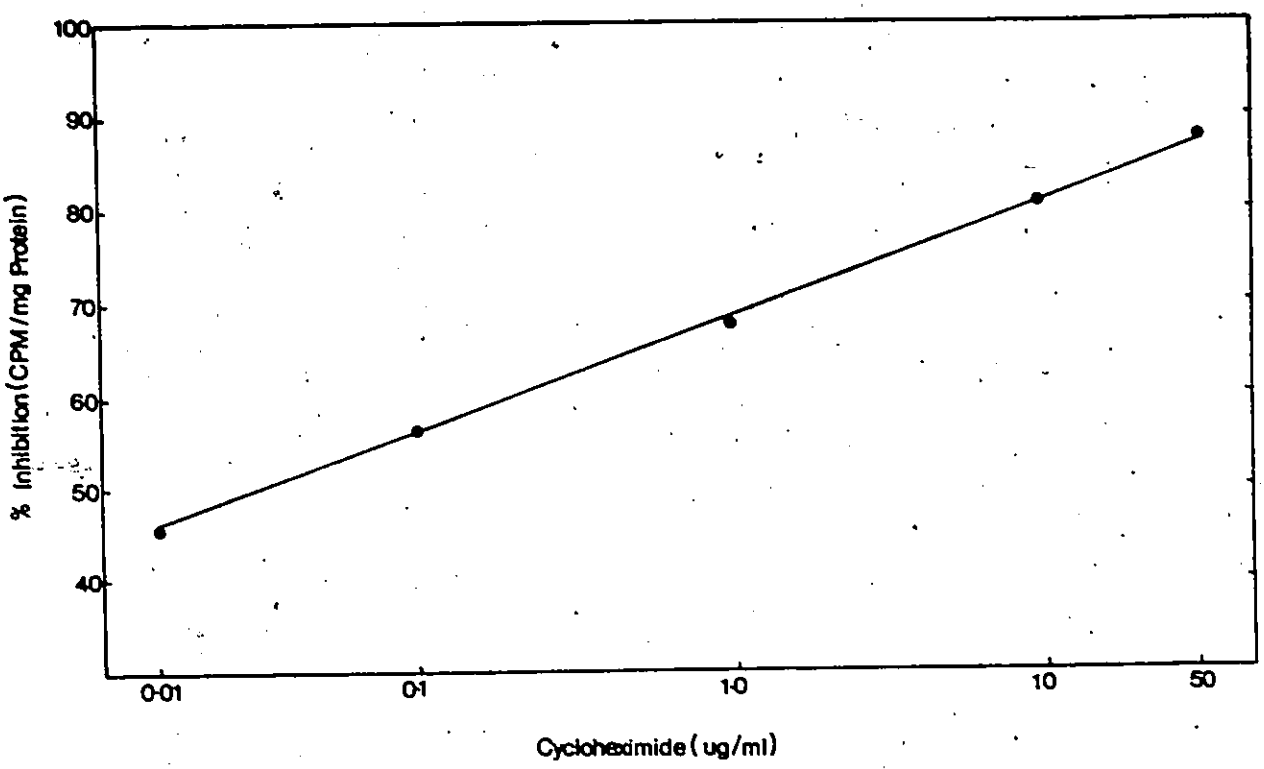

FIGURE 15. Effect of cycloheximide (50, 10, 1, 0.1 and 0.01 µg/ml) on the incorporation of ^{3}H-amino acids into follicular protein synthesis. Results are expressed as mean of duplicate incubations (6 follicles/incubation) and are expressed as percent inhibition of control (100%).

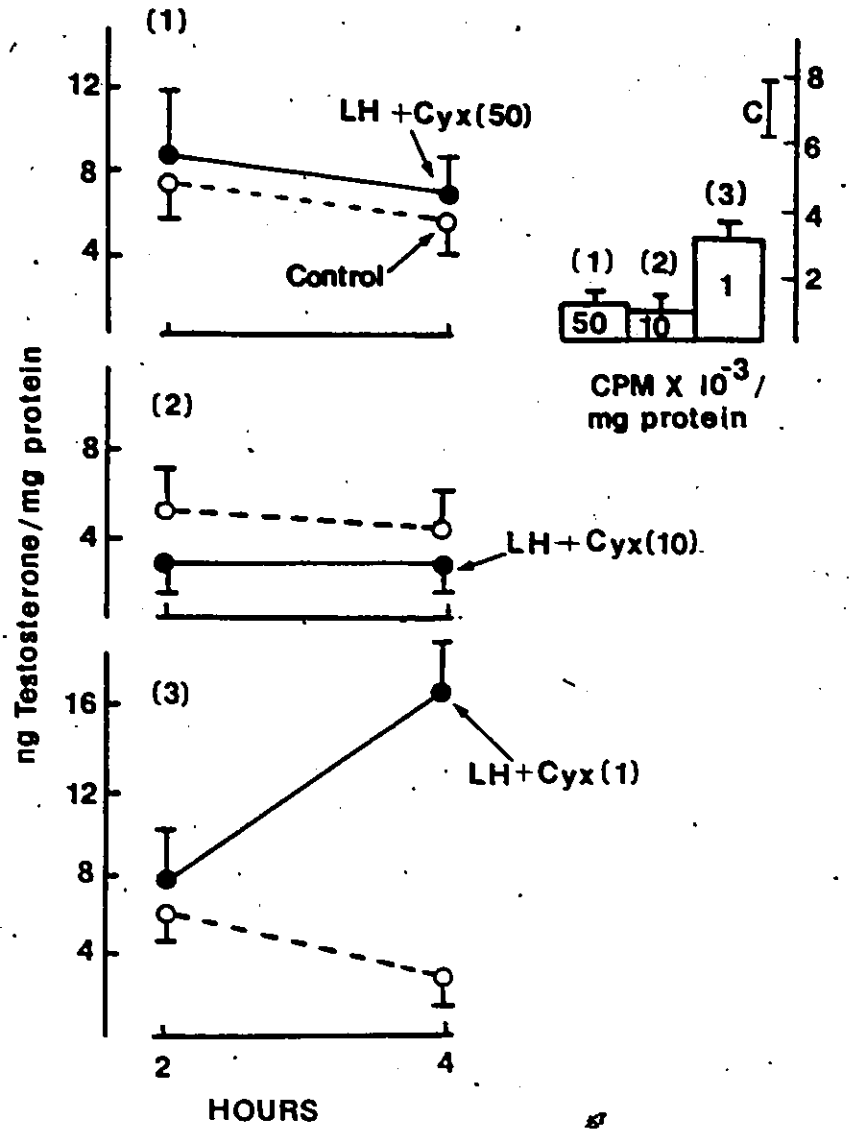

FIGURE 16. Effect of LH (5 µg/ml) plus cycloheximide (50, 10 and 1 µg/ml) on testosterone production and the uptake of ^{3}H-amino acids into follicular protein after 4 hours in vitro. Lines on left represent mean ± SEM (n=4; 6 follicles/incubation) of testosterone production (medium), in the absence (o---) or presence (●——) of LH plus cycloheximide. Histograms at upper right represent the uptake of ^{3}H-amino acids into protein, in the absence (C) or presence (numerical insets) of LH plus cycloheximide. The protein content (262 ± 24 µg/6 follicles) between groups remained constant. Since no statistical difference between control groups was determined by analysis of variance, the controls were pooled and statistical analysis was determined by Duncan's New Multiple Range Test.

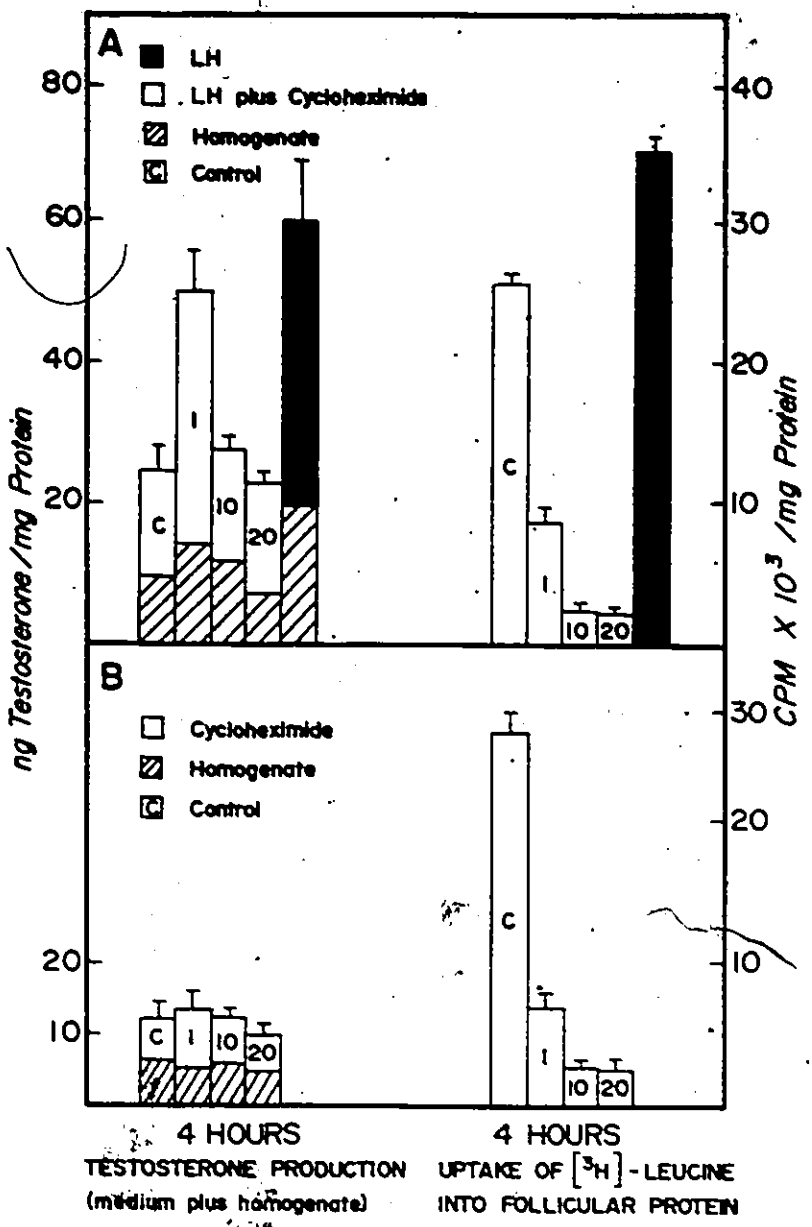

FIGURE 17. Effects of LH (5 µg/ml), LH (5 µg/ml) plus cycloheximide (1, 10 and 20 µg/ml) and cycloheximide (1, 10 and 20 µg/ml) alone on testosterone production and uptake of ^{3}H-leucine into follicular protein after 4 hours in vitro. Histograms represent mean ± SEM (n=5; 1 follicle/incubation). For details see Figure 13. Symbol "C" and "numerical insets" represent control and cycloheximide treatments respectively, either alone (Figure 17B) or together with LH (Figure 17A). Follicular size (1.9 ± 0.2 mm/follicle) and protein content (118 ± 1.5 µg/follicle) remained constant between treatment groups. Statistical analysis was determined by Duncan's New Multiple Range Test.

17B, testosterone production remained basal and similar to controls while protein synthesis was inhibited by more than 70% ($p < .01$), at all concentrations tested.

The electrophoretic pattern and distribution of ^{35}S-methionine incorporation into individual follicular proteins following a 2 hour incubation with LH (5 µg/ml) plus cycloheximide (0.1, 1.0, 10 and 20 µg/ml) are shown in Figure 18. LH had no apparent effect on the distribution pattern or the appearance of a new protein band different from control. The incorporation of ^{35}S-methionine into protein was completely inhibited by cycloheximide concentrations of 20, 10 and 1 µg/ml, whereas 0.1 µg/ml showed decreasing inhibitory effects. The inhibition by cycloheximide on follicular protein synthesis seemed to be a generalized phenomenon as the disappearance of a distinct protein band was not evident.

Effect of puromycin on LH-induced testosterone and protein synthesis

A discrepancy between puromycin-inhibition of follicular protein synthesis and LH-induced testosterone production was also observed. Following a 2 hour pre-incubation in MEM, isolated follicles (1 per incubation) were incubated with ^{3}H-leucine together with LH (5 µg/ml), LH (5 µg) plus puromycin (40, 10, 1 and 0.1 µg/ml) or puromycin (40, 10, 1 and 0.1 µg/ml) alone for 2 hours. As shown in Figure 19A, LH (5 µg/ml) significantly enhanced testosterone production above control values ($p < .01$) and the incorporation of ^{3}H-leucine into follicular protein ($p < .05$). Puromycin (40 µg/ml) inhibited LH-induced testosterone production by 66%, and the uptake of ^{3}H-leucine into follicular protein by 74% ($p < .01$). However, puromycin (10, 1, or 0.1 µg/ml) did not

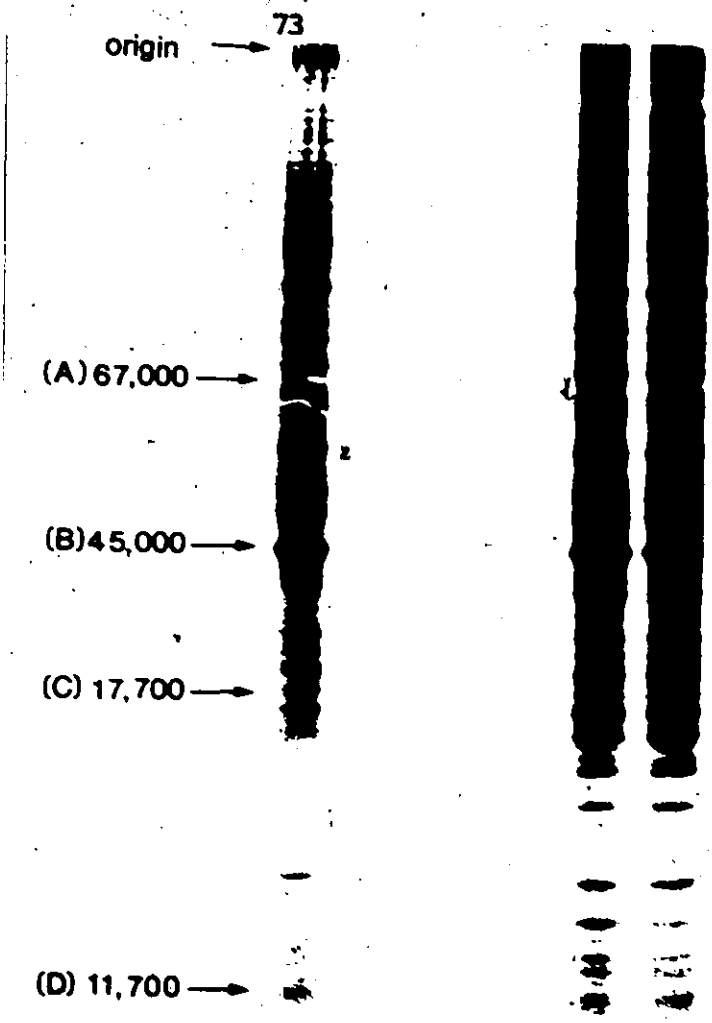

FIGURE 18. Effect of LH plus cycloheximide on the incorporation of ^{35}S-methionine into follicular protein synthesis. Following a 2 hour preincubation in methionine free MEM, follicles (3 per incubation) were incubated with ^{35}S-methionine in the presence or absence of LH (5 μg/ml) alone or LH (5 μg/ml) plus cycloheximide (20, 10, 1 and 0.1 μg/ml) for an additional 2 hours in vitro. After extraction, total cellular proteins were fractionated on a 7.5 - 15% discontinuous sodium dodecyl sulphate/polyacrilamide gel. Pictures of radioautograms (14 days exposure) of fractionated proteins are shown. Standards used as molecular weight markers were: (A) albumin MW 67000; (B) ovalbumin, MW 45000; (C) myoglobin, MW 17800; (D) cytochrome C, MW 125000.

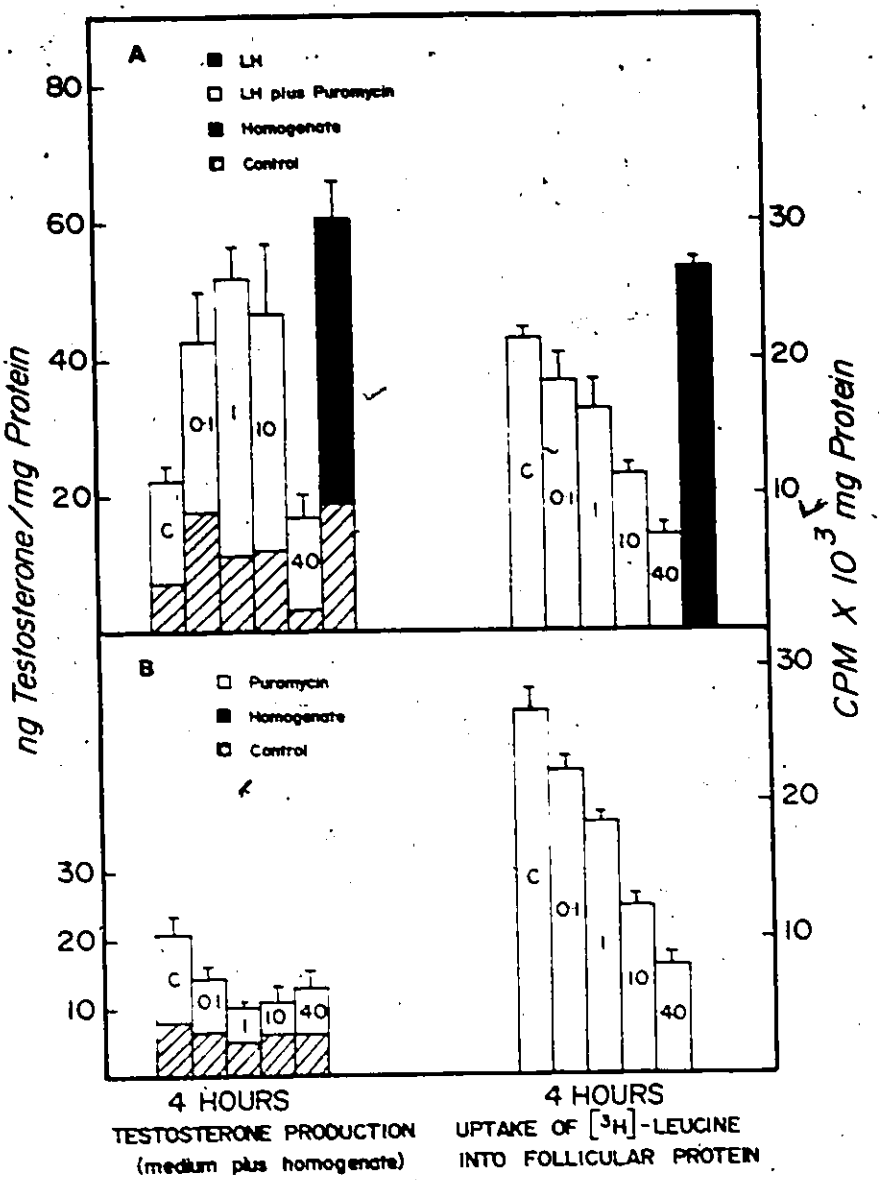

FIGURE 19. Effects of LH (5 µg/ml), LH (5 µg/ml) plus puromycin (0.1, 1.0, 10 & 40 µg/ml) or puromycin (0.1, 1.0, 10 & 40 µg/ml) alone on testosterone production and uptake of ^{3}H-leucine into protein. Histograms represent mean ± SEM (n=4; 1 follicle/incubation). For details see Figure 13. Symbol "C" and "numerical insets" correspond to control and puromycin treatments, respectively, either alone (Figure 19B), or together with LH (Figure 19A). Solid black area represents the LH positive control. Follicular size (1.5 ± 0.1 mm/follicle) and protein content (83 ± 3 µg/follicle) remained constant between treatment groups. Statistical analysis was done using Duncan's New Multiple Range Test.

inhibit LH-induced testosterone production, yet ^{3}H-leucine incorporation into protein was inhibited by 58, 37 and 31%, respectively ($p < .01$).

In the presence of puromycin (40, 10, 1 and 0.1 µg/ml) alone (Figure 19B), the incorporation of ^{3}H-leucine into follicular protein was inhibited by 70, 55, 31 and 17%, respectively ($p < .01$). Testosterone production was also inhibited, compared to controls ($p < .05$), at puromycin concentrations of 40, 10 and 1 µg/ml, but not 0.1 µg/ml.

Reversible effects of cycloheximide

Preliminary experiments suggested that the inhibitory effects of cycloheximide on LH-induced steroidogenesis were reversible. A series of 8 hour incubations were therefore performed to determine: (i) if the effects of cycloheximide were reversible after the antibiotic was removed from the incubation medium; and (ii) was the resumption of steroidogenesis accompanied by an increased incorporation of labeled amino acids into follicular protein.

The effects of LH (5 µg/ml) plus cycloheximide (50 µg/ml) on testosterone production and the uptake of ^{3}H-amino acids into follicular protein after 8 hours in vitro are shown in Figure 20. A brief description of the incubation protocol is as follows. Following a 2 hour pre-incubation in MEM, isolated follicles (6 per incubation) were incubated with LH plus cycloheximide from 2 to 4 hours of incubation. The follicles were then thoroughly washed (6 times) and reincubated in MEM for the remaining 4 hours. The ^{3}H-amino acid mixture was present in the medium from 2 to 4 hours (Figure 20, Panel I), from 4 to 8 hours (Panel II) and 2 to 8 hours of incubation (Panel III).

As shown in Figure 20, cycloheximide (50 µg/ml) inhibited LH-

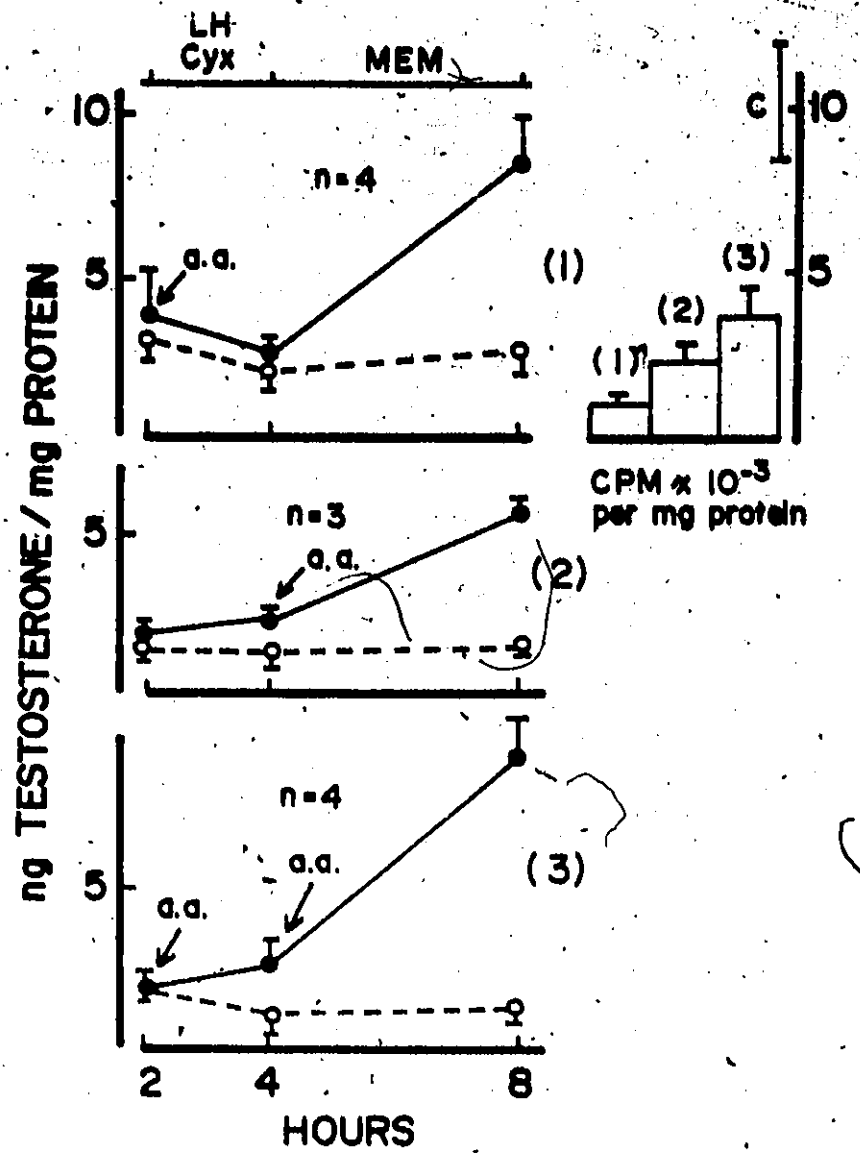

FIGURE 20. Effect of LH (5 µg/ml) plus cycloheximide (50 µg/ml) on testosterone production and uptake of ^{3}H-amino acids into follicular protein after 8 hours in vitro. Lines on left represent mean ± SEM (n=3 or 4; 6 follicles/ incubation) of testosterone production (medium), in the absence (o---) or presence (●——) of LH plus cycloheximide. Histograms on upper right represent the uptake of ^{3}H-amino acids into follicular protein from these experiments when labeled amino acids were added from 2 to 4 hours of incubation (Figure 20, Panel 1), from 4 to 8 hours (Figure 20, Panel 2) or from 2 to 8 hours (Figure 20, Panel 3). No statistical difference in protein content (258 ± 18 µg/6 follicles) between groups was determined. Since no statistical difference between control groups was observed by analysis of variance, the controls were pooled and statistical analysis was determined by Duncan's New Multiple Range Test.

induced testosterone production from 2 to 4 hours of incubation. After removal of cycloheximide, testosterone production significantly increased ($p < .01$) from 4 to 8 hours, in the presence of more than 60% inhibition of amino acid incorporation into follicular protein. However, if one compares the incorporation of ^{3}H-amino acids in the presence of cycloheximide (Figure 20, Panel I: 2 to 4 hours) *versus* the increased incorporation after the follicles were washed and inhibitor removed (Panel II, 4 to 8 hours), a significant difference ($p< .01$) is observed. This increased amino acid incorporation may correspond to the late appearance of testosterone.

To overcome a criticism that the previous experiment may not have been adequately controlled, a similar 8 hour experiment was undertaken. Isolated follicles (3 per incubation) were incubated with LH (5 µg/ml), LH (5 µg/ml) plus cycloheximide (10 µg/ml), or cycloheximide (10 µg/ml) alone from 2 to 4 hours of incubation. The follicles were then washed (6 times) and reincubated in MEM plus ^{3}H-amino acids for the remaining 4 hours (Figure 21). As before, the results clearly demonstrate that the steroidogenic effects of LH are long lasting with complete recovery of cycloheximide-inhibited testosterone production occurring 2 to 4 hours after removal of the antibiotic. LH (5 µg/ml) alone was without stimulatory effect on the incorporation of ^{3}H-amino acids into follicular protein. Cycloheximide (10 µg/ml) inhibited the incorporation of ^{3}H-amino acids into follicular protein by more than 50% in the presence or absence of LH ($p < .01$). However, when the incorporation of ^{3}H-amino acids was compared in the presence of LH plus cycloheximide *versus* cycloheximide alone a significant increase in the incorporation of labeled amino acids

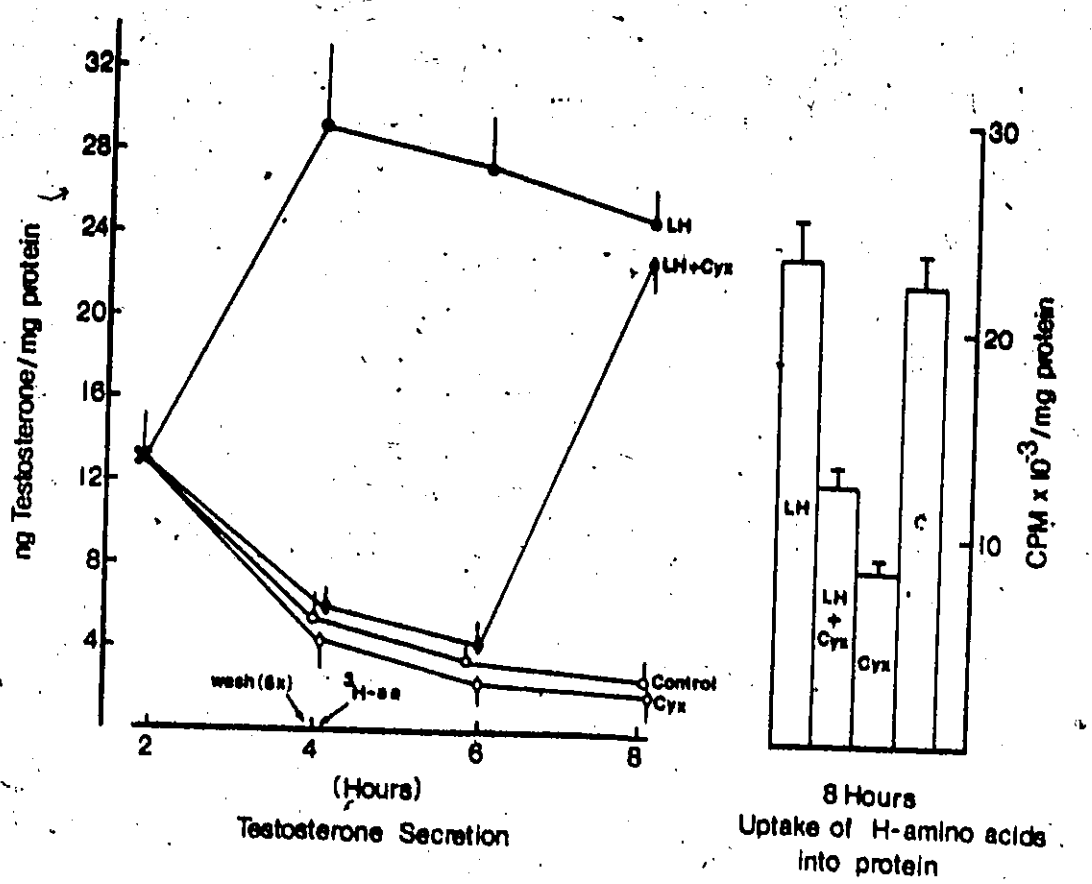

FIGURE 21. Effect of LH (5 µg/ml), LH (5 µg/ml) plus cycloheximide (10 µg/ml) or cycloheximide (10 µg/ml) alone on testosterone production and uptake of ^{3}H-amino acids into follicular protein after 8 hours in vitro. Lines on left represent mean ± SEM (n=5; 3 follicles/ incubation) of testosterone production (medium) over 8 hours, in the absence (o—), or presence of LH (●—), LH Plus cycloheximide (◆—) or cycloheximide (◇—) alone. Histograms on right represent uptake of labeled amino acids into follicular protein synthesis, determined at the end of 8 hours. Test substances (LH or cycloheximide) were present only during the 2 to 4 hour incubation period. Labeled amino acids were added at the end of 4 hours. For details see text. No statistical difference in follicular protein content (258 ± 41 µg/3follicles) was observed between treatment groups. Statistical analysis was determined by Duncan's New Multiple Range Test).

was observed ($p < .01$). This increased amino acid incorporation may be attributed to LH and corresponds to the appearance of testosterone production

Effect of cycloheximide on cyclic AMP-induced testosterone and protein synthesis

A discrepancy was found between the inhibition of cyclic AMP-induced testosterone production and protein synthesis in the isolated rabbit follicle.

Following the usual 2 hour pre-incubation in MEM, follicles (1 per incubation) were incubated with a ^{3}H-amino acid mixture together with cyclic AMP (5 mM), cyclic AMP (5 mM) plus cycloheximide (50 and 1 µg/ml), or cycloheximide (50 and 1 µg/ml) alone for an additional 2 hours (Figure 22). Cycloheximide (50 µg/ml) inhibited cyclic AMP-induced testosterone production (measured in medium), and the incorporation of ^{3}H-amino acids into follicular protein by 89.6 and 84%, respectively ($p < .01$). However, cycloheximide (1 µg/ml) did not inhibit cyclic AMP-induced testosterone production, while the ^{3}H-amino acid incorporation into follicular protein was inhibited by 77.7% ($p < .01$).

In the presence of cycloheximide (50 and 1 µg/ml) alone (Figure 22B), testosterone production remained basal and similar to control values, while protein synthesis was inhibited by 87.2 and 68.1%, respectively ($p < .01$).

Effect of puromycin on cyclic AMP-induced testosterone and protein synthesis

The effects of cyclic AMP (5 mM), cyclic AMP (5 mM) plus puromycin (40, 10 and 1 µg/ml) and puromycin (40, 10 and 1 µg/ml) alone on testosterone production and the uptake of ^{3}H-amino acids into follicular protein were examined (Figure 23). After 4 hours, puromycin (40 µg/ml)

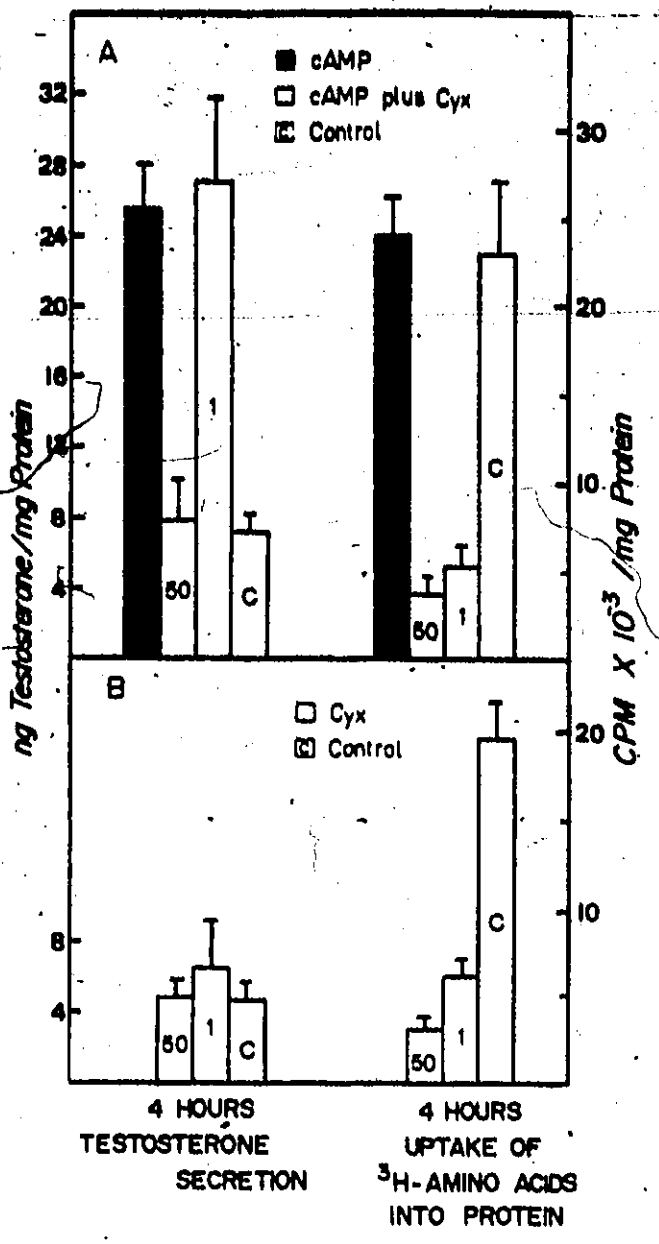

FIGURE 22. Effect of cyclic AMP (5 mM), cyclic AMP (5 mM) plus cycloheximide (50 & 1 µg/ml) and cycloheximide (50 & 1 µg/ml) alone on testosterone production and uptake of ^{3}H-amino acids into protein after 4 hours in vitro. Histograms on left represent testosterone, measured in medium. Histograms on right represent uptake of ^{3}H-amino acids into follicular protein. Results are expressed as mean ± SEM (n=4; 6 follicles/incubation). Symbol "C" and "numerical insets" correspond to control and cycloheximide treatments, respectively, either alone (Figure 22B) or together with cyclic AMP (Figure 22A). Solid black areas represent cyclic AMP positive controls. No statistical difference in protein content (271± 19 µg/6 follicles) was observed between treatment groups. Statistical analysis was done by Duncan's New Multiple Range Test.

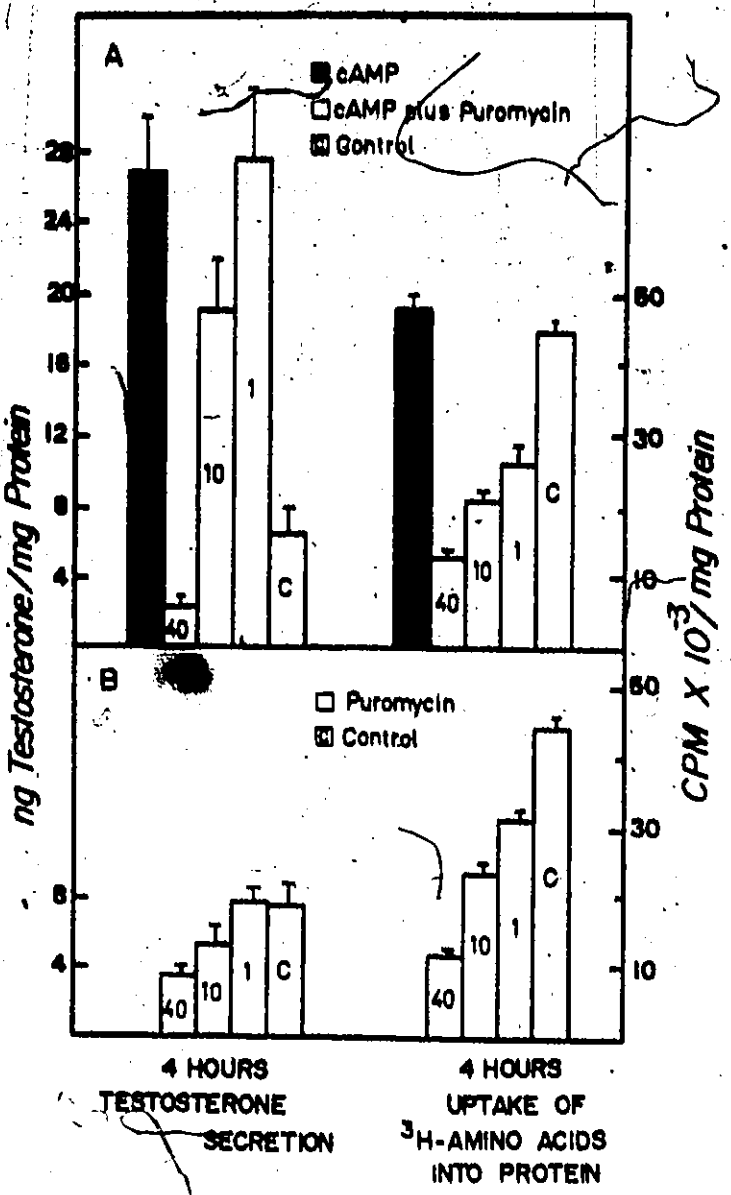

FIGURE 23. Effect of cyclic AMP (5 mM), cyclic AMP plus puromycin (40, 10, 1 µg/ml) or puromycin alone on testosterone production and uptake of ^{3}H-amino acids into follicular protein after 4 hours in vitro. Histograms on left represent testosterone in medium. Histograms on right represent uptake of ^{3}H-amino acids into follicular protein. Results are expressed as mean ± SEM (n=8, 1 follicle/incubation). No statistical difference in follicular size (1.9 ± 0.2 mm/follicle) or protein content (129 ± 5.7 µg/follicle) was observed. Symbol "C" and "numerical insets" correspond to Control and puromycin treatments, either alone (Figure 23B) or together with cyclic AMP (Figure 23A). Solid black areas represent cyclic AMP positive controls. Statistical analysis was done using Duncan's New Multiple Range Test.

inhibited both cyclic AMP-induced testosterone production and the incorporation of ^{3}H-amino acids by 92 and 74%, respectively ($p < .01$). However, puromycin (10 and 1 µg/ml) did not inhibit cyclic AMP-induced testosterone production while ^{3}H-amino acid incorporation was inhibited by 56 and 45%, respectively ($p < .01$).

In the presence of puromycin (40, 10 and 1 µg/ml) alone (Figure 23B), the incorporation of ^{3}H-amino acids into follicular protein was inhibited by 73.4, 48.2 and 30%, respectively ($p < .01$). Testosterone production was also inhibited, compared to controls, at puromycin concentrations of 40 µg/ml ($p < .05$), but not at 10 and 1 µg/ml.

Effect of Actinomycin D, an inhibitor of RNA synthesis

In view of previous reports[122, 131] and the fact that neither LH (0.1 - 10 µg/ml) nor cyclic AMP (0.1 - 10 mM) directly enhanced the incorporation of ^{3}H-uridine into follicular RNA (Figure 11B ; Table 3), it was of interest to investigate the effect of Actinomycin D on LH- and cyclic AMP-induced steroidogenesis and the incorporation of ^{3}H-uridine into follicular RNA.

On LH-induced steroidogenesis and follicular RNA synthesis

Following a 2 hour pre-incubation in MEM, follicles (1 per incubation) were incubated for 2 hours with ^{3}H-uridine and Actinomycin D (1, 20, 80 and 160 µg/ml) alone, or together with LH (5 µg/ml). As can be seen in Figure 24A, LH (5 µg/ml) had no effect on incorporation of ^{3}H-uridine into follicular RNA. Actinomycin D (1, 20, 80, 160 µg/ml) alone, or together with LH (5 µg/ml), inhibited RNA synthesis by 86, 85, 79 and 50%, respectively ($p < .01$). Paradoxically, Actinomycin D

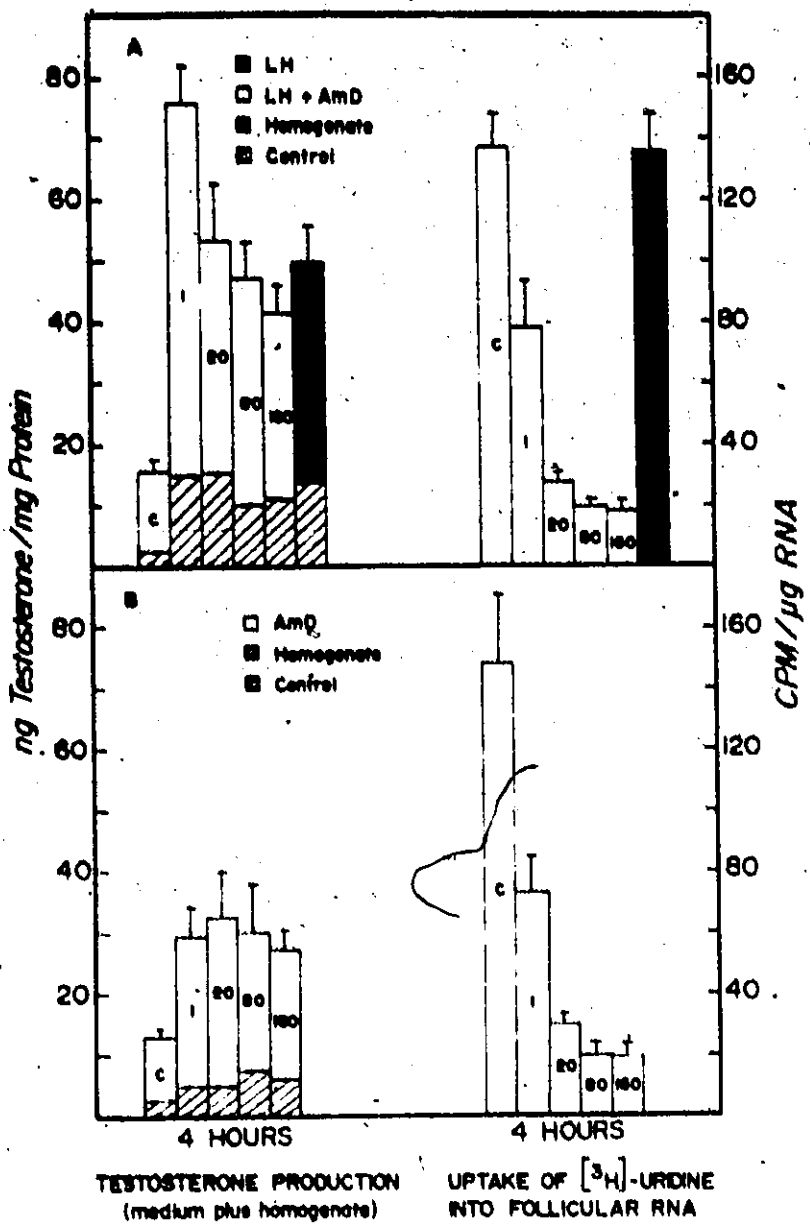

FIGURE 24. Effects of LH (5 µg/ml), LH plus Actinomycin D (1, 20, 80 & 160 µg/ml) or Actinomycin (1, 20, 80 & 160 µg/ml) alone on testosterone production and uptake of ^{3}H-uridine into follicular RNA after 4 hours in vitro. Histograms represent mean ± SEM (n-5; 3 follicles/incubation). Control or Actinomycin D treatments, respectively, either alone (Figure 24B) or together with LH (Figure 24A). Solid black area represents LH positive control. No statistical difference in follicular size (1.5 ± 0.1 mm/follicle), protein content (230 ± 22 µg/3 follicles) or RNA content (28 ± 1.1 µg/3 follicles) was observed. Statistical analysis was carried out using Duncan's New Multiple Range Test.

(1 µg/ml), in the presence of LH (5 µg/ml), enhanced testosterone production above that elicited by LH alone ($p < .01$). This profile was consistent when measured in both the incubation medium and follicular homogenates.

The above findings indicated that Actinomycin D may act on testosterone formation by affecting either the steroid biosynthetic or metabolic processes. The latter was rejected since Actinomycin D (1 µg/ml) had no apparent effect on further ^{3}H-testosterone metabolism (Table 1). In order to investigate the former possibility, i.e. biosynthetic stimulation, a steroidogenic profile (androstenedione, dehydroepiandrosterone, estradiol-17β and progesterone) was obtained from the same samples corresponding to the testosterone results shown in Figure 24A. In the presence of LH (5 µg/ml) plus Actinomycin D (1 µg/ml), progesterone decreased while dehydroepiandrosterone increased (Table 5). Conversely, progesterone, but not dehydroepiandrosterone, was elevated in the presence of LH alone and LH plus Actinomycin D (80 and 160 µg/ml). Androstenedione was elevated with all treatments: LH (5 µg/ml) or LH (5 µg/ml) plus Actinomycin D (1, 20, 80, 160 µg/ml). No change in estradiol-17β accumulation ($\bar{x}$ 6.5 $\pm$ 2.3 ng/mg protein) was observed between treatment groups.

In the presence of Actinomycin D (1, 20, 80, 160 µg/ml) alone (Figure 24A), testosterone was elevated, however, this was not statistically significant. No changesin progesterone ($\bar{x}$ 15.3 $\pm$ 5.6 ng/mg protein), dehydroepiandrosterone ($\bar{x}$ 8.3 $\pm$ 2.9 ng/mg protein) or estradiol-17β ($\bar{x}$ 3.4 $\pm$ 1.3 ng/mg protein) were observed when compared to control values.

TABLE 5

Effect of LH (5 µg/ml) and LH (5 µg/ml) plus Actinomycin D (1, 20, 80, 160 µg/ml) on steroid production in the isolated rabbit ovarian follicle after 4 hours in vitro.

Treatment (µg/ml)	Progesterone (ng/mg protein)	Androstenedione (ng/mg protein)	Dehydroepi-androsterone (ng/mg protein)
LH (5)	21.6 ± 5.7**	17.7 ± 1.7*	7.8 ± 2.3
LH (5) + AMD (1)	11.7 ± 1.2+	22.4 ± 3.8**	14.6 ± 1.8**++
LH (5) + AMD (20)	12.1 ± 1.5+	22.7 ± 2.0**	12.2 ± 0.9**+
LH (5) + AMD (80)	17.5 ± 2.8**	23.6 ± 4.9**	8.1 ± 0.8
LH (5) + AMD (160)	18.7 ± 2.3**	17.3 ± 1.6**	6.6 ± 1.4
Control	5.0 ± 0.77++	5.30± 0.7++	3.6 ± 0.7++

* $p < 0.05$ vs control

** $p < 0,01$ vs control

\+ $p < 0.05$ vs LH (5µg/ml)

++ $p < 0.01$ vs. LH (5µg/ml)

Results are expressed as mean ± SEM (n=5; 3 follicle incubation) for total steroid production (medium plus homogenate). No statistical difference in follicular size (1.5 ± 0.1 mm/follicle), protein content (230 ± 22 µg/3 follicles) or RNA content (28.2 ± 1.1 µg/3follicles) was observed.

When female rabbits were first injected intraperitoneally with Actinomycin D (20 µg/100 g.b.w.) at 24 and 12 hours prior to sacrifice (Table 6), Actinomycin D (1 µg/ml) significantly enhanced follicular testosterone production above control values ($p < .05$) after 2 hours in vitro.

The in vivo administration of high concentrations of Actinomycin D (Table 6) had drastic physiological effects on the female rabbit. Twenty-four hours after injection the rabbits appeared sick, displaying a marked loss in body weight (approximately 240 gm/rabbit), acute diarrhea, irregular breathing and extreme docility.

On cyclic AMP-induced testosterone and RNA synthesis

Following a 2 hour pre-incubation in MEM, follicles (6 per incubation) were incubated for 2 hours with ^{3}H-uridine and Actinomycin D (160 µg/ml) alone, or together with cyclic AMP (5 mM). Actinomycin D (160 µg/ml) alone, or together with cyclic AMP (5 mM), inhibited the incorporation of ^{3}H-uridine into follicular RNA by more than 89% of control values ($p < .01$), Figure 25. However, Actinomycin D had no apparent effect on cyclic AMP enhanced testosterone production. In the presence of Actinomycin (160 µg/ml) alone, testosterone was not statistically elevated above control values.

Effect of inhibitors of phosphodiesterase: methylxanthines

The methylxanthines, theophylline and 1,3-isobutyl-methylxanthine (MIX), are potent inhibitors of phosphodiesterase, the enzyme responsible for hydrolysis of cyclic AMP to 5'-AMP. Since cyclic AMP mimicked the effects of LH on protein synthesis (Tables 3 and 4), it should

TABLE 6

Effect of incubation with LH (5 µg/ml) alone or with Actinomycin D (1 µg/ml) and Actinomycin D (1 µg/ml) alone on testosterone production after pretreatment of rabbits with Actinomycin D (20 µg/100g) *in vivo* 24 and 12 hours prior to sacrifice.

Treatment (µg/ml)	Testosterone produced (ng/mg protein)
LH (5)	40.7 ± 1.7**
LH (5) + AMD (1)	47.8 ± 1.6**+
AMD (1)	28.7 ± 0.8**++
Control	12.7 ± 1.8++

** $p < 0.01$: Compared with control follicles.

\+ $p < 0.05$; ++ $p < 0.01$: Compared with LH treated follicles.

Results are expressed as mean ± SEM (n=4; one follicle/incubation).

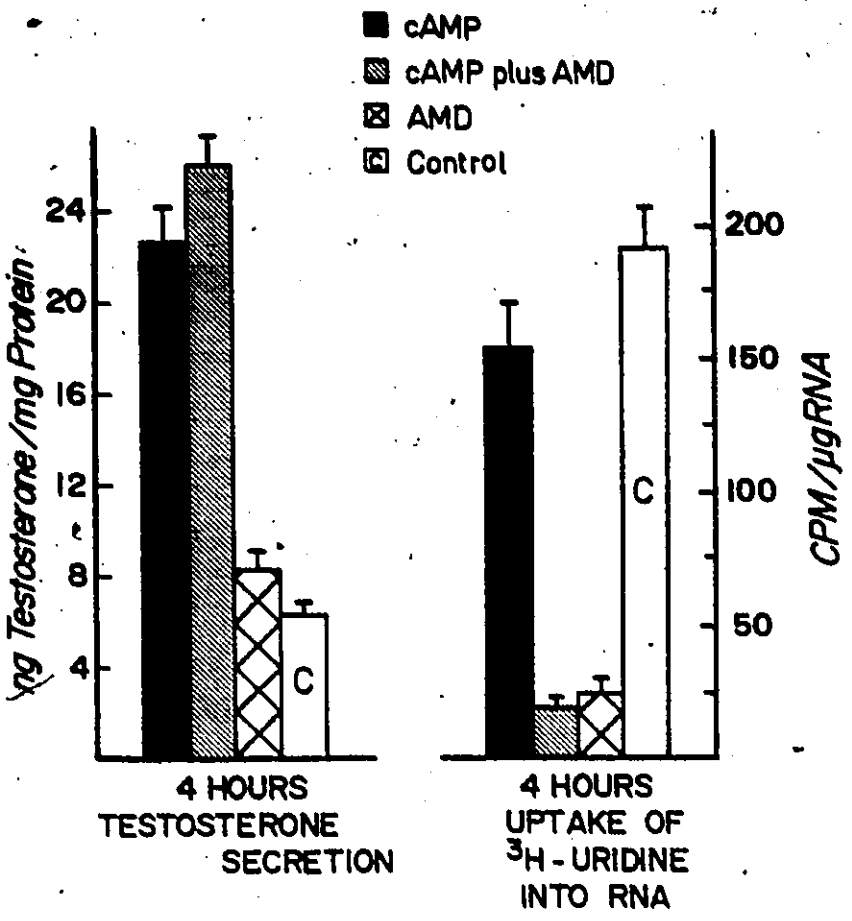

FIGURE 25. Effect of cyclic AMP (5 mM), cyclic AMP (5 mM) plus Actinomycin D (160 µg/ml) or Actinomycin D (160 µg/ml) alone on testosterone production and uptake of ^{3}H-uridine into follicular RNA after 4 hours in vitro. Histograms represent mean ± SEM (n=4; 6 follicles/incubation). Follicular protein (224 ± 13 µg/6 follicles) and RNA (32 ± 2 µg/6 follicles) content remained constant between treatment groups. Statistical analysis was determined by Duncan's New Multiple Range Test.

follow that inhibition of the natural breakdown of cyclic AMP would increase the intracellular cyclic AMP content, thereby increasing steroidogenesis. It was felt that by determining the incorporation of ^{3}H-uridine and ^{3}H-amino acids into follicular RNA and protein in the presence of methylxanthines, some insight could be gained into their necessity as prerequisite for follicular steroidogenesis.

Following a 2 hour pre-incubation in MEM, isolated follicles (6 per incubation) were incubated for 2 hours with either a ^{3}H-amino acid mixture or ^{3}H-uridine, plus theophylline (25, 10 and 1 mM) or MIX (5 and 0.5 mM) alone, or together with cyclic AMP (5 mM). As shown in Table 7, cyclic AMP significantly stimulated testosterone production above control values ($p < .01$), but had no effect on the uptake of either ^{3}H-uridine into follicular RNA or ^{3}H-amino acids into protein. The methylxanthines, theophylline (25, 10 and 1 mM) and MIX (5 and 0.5 mM) alone, or together with cyclic AMP (5 mM), inhibited the incorporation of ^{3}H-uridine (35 to 68%) and ^{3}H-amino acids (45 to 69%) into follicular RNA and protein at all concentrations tested. Theophylline 25 mM, but not 1 and 10 mM, enhanced testosterone production above control values ($p < .05$). This stimulatory effect of theophylline was not synergistic with the effects of cyclic AMP on testosterone production. In the presence of 5 mM MIX, but not 0.5 mM, testosterone production was significantly inhibited when compared to cyclic AMP treated follicles ($p < .01$).

Effect of cyclic GMP on follicular RNA, protein and testosterone production

Very little is known about the role of cyclic GMP on ovarian

TABLE 7

Effect of theophylline (25, 10, 1 mM) and MIX (5, 0.5 mM) on cyclic AMP (5 mM) enhanced testosterone production and the uptake of ^{3}H-amino acids and ^{3}H-uridine into follicular protein and RNA.

Treatment	n	Testosterone (ng/mg protein)	n	Uptake of ^{3}H-amino acids into protein cpm x 10^{-3}/mg protein	n	Uptake of ^{3}H-uridine into RNA cpm/ µg RNA
cAMP (5 mM)	8	23.6 ± 1.2**	4	21.7 ± 1.8	4	153.7 ± 18
cAMP + Theo (25 mM)	4	20.6 ± 2.4	4	11.9 ± 1.9**+-		N.D.
+ Theo (10 mM)	8	22.3 ± 2.5**	4	10.2 ± 0.5**++	4	93.8 ± 12**+
+ Theo (1 mM)	8	21.6 ± 2.2**	4	10.9 ± 0.2**++	4	103 ± 7.1**+
cAMP + MIX (5.0 mM)	8	11.2 ± 1.7*++	4	8.8 ± 0.6**++	4	90.5 ± 12.8**+
+ MIX (0.5 mM)	7	21.5 ± 3.1**	3	10.9 ± 1.6**++	4	84.8 ± 7.7**+
Theo (25 mM)	5	11.6 ± 3.5*	5	12.4 ± 1.4**		N.D.
(10 mM)	8	9.9 ± 1.2	4	11.1 ± 1.2**	4	54.3 ± 6.3**
(1 mM)	7	7.6 ± 1.2	3	7.2 ± 2.0**	4	110 ± 4.2**
MIX (5.0 mM)	7	8.0 ± 0.8	4	10.4 ± 0.9**	4	76.5 ± 11.8**
(0.5 mM)	7	10.0 ± 1.2	4	10.3 ± 0.9**	3	107 ± 5.3**
Control	77	6.7 ± 0.3++	42	21.8 ± 0.8	35	170.2 ± 7.0

N.D. = Not Determined

* $p < 0.05$ vs control ** $p < .01$ vs control + $p < .05$ vs cAMP (5 mM) ++ $p < .01$ vs cAMP (5 mM)

Results are expressed as mean ± SEM. No significant difference in protein (392 ± 5 µg/6 follicles) or RNA (36.7 ± 3.0 µg/6 follicles) was observed between groups.

function. The present study was undertaken to investigate the role of cyclic GMP in testosterone, protein and RNA synthesis in the isolated rabbit ovarian follicle. Isolated follicles (6 per incubation) were incubated for 2 hours with cyclic GMP (25, 10 and 1 mM) plus a ^{3}H-amino acid mixture or ^{3}H-uridine following a 2 hour pre-incubation in MEM alone. As shown in Table 8, at all concentrations of cyclic GMP tested, testosterone production and the uptake of ^{3}H-amino acids into protein showed no difference from controls. On the other hand, at concentrations of 10 and 25 mM, but not 1 mM, cyclic GMP significantly enhanced the uptake of ^{3}H-uridine into RNA ($p < .01$).

TABLE 8

Effect of cyclic GMP (25, 10, 1 mM) on testosterone production and uptake of ^{3}H-amino acids and ^{3}H-uridine into follicular protein and RNA after 4 hours *in vitro*.

Treatment (mM)	Testosterone (ng/mg protein)	Uptake of ^{3}H-amino acids into protein (cpm x 10^{-3}/mg protein)	Uptake of ^{3}H-uridine into RNA (cpm/µg RNA)
cGMP 25	6.2 ± 0.9 (8)	11.6 ± 2.8 (4)	*161.8 ± 27.5 (4)
10	6.6 ± 0.5 (8)	13.5 ± 1.4 (4)	*179.2 ± 37.7 (4)
1	7.2 ± 0.6 (8)	14.7 ± 0.7 (4)	116.6 ± 12.2 (4)
Control	6.4 ± 0.4 (24)	14.8 ± 1.2 (12)	77.9 ± 5.5 (12)

* $p < .01$ vs control

Results are expressed as mean ± SEM. In determining the incorporation of ^{3}H-uridine and ^{3}H-amino acids into RNA and protein, each experiment was carried out separately with a paired control. There was no significant difference in follicular protein content (400 ± 20 µg/6 follicles) or RNA content (55 ± 4.9 µg/6 follicles) between groups.

DISCUSSION

In current models of LH action on steroidogenesis (Figure 3), LH is depicted as binding to specific plasma membrane receptors, thereby stimulating adenylate cyclase (reviewed by Channing and Tsafriri[3]). The resulting increased cyclic AMP activates a protein kinase which can: (i) enhance messenger RNA synthesis essential for the production of a labile, regulatory protein; or (ii) phosphorylate a pre-existing enzyme required to mediate the action of LH. This regulatory protein may then catalyze the mitochondrial conversion of cholesterol to pregnenolone, or facilitate pregnenolone transport out of the mitochondria.

A role for RNA and protein synthesis in gonadal steroidogenesis is suggested by the fact that transcriptional and translational inhibitors prevent LH-induced steroidogenesis without effecting cyclic AMP formation (reviewed by Marsh[155]; Wicks[190]). However, researchers have failed to demonstrate a direct causal relationship between de novo RNA or protein synthesis with steroidogenesis[32, 41].

The present investigation has attempted to elucidate the role of RNA and protein synthesis in LH-induced steroidogenesis using the intact isolated rabbit ovarian follicle as an experimental model. The isolated rabbit follicle offers an attractive model whereby the concentrations of various test substances can be carefully controlled and their effects on steroidogenic processes measured.

The effect of LH and other test substances on follicular transcriptional and translational processes was determined by measuring the incorporation of labeled uridine and amino acids into RNA and protein. Testosterone production was used as a marker for measuring the effects of LH or other test substances on follicular steroidogenesis. Testosterone production was used for two reasons: (i) testosterone is the major steroid produced upon LH stimulation of the isolated rabbit follicle (Figure 10); and (ii) the testosterone produced in vitro was not further metabolized (Table 1).

Role of RNA Synthesis in Follicular Steroidogenesis

RNA and testosterone production

In order to investigate the role of RNA synthesis in follicular steroidogenesis, isolated follicles were incubated with labeled uridine plus LH or cyclic AMP, either alone or in combination with Actinomycin D (an inhibitor of DNA-dependent RNA synthesis[191]).

Both LH (5 µg/ml) and cyclic AMP (0.1 to 10 mM) caused a significant increase in testosterone production by the follicles, yet there was no concomitant increase in the uptake of labeled uridine into follicular RNA (Figures 11B, 24A, 25 and Table 3). Also, Actinomycin D (160 µg/ml) completely inhibited the incorporation of ^{3}H-uridine into follicular RNA, but had no effect on LH or cyclic AMP-induced testosterone production (Figures 24A and 25). These data suggest that, in the rabbit follicle, RNA synthesis is not required for acute LH-induced steroidogenesis.

The non-involvement of RNA synthesis in steroidogenesis has similarly been noted by YoungLai[122] in the isolated rabbit ovarian follicle. This observation was based on the inability of Actinomycin D to prevent LH-induced steroidogenesis. However, the incorporation of labeled ribonucleosides into follicular RNA was not examined. The above results, therefore, confirm and extend YoungLai's[122] earlier observation.

These results also point to an important distinction between the rabbit and rat in the control mechanism of steroidogenesis. In the rat testis[133, 192, 193], ovary[146] and Graafian follicle[32, 41], low concentration of Actinomycin D ($\leq$ 8 µg/ml) inhibited LH-induced steroidogenesis, thereby implicating transcriptional control processes and a role for RNA in steroidogenesis. In contrast, in the isolated rabbit follicle, extremely high concentrations of Actinomycin D (160 µg/ml) had no inhibitory effect on LH-induced testosterone production (Figures 24 and 25). A translational control mechanism is implied, as was previously suggested by YoungLai[122].

If one accepts that the induction of an essential protein requires prior RNA synthesis, then the failure of a specific protein to appear implies the absence of essential RNA species. Since LH did not induce the incorporation of ^{35}S-methionine into a specific protein band (Figure 14), these data further suggest that de novo RNA synthesis is not required for acute steroidogenesis in the rabbit follicle.

The above interpretation, however, does not exclude a number of other possibilities concerning LH-or cyclic AMP-induced steroidogenesis.

It is possible that: (i) only a few essential RNA species are synthesized and that their detection is masked by the overall incorporation of labeled uridine into total RNA; and (ii) although Actinomycin D inhibited overall RNA synthesis, this does not rule out the possibility of the action of a stable pre-formed messenger RNA.

Nevertheless, the present results collectively suggest that de novo RNA synthesis is not a necessary pre-requisite for LH-induced steroidogenesis in the rabbit Graafian follicle.

Paradoxical effects of Actinomycin D

In recent years, attention has focused on the non-specific and cytotoxic effects of Actinomycin D as well as the wide range of results observed when different tissues are used (reviewed by Bransome[194]).

Actinomycin D is a potent inhibitor of RNA synthesis in eucaryotic cells. Actinomycin D is a cyclic polypeptide which is thought to complex with the deoxyguanosine residue of the DNA template and so prevents DNA-directed RNA synthesis by RNA polymerase[191]. In cell free systems, low concentrations of Actinomycin D (<1ug/ml) preferentially inhibit ribosomal RNA while higher concentrations inhibit both ribosomal and messenger RNA synthesis[195].

In the isolated rabbit follicle the antibiotic exhibits paradoxical effects. Actinomycin D (1 µg/ml) stimulates follicular steroidogenesis, alone or in combination with LH (superinduction), while at the same time inhibits the incorporation of ^{3}H-uridine into follicular RNA by 45 to 85% (Figure 24).

The term "superinduction" was first used by Thompson et al.[196] to describe the potentiating effect of Actinomycin D on corticoid-induced liver enzymes. These authors postulated that superinduction occurs if Actinomycin D inhibits the synthesis of a messenger RNA species which is responsible for the production of a repressor substance normally required for translational control mechanisms. In our system, such an effect could operate by either stimulating testosterone biosynthesis or inhibiting its metabolism. The latter was rejected because only 7% of the ^{3}H-testosterone was unaccounted for in in vitro incubation studies and this amount was independent of treatment (Table 1). Actinomycin D (1 µg/ml)-superinduction of testosterone production (Figure 24) was also accompanied by a decrease in progesterone (Table 5) and an increase in androstenedione and dehydroepiandrosterone production when compared to the positive LH control. It is inferred that Actinomycin may be activating 17α-hydroxylase and/or 17-20 desmolase. A similar mechanism for Actinomycin-superinduction of LH stimulated steroidogenesis has been suggested for the rat follicle[41] and ovary[146].

The finding that Actinomycin D alone can stimulate testosterone production (Table 6), combined with the observed LH-superinduction (Figure 24), suggests that Actinomycin D acts at a site independent of LH. Similar findings for Actinomycin D enhanced corticosteroidogenesis have been reported in the human[197] and guinea pig adrenal cortex[194]. In the isolated rabbit follicle, however, there is unsufficient evidence to determine the mechanism by which Actinomycin D induces steroidogenesis, either alone or in the presence of LH.

Role of Protein Synthesis in Steroidogenesis

Initial studies were aimed at directly investigating the role of protein synthesis in steroidogenesis by the rabbit ovarian follicle in vitro.

While it was not possible to show an effect of LH on the uptake of ^{3}H-amino acid mixture into follicular protein (Figures 11, 22 and 23), a significant incorporation was noted in experiments using a single amino acid, ^{3}H-leucine (Figures 12, 17, 19 and Tables 2-4). This discrepancy may be attributed to a general masking of any effect by the variety of labeled amino acids in the mixture.

Dose response and time course studies

In dose response studies, LH (0.1 µg/ml) stimulated testosterone production ($p<.01$) after 2 hours in vitro. However, the incorporation of ^{3}H-leucine into follicular protein was only observed at concentrations of 2.5 µg LH/ml or greater (Figure 13). In contrast, cyclic AMP enhanced both testosterone production and ^{3}H-leucine incorporation in parallel dose dependent fashions (Table 3). The difference between the dose dependent effects of LH and cyclic AMP may be due to the inability of low concentrations of cyclic AMP to penetrate the cell membrane.

In time course studies, although LH enhanced both testosterone production and the incorporation of ^{3}H-leucine into follicular protein, the appearance of testosterone preceded protein synthesis by 75 minutes (Table 2). Similar results were obtained in cyclic AMP time course

studies where testosterone production preceded protein synthesis by 45 minutes (Table 4). On the basis of these results, one could reasonably conclude that there appears to be no causal relationship between de novo protein synthesis and acute LH-or cyclic AMP-induced steroidogenesis in the rabbit follicle.

Grower and Branscme[198] reported similar results in cultured mouse adrenocortical tumor cells where ACTH and cyclic AMP-induced corticosteroidogenesis preceded the incorporation of ^{3}H-leucine into total cellular protein by 45 to 60 minutes.

In the isolated rabbit follicle, polyacrilamide gel electrophoresis followed by radioautographic examination of follicular proteins synthesized over a 2-hour incubation period, revealed that while a large number of protein bands incorporated ^{35}S-methionine, LH had no observable stimulatory effect different from controls (Figure 14). These findings further support the theory that de novo protein synthesis is not a necessary pre-requisite for acute steroidogenesis in the rabbit follicle.

In cultured Leydig cells, Janszen et al.[56] demonstrated by electrophoresis and radioautography, that although LH enhanced the incorporation of ^{35}S-methionine into a specific protein band, termed "LH-IP" (MW 33,000), the appearance occurred only 2 to 3 hours after LH-induced testosterone was first measured. It was similarly concluded that LH-IP was not involved in acute LH-induced testosterone production in the testis.

Inhibitor studies: cycloheximide and puromycin

The antibiotics, cycloheximide and puromycin, have been extensively used to delineate the role of protein synthesis in steroidogenesis. Cycloheximide inhibits protein synthesis by slowing the overall rate of translation whereas puromycin, an antibiotic chemically unrelated from cycloheximide, causes premature polypeptide termination[189].

A role for protein synthesis was implicated in adrenal[140] and Leydig[132] cell suspensions, when cycloheximide inhibited both cellular protein synthesis and steroidogenesis in a parallel dose dependent fashion. Attempts were therefore made to demonstrate a causal relationship between the inhibition of protein synthesis and steroidogenesis in the isolated rabbit ovarian follicle. When cycloheximide and puromycin dose response studies were used to evaluate such a relationship, a discrepancy was found between the inhibition of follicular protein synthesis with LH and cyclic AMP induced steroidogenesis in the rabbit follicle.

Cycloheximide inhibited by 50% the incorporation of ^{3}H-amino acids into follicular protein at a concentration between 0.1 to 0.01 µg/ml (Figure 15). At a concentration of 1 µg/ml, cycloheximide inhibited the incorporation of ^{3}H-leucine and ^{3}H-amino acids into follicular protein by 75 to 80%, yet had no significant inhibitory effect on the steroidogenic responses to LH (Figures 16 and 17) or cyclic AMP (Figure 22). Puromycin produced similar results; 10 µg/ml inhibited the incorporation of labeled amino acids into follicular

protein by 50%, yet had no inhibitory effect on LH-or cyclic AMP-induced testosterone production (Figures 19 and 23). The fact that the inhibitory effects of cycloheximide and puromycin on protein synthesis and testosterone production were not parallel, and these two chemically different translational inhibitors produced identical results, lead one to further question the existence of a causal relationship between these two events.

It may be argued that whereas the inhibition of protein synthesis was only partially complete, the remainder was sufficient to allow synthesis of a protein regulator necessary for steroidogenesis. Two pieces of evidence suggest that this is not the case in the rabbit follicle. Fractionation by polyacrilamide gel electrophoresis of total follicular protein, labeled with ^{35}S-methionine, demonstrated that: (i) LH did not visibly enhance the synthesis of a new protein band (Figure 14); and (ii) cycloheximide appeared to inhibit all protein bands to the same extent (Figure 18).

The recovery of LH-induced steroidogenesis from cycloheximide inhibition 2 to 4 hours after removal of LH and cycloheximide (Figures 20 and 21) suggests that: (i) LH initiates an irreversible series of events which are inhibited by cycloheximide but reversed after washing and removal of the inhibitor; or (ii) LH may be still actively bound to the receptor and stimulates steroidogenesis after removal of cycloheximide. In addition, the fact that the incorporation of ^{3}H-amino acids and LH-induced steroidogenesis both increased 2 to 4 hours after removal of cycloheximide, may suggest that de novo protein synthesis

is necessary for steroidogenesis. However, in view of the previous cycloheximide results (Figures 16-18), it is more likely that following cycloheximide inhibition, LH is stimulating a renewal of all cellular metabolic processes, including protein synthesis and steroidogenesis, and that the two are independent events. In order to clarify this point with certainty, an electrophoretic and radioautographic profile of all cellular proteins would have to be determined in these 8-hour experiments.

Regulatory Protein in Steroidogenesis: Hypothetical Models

Three hypothetical models are illustrated in Figure 4 which depict the regulation of steroidogenesis by a protein with a short half life. Garren et al.[138] originally postulated that adrenal steroidogenesis was mediated by the de novo synthesis of a protein with a short half life of 7 to 13 minutes (Figure 4; Model I). Lowry and Martin[139] and Schulster et al.[140] rejected this hypothesis as it was seemingly impossible for de novo synthesis of a regulatory protein to occur within the time (24 to 60 seconds) in which ACTH-induced steroidogenesis first became apparent. Rather, these authors[139, 140] suggested that ACTH activated a pre-existing protein with a short half life (Figure 4; Model III). Similarly in Leydig cell suspensions, although Janszen et al.[56] isolated an LH inducible protein (LH-IP), Model I was also rejected since the appearance of LH-IP occurred 2-to 3-hours after LH addition to the Leydig cells compared with 5-to 15-minutes for LH stimulation of testosterone production[132].

In the isolated rabbit ovarian follicle, present results also do not support Model I as a control mechanism for LH-induced steroidogenesis. The fact that steroidogenesis preceded the incoporation of ^{3}H-leucine into total cellular protein by 45 to 75 minutes (Tables 2 and 4), coupled with the finding that the induction of a specific protein(s) by LH was not observed (Figures 12 and 18) suggests that de novo protein synthesis is not obligatory for acute steroidogenesis in the rabbit follicle. The observed increase incorporation of ^{3}H-leucine may simply be due to the overall growth of the follicle known to be induced by LH[30, 41].

An alternative hypothesis (Model II, Figure 4) is proposed for the rat Leydig cells where LH-induced testosterone production is indirectly mediated by an LH-independent regulator protein with a half life of 7 to 13 minutes[56, 132]. Janszen et al.[56] isolated by polyacrilamide gel electrophoresis a cycloheximide sensitive "protein 33" (MW 33,000) which appeared in both LH and control samples, 2 hours after the start of the incubation. A regulatory protein with similar physical characteristics as protein 33 has been isolated from the rat testis which specifically inhibits cyclic AMP-dependent protein kinase and phosphodiesterase[199]. Although this hypothesis (Model II) has not been thoroughly tested in the rabbit follicle, the synthesis of such an LH-insensitive regulatory protein was not apparent from radioautographs (Figures 14 and 18). It is possible that incubation periods longer than 2 hours are necessary for the synthesis of such a regulatory protein in the rabbit follicle. This being the case,

then the detection of such a protein would not be observed with the experimental protocol described here.

The regulation of steroidogenesis *via* cyclic AMP dependent protein kinase activation of pre-existing protein (Model III, Figure 4) has been suggested for the adrenal cortex[140, 143], corpus luteum[117], Graafian follicle[200], ovary[145], and testis[56, 115, 118]. A positive correlation (LH dose or time dependent) between protein kinase activation and steroid production has been demonstrated in the testis[115, 118], ovary[116], and corpus luteum[117]. In the bovine corpus luteum, a cyclic AMP-dependent protein kinase catalyzes the phosphorylation of a number of cytoplasmic, ribosomal and membrane phosphoproteins[201, 202], as well as the activation of cholesterol side chain cleavage enzymes[120]. In the bovine adrenal, the phosphorylation of cholesterol esterase *via* cyclic AMP-dependent protein kinase resulted in enhanced cholesterol ester hydrolysis, suggesting that the enzyme existed prior to stimulation in a dephosphorylated form[143].

In Leydig cell cultures, Janszen *et al.*[56], after labeling cellular proteins with ^{35}S-methionine, demonstrated the appearance of an LH-sensitive protein band (MW 21000) and a LH-insensitive band (MW 33000) only after 2 hours. In identical studies, LH *via* protein kinase enhanced the phosphorylation of three stable phosphoproteins (MS 14300, 57000 and 76000) within 5 to 10 minutes, a time consistent with the first appearance of testosterone production. Since the *de novo* synthesis of these phosphoproteins did not appear to be required,

it was suggested that phosphorylation of pre-existing proteins play a regulatory role in LH-induced testicular steroidogenesis[56, 118].

It is entirely possible that follicular steroidogenesis is regulated by protein kinase phosphorylation of a pre-existing protein-enzyme. If so, there would be no need for de novo protein synthesis upon acute LH- or cyclic AMP-induced steroidogenesis. Although LH has been shown to stimulate steroidogenesis[29, 122] and to activate protein kinase[112-114] within 5 - 15 minutes in the rabbit ovarian follicle, appropriate studies have not been carried out to correlate these events with follicular protein synthesis or the phosphorylation of endogenous phosphoproteins. Further work is warranted.

The present results suggest that de novo protein synthesis is not necessary for the acute effects of LH on follicular steroidogenesis and that the activation of a pre-existing enzyme is a more plausible control mechanism.

Role of cyclic GMP

The role of cyclic GMP in the ovary has not been defined. In order to investigate the role of this cyclic nucleotide in follicular steroidogenesis, isolated follicles were incubated with cyclic GMP (25, 10 and 1 mM) together with ^{3}H-uridine or ^{3}H-amino acids from 2- to 4-hours of incubation, following a 2-hour preincubation in MEM. The data in Table 8 shows that cyclic GMP (25 and 10 mM) enhances follicular RNA synthesis without stimulating protein or testosterone production. These results suggest that cyclic GMP is not involved

in follicular steroidogenesis. Furthermore, these data support the notion that de novo RNA is not required for acute steroidogenesis.

In the rabbit follicle, the fact that cyclic GMP increased follicular RNA synthesis (Table 7), which could not be mimiced by LH (Figure 11B), suggests that cyclic GMP is not stimulated by LH. However, the demonstration that FSH and LH can stimulate cyclic GMP production[82] in the isolated hamster follicle suggests that cyclic GMP may be involved in follicular functions. Zor et al.[108] have demonstrated that 8-bromo-cyclic GMP stimulated prostaglandin E production in the isolated rat Graafian follicle and suggested that cyclic GMP may play a role in ovulation.

Effects of methylxanthines: theophylline and MIX

The methylxanthines are potent inhibitors of phosphodiesterase[95]. Methylxanthines presumably act by forming an irreversible complex with phosphodiesterase, thereby preventing cyclic AMP degradation[92].

The inhibition of phosphodiesterase by methylxanthines was thought to provide a useful means to study the intracellular effects of cyclic AMP in the control of steroidogenesis. The effects of theophylline and MIX on follicular RNA, protein and testosterone production were investigated.

The demonstration that theophylline (25 mM) alone significantly stimulated follicular testosterone production (Table 7) is consistent with the known effects of the inhibitor on phosphosphodiesterase, and satisfies one of the criteria for the role of cyclic AMP in steroid-

ogenesis. The lack of synergism between theophylline and cyclic AMP can be attributed to the fact that cyclic AMP (5 mM) alone was sufficient for maximal stimulation of testosterone production. The inhibition of cyclic AMP-induced testosterone production by MIX (5mM) may be due to cytotoxic effects observed with high concentrations of the inhibitor[203].

There is increasing evidence that methylxanthesis may be acting at sites other than on the inhibition of phosphodiesterase In the rabbit follicle, this was demonstrated when theophylline (25, 10 and 1 mM) and MIX (0.5 mM) inhibited the incorporation of ^{3}H-amino acids and ^{3}H-uridine into follicular RNA and protein by 35 to 68%, respectively (Table 8). At the same time, the methylxanthines had no inhibitory effect on cyclic AMP-induced testosterone production. The inhibition of protein synthesis by methylxanthines was similarly found in the rat adrenal[204] and testis[205]. Since steroidogenesis was also inhibited, these authors concluded that protein synthesis was necessary for steroidogenesis. However, there is no evidence that this is the case in the rabbit follicle. The inhibition of follicular RNA and protein synthesis by methylxanthines appears to be a non-specific effect of the inhibitor. Other reported anomalous effects of methylxanthines include; the inhibition of glucose[206] and nucleoside[207] transport into the cell, and inhibition of protein kinase activity and phosphorylation[208]. Such non-specific effects of methylxanthines make their use as metabolic inhibitors questionable.

SUMMARY

The isolated rabbit ovarian follicle has been used as a model to study the roles of RNA and protein synthesis in LH-induced steroidogenesis. One major conclusion can be drawn from these studies -- that neither de novo RNA nor protein synthesis are necessary pre-requisites for acute LH-induced steroidogenesis by the rabbit ovarian follicle. Eight observations support this conclusion:

1. Neither LH nor cyclic AMP enhanced the incorporation of labeled uridine into follicular RNA at concentrations which significantly stimulated testosterone production.
2. While follicular testosterone production and protein synthesis were both stimulated by LH and cyclic AMP, steroidogenesis preceded the incorporation of ^{3}H-leucine into follicular protein by 75 and 45 minutes, respectively.
3. Testosterone production responded to low doses of LH which had no stimulatpry effect on protein synthesis.
4. LH had no observable effect on the incorporation of ^{35}S-methionine into new and different protein bands.
5. Actinomycin D inhibited follicular RNA synthesis by 85% without preventing LH- or cyclic AMP-induced steroidogenesis.

6. Cycloheximide and puromycin, at concentrations which inhibited the incorporation of labeled amino acids into follicular protein by more than 70%, had no inhibitory effect on LH- or cyclic AMP-enhanced steroidogenesis.
7. Cyclic GMP significantly enhanced follicular RNA synthesis without stimulating protein synthesis or testosterone production.
8. The methylxanthines (theophylline and MIX) inhibited both follicular RNA and protein synthesis by 35 to 68%, without effecting cyclic AMP-enhanced testosterone production.

Two qualifications must be made in the interpretation of this data: First, the isolated rabbit follicle was treated as if it were an individual cell, while in fact, it is composed of at least two cell types (theca and granulosa) each capable of steroid production. Second, no attempt was made to delineate the subcellular site where a regulatory protein may be acting. Despite such limitations, these observations collectively suggest that de novo RNA and protein synthesis are not required for the acute effects of LH on steroidogenesis.

Future studies on the role of RNA and protein synthesis in LH-induced steroidogenesis in the follicle could be directed toward (i) examining the effect of LH and cyclic AMP on the specific pattern of RNA and protein species, (ii) correlating the activation of cyclic AMP-dependent protein kinase with phosphorylation of phosphoprotein and steroidogenesis, and (iii) verify the observed changes in individual cell types (theca and granulosa) of the follicle.

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MOLECULAR CONTROL OF RABBIT FOLLICULAR TESTERONE PRODUCTION,

ROLE OF PROTEIN AND RNA AFTER STIMULATION WITH LUTEINIZING HORMONE.

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