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**Purine involvement in corpus luteum function in non-pregnant
sheep**

Patrick, Kimberly Miller, Ph.D.

University of Hawaii, 1992

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PURINE INVOLVEMENT IN CORPUS LUTEUM FUNCTION
IN NON-PREGNANT SHEEP

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES (ANATOMY AND REPRODUCTIVE BIOLOGY)

MAY 1992

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DEDICATION

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I would like to dedicate this dissertation to my husband, Kevin Patrick. For his patience, support, guidance, critiques, and love, I am especially grateful. I was never generous enough in expressing my appreciation for all of the times he had dinner waiting for me when I got home (late, of course), vacuumed, did laundry, dishes, and the shopping. He could be counted on for "hug breaks," and providing encouragement and inspiration in my darkest hours. We've survived three degrees together, and there is only one more to go. Hang in there Kev, we've almost made it!

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Five experiments were done to discern the involvement of de novo synthesis of purines on ovine corpus luteum (CL) function, and to establish a model for assessing the in vivo effects of purines on CL function. In order to identify specific purine involvements, the adenylic, guanylic, and inosinic biosynthesis pathways were isolated using the de novo synthesis pathway inhibitors hadacidin, mycophenolic acid, and azaserine.

In three in vivo experiments, these treatments were delivered into the sheath surrounding the ovarian vascular pedicle (OVP) ipsilateral to the ovary bearing the corpus hemorrhagicum via an exteriorized indwelling catheter. Delivery of the drugs to the CL by uptake into the vasculature was confirmed indirectly by HPLC analysis of luteal AMP, IMP, and GMP levels.

Azaserine, hadacidin, or mycophenolic acid, or these drugs plus replacement compounds (inosine, adenosine, or guanosine, respectively), were delivered in phosphate buffered saline (PBS) via the OVP catheter at 4 or 6 hour intervals over days 1-7 or 1-8 post-estrus. Daily jugular blood samples were taken over the treatment period and quantified for estradiol-17 β and progesterone by RIA. CL, collected on day 7 or 8 post-estrus, were dissociated, and luteal cell populations and live/dead ratios of luteal cells were noted. Augmented profiles of progesterone and estradiol-17 β were seen in ewes treated with 150 μ g of azaserine, or mycophenolic acid, respectively, as compared to ewes treated with 500 μ g of any of the drugs or controls. Estradiol-17 β and progesterone

profiles for drug + replacement compound-treated animals were not different from control ewes.

In vitro experiments involving incubation of luteal slices in PBS + luteinizing hormone (LH) with azaserine alone or combined with one of the pathway replacement compounds revealed that azaserine-treated luteal slices were able to produce progesterone levels that were not different from those produced either by controls, or adenosine-treated slices (amplifies LH-stimulated progesterone production). These data suggest that either a) the CL is less dependent on de novo synthesized purines than the readily available salvage pathway-derived purines, or that b) there may be a non-purinergic dependent second messenger system controlling biosynthesis of steroids in luteal cells.

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LIST OF ABBREVIATIONS

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The following may be used interchangeably in the text:

ADEN = adenosine

AMP = adenosine monophosphate

ATP = adenosine triphosphate

AZA = azaserine

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

CL = corpus luteum

DNase = deoxyribonuclease

DNA = deoxyribonucleic acid

FGAR = formylglycinamide ribotide

FSH = follicle stimulating hormone

GMP = guanosine monophosphate

GnRH = gonadotropin releasing hormone

GTP = guanosine triphosphate

GUAN = guanosine

HAD = hadacidin

HG-PRTase = hypoxanthine-guanine phosphoribosyltransferase

IMP = inosine monophosphate

INO = inosine

LH = luteinizing hormone

MA = mycophenolic acid

MEM = minimal essential medium

PBS = phosphate buffered saline

PGE₁ = prostaglandin E₁

PGE₂ = prostaglandin E₂

PGF_{2α} = prostaglandin F_{2α}

xxi

RNA = ribonucleic acid

TFA = trifluoroacetic acid

μg = micrograms

μM = micromoles

CHAPTER 1

THE ESTROUS CYCLE

1.A JUSTIFICATION: LUTEAL FUNCTION AND REPRODUCTIVE SUCCESS

Animals that have reached puberty are capable of becoming pregnant if they display estrous cyclicity and are mated. Once during each cycle, mature ova are released from follicles on the ovary. After the follicle has ruptured, it begins the transformation to a transitory structure known as the corpus luteum (CL). The main function of the corpus luteum is to secrete the hormone progesterone, which is necessary for the establishment and maintenance of pregnancy in most species. In the ewe, for example, a functional corpus luteum is required for at least the first 60 days of their 145-150 day gestation period (Casida and Warwick, 1945). Consequently, this organ, if functioning improperly, can have a profound negative effect upon the fertility of a female.

Subnormal luteal function is recognized as a significant cause of infertility in many domestic species, including sheep (Ashworth et al., 1987) and cattle (Gaverick and Smith, 1986). Infertility related to luteal insufficiency may account for a large percentage of the reduction in net calf/lamb crops (Parr et al., 1982). Dziuk and Bellows (1983) note that in a "healthy" beef herd bred by natural service, 17.4% of the net calf crop reduction is due to the inability of the cows to conceive or maintain a pregnancy. Edey (1969) reports that prenatal losses in sheep may be as high as 20-30%, most of which is during the first trimester of pregnancy.

Common stressors like excessive heat adversely affect fertility and early embryo survival in many species. Decreased pregnancy rates due to heat stress have been noted in cows (Dunlap and Vincent, 1971), sheep (Dutt, 1963), and mice (Alfred et al., 1961). There is evidence that heat stress-induced fertility problems may be a result of insufficient progesterone production by the corpus luteum.

Rosenberg et al. (1982) reported that blood progesterone concentrations are lower in heat-stressed cows, while Alfred et al. (1961) presented evidence that administration of progesterone to mice helped to overcome heat stress-induced early embryonic loss. Whatever the reason for corpus luteum failure, luteal insufficiency has a significant negative effect on successful reproduction in many species. Economically, this reduction in reproductive efficiency has been targeted by the beef, dairy, and sheep industries as a major problem that needs to be rectified (Parker and Pope, 1983; Dziuk and Bellows, 1983).

1.B THE ESTROUS CYCLE OF THE SHEEP

In order to further assess this problem, a clear understanding of the estrous cycle and luteal function is necessary. The sheep provides an excellent model for the study of luteal function, so most of the information presented will concern the ovine species. Most sheep are seasonal breeders, and the timing of their estrous cycles is highly dependent on variations in photoperiod. Decreasing day length is stimulatory to the onset of the reproductive season and process in sheep, determining whether animals are seasonally monoestrous or polyestrous (Hafez, 1952; Hansel and Echterkamp, 1972).

Most sheep in the United States display estrous cyclicity from late summer to early spring (Hansel and Echternkamp, 1972; Lamberson and Thomas, 1982; Hafez, 1952), in response to a decreasing day length during this time of year. As the days grow longer in early spring, ewes may stop cycling and become anestrus, due to light-induced changes in hormone sensitivity at the hypothalamo-hypophyseal axis (Legan et al., 1977; Karsch et al., 1980). In subtropical climates like Hawaii, where daylight changes are less dramatic, sheep tend to display estrous cyclicity throughout the entire year (Hafez, 1952).

Sheep reach puberty and begin displaying estrous cyclicity at an average age of 36 weeks (Hafez, 1952). The average ovine estrous cycle is 17 days in length (Hafez, 1952; Hansel and Echternkamp, 1972). Onset of puberty may vary greatly depending on the breed, season of birth, and nutritional status of the animal (Hafez, 1952; Lamberson and Thomas, 1982). Evidence of estrous cyclicity is the display of estrous behavior. Estrus lasts for a period of approximately 28 hours (Hafez, 1952; Hansel and Echternkamp, 1972), where the ewe displays sexual receptivity, and will stand to allow a ram to mate with her. This behavioral phenomenon represents the imminent internal release of mature oocytes from the ovaries 24-28 hours after the onset of behavioral estrus (Murdoch and Dunn, 1982) in preparation for fertilization. Estrus is the only outward sign that a ewe is cycling, since the remainder of the internal events of the estrous cycle are "silent," with no outwardly visible signs of their occurrence.

1.C FOLLICULOGENESIS

1.C.1 Follicle Growth and Hormonal Requirements

The estrous cycle has two phases - the follicular phase, and the luteal phase. The follicular phase begins approximately 3 days prior to ovulation. It is during this time, that the ova which will eventually ovulate are selected and undergo final maturation (Smeaton and Robertson, 1971).

The ovary contains a multitude of follicles, which contain a single oocyte. These follicles, all present from birth, may be in various stages of development (Figure 1). The earliest follicle type present in folliculogenesis is called the non-growing primordial follicle, which consists of an oocyte surrounded by a single layer of squamous epithelial cells called the granulosa cells (Ross and Reith, 1985). Under the influence of a stimulus(i), as yet undefined, primordial follicles leave the reserve non-growing follicular pool and enter a preantral growth phase where the oocyte increases in size and the number of granulosa cells is multiplied (Cahill, 1981) (Figure 2).

As the preantral follicle reaches a diameter of 0.2 mm, intercellular spaces begin to form and fill with fluid. Once a distinct cavity (antrum) has formed, the follicle is classified as a tertiary or antral follicle (Cahill, 1981). Follicle stimulating hormone (FSH) is one of the main hormones required for growth of antral follicles (Evans et al., 1932; Baird and McNeilly, 1981). FSH is a glycoprotein released into the bloodstream by the anterior pituitary gland under the influence of tonic gonadotropin releasing hormone (GnRH) secretion from the hypothalamus (Baird and McNeilly, 1981).

Figure 1. Schematic representation of various stages of follicular development in the mammalian ovary (Ross and Reith, 1985).

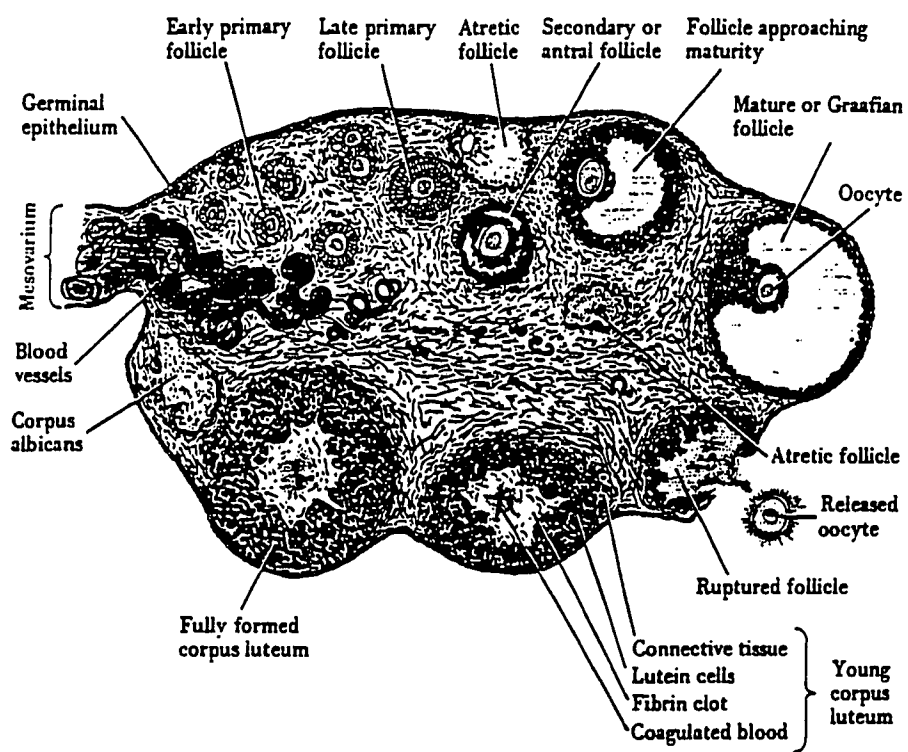
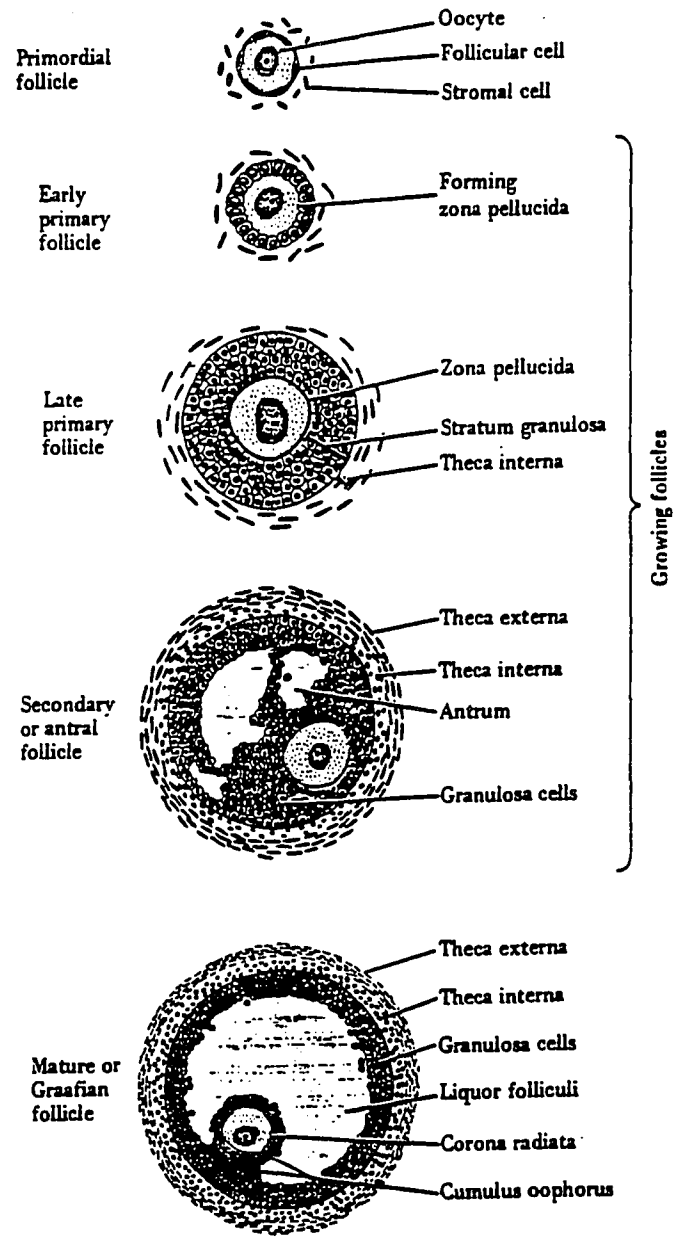


Figure 2. Histological representation of follicular development from primordial to Graafian follicles (Ross and Reith, 1985).



Cellular receptors for FSH are present on the granulosa cells of late pre-antral and antral follicles (Carson et al., 1979; Armstrong et al., 1981; Ireland, 1987). FSH induction of these receptors is vital for the stimulation of cell division and increasing the mitotic index of the granulosa cells, which allows the follicles to grow in size (Baird and McNeilly, 1981). In addition, FSH activation of the granulosa cells' adenylate cyclase systems through binding to high affinity receptors ultimately results in the secretion of estradiol 17- β by the granulosa cells (Armstrong et al., 1981).

While the avascular granulosa cells are increasing in numbers, the vascular theca layer surrounding the granulosa cell layer is also developing. The theca cell layer is composed of two parts - the theca interna and the theca externa. These cells are responsive to the gonadotropin luteinizing hormone (LH), which, like FSH, is secreted by the anterior pituitary gland in response to stimulation by hypothalamic GnRH (Clarke and Cummins, 1982). LH is secreted in a pulsatile fashion, due to the tonic secretion of GnRH by the hypothalamus (Reeves et al., 1971; Clarke and Cummins, 1982). The cellular receptors for LH are not present until the follicle has formed an antrum (Ireland, 1987).

1.C.2 Follicular Steroid Production

LH acts on the theca cells to cause production of androgens by stimulating the conversion of cholesterol to pregnenolone (Hall and Young, 1968; Marsh, 1976). The enzyme 3 β -hydroxysteroid dehydrogenase further converts pregnenolone to progesterone within the thecal cells. Both pregnenolone and progesterone can also be converted into the androgens dehydroepiandrosterone,

androstenedione, and testosterone through hydroxylation and side chain removal (Figure 3).

Androgen production by the theca cells (England et al., 1981) is the basis for an intimate relationship between the granulosa and theca cell layers which ultimately results in the production of estrogens by maturing follicles (Baird, 1977). Granulosa cells, unable to produce their own androgens, receive androgens from theca cells by diffusion across the basement membrane which separates the granulosa and theca layers (Armstrong et al., 1981; England et al., 1981). Once the androgens are within the granulosa cells, FSH binding to its receptors stimulates the activity of the enzyme aromatase which catalyzes the conversion of androgens to estradiol-17 β (Armstrong et al., 1981) (Figure 4).

1.C.3 Selection of Dominant Follicles

As the follicles mature, their ability to produce estradiol-17 β is vital in determining which follicles will proceed to become fully mature Graafian follicles, and those that will "drop out" of the growing pool and become atretic follicles (Ireland, 1987). This is significant because, of the 500-1000 primordial follicles that are recruited into the growing pool each estrous cycle, 99.9% of them fail to fully mature and ovulate. Waves of follicular growth and degeneration are ongoing, and occur during both phases of the estrous cycle (Ireland, 1987). In the ewe, the three waves of follicular development that normally occur, correlate with three periods of elevated estradiol-17 β in plasma (Scaramuzzi et al., 1970).

Estradiol-17 β , primarily produced by antral follicles, has a number of roles in the process of folliculogenesis. It acts at both the

Figure 3. The steroid biosynthesis pathway (Short, 1985).

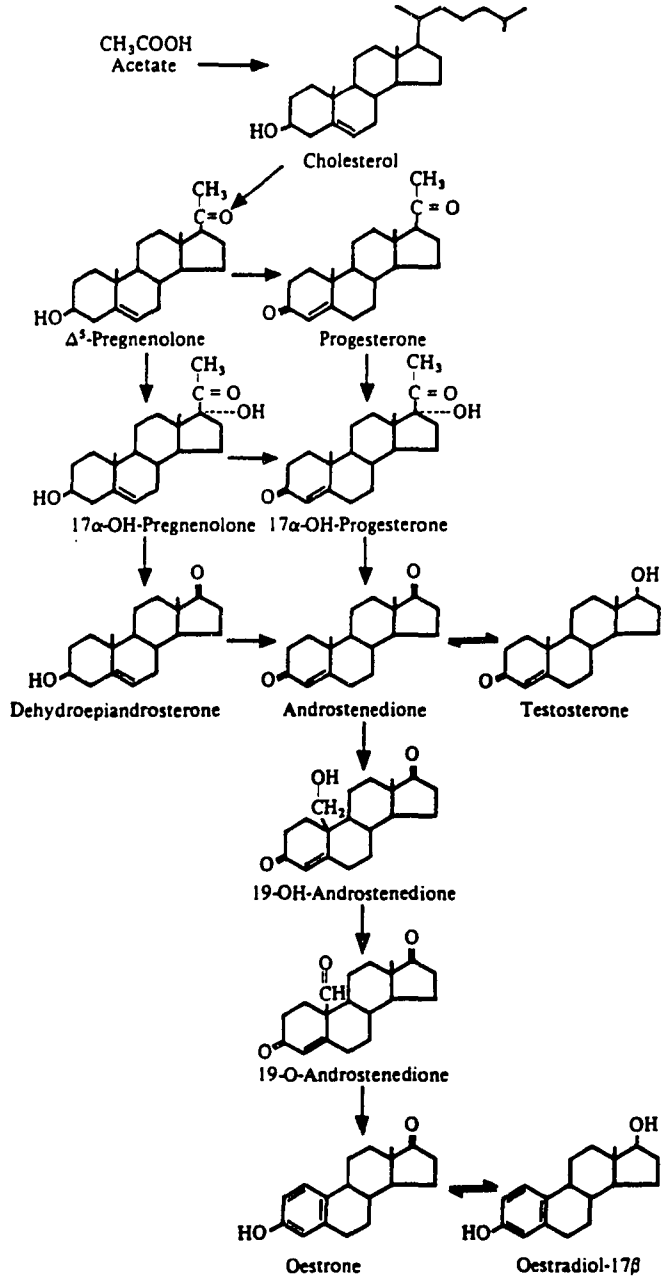
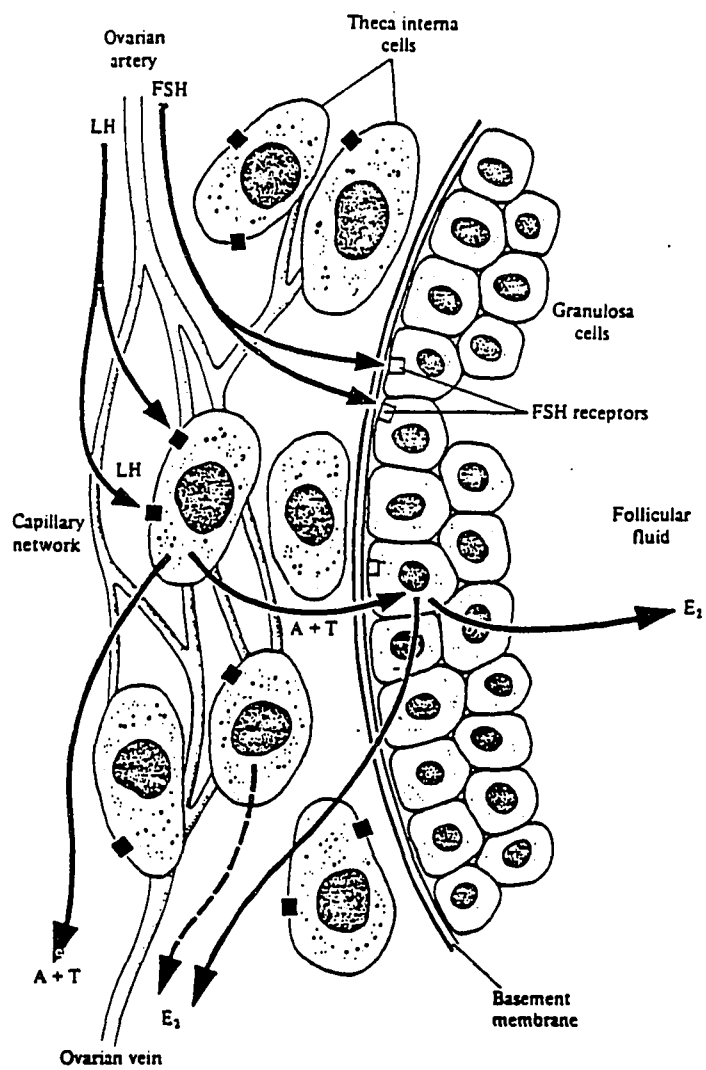


Figure 4. The "two-cell" theory of follicular steroid production. A = androstenedione; T = testosterone; E₂ = estradiol-17β. Androstenedione and testosterone are produced by the theca cells in response to LH stimulation. The androgens are transported to the granulosa cells where FSH stimulates their conversion to estradiol-17β (Short, 1985).



follicular and hypothalamo-hypophyseal levels. Research with rats has shown that, at the follicular level, estradiol-17 β is a required potentiator for FSH-stimulated growth of granulosa cells and estradiol-17 β production (Tonetta et al., 1985). Estradiol-17 β is also able to increase the numbers of its own granulosa cell receptors, thereby increasing aromatization of androgens to estrogen and amplifying its own production (Richards, 1975; Zhuang et al., 1982). Estradiol-17 β is actually sequestered in the follicular fluid of large ovine and bovine antral follicles, creating a high estrogen microenvironment within the follicle (Moor et al., 1978; Padmanahban et al., 1984).

This sequestration of estradiol-17 β in the follicle may help to overcome the deleterious effects of the decrease in FSH secretion due to the negative feedback of circulating estradiol-17 β and increasing inhibin output from the dominant follicle at the hypothalamo-pituitary axis (Baird and McNeilly, 1981; Goodman et al., 1981; Ireland et al., 1983; Tonetta and diZerega, 1989). Therefore, as a consequence of the high estradiol-17 β and inhibin production by the largest antral follicles, these follicles control not only their own growth and development, but also the growth and development of all other cohort follicles in the growing pool due to their ability to directly decrease FSH secretion by the anterior pituitary gland as a result of negative feedback (Goodman et al., 1981; Cahill, 1981; Ireland, 1987). In this manner, it is possible for a follicle to become the "dominant" follicle, destined to ovulate, while eliminating its competition. GnRH-like Protein (Hsueh and Jones, 1981; Behrman et al., 1980), a known inhibitor of folliculogenesis, may also assist the dominant follicle in succeeding over its cohorts.

1.C.4 The Ovulatory Process

Once a dominant follicle has been established, events progress rapidly toward ovulation. The high amounts of estradiol-17 β secreted by the dominant follicle serve to fulfill several purposes. Estradiol-17 β acts at the uterus to promote secretion of a uterine luteolysin known as prostaglandin F_{2 α} (PGF_{2 α}) (Ford et al., 1975), which helps to cause the regression of any corpus luteum which may have been present from a previous cycle. With this source of progesterone declining rapidly, the negative feedback of progesterone on the anterior pituitary secretion of LH is reduced. LH pulses become more frequent, enhanced by the estradiol-17 β priming of the hypothalamus and hypophysis (Karsch et al., 1979; Hansel and Convey, 1983). Estradiol-17 β and FSH also serve to increase the appearance of LH receptors at the follicular level, making a greater response to increasing levels of LH possible (Carson et al., 1979). In fact, increasing basal LH secretion in turn enhances estradiol-17 β secretion, creating a preovulatory estradiol peak (Karsch et al., 1979). Finally, one of the most important effects of the high estradiol-17 β output from the dominant follicle, is that it induces estrous behavior, so that mating can occur and the impending ovulation will not have been in vain (Baird and McNeilly, 1981).

The estradiol-17 β -provoked preovulatory surge of LH, which occurs about 24 hours prior to ovulation, is very important in causing the final maturation of the Graafian follicle (Cumming et al., 1971). This surge is responsible for the induction of follicular rupture and luteinization of granulosa cells (Yoshimura and Wallach, 1987). In addition, the pre-ovulatory surge of LH is the stimulus which causes

the preovulatory follicle to switch from an estrogen producing structure to a progesterone producing structure (Murdoch and Dunn, 1982).

Ovulation, which involves the rupture of the wall of the Graafian follicle at the stigma and the release of a mature oocyte, marks the onset of the luteal phase of the estrous cycle. As soon as ovulation occurs, the wall of the ruptured Graafian follicle collapses, forming a corpus hemorrhagicum (Short, 1985). Former follicular granulosa and thecal cells are transformed into large (average diameter = 23.6 microns) and small (average diameter = 15.6 microns) luteal cells, respectively, during this period of rapid change (Alila and Hansel, 1984; Rodgers et al., 1984).

1.D THE CORPUS LUTEUM

1.D.1 Luteal Cell Populations

While the total volume density of steroidogenic cells (large and small luteal cells) in the CL remains constant at approximately 55% over the estrous cycle, the ratio of small to large luteal cells is dynamic (Farin et al., 1986). A study by Farin et al. (1986) concludes that the number of small luteal cells increases four fold over the developing period of the CL (the CL reaches its maximum development between days 7-9 post-estrus), while there appears to be no change in the size of individual cells. In contrast, the large luteal cell population numbers remain static, but the cells increase in size over the CL developmental period. As a result, small luteal cell to large luteal cell number ratios shift from 1:1 on day 4 post-estrus to 4:1 at CL maturity (Farin et al., 1986).

1.D.2 Luteal Cell Steroid Production

These population changes are intricately related to the function of the CL, since the two cell types produce progesterone in response to different stimuli, and in differing amounts (Hoyer et al., 1984). The basal rate of progesterone secretion by large luteal cells is nine fold higher than that of small luteal cells (Hoyer and Niswender, 1985). Currently, the stimulus for this high progesterone output by large luteal cells is unknown, but it has been discerned that large bovine luteal cells produce progesterone in response to the stimulation of their inositol triphosphate second messenger system (Hansel and Dowd, 1986).

Small luteal cells are dependent on LH activation of their cyclic AMP (cAMP) second messenger systems for stimulation of progesterone production (Hoyer and Niswender, 1985). Unlike large ovine luteal cells which have few LH receptors and appear to be unresponsive to LH, the small luteal cells possess the majority of the LH receptors present in the CL (Hoyer et al., 1984). There is a forty fold increase in LH receptor numbers per luteal cell between days 4 and 14 post-estrus (Niswender, et al., 1981). According to Niswender et al. (1981), the total luteal cell content of LH receptors and the proportion of receptors with bound LH are highly correlated with serum levels of progesterone.

1.D.3 Luteotropins

Other factors that may play a role in CL growth or function include the E series of prostaglandins (PGE₁ and PGE₂) (Weems et al., 1985a) and purine nucleotides (Hall et al., 1981; Weems et al., 1989; Behrman et al., 1983). PGE₁ is known to increase the amount

of endogenous LH bound to the luteal cells (Weems et al., 1985a). PGE₁, PGE₂, and adenosine, all vasodilators (Bergstrom et al., 1968; Cassis et al., 1987), have demonstrated the ability to stimulate the luteal cells to secrete progesterone in vivo and to be antiluteolytic in vivo in the non-pregnant ewe (Weems et al., 1985a; Weems et al., 1989). In the face of a luteolytic challenge by PGF_{2α} (a vasoconstrictor) (Bergstrom et al., 1968), the E series prostaglandins and adenosine may help to maintain blood flow to the CL and, by direct actions on the CL, avoid the occurrence of luteolysis during early pregnancy (Reynolds et al., 1981; Weems et al., 1985b; Hoyer et al., 1985; Weems et al., 1989). Adenosine directly amplifies LH action on luteal cells, which may aid in overcoming the effects of PGF_{2α} (Behrman et al., 1982; Behrman et al., 1983; Weems et al., 1987).

1.D.4 Prostaglandin F_{2α}

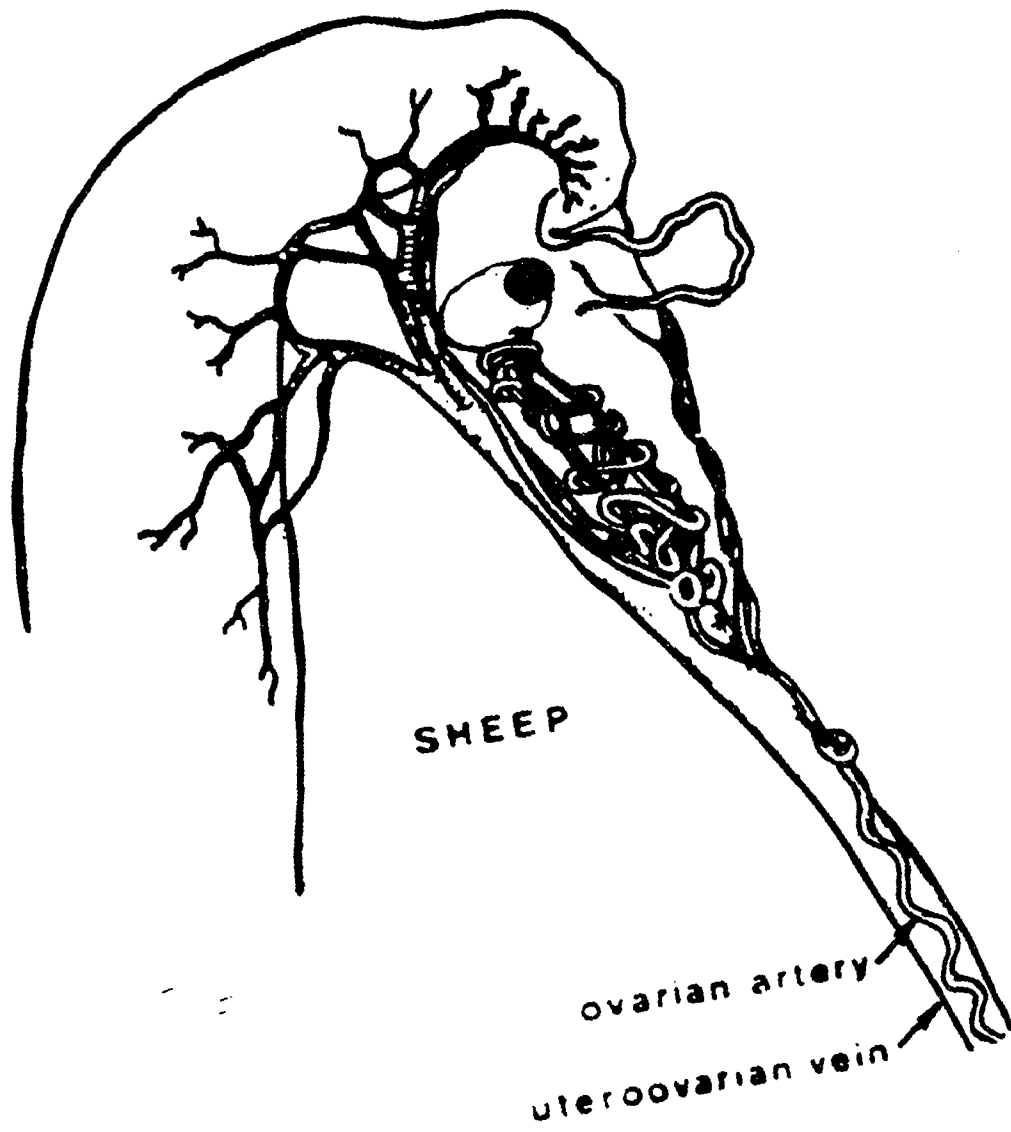
After day 4 post-estrus, the CL first becomes sensitive to PGF_{2α}, which exerts its effects by acting on estrogen and progesterone-primed large luteal cells to decrease progesterone production and increase luteal cell mortality (Silvia et al., 1984). PGF_{2α} appears to suppress the ability of LH to stimulate progesterone secretion by luteal cells (Evrard et al., 1978), although the small luteal cells which contain the majority of the LH receptors in the CL, have few, if any receptors for PGF_{2α}, PGE₁, or PGE₂ (Fitz et al., 1982; Silvia et al., 1984). PGF_{2α} may act on the small luteal cells in an indirect manner, by causing the large luteal cells to secrete a factor that disrupts the ability of LH to stimulate adenylate cyclase in the small luteal cells (Silvia et al., 1984).

PGF_{2α}, of uterine endometrial origin, is delivered to the ovary locally by a veno-arterial exchange (figure 5) between the uterine branch of the ovarian vein and the ovarian artery (Ginther et al., 1973; Ginther, 1974; Mapletoft et al., 1976). Endometrial PGF_{2α} travels to the uterine branch of the ovarian vein via the uterine venous drainage system. At the uterine branch of the ovarian vein near the ovary, the ovarian arteries are extensively coiled around it, forming close anastomoses between the vessels. This relationship allows molecular materials to be exchanged between the arterial and venous systems in a "counter-current" exchange manner (Ginther, 1974).

Release of PGF_{2α} from the uterus is enhanced by progesterone (Vincent and Inskeep, 1986; Baird et al., 1976), and may be further amplified when followed by estradiol-17β (Ford et al., 1975; Barcikowski et al., 1974), potentially arising from the development of a new wave of follicles at mid-cycle. This rise in estradiol-17β also serves to prime oxytocin receptors in the uterus, which enables oxytocin to further stimulate uterine secretion of PGF_{2α} (Fairclough et al., 1984; Soloff, 1975; Roberts et al., 1975). Levels of PGF_{2α} in the sheep begin to rise about day 10 post-estrus and reach maximal levels at around day 17, which coincides with the completion of luteal regression (Ottobre et al., 1984). The vasoconstrictive properties of PGF_{2α} may suppress blood flow to the ovary bearing the CL, and probably further intensify its luteolytic actions (Niswender et al., 1976).

In the event that fertilization of an ovum has occurred, PGF_{2α} would be ineffective in causing the regression of the corpus luteum

Figure 5. The vasculature of the ovine ovarian pedicle region. Blood drains the uterine horn via the uterine branch of the ovarian vein ("uteroovarian vein"), which forms anastomoses with the ovarian artery (Ginther, 1974).



(Silvia and Niswender, 1984). There is evidence that the embryo secretes a factor that mediates corpus luteum maintenance and progesterone production during early pregnancy (Rowson and Moor, 1967). Godkin et al. (1984) have shown that a protein secreted by the day 13 embryo, oTP-1, is able to prolong successful luteal function even in cycling sheep when infused directly into the uterine lumen. Increases in PGE₂ concentration have also been noted in utero-ovarian blood around day 13 post-estrus in pregnant ewes (Ottobre et al., 1984). There is evidence that the embryo itself produces PGE₂ (Hyland et al., 1982). PGE₁ (Weems et al., 1985a), and PGE₂ (Henderson et al., 1977; Reynolds et al., 1981; Weems et al., 1985a), in turn, reduce luteal sensitivity to the effects of PGF_{2α} so that optimal progesterone output, and pregnancy, may be maintained.

CHAPTER 2

PURINE NUCLEOTIDES

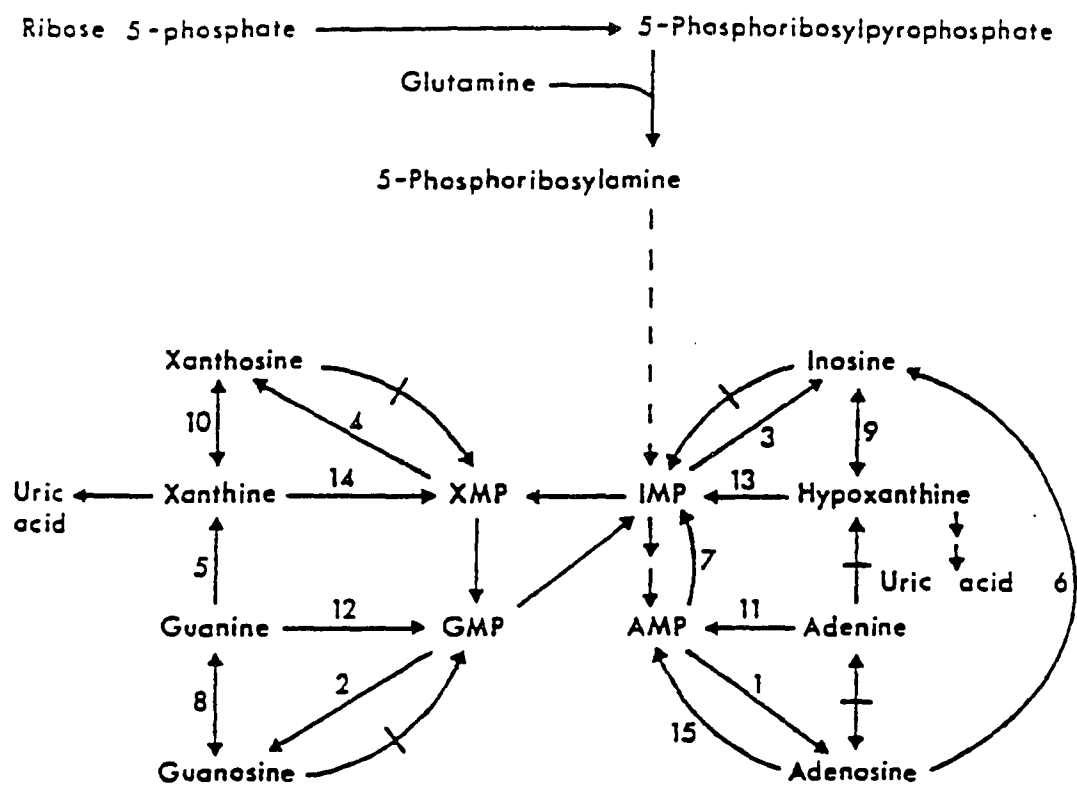
2.A PURINE SYNTHESIS

Purines are synthesized de novo primarily in the liver (Smellie et al., 1956). Glucose 6-phosphate, formed during glycolysis, is dehydrogenated to become an intermediate subsequently hydrolyzed to form 6-phosphogluconate as part of the oxidative phase of the pentose phosphate pathway. The 6-phosphogluconate is then oxidatively decarboxylated by 6-phosphogluconate dehydrogenase to yield ribulose 5-phosphate, which is isomerized to form ribose 5-phosphate (Seegmiller and Horecker, 1952).

A ribose phosphate, made from 5-phosphoribosyl-1-pyrophosphate (PRPP), is added to a hypoxanthine purine ring to form the first product in the synthesis of purines - inosinate (IMP) (figure 6). Adenylate (AMP) and guanylate (GMP) are both formed from IMP by insertion or substitution of amine groups. Adenine nucleotides and guanine nucleotides are intimately linked in that guanine triphosphate (GTP) is a substrate in the synthesis of adenylic ribonucleotides, and adenosine triphosphate (ATP) is a substrate for synthesis of guanylic ribonucleotides (Henderson, 1972; Lehninger, 1975; Stryer, 1988).

Purines are also made available to cells via a "salvage" pathway, which utilizes preformed bases or nucleosides (figure 6). In vivo, hypoxanthine is the main purine available for the salvage pathway (Mager et al., 1967; Murray et al., 1970). Erythrocytes take up adenine, guanine, hypoxanthine, and xanthine, and convert them into nucleotides, with hypoxanthine and xanthine being the only purines which are released from the cells (Mager et al., 1967). These purine precursors are

Figure 6. Purine biosynthesis and metabolic pathways. Numbers correspond to the following enzymes: 1, 2, 3, and 4: 5'-nucleotidase; 5: guanine deaminase; 6: adenosine deaminase; 7: AMP deaminase; 8, 9, 10: purine nucleoside phosphorylase; 11: adenine phosphoribosyltransferase; 12, 13, and 14: hypoxanthine-guanine phosphoribosyltransferase; 15: adenosine kinase. Reactions that are identified with a crossed arrow are not considered to be significant in animal tissues (Murray et al., 1970).



transported in the erythrocytes until a suitable local signal prompts their release. According to Henderson and LePage's (1959) work, raised oxygen levels tend to stimulate purine release from the erythrocytes.

2.B PURINE INVOLVEMENT IN PHYSIOLOGICAL SYSTEMS

The adenine-related compound adenosine is utilized in nearly every physiological system; neural (Phillis and Wu, 1983); cardiac (Burnstock and Brown, 1981); excretory (Spielman and Thompson, 1982); circulatory (Baer and Drummond, 1979; Sparks et al., 1984); metabolic (Baer and Drummond, 1979); and reproductive (Hall et al., 1981; Maguire et al., 1990). Sources of adenosine include diet-derived adenosine, de novo synthesis, and salvage pathways. De novo synthesis in the liver and diet are the primary sources of adenylic compounds, since the liver is one of the few tissues able to produce large quantities of purines to meet metabolic needs (Smellie et al., 1956; Lajtha and Vane, 1958). In addition, adenine is the only dietary purine that is conserved by the body during the digestive process (Salati et al., 1984). Other adenine-related compounds, xanthine and hypoxanthine (originating from the liver), are processed primarily into ATP by the red blood cells, and transported to peripheral tissues (Henderson and LePage, 1959; Mager et al., 1967). The endothelial cells are capable of collecting adenylic compounds from the blood, and storing them until they are needed by surrounding tissues (Sparks et al., 1984).

Adenosine is produced and regulated by a variety of tissues, although the liver synthesizes the bulk of adenosine. There are a myriad of actions associated with adenosine including: vasodilation (Cassis et al., 1987); hypotension due to vasodilation (Drury and Szent-Gyorgyi, 1929); bronchoconstriction (Holgate et al., 1987); inhibition of platelet

aggregation (Baer and Drummond, 1979); and oocyte maturation inhibition (Downs and Eppig, 1987; Miller and Behrman, 1985). In addition, it is antilipolytic (Baer and Drummond, 1979), antispasmodic (Burnstock, 1972), inhibitory to the release of cholinergic and noradrenergic transmitters (Dobson et al., 1987), and has been shown to cause a marked amplification of both cAMP and steroidogenesis in rat luteal cells in response to LH (Behrman et al., 1983).

2.C MECHANISMS OF ACTION: ADENOSINE

There are two demonstrated mechanisms of adenosine action. The first is a metabolic coupling process during which adenosine acts intracellularly to increase ATP levels (Arch and Newsholme, 1980). Secondly, adenosine may either inhibit or activate adenylate cyclase activity in a cell, depending on the type of external surface receptor with which it interacts (Daly, 1982). There are two classes of purine receptors that exist in cell membranes. The P₁ receptor binds adenosine, and primarily controls adenylate cyclase activity, while P₂ receptors (ATP receptors) bind adenine nucleotides and appear to control ion fluxes (Daly, 1982).

The adenosine specific receptors can be further classified into A₁/R_i and A₂/R_s subtypes (Van Calker et al., 1979; Londos et al., 1980). The A₁/R_i adenosine receptors couple to adenylate cyclase in an inhibitory manner, while the A₂/R_s receptors stimulate adenylate cyclase activity. Generally, A₁ responses result in a reduction in oxygen demand by inducing vasoconstriction, or inhibition of lipolysis, for example, while A₂ responses result in events that increase oxygen supply such as vasodilation, or inhibition of platelet aggregation (Bruns et al., 1987).

It has been possible to identify many of the receptor actions by the use of adenosine and ATP structural analogs. For example, adenosine receptors (P_1) can be differentiated from ATP receptors (P_2) by using theophylline, caffeine, and other xanthines, which block P_1 receptors but not P_2 receptors (Daly, 1982). However, theophylline also inhibits phosphodiesterase activity and permits cyclic AMP to be active longer. Analogs of theophylline and caffeine can further aid in the distinction of receptor types by selectively blocking the A_1 and A_2 receptors, respectively (Daly et al., 1987).

2.D GUANINE INTERACTIONS

Guanine triphosphate (GTP) has been linked to the successful elevation of adenylate cyclase activity by adenosine at the P_1 receptors (Behrman et al., 1984). GTP is also involved with the "G protein" intermediate which aids in the coupling of the receptor-adenylate cyclase complex. Since GTP increases with elevating ATP levels (Brennan et al., 1983), it appears that these compounds have a "working relationship." In fact, GTP analogs cause increases in adenylate cyclase in luteal cells similar to those produced by adenosine (Behrman et al., 1984).

2.E. PURINE INVOLVEMENT IN REPRODUCTIVE PROCESSES

2.E.1 Oocyte Maturation

Adenine/guanine control mechanisms have also been implicated in oocyte maturation. Miller and Behrman (1986) found that adenosine inhibited oocyte maturation, while adenine, guanine, inosine, and hypoxanthine did not at equivalent doses. Downs and Eppig (1987) followed with an experiment that indicates guanyl compounds (especially guanosine) are involved in the inhibition of oocyte maturation in mouse oocytes.

2.E.2 The Corpus Luteum

Purines play a reverse role in the corpus luteum. Rather than having an inhibitory action, some of the purines may be involved in the growth and development of the CL. Not only has adenosine been shown to be involved in increasing the blood flow to tissues (Baer and Drummond, 1979) (the developing CL is one of the most vascular tissues in the body; Niswender et al., 1976), but it also has actions directly on the luteal cells.

To date, a majority of the research on the relationship between adenosine and luteal cells has been conducted in vitro using cultured luteal cells. In vivo work in large animals involving the interaction of purinergic compounds other than adenylic compounds (Weems et al., 1989) and luteal cells has not yet been done. Hall et al. (1981) and Brennan et al. (1983) have shown that administration of adenosine will amplify LH-stimulated increases in cAMP, ATP, adenylate cyclase activity and subsequently, steroidogenesis (production of progesterone) in cultured luteal cells. Billig et al. (1989) proposes an autocrine/paracrine loop by which adenosine regulates luteal progesterone secretion.

Soodak et al. (1988) suggests that amplification of LH-induced progesterone secretion is dependent on adenosine-mediated increased receptor availability, or increased coupling between receptors and adenylate cyclase. Other purines; inosine, adenine, ADP, AMP and hypoxanthine have an effect on luteal cells similar to that of adenosine, while guanosine and xanthine are inactive (Brennan et al., 1983). Dorflinger et al. (1983) and Behrman et al. (1984), however, report the GTP analogs produce intra-luteal cell increases in cAMP similar to levels provoked by adenosine. Brennan et al. (1983) discovered that the

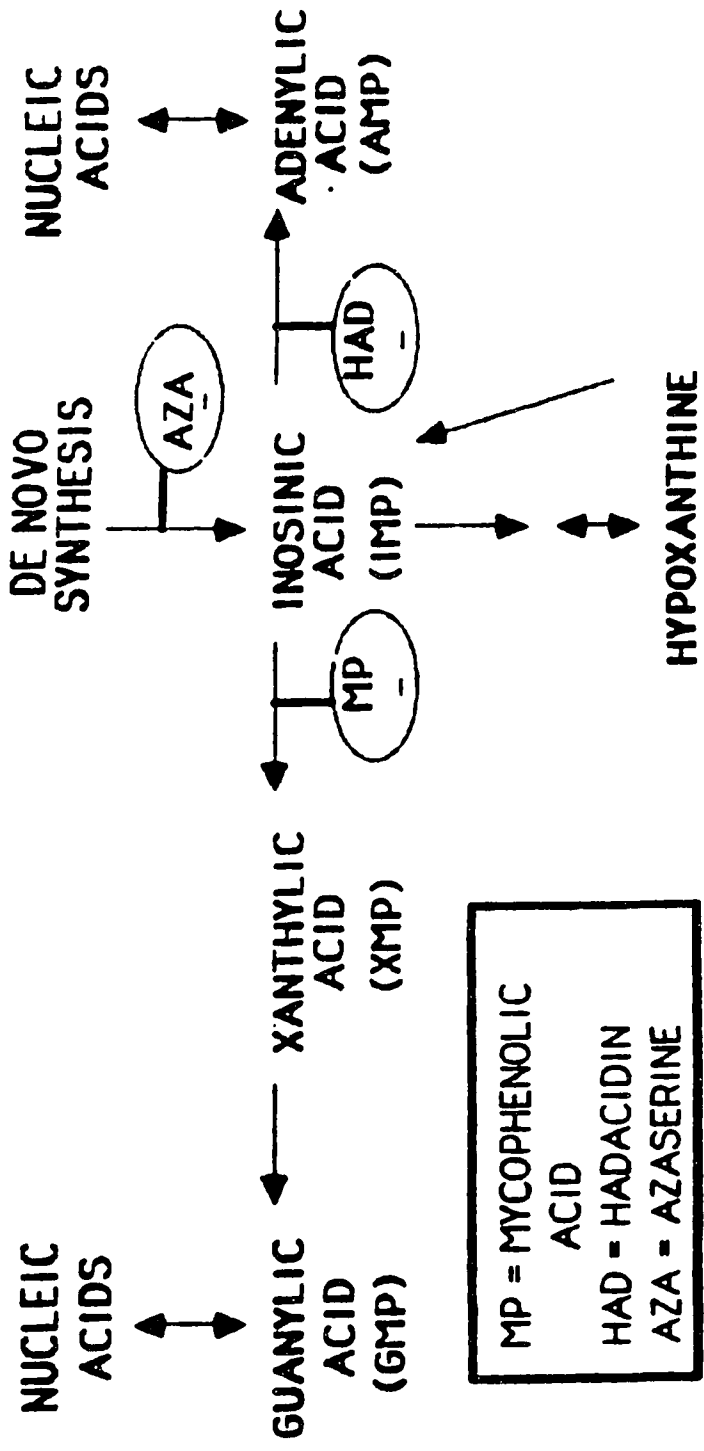
reason most of these other purines cause effects similar to adenosine, is that they are actually converted to adenosine intracellularly before any of the "adenosine-like effects" occur.

This conversion would seem to indicate that adenosine plays an important regulatory role in the function of the corpus luteum. Its role in the promotion of steroidogenesis is in direct opposition to the role of the natural luteolysin, $\text{PGF}_{2\alpha}$. Adenosine and $\text{PGF}_{2\alpha}$ are competitive antagonists of LH-stimulated cAMP accumulation (Behrman et al., 1982). In addition, adenosine has been shown to delay natural (Weems et al., 1989) or prevent early $\text{PGF}_{2\alpha}$ -induced luteolysis in sheep *in vivo* (Weems et al., 1987). The receptiveness of the luteal cells to respond to either purinergic luteotropic agents or luteolytic prostaglandins changes with luteal lifespan, as evidenced by their decreased ability to respond to LH on day 11 post-ovulation in pseudopregnant rats, as compared to a higher response on day 4 (Behrman et al., 1982).

2.F CONTROL OF PURINE SYNTHESIS PATHWAYS

In order to ascertain the consequences of exposure of luteal cells to selected purines *in vivo*, it is necessary to have some control over the synthesis pathways, both de novo and salvage. In this particular study, the de novo biosynthesis pathway will be targeted. As shown by Downs and Eppig (1987), azaserine, hadacidin, and mycophenolic acid are able to selectively control the purine de novo synthesis pathways (Figure 7). Azaserine interferes with the de novo synthesis of inosinic acid by reacting irreversibly with formylglycinamide ribotide (FGAR) (Levenberg et al., 1957; Moore and LePage, 1957). In order for FGAR to promote the production of inosinic acid, it must form a complex with ATP FGAR amidotransferase. Azaserine, a structural analog of glutamine (Held et

Figure 7. Schematic representation of the sites of action of the purine de novo biosynthesis pathway inhibitory drugs azaserine, hadacidin, and mycophenolic acid (Downs and Eppig, 1987).



yal., 1969), interferes by binding to a glutamine binding site on the enzyme, rendering the complex too unstable to proceed (Mizobuchi et al., 1968).

Hadacidin inhibits the de novo biosynthesis of adenylic and deoxyadenylic acids by reversibly blocking the action of adenylosuccinate synthetase (Shigeura and Gordon, 1962). This enzyme is responsible for the conversion of inosinic acid to adenylosuccinic acid. Hadacidin has also been reported to inhibit tumor growth (Kaczka et al., 1962). Mycophenolic acid is an antibiotic with antineoplastic characteristics which strongly inhibits DNA synthesis (Franklin and Cook, 1969). It specifically blocks the guanylic biosynthetic pathway by inhibiting the actions of inosinic acid dehydrogenase (Carter et al., 1969; Franklin and Cook, 1969). Inosinic acid dehydrogenase normally promotes the conversion of inosinic acid to xanthylic acid.

CHAPTER 3

PREFACE TO EXPERIMENTS: OBJECTIVES

The purpose of these experiments was not only to discern the involvement of specific purines in progesterone secretion by the developing CL, but also to establish a model system for assessing the in vivo effects of purinergic compounds at the level of the CL. These goals were addressed in a series of in vivo and in vitro experiments.

Three in vivo experiments were done, with the purpose of establishing: a) a luteal response to treatment, with plasma progesterone levels as an index (experiment 1); b) a comparison of the effectiveness of 2 different doses for each treatment and an assessment of possible toxic effects (experiment 1); c) using a drug dosage determined from experiment 1, test the specificity of the blocking drug and its effects on the purine biosynthesis pathway as a whole, by administering purine building block replacement compounds in addition to the pathway blocking drugs (experiment 2); and d) evidence that the purine synthesis pathway blocking drugs were delivered to the CL by quantifying AMP, IMP, and GMP levels in the tissue, which are altered by the actions of the drugs (experiment 3).

Two in vitro experiments were run to determine: a) a modified dose response curve to the purine biosynthesis pathway blocking drugs, using progesterone secretion by the corpus luteum tissue slices and live/dead staining techniques as indicators of drug effectiveness and cytotoxicity, respectively (experiment 4); and b) whether the effects of the purine pathway blocking drug azaserine (blocks synthesis of all three purines) on progesterone secretion could be altered by the addition of

compounds which replace those biosynthesis pathway precursors
blocked by the drug (experiment 5).

CHAPTER 4

EXPERIMENTS 1, 2, and 3: IN VIVO DETERMINATIONS OF LUTEAL RESPONSE TO DIFFERENT DOSES OF AZASERINE, HADACIDIN, AND MYCOPHENOLIC ACID, WITH OR WITHOUT PURINE BIOSYNTHESIS PATHWAY REPLACEMENT COMPOUNDS, AND ASSESSMENT OF DRUG DELIVERY

4.A MATERIALS AND METHODS

Crossbred ewes, housed at the University of Hawaii Waialea Research Station, were checked for estrus twice daily (0700 and 1500) using brisket-painted vasectomized rams. Ewes in estrus (day 0) were identified by grease paint marks on their rumps left by the rams after the ewes have allowed the rams to mount them. Marked ewes were verified to be in estrus by checking with a different vasectomized ram. Only ewes which displayed 2 previous 16-18 day estrous cycles were utilized in the experiments.

Within 24 hours of detection of standing estrus, a jugular vein catheter was installed and ewes were laparotomized under sodium pentobarbital anesthesia. A 1.02 mm bore polyvinyl catheter (Cole Parmer, Chicago) was installed into the sheath surrounding the ovarian vascular pedicle ipsilateral to the corpus hemorrhagicum, and exteriorized through the flank (see Appendix D for detailed procedure).

Treatments were administered at assigned intervals via the ovarian vascular pedicle catheter. A 1cc syringe was used to administer 0.3 ml of PBS containing the appropriate amount of dissolved treatment drug, which was immediately followed by 0.7 ml of PBS (pH = 7.0) administered via a second 1cc syringe. The 0.7 ml PBS was used to wash the drug treatment through the catheter and into the ovarian

vascular pedicle to: a) ensure delivery of the entire treatment amount and b) reduce the possibility of the drugs being absorbed by the polyvinyl catheter. Complete delivery of both the treatment and the PBS was accomplished in less than 45 seconds, further reducing the risk of drug absorption by the catheter.

Experiment 1 was designed to establish a luteal response to treatment by comparing 2 doses of each of the purine de novo synthesis pathway blocking drugs and also to determine cytotoxic effects due to treatment. Treatments were administered every 6 hours (0600, 1200, 1800, 2400) on days 1-7 post-estrus. The randomly assigned treatments (5 ewes/treatment group) were as follows:

1. Control (0.1 M PBS, pH = 7.0)
2. 150 μ g Azaserine
3. 500 μ g Azaserine
4. 150 μ g Hadacidin
5. 500 μ g Hadacidin
6. 150 μ g Mycophenolic Acid
7. 500 μ g Mycophenolic Acid

For each of the three in vivo experiments, drug treatments were prepared fresh daily and refrigerated at 4° C until use. No pH correction was made following the addition of the drugs to the 0.1 M PBS vehicle at pH = 7.0.

The second experiment was designed to test the specificity of action of the purine de novo synthesis pathway blocking drugs by administering purine biosynthesis pathway replacement compounds concurrently with the blocking drugs. The randomly assigned treatment combinations (5 ewes/treatment) were administered every 4 hours

(0400, 0800, 1200, 1600, 2000, 2400) on days 1-8 post-estrus. The following treatments were used:

1. control (0.1 M PBS, pH = 7.0)
2. 500 μ g azaserine
3. 500 μ g azaserine + 500 μ g inosine
4. 500 μ g hadacidin
5. 500 μ g hadacidin + 500 μ g adenosine
6. 500 μ g mycophenolic acid
7. 500 μ g mycophenolic acid + 500 μ g guanosine

The third experiment was done to determine whether the pathway blocking drugs affected luteal levels of AMP, GMP, or IMP by blocking the enzymes specific to each of the synthesis pathways. The following treatments were randomly administered (5 ewes/treatment group) as described previously, every 4 hours on days 4-7 of the estrous cycle:

1. 500 μ g azaserine
2. 500 μ g hadacidin
3. 500 μ g mycophenolic acid
4. control (PBS buffer)

Blood samples were taken via the jugular vein catheter prior to laparotomy, and at 1600 or 1800 hours daily during the treatment period for experiments 1 and 2. Following centrifugation of the blood, the plasma was collected and stored at -20° Celsius until assayed for estradiol-17 β and progesterone by radioimmunoassay (see appendix A). Ewes were kept in digestion crates and fed hay and water ad libitum throughout the treatment period. Food and water were withheld 18-24 hours prior to surgery.

On day 7 (experiments 1 and 3) or day 8 (experiment 2) post-estrus, ewes were laparotomized under sodium pentobarbital anesthesia to collect the corpus luteum, and obtain samples of ovarian vein blood (experiment 1 and 2) and ovarian pedicle vasculature (experiment 1). Following collection of samples, ewes were euthanized by an overdose of anesthetic, and incinerated. Blood samples were processed as mentioned previously, and later assayed for estradiol-17 β and progesterone by radioimmunoassay (see appendix A). Ovarian arteries were collected and placed in 10% buffered formalin until paraffin embedding, histological sectioning, and staining using the Feulgen procedure (Chayen et al., 1973; Humason, 1979) to detect pycnotic nuclei and morphological changes (see appendix C).

CL were weighed immediately upon removal, and then placed in warm Minimal Essential Medium (MEM) (Gibco #320-2561 AG) until dissociation (approximately 15 minutes). CL used in experiment 3 were flash frozen in cryovials in a slurry of methanol and dry ice, and stored at -70° C until processing for analysis of AMP, GMP, and IMP levels by high pressure liquid chromatography (HPLC).

In order to assess luteal cell populations, CL were dissociated using the method of Brennan et al. (1983) (see appendix B for detailed procedure). Luteal tissue was minced and incubated in MEM containing DNase and collagenase (Worthington Biochemicals, Freehold, NJ) in a 37° Celsius shaking water bath for 1 hour. Following incubation, fresh MEM + 1% bovine serum albumin (BSA) (Sigma Chemical Co.; St. Louis, MO) was combined with the existing DNase/collagenase/MEM mixture and the tissue was centrifuged for 5 minutes. After centrifugation, the supernatant was discarded and the tissue pellet resuspended in 9 ml of

MEM + 1% BSA with 0.1 ml of 100 mM disodium ethylenediamine tetraacetate-2 (EDTA) (Fisher Scientific Co.; Pittsburgh, PA) before a second 5 minute centrifugation.

At the completion of the second centrifugation, the supernatant was again discarded, and the tissue pellet was resuspended in a known amount of fresh MEM + 1% BSA. Aliquots of this mixture were taken with a red blood cell diluting pipette, and loaded onto a hemacytometer. One of the 2 aliquots on the hemacytometer was stained with trypan blue (1% in 0.9% saline) (Fisher Scientific Co.; Pittsburgh, PA). With a light microscope, luteal cell types layered on the central grid were classified into one of three categories: large luteal cells (23.6 microns in diameter); small luteal cells (15.6 microns in diameter); and "other" luteal cells (including endothelial cells, pericytes, blood cells, etc.), based on the criteria of Rodgers et al. (1984). Percentages of live/dead cells were determined for each luteal cell type in the trypan blue stained aliquot, while the unstained aliquot was used to determine relative percentages of the cell types.

In preparation for HPLC analysis of the purine biosynthesis pathway components AMP, GMP, and IMP, the frozen corpora lutea were weighed and then immediately homogenized in 1 ml of 5% trifluoroacetic acid (TFA) (EM Science, Cherry Hill, NJ) using a Dounce tissue grinder. The TFA supernatant was pipetted into a plastic microcentrifuge tube and set aside. The remaining ground tissue was homogenized once more using another 1 ml TFA, which was pipetted off and put into a second microcentrifuge tube. Both tubes were centrifuged for 10 minutes at 5000 x g in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY).

Following centrifugation, the supernatant was collected from both microcentrifuge tubes and combined in a 12 x 75 mm borosilicate glass test tube. Purified nitrogen was used to remove the solvent, producing a syrupy residue. The residue was reconstituted in exactly 50 μ l of double distilled water. The reconstituted syrup (20 μ l) was manually injected into a Shimadzu HPLC apparatus (model 6A) (Shimadzu Corporation, Kyoto, Japan). The HPLC apparatus was fitted with a 25 cm, 5 micron pore size Whatman C-18 Partisphere column (#4611-1502, Fisher Chemical Company, Pittsburgh, PA) and a Whatman Partisphere guard column.

Solvent A consisted of 1 mM KH_2PO_4 monobasic (Fisher Chemical Company, Pittsburgh, PA) + 5 mM tetrabutylammonium hydroxide (TBAH) (Fisher Chemical Company, Pittsburgh, PA) in double distilled water. The pH was adjusted to 4.0 using a 10% phosphoric acid solution. Solvent B was 100% methanol (Fisher Chemical Company, Pittsburgh, PA). At a flow rate of 1.5 ml/minute, elution conditions were as follows (0-70 minutes): 0-30 minutes of 100% solvent A, and 30-70 minutes of a linear gradient resulting in a final ratio of 70% solvent A and 30% solvent B. GMP, IMP, and AMP eluted at 26.5, 28.0, and 33.5 minutes respectively. HPLC standards for AMP, IMP, and GMP were obtained from Sigma Chemical Co., St. Louis, MO..

Data for CL weights, concentrations of progesterone and estradiol- 17β in ovarian vein blood, total luteal cell percentages, live/dead luteal cell percentages, ovarian vascular cell staining percentages, and luteal AMP, IMP, and GMP levels were subjected to a one-way analysis of variance for a completely randomized design. Percentages of total luteal cells, ovarian vascular stained cells, and live/dead luteal cells were

transformed (arc sine) prior to analysis (Little and Hills, 1978) in order to conform to assumptions of homogeneity of variance. Estradiol-17 β and progesterone concentrations from the daily jugular blood samples were compared using a general linear model analysis of variance for repeated measures using a split plot design. Treatments served as the mainplots, and days within treatment as the subplots. Assumption of independent variances of subplots within plots were tested by the method of Gill and Hafs (1971).

4.B RESULTS

In the first experiment, daily jugular progesterone profiles for ewes treated with 150 μ g azaserine showed generally higher concentrations than controls ($P \leq 0.01$, day*treatment) (figure 8). There was a trend ($P \leq 0.1$) in experiment 2 for ewes treated with azaserine alone to have a lower progesterone profile (day*treatment effect) than controls (figure 15). Other treatments in both experiments 1 and 2 were not different from controls (figures 9, 10, 16, and 17). Daily progesterone profiles showed significant "day" effects for all treatments (figures 8, 9, 10, 15, 16, and 17).

Estradiol-17 β profiles from daily jugular blood samples showed differences only between the two doses of mycophenolic acid ($P \leq 0.05$) (figure 13) in which ewes treated with 150 μ g of mycophenolic acid had higher average estradiol-17 β levels than those treated with 500 μ g of mycophenolic acid. No other day*treatment effects were found, but "day" effects ($P \leq 0.01$), indicating a significant day to day change in the plasma estradiol-17 β (likely due to recruitment or atresia in the follicle pool), were noted in daily estradiol-17 β profiles from experiment 2 (figures 11, 12, 18, 19, and 20). Ovarian vein progesterone and

estradiol-17 β were not significantly different from levels in control animals for either experiment 1 or experiment 2 ewes (Tables 4.1, 4.2, 4.10, and 4.11).

Total percentages of small and other luteal cells were consistent among treatments (Tables 4.4, 4.6, 4.8, 4.14, 4.16, 4.18), while large luteal cell percentages for animals treated with 150 μ g of hadacidin were greater than controls ($P \leq 0.05$) (Table 4.6). There were no differences in live/dead staining of "other" luteal cells regardless of treatment, but CL from ewes treated with 500 μ g of mycophenolic acid had more dead small luteal cells than controls ($P \leq 0.05$) (Table 4.7). There was a trend ($P \leq 0.07$) among hadacidin + adenosine-treated ewes to have a lower number of live large luteal cells than control ewes (Table 4.15). Total percentages and percent live cells for all others were not different (Tables 4.3, 4.5, 4.13, and 4.17) CL weights were not significantly different among treatment groups (Tables 4.9 and 4.12).

Data from the Feulgen staining procedure used in experiment 1 compared vascular stainability between not only the treatments and controls, but also between the cannulated and contralateral uncannulated vascular samples from each animal within treatments. There were no differences between the average number of live cells for the cannulated versus the contralateral non-cannulated vasculature within each ewe for the azaserine, hadacidin, or mycophenolic acid treatments ($P \geq 0.1$, $P \geq 0.3$, and $P \geq 0.1$, respectively). Within cannulated vasculature samples among the treatment groups, azaserine-treated animals had higher numbers of live cells than control animals ($P \leq 0.05$), while the other treatments were not significantly different from controls (figure 14).

In experiment 3, luteal levels of AMP were significantly lower in hadacidin-treated animals than in controls ($P \leq 0.01$). In mycophenolic acid-treated ewes, there was a trend ($P \leq 0.1$) towards a lower GMP:AMP ratio than in control animals, but GMP levels were not different from controls. Azaserine-treated ewes showed a trend towards a decreased GMP:AMP ratio ($P \leq 0.1$) compared to controls, and IMP levels tended to be lower than in controls ($P \leq 0.17$).

4.C DISCUSSION

The antineoplastic and antipurinergic biosynthetic enzyme specific activities of the treatment drugs azaserine, hadacidin, and mycophenolic acid are well documented (Mizobuchi et al., 1968; Shigeura and Gordon, 1962; Franklin and Cook, 1969). These drugs specifically block de novo purine biosynthetic pathways, while leaving salvage pathways intact. The fact that salvage pathways remain operational appears to have an indirect effect on the drugs' abilities to alter purine availability at the site of the corpus luteum.

While a day effect was noted in daily jugular progesterone concentrations due to normal corpus luteum development during this period, treatment and day*treatment effects were also observed in the daily estradiol-17 β and progesterone profiles. It is likely that these differences, along with a lack of other differences can be attributed to biosynthesis pathway alterations induced by the drug treatments.

In experiment 1, azaserine treatments at lower doses (150 μ g), for example, enhanced progesterone secretion as compared to controls. While this seems unlikely, considering the nature of the drug, Levenberg et al. (1957) found that the degree of purine biosynthetic pathway inhibition by low doses of azaserine is directly related to local levels of

glutamine, and the inhibitory effects (prevention of IMP de novo synthesis) can be overcome by methionine or purines. Taking into account the fact that both adenylic and guanylic salvage pathways are controlled by feedback inhibition (Rosenbloom, 1968), and that azaserine reduces the levels of both adenylic and guanylic compounds, the intact salvage pathways could be highly activated.

Since purinergic compounds are readily taken up, transported, and deposited by the erythrocytes (Mager et al., 1967), a highly vascular organ such as the corpus luteum should receive a large source of compensatory purines via the vascular system. A high influx of compensatory purines into the corpus luteum may "overcompensate" for the azaserine-induced decline in local purine production, thereby enhancing local receptor actions requiring the involvement of purinergic compounds (i.e., adenylate cyclase system).

Arch and Newsholme (1980) have described how local increases in cyclic guanylic and adenylic monophosphates and other related compounds are stimulatory to adenylate cyclase activity. Billig et al. (1989) have also shown that adenosine analogs stimulate adenylate cyclase activity in cell homogenates of isolated luteal cells. Guanine nucleotides appear to play an additional role in regulating the actions of hormones on adenylate cyclase activity (Spiegel and Downs, 1981).

Adenylate cyclase activity is closely related to steroid production by both the corpus luteum and the ovarian follicles. Hall et al. (1981) showed that adenosine and inosine stimulated intracellular cAMP levels, and subsequently caused an increase in progesterone synthesis by rat luteal cells in vitro. Using this information, it is possible to see how purine salvage pathways not only enable the treated tissue to overcome

low doses of drugs (i.e., the 150 μg dose of azaserine), but also they can serve as a stimulus to purine-related tissue functions.

The hormone profiles for the 500 μg dose-treated ewes for all drugs were not different from those of controls. Combined drug and replacement compound-treated animals were not different from controls either, although they seem to have a tendency to produce less progesterone than controls. While the initial premise of the drug + replacement compound reaction was that the replacement compound would compensate for the pathway blockage caused by the drug and perhaps even enhance luteal progesterone output, this may not be the case.

From the 500 μg drug dose data, one would assume that since the corpus luteum is able to overcome the effects of this dose without the aid of exogenous purinergic compounds (via its own salvage pathway supplies), that the addition of an exogenous pool of purines would possibly enhance luteal steroid production. Hall et al. (1981) have reported that adenosine and inosine, for example, enhance LH-induced production of progesterone by cultured rat luteal cells.

Hall et al. (1981) also found, though, that increasing quantities of adenosine produced a clear dose-dependent inhibition of adenosine uptake by cultured rat luteal cells. Arch and Newsholme (1980) report that exogenous inosine can inhibit cellular uptake of adenosine. The replacement compounds in this experiment were meant to enhance purine supply in the area, whereas, considering the findings of Hall et al. (1981), they may actually be causing a decrease in local purine biosynthesis and uptake by activating the negative feedback inhibitory system. This may be why drug + replacement compound-treated

animals seem to have generally lower (albeit not significantly lower) levels of progesterone.

The higher general profile of estradiol-17 β production found in ewes treated with the 150 μ g mycophenolic acid dose over control ewes is probably affected similarly by salvage pathway intervention. Franklin and Cook (1969) have reported that the effect that mycophenolic acid has on tissues is dependent on the tissues' ability to meet its requirement for guanine nucleotides by conversion of guanine to GMP via the salvage pathway enzyme hypoxanthine-guanine phosphoribosyltransferase (HG-PRTase). In addition, Hodges et al. (1989) have shown that not only do cells exposed to mycophenolic acid (which decreases intracellular GTP) have increased rates of purine synthesis and catabolism, apparently as a result of a deficiency of guanine nucleotides, but also that inhibition of IMP dehydrogenase (a primary action of mycophenolic acid) results in compensatory purine overproduction.

Based on the information from Franklin and Cook (1969) and Hodges et al. (1989), low doses of mycophenolic acid (i.e., the 150 μ g dose) may be overcome by salvage pathway compensation, and steroid hormone production enhanced by increased local concentrations of purinergic compounds stimulatory to the adenylate cyclase system. Frequent infusion of higher doses of mycophenolic acid and azaserine may be harder to overcome as far as local production of purinergic products, so simple compensation by salvage pathways and importation of purines from other bodily sources via the blood stream enable the corpus luteum and the follicles to at least maintain normal levels of

function, as opposed to enhanced function induced by overcompensation.

In experiment 1, the higher 500 μg dose of mycophenolic acid proved to have a negative effect on the percentage of live small luteal cells as compared with the control ewes and those receiving 150 μg mycophenolic acid doses. When considering that the small luteal cells are rapidly proliferating during the treatment period, it is not surprising that an antimitotic compound such as mycophenolic acid would have a detrimental effect on cells that normally would be undergoing high amounts of mitotic activity. It is doubtful that mycophenolic acid treatment impaired corpus luteum function as a whole, as evidenced by a lack of difference between progesterone levels in mycophenolic acid-treated animals versus control animals. However, chronic inhibition of mitotic activity appears to slightly increase cell mortality in rapidly proliferating cell populations. In these situations, purinergic building blocks are in high demand, as is the need for what would normally be excess purine building blocks to overcome mitotic blocks imposed by mycophenolic acid.

The percentage of total large luteal cells was higher in ewes treated with hadacidin than in control animals, and in fact, ewes treated with the 150 μg hadacidin dose tended to have a greater percentage of both large and small steroidogenic luteal cells than did 500 μg hadacidin or control treated animals. Hadacidin, a relatively weak inhibitor of mitosis (Kaczka et al., 1962), apparently does not have an inhibitory effect on the rate of cell proliferation. In fact, hadacidin actions can stimulate the conversion of AMP to adenylosuccinate by adenylosuccinase in the adenylic salvage pathway, which is activated by

a lack of inhibition due to initial actions of hadacidin on levels of cellular adenylic compounds (Shigeura and Gordon, 1962). Salvage pathway derived adenylic compounds are then available to the steroidogenic cell populations, and may be utilized for growth and proliferation.

In experiment 2, hadacidin + adenosine-treated ewes showed a trend towards lower numbers of live large luteal cells, although the total number of large luteal cells was not different from that of controls. The switching off of the cells' purine biosynthetic mechanisms due to high local adenosine levels may be involved in the demise of these cells that produce far more progesterone than their small luteal cell counterparts. These large luteal cells may be overtaxed by high hormonal output and dwindling purine supplies, perhaps resulting in complete exhaustion of the cell and eventually death.

In the case of significantly greater numbers of live vascular cells found in azaserine-treated animals versus control animals, a similar feedback situation exists. Considering the carcinogenic properties of azaserine this seems unusual, but Held et al. (1969) found that azaserine-treated cells decreased initially, followed by a sharp increase in the incorporation of glycine, adenine, and other preformed purine bases into RNA. This provides evidence that when cells are stressed by the drug-provoked pathway inhibitions, they are stimulated to compensate for these actions presumably by utilizing salvage pathway components.

The results gained from experiment 3 verify the ability of this in vivo model system to deliver the purine pathway blocking drugs to the corpus luteum effectively. Hadacidin blocks the adenylic de novo biosynthetic pathway by competitively inhibiting the activity of adenylosuccinate synthetase, which prevents formation of

adenylosuccinate, and subsequently, AMP (Shigeura and Gordon, 1962). This is borne out here by the fact that corpora lutea from hadacidin-treated ewes had a lower AMP content than did corpora lutea from control ewes.

Mycophenolic acid acts by inhibiting the action of IMP dehydrogenase, thereby preventing de novo biosynthesis of GMP. While GMP levels in mycophenolic acid-treated ewes appeared to be somewhat lower than in control animals, albeit not significantly lower, there is definitely a trend for GMP:AMP ratios to be lower in mycophenolic acid-treated ewes than in controls. This is consistent with lower GMP levels.

This same trend for a decreased GMP:AMP ratio relative to controls was present in azaserine-treated animals. Although azaserine, a glutamine antimetabolite, most specifically prevents the conversion of formylglycinamide ribotide to formylglycinamide ribotide, it also can have a competitive action on glutamine in the enzymatic conversion of XMP to GMP at high concentrations (Levenberg et al., 1956).

These collective alterations in corpus luteum purine concentrations may reflect stresses placed on the local purine pools in the corpus luteum. Tissue samples for experiment 3 were taken within 2 hours of the last drug infusion, so these decreases and alterations may reflect either an acute response to drug interference, or the results of long term high stresses on the purine storage pool. This does not necessarily imply that there is not compensation either at the corpus luteum level or by importation of purinergic products via the circulation.

With azaserine, for example, there is an initial decrease in purine utilization, followed by an above normal increase in purine incorporation into RNA (Held et al., 1969). Held et al. (1969) also reports an increased

turnover of the purine nucleotide pool subsequent to azaserine's initial inhibitory effect on the de novo synthesis pathway, and that azaserine does not adversely affect the utilization of salvage pathway adenylic products in vivo. Consequently, salvage pathways could still be a source of purines, along with a ready supply from the vascular system.

Although pool levels of the various purines may be lowered, these levels still are probably sufficient for normal luteal function as evidenced by progesterone outputs similar to controls by animals in the second experiment receiving equivalent doses of azaserine, hadacidin and mycophenolic acid similar to those in the third experiment. Additionally, the possibility that the luteal cells are relying on non-purinergic second messengers is highly likely. Cooke et al. (1976) have shown that LH-stimulated steroidogenesis in Leydig cells can reach 50 percent of maximum output without causing an increase in intracellular cAMP levels. It has been suggested that increasing intracellular calcium levels and lipoxygenase pathway products of arachidonic acid metabolism directly stimulate LH-related steroidogenesis, circumventing the traditionally accepted cAMP second messenger (Dix et al., 1984; Sullivan and Cooke, 1985; Cooke, 1990) (see General Discussion).

Figure 8. Daily jugular progesterone profiles for ewes treated with 150 or 500 μg of azaserine versus controls (day, treatment*day $P \leq 0.01$). Five ewes per treatment group.

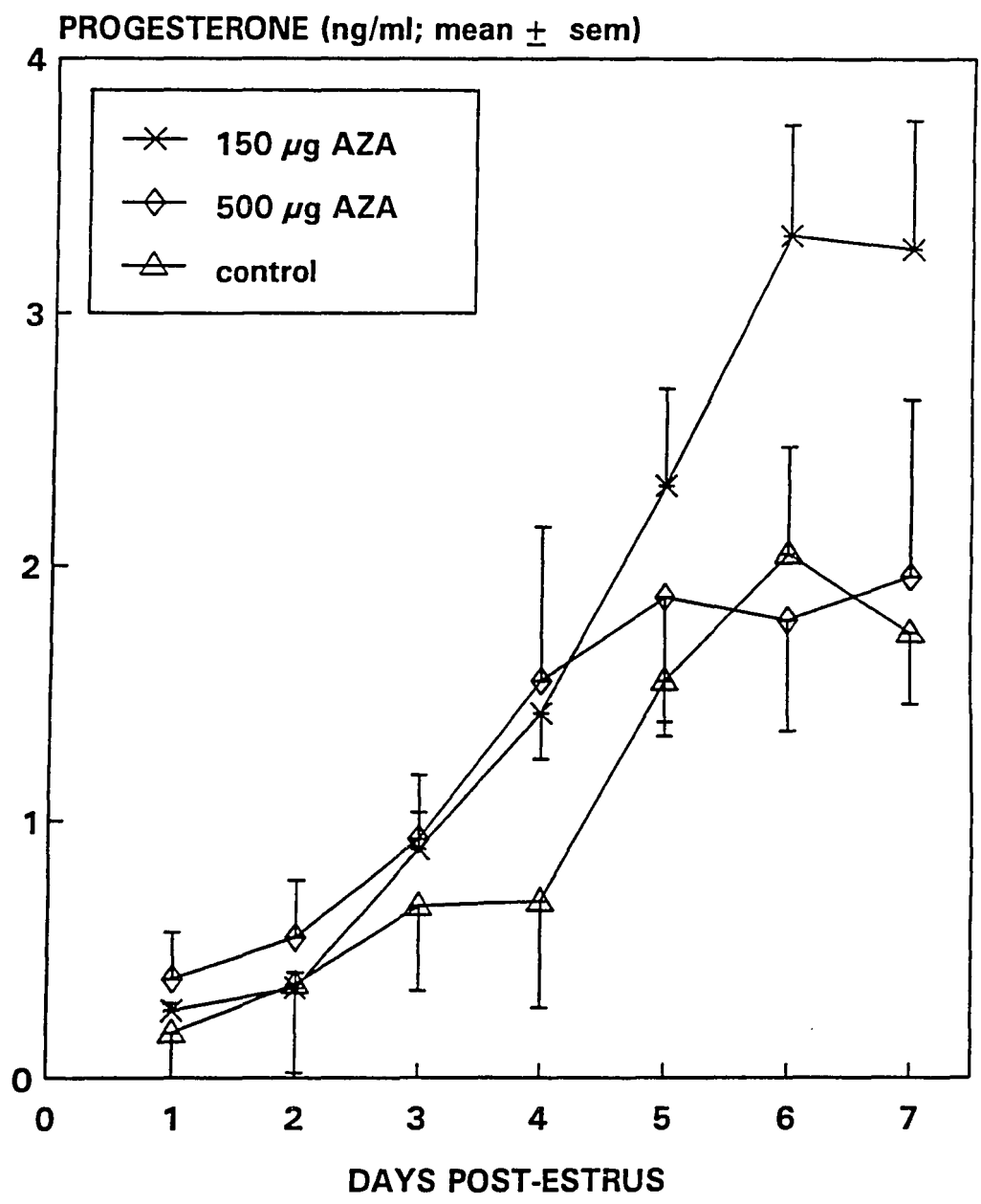


Figure 9. Daily jugular progesterone profiles for ewes treated with 150 or 500 μg of hadacidin versus controls (day $P \leq 0.01$). Five ewes per treatment group.

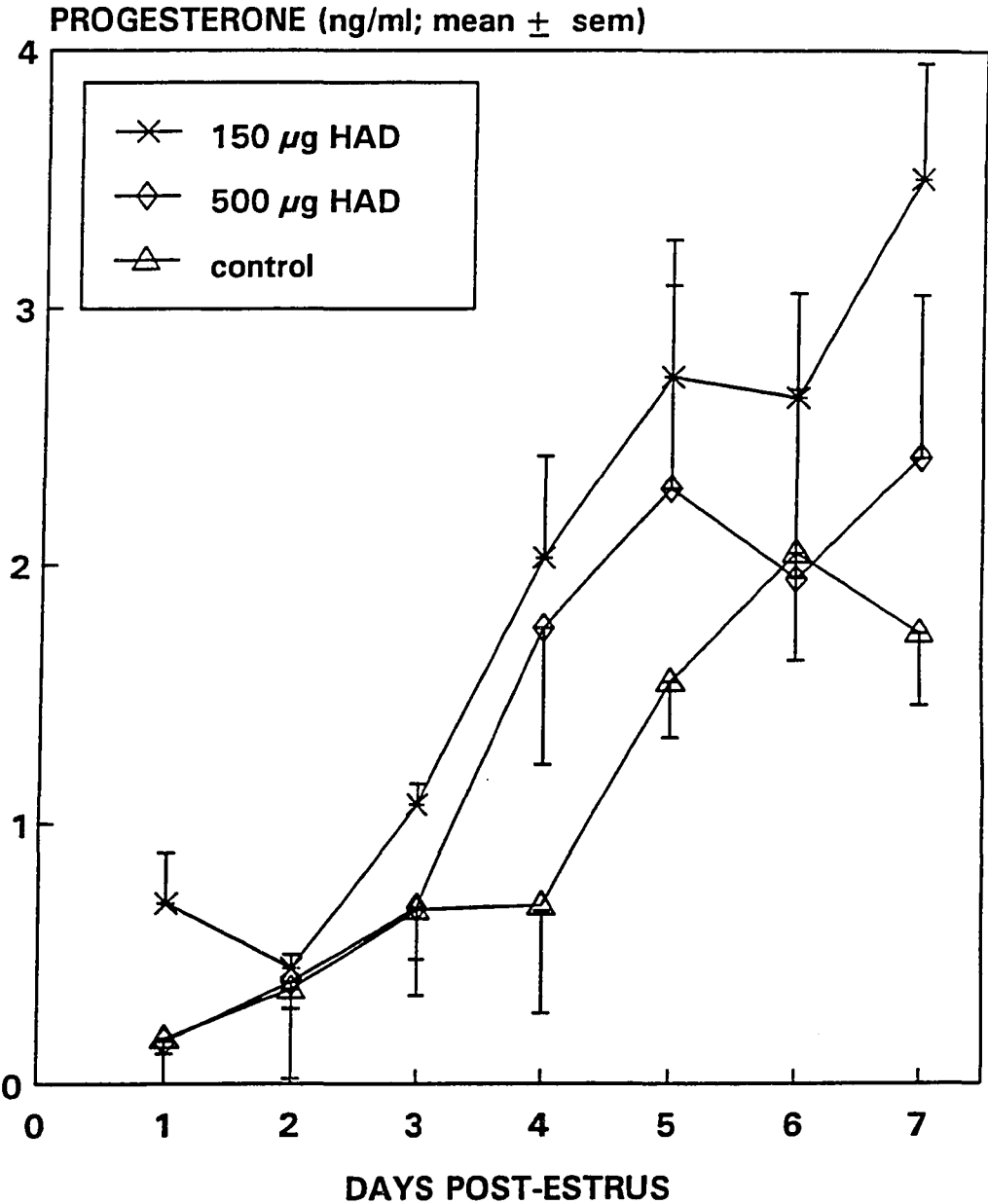


Figure 10. Daily jugular progesterone profiles for ewes treated with 150 or 500 μg of mycophenolic acid versus controls (day $P \leq 0.01$). Five ewes per treatment group.

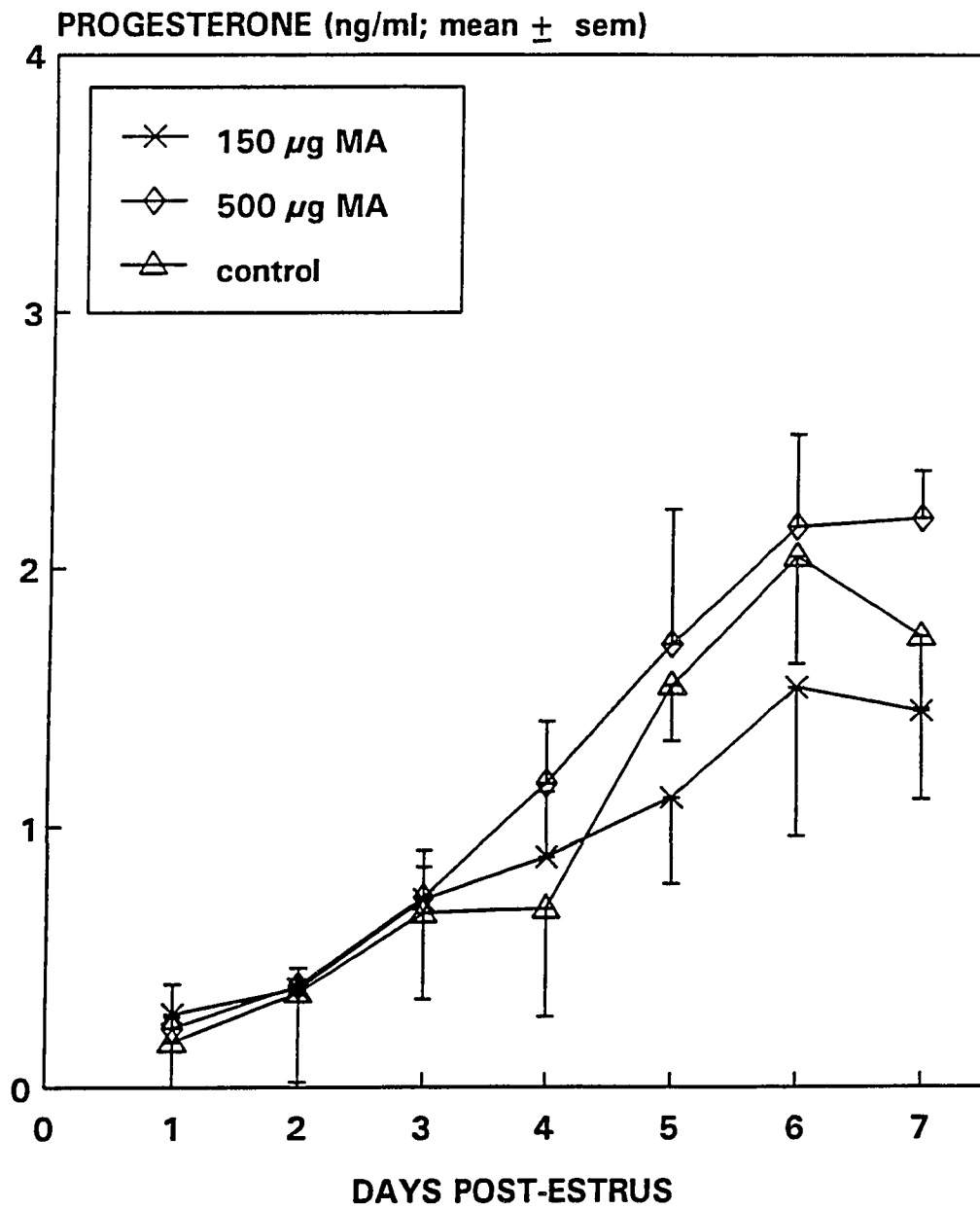


Figure 11. Daily jugular estradiol-17 β profiles for ewes treated with 150 or 500 μ g of azaserine versus controls (no significant differences). Five ewes per treatment group.

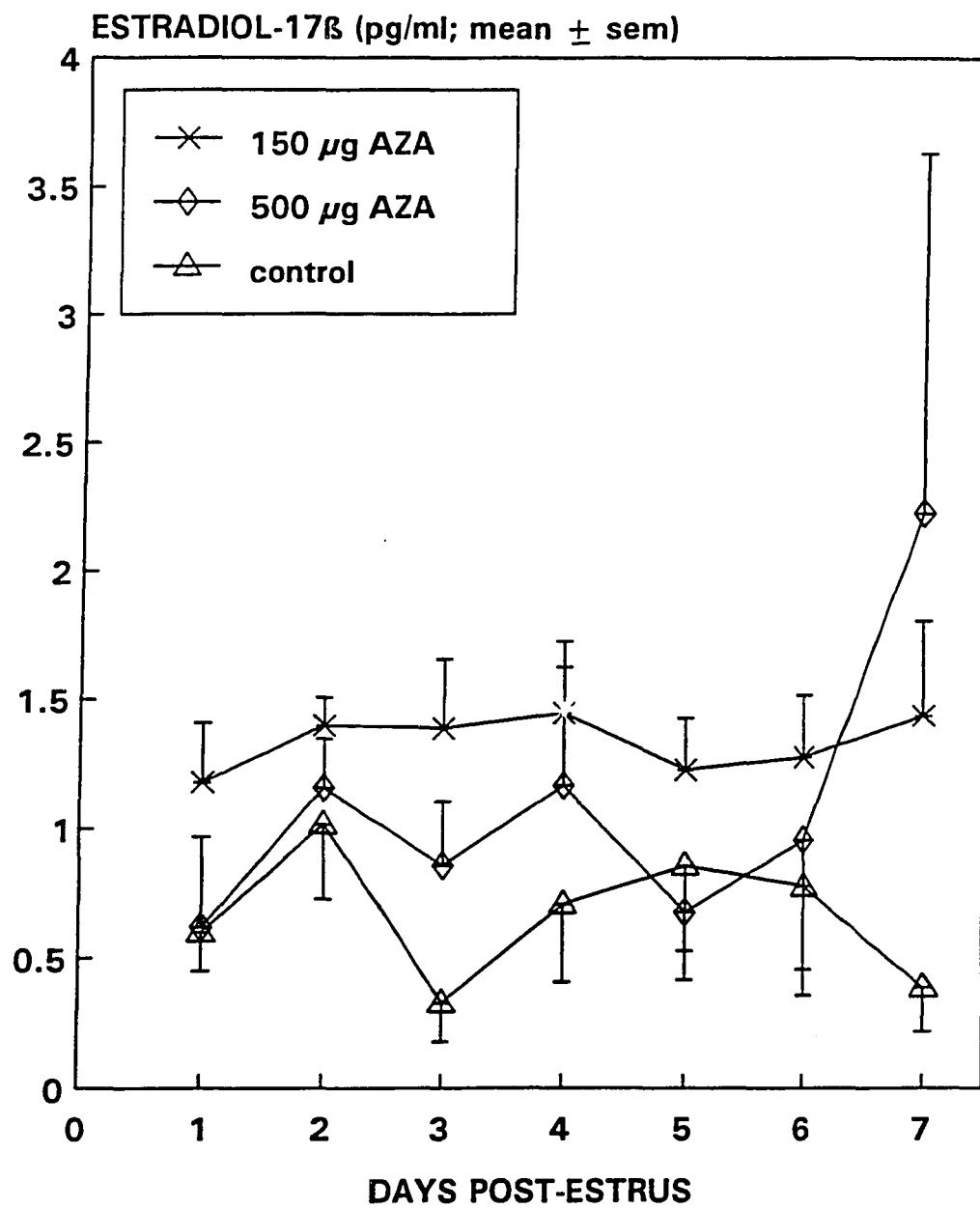


Figure 12. Daily jugular estradiol-17 β profiles for ewes treated with 150 or 500 μ g of hadacidin versus controls (no significant differences). Five ewes per treatment.

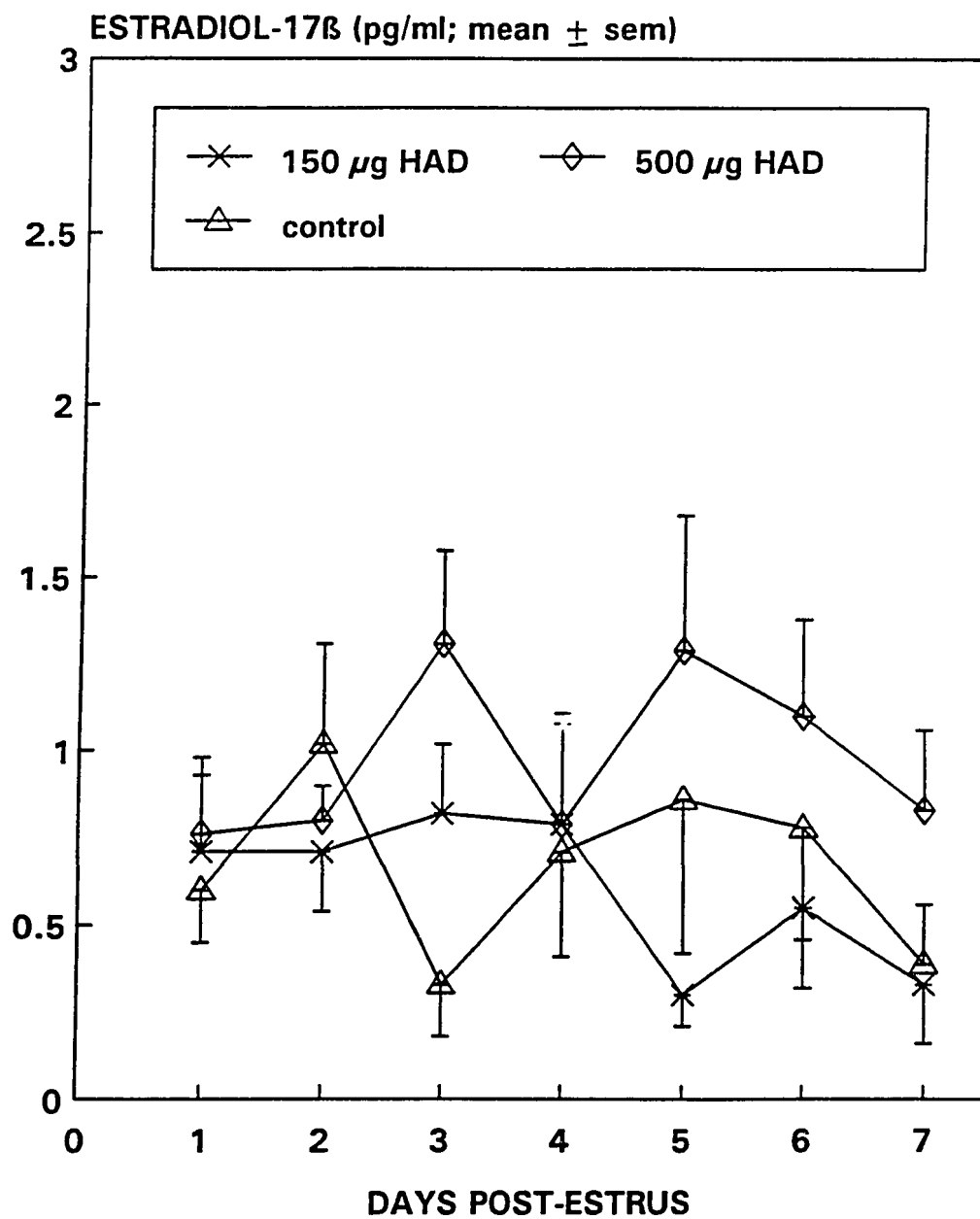


Figure 13. Daily jugular estradiol-17 β profiles for ewes treated with 150 or 500 μ g of mycophenolic acid versus controls (treatment $P \leq 0.05$). Five ewes per treatment group.

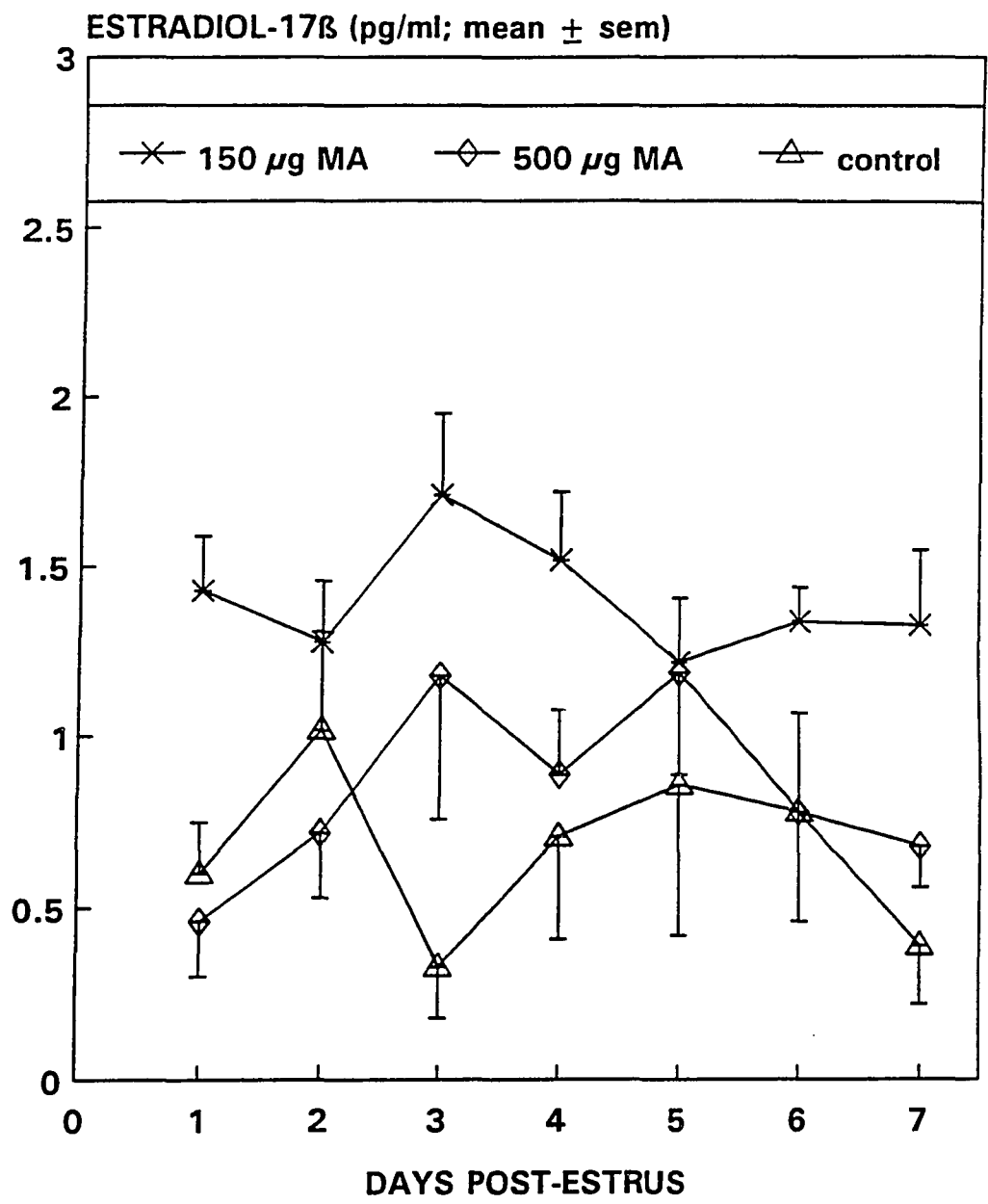
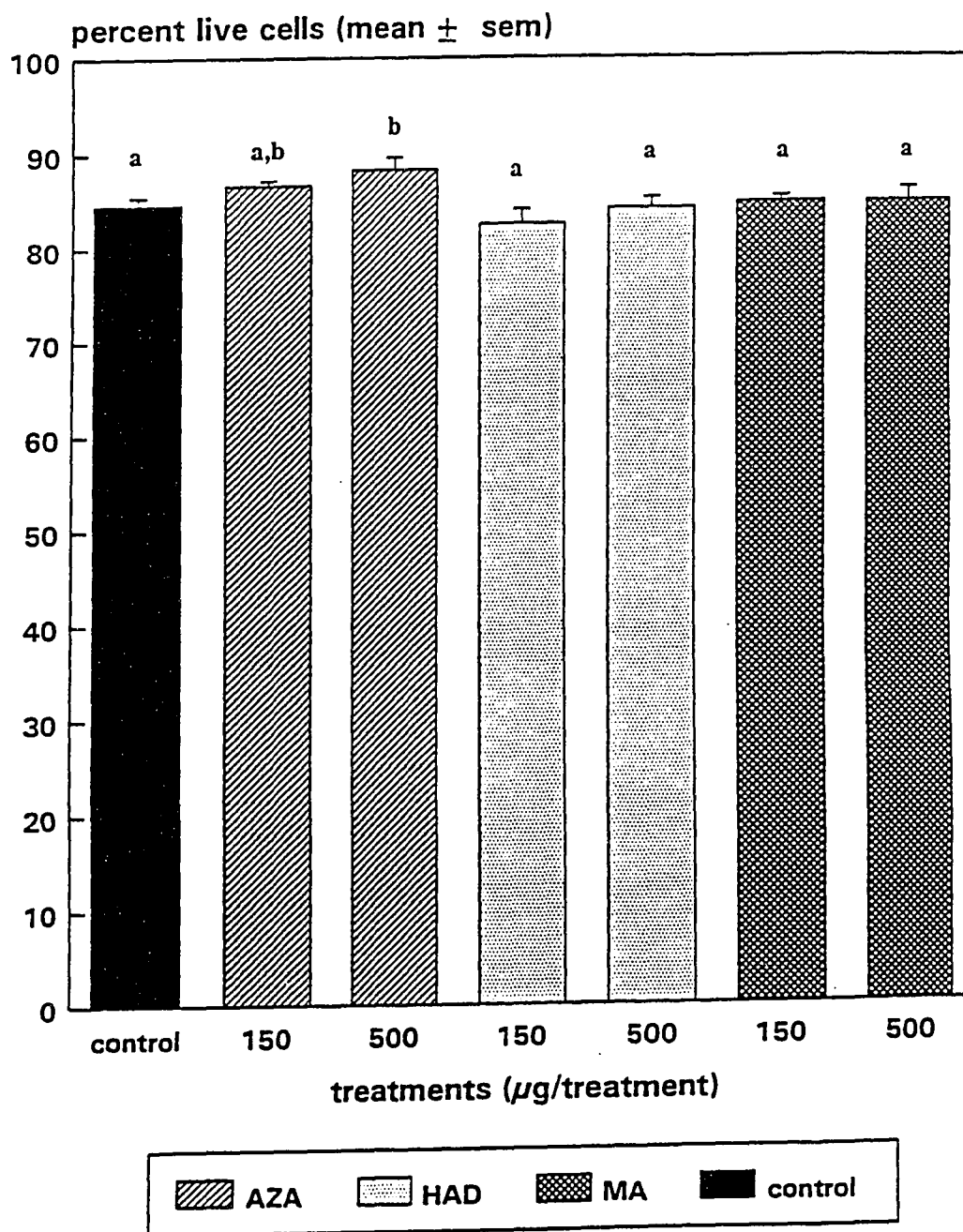


Figure 14. Determination of percent live endothelial cells by Feulgen staining in ovarian pedicle arterial vasculature from the cannulated side of azaserine, hadacidin, mycophenolic acid, or control treated animals. Five animals per treatment group.



a,b differ at $P \leq 0.05$ within treatment

Figure 15. Daily jugular progesterone profiles for ewes treated with azaserine, azaserine + inosine, or control treatments (day $P \leq 0.01$, day *treatment $P \leq 0.1$). Five ewes per treatment group.

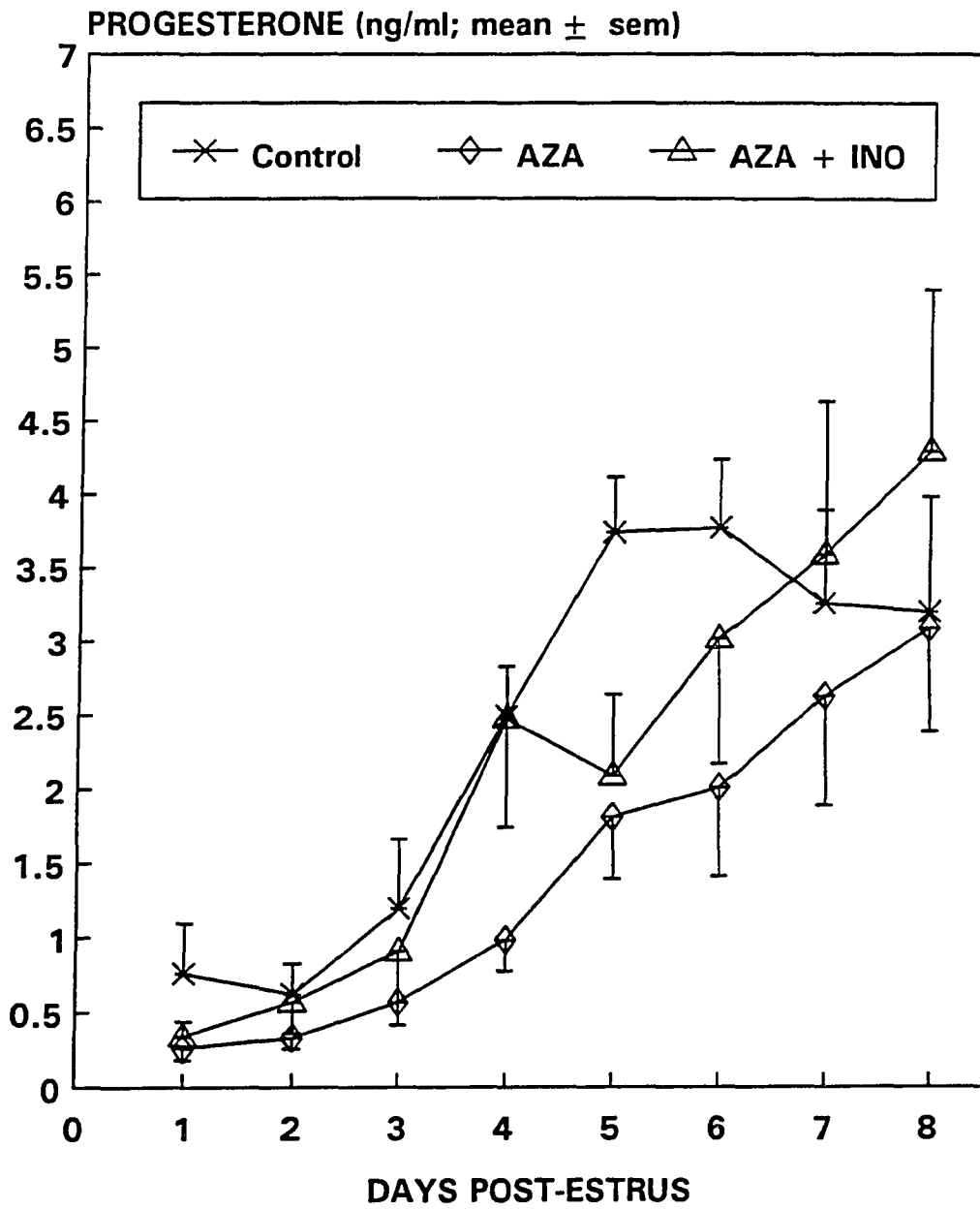


Figure 16. Daily jugular progesterone profiles for ewes treated with hadacidin, hadacidin + adenosine, or control treatments (day $P \leq 0.01$). Five ewes per treatment group.

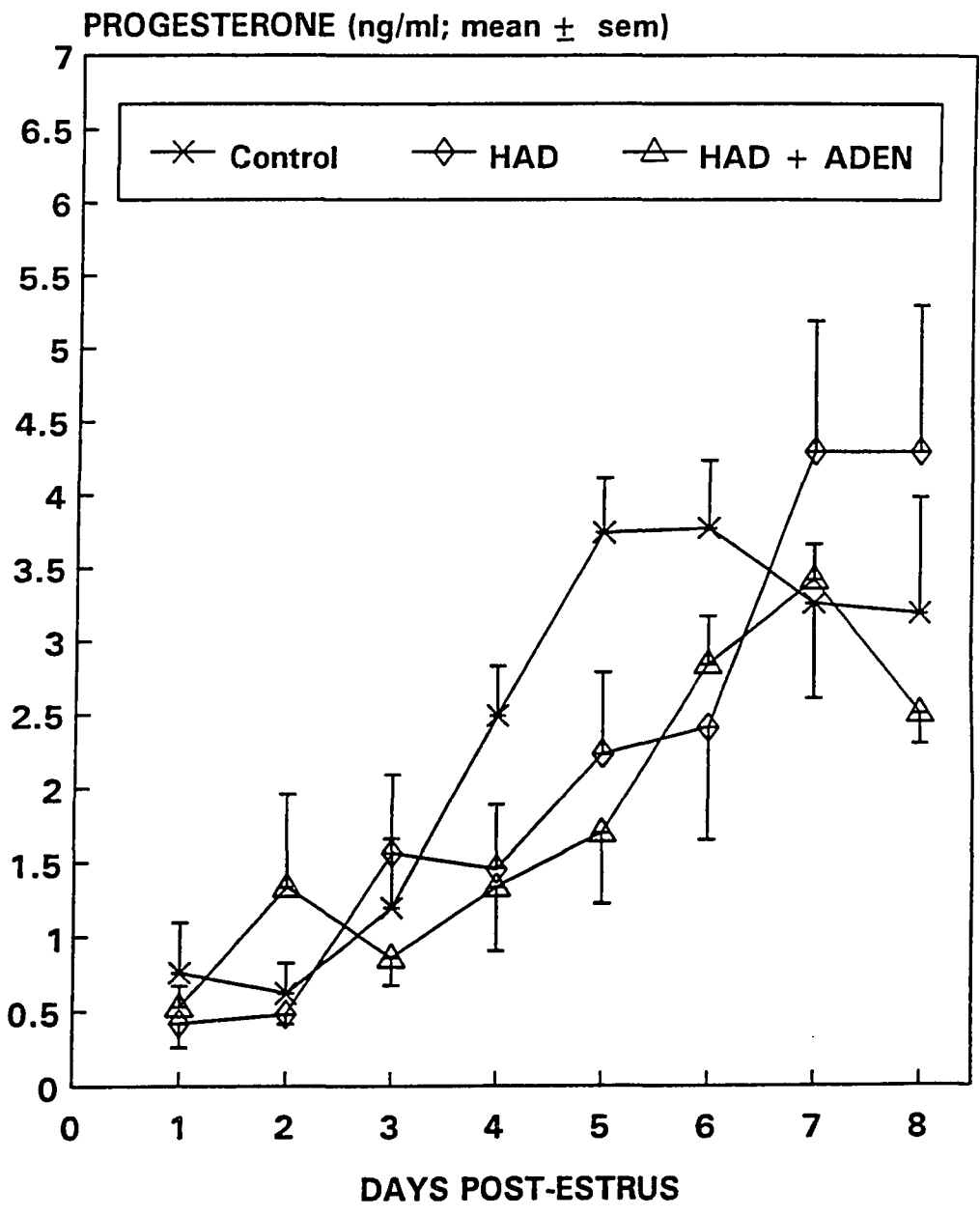


Figure 17. Daily jugular progesterone profiles for ewes treated with mycophenolic acid, mycophenolic acid + guanosine, or control treatments (day $P \leq 0.01$). Five ewes per treatment group.

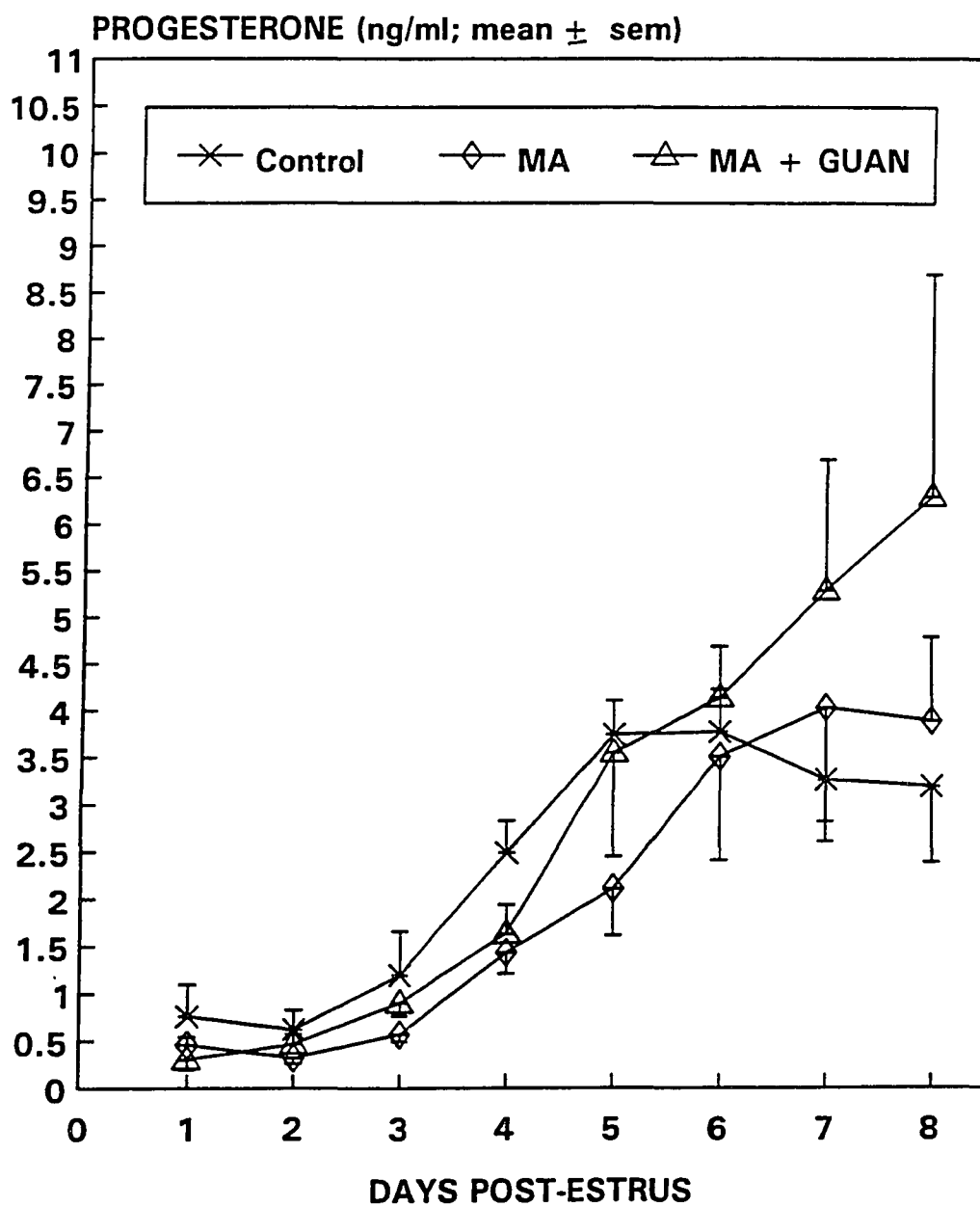


Figure 18. Daily jugular estradiol-17 β profiles for ewes treated with azaserine, azaserine + inosine, or control treatments (day P \leq 0.01). Five ewes per treatment group.

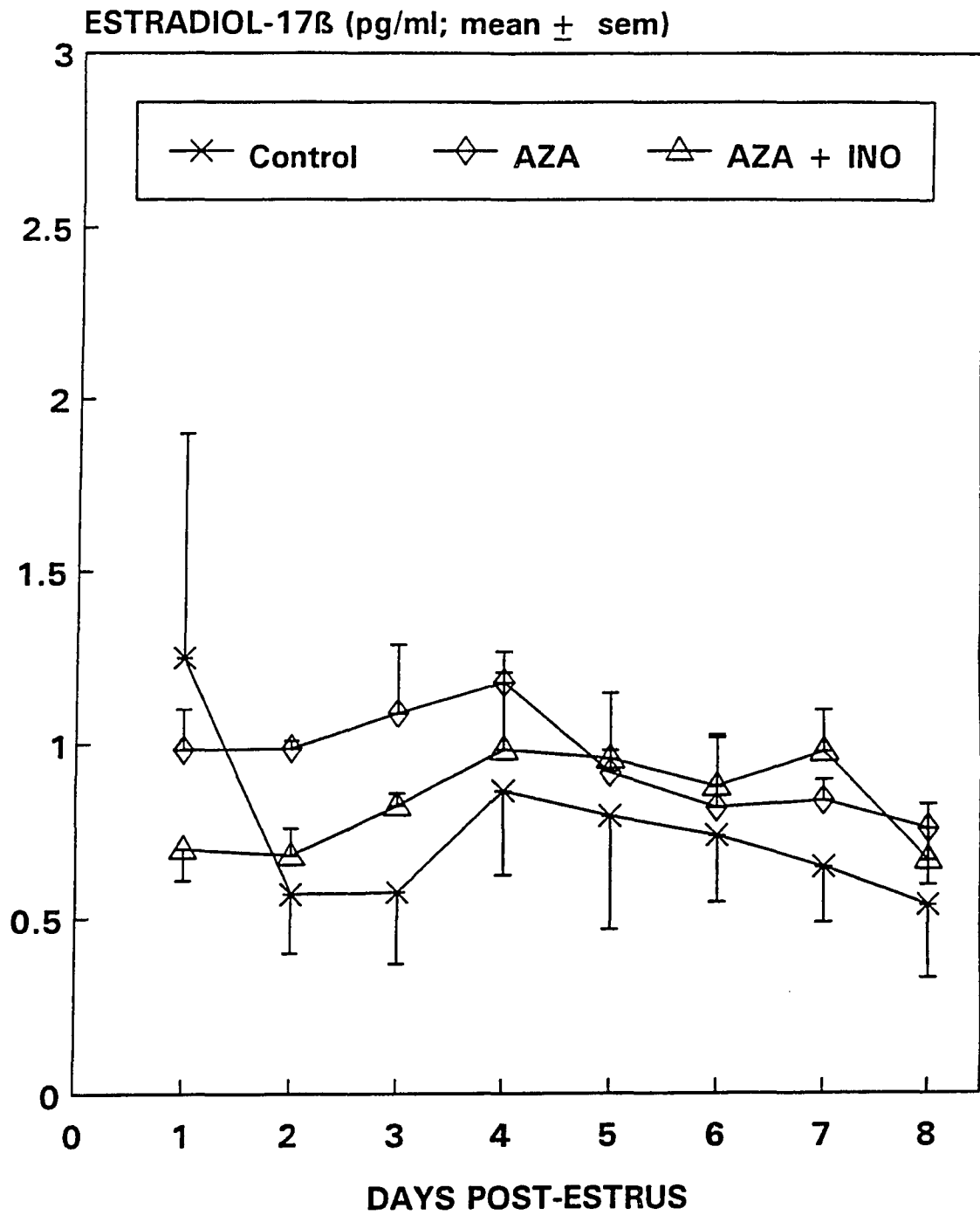


Figure 19. Daily jugular estradiol-17 β profiles for ewes treated with hadacidin, hadacidin + adenosine, or control treatments (day P \leq 0.01). Five ewes per treatment group.

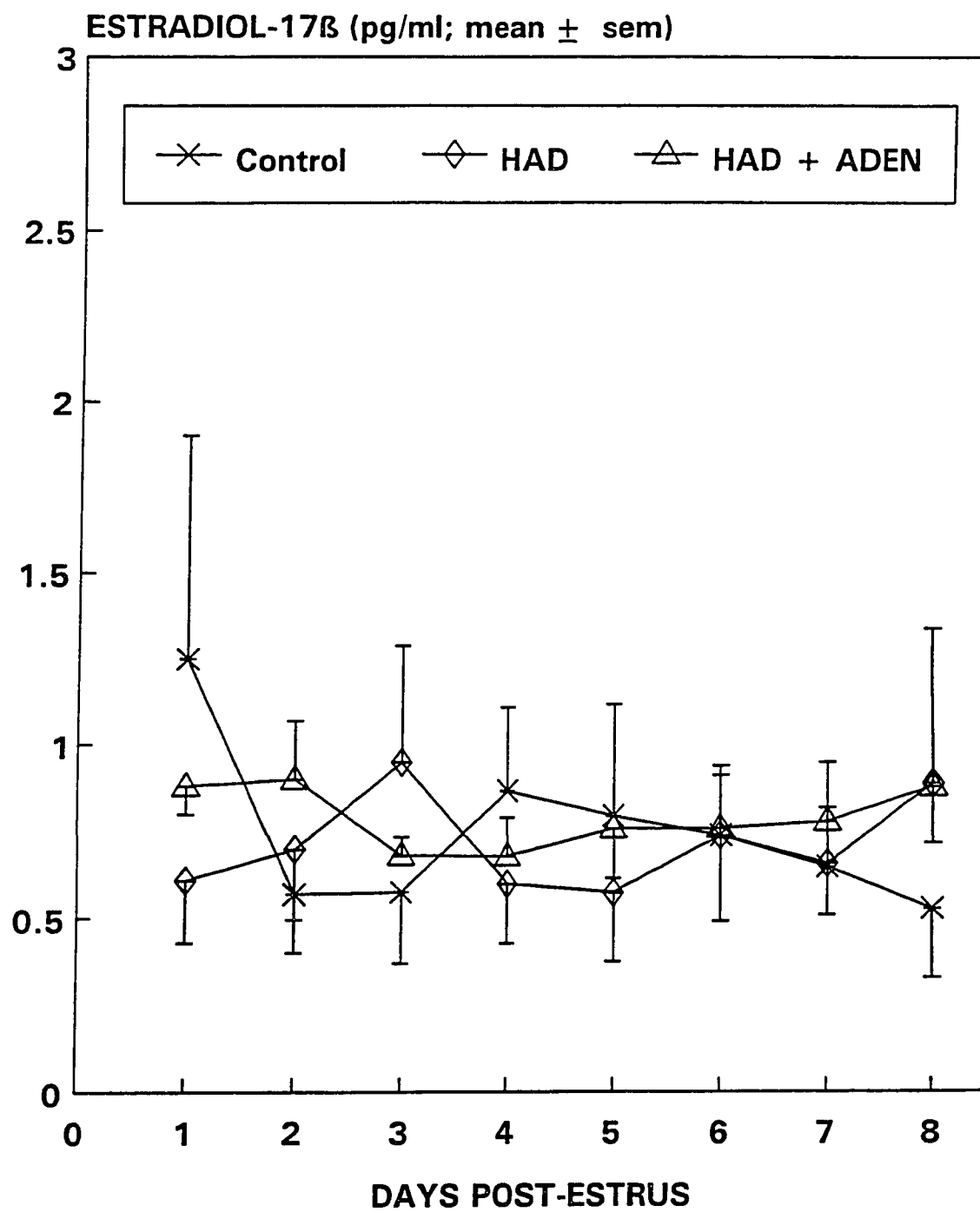


Figure 20. Daily jugular estradiol-17 β profiles for ewes treated with mycophenolic acid, mycophenolic acid + guanosine, or control treatments (day $P \leq 0.01$). Five ewes per treatment group.

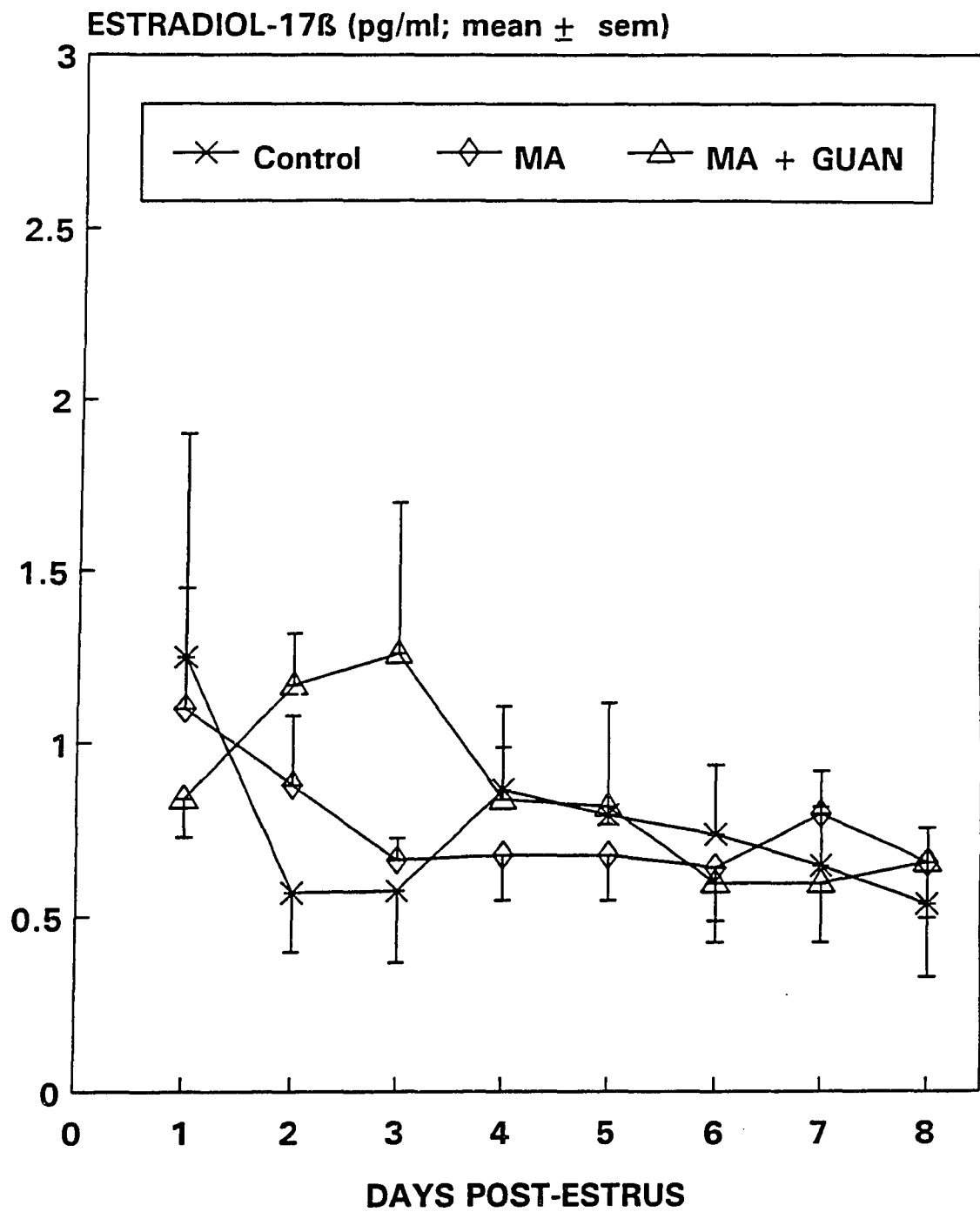


Figure 21. A representative chromatograph of AMP, IMP, and GMP levels in a corpus luteum treated in vivo with control treatments.

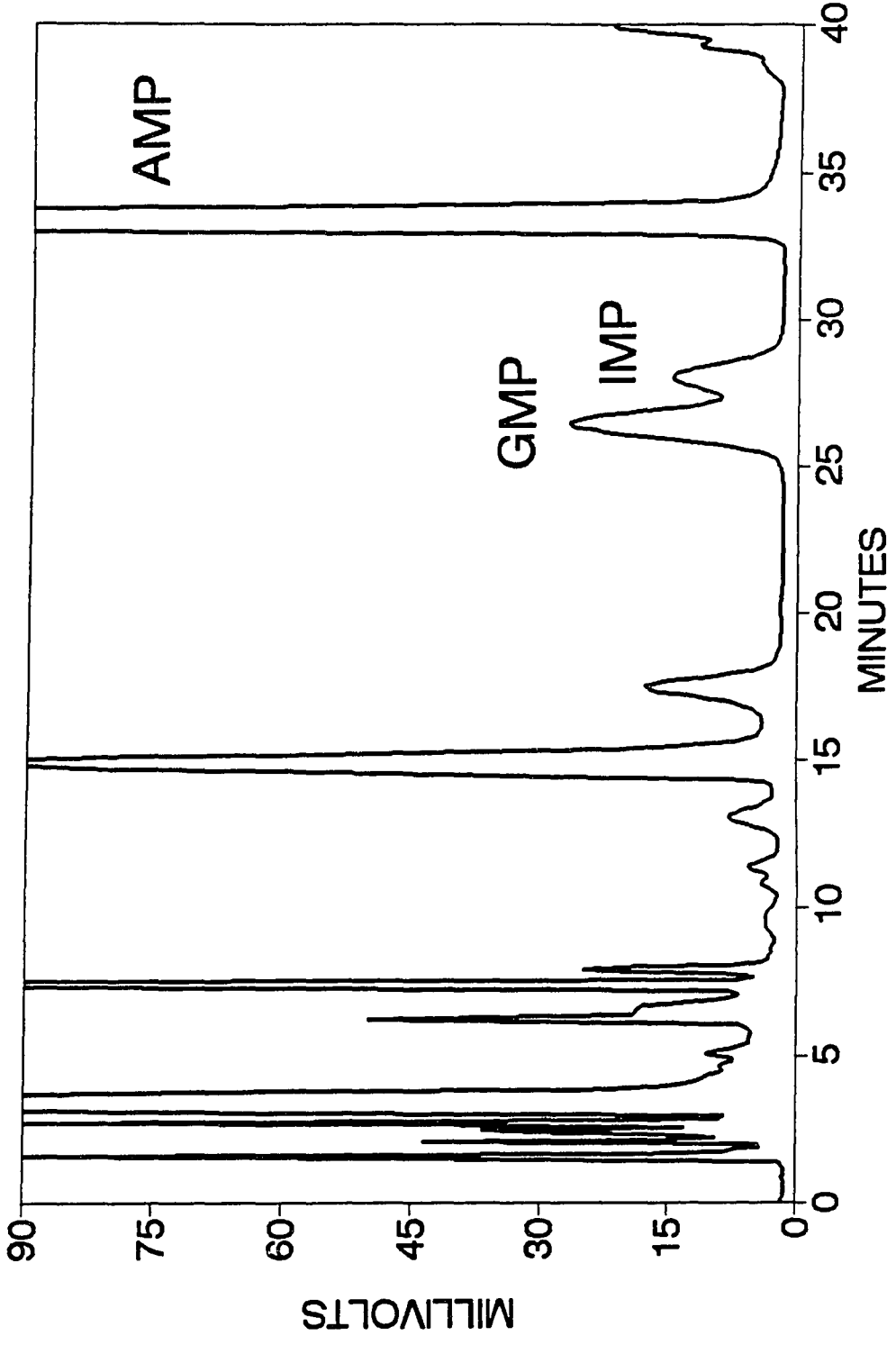


Figure 22. A representative chromatograph of AMP, IMP, and GMP levels in a corpus luteum treated in vivo with azaserine.

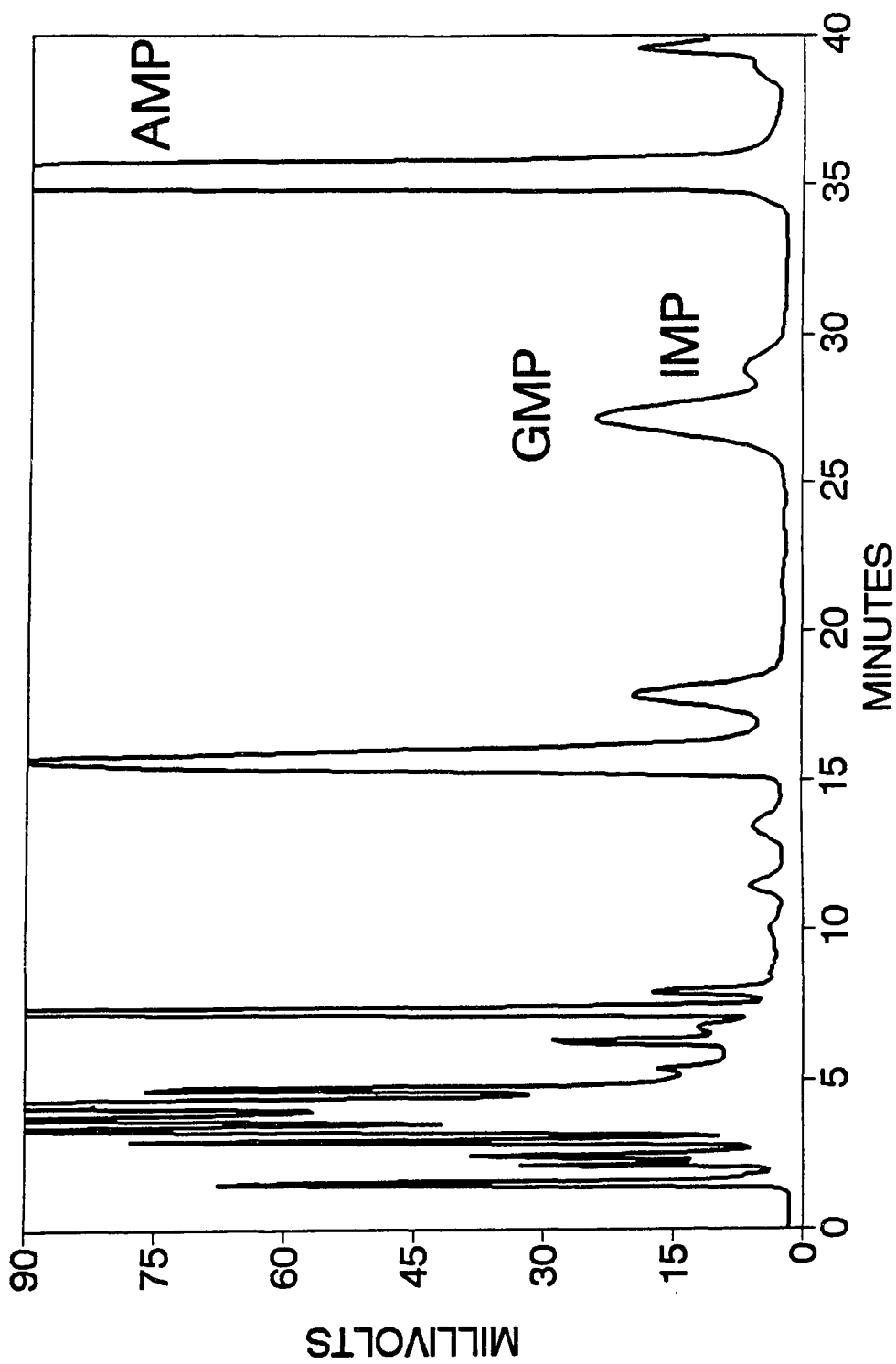


Figure 23. A representative chromatograph of AMP, IMP, and GMP levels in a corpus luteum treated in vivo with hadacidin.

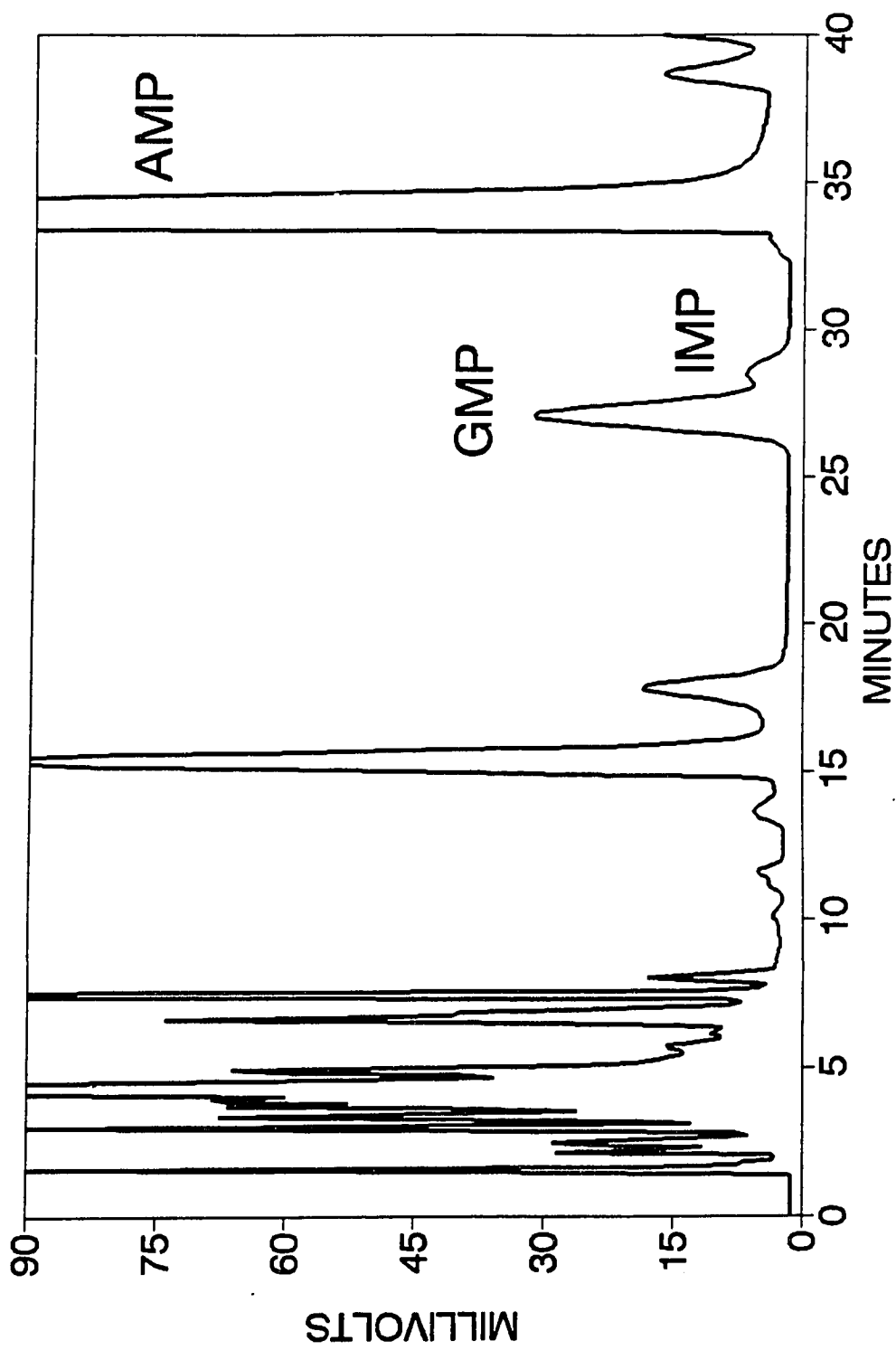


Figure 24. A representative chromatograph of AMP, IMP, and GMP levels in a corpus luteum treated in vivo with mycophenolic acid.

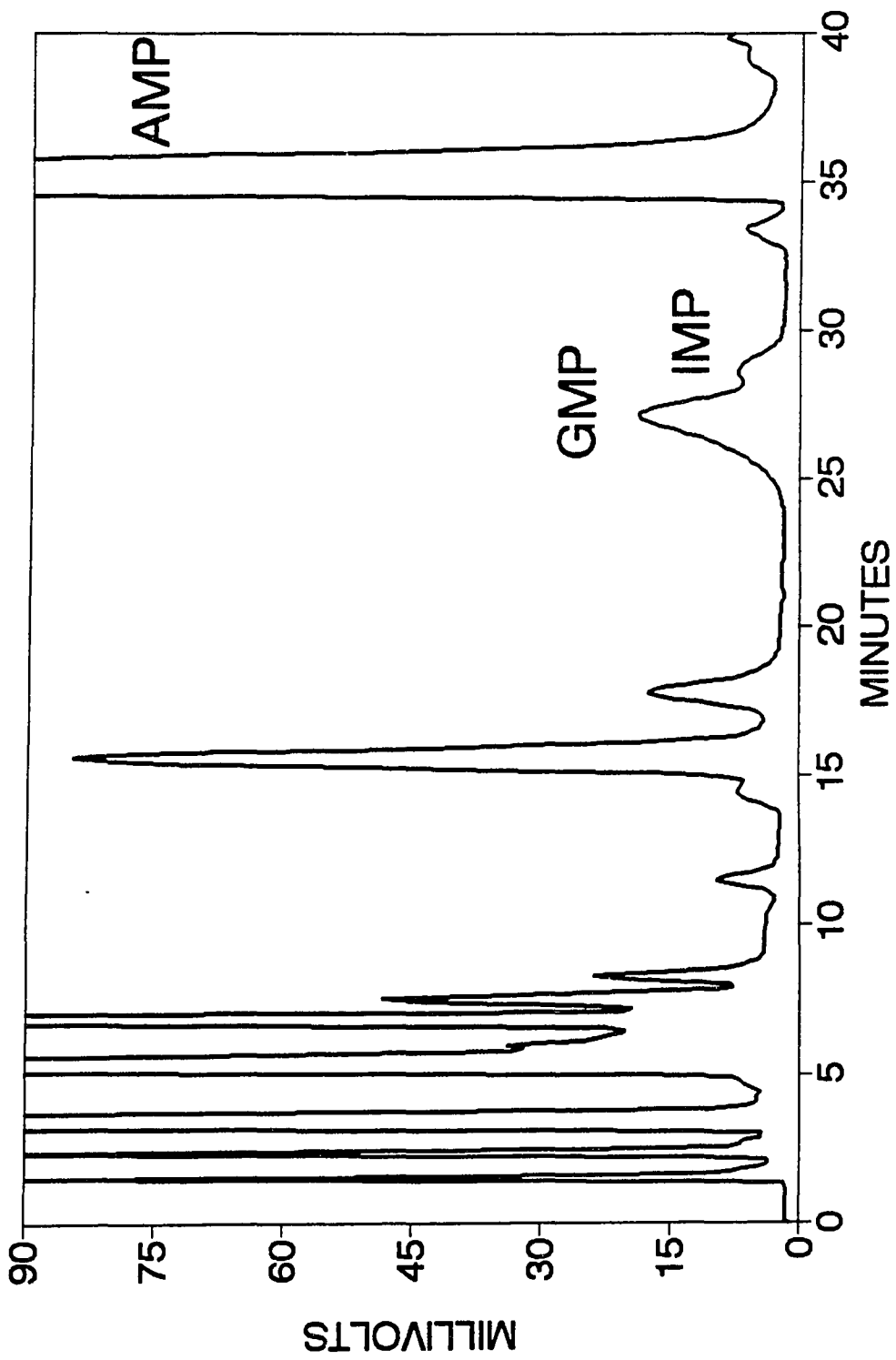


Table 4.1. Mean (\pm SEM) ovarian venous progesterone concentrations obtained at the second laparotomy from ewes treated in vivo with 150 or 500 μ g of azaserine, hadacidin, or mycophenolic acid, or control treatment. Five ewes per treatment group. 87

TREATMENT	PROGESTERONE (ng/ml)
Control	1853 \pm 581 ^a
150 μ g Azaserine	1189 \pm 1021 ^a
500 μ g Azaserine	437 \pm 301 ^a
150 μ g Hadacidin	2442 \pm 676 ^a
500 μ g Hadacidin	1672 \pm 901 ^a
150 μ g Mycophenolic Acid	863 \pm 458 ^a
500 μ g Mycophenolic Acid	1038 \pm 487 ^a

a Values with the same superscripts are not different

Table 4.2. Mean (\pm SEM) ovarian venous estradiol-17 β concentrations obtained at the second laparotomy from ewes treated *in vivo* with 150 or 500 μ g of azaserine, hadacidin, or mycophenolic acid, or control treatment. Five ewes per treatment group.

TREATMENT	ESTRADIOL (pg/ml)
Control	6.7 \pm 2.9 ^a
150 μ g Azaserine	176.2 \pm 154.0 ^a
500 μ g Azaserine	5.9 \pm 4.7 ^a
150 μ g Hadacidin	48.7 \pm 41.1 ^a
500 μ g Hadacidin	36.3 \pm 28.9 ^a
150 μ g Mycophenolic Acid	13.2 \pm 6.6 ^a
500 μ g Mycophenolic Acid	5.5 \pm 4.3 ^a

a Values with the same superscripts are not different

Table 4.3. Percent of live luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg azaserine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells, and others. Five ewes per treatment group.

TREATMENT	LLC	LSC	LOC
Control	95.0 \pm 8.1 ^a	93.0 \pm 3.7 ^a	55.0 \pm 3.6 ^a
Azaserine (150 μg)	83.0 \pm 7.3 ^a	83.5 \pm 4.3 ^a	72.0 \pm 4.3 ^a
Azaserine (500 μg)	97.0 \pm 5.5 ^a	87.0 \pm 1.4 ^a	72.0 \pm 5.8 ^a

a Values with the same superscripts are not different

Table 4.4. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg azaserine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	2.0 \pm 0.5 ^a	16.0 \pm 3.0 ^a	82.0 \pm 3.0 ^a
Azaserine (150 μg)	4.0 \pm 1.0 ^a	11.0 \pm 2.0 ^a	85.0 \pm 2.0 ^a
Azaserine (500 μg)	2.0 \pm 1.0 ^a	16.0 \pm 3.0 ^a	81.0 \pm 4.0 ^a

a Values with the same superscripts are not different

Table 4.5. Percent of live luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg hadacidin, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells, and others. Five ewes per treatment group.

TREATMENT	LLC	LSC	LOC
Control	95.0 \pm 8.1 ^a	93.0 \pm 3.7 ^a	55.0 \pm 3.6 ^a
Hadacidin (150 μg)	81.0 \pm 9.1 ^a	86.0 \pm 2.4 ^a	69.0 \pm 3.2 ^a
Hadacidin (500 μg)	94.0 \pm 6.8 ^a	86.0 \pm 2.4 ^a	59.0 \pm 3.2 ^a

a Values with the same superscript are not different

Table 4.6. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg hadacidin, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	2.0 \pm 0.5 ^a	16.0 \pm 3.0 ^c	82.0 \pm 3.0 ^c
Hadacidin (150 μg)	4.0 \pm 0.5 ^b	26.0 \pm 2.0 ^d	70.0 \pm 3.0 ^d
Hadacidin (500 μg)	4.0 \pm 0.5 ^b	18.0 \pm 7.0 ^c	78.0 \pm 3.0 ^c

a, b Values in a column with different superscripts are different ($P \leq 0.05$)
c, d Values in a column with different superscripts are different ($P \leq 0.1$)

Table 4.7. Percent of live luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg mycophenolic acid, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells, and others. Five ewes per treatment group.

TREATMENT	LLC	LSC	LOC
Control	95.0 \pm 8.1 ^a	93.0 \pm 3.7 ^c	55.0 \pm 3.6 ^a
Mycophenolic acid (150 μg)	76.0 \pm 5.3 ^b	83.0 \pm 3.6 ^d	61.0 \pm 4.1 ^a
Mycophenolic acid (500 μg)	73.0 \pm 6.0 ^b	78.0 \pm 2.9 ^d	64.5 \pm 1.9 ^a

a, b Values in a column with different superscripts are different ($P \leq 0.1$)
c, d Values in a column with different superscripts are different ($P \leq 0.05$)

Table 4.8. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with 150 or 500 μg mycophenolic acid, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	2.0 \pm 0.5 ^a	16.0 \pm 3.0 ^a	82.0 \pm 3.0 ^a
Mycophenolic acid (150 μg)	4.0 \pm 1.0 ^b	16.0 \pm 2.0 ^a	80.0 \pm 3.0 ^a
Mycophenolic acid (500 μg)	2.0 \pm 0.5 ^a	16.0 \pm 3.0 ^a	82.0 \pm 3.0 ^a

a, b Values in a column with different superscripts are different ($P \leq 0.1$)

Table 4.9. Mean (\pm SEM) weights of corpora lutea treated *in vivo* with 150 or 500 μ g of azaserine, hadacidin, or mycophenolic acid, or control treatment. Five ewes per treatment group.

TREATMENT	WEIGHT (mg)
Control	380.0 \pm 20.0 ^a
150 μ g Azaserine	446.0 \pm 60.0 ^a
500 μ g Azaserine	276.0 \pm 50.0 ^a
150 μ g Hadacidin	344.0 \pm 30.0 ^a
500 μ g Hadacidin	290.0 \pm 60.0 ^a
150 μ g Mycophenolic Acid	356.0 \pm 40.0 ^a
500 μ g Mycophenolic Acid	400.0 \pm 60.0 ^a

a Values with the same superscript are not different

Table 4.10. Mean (\pm SEM) ovarian venous progesterone concentrations obtained at the second laparotomy from ewes treated *in vivo* with azaserine, azaserine + inosine, hadacidin, hadacidin + adenosine, mycophenolic acid, or mycophenolic acid + guanosine, or control treatment. Five ewes per treatment group.

TREATMENT	PROGESTERONE (ng/ml)
Control	312 \pm 151 ^a
Azaserine	1139 \pm 331 ^a
Azaserine + inosine	1147 \pm 92 ^a
Hadacidin	839 \pm 542 ^a
Hadacidin + adenosine	541 \pm 367 ^a
Mycophenolic Acid	1492 \pm 388 ^a
Mycophenolic Acid + guanosine	871 \pm 312 ^a

a Values with the same superscripts are not different

Table 4.11. Mean (\pm SEM) ovarian venous estradiol-17 β concentrations obtained at the second laparotomy from ewes treated *in vivo* with azaserine, azaserine + inosine, hadacidin, hadacidin + adenosine, mycophenolic acid, or mycophenolic acid + guanosine, or control treatment. Five ewes per treatment.

TREATMENT	ESTRADIOL (pg/ml)
Control	3.1 \pm 1.1 ^a
Azaserine	20.3 \pm 16.2 ^a
Azaserine + inosine	5.7 \pm 1.5 ^a
Hadacidin	1.8 \pm 0.4 ^a
Hadacidin + adenosine	16.3 \pm 13.2 ^a
Mycophenolic Acid	18.9 \pm 9.8 ^a
Mycophenolic Acid + guanosine	1.5 \pm 0.2 ^a

a Values with the same superscript are not different

Table 4.12. Mean (\pm SEM) weights of corpora lutea treated *in vivo* with 98 azaserine, azaserine + inosine, hadacidin, hadacidin + adenosine, mycophenolic acid, or mycophenolic acid + guanosine, or control treatment. Five ewes per treatment group.

TREATMENT	WEIGHT (mg)
Control	336.0 \pm 30.0 ^a
Azaserine	334.0 \pm 50.0 ^a
Azaserine + inosine	340.0 \pm 20.0 ^a
Hadacidin	415.0 \pm 40.0 ^a
Hadacidin + adenosine	304.0 \pm 30.0 ^a
Mycophenolic Acid	388.0 \pm 60.0 ^a
Mycophenolic Acid + guanosine	376.0 \pm 60.0 ^a

a Values with the same superscript are not different

Table 4.13. Percent of live luteal cells after dissociation of corpora lutea treated *in vivo* with azaserine, azaserine + inosine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells and others. Five ewes per treatment group. 99

TREATMENT	LLC	LSC	LOC
Control	91.0 \pm 7.5 ^a	84.0 \pm 0.9 ^a	64.0 \pm 1.8 ^a
Azaserine	87.0 \pm 5.3 ^a	84.0 \pm 3.4 ^a	60.0 \pm 5.2 ^a
Azaserine + inosine	71.0 \pm 2.1 ^a	83.0 \pm 3.3 ^a	65.0 \pm 4.0 ^a

a Values with the same superscripts are not different

Table 4.14. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with azaserine, azaserine + inosine, or control (mean + SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	3.0 ± 1.7 ^a	34.0 ± 2.3 ^a	63.0 ± 2.8 ^a
Azaserine	3.0 ± 1.3 ^a	28.0 ± 3.7 ^a	69.0 ± 4.0 ^a
Azaserine + inosine	3.0 ± 1.0 ^a	24.0 ± 1.6 ^a	73.0 ± 1.9 ^a

a Values with the same superscripts are not different

Table 4.15. Percent of live luteal cells after dissociation of corpora lutea ¹⁰¹ treated in vivo with hadacidin, hadacidin + adenosine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells, and others. Five ewes per treatment group.

TREATMENT	LLC	LSC	LOC
Control	91.0 \pm 7.5 ^a	84.0 \pm 0.9 ^a	64.0 \pm 1.8 ^a
Hadacidin	78.5 \pm 3.4 ^{a,b}	84.0 \pm 1.5 ^a	60.0 \pm 0.8 ^a
Hadacidin + adenosine	62.0 \pm 5.2 ^b	86.0 \pm 1.7 ^a	61.0 \pm 3.6 ^a

a,b Values within a column with different superscripts differ at $P \leq 0.1$

Table 4.16. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with hadacidin, hadacidin + adenosine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	3.0 \pm 1.7 ^a	34.0 \pm 2.3 ^a	63.0 \pm 2.8 ^a
Hadacidin	2.0 \pm 1.1 ^a	28.0 \pm 1.9 ^a	70.0 \pm 1.6 ^a
Hadacidin + adenosine	3.0 \pm 0.9 ^a	30.0 \pm 1.6 ^a	67.0 \pm 1.4 ^a

a Values with the same superscripts are not different

Table 4.17. Percent of live luteal cells after dissociation of corpora lutea treated *in vivo* with mycophenolic acid, mycophenolic acid + guanosine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance. LLC = live large luteal cells, LSC = live small luteal cells, and LOC = live "other" luteal cells. "Other" refers to non-steroidogenic luteal cells, including blood cells, fibrocytes, endothelial cells, and others. Five ewes per treatment group.

TREATMENT	LLC	LSC	LOC
Control	91.0 \pm 7.5 ^a	84.0 \pm 0.9 ^a	64.0 \pm 1.8 ^a
Mycophenolic acid	91.0 \pm 7.1 ^a	88.0 \pm 3.0 ^a	62.5 \pm 3.6 ^a
Mycophenolic acid + guanosine	78.0 \pm 7.7 ^a	88.0 \pm 2.8 ^a	73.0 \pm 5.0 ^a

a Values with the same superscripts are not different

Table 4.18. Percent of total luteal cells after dissociation of corpora lutea treated *in vivo* with mycophenolic acid, mycophenolic acid + guanosine, or control (mean \pm SEM). Values were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. TLC = total percent of large luteal cells, TSC = total percent of small luteal cells, TOC = total percent of "other" luteal cells. "Other" refers to non-steroidogenic luteal cells such as endothelial cells, fibrocytes, blood cells, and others. Five ewes per treatment group.

TREATMENT	TLC	TSC	TOC
Control	3.0 \pm 1.7 ^a	34.0 \pm 2.3 ^a	63.0 \pm 2.8 ^a
Mycophenolic acid	5.0 \pm 2.4 ^a	24.0 \pm 2.9 ^a	71.0 \pm 3.0 ^a
Mycophenolic acid + guanosine	3.0 \pm 1.2 ^a	26.0 \pm 2.1 ^a	71.0 \pm 2.2 ^a

a Values with the same superscripts are not different

Table 4.19. Levels of GMP, IMP, AMP ($\mu\text{g/g}$ tissue), and ratios of GMP-AMP, IMP-AMP, and GMP-IMP in corpora lutea receiving azaserine or control treatments *in vivo* (mean \pm SEM). Five ewes per treatment group.

PARAMETER	CONTROL	AZASERINE
GMP	12.30 \pm 1.90 ^a	10.30 \pm 1.00 ^a
IMP	3.90 \pm 0.80 ^c	2.10 \pm 0.40 ^d
AMP	90.60 \pm 14.10 ^a	83.00 \pm 8.30 ^a
GMP-AMP	0.14 \pm 0.01 ^a	0.12 \pm 0.01 ^b
IMP-AMP	0.05 \pm 0.01 ^a	0.03 \pm 0.01 ^a
GMP-IMP	4.30 \pm 1.40 ^a	5.50 \pm 1.10 ^a

a,b values within row with different superscripts differ at $P \leq 0.1$.
c,d values within row with different superscripts differ at $P \leq 0.17$.

Table 4.20. Levels of GMP, IMP, AMP ($\mu\text{g/g}$ tissue), and ratios of GMP-AMP, IMP-AMP, and GMP-IMP in corpora lutea receiving hadacidin or control treatments *in vivo* (mean \pm SEM). Five ewes per treatment group. 106

PARAMETER	CONTROL	HADACIDIN
GMP	12.30 \pm 1.90 ^a	9.10 \pm 2.50 ^a
IMP	3.90 \pm 0.80 ^a	3.00 \pm 0.80 ^a
AMP	90.60 \pm 14.10 ^a	64.30 \pm 15.10 ^b
GMP-AMP	0.14 \pm 0.01 ^a	0.14 \pm 0.01 ^a
IMP-AMP	0.05 \pm 0.01 ^a	0.05 \pm 0.01 ^a
GMP-IMP	4.30 \pm 1.40 ^a	3.60 \pm 0.90 ^a

a,b values within row with different superscripts differ at $P \leq 0.01$.

Table 4.21. Levels of GMP, IMP, AMP ($\mu\text{g/g}$ tissue), and ratios of GMP-AMP, IMP-AMP, and GMP-IMP in corpora lutea receiving mycophenolic acid or control treatments *in vivo* (mean \pm SEM). Five ewes per treatment group. ¹⁰⁷

PARAMETER	CONTROL	MYCOPHENOLIC ACID
GMP	12.30 \pm 1.90 ^a	9.20 \pm 1.20 ^a
IMP	3.90 \pm 0.80 ^a	2.80 \pm 0.70 ^a
AMP	90.60 \pm 14.10 ^a	74.30 \pm 5.20 ^a
GMP-AMP	0.14 \pm 0.01 ^a	0.12 \pm 0.01 ^b
IMP-AMP	0.05 \pm 0.01 ^a	0.04 \pm 0.01 ^a
GMP-IMP	4.30 \pm 1.40 ^a	4.10 \pm 0.90 ^a

a,b values within row with different superscripts differ at $P \leq 0.1$.

CHAPTER 5

EXPERIMENTS 4 AND 5: DETERMINATION OF DOSE RESPONSES OF
MATURE LUTEAL TISSUE TO AZASERINE, HADACIDIN, AND
MYCOPHENOLIC ACID IN VITRO, AND ACUTE RESPONSE OF MATURE
OVINE CORPORA LUTEA TO AZASERINE AND PURINE BIOSYNTHESIS
PATHWAY REPLACEMENT COMPOUNDS IN VITRO

5.A MATERIALS AND METHODS

CL were collected on day 9 post-estrus from cycling non-pregnant ewes laparotomized under sodium pentobarbital anaesthesia. Immediately following removal, CL were weighed, quartered with a razor blade, and then the quarters were weighed individually (wet weight). Prior to incubation with the drug treatments, the quarters were cut into four pieces each to increase surface area, and each total quarter was pre-incubated in Minimal Essential Medium (MEM) (Gibco Ca⁺⁺ free medium #320-2561AG) for 30 minutes in a shaking water bath at 37° Celsius. Pre-incubation was done to enable the CL slices to clear progesterone previously stimulated to be released, prior to the application of treatments.

Following pre-incubation the CL quarters were divided, one quarter per each of 4 sterile culture tubes containing PBS and one of the treatment regimens. The treatments for experiment 4 included (total volume per tube = 3 ml):

1. control (PBS), AZA (1 μ M, 10 μ M, 100 μ M)
2. control, AZA (100 μ M, 1,000 μ M, 10,000 μ M)
3. control, HAD (1 μ M, 10 μ M, 100 μ M)
4. control, HAD (100 μ M, 1,000 μ M, 10,000 μ M)
5. control, MA (1 μ M, 10 μ M, 100 μ M)

6. control, MA (100 μ M, 1,000 μ M, 10,000 μ M)

Experiment 4 was done to determine a dose response curve for each of the 3 purine biosynthesis pathway blocking drugs.

For experiment 5, a determination of acute response to azaserine in combination with individual purine biosynthesis pathway replacement compounds, the four quarters were each randomly assigned (5 quarters per treatment group) to one of the following treatments (total volume = 3 ml of PBS vehicle):

1. PBS
2. PBS + 100 ng/ml LH
3. 10,000 μ M azaserine + 100 ng/ml LH
4. 10,000 μ M azaserine + 100 ng/ml LH + 10,000 μ M adenosine
5. 10,000 μ M adenosine + 100 ng/ml LH
6. 10,000 μ M azaserine + 100 ng/ml LH + 10,000 μ M guanosine
7. 10,000 μ M guanosine + 100 ng/ml LH
8. 10,000 μ M azaserine + 100 ng/ml LH + 10,000 μ M inosine
9. 10,000 μ M inosine + 100 ng/ml LH

For both experiments, incubations were carried out for 4 hours under the same conditions as the pre-incubation. In order to determine an appropriate incubation time, a pilot LH time course study was done (see appendix E). All culture tubes contained ovine luteinizing hormone (LH) (LER-1374A) in a concentration of 100 ng/ml along with the treatments dissolved in PBS. No pH corrections were made to the 0.1 M PBS (pH = 7.0) once the treatments were added. At the end of the

incubation period, the medium was collected and frozen at -20° Celsius until analysis for progesterone concentration by radioimmunoassay (see appendix A). For luteal slices treated with doses of $100 \mu\text{M}$ or higher, samples of the slice were dissociated and the cells were stained with trypan blue to determine the percent of live cells (see appendix B).

Progesterone concentrations in incubation media obtained by radioimmunoassay were corrected for tissue weight differences, and then subjected to a simple analysis of variance using a general linear model. Comparisons of percent change from control were made for progesterone concentrations from experiment 4, and these data were transformed (arc sine) prior to a simple analysis of variance (general linear model) in order to conform to rules of homogeneity of variance.

5.B RESULTS

In experiment 4, progesterone levels in the incubation medium were not different from controls for azaserine- ($P \leq 0.9$), hadacidin- ($P \leq 0.5$), or mycophenolic acid-treated slices ($P \leq 0.6$) at any of the doses (Table 5.1). When percent change from control was compared, however, the $10,000 \mu\text{M}$ dose of azaserine was the only dose of any of the drugs to show significant change ($P \leq 0.05$) (Table 5.2, Figure 25). Live/dead staining of corpora lutea treated with drug doses of $100 \mu\text{M}$ or greater showed no differences in the percent of live cells present ($P \leq 0.7$, 0.8 , and 0.3 for mycophenolic acid, hadacidin, and azaserine, respectively).

In experiment 5, corpus luteum slices treated with adenosine + LH and adenosine + azaserine + LH, secreted significantly higher concentrations of progesterone than control PBS + LH slices ($P \leq 0.01$) (Table 5.3). Corpus luteum slices incubated in inosine + LH, azaserine

+ inosine + LH, azaserine + guanosine + LH, and guanosine + LH, did not have progesterone levels that were different from either control or PBS-treated slices.

5.C DISCUSSION

Since the 10,000 μ M dose of azaserine appeared to be the most effective in causing changes in progesterone secretion from that of the control-treated luteal slices, it was decided that this dose would be used for treatment in the subsequent in vitro experiment (experiment 5). Azaserine blocks de novo synthesis of all three purine products, so individual pathway importance would be able to be determined by the administration of the drug, plus one of the pathway replacement compounds (IMP, AMP, or GMP). It should be noted that the pH of treatments was not adjusted after addition of the drugs to the PBS incubation medium, and this may have had some influence upon the steroidogenic capabilities of the luteal tissue.

Consistent with studies done using human and rat luteal tissue (Behrman et al., 1983; Hall et al., 1981), luteal slices that were incubated in medium containing both adenosine and LH produced the highest quantities of progesterone. Slices treated with inosine or azaserine alone produced somewhat lower levels of progesterone, followed by guanosine-, control- (PBS + LH), and PBS-treated slices having the lowest progesterone concentrations. These results concur with those of Behrman et al. (1983), who note that adenine nucleotides and adenosine caused the largest production of LH-stimulated progesterone production by cultured luteal cells, followed by inosine, adenine, and hypoxanthine, showing decreasing activity. Guanosine,

guanine, xanthine, and pyrimidines were inactive in promoting progesterone production.

Adenosine is proposed to mediate its effects on progesterone secretion by amplification of intracellular cAMP accumulation, which is shown to occur in rat luteal tissue (Hall et al., 1981). Brennan et al. (1983) have shown that purine amplification of LH-stimulated cAMP accumulation is due to an increase in intracellular ATP levels. This increased ATP may be a result of the conversion of purines after cellular uptake, which increases ATP availability for adenylate cyclase or other cAMP enhancing functions (Brennan et al., 1983).

Studies by Hall et al. (1981) have shown that the effects of the purines are mediated via an intracellular mechanism, and not interaction with LH receptor binding or the cellular membrane transport systems. They suggest that once adenosine is within the luteal cell, it may rapidly be converted to inosine, which is consistent with the fact that progesterone levels from inosine-treated luteal slices were not significantly different from progesterone levels produced by adenosine treated slices in this experiment.

The effects of azaserine, in combination with the replacement compounds were not different from the effects of the replacement compounds alone. This would seem to indicate that the de novo purine synthesis pathway is of less importance to progesterone production by the luteal slices in vitro, than is the salvage pathway which utilizes preformed compounds. This is also borne out by the fact that azaserine + LH treated slices, although not significantly different from PBS + LH treated slices, produce the third highest progesterone concentrations among all of the treatments.

Table 5.1. *In vitro* progesterone production (ng/g of tissue) by luteal slices in response to increasing doses of azaserine, hadacidin, or mycophenolic acid (mean \pm SEM). N = number of ewes per treatment group.

DOSES (μ M)	AZASERINE	HADACIDIN	MYCOPHENOLIC ACID
0 (n=10)	638.8 \pm 58.1 ^a	934.9 \pm 135.5 ^a	1010.6 \pm 82.5 ^a
1 (n=5)	665.5 \pm 119.6 ^a	1417.3 \pm 209.1 ^a	1283.7 \pm 318.0 ^a
10 (n=5)	670.9 \pm 126.5 ^a	1270.9 \pm 186.7 ^a	1238.0 \pm 52.1 ^a
100 (n=10)	673.7 \pm 93.4 ^a	1183.3 \pm 254.8 ^a	898.9 \pm 101.6 ^a
1,000 (n=5)	584.6 \pm 66.1 ^a	900.2 \pm 140.2 ^a	969.6 \pm 95.2 ^a
10,000 (n=5)	758.7 \pm 48.0 ^a	891.8 \pm 136.5 ^a	911.3 \pm 184.6 ^a

a Values with the same superscript are not different

Table 5.2. Percent change from control values for progesterone levels produced by luteal slices incubated *in vitro* with azaserine, hadacidin, or mycophenolic acid (mean \pm SEM). Percentages were subjected to arc sine transformation prior to statistical analysis to conform to rules of homogeneity of variance. Values have been converted back to original percentages. N = number of ewes per treatment group.

DOSES (μ M)	AZASERINE	HADACIDIN	MYCOPHENOLIC ACID
0 (n=10)	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a
1 (n=5)	99.0 \pm 16.1 ^a	122.0 \pm 31.0 ^a	101.0 \pm 23.3 ^a
10 (n=5)	97.0 \pm 13.0 ^a	110.0 \pm 28.4 ^a	101.0 \pm 15.4 ^a
100 (n=10)	101.0 \pm 12.0 ^a	106.0 \pm 17.3 ^a	96.0 \pm 7.0 ^a
1,000 (n=5)	102.0 \pm 10.8 ^{a,b}	118.0 \pm 43.6 ^a	104.0 \pm 4.1 ^a
10,000 (n=5)	135.0 \pm 16.6 ^b	124.0 \pm 48.4 ^a	98.0 \pm 12.7 ^a

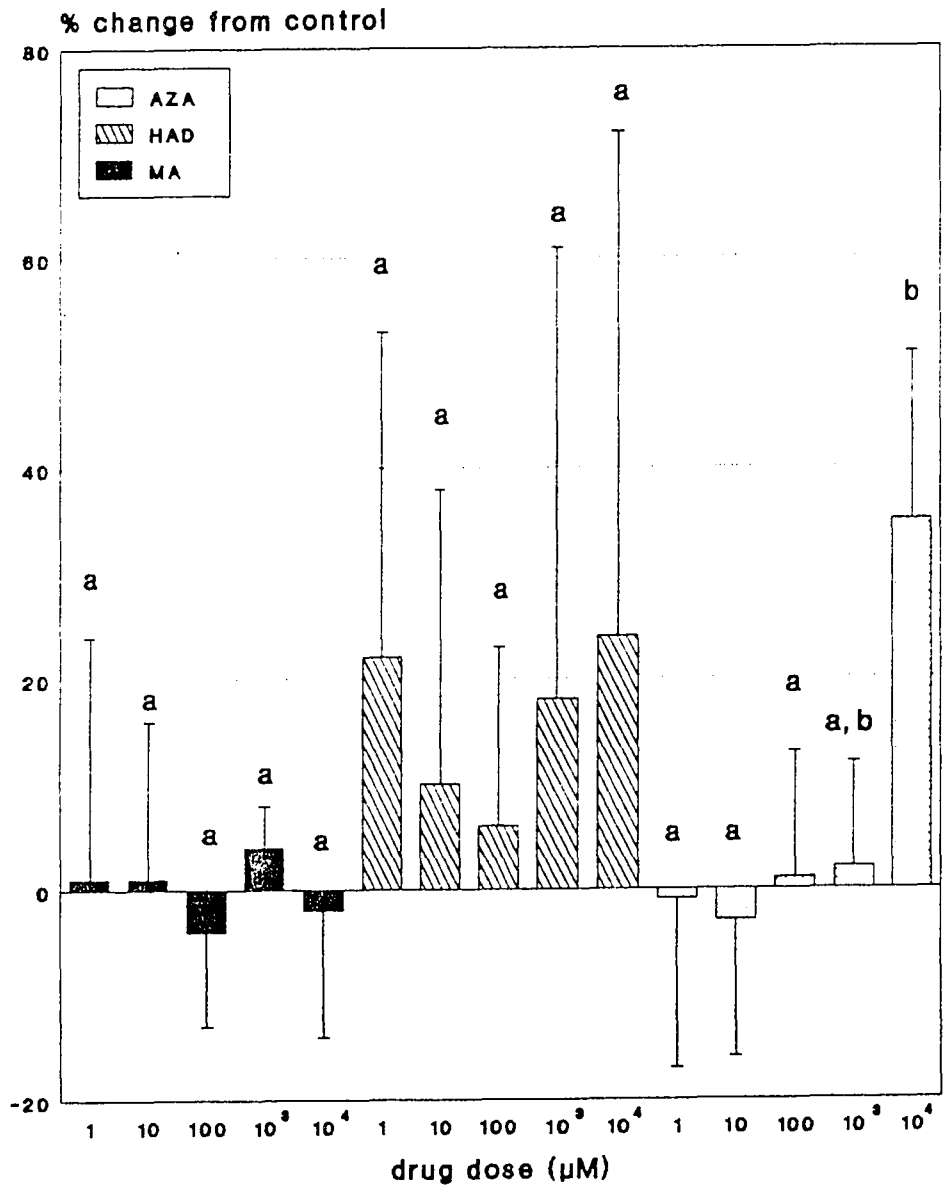
a,b values within columns with different superscripts are different at $P \leq 0.05$.

Table 5.3. Progesterone concentrations (ng/g of tissue) in culture medium after incubation of luteal slices for 4 hours. Concentrations were adjusted for variations in tissue weight among treatments (mean \pm SEM). N=5 for each treatment.

TREATMENT	PROGESTERONE
Control (PBS + LH)	950.4 \pm 68.0 ^{c,d}
PBS	834.9 \pm 131.4 ^{c,d}
Adenosine + LH	1712.3 \pm 107.4 ^a
Inosine + LH	1216.5 \pm 185.6 ^{a,b,c,d}
Guanosine + LH	876.9 \pm 108.5 ^{c,d}
Azaserine + LH	1262.9 \pm 165.8 ^{a,b,c}
Azaserine + adenosine + LH	1567.4 \pm 197.4 ^{a,b}
Azaserine + inosine + LH	1171.0 \pm 184.8 ^{b,c,d}
Azaserine + guanosine + LH	713.8 \pm 164.1 ^d

a,b,c,d values within columns with different superscripts are different at $P \leq 0.01$.

Figure 25. Percent change from control for progesterone concentrations produced in vitro by luteal slices incubated in varying doses of azaserine, hadacidin, or mycophenolic acid (mean \pm SEM). Percentages were subjected to arc sine transformation prior to statistical analysis in order to conform to rules of homogeneity of variance (azaserine doses $P \leq 0.05$).



a,b w/in trt. differ at P<0.05

CHAPTER 6

GENERAL DISCUSSION

In general the purine de novo biosynthesis pathway blocking drugs had relatively little effect on progesterone output by the corpus luteum in vivo. The only instances where steroid secretion profiles were altered from those of controls were in experiment 1, where the 150 μg azaserine and 150 μg mycophenolic acid doses resulted in generally elevated profiles of progesterone and estradiol-17 β respectively. In both cases, low doses of each of the drugs were noted to stimulate overcompensatory purine production (Shigeura and Gordon, 1962; Held et al., 1969; Hodges et al., 1989).

The lack of differences among drug treatments and controls, and drug treatments plus replacement compounds and controls may be due to one of several reasons. As suggested in the discussions for experiments 1 and 2, the purine biosynthesis "salvage" pathways may be playing a large role in the lack of response of luteal tissue to the purine de novo biosynthesis pathway blocking drugs. These pathway blocking drugs are very specific to the de novo synthesis pathways, so they have no effect on the ability of the salvage pathways to function. Consequently, the salvage pathways are able to use purinergic products delivered via the corpus luteum's rich vascular supply to override the effects of the de novo synthesis blocking drugs by making a ready supply of purines available to the cells.

Another inference that may be drawn from these data is that the steroidogenic cells of the corpus luteum just do not depend on the purine de novo biosynthetic pathways for their major source of purines. They may rely much more heavily on salvage pathway conversion of the ready

supply of purinergic materials made available by the extensive vasculature or from within the luteal cells.

Such is the case with bone marrow cells. Lajtha and Vane (1958) have shown that bone marrow cells are extremely dependent on the liver for their purine supply (with delivery via the erythrocytes), and make little if any of their own purines. This is also the case for many neoplastic cell types, which prompted Smellie et al. (1956) to suggest that there might exist two types of tissues: those that can make sufficient purine nucleotides to meet their requirements (i.e., liver), and those that depend on a supply of purines formed in other tissues (i.e., bone marrow cells).

Since the corpus luteum is a rapidly proliferating tissue, it may also share the dependence on an external purine source that other rapidly proliferating and highly metabolic cells have. It makes sense that such active cells would import purines from a readily available outside source, since they are already extremely metabolically active due to high levels of mitotic and/or secretory activity.

The possibility also exists that the steroidogenic cells of the corpus luteum may not be as heavily dependent upon purine based messenger systems as originally anticipated. The large luteal cells, while smaller in number than the small luteal cells, produce nine times as much progesterone as the small luteal cells (Hoyer and Niswender, 1985). Large luteal cells utilize a second messenger system of receptor transduction (inositol triphosphate) that is different from that of the small luteal cells (adenylate cyclase system) (Hoyer et al., 1984). Large luteal cells may be producing a much greater amount of progesterone than normally attributed to them.

It has also been suggested that activated LH receptors (found on the small luteal cells) are able to stimulate steroid production without depending solely on cAMP as a second messenger, and that there may be other effectors (Asem et al., 1987). Cooke (1990) attributes this ability to alterations in intracellular calcium levels, and Ca^{++}/Cl^{-} channel effects. Veldhuis and Klase (1982) have shown that calcium ions significantly modulate LH-stimulated progesterone biosynthesis in isolated swine ovarian granulosa cells *in vitro*, and Sullivan and Cooke (1985) found that calmodulin inhibitors blocked LH- and LHRH-agonist stimulated steroidogenesis in rat Leydig cells. Asem et al. (1987) also found rapid intracellular calcium mobilization in chicken granulosa cells in response to LH, prompting the conclusion that rapid intracellular calcium mobilization may represent one of the initial steps in the mechanism of action of LH.

The stimulatory effects of increased intracellular calcium on steroidogenesis, without any associated rise in cAMP, are consistent with earlier findings by Cooke et al. (1976). They suggested that cAMP was not involved in normal LH-stimulated steroidogenesis in Leydig cells since more LH was needed to cause an increase in intracellular cAMP than was necessary to sustain normal testosterone production. Levels of intracellular cAMP do not begin to rise until LH levels are high and levels of steroidogenesis are greater than 50 percent of maximum output, indicating that cAMP may play more of a sensitizing role in steroidogenesis.

Lipoxygenase pathway products, in addition to calcium, may play more of a direct role in the stimulation of steroidogenesis. Dix et al. (1984) found that inhibition of the lipoxygenase pathway, but not the

cyclo-oxygenase pathway, caused an inhibition of both LH- and dibutyryl cAMP-stimulated steroidogenesis, indicating a stimulatory role for lipoxygenase pathway products of arachidonic acid metabolism in steroidogenesis. Consequently, the ability of the corpus luteum to produce normal levels of progesterone in the face of a challenge by purine biosynthesis pathway blockers may be due to the cells' ability to utilize an alternative steroidogenic stimulating pathway outside of the cAMP dependent pathway to which we normally attribute stimulation of steroid synthesis.

This hypothesis can also be supported by the in vitro data obtained in experiment 5, where luteal slices incubated with azaserine + LH produced the third highest levels of progesterone, behind slices treated with adenosine + LH, and azaserine + adenosine + LH. While adenosine is known to increase progesterone production in cultured luteal cells by amplification of LH-stimulated cAMP accumulation (Hall et al., 1981), the fact that luteal slices treated with the purine de novo biosynthesis pathway inhibitor azaserine can produce progesterone levels that are not different from those induced by adenosine indicates that either there is an alternative non-purine stimulated pathway being utilized by the luteal cells, or that the cells have sufficient cellular pools of purinergic compounds and salvage pathway enzymes to overcome the acute challenge.

Azaserine + LH-treated luteal slices did not differ significantly from PBS + LH-treated luteal slices in progesterone output, although the azaserine + LH-treated luteal slices had a higher mean level of progesterone secretion. This implies that azaserine treatment was stimulatory to progesterone production, which indicated that this drug

was able to create a purine deficit leading to salvage pathway compensation, or that it could be a "stressor" that causes the cells to switch to an alternative non-purine mode of second messenger activation such as calcium, phosphatidylinositide, or protein kinase C (Wiltbank et al., 1991).

There are a number of ways to test the hypotheses suggested in this discussion. In order to test the importance of salvage pathway purines in luteal function, it would be necessary to use a salvage pathway blocking drug such as 6-mercaptopurine (6-MP) (Tomisek et al., 1965) in combination with azaserine to control both the de novo and salvage purine biosynthesis pathways (Kimball et al., 1966). Intrapedicule administration of 6-MP would prevent the luteal cells from being able to convert purine building blocks provided via the bloodstream into other purine products.

Another possibility would be to introduce a radiolabelled hypoxanthine or xanthine into the blood, and note uptake of radioactive products into the CL, and in which purinergic compounds the radioactive products are utilized. This could be done in 6-MP/azaserine-treated animals to determine the effectiveness of the drugs for blocking purine salvage product utilization.

The involvement of calcium as an alternative stimulus for steroidogenesis could be addressed by utilizing calmidazolium (a calmodulin inhibitor) either in vivo or in vitro. Calmidazolium increases intracellular calcium levels by inhibiting the calmodulin-dependent calcium/magnesium ATPase which pumps calcium out of the cell. In vitro experiments could also be done, comparing progesterone production by luteal cells in medium with and without calcium.

APPENDIX A: RADIOIMMUNOASSAYS FOR PROGESTERONE AND
ESTRADIOL 17- β

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The procedure for the progesterone assay has been validated in our laboratory and described by Weems et al. (1991). Contents of all assay reagents are listed after the general procedure.

Progesterone Assay

Duplicate 200 μ l aliquots of plasma samples (samples from ovarian vein blood and incubation media were diluted 1:100 with buffer), along with duplicate 200 μ l quantities of pooled plasma and pool plasma fortified with 100 μ l of 1 ng/ml progesterone standard were extracted twice using 3 ml of petroleum ether (Fisher Scientific Products, Fair Lawn, NJ) each time. Tubes were placed in a slurry of dry ice and methanol to freeze the aqueous layer, and the ether was decanted into a second assay tube. The assay tubes were dried down in a 37° Celsius water bath using purified nitrogen gas (Big Three Co., Honolulu, HI). Following the extraction procedure, 2 standard curves were pipetted out (duplicates were done for each quantity), with one placed at the front, and one at the back of the assay. The methanol based progesterone standards for the curves amounted to 10, 20, 50, 100, 200, and 400 pg per assay tube (done in duplicate). The standard curves were dried down in a 37° Celsius water bath under purified nitrogen gas.

After the standard curves were dried down, all of the assay tubes received 100 μ l of Illinois Assay Buffer. The total and non-specific binding tubes received an additional 100 μ l of buffer in lieu of 100 μ l of progesterone anti-serum, which was added to all of the other tubes following the addition of the buffer. The tubes were all vortexed to mix the contents, and then either placed in a 37° Celsius water bath for 5

minutes followed by a 45 minute incubation in a 4° Celsius refrigerator, or incubated in a 4° Celsius refrigerator overnight.

After incubation, all of the remaining steps were done at 4° Celsius. Dextran-coated charcoal (0.5 ml/tube) was added to all but the total tubes to remove excess tritiated progesterone. The total tubes received 0.5 ml of double distilled water. Following charcoaling, all of the tubes were vortexed and incubated for 15 minutes prior to a 10 minute centrifugation (Sorvall RC5C centrifuge, Newtown, CT) at 3000 rpm. The resulting supernatant fluid was decanted into omnivials (Wheaton Scientific, Millville, NJ) containing 2.5 ml of scintillation cocktail at room temperature, and each vial was counted in a liquid scintillation counter (Packard Tri-Carb 4550, Downers Grove, IL) for 2 minutes.

Coefficients of intra- and interassay variation were 5% (n = 10) and 7.1% (n = 12) respectively. Assay sensitivity was 0.05 ng/ml, and average binding was 40.8% (n = 12). Progesterone concentrations were adjusted for procedural losses, which were estimated by recovery of radiolabelled progesterone at 89.1%. Parallelism was determined by measuring quantities of progesterone in different volumes of pooled plasma, and comparing them to standard curve values.

Antibody crossreactivity with 18-hydroxyprogesterone and 17 α -hydroxyprogesterone was 11% and 2.1%, while crossreactivity with the following was less than 2%: allopregnenolone, androstenedione, androstenediol, androstanediol, androsten-3 β ,17 β -diol, 5 α -androstan-3 β ,17 β -diol, cholesterol, corticosterone, dehydroepiandrosterone, 6-dehydrotestosterone, dihydrotestosterone, 17 α -estradiol, 17 β -estradiol,

estriol, 6-keto-17 β -estradiol, 11 α -hydroxyprogesterone, 19-nortestosterone, 4-pregnen-20 α -ol-3-one, and testosterone.

Progesterone Assay Reagents

Illinois Assay Buffer

Two stock solutions, A and B, are made in 2 liter volumetric flasks, using 2X distilled water as a solvent:

Solution A

5.36 g Sodium Phosphate dibasic

16.36 g NaCl

Solution B

2.76 g Sodium Phosphate monobasic

16.36 g NaCl

Combine all of the dibasic solution with about 1400-1450 ml of the monobasic solution until a pH of 7.0. Add enough Knox gelatin to make a 0.1% wt/vol solution, and warm to dissolve. Cool, and add sufficient thimerosal (Sigma Chemicals, St. Louis, MO) to make a 0.01% wt/vol solution. Store in refrigerator.

Progesterone Antiserum (1:2000 dilution)

Progesterone antibody supplied by Dr. R. L. Butcher, Dept. of Obstetrics and Gynecology, West Virginia University.

In a 200 ml volumetric flask, combine 100 μ l of Anti-progesterone ("EWE #123 10-21-74") and enough Illinois Assay Buffer to bring to volume.

Mix well and store in refrigerator.

³H-Progesterone (0.01 μ Ci/100 μ l)

In a 200 ml volumetric flask, add 25 μ l of ³H-progesterone (1,2,6,7-³H(n)) (#NET-381, New England Nuclear, Boston, MA), and fill to volume with Illinois Assay Buffer. Mix well. Final binding should be 35-40%.

Dextran-Charcoal Suspension

Combine 2.5 g Fisher Carbon Decolorizing Alkaline Norit-A (Fair Lawn, NJ) and 0.25 g Kodak low fraction dextran (Rochester, NY) in a 1 liter flask, and add 1 liter of 2X distilled water. Mix well using a magnetic stir bar, and store refrigerated.

Progesterone Standards

Two concentrations of progesterone standards are required:

1 ng/ml

Combine 200 μ l of a 1 μ g/ml stock solution of progesterone (Sigma Chemicals, #P0130, St. Louis, MO) with enough methanol (Fisher Scientific, Fair Lawn, NJ) to bring to a final volume of 200 ml.

100 pg/ml

Into a 100 ml volumetric flask, pipette 10 ml of 1 ng/ml progesterone standard, and fill to volume with methanol. Both standard solutions should be refrigerated.

Assay for Estradiol-17 β

Plasma samples (0.5 ml) in duplicate (samples for the ovarian vein blood were diluted 1:100 with buffer), along with the same amount of pooled plasma and fortified pool plasma (pooled plasma + 500 μ l of 5 pg/ml estradiol-17 β standard), were vortexed for two separate 1 minute periods after the addition of 4 ml of fresh (opened less than 48 hours earlier) anhydrous diethyl ether (Mallinckrodt #UN1155, Paris, KY). The tubes were placed in a slurry of methanol and dry ice to freeze the aqueous layer, and the ether layer was decanted into separate assay tubes. Four ml of diethyl ether was added to the assay tubes assigned to receive the standard curve, and then all of the tubes were dried down

in a 37° Celsius water bath under purified nitrogen gas (Big Three Co., Honolulu, HI).

The standard curve, consisting of 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 pg per tube done in duplicate, was subsequently dried down under the same conditions as the ether. All assay tubes then received 100 μ l of 1% Bovine Serum Albumen (A-2153 fraction 5, Sigma Chemicals, St. Louis, MO), followed by 100 μ l of estradiol-17B antibody with the exception of the total and non-specific binding tubes, which received 100 μ l of serum assay buffer in lieu of antibody. The tubes were vortexed, placed in a 37° Celsius water bath for 5 minutes, and then incubated in a 4° Celsius refrigerator for 1 hour.

Following the 1 hour incubation, 100 μ l of 125 I-Estradiol-17 β (#2258, ICN Biomedicals, Costa Mesa, CA) (0.01 uCi /100 μ l) was added to each tube, which was subsequently vortexed and incubated at 4° Celsius for a minimum of 15 hours. At that point, 0.8 ml of a dextran coated charcoal mixture was added to all tubes except total tubes, which received 0.8 ml of 2X distilled water. Tubes were vortexed, incubated for 10 minutes at 4° Celsius, and then centrifuged (Sorvall RC5C, Newtown, CT) at 3,000 rpm for 10 minutes. The resulting supernatant was decanted into omnivials, which were counted in a gamma counter (Auto-Gamma 5000 series, Packard Inst., Downers Grove, IL).

The sensitivity of this assay was determined to be 1 pg/ml. Intra- and interassay coefficients of variation were 1.6% (n = 6) and 5.2% (n = 17) respectively. Average total percent binding was 68.3% (n = 17).

Reagents for Estradiol-17 β Assay

Serum Assay Buffer

Combine 9 g NaCl and 4 ml of 2.5 M Sodium Phosphate stock solution in a 1 liter volumetric flask, and fill to volume with 2X distilled water. Add 1 g Knox gelatin, and stir on hot plate until gelatin is dissolved. Cool, and add 0.1 g thimerosal (Sigma Chemicals, St. Louis, MO), mix well, and adjust the pH to 7.0-7.2. Store in refrigerator.

Estradiol-17 β Antibody (1:100,000 dilution)

Anti-estradiol-17 β -3-hemisuccinyl-human serum albumin (EWE 12 RLB, 1-17-72) was provided by Dr. R. L. Butcher, Dept. of Obstetrics and Gynecology, West Virginia University. In a 250 ml flask, add 20 ml of 1:10,000 anti-estradiol stock and 180 ml of serum assay buffer. Mix well and refrigerate.

¹²⁵I-Estradiol (0.01 μ Ci/100 μ l)

¹²⁵I-estradiol (#2285, ICN Biomedicals, Costa Mesa, CA) was diluted in serum assay buffer to achieve a final concentration of 0.01 μ Ci/100 μ l, and stored under refrigeration. Binding should be about 60%.

Dextran-Charcoal Suspension

In a 500 ml flask, combine 1.25 g Carbon decolorizing alkaline norit-a (Fisher Scientific, Fair Lawn, NJ) and 5 g of dextran (Kodak, Rochester, NY) with 500 ml 2X distilled water. Mix with a magnetic stir bar, and store in the refrigerator.

Estradiol Standards

Two standard solutions are necessary, and can be made by diluting a stock solution of 1 μ g/ml estradiol (Steraloids, Inc., Wilton,

NH). Dilute 50 μ l of the 1 μ g/ml stock with methanol in a 50 ml volumetric flask. This forms a 1 ng/ml concentration. To make a 50 pg/ml dilution, pipette 5 ml of 1 ng/ml stock into a 100 ml volumetric flask, and fill to volume with methanol. To make the second assay dilution, pipette 10 ml of the 50 pg/ml dilution into a 100 ml volumetric flask, and add methanol to volume. These dilutions should be stored in the refrigerator.

At least 1 hour before luteal dissociation, the enzyme solution that will be used to dissociate the CL must be made up and allowed to come to room temperature. To make the enzyme solution, combine 2000 IU of collagenase (#4196 Worthington Chemicals, Freehold, NJ), 3000 IU of DNase (#2007, Worthington Chemicals, Freehold, NJ), and 5 ml of Minimal Essential Medium (MEM) (#320-2561AG, Gibco, Grand Island, NY) per 1 g of tissue in a small flask. Once the CL has been cleaned of extraneous tissue and is ready for processing, mince it with a straight razor blade, then add the tissue to the appropriate amount of enzyme solution in relation to the amount of tissue. Incubate the tissue in a 37° Celsius shaking water bath (Lab-Line Instruments, Melrose Park, IL) for 1 hour.

After incubation, transfer the entire mixture to a 15 ml round bottom polypropylene centrifuge tube, and dilute it with 1 ml MEM + 0.1% Bovine Serum Albumen (#A2153, Sigma Chemicals, St. Louis, MO) (BSA). Agitate tissue by repeatedly drawing the tissue up into a pasteur pipette which has had the tip broken off. Centrifuge (IEC Centra-4B, Needham Heights, MA) at 100 x g for 5 minutes. Pipette off the excess media and resuspend the tissue pellet in a warm solution of 9 ml MEM + 0.1% BSA and 0.1 ml of a 100 mM EDTA solution (#E5134, Sigma Chemicals, St. Louis, MO) (stock made in distilled water). Centrifuge at 100 x g for 5 minutes.

Resuspend the tissue pellet in several ml (a known amount, so concentrations can be determined if necessary) of warm MEM. Use a red blood cell diluting pipette to draw up an aliquot of the dissociated cell mixture. Load the mixture onto a hemacytometer (#1483, Cambridge

Instruments, Buffalo, NY) and count the cells which appear on the central grid. An aliquot can be pre-stained with a 1% trypan blue (Kodak, Rochester, NY) in 0.9% saline solution to determine live/dead cell ratios. This procedure is adapted from Brennan et al.(1983).

This procedure, which preferentially stains DNA, is based on the protocols of Humason (1979) and Chayen, Bitensky, and Butcher (1973).

Tissue, previously fixed in 10% buffered formalin, was dehydrated over a period of 20 hours using an Autotechnicon (model 2A, The Technicon Co., Chauncey, NY) containing the following solutions:

1. distilled water pre-soak
2. 60% alcohol (2 hours)
3. 80% alcohol (2 hours)
4. 95% alcohol (2 hours)
5. 95% alcohol (1 hour)
6. 100% alcohol (1 hour)
6. 100% alcohol (2 hours)
8. 100% alcohol (2 hours)
9. xylol (1 hour)
10. xylene (1 hour)
11. xylene (1 hour)
12. paraffin (2 hours)
13. paraffin (3 hours)

Tissue was embedded in paraffin blocks directly from the Autotechnicon. Blocks were sectioned at 8 microns, and the sections were floated from a warm water bath onto subbed slides and dried for 24 hours on a slide warmer. Slides were placed in staining racks and processed as follows:

1. xylene (5 min.)
2. xylene (3 min.)
3. 100% alcohol (3 min.)
4. 100% alcohol (3 min.)

5. 95% alcohol (3 min.)
6. 70% alcohol (3 min.)
7. distilled water (2 min.)
8. distilled water (2 min.)
9. 3.5 N HCl (37°C) (30 min.)
10. distilled water (30 sec.)
11. distilled water (30 sec.)
12. fortified Schiff's (1 hour)
13. bleach solution (4 min.)
14. bleach solution (4 min.)
15. bleach solution (4 min.)
16. bleach solution (4 min.)
17. tap water (30 sec.)
18. distilled water (30 sec.)
19. 70% alcohol (30 sec.)
20. 95% alcohol (30 sec.)
21. 100% alcohol (2 min.)
22. xylene (5 min.)
23. xylene (5 min.)
24. mount coverslips using Permount (6:1 xylene)

Slides were allowed to dry at least 24 hours before placement into slide boxes. Slides were observed using a light microscope to differentiate between "average" and "dark" staining cells. The second section on each of 3 slides of serial sections was used consistently, and ovarian arterial endothelial cells were identified. One hundred endothelial cells were counted in a clockwise direction from a random starting point. These cells were classified as "normal" or "dark" staining, and

stainability was such that three separate individuals were able to independently duplicate sample cell counts to within ± 1 cell of each other.

Reagents required for the Feulgen procedure:

1.0 N HCl solution

3.5 N HCl solution

10% Sodium metabisulfite solution

Schiff's solution

7.5 g basic fuchsin (pararosaniline hydrochloride)

19.0 g sodium metabisulfite

850 ml distilled water

150 ml 1 N HCl

2 g activated charcoal

Dissolve fuchsin in a foil wrapped flask containing 850 ml boiling distilled water. Cool to 50°C and then add 1 N HCl and sodium metabisulfite. Stopper flask, shake well, and refrigerate for 24 hours.

After the 24 hour refrigeration, add the charcoal and shake for 2 minutes. In a dark room, filter through coarse filter paper into another foil wrapped flask. The solution should be clear and colorless. It is light sensitive, but well wrapped, it can be stored bottled in the refrigerator for several months.

Before use, Schiff's solution must be fortified with 10% sodium metabisulfite in a 4:1 ratio.

Bleach Solution

100 ml 1.0 N HCl

100 ml 10% sodium metabisulfite solution

1080 ml distilled water

Combine all ingredients 10 minutes before use.

Avoid excessive exposure to light. Solution is good for 2-3 hours.

APPENDIX D: INSTALLATION OF OVARIAN VASCULAR PEDICLE CATHETER

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Within 24 hours of the onset of estrus, ewes were anesthetized with sodium pentobarbital. The abdomen and left flank were shaved, scrubbed with Betadine (Purdue-Frederick, Norwalk, CT), and rinsed with water four times. Sterile procedures were followed during the surgical installation of the catheter.

A midventral incision large enough to allow easy passage of a hand was made beginning about 2 cm anterior to the mammary tissue. The uterus was exteriorized, held with saline moistened gauze by a sterile "nurse," and the ovary with the corpus hemorrhagicum (CH) was identified. Care was taken to keep the exteriorized organs moistened with sterile physiological saline. While anchoring the ovarian pedicle sheath with moistened gauze, a blunt rod (1 mm diameter) was used to tease a small hole in the sheath just below the ovary with the CH. The rod was carefully used to clear a path through the anastomosing vasculature for subsequent catheterization.

After removal of the rod, a blunt ended 1.02 mm bore polyvinyl catheter (Cole Parmer, Chicago) that had been flushed with heparinized saline (250,000 IU/liter) was threaded through the opening to a depth of at least 4 cm. The opening surrounding the catheter was closed using 5-0 cardiovascular silk (Ethicon, Sommerville, NJ) and a purse-string stitch. The catheter was anchored to the oviduct at two points, approximately 5 and 7 cm from the opening in the sheath using 3-0 silk.

Following the anchoring of the catheter, the uterus was moistened with physiological saline and carefully replaced into its original position in the abdominal cavity. In order to exteriorize the catheter through the left

flank, the end of the catheter was securely clamped in a large uterine hemostat, with the excess wrapped around the neck of the instrument. Taking care not to catch any organs, the hemostat was inserted into the incision and passed through the abdominal cavity until the point made contact with the body wall of the left flank. Pressure was applied to the hemostat, causing its position to be visible from the exterior. A scalpel was used to make a small incision at this location, allowing the catheter and hemostat to pass through.

A second hemostat was used to grab the end of the catheter from the outside, and the large uterine hemostat was disengaged and carefully removed. The catheter was pulled out through the incision in the body wall, leaving about 20 cm of slack within the abdominal cavity. A purse string stitch using 1-0 nylon suture (S. Jackson Inc., Washington, DC) was done to close the incision around the catheter, which was also secured to the skin approximately 3 cm from the opening. The crushed end of the catheter was trimmed off, and a blunted 18 gauge needle with the cut tip of a 1 cc syringe in place was used to plug the catheter. Patency of the catheter was checked by removing the cut off syringe (the "plug"), injecting 1 cc of saline through the blunted needle, and then replacing the plug. The abdominal wall was sutured with 3-0 nylon suture (S. Jackson Inc., Washington, DC), and the skin was closed using 1-0 nylon suture (S. Jackson Inc., Washington, DC). Betadine was applied to the incisions, and the animal was given 10 cc of a combination antibiotic subcutaneously.

Luteal slices (3/treatment/time), prepared as for experiments 4 and 5, were incubated in either PBS, or 100 ng/ml oLH + PBS (Fitz et al., 1982) for 0 (slice just "dipped" into incubation tube with medium), 1, 2, 4, and 6 hour intervals at 37° Celsius in a shaking water bath. It was found that progesterone concentrations secreted by the luteal slices peaked at 4 hours and then plateaued, and that progesterone profiles over time for slices treated with or without oLH were not different ($P \geq 0.17$). Unexpectedly, luteal slices treated with oLH produced significantly lower progesterone concentrations in general than did luteal slices incubated in PBS alone ($P \leq 0.01$) (figure 26).

To test the viability of the oLH, a study was done comparing human chorionic gonadotropin (hCG) and oLH in a dose comparison experiment. Doses of hCG (0.008, 0.08, and 0.8 IU/3 ml PBS) and oLH (10, 100, and 1,000 ng/ml) were chosen based on doses utilized previously by Bourdage et al. (1984). Luteal slices (4/treatment) were incubated in one of the seven treatments (PBS, or one of the 6 treatments listed above) under the same conditions as in experiments 4 and 5. Progesterone secretion was measured by RIA and luteal slices treated with hCG or oLH were found to produce levels of progesterone that were not different ($P \geq 0.26$) (figure 27). Consequently, it was decided to use the 100 ng/ml dose of oLH in order to be consistent with much of the literature dealing with in vitro incubation with oLH (Fitz et al., 1982), although the activity of the oLH that was used was 2.74 x NIH s-1 standard (may have resulted in some down regulation of receptors).

Figure 26. Progesterone secretion in vitro by luteal slices incubated with and without LH (ng/g tissue).

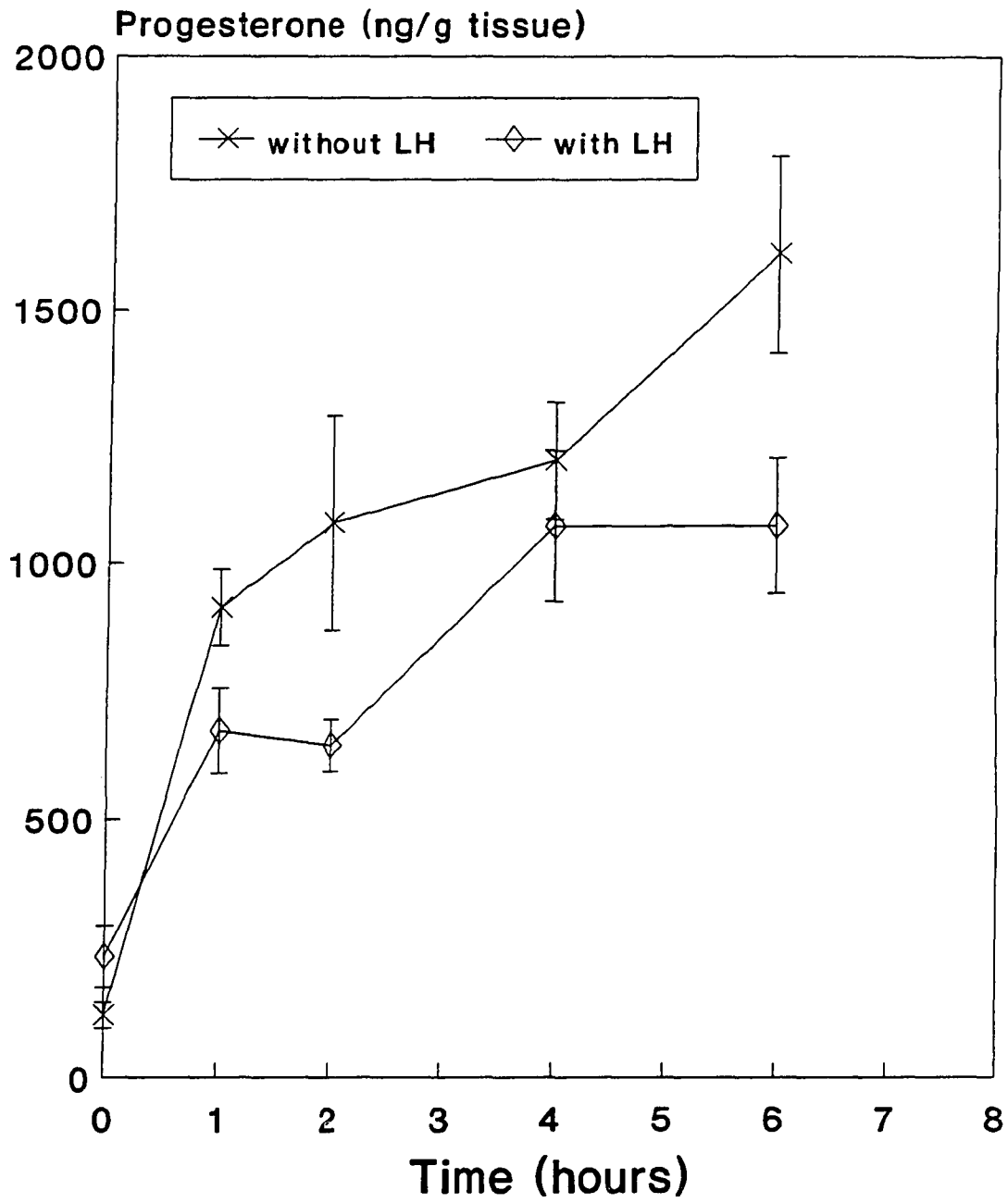
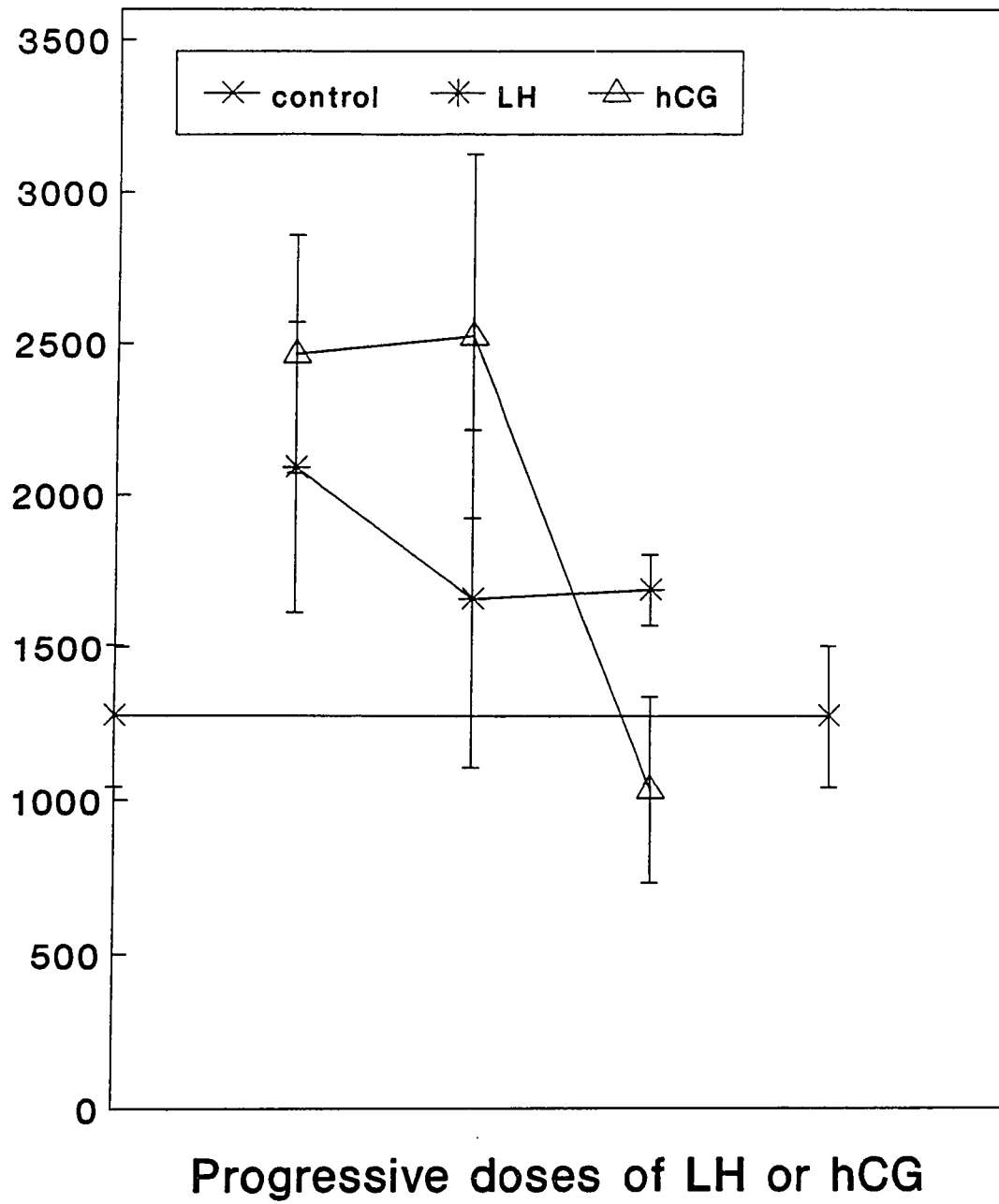


Figure 27. Progesterone secretion in vitro by luteal slices incubated with LH, hCG, or control treatments (ng/g tissue).



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