PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF GONADOTROPIN INDUCED OVULATION IN THE NONHUMAN PRIMATE

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF GONADOTROPIN INDUCED OVULATION IN THE NONHUMAN PRIMATE

By

Jon M.R. Rawson

These studies were conducted using two species of nonhuman primates (Macaca fascicularis and Saimiri sciureus) as models to evaluate the effects of systemic administration of gonadotropins on ovulation induction, to develop a technique which would allow the determination of the effects of local administration of gonadotropins on the ovarian follicle, to assess the ovulation-inducing properties of gonadotropins from primate and nonprimate sources when administered at the follicular level, and to analyze the ability of proposed biochemical intermediates of LH action to induce ovulation when administered at the follicular level.

In the Saimiri sciureus Human Chorionic Gonadotropin (HCG) was able to stimulate ovulation in 70% of females without administration of Follicle Stimulating Hormone (FSH) eight days following a previously synchronized ovulation.

There was a trend towards a second increase in ovulatory response rate around days 14 to 18 following synchronization, which was in agreement with the concept of a 7 to 9 day cycle length for this species.

In 458 menstrual cycles from a colony of 28 female Macaca fascicularis, the average cycle length was 30.9 ± 0.21 days; with an average menstrual flow duration of 2.6 ± 0.1 days. Of the 51 cycles where ovulation could be accurately timed the follicular phase length was 14.7 ± 0.27 days.

Four daily intramuscular injections of 1 mg of FSH were superior to either two or three daily injections for producing normal appearing follicular growth in the squirrel monkey. In the macaque, FSH (5 mg/day for 5 days) induced morphologically normal follicular growth in only 1 of 20 animals with the others exhibiting a high incidence of cystic and hemorrhagic follicles.

Filter paper disks (2 mm diameter) saturated with either FSH or Luteinizing Hormone (LH) had no effect on the occurrence of ovulation in the macaque when administered directly to the follicular surface. Subsequent experiments suggested that the one hour interval, necessitated by anesthetic limitations, was not sufficient to allow an effect.

By using laparoscopy, a technique was developed for injection of substances directly into the follicular antrum. Injection of saline did not result in ovulation, and injection of India ink revealed no observable leakage from

the injection site. The ovulation rate following intrafollicular injection of LH into squirrel monkeys who were not pretreated with FSH, while higher than that of females receiving intrafollicular saline, FSH pretreatment only, or the FSH pretreatment followed by intrafollicular saline, does not compare with the rates of animals receiving an FSH pretreatment designed to supply follicles at a preovulatory stage. Furthermore, there was no difference in ovulation rate among animals pretreated with FSH and then given systemic HCG, intrafollicular HCG or intrafollicular LH.

Intrafollicular injection of HCG in the Macaca fascicularis pretreated with FSH neither induced ovulation nor caused a rise in the peripheral plasma progesterone levels. However, this treatment caused a significant lengthening of the menstrual cycle, with a return to normalcy in the immediate post-treatment cycle.

Intrafollicular injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced ovulation in FSH primed squirrel monkeys at a rate similar to that following LH. Neither PGE_2, cyclic 3',5' AMP, or dibutyryl cyclic 3',5'AMP caused ovulation in a significant number of animals when administered into the ovarian follicle.

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By

Jon M.R. Rawson

A THESIS

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To those less fortunate

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INTRODUCTION

Regulation of mammalian reproduction offers a rich opportunity for the maintenance of the quality of life in our environment. Methods of increasing the fertility of food producing species must be found which have no side effects on the consumer. Similarly, adequate procedures for controlling human fertility must be developed, which are more desirable than the present methods.

Control of ovulation offers one of the best opportunities for this biphasic effect on fertility, in that its occurrence can be either increased or decreased as desired. However, the present methods for ovulation control do not satisfy many of the necessary conditions for widespread usage, since they are too costly or ineffective for the majority of the world's population, and in short supply relative to the overall need. In addition, many produce side effects in some of the users.

Various nonhuman primates have been studied for their applicability as models for the control of human reproduction. As early as 1897, Heape had studied the menstrual

cycle lengths of two species of Macaques and found that their cycles closely resembled that of the human female. Hartman's studies in the 1930's pioneered our knowledge of the macaque menstrual cycle, and contributed much of the background for our present studies. Regardless, the true value of these species as models of human reproductive physiology will only be realized when we have a complete understanding of the complex interactions occurring during their reproductive cycles.

Within the past decade another species of nonhuman primate, the squirrel monkey (Saimiri sciureus) has become popular as a research animal. This species is easier to handle than the larger macaques and can be maintained for a fraction of the cost. These facts, coupled with their availability, have caused the squirrel monkey to become the second most widely used nonhuman primate in biomedical research. Despite this wide usage, literature estimates of the reproductive cycle length of the females of this species vary from 7 to 54 days. Understandably, documentation of their actual cycle length would play a significant role in the definition of the value of this species as a model of human reproductive function.

One of the common methods for studying the physiology of the ovary and its components has involved exogenous treatments with gonadotropins from various sources. Historically, the response of the primate species to such treatments has been less than satisfactory, an observation

attributed to a species specificity for the various hormones (van Wagenen, 1968). Clearly, as the use of gonadotropic hormones is increased both in the scientific and the clinical fields, efforts must be made to assess the normality of induced ovulations. Further, experiments employing systemic gonadotropin treatments fail to reveal the actual effects of these hormones at the level of the ovarian follicle, since plasma protein binding, molecular modification in the circulatory system, the presence of a countercurrent multiplication in the ovarian vasculature or simply an uneven distribution of ovarian blood flow may act to alter the actual effects of these substances on the ovarian follicle.

There has recently been a number of investigators who have implicated prostaglandins or cyclic AMP as mediators in the ovulation-inducing activity of LH. Such studies have usually involved the systemic administration of these compounds or their various inhibitors while subsequently noting the effect upon ovulation. Other studies have demonstrated the effects of LH sources on ovarian levels of these compounds. Results of such studies are not sufficient to conclusively describe the biochemical sequence of events leading to and culminating in rupture of the mature ovarian follicle. Further, few studies have been reported using primate models for the study of follicular physiology. Because of the importance of an understanding of the physiology of the primate ovarian follicle, the

present studies were undertaken with the following objectives:

1. To analyze the responsiveness of the Saimiri sciureus ovary to systemic HCG following a previously synchronized ovulation, in order to further define the reproductive cycle length of this species.
2. To evaluate important cyclic ovarian phenomena occurring in a stable colony of Macaca fascicularis.
3. To examine the ability of exogenous FSH to duplicate the induction of follicular growth and normal morphological development in these species.
4. To develop a technique which would allow the evaluation of the ovulation-inducing effects of substances when administered directly to the ovarian follicle.
5. To determine the efficacy of locally administered gonadotropins for ovulation induction in the squirrel monkey.
6. To assess the ovulation-inducing capabilities of locally administered gonadotropin sources from primate and nonprimate species, as well as the effects of several biochemical intermediates of LH action.

LITERATURE REVIEW

A thorough understanding of the ovulatory process in the human is necessary in order to facilitate the introduction of new biomedical therapies designed for the alleviation of many fertility problems presently encountered. It is evident that the basic research required to provide this understanding can not employ human subjects and, therefore, an attempt must be made to find an acceptable model for this system. The nonhuman primate presents one such possible model. Unfortunately, the available primate literature is not extensive due to the rarity and the value of such species.

In the following discussion of pertinent literature, references to experiments with primates (especially the genera Macaca and Saimiri) are included where possible. Other references have been included which cite work in nonprimate species which provide the foundation for the present studies in the nonhuman primate.

Reproductive Cycle Characteristics

Macaca spp.

The burgeoning use of nonhuman primate species for research purposes has resulted in a great wealth of data regarding the reproductive cycles of the more commonly used species such as the various members of the genus Macaca. In 1973, this author reviewed the literature pertaining to the reproductive cycle characteristics and discussed the normality of the colony of Macaca fascicularis used in the present experiments (Rawson, 1973). Recently, Butler (1974) presented a review of this area which compared various reproductive parameters among all of the Families of the Order Primates.

Saimiri sciureus

One of the earliest reported attempts at the definition of the squirrel monkey reproductive cycle was reported in a technical note by Denniston (1964), who suggested a 25.2 day cycle based on vaginal cornification. He noted the occasional presence of erythrocytes in vaginal smears but was unable to correlate these cycles with an increase in sexual receptivity or attractiveness to the males. In 1967, Lang utilized observations of the external genitalia, presence of a vaginal plug and presence of sperm in vaginal smears as well as vaginal cytology to describe a 12.5 day estrous cycle. Rosenblum, Nathan, Nelson, and Kaufman (1967) detected a cyclic appearance of erythrocytes in the

vaginal smears of 11 of 15 squirrel monkeys and, along with data on the presence of sperm in vaginal smears, defined a seven day estrous cycle. In 1968 Costellanos and McCombs described a cyclic swelling of the external genitalia and a prominent vulvar hyperemia which suggested a cycle length of 12 days. No evidence of menstruation was detected by this study. In a similar study Hutchinson (1970) found no evidence of erythrocytes in the vaginal smears of 26 female squirrel monkeys. Using vaginal cytology, he described a 12.3 day cycle. In 1970, Srivastava, Cavazos, and Lucas found no evidence of cyclic menstrual flow or swelling of the external genitalia. They observed a vulvar plug of desquamated vaginal cells which appeared in a cyclic fashion. This, combined with cytological data, suggested an 18 to 20 day estrous cycle. Another study of this type (Gould et al., 1973) reported an 8 day cycle in the percentage of cornified cells in the vaginal smear.

Studies up to this time had made no attempt at direct ovarian observation. Harrison and Dukelow (1974) reported laparoscopic evidence suggesting a nine to eleven day cycle length. Their procedure involved serial laparoscopies at 2 day intervals beginning 5 days after an artificially induced ovulation. Subsequently, ovulation was diagnosed in 4 of the 6 animals at an interval of between 9 and 11 days.

Ovulation Induction and Follicular Growth

In 1973, van Wagenen and Simpson published a text on the subject of ovulation induction in the macaque which included an extensive review of the early experiments in this area. The consistent result of such early studies using heterologous pituitary gonadotropins was successful follicular growth with only a few reports of ovulation and corpus luteum formation. Administration of anterior pituitary extracts with HCG supplementation was found to cause luteinization of the thecal layers but again, without ovulation (Engle, 1933, 1934; Engle and Hamburger, 1935). Hartman (1938, 1942, 1943) observed marked follicular development in response to equine gonadotropin, partially purified pituitary extracts, or transplantation of heterologous pituitaries but only sporadic ovulations were seen. The majority of these early studies stated that, while equine gonadotropin resulted in follicular development, it rarely caused ovulation, and that HCG given alone had doubtful beneficial effects (Engle and Hamburger, 1935; Hartman, 1938; Sielger and Fein, 1939; and van Wagenen and Cole, 1938).

In 1956, Knobil, Morse and Greep suggested a species specificity of monkey pituitary growth hormone for the repair of the metabolic abnormalities observed in hypophysectomized rhesus monkeys since beef growth hormone failed in this respect. The following year van Wagenen and Simpson (1957) demonstrated the successful induction

of multiple ovulation in the immature and adult rhesus monkey using a follicle stimulating substance prepared from rhesus monkey pituitaries, followed by a mixture of this substance with human chorionic gonadotropin. They suggested that the primate source of the gonadotropin may have accounted for their repeated successful experimental ovulations, and inferred that a species specificity existed in primates for the ovulating hormone.

Knobil, Kostyo and Greep (1959) attempted to induce ovulation in a group of 13 hypophysectomized rhesus monkeys, and found that follicle stimulation was readily accomplished with FSH preparations of non-primate origin and further, that excessive follicle stimulation achieved too rapidly inhibited ovulation in response to subsequent doses of chorionic gonadotropin. Their recommendation was that a gradual stimulation of follicles with FSH followed by large doses of HCG in a time span corresponding to the follicular phase of the menstrual cycle was most likely to result in ovulation.

The successful induction of ovulation in the rhesus monkey using human urinary gonadotropins was reported in 1962 by Simpson and van Wagenen. This was a significant accomplishment as these hormones are a more readily available source of gonadotropic activity than either human or monkey pituitaries. Their regime included five days of human menopausal gonadotropin (HMG) followed by 3 to 4 days of HMG + HCG. This produced from 3 to 6 ovulations per ovary in four adult females.

Dede and Plentl (1966) subjected a group of rhesus monkeys to 8 to 10 days of HMG with the addition of 2000 units of HCG to the HMG for 2 more days. Although they do not state the total number of cycles tested, they did report that ovulation was induced in 31 cycles, with five pregnancies resulting.

Administration of 3 mg/kg clomiphene citrate by gastric intubation, was observed by Valerio and Courtney (1968) to increase the incidence of pregnancy from 15% in a group of 30 infertile control females, to 56% in 27 infertile clomiphene females. This was assumed to have been due to a correspondingly increased ovulation rate.

In 1968, van Wagenen suggested that successful ovulation induction in the nonhuman primate was possible with macaque pituitary gonadotropin, human pituitary gonadotropin, or human menopausal gonadotropin when combined with human chorionic gonadotropin. It was further stated that pituitary preparations from other species (specifically ovine) gave sporadic and inconsistent ovulations.

Results of a study designed to compare the relative advantages of the various methods of ovulation induction (Wan and Balin, 1969) suggested that while 60% of the treated cycles were ovulatory in response to intramuscular HMG (75 IU for 5 to 8 days) followed by HMG (35.5 IU) and HCG (2000 IU for 3 days), 46% of these produced multiple ovulations. In addition they found that 59% of clomiphene

treated cycles were ovulatory and that all clomiphene induced ovulations were single.

Using clomiphene in Macaca fascicularis, Mahoney (1970) found ovarian enlargement with 5 of 14 cycles being ovulatory. Wan and Balin (1971) reported that 94% of their rhesus monkeys treated with clomiphene responded with ovulation in 59% of the treated cycles. The clomiphene cycles were said to be characterized by normal ovarian size and appearance with single-site ovulations in every instance where ovulation occurred. Using an HMG/HCG induction regime, the same authors reported a total of six instances of ovarian hyperstimulation, noted by exaggerated cystic enlargements.

In 1971, Breckwoldt, Bettendorf, and Garcia published a study of the effects of various FSH/LH ratios in amenorrheic or hypophysectomized rhesus monkeys. They concluded that the minimum dose of LH required for full follicular maturation was 35 IU/day for 10 to 12 days in amenorrheic animals and 75 IU/day in hypophysectomized animals. Further, they suggested that ovarian hyperstimulation might be avoided by reducing the dose of FSH during follicular development, and using purified human pituitary LH for the induction of ovulation.

Ovadia, McArthur, Smith, and Bashir-Farahmand (1971) suggested that each animal should be considered individually when inducing ovulation, and by monitoring several clinical signs (total urinary estrogens, cervical mucus ferning, vaginal smear, and sex skin coloration) they developed a

method involving "staircase" increases in HMG until the total urinary estrogens had increased to 6 μg/24 hours following which they withheld treatments for 2 days and gave a single injection of 500 IU of HCG to simulate the LH surge. Using this technique they reported the reliable induction of single ovulations.

A much lower ovulation rate was obtained by Batta and Brackett (1974), who found that only 2 of 6 animals receiving PMS (900 IU over 6 to 8 days) followed by HCG (2000 IU or 8000 IU) responded with ovulation. The addition of 5 mg of prostaglandin E_1 or E_2 on the day following the last HCG injection, however, was sufficient to increase the observed ovulatory response to 100 % (18/18).

The first report of ovulation induction in a New World primate species was published in 1967 by Bennett who used a five day administration of pregnant mares serum gonadotropin (PMSG) followed by 4 days of a combination of PMSG and HCG. Superovulation (5.3 ovulations per ovary) resulted from this regime. In 1970, Dukelow extended the observations of Bennett with a study which compared the follicle stimulating ability of FSH sources in the squirrel monkey. His results indicated that 4 single daily injections of PMSG alone were not sufficient to cause large numbers of follicles to develop. The optimal procedure indicated by this study involved a pretreatment with progesterone for 5 days, to simulate the animal's luteal phase, followed by 4 days of FSH and an injection of 500 IU

of HCG. With this procedure an ovulation rate of 56% was reported, 75% of which were single ovulations. The ovulation latency ranged from 10 to 12 hours following the HCG stimulus.

In 1971, Fajer and Bechini noted a decline in ovarian venous progesterone concentrations in the squirrel monkey, within 5 days of an ovulation induction regime. This suggested that either these induced ovulations were abnormal or that indeed this may relate to an inherently short cycle length.

The observations of Dukelow (1970) were extended in 1973 by Harrison who used the technique of laparoscopy. This method allowed more frequent observation of ovarian condition. Harrison's experiments confirmed the previous reports of an approximate 60% ovulation rate following the use of a Progesterone-FSH-HCG regime for ovulation induction in the squirrel monkey. Further, it was discovered that an identical ratio was found when the progesterone pretreatment was deleted. Ovulation occurred in a range from 6 to 14 hours after the HCG.

In the absence of any primate gonadotropin source, Gould, Cline and Williams (1973) reported induction of ovulation in 12 of 16 S. sciureii approximately 45 hours after the injection of 100 IU of PMSG. Recently laparoscopic techniques were used to define ovulatory morphology of the Saimiri sciureus following the standard FSH-HCG regime for that species (Harrison & Dukelow, 1974). These

authors defined the preovulatory morphological development of the ovarian follicle, and reported that laparoscopy made diagnosis of ovulation in this species a relatively simple task. Spontaneous ovulation was also observed during this study and there were no morphological differences detected between natural and induced ovulations.

Physiology of the Mammalian Ovarian Follicle

The studies reviewed in the preceeding section have dealt with the induction of ovulation in the nonhuman primate through administration of gonadotropic hormones from various sources. The consensus view favors the necessity of gonadotropins having both follicle stimulating and luteinizing activity, the latter of which should be from a primate source. In order to understand the individual roles of FSH and LH sources in the processes leading to oocyte maturation and ovulation, specific histological and physiological studies are required which have not previously been feasible with primates. The following discussion will focus on the current knowledge of the biochemical and physiological events occurring in the mammalian ovarian follicle from the start of its preovulatory growth through actual ovulatory rupture.

Follicle Growth

Recently, Ryle (1972), using *in vitro* culture methods, corroborated what had been an accepted principle of

gonadotropic action -- that FSH was necessary in culture to maintain the growth rate of one and two cell layer follicles. Goldenberg et al. (1973) found that in the hypophysectomized female rat, PMSG or a combination of FSH and HCG were necessary for the optimal uptake of ^{3}H-HCG by the ovarian tissue in vivo. Although FSH or HCG alone was able to increase the ovarian uptake of the labeled HCG, they found that the maximum stimulation occurred only following treatment which resulted in ovulation and formation of corpora lutea. They suggested that the effect of FSH on ^{3}H-HCG uptake was dependent on its role in stimulating the maturation of the follicle as a prelude to ovulation. This concept is supported by Channing and Kammerman (1974) whose studies show a 10 to 1000 fold greater affinity of large follicles to HCG than is exhibited by smaller follicles.

That endogenous FSH is not essential for the induction of ovulation in the preovulatory follicle was shown by Schwartz et al. (1973) when administration of an FSH antiserum failed to inhibit ovulation or estrogen secretion in the female rat. A necessity of FSH for follicular growth, however, was supported by the observation that treatment with this antiserum exerted a negative effect on follicle growth and development in subsequent cycles.

Seleznik et al. (1974) found that one of the roles of FSH in the rat may be to stimulate the maturation of the follicular granulosa cells thus inducing or activating

receptors for LH (HCG). This idea is based on their experiments which illustrated an increased binding of HCG following FSH administration.

Follicular stimulating activity has been shown to be essential to ovulation induction in the hypophysectomized immature female rat by Nuti et al. (1974) who demonstrated that while the administration of 2 μg FSH/day for 4 days had no effect on serum progesterone levels, this priming was essential for an ovulating hormone to be effective.

That FSH alone has little effect on the preovulatory follicle was recently illustrated by Lipner et al. (1974). Their experiments used purified FSH as well as a highly specific LH antiserum in hypophysectomized, immature female rats to insure that the animals were totally devoid of LH influence. Their results indicate that LH-free FSH has minimal intrinsic ovulating ability.

Inferences that the actions of various gonadotropins are similar in the primate species must presently be considered speculative, due largely to the paucity of data in these species. Reviewing the clinical literature relating to human ovarian follicular morphology following spontaneous or pharmacologically induced suppression of pituitary function, Ross (1973) stated that the literature supports the concept that pituitary gonadotropic stimulation is required for advancement of follicular development beyond the primary follicle stage.

Another recent review (Koering, 1974) showed that follicular development appears to be similar in most primate species. This author cites the continuous development and atresia of ovarian follicles in the prepubertal monkey (in the absence of ovulation) as evidence that the gonadotropic stimulus necessary for further maturation is lacking.

Theories of Ovulation

Espey in 1974 reviewed the modern theories of the mechanism of ovulation. He points out that until the early 1960's there existed two general schools of thought, one of which suggested an active rupturing process via an increased intrafollicular pressure. The second theory proposed that ovulation was a passive process brought about by the enzymatic degradation of the follicular wall or possibly by necrotic changes in the follicular tissue.

The presence of smooth muscle fibers in the ovary lead to the hypothesis as early as 1858 (Rouget, 1858) that follicular rupture was caused by vascular congestion due to a restriction of venous return following constriction of these elements. This theory was supported by the study of Guttmacher and Guttmacher (1921) who demonstrated contractions in the wall of sow tertiary follicles that appeared similar to smooth muscle contractions. Aside from direct evidence, one of the most frequent arguments in favor of this concept prior to 1960, was based on the inferences

made following observation of the dynamic nature of the rupture of the follicular wall (Kelly, 1931).

In 1947, Claesson published a study in which he was unable to show the presence of smooth muscle cells in the follicular wall of the cow, pig, rabbit or guinea pig. Further ultrastructural studies have substantiated this lack of smooth muscle in the rabbit ovarian follicle (Espey, 1967). In addition, several of the classical stimulants of smooth muscle activity have been found to have no effect on the activity of sow follicles (Espey, 1964).

Despite evidence to the contrary, there has recently been revival of interest in the role of smooth muscle cells in ovulation. One recent report cites the demonstration of an *in vitro* spontaneous contractile activity of the ovary (Palti and Freund, 1973), while the electron micrographs of another have been reported to show myofibrils in the thecal layer of the ovarian follicles of several mammalian species (O'Shea, 1970; Okamura et al., 1972). In addition, Virutamasen et al. (1971) were able to demonstrate significant effects of several adrenergic drugs on the occurrence of ovulation in the rabbit. These effects were attributed to actions on ovarian smooth muscle contractile activity. The same authors have subsequently demonstrated effects of prostaglandins on both the *in vivo* and the *in vitro* contractile activity of the rabbit ovary (Virutamasen et al., 1972). Regardless, this question is

still being disputed and will be settled only when new evidence is presented.

Increases in intrafollicular pressure have also been postulated to arise from other sources. Zachariae in 1958 was able to show an increase in the permeability to Evans Blue of the blood-follicle barrier in rabbits after an ovulatory stimulus. This investigator concluded that ovulation was due to a breakdown of intrafollicular mucopolysaccharides, thus causing an increase in the intrafollicular osmotic pressure and a resultant increase in follicular pressure sufficient to reach the rupture point.

Regardless of source, increased intrafollicular pressure was eliminated as a cause of ovulation by three separate laboratories by the demonstration that intrafollicular pressure did not increase prior to or during ovulation in the rat (Blandau and Rumery, 1963) or rabbit (Espey and Lipner, 1963; Rondell, 1964). These studies demonstrated a slight fall in pressure as rupture approached. The absence of a pressure increase prior to follicle rupture is indirect evidence favoring some type of degradation of the follicular wall, since it seems unlikely that the contraction of myofibrils, the venous constriction or an increase in osmotic pressure could occur without a corresponding increase in follicular pressure.

The theory relating ovulation to a sequential degradation of the layers of the follicle was postulated in 1916 by Schocket. Corroborating evidence was reported by Rugh

(1935) when, working with frogs, he found that a combination of pepsin and hydrochloric acid would initiate follicle rupture.

Working with rabbits, Espey and Lipner (1965) identified several enzymes which would cause morphological changes similar to normal swelling, stigma formation, and ovulation when injected into the mature follicle. The most effective of these preparations were nagarse, pronase and a bacterial collagenase.

Rodbard (1968) warned against the assumption that intrafollicular administration of enzymes was causing ovulation through a degradation of the follicular wall and suggested that this treatment would also raise the intrafollicular osmotic pressure. Subsequently, Rondell (1970) showed, using a mathematical evaluation of the relationships between the wall tension and luminal pressure of thin-walled spheres, that it is possible to increase follicular volume (via osmotic or secretory phenomena) to the breaking point without measurably increasing the intrafollicular pressure.

Another observation of importance was the histological demonstration that the collagen network in the apex of the rabbit follicle becomes dissociated as ovulation approaches (Espey, 1967b). Espey (1974) has provided evidence that this breakdown of the framework of the follicle is caused by collagenolytic or proteolytic enzymes in response to gonadotropic stimulation. Others believe that these degradative changes in the follicular wall may be due to

necrotic changes, such as an interruption of the vascular supply to this area (Blandau, 1966).

It is obvious that the exact nature of the stimulus for follicular rupture remains to be elucidated. It is conceivable that the ovulatory mechanism may vary among species, and that this mechanism may actually reflect a combination of physiological events.

Involvement of Prostaglandins in Ovulation

With the indication that the prostaglandins might be important mediators of luteinizing hormone action on the ovary (Kuehl, et al., 1970), an intense investigation into the role of these potent physiological compounds in the process of ovulation began. In 1971, Coutinho and Maia reported a series of rapid changes in human intraovarian pressure following the administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Such pressure fluctuations were attributed to muscular contractions and were not observed following administration of PGE_2. Similar results in the rabbit were noted the following year by Virutamasen, Wright and Wallach (1972). The latter report detected an increased level of ovarian contractility following $PGF_{2\alpha}$ administration either *in vivo* or *in vitro*. This effect was inhibited by PGE_2.

A direct role in follicle rupture was theorized by Armstrong et al. (1972) following experiments in which Indomethacin, a potent inhibitor of prostaglandin synthesis, was shown to effectively block LH-induced ovulation in the

rabbit. These authors did not observe a prevention of luteinization in those follicles where ovulation was blocked.

Working with rhesus monkey granulosa cell cultures, Channing (1972a) described a stimulation of progestin secretion and morphological luteinization by several prostaglandins with the order of effectiveness being PGE_2 or PGE_1 much greater than PGA_1, which was greater than $PGF_{2\alpha}$. Channing (1972b) also reported that prostaglandin inhibitors could block the stimulatory action of gonadotropins on luteinization and progesterone secretion. From these observations, she concluded that prostaglandins may be an intermediate in the action of LH or HCG upon granulosa cell luteinization in the primate.

Similar studies with the rat caused Tsafriri et al. (1972a) to agree with the concept of prostaglandins being intermediates of LH action. They reported that PGE_2 would induce ovulation in adult rats whose endogenous ovulatory surge of LH had been blocked with sodium pentabarbital. Treatment of animals with indomethacin was observed to inhibit follicular rupture but no ovum maturation following an ovulatory dose of LH. Additionally PGE_2 was capable of inducing ovum maturation, although not essential for this process. Another study reported by the same authors (Tsafriri et al., 1972b) attempted to ascertain the effect of LH on the maturational development of the follicle-enclosed rat oocyte. Their results indicated that the

addition of either LH, HCG, FSH or PGE_2 to an *in vitro* culture system would induce completion of the first meiotic division. A further observation was that cyanoketone, an inhibitor of steroid synthesis, would not inhibit the maturation-inducing action of LH. From such studies these authors proposed that the action of LH on oocyte maturation possibly involved the prostaglandins.

Further support for the concept that prostaglandins play a role in the process of ovulation came in 1973 when it was noted that ovulatory stimulation in the rabbit would produce a marked increase in the follicular levels of prostaglandins E and F (Yang et al., 1973). Also reported was the ability of intravenous indomethacin to inhibit the follicular prostaglandin increases. In a continuation of this study, Yang et al. (1974) demonstrated that these increases were limited to the follicles that actually ovulate.

Labhsetwar (1973) suggested that $PGF_{2\alpha}$ treatment may cause an estrogen positive feedback which stimulates the release of the ovulating hormone through the hypothalamo-pituitary system.

Richman et al. (1974) used the procedure of infusing PGE_2 or $PGF_{2\alpha}$ into the ovarian arteries of rabbits seven hours following an intravenous injection of HCG. Animals receiving $PGF_{2\alpha}$ infusion responded with ovulation at the same rate as the control animals who received HCG only. Treatment with PGE_2, on the other hand, decreased the

number of follicles rupturing, an effect attributed by these authors to a direct effect of the prostaglandins on the ovary.

In mice, the injection of an antiserum specific for $PGF_{2\alpha}$ caused an 80% decrease in the number of animals exhibiting spontaneous ovulations (Lau, et al., 1974). When $PGF_{2\alpha}$ was administered along with the anti-$PGF_{2\alpha}$ the ovulation percentage was increased by 50%. In other studies in the mouse, PGE_2 was shown to reverse the inhibition of ovulation seen following systemic indomethacin, while $PGF_{2\alpha}$ was only partially effective (Saksena et al., 1974). These experiments indicated that both the E and the F series of prostaglandins were probably involved in ovulation. The blocking of ovulation by indomethacin was attributed to an interference with LH action at the ovarian level as well as to its inhibition of PG synthesis.

Utilizing the Ovarian Ascorbic Acid Depletion bioassay for LH activity, Sato et al. (1974) found a response dichotomy following prostaglandin treatment. On the one hand, either PGE_1, E_2 or $F_{2\alpha}$ in hypophysectomized immature rats would produce the significant depletion of ovarian ascorbic acid characteristic of compounds with LH-like activity. However, when given to PMS pretreated animals, these prostaglandins were not able to mimic the ovulation-inducing capabilities of HCG.

Another possible site of the ovulation-inducing action of systemic prostaglandins is the hypothalamo-hypophyseal

axis. A recent study of Harms et al. (1974) suggests that, at this level, PGE_2 may have a specific effect in stimulating the release of LH by the adenohypophysis. Prostaglandins E_1, $F_{1\alpha}$ and $F_{2\alpha}$ were ineffective in causing LH release.

Similar effects of prostaglandins may be expected in the nonhuman primate. Batta and Brackett (1974) demonstrated that systemic administration of prostaglandins of the E series to rhesus macaques could complement PMS and HCG treatments and result in a more consistent induction of ovulation than is possible through gonadotropin therapy alone. The mode of action was not defined by these authors nor by Shaikh and Klaiber (1974), who indicated that sequential treatment with estradiol and $PGF_{2\alpha}$ would cause a shortened menstrual cycle length in the rhesus.

Involvement of Cyclic AMP in Ovulation

In 1960, Haynes et al. implicated cyclic 3',5' adenosine monophosphate (cAMP) as a mediator in the action of adrenocorticotropic hormone on the adrenal cortex. Shortly thereafter, Marsh and Savard (1966) found that the addition of cAMP to slices of bovine corpora lutea *in vitro* would significantly stimulate steroid synthesis. They also observed that the effects of cAMP closely resembled the ability of luteinizing hormone to stimulate the utilization of acetate-1-C^{14} and cholesterol-7-H^3 for progesterone synthesis. They concluded that this study was evidence

that cAMP was a mediator of the steroidogenic action of LH in the bovine corpus luteum.

Although at first appearing contradictory, the reported inability of either cAMP or its dibutyryl derivative to initiate luteinization in cultured rabbit follicles was attributed to inadequate penetration of the granulosa cells (Keyes, et al., 1972). This observation was supported by Tsafriri et al. (1972 a, b), who reported that while the addition of LH or HCG to a culture medium including explanted rat follicles would stimulate meiotic division, such stimulation did not occur with either cAMP or dibutyryl cAMP. However, it was found that when dibutyryl cAMP was administered directly into the follicle, meiotic activity would resume. Thus, incorporating their earlier results, these authors proposed that the action of luteinizing hormone on oocyte maturation involved the mediation of the adenyl cyclase/cAMP system and possibly of the prostaglandins.

The relationship of cAMP to the process of ovulation was suggested by Marsh et al. (1972) following studies indicating that there was a steady decline in the accumulation of this nucleotide in the rabbit tertiary follicle between the ovulatory stimulus and actual rupture.

The poor response of the follicle to cAMP demonstrated by Keyes et al. in 1972 was improved in 1974 by Miller and Keyes simply by eversion of the follicle to expose the granulosa cells to the culture medium. Following incubation with LH or dibutyryl cAMP and autotransplantation, corpora

lutea were seen to develop, which was a response not observed in follicles incubated in a control media.

Extension of the hypothesized intermediate role of cAMP in the action of LH to a primate species has been reported by Channing (1974). Her studies indicate that a two day in vitro culture of granulosa cells from the follicles of monkeys in a preovulatory state, a midfollicular state or a gonadotropin (HMG or PMSG) treated state, would cause luteinization and progestin secretion if LH or dibutyryl cAMP was present in the culture media.

Mediation of Gonadotropic Action by cAMP and/or Prostaglandins

In 1965, Savard et al. reported that 0.02 M cAMP would cause a stimulation of progesterone synthesis from slices of bovine corpus luteum that was comparable to that produced by saturating amounts of luteinizing hormone. The following year, the same laboratory demonstrated the rapid accumulation of cAMP in response to the addition of luteinizing hormone to the incubated slices from bovine corpora lutea (Marsh et al., 1966). This response was not seen following the addition of substances shown to be ineffective in stimulating progesterone synthesis (hydrogen peroxide inactivated LH, prolactin, ACTH, epinepherine, or glucagon). These authors also observed a marked accumulation of this nucleotide in incubated slices of human corpora lutea following the addition of HCG. The increased cAMP

brought about by LH precedes the increase in progesterone synthesis which is additional evidence of a role of this nucleotide in the steroidogenic action of LH.

Further complication of the issue was demonstrated in 1970 by Kuehl et al., from a study of the kinetics involved in the stimulation of ovarian cAMP accumulation by LH, PGE_1 and PGE_2. Their evidence suggested that prostaglandins stimulated adenyl cyclase as their role in hormone action, and that the prostaglandin receptor was an essential component of the LH effect.

In 1971 these observations were extended to the tertiary follicle of the proestrous rat, which, in a culture system, was also seen to respond to the addition of LH or PGE_2 with the production of cAMP (Lamprecht et al., 1971). A study by Marsh et al. (1971) showed that the follicle of the rabbit could synthesize cAMP and that its accumulation is greatly enhanced by luteinizing hormone. Employing a prostaglandin antagonist, Ellsworth and Armstrong (1974) stated that this inclusion in a culture system prevented the luteinization of rat ovarian follicles in response to the luteinizing agents LH, PGE_2 or dibutyryl cAMP. They concluded that LH and PGE_2 have a common mode of action via cAMP.

Similar conclusions were drawn from a study of LH and FSH-induced luteinization of granulosa cells harvested from rhesus monkey preovulatory follicles (Channing & Crisp, 1972). These workers stated that, as in other species,

gonadotropin-induced luteinization of primate granulosa cells was mediated by cAMP with the possible involvement of prostaglandins. Also working with a primate species, Wilkes et al. (1972) demonstrated the active synthesis of $PGF_{2\alpha}$ by ovarian tissue in vitro. This response increased following the addition of LH to the culture system.

In 1973, Yang et al. indicated that systemic administration of HCG or LH would stimulate follicular levels of both PGF and PGE. These increases were due to increased synthesis of prostaglandins since the increases were abolished following pretreatment with indomethacin. They concluded that increasing levels of prostaglandins within the Graafian follicle are associated with the normal physiological process of ovulation. This conclusion is substantiated by a report indicating that various prostaglandins can stimulate ovarian cAMP accumulation in a manner similar to LH (Mason et al., 1973).

It has been suggested, however, that prostaglandins may not be mediators of gonadotropic action upon cAMP formation in some systems (Kolena & Channing, 1972). Evidence against the theory that PGE_2 is an obligatory intermediate of LH action on the ovary was presented by Lamprecht et al. (1973) who found an additive effect of maximally stimulatory levels of LH and PGE_2 on cAMP formation in whole ovary cultures. These authors observed a refractory state following LH incubation during which the ovaries could no longer be stimulated by LH yet

remained fully responsive to PGE_2. In another study reported the same year, incubation of juvenile rat ovaries with LH caused an increased cAMP accumulation without increasing the activity of prostaglandin synthetase. Inhibitors of this synthetase activity were also found to abolish the prostaglandin-induced increases in cAMP formation in vitro, without preventing the stimulatory effect of LH on formation of this nucleotide (Zor et al., 1973). These authors argue against the hypothesis that prostaglandins of the E series are involved as obligatory intermediates of the cAMP dependent actions of LH on the ovary.

Recently, LeMaire and Marsh (1975) have attempted to reconcile the contradictory proposals regarding the interrelationship of these substances with ovulation. They find progressively increasing levels of both PGE and PGF as ovulation progresses in the face of a declining cAMP synthesis. Current speculation involves the relationship of prostaglandins to follicle rupture rather than to ovum maturation or to luteinization.

Intrafollicular Injection Techniques

The first procedure reported for the in vivo monitoring of intrafollicular phenomena was the microcannulation technique used by Espey and Lipner (1963) to record pressure changes inside the rabbit follicle. Their procedure employed capillary glass tubing with a small diameter tip.

These authors modified this technique in 1965 for the intrafollicular injection of various enzymatic solutions. Local administration of collagenase, pronase or nagarse in 1 microliter volumes induced follicle rupture in a manner similar to that produced by mating. There was a total absence of effect with injections of up to five microliters of saline. These relatively large volumes of saline would neither induce nor inhibit ovulation in coitally stimulated females and was good evidence that the observed effects were not artifacts of the technique.

Blanchette (1966) reported the use of a similar technique for the study of the morphological differentiation of follicular granulosa cells into luteal cells. In this study, injections were made through either drawn capillary pipettes or 30 gauge needles. Leakage of the injected substance was prevented by withdrawing the needles slowly, approximately a minute following the completion of the injection. Injection of volumes of one to five µl were studied with the adoption of one µl as the standard. The results of this study demonstrated that the intrafollicular administration of 8 µg of purified ovine LH (NIH-LH-S7) or 1 IU of HCG (APL-Ayerst) would cause the cytological events normally occurring in follicular granulosa cells undergoing differentiation into lutein cells.

Ferrando and Nalbandov (1969) reported the successful inhibition of ovulation from alternate follicles on one ovary in the rabbit following the injection of an adrenergic

blocking drug, dibenzyline. They used 27 gauge needles and confirmed the absence of effect of saline intrafollicular injections.

The presence of corpora lutea five days following intrafollicular administration of as little as 0.1 ng of LH was reported by LeMaire et al. (1972). When 0.4 μ mole of cAMP or dibutyryl cAMP was administered concurrently with the LH a marked decline in corpora lutea production was observed. Furthermore, intrafollicular administration of 25 μg of phosphodiesterase, which was thought to decrease the endogenous levels of cAMP, would also produce luteinization. These authors concluded that the normal function of cAMP might be to inhibit premature luteinization. Their technique had utilized a "very fine" needle to deliver one μl volumes from a ten μl Hamilton syringe. Methylene blue was added to the solutions to monitor follicular leakage which was rarely observed.

Although the preceeding report made no mention of ovulation, the production of corpora lutea can be considered as presumptive evidence in its favor. Follicle rupture in the rabbit was demonstrated in response to intrafollicular administration of either 10 ng LH or 100 ng FSH by Jones a and Nalbandov in 1972. Their technique involved entry into the follicle through the ovarian stroma to facilitate tissue sealing. Follicle locations were sketched for subsequent identification. The ovaries were re-exposed following 18 to 24 hours, and the number of follicle rupture points were

observed and counted. Luteinization was invariably seen following the injection of subovulatory doses of LH or FSH, and these luteal structures were competent in progesterone production. This technique produced additional evidence implicating the sympathetic nervous system in the process of ovulation, following the demonstration that the alpha adrenergic blocking drug, phentolamine, would inhibit ovulation. It was further shown that this blockade could be overcome by catecholamines, cAMP, or a mixture of FSH and LH. The theory was proposed that the effect of LH was to stimulate the cAMP system which then may activate synthesis of the catecholamines and, in turn, activate an ovarian collagenase resulting in ovulation (Nalbandov et al., 1973).

The ability of cAMP to induce ovulation when administered locally was disputed by Das and Talwar (1974). No ovulations were observed following injection of from 5 to 500 μg of cAMP even though LH would induce ovulation when given in this manner. Their results indicated an inability of cAMP to mediate the ovulation-inducing activity of LH. This conclusion may not be comparable to the other studies involving intrafollicular injections, however, as they made no mention of administering their compounds into the ovarian follicle. Rather their substances were injected directly "into one of the ovaries."

Prostaglandins E_2 and $F_{2\alpha}$ were administered via intrafollicular injection by Moon and Armstrong (1974), resulting in their implication of the latter in the process of

ovulation, possibly by its action on the follicular contractile process. Further evidence of the involvement of prostaglandins with follicular rupture was the report that antisera to $PGF_{2\alpha}$ would consistently inhibit ovulation (Moon and Armstrong, 1974). PGE_2 antisera was also inhibitory, but to a lesser extent, while intrafollicular injections of indomethacin, an inhibitor of prostaglandin synthesis, proved 100% effective in blocking LH induced ovulation in the rabbit.

MATERIALS AND METHODS

Experimental Units

The nonhuman primates used in this study were from the permanent colony maintained at the Endocrine Research Unit on the campus of Michigan State University. This colony is made up of two species, *Macaca fascicularis* and *Saimiri sciureus*, which are used exclusively in non-terminal studies of various physiological aspects of primate reproduction. Their environmental conditions include a 12 hour light-dark cycle, a temperature maintained between 21° and 26°C, and fluctuations in relative humidity from approximately 40 to 60%. All animals received water ad libitum and a commercially prepared monkey diet (Wayne Co.).

The macaques were housed individually in 48-inch double unit stainless steel cages. Cages were equipped with slotted floors, a back mounted perch, and a squeeze-back apparatus to facilitate animal handling. The squirrel monkeys were kept either in a 280 cubic foot community type cage or were kept as small groups in the same type of cage as that used for the macaques. The cages were washed

daily, at which time a visual inspection of the macaque cages was made for the observation of menses.

Induction of Follicle Growth

Since ovulation served as the endpoint for the majority of these studies, it was necessary to have a procedure for the reliable production of mature follicles. Such a technique was first described in the squirrel monkey by Dukelow in 1970. It involves four daily injections of 1 mg follicle stimulating hormone (FSH-P), Armour Baldwin Laboratories, Omaha, Nebraska), followed by an ovulation-inducing injection of human chorionic gonadotropin (HCG, 500 IU, A.P.L., Ayerst Co.). This regime will yield ovulation approximately 6 to 14 hours after the HCG injection in 60% of the animals (Harrison, 1973).

Although the production of ovulation in the macaques has been less reliable than in the saimiri, extrapolation of the effective dose level suggested that 5 mg/day would be sufficient for the macaque. For an ovulation induction system in the rabbit, this dosage has been shown to be equipotent with 400 IU PMSG (Kennelly and Foote, 1965). This level is higher than that necessary for the stimulation of mature follicular growth in the cynomolgus macaque (Jainudeen and Hafez, 1973). Assessment of follicular growth and quantification of ovulation sites was made possible through visual observation of the ovaries with the laparoscope.

Cyclicity in Saimiri sciureus

To detect the presence of a cyclical pattern of ovarian response to exogenous gonadotropins, a total of 70 female squirrel monkeys were treated with the regime consisting of 4 daily FSH administrations followed by an ovulation inducing treatment on day 5. Without further FSH treatments, the animals received intramuscular injections of 500 IU HCG at two day intervals beginning 5 to 17 days following the synchronized ovulation. Subsequent laparoscopies were performed to determine the number of animals ovulating at each interval.

Laparoscopic Techniques

Surgical anesthesia was produced in the squirrel monkey by intraperitoneal injection of 16.2 mg sodium pentobarbital (Halatal, Jensen-Salsberry Laboratories, Richardson-Merrill Inc., Kansas City, Mo.). In the macaque, intramuscular administration of 15 mg of phencyclidine-HCl (Sernylan, Bio-Ceutic Laboratories) produced adequate anesthesia for up to 45 minutes. The animals were placed on a tilted surgical table with their feet elevated and secured with adjustable leg straps. The abdominal region was shaved and prepared with benzalkonium chloride (Zephiran, Winthrop, New York). Insertion of the trocar-cannula was made through a 5 mm periumbilical incision following which the trocar was removed and replaced by the laparoscope. The laparoscope used in these studies was a 135 degree pediatric

model (Richard Wolf Co., Knittlingen, W. Germany) in conjunction with a Wolf model 4000 light source and flexible fiber optic cable. Transillumination of the abdominal wall insured proper placement of a Verres-cannula which has been found to serve adequately as a probe for intra-abdominal manipulations. A CO_2-produced pneumoperitoneum facilitated observation. When experiments were expected to last longer than 30 minutes, uretheral catheterization was performed to prevent obstruction of the procedure by the urinary bladder.

For laparoscopic photography a Canon TL 35 mm single lens reflex camera was utilized since this allowed simultaneous observation and photography. Shutter speeds ranging from 1/2 to 1/8 of a second were found to produce the most desirable exposures with Ektachrome DHB film (ASA 120).

When the procedure was completed, the laparoscope was withdrawn, leaving the cannula in place to allow ready escape of the CO_2. Following removal of the cannula and probe, nitrofurazone powder (Furacin, Eaton Labs) was applied as a hemostatic and the incision was closed with subcutaneous suturing. Topical Furacin ointment and systemic penicillin administrations were used to combat infection.

Studies involving local ovarian application of compounds required the laparoscopic placement of disks of 2 mm in diameter on the surface of the ovary. For these studies, a 3 mm laparoscopic forceps with a fiberglass cannula was substituted for the Verres cannula. This forceps allowed placement and recovery of the disks without requiring major

abdominal surgery. Disks were left in place for one hour except in cases where anesthetic limitations required early removal. Such cases were not considered to be completed experiments and were not included in the results.

The ovarian disks were made from either (a) #42 filter paper (Whatman Co.) or (b) Gelfoam (Upjohn Co., Kalamazoo). Preliminary comparisons revealed that, although the Gelfoam disks would hold more liquid than would the filter paper, they were less stationary when on the ovarian surface, and therefore all subsequent studies were carried out using the filter paper disks. Identical disks were used in the in vitro experiment to be described later (Appendix I).

A technique for injecting substances directly into the ovarian follicle of the squirrel monkey was developed. Initially, compounds were injected with a 10 microliter Hamilton syringe equipped with a Chaney adaptor and custom made 37 gauge needles (Hamilton Co., Whittier, Cal.). Follicles were randomly chosen and entry was made at the base of the transilluminated follicular dome. Control and experimental injections were made in 0.2 µl volumes into contralateral ovaries. The needle was allowed to remain in the follicle for approximately 30 seconds and was then slowly withdrawn. This procedure prevented any obvious flow of fluid from the follicle. Follicular location was carefully mapped with notations referring to the normal orientation of the ovary in the animal. Subsequent

laparoscopic examinations were performed using observer-blind analysis of the ovarian surface.

Adaptation of the intrafollicular injection technique to laparoscopy required a syringe that could be manipulated with one hand and, therefore, the Gilmont Micrometer Syringe was used. This syringe could more accurately deliver the small volumes necessary for these studies. At this time the injection volume was increased to 1 µl to further increase delivery precision. As the 37 gauge needles bent frequently, 32 gauge needles were substituted with no detrimental effects and with the added benefit of less frequent clogging. Twenty-four gauge needles (½ inch long) were inserted with laparoscopic guidance, to serve as cannulae for the fine injection needles. As needle clogging occasionally occurred, patency was tested prior to entry into the abdomen and again immediately before follicular penetration. Experimental injections were accompanied by vehicle injections into contralateral ovaries using separate syringes and needles. Some follicles were marked with either India ink or Trypan Blue (0.05%) to assess the reliability of the delivery technique, as well as to check for follicular leakage.

Laparoscopic examinations were carried out at intervals sufficient to detect any post-ovulatory changes (12 to 36 hours). Ovulation was diagnosed using the criteria previously described for the squirrel monkey (Harrison & Dukelow, 1974) and for the cynomolgus monkey (Jewett & Dukelow, 1972; Rawson & Dukelow, 1973; Dukelow, 1975).

Experimental Compounds

All substances for the ovarian disk or intrafollicular injection studies were kept frozen and thawed shortly prior to use. The gonadotropins used were luteinizing hormone (NIH-ovine-LH-S18) and human chorionic gonadotropin (A.P.L., Ayerst Co.). The LH was dissolved with sterile saline while the HCG was prepared using the commercially supplied diluent. Prostaglandins E_2 and $F_{2\alpha}$ (The Upjohn Company) were prepared by initial dissolution in 95% ethanol with subsequent dilution by sterile saline containing sodium carbonate resulting in a final PG concentration of 10 mg/ml. Cyclic 3',5' AMP and its dibutyryl derivative (Cal Biochem, Los Angeles) were dissolved in sterile saline. Dilution was such that 0.6 μM of the nucleotide could be delivered in 1 μl volume.

The pH of all solutions was between 6.8 and 7.1 and the described vehicles were used as controls in all cases.

Progesterone Assay

The assay of peripheral plasma progesterone has been described previously (Louis et al., 1973). Basically, the procedure involved the extraction of 200 μl of duplicate samples of plasma with 2 ml benzene-hexane (1:2). Following removal of organic solvent, 200 μl of rabbit antiprogesterone (prepared against 6 β-succinylprogesterone conjugated to bovine serum albumin and supplied by Dr. G.D. Niswender, Colorado State Univ.) was added to the aqueous phase and

samples were incubated at room temperature. Next, ^{3}H-progesterone (approximately 24,000 CPM) was added to each sample, and the tube contents were mixed, and incubated at 4 for 4 to 20 hours. Dextran-coated charcoal was then added to each sample, while maintaining them in an ice bath. The samples were then mixed and centrifuged (2500 g for 10 min.). This step effectively separates the bound progesterone from the free. A liquid scintillation spectrometer was then used to measure the amount of ^{3}H-progesterone bound to the antibody in 0.5 ml of the supernatant.

Previous studies (Louis et al., 1973) have demonstrated that the efficiency of the extraction procedure is 80 ± 1% while the sensitivity of the assay is less than 25 pg of progesterone.

Statistical Analysis and Resource Allocation

The studies contained in this thesis have dealt with two types of data; quantitative data and binomial data. In experiments where the data generated fit a continuous, numerical scale (quantitative data), statistical analysis was by analysis of variance. Such data are exemplified by the analysis of the cycle characteristics of the Macaca fascicularis. In studies where the data involved the presence or absence of an animal's response (binomial data), Chi-Square, 2 x 2 contingency tables were used for analysis (Pearson and Hartley, 1958). Examples of these data include

the experiments designed to assess the ovulation inducing ability of locally administered compounds. Other experiments generating binomial data involved the need to compare treatment effects to a hypothetical probability of event occurrence, as well as the evaluation of the difference between sample percentages. These experiments were analyzed using the procedures for handling such data which were described by Goldstein (Chapter 3, 1964). For all statistical considerations in this thesis, an alpha of 0.05 was presumed to reflect significant differences.

Recommended animal numbers for binomial experiments were based on the probability of the occurrence of the event being observed. In this case, that event referred to the presence of ovulation, which occurs in approximately 60% of the Saimiri sciureus submitted to an ovulation induction regime (Dukelow, 1970; Harrison, 1973). The formula for determining recommended numbers is given by Goldstein (p. 94, 1964) to be $np = 5$, where p represents the probability of the occurrence of an event, and n refers to the animal numbers. If the value of 50% is assigned to p (making the estimate more conservative), the resultant n equals 10 animals. Accordingly, the majority of these experiments were carried out using 10 animals in each group.

RESULTS

In order to define the reproductive cycle length of the squirrel monkey, an experiment was undertaken to test the ovarian responsiveness of animals synchronized by an ovulation inducing treatment, to a subsequent dose of HCG (500 IU). The results are displayed in Table 1. The greatest ovulatory response (70%) was in the group of

TABLE 1

Ovulatory Response of Saimiri sciureus to 500 IU HCG at Varying Intervals Following Synchronization

	Day[a]						
	6	8	10	12	14	16	18
Animals ovulating/ total	3/10	7/10	2/10	0/10	4/10	1/10	4/10

[a]Interval between bynchronization and subsequent ovulation induction (in days)

animals induced to ovulate eight days following their previously synchronized ovulation. When these responses are compared to the expected level (60%) for this species

(Dukelow, 1970; Harrison, 1973), the responses observed on days 10, 12 and 16 are significantly lower ($p < 0.05$) while the remaining responses are not different. It is interesting to note that eight days following a synchronized ovulation, there appears to be no requirement for exogenous FSH in order to stimulate levels of ovulation similar to those seen following the standard regime which includes 4 daily FSH treatments.

Macaca fascicularis

When studying Old World primate species such as the Macaca fascicularis it is much easier to discern reproductive cycle parameters as these animals exhibit regular menstrual cycles. During the course of a four year study (January 1970 to December 1973), several such parameters were recorded for a colony of 28 female M. fascicularis. Table 2 presents the mean duration of menstrual flow and the cycle lengths for each individual female. As illustrated by these data the mean cycle length for this colony was 30.9 ± 0.21 days, while the mean duration of menstrual flow was 2.6 ± 0.1 days. The median cycle length for this period was 30 days, while the modal interval was 29 days. It should be noted that the low numbers of cycles from several of the females can be attributed to pregnancies, lactation, experimental treatments or amenorrheic conditions.

Although there was apparently no effect of season on the duration of menstrual flow (Figure 1) there was a

TABLE 2

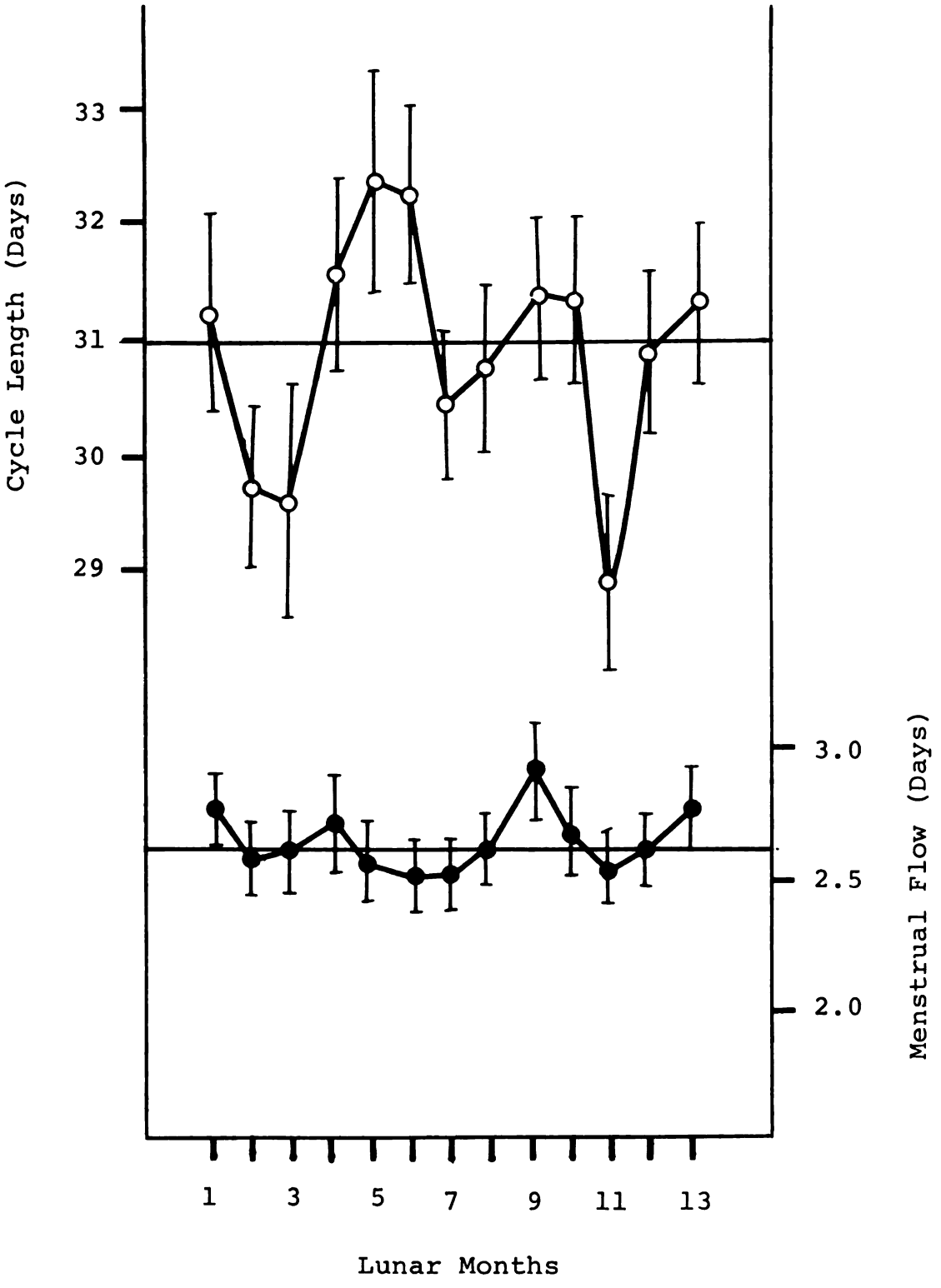
Individual Mean Cycle Lengths and Menstrual Flow Duration for *Macaca fascicularis* Used in This Study

Animal	No. of Cycles	Mean Cycle Length (S.E.)	Mean Flow Duration (S.E.)
7	4	28.8 (1.5)	1.2 (0.1)
8	3	27.7 (0.3)	1.4 (0.3)
9	32	34.4 (1.0)	3.2 (0.2)
10	1	27.0	2.0
11	20	30.6 (0.9)	4.1 (0.4)
12	29	32.5 (0.6)	4.2 (0.5)
14	19	30.4 (0.6)	2.0 (0.2)
15	7	33.7 (0.6)	1.5 (0.2)
16	1	29.0	3.0
17	12	32.3 (2.9)	2.1 (0.2)
18	9	31.4 (1.7)	2.0 (0.3)
25	14	29.1 (1.5)	1.5 (0.2)
26	1	33.0	1.0
27	7	35.1 (1.7)	2.4 (0.3)
31	26	29.1 (0.6)	3.2 (0.4)
39	19	36.2 (0.8)	2.8 (0.2)
40	24	30.3 (0.8)	3.0 (0.2)
41	27	32.6 (0.9)	2.0 (0.2)
42	29	29.3 (0.8)	2.9 (0.2)
43	35	28.5 (0.4)	2.8 (0.2)
44	36	29.4 (0.5)	3.7 (0.2)
45	1	29.0	1.0
46	2	30.5 (2.5)	1.5 (0.5)
51	21	29.4 (0.3)	3.0 (0.2)
52	11	31.7 (1.2)	2.0 (0.3)
53	27	30.3 (0.8)	2.0 (0.1)
54	22	31.4 (1.2)	2.1 (0.2)
55	19	29.1 (0.9)	1.8 (0.2)
28	458	30.9 (0.21)	2.6 (0.1)

FIGURE 1

Variation of Menstrual Flow and Cycle Lengths of *Macaca fascicularis* During the Year

FIGURE 1


Cycle Length (Days)
33
32
31
30
29
3.0
2.5
2.0
Menstrual Flow (Days)
1
3
5
7
9
11
13
Lunar Months

significant variation in cycle length during the year, the longest cycles occurring during the fifth and sixth lunar months while the shortest were seen during the third and eleventh months.

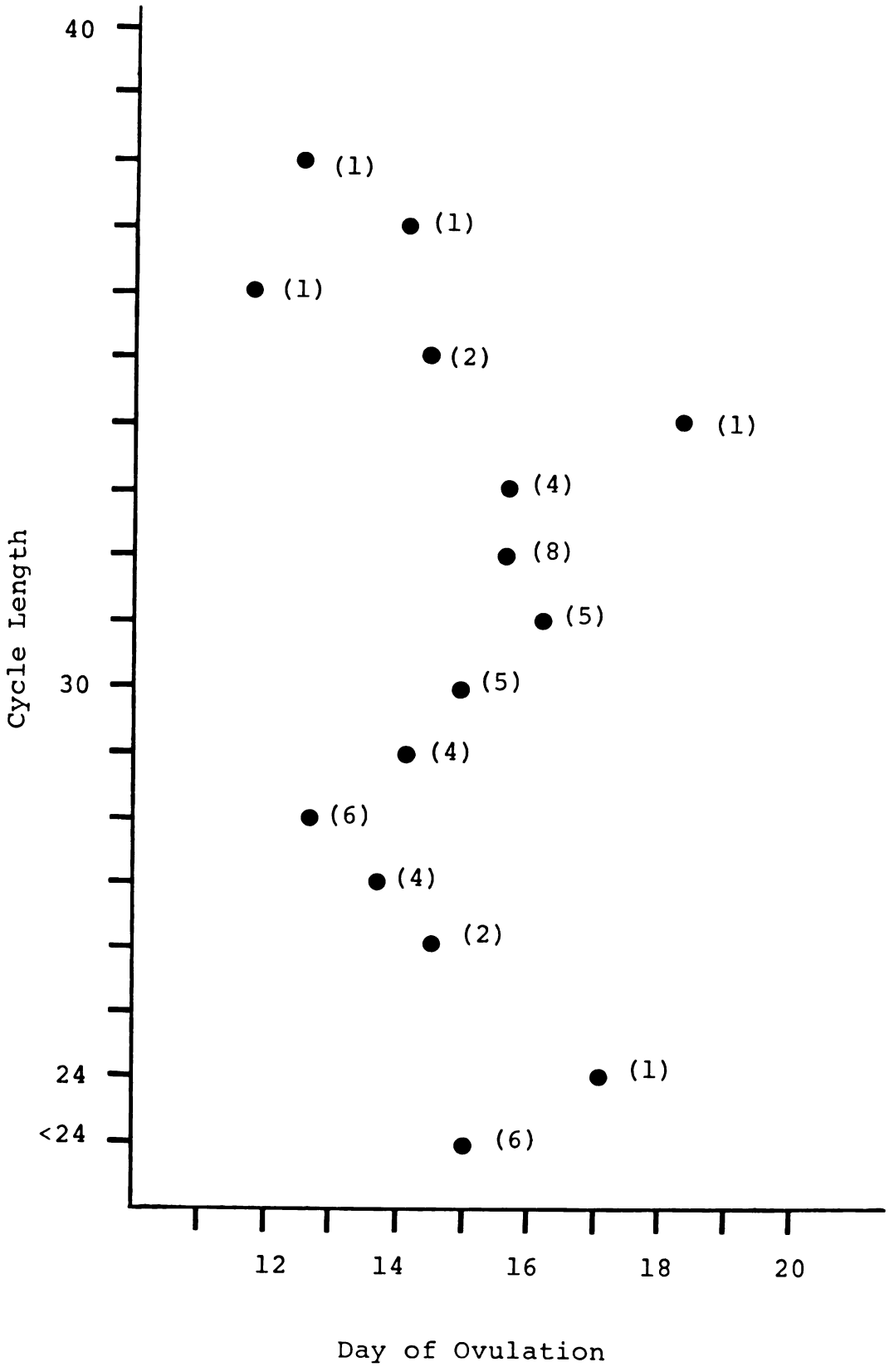
During 104 menstrual cycles in this species, laparoscopic examinations allowed the definition of ovulatory status. In 93 of these cycles (89.4%), the ovarian morphology indicated that ovulation had taken place. The other 11 cycles (10.6%) were anovulatory. Ovulation was observed on the left ovary in 62.3% of the cases and in the right ovary in 37.7% of the cases. During 27 pairs of consecutive cycles, the second ovulation occurred on the contralateral ovary 70.4% of the time, and on the ipsilateral ovary 29.6% of the time. In 51 cycles laparoscopy was utilized to define the precise time of ovulation. Figure 2 reveals the relationship between the day of ovulation and the length of the menstrual cycle. If we disregard the top three points, which represent only one cycle each, we can begin to see a pattern with the cycles ranging from 28 to 34 days in length. There is, however, no significant relationship between these two variables.

Twin ovulations were infrequent with contralateral twin ovulations noted only three times while twin ovulations on the same ovary were seen only once. Actual follicular rupture was witnessed on four separate occasions. In three of these ovulations, the site of follicular rupture was located near the apex of the ovarian stigma.

FIGURE 2

Relationship Between Follicular Phase Length and Menstrual Cycle Length of *Macaca fascicularis*

FIGURE 2


40
30
24
<24
Cycle Length
(1)
(1)
(1)
(2)
(1)
(4)
(8)
(5)
(5)
(4)
(6)
(4)
(2)
(1)
(6)
12
14
16
18
20
Day of Ovulation

The fourth rupture occurred from the side of the stigma, near the base of the follicle. Considerable bleeding was noted from the ovulation site at the time of rupture in three of these cases, making the cumulus mass take on a hemorrhagic appearance.

Induction of Follicular Growth

Preliminary trials of local gonadotropin application in squirrel monkeys utilized animals with no pretreatment, 2 days of FSH pretreatment (1 mg/day), or 3 days of FSH pretreatment. In five animals receiving no pretreatment there was only one follicle observed which had the appearance of a preovulatory follicle. Following two days of FSH, four of seven animals (57%) were observed to have normal preovulatory follicular development on the fifth day after start of the treatments. Addition of a third FSH administration did not increase the number of animals exhibiting normal follicular development (six out of twelve). Four days of FSH pretreatment, however, significantly ($p < 0.05$) increased the number of animals responding with normal preovulatory development (50 out of 64, or 78%). Thus, the four day FSH regime was adopted for all studies.

In the macaques a regime consisting of 5 mg FSH/day was administered for each of the first five days of the animals menstrual cycle. Of twenty animals receiving this treatment, only one exhibited the normal, preovulatory follicular development characteristic of this species

(Table 3). The most common response included the development of multiple follicles in 15 (75%) of these animals. Other abnormal occurrences included the presence of large follicles appearing to be filled with fluid, therefore referred to as cystic, in three of these animals and the development in two animals of follicles having the appearance of containing coagulated blood. The latter type follicles are frequently observed in the rabbit following FSH administration and have been referred to as "blood" follicles. This treatment had no significant effect on the menstrual cycle length of either the treated cycle or the post-treatment cycle in these animals.

Local Administration Experiments

Gonadotropin Studies

At the inception of this project it was decided that the technique of local administration of substances to the ovarian follicle which involved the least amount of experimental manipulation would be advantageous. We, therefore, elected to administer purified gonadotropins to the ovarian surface with the laparoscopic procedures previously described. Table 4 illustrates the results of experiments in which 2 mm hormonal saturated disks were placed on the ovarian surface in the macaque. These data reveal no apparent effects of either FSH or LH on the occurrence of ovulation. The two ovulations in the FSH treated group were thought to represent chance observations since they were not

TABLE 3

Effects of FSH and Intrafollicular HCG in Macaca fascicularis

Animal	Treatment	Follicular Development	Effects of IF HCG			Effect on Cycles[g]	
			Ovulation	Luteinization[a]	Vascularization	Treated Cycles	Next Cycle
12	FSH[b]	multiple[e]					
40	FSH	multiple[f]				23/30	33/30
44	FSH	multiple[e]				37/29	29/29
52	FSH	multiple				42/32	34/32
53	FSH	multiple[e]				21/30	30/30
41	FSH	poor				23/33	30/33
51	FSH	poor				25/29	25/29
52	FSH	poor				52/32	34/32
9	FSH + IF HCG[c]	multiple[e]		0	0	27/34	28/34
17	FSH "	enlarged ovary		+	0	60/32	38/32
25	FSH "	normal		0	+	47/29	66/29
42	FSH "	multiple		0	0	50/29	44/29
43	FSH "	multiple		++	0	35/29	32/39
44	FSH "	poor		++	+	25/29	31/29
14	FSH[d] "	multiple					
17	FSH "	multiple		++		50/32	32/32
39	FSH "	multiple[e]		++	0	57/34	27/34

TABLE 3 (cont'd.)

Animal	Treatment	Follicular Development	Effects of IF HCG			Effect on Cycles	
			Ovulation	Lutein-ization[a]	Vascular-ization	Treated Cycles	Next Cycle
41	FSH + IF HCG	multiple[e]		+	+	55/36	36/36
51	FSH "	multiple	4 days after HCG	+	0	58/29	28/29
53	FSH "	multiple			++	19/30	30/30

[a]Apparent "morphological luteinization" (i.e., luteal coloration of treated follicle)

[b]5 days, 5 mg/day

[c]HCG injected into one follicle on one ovary only

[d]HCG injected into one follicle on each ovary

[e]Follicles appear large, and fluid filled, resembling cystic follicles.

[f]Follicles appear to contain coagulated blood, resemble "blood follicles" in rabbit

[g]Cycle lengths in days/previous average cycle length

TABLE 4

Effects of Local Administration of Ovarian Disks in the Macaque

Treatment	Ovulation	Morphological Alteration		No Effects
		Vascularization[a]	Luteinization[b]	
FSH	2/10 (20%)	3/10 (30%)	0/10	5/10 (50%)
LH	0/9	4/9 (44%)	2/9 (22%)	3/9 (33%)

[a]Subjective evaluation of increases in vascularization above levels observed during the normal cycles

[b]Subjective evaluations of increases in luteal coloration

repeatable. Subjective evaluations of the degree of vascularity and luteal coloration were also recorded. These observations suggest that LH administered by the topical ovarian disk may have had an effect to increase vascularization over that seen in a normal cycle.

Due to the lack of effects of these treatments, it was hypothesized that either the ovarian membrane was impermeable to the gonadotropin or that the one hour exposure was not sufficient to allow an effect. In vitro porcine follicle culture experiments suggested the latter to be the case (Appendix I) and, therefore, it was decided that another technique for local administration of these compounds was needed. It was on this basis that the technique of intrafollicular injection was developed.

Initial experiments using the 10 µl Hamilton syringe were done at laparotomy in the unprimed squirrel monkey. Ten ng of purified LH were injected into all externally observable antral follicles (2 to 4 per animal) with the procedure previously described. Control animals received saline injections. The results indicate that while this treatment appears to cause a luteal-like coloration of the injected follicle, in only one case did this cause ovulation (Table 5). The follicle that ovulated in response to this treatment was the largest follicle observed in any of the animals and closely approximated in size the follicles seen in FSH primed females. It was assumed to be a natural preovulatory follicle. When given the FSH pretreatment,

TABLE 5

Response of Saimiri sciureus to Intrafollicular Injections at Laparotomy

Treatment	Ovulation		Morphological Alterations[a]	
	Animal Response (%)	Follicle Response	Animal Response (%)	Follicle Response
Intrafollicular saline	0/5	0/8	0/5	0/8
Intrafollicular LH	1/5 (20%)	1/15	5/5 (100%)	14/15
FSH pretreatment + IF LH	3/5 (60%)	4/11	4/5 (80%)	5/11

[a]Apparent changes in vascularization or luteinization of the treated follicles in the absence of ovulation

these animals exhibited a marked but non-significant increase in ovulation rate.

Adaptation of this procedure to be used with laparoscopy required only a few minor technical adjustments as described earlier. In order to insure the accuracy of delivery of this procedure, five squirrel monkeys received intrafollicllar injections of India ink. This substance was seen to rapidly disperse following injection (Figure 3a,b) and would clearly define the follicular boundaries. Blood loss can be seen from the site of follicular puncture, probably from the extrafollicular capillary network as no ink leakage was observable. Injection of LH dissolved into 0.05% Trypan Blue in saline resulted in ovulation from two of five animals treated. Similar injections of the vehicle alone failed to cause follicular rupture. These injections of dye markers demonstrated that the ovarian mapping would allow accurate identification of treated follicles since in the laparoscopic position the ovaries had a characteristic orientation in the abdominal cavity.

Table 6 shows the results obtained when 10 ng of purified LH were injected into the ovarian follicle using laparoscopy. These results are not different from those seen when the injections were made into exteriorized ovaries under a dissecting microscope.

In order to test the ability of an intrafollicular injection of a primate LH source to induce ovulation in the squirrel monkey, this procedure was repeated with the

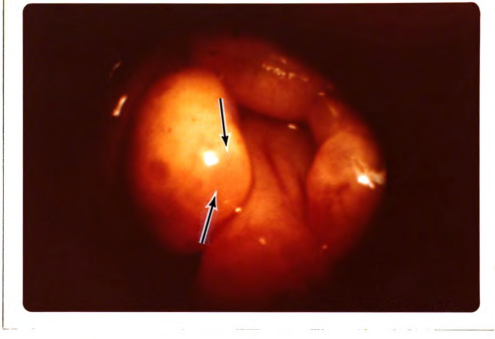


Figure 3a. Mature follicle on ovary of Saimiri sciureus following 4 days of FSH

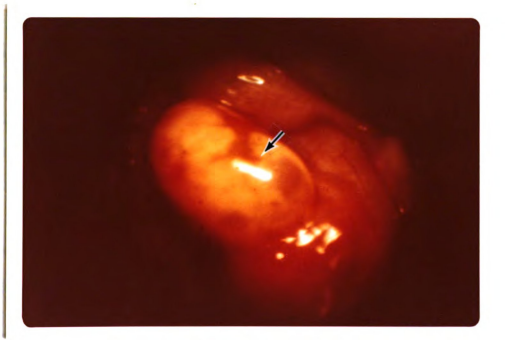


Figure 3b. Mature follicle on ovary of Saimiri sciureus following intrafollicular injection of India ink. Bleeding can be observed from the site of injection without loss of the dye.

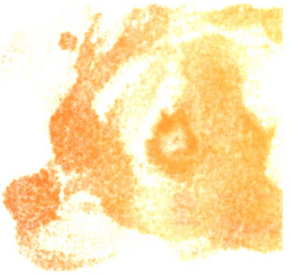


TABLE 6

Comparison Between Intrafollicular Injection Technique at Laparotomy and at Laparoscopy

Treatment	Ovulation		Morphological Alterations[a]	
	Animal Response (%)	Follicle Response	Animal Response (%)	Follicle Response
FSH pretreatment + IF LH (Laparoscopy)	3/6 (50%)	3/11	3/6 (50%)	3/11
FSH pretreatment + IF LH (Laparotomy)	3/5 (60%)	4/11	4/5 (80%)	5/11

[a]Apparent changes in vascularization or luteinization of the treated follicles in the absence of ovulation

injection of 0.5 IU of HCG. It can be seen from Table 7 that this procedure resulted in the same ovulation rate observed previously with the ovine LH. Further, both the ovulation rates and the levels of morphological alterations following the intrafollicular injection of either 10 ng of ovine LH or 0.5 IU of HCG were significantly greater than the respective levels seen following intrafollicular saline injections. Support of the concept of a species-specific requirement of a gonadotropin source to induce ovulation was demonstrated by the inability of systemic ovine LH to stimulate ovulation in the FSH pretreated squirrel monkey, since systemic LH from a primate source, HCG, was effective in stimulating ovulation in these animals.

As this procedure had proven to be satisfactory for the local administration of compounds to the ovary in the squirrel monkey, and as a frequent effect of the injection of an LH source was the appearance of a luteal coloration of the follicle, it was decided to use this technique in the macaques where menstrual cyclicity and circulating progesterone levels could be monitored.

The results of either FSH administration alone or FSH administration followed by intrafollicular injection of 2 IU of HCG are presented in Table 3. Analysis of Variance revealed no significant differences between the lengths of treated cycles and the previous mean cycle lengths of 8 animals receiving 5 daily, intramuscular injections of 5 mg FSH. Neither did FSH alone have any effect on the length

TABLE 7

Response of Saimiri sciureus to Primate and Nonprimate Gonadotropin Sources

Treatment	Ovulation		Morphological Alterations[a]	
	Animal Response (%)	Follicle Response	Animal Response (%)	Follicle Response
FSH pretreatment only	1/10 (10%)	N.A.[b]	N.A.	N.A.
FSH pretreatment + intramuscular ovine LH (2 mg)	0/5	N.A.	N.A.	N.A.
FSH pretreatment + IF saline	0/23	0/39	0/23	0/39
Total FSH pretreatment + IF LH	6/11 (55%)	7/22	7/11 (64%)	8/22
Total FSH pretreatment + IF HCG	5/11 (45%)	5/20	7/11 (64%)	7/20

[a]Apparent changes in vascularization or luteinization of the treated follicles in the absence of ovulation

[b]Not applicable

[c]Control group

[d]Significant difference ($p < 0.05$) from group marked c

of subsequent cycles. When HCG was injected into FSH-stimulated follicles, there was no induction of ovulation observed except in one animal whose ovulation occurred four days following the treatment making any correlation between injection and effect questionable. There was, however, a significant increase in the menstrual cycle length of the animals receiving intrafollicular HCG when compared to the group mean of their previous cycle lengths. Table 8 illustrates these effects of treatment on menstrual cyclicity. Furthermore, the injection of HCG into one follicle in each ovary (two follicles per animal) did not significantly increase the cycle length over that seen in animals having only one treated follicle. As observed previously in the squirrel monkey, the apparent increases in follicular vascularization and luteal coloration were also quite evident following this treatment (Table 3).

These results suggested the possibility that the intrafollicular gonadotropin administration was causing follicular luteinization (in the macaques in the absence of ovulation) which may then be lengthening the cycle. To test this hypothesis, peripheral plasma levels of progesterone were assayed.

Figure 4 illustrates the plasma progesterone levels in the normal cycle of six Macaca fascicularis females. During these cycles the animals were laparoscoped 4 to 10 times each (mean = 6.3), and the cycles were adjusted to the day of ovulation (mean = 14.6). Following intrafollicular

TABLE 8

Effects of Exogenous Gonadotropin Administrations on the Length of the *Macaca fascicularis* Menstrual Cycle

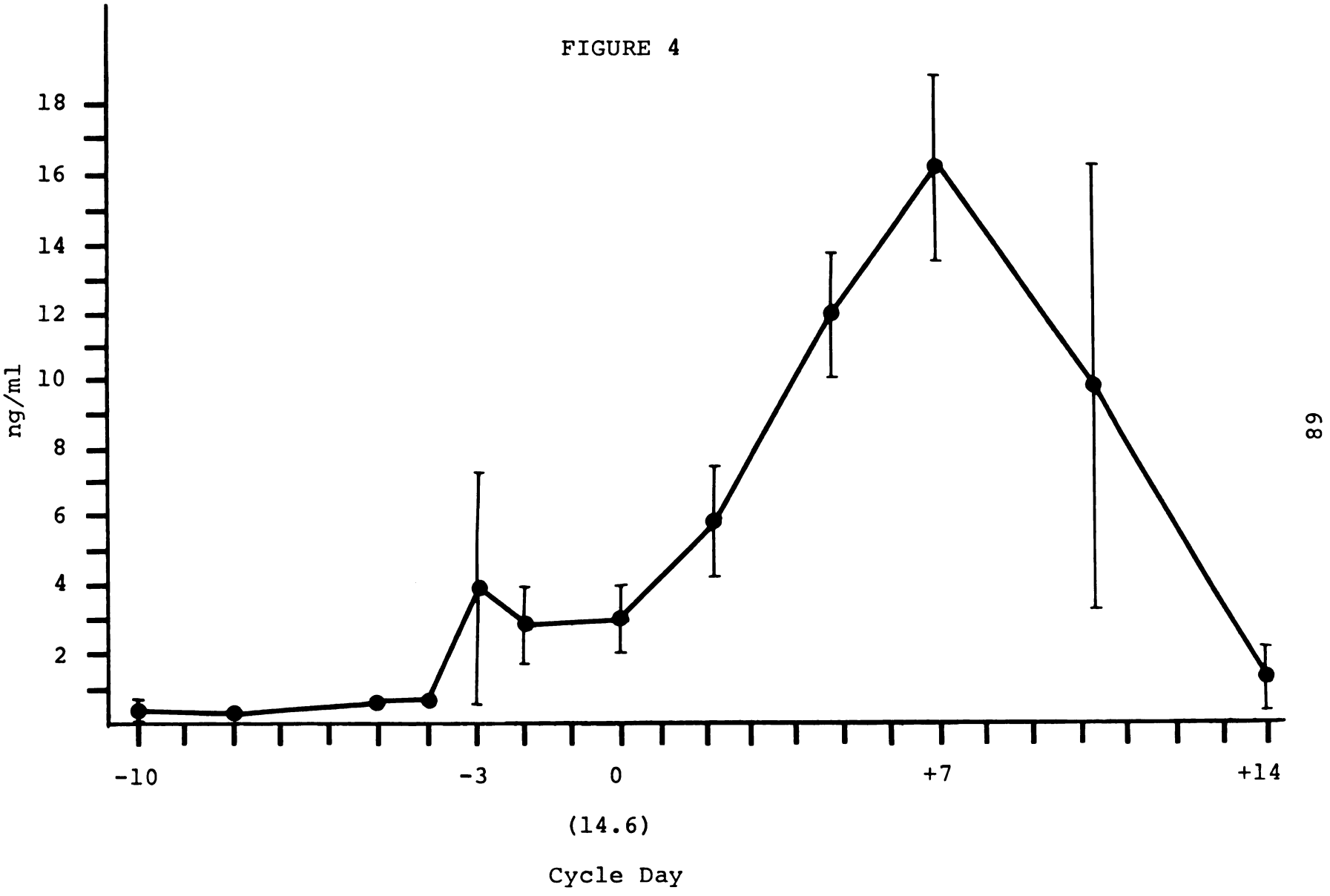
Treatment	N	Treated Cycle Length	Subsequent Cycle Length
FSH only (5 days/5 mg per day)	7	31.9/30.7	30.7/30.7
FSH + IF HCG 2 IU into one follicle per animal	6	40.7/32.2	30.6/32.2
FSH + IF HCG 2 IU into one follicle per ovary	5	47.8/30.33	39.8/30.33
Total FSH + IF HCG	11	43.9[a]/31.2[a]	35.6/31.2

[a]Significant differences exist ($p < 0.05$)

FIGURE 4

Plasma Progesterone Levels in Macaca fascicularis $\bar{x}$ ± S.E. (in 6 animals)

FIGURE 4



injection of 2 IU of HCG the plasma progesterone levels of six macaques were not different (Figure 5) from the normal follicular plasma levels seen in this species.

Studies Using Biochemical Intermediates

The success of the laparoscopic intrafollicular injection technique in the squirrel monkey allowed utilization of this procedure for the evaluation of the ovulation-inducing ability of several biochemical compounds which are currently thought to serve as mediators of the follicular rupture inducing properties of luteinizing hormone. Table 9 demonstrates the results obtained with several of these compounds. From these data it can be seen that the intrafollicular administration of $PGF_{2\alpha}$ induced ovulation in a significantly higher proportion of treated animals than the control injections. This treatment also resulted in the frequent observation of an increased level of follicular vascularity. PGE_2 did not stimulate follicular rupture in a significant number of animals nor did it cause the profound alterations of follicular morphology seen following $PGF_{2\alpha}$. Similarly, neither cyclic AMP nor dibutyryl cyclic AMP caused ovulation of the mature ovarian follicle in this animal. Dibutyryl cAMP did appear to induce a luteal coloration of the treated follicles in a high proportion of the animals. The majority of the ovulations observed following prostaglandin treatments occurred within 24 hours of the treatment.

FIGURE 5

Plasma Progesterone Levels Following Intrafollicular Injection of HCG (2 IU) in *Macaca fascicularis*
$\bar{x}$ ± S.E. (in 6 animals)

FIGURE 5

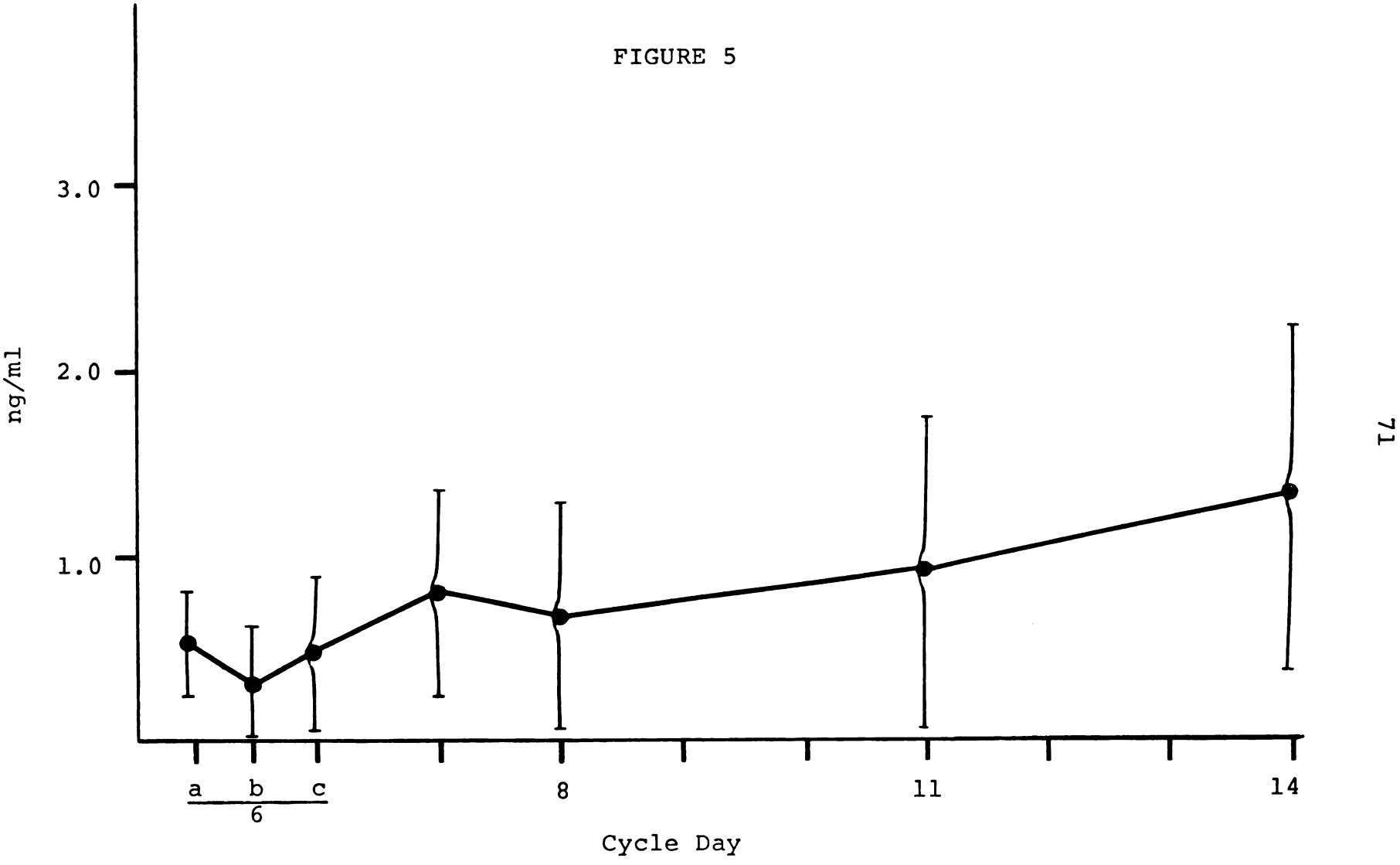


TABLE 9

Effects of Intrafollicular Injections of Biochemical Intermediates in the Saimiri sciureus

Treatment	Ovulation	Morphological Alterations[a]
FSH ± IF $PGF_{2\alpha}$ (10 µg)	4/10[b]	7
Control follicles	0/10	1
FSH ± IF PGE_2 (10 µg)	3/10	2
Control follicles	1/10	1
FSH ± IF cyclic AMP (0.6 µM)	0/10	3
Control follicles	0/10	1
FSH ± IF db cyclic AMP (o.6 µM)	1/10	6
Control follicles	0/10	0

[a]Apparent changes in vascularization or luteinization of the treated follicles in the absence of ovulation

[b]$p < 0.05$

DISCUSSION

The cynomolgus macaques used in these studies were members of a colony being observed for the assessment of normal reproduction of this species. Four years of data analysis has demonstrated that the mean menstrual cycle length of the members of this colony is 30.9 days (± 0.21 days). This value agrees with those reported by Fujiwara et al. (1969), Mahoney (1970) and MacDonald (1971). Therefore, Macaca fascicularis has a longer cycle than either its well-studied relative, Macaca mulatta, or the human female.

Laparoscopy has been shown to be an effective technique for the prediction and timing of ovulation in the nonhuman primate, and the criteria for morphological definition of ovulation has been well documented (Rawson & Dukelow, 1973; Harrison & Dukelow, 1974; Dukelow, 1975). Additionally, it has been shown that frequent laparoscopic observations do not impose adverse effects on either reproductive cyclicity or the occurrence of ovulation (Rawson, 1973; Rawson & Dukelow, 1973). Further evidence that laparoscopy has little effect on the endocrine cyclicity of the

macaque, is the present observation that progesterone profiles in 6 animals receiving an average of 6.5 laparoscopic examinations per cycle are similar to those seen in untreated animals. The present study utilized laparoscopy to demonstrate that at least 90% of all menstrual cycles observed were ovulatory, with the left ovary ovulating more frequently than the right.

The lack of a significant relationship between the day of ovulation (i.e., the length of the follicular phase) and the length of the menstrual cycle suggests that the luteal phase length of this species is no more consistent than is its follicular phase length, since we would expect a linear plot with a positive slope if the luteal phase length was more regular than the follicular phase length.

The occurrence of a peak ovulatory response of the squirrel monkey eight days following their previous ovulation is in agreement with the concept of a 7 to 9 day cycle length in this species. This interval agrees with the indirect evidence presented by Rosenblum et al. (1967) and Gould et al. (1973), as well as the previous laparoscopic observations of Harrison and Dukelow (1974). The present study demonstrates that HCG, when administered at the appropriate interval to nonprimed animals, will induce the same ovulation rate seen following the standard regime including FSH. If this is the case, one would expect to see a return to this level of ovulation at periodic 8 to 9 day intervals. The lack of a clear return to such levels

on days 16 to 18 of this study may represent interanimal variation as the effect of the synchronizing ovulatory regime becomes further away and the animals begin to exhibit the cycle lengths characteristic of each individual. Regardless, although a full recovery is not observed, there is a trend towards an increased response at day 18.

Laparoscopic evaluations of follicular development following FSH administration to the squirrel monkey, demonstrated that neither 2 nor 3 days of FSH pretreatment were sufficient to induce normal follicular development, while 4 days proved adequate. It was also shown that FSH pretreatment would not stimulate ovulation alone, making this a good model for the study of ovulation induction. The superiority of the 4 day FSH treatment lent further support to the ovulation-induction regime of Dukelow (1970).

Unlike the case in the squirrel monkey, where the FSH regime was begun on a randomly selected day in the cycle, the FSH was administered beginning on day 1 of the macaques menstrual cycle. This allowed differentiation of the treatment effects from spontaneous follicular growth and ovulation. The inability of this regime to stimulate normal appearing follicular growth was unexpected. Unfortunately, our animal numbers were too low to allow the establishment of the cause for this inactivity, an important difference between the ovarian physiology of the macaque and Saimiri.

The lack of local effect of gonadotropin saturated disks is believed due to the short period of contact with

the ovary. Aratei (1972) proposed a theoretical analysis of the permeability of the ovarian follicular membranes and suggested that the transfer of a protein across these membranes was time dependent. This concept would suggest that if the disks could be held in place for a longer interval the expected effects might be seen. The results of an experiment utilizing in vitro culture of porcine follicles (Appendix I) are supportive of this hypothesis.

Injection directly into the ovarian follicle was a method which resulted in exposure of the follicle to gonadotropin sufficient to produce an effect. The ovulation rate in non-pretreated squirrel monkeys following intrafollicular LH; while higher than females receiving intrafollicular saline, FSH pretreatment only, or the FSH pretreatment followed by intrafollicular saline; did not compare with the rates of animals receiving an FSH pretreatment followed by intrafollicular administration of LH. This is further evidence of the necessity of both FSH and LH as synergists for the induction of ovulation in the primate.

The inability of saline injections to cause mechanically induced follicle rupture is consistent with the reports of earlier advocates of similar techniques (Espey & Lipner, 1963; Blanchette, 1966; Ferrando & Nalbandov, 1969) all of whom utilized either greater injection volumes or larger gauge needles than the present studies. Injection of an ink marker demonstrated that injected

substances do enter the follicular antrum and are trapped there without escape. Thus, this system allows the evaluation of the effect of various substances on ovulation when applied directly at the follicular level.

The sufficiency of ovine-LH for ovulation induction in the squirrel monkey administered by this method suggests two possible conclusions. The first, that this species does not have a specific requirement for an LH source of primate origin. This is refuted by the inability of systemic ovine-LH to stimulate ovulation in similar animals. Also, this hypothesis ignores the previous experience with ovulation induction in the nonhuman primate. The second hypothesis is that this species-specificity is at an extrafollicular level, possibly relating to binding in the circulatory system or some type of reactivity at the blood-follicle barrier.

As stated previously, the poor response of the macaque to this procedure demonstrates an important difference between these two species. An effect was definitely indicated by the significant increase in the length of the menstrual cycles, but there did not appear to be the increase in circulating progesterone levels that would be expected if luteinization were being induced by the procedure. It is possible that the poor response observed in macaques relates to an inappropriate stimulation of follicular growth seen following FSH. This would agree with the conclusion of Knobil et al. (1959), who suggested that

excessive follicle stimulation achieved too rapidly would inhibit ovulation in response to a subsequent ovulating dose of chorionic gonadotropin.

Whether follicular rupture results from a degradation of the wall of the follicle or from the contraction of smooth muscle elements, the initial stimulus is known to be LH. The mechanism of this action of LH at the ovarian level has recently been an area of extensive investigation. In 1970, it was suggested that prostaglandins might be obligatory intermediates of the action of LH on the mammalian ovary (Kuehl, et al., 1970). Subsequently, Armstrong et al. (1972) demonstrated a role of these compounds in ovulation, when an inhibitor of prostaglandin synthesis (indomethacin) was shown to inhibit follicular rupture in the rabbit. The present studies have shown that $PGF_{2\alpha}$ administered directly into the ovarian follicle of the squirrel monkey mimicked the induction of follicular rupture observed following administration of an LH source. This is evidence corroborating the possibility of an intermediary role of $PGF_{2\alpha}$ in the LH induction of ovulation in the nonhuman primate. This concept is supported recently by studies in the mouse (Lau, et al., 1974) and in the rabbit (Richman, et al., 1974). Although PGE_2 caused an increase in the ovulation rate following intrafollicular administration, it was unable to stimulate significantly greater levels than the control values.

This partial effect of PGE_2 has also been observed by Moon and Armstrong (1974) who demonstrated that while $PGF_{2\alpha}$ antiserum effectively inhibited ovulation in the rabbit, anti-PGE_2 was effective in only 43% of the animals. In the mouse Saksena et al. (1974) found PGE_2 to be more effective than $PGF_{2\alpha}$ in the reversal of an indomethacin blockade of ovulation. The latter experiment, however, was conducted with systemic administration of the drugs and may have represented a different level of activity.

The demonstration that cAMP caused an LH-like stimulation of progesterone synthesis by slices from bovine corpora lutea (Savard et al., 1965) was strong evidence that cAMP served as an intermediate in the action of this hormone. That prostaglandins would stimulate cAMP accumulation by the ovaries suggested that prostaglandins may also be implicated in this hormone action (Kuehl, et al., 1970). There is good evidence supporting the role of cAMP in the LH-stimulation of luteinization (Marsh & Savard, 1966; Miller and Keyes, 1974; Channing, 1974). There is, however, little direct evidence supporting a role of cAMP in the process of ovulation. The experiments reported here refute the proposed intermediary role of cAMP in LH-induced ovulation in the primate. Our results concur with the observations of LeMaire et al. (1972) who demonstrated an inhibition of LH-induced ovulation in the rabbit by the intrafollicular administration of either cAMP or dibutyryl

cAMP. These authors proposed that the intrafollicular cAMP may act normally to inhibit luteinization of the follicle.

The present experiments have involved the local administration of various substances to the primate ovarian follicle to evaluate the direct effect of each compound. The results suggest that prostaglandins (especially $PGF_{2\alpha}$) may be involved in the biochemical sequence of events culminating in follicular rupture. Although cAMP did not appear to be involved in this process, there can be no doubt that it is present and functional in the ovarian follicle. The present studies support the theory proposed by LeMaire that the ovulation-inducing properties of LH may be mediated by prostaglandins while cAMP may serve as an intermediate for the action of LH on oocyte maturation or luteinization.

The laparoscopic technique developed and used in these studies offers a method for evaluating the influence of various chemical compounds on the processes involved in ovulation in the nonhuman primate without submitting the animal to the stress of a major surgical procedure. Further use of this technique will increase our understanding of the follicular mechanisms involved in ovulation.

SUMMARY AND CONCLUSIONS

Several parameters of reproductive function were studied in two species of nonhuman primates, with an emphasis on the development of a primate model for the study of follicular physiology. The results of the present experiments indicate the following conclusions.

In the squirrel monkey, ovulation can be induced with HCG in the absence of an FSH pretreatment at 7 to 9 day intervals following synchronization. There is a trend toward another increase in ovulation rate between 16 and 18 days following synchronization which would agree with the concept of a short reproductive cycle length (7 to 9 days) for this species.

Analysis of data on the reproductive cycle of the Macaca fascicularis, collected over a four year span, is in accord with previous observations of a 30 day menstrual cycle, having a 14 day follicular phase. Almost 90% of the cycles studied were shown to be ovulatory, with more ovulations occurring on the left ovary than on the right. Follicle rupture was observed in four cases, three of which were accompanied by bleeding from the site of ovulation.

Treatment of the Saimiri sciureii with 4 daily, 1 mg injections of FSH was shown to result in apparently normal follicular development. Similar treatment (5 days, 5 mg/day) in the Macaca fascicularis did not induce normal appearing follicular growth, as there was a high incidence of cystic and hemorrhagic follicles.

A technique was developed for the assessment of the ability of various compounds to mimic the ovulation-inducing properties of luteinizing hormone. This procedure involved the injection of these substances directly into the antrum of the preovulatory follicle. When administered in this manner, ovine LH was shown to stimulate an ovulation rate similar to that seen following systemic administration of human chorionic gonadotropin. Intrafollicular injections of an LH from a primate source (HCG) did not cause a higher ovulation rate than an LH from a nonprimate source, which suggested that the reported species specificity of response of the primates to LH was at a suprafollicular level.

Intrafollicular injections of $PGF_{2\alpha}$ were able to induce ovulation rates that were similar to those seen following LH administration. This is in agreement with the proposed mediation of this prostaglandin in the ovulation-inducing mechanism of LH. Prostaglandins E_2, cyclic 3',5' AMP, and dibutyryl cyclic 3',5' AMP were unable to stimulate such levels.

Intrafollicular injection of HCG in the FSH-primed macaque would not induce ovulation, apparently due to the

abnormal follicular development induced by the pretreatment. Neither did this treatment have any effect on the plasma progesterone levels of this species. Menstrual cycle lengths of these animals were, however, significantly longer than their previous cycles with a return to normalcy during the first post-treatment cycle.

REFERENCES

REFERENCES

Aratei, H. Ovarian follicle and permeability of the membrane. Rev. Roum. Endocrinol 9:79-85, 1972.

Armstrong, D.T., D.L. Grinwich, Y.S. Moon, and J. Zamecnik. Inhibition of ovulation in rabbits by intrafollicular injection of indomethacin and prostaglandin F antiserum. Life Sciences 14:129-140, 1974.

Armstrong, D.T., Y.S. Moon, and D.L. Grinwich. Possible role of prostaglandins in ovulation. Advances in the Biosciences 9:709-715, 1972.

Balin, H., and L.S. Wan. A study of induction of ovulation in Macaca mulatta. The Journal of Reproductive Medicine: Lying-In 2:273-284, 1969.

Batta, S.K., and B.G. Brackett. Ovulation induction in rhesus monkeys by treatment with gonadotropins and prostaglandins. Prostaglandins 6:45-54, 1974.

Bennet, J.P. The induction of ovulation in the squirrel monkey (Saimiri sciureus) with pregnant mares serum (PMS) and human chorionic gonadotrophin (HCG). J. Reprod. Fert. 13:357, 1967.

Betteridge, K.J. Unilateral stimulation of bovine ovaries by local injection of pregnant mare's serum gonadotrophin. J. Reprod. Fert. 37:101-104, 1974.

Blanchette, E.J. Ovarian steroid cells. I. Differentiation of the lutein cell from the granulosa follicle cell during the preovulatory stage and under the influence of exogenous gonadotropins. J. Cell Biol. 31:501-516, 1966.

Blandau, R. in Greenblatt, R.B., ed. Ovulation, J.B. Lippincott Company, Philadelphia, 1966.

Blandau, R.J., and R.E. Rumery. Measurements of intrafollicular pressure in ovulatory and preovulatory follicles of the rat. Fert. Ster. 14:330-341, 1963.

Breckwoldt, M., G. Bettendorf, and C-R. Garcia. Induction of ovulation in noncycling and hypophysectomized rhesus monkeys with various human gonadotropins. Fert. Ster. 22:451-455, 1971.

Butler, H. Evolutionary trends in primate sex cycles. Contrib. Primat. 3:2-35, 1974.

Castellanos, H., and H.L. McCombs. The reproductive cycle of the new world monkey: Gynecological problems in a breeding colony. Fert. Ster. 19:213-227, 1968.

Channing, C.P. Stimulatory effects of prostaglandins upon luteinization of rhesus monkey granulosa cell cultures. Prostaglandins 2:331-349, 1972.

Channing, C.P. Effects of prostaglandin inhibitors, 7-OXA-13-prostynoic acid and eicosa-5,8,11,14-tetraynoic acid, upon luteinization of rhesus monkey granulosa cells in culture. Prostaglandins 2:351-367, 1972.

Channing, C.P. Temporal effects of LH, HCG, FSH and dibutyryl cyclic 3'-5'-AMP upon luteinization of rhesus monkey granulosa cells in culture. Endocrinol. 94:1215-1223, 1974.

Channing, C.P., and T.M. Crisp. Comparative aspects of luteinization of granulosa cell cultures at the biochemical and ultrastructural levels. General and Comparative Endocrinology, Suppl. 3:617-625, 1972.

Channing, C.P., and S. Kammerman. Binding of gonadotropins to ovarian cells. Biol. of Reprod. 10:179-198, 1974.

Claesson, L. Is there any smooth musculature in the wall of the Graafian follicle? Acta Anat. 3:295-311, 1947.

Coutinho, E.M., and H.S. Maia. The contractile response of the human uterus, fallopian tubes, and ovary to prostaglandins in vivo. Fert. Ster. 22:539-543, 1971.

Das, C., and G.P. Talwar. Inability of adenosine 3':5'-cyclic monophosphate to stimulate fully the effects of LH in rabbit ovaries. J. Reprod. Fert. 37:413-416, 1974.

Dede, J.A., and A.A. Plentl. Induced ovulation and artificial insemination in a rhesus colony. Fert. Ster. 17:757-764, 1966.

Denniston, R.H. Notes on the vaginal cornification cycle of captive squirrel monkeys. J. Mammal. 46:471, 1964.

Dukelow, W.R. Induction and timing of single and multiple ovulations in the squirrel monkey (Saimiri sciureus). J. Reprod. Fert. 22:303-309, 1970.

Dukelow, W.R. The morphology of follicular development and ovulation in nonhuman primates. J. Reprod. Fert., Suppl. 22:

Ellsworth, L.R., and D.T. Armstrong. Inhibition of luteinization of transplanted rat ovarian follicles by polyphloretin phosphate. Endocrinol. 94:892-896, 1974.

Engle, E.T. Biological differences in response of the female macacus monkey to extracts of the anterior pituitary and of human pregnancy urine. Am. J. Physiol. 106:145-155, 1933.

Engle, E.T. Luteinization of the ovary of the monkey by means of combined use of anterior pituitary extract and an extract of pregnancy urine. Endocrinol. 18:513-520, 1934.

Engle, E.T., and C. Hamburger. Action of gonadotropic hormone from pregnant mare's serum on ovaries of Rhesus monkeys. Proc. Soc. Exp. Biol. Med. 32:1531-1533, 1935.

Espey, L.L. The Mechanism of Mammalian Ovulation, Ph.D. Thesis, Florida State University, 1964.

Espey, L.L. Ultrastructure of the apex of the rabbit Graafian follicle during the ovulatory process. Endocrinol. 81:267-276, 1967.

Espey, L.L. Ovarian proteolytic exzymes and ovulation. Biol. of Reprod. 10:216-235, 1974.

Espey, L.L., and H. Lipner. Measurements of intrafollicular pressures in the rabbit ovary. Amer. J. Physiol. 205: 1067-1072, 1963.

Espey, L.L., and H. Lipner. Enzyme-induced rupture of rabbit Graafian follicle. Amer. J. Physiol. 208: 208-213, 1965.

Fajer, A.B., and D. Bechini. Pregnenolone and progesterone concentrations in ovarian venous blood of the artificially ovulated squirrel monkey (Saimiri sciureus). J. Reprod. Fert. 27:193-199, 1971.

Ferrando, G., and A.V. Nalbandov. Direct effect on the ovary of the adrenergic blocking drug dibenzyline. Endocrinol. 85:38-42, 1969.

Fujiwara, T., S. Honjo, and K. Imaizumi. Practice of the breeding of cynomolgus monkeys (Macaca irus) under laboratory conditions. Exp. Animals 18:29-40, 1969.

Goldenberg, R.L., E.O. Reiter, J.L. Vaitukaitis, and G.T. Ross. Hormonal factors influencing ovarian uptake of human chorionic gonadotropin. Endocrinol. 92:1565-1567, 1973.

Goldstein, A. Biostatistics. An Introductory Text, The Macmillan Company, New York, 1964.

Gould, K.G., E.M. Cline, and W.L. Williams. Observations on the induction of ovulation and fertilization in vitro in the squirrel monkey (Saimiri sciureus). Fert. Ster. 24:260-268, 1973.

Guttmacher, M.S., and A.F. Guttmacher. Morphological and physiological studies of the musculature of the mature Graafian follicle of the sow. Bull. Johns Hopkins Hosp. 32:394-399, 1921 (from Espey, 1974).

Harms, P.G., S.R. Ojeda, and S.M. McCann. Prostaglandin-induced release of pituitary gonadotropins: Central nervous system and pituitary sites of action. Endocrinol. 94:1459-1464, 1974.

Harrison, R.M. Ovulation in Saimiri sciureus: Induction, Detection and Inhibition, Ph.D. Thesis, Michigan State University, 1973.

Harrison, R.M., and W.R. Dukelow. Morphological changes in the Saimiri sciureus ovarian follicles as detected by laparoscopy. Primates 15:305-309, 1974.

Hartman, C.G. The use of gonadotropic hormones in the adult rhesus monkey. Bull. Johns Hopkins Hosp. 63: 351, 1938.

Hartman, C.G. Further attempts to cause ovulation by means of gonadotropes in the adult rhesus monkey. Contr. Embryol. 30:113, 1942.

Haynes, R.C., Jr., E.W. Sutherland, and T.W. Rall. The role of cyclic adenylic acid in hormone action. Recent Progr. Hormone Res. 16:121-132, 1960.

Hutchinson, T.C. Vaginal cytology and reproduction in the squirrel monkey (Saimiri sciureus). Folia primat. 12:212-223, 1970.

Jainudeen, M.R., and E.S.E. Hafez. Gonadotrophin-induced multiple ovulation in the crab-eating monkey, Macaca fascicularis. J. Reprod. Fert. 33:151-154, 1973.

Jewett, D.A., and W.R. Dukelow. Follicular morphology in Macaca fascicularis. Folia Primat. 16:216-220, 1971.

Jones, E.E., and A.V. Nalbandov. Effects of intrafollicular injection of gonadotrophins on ovulation or luteinization of ovarian follicle. Biol. Reprod. 7:87-93, 1972.

Kelly, G.L. Direct observations of rupture of Graafian follicles in the mammal. J. Fla. Med. Ass. 17:422-423, 1931 (from Espey, 1974).

Kennelly, J.J., and R.H. Foote. Superovulatory response of pre- and post-pubertal rabbits to commercially available gonadotropins. J. Reprod. Fertil. 9:177-188, 1965.

Keyes, P.L., G.D. Canastar, and J.B. Miller. Studies on luteinization of transplanted rabbit follicles exposed to LH, Actinomycin D, or Adenosine 3',5'-cyclic monophosphate in vitro. Endocrinol. 91:197-205, 1972.

Knobil, E., J.L. Kostyo, and R.O. Greep. Production of ovulation in the hypophysectomized rhesus monkey. Endocrinol. 65:487-493, 1959.

Knobil, E., A. Morse, and R.O. Greep. The effects of beef and monkey pituitary growth hormone on the costochondral junction in the hypophysectomized rhesus monkey. Anat. Record 124:320, 1956.

Koering, M.J. Comparative morphology of the primate ovary. Contrib. Primat. 3:38-81, 1974.

Kolena, J., and C.P. Channing. Stimulatory effects of LH, FSH and prostaglandins upon cyclic 3',5'-AMP levels in porcine granulosa cells. Endocrinol. 90:1543-1550, 1972.

Kuehl, F.A., Jr., J.L. Humes, J. Tarnoff, V.J. Cirillo, and E.A. Ham. Prostaglandin receptor site: Evidence for an essential role in the action of luteinizing hormone. Science 169:883-885, 1970.

Labhsetwar, A.P. Neuroendocrine basis of ovulation in hamsters treated with prostaglandin $F_{2\alpha}$. Endocrinol. 92:606-610, 1973.

Lamprecht, S.A., U. Zor, A. Tsafriri, and H.R. Lindner. Action of prostaglandin E_2 and luteinizing hormone on cyclic adenosine 3',5'-monophosphate production and protein kinase activity in fetal, early postnatal and adult rat ovaries. Israel Journal of Medical Sciences 7:704, 1971.

Lamprecht, S.A., U. Zor, A. Tsafriri, and H.R. Lindner. Action of prostaglandin E_2 and of luteinizing hormone on ovarian adenylate cyclase, protein kinase and ornithine decarboxylase activity during postnatal development and maturity in the rat. J. Endocrinol. 57:217-233, 1973.

Lang, C.M. The estrous cycle of the squirrel monkey (Saimiri sciureus). Lab. Anim. Care 17:442-451, 1967.

Lau, I.F., S.K. Saksena, and M.C. Chang. Prostaglandins F and ovulation in mice. J. Reprod. Fert. 40:467-469, 1974.

LeMaire, W.J., and J.M. Marsh. Interrelationship between prostaglandins, cyclic AMP and steroids in ovulation. J. Reprod. Fert., Suppl. 22:

LeMaire, W.J., T. Mills, Y. Ito, and J.M. Marsh. Inhibition by 3'-5'-cyclic AMP of luteinization induced by intrafollicular injection of luteinizing hormone. Biol. Reprod. 6:109-116, 1972.

LeMaire, W.J., N.S.T. Yang, H.H. Behrman, and J.M. Marsh. Ovulatory changes in the concentration of prostaglandins in rabbit Graafian follicles. Prostaglandins 3:367-375, 1973.

Lipner, H., M.A. Hirsch, N.R. Moudgal, G.J. MacDonald, S-Y. Ling, and R.O. Greep. Ovulation-inducing activity of FSH in the rat. Endocrinol. 94:1351-1358, 1974.

Louis, T.M., H.D. Hafs, and B.E. Seguin. Progesterone, LH, estrus and ovulation after prostaglandin $F_{2\alpha}$ in heifers. Proc. Soc. Exp. Biol. Med. 143:152-155, 1973.

MacDonald, G.J. Reproductive patterns of three species of macaques. Fert. Ster. 22:373-377, 1971.

Mahoney, C.J. Induction of ovulation in Macaca irus with clomiphene citrate and gonadotrophic hormones. J. Reprod. Fert. 23:529-531, 1970.

Marsh, J.M., R.W. Butcher, K. Savard, and E.W. Sutherland. The stimulatory effect of luteinizing hormone on adenosine 3',5'-monophosphate accumulation in corpus luteum slices. The Jour. of Biol. Chem. 241:5436-5440, 1966.

Marsh, J.M., T.M. Mills, and W.J. LeMaire. Cyclic AMP synthesis in rabbit Graafian follicles and the effect of luteinizing hormone. Biochim. et Biophys. Acta 273:389-394, 1972.

Marsh, J.M., T.M. Mills, and W.J. LeMaire. Preovulatory changes in the synthesis of cyclic AMP by rabbit Graafian follicles. Biochim. Biophys. Acta 304: 197-202, 1973.

Marsh, J.M., and K. Savard. The stimulation of progesterone synthesis in bovine corpora lutea by adenosine 3',5'-monophosphate. Steroids 8:133-148, 1966.

Mason, N.R., R.J. Schaffer, and R.E. Toomey. Stimulation of cyclic AMP accumulation in rat ovaries in vitro. Endocrinol. 93:34-41, 1973.

Miller, J.B., and P. Landis Keyes. Initiation of luteinization in rabbit Graafian follicles by dibutyryl cyclic AMP in vitro. Endocrinol. 95:253-259, 1974.

Moon, Y.S., and D.T. Armstrong. Evidence for a role of prostaglandin $F_{2\alpha}$ in ovulation. Fed. Proc. 33:435, 1974.

Nalbandov, A.V., L.W.L. Kao, and E.E. Jones. Effects of intrafollicular injection of hormones and drugs. J. Reprod. Fert., Suppl. 18:15-22, 1973.

Nuti, L.C., W.H. McShan, and R.K. Meyer. Effect of ovine FSH and LH on serum steroids and ovulation in hypophysectomized immature female rats. Endocrinol. 95:682-689, 1974.

Okamura, H., P. Virutamasen, K.H. Wright, and E.E. Wallach. Ovarian smooth muscle in the human being, rabbit and cat. Histochemical and electron microscopic study. Amer. J. Obstet. Gynec. 112:183, 1972.

O'Shea, J.D. An ultrastructural study of smooth muscle-like cells in the theca externa of ovarian follicles in the rat. Anat. Rec. 167:127, 1970.

Ovadia, J., J.W. McArthur, O.W. Smith, and J. Bashir-Farahmand. An individualized technique for inducing ovulation in the bonnet monkey. Macaca radiata. J. Reprod. Fert. 27:13-23, 1971.

Palti, Z., and M. Freund. Spontaneous contractions of the human ovary in vitro. J. Reprod. Fert. 28:113-115, 1972.

Pearson, E.S., and H.O. Hartley, eds. Biometrika Tables for Staticians, Vol. I, Cambridge University Press, 1958.

Rawson, J.M.R. Ovulation in Macaca fascicularis, M.S. Thesis, Michigan State University, 1973.

Rawson, J.M.R., and W.R. Dukelow. Effect of laparoscopy and anesthesia on ovulation, conception, gestation, and lactation in a Macaca fascicularis. Lab. Primate Newsl. 12:4, 1973a.

Rawson, J.M.R., and W.R. Dukelow. Observation of ovulation in Macaca fascicularis. J. Reprod. Fert. 34:187, 1973b.

Richman, K.A., K.H. Wright, and E.E. Wallach. Local ovarian effects of prostaglandins E_2 and $F_{2\alpha}$ on human chorionic gonadotropin-induced ovulation in the rabbit. Obstet. Gynec. 43:203-210, 1974.

Rodbard, D. Mechanics of ovulation. J. Clin. Endocrinol. 28:849-861, 1968.

Rondell, P. Follicular pressure and distensibility in ovulation. Amer. J. Physiol. 207:590-594, 1964.

Rondell, P. Biophysical aspects of ovulation. Biol. of Reprod., Suppl. 2:64-89, 1970.

Rosenblum, L.A., T. Nathan, J. Nelson, and I.C. Kaufman. Vaginal cornification cycles in the squirrel monkey (Saimiri sciurea). Folia primatol. 6:82, 1967.

Ross, G.T. Gonadotropins and preantral follicular maturation in women. Fert. Ster. 25:522-543, 1974.

Rouget, C.I. Recherches sur les organes erectiles de la femme et sur l'appareil musculaire tubo-ovarian dans leurs rapports avec l'ovulation et la menstruation. J. Physiol (Paris) 1:320-343, 1958 (from Espey, 1974).

Rugh, R. Ovulation in the frog. II. Follicular rupture to fertilization. J. Exp. Zool. 71:163-193, 1935.

Ryle, M. The growth in vitro of mouse ovarian follicles of different sizes in response to purified gonadotropins. J. Reprod. Fert. 30:395-405, 1972.

Saksena, S.K., I.F. Lau, and A.A. Shaikh. Cyclic changes in the uterine tissue content of F-prostaglandins and the role of prostaglandins in ovulation in mice. Fert. Ster. 25:636, 1974.

Sato, T., T. Iesaka, T. Jyujo, K. Taya, J. Ishikawa, and M. Igarashi. Prostaglandin-induced ovarian ascorbic acid depletion. Endocrinol. 96:417-420, 1974.

Savard, K., J.M. Marsh, and B.F. Rice. Gonadotropins and ovarian steroidogenesis. IV. Steroid sex hormones. Recent Progress in Hormone Research 21:285-365, 1965.

Schochet, S.S. A suggestion as to the process of ovulation and ovarian cyst formation. Anat. Rec. 10:447-457, 1916 (from Espey, 1974).

Schwartz, N.B., K. Krone, W.L. Talley, and C.A. Ely. Administration of antiserum to ovine FSH in the female rat: Failure to influence immediate events of cycle. Endocrinol. 92:1165-11974, 1973.

Shaikh, A.A., and E.L. Klaiber. Effects of sequential treatment with estradiol and $PGF_{2\alpha}$ on the length of the primate menstrual cycle. Prostaglandins 6:253-262, 1974.

Siegler, S.L., and M.J. Fein. Studies on artificial ovulation with the hormone of pregnant mare's serum. Am. J. Obstet. Gynec. 38:1021-1036, 1939.

Simpson, M.E., and G. van Wagenen. Induction of ovulation with human urinary gonadotrophins in the monkey. Fert. Ster. 13:140-152, 1962.

Srivastava, P.K., F. Cavazos, and F.V. Lucas. Biology of reproduction in the squirrel monkey (Saimiri sciureus): I. The estrus cycle. Primates 11:125-134, 1970.

Tsafriri, A., H.R. Lindner, U. Zor, and S.A. Lamprecht. In vitro induction of meiotic division in follicle-enclosed rat oocytes by LH, cyclic AMP and prostaglandin E_2. J. Reprod. Fert. 31:39-50, 1972.

Tsafriri, A., H.R. Lindner, U. Zor, and S.A. Lamprecht. Physiological role of prostaglandins in the induction of ovulation. Prostaglandins 2:2-10, 1972.

Valerio, D.A., and K.D. Courtney. Treatment of infertility in Macaca mulatta with clomiphene citrate. Lab. Anim. Care 18:339-345, 1968.

van Wagenen, G. Induction of ovulation in Macaca mulatta. Fert. Ster. 19:15-29, 1968.

van Wagenen, G., and H.H. Cole. Failure of continued injections of gonadotrophic hormones to prevent menstrual cycles and pregnancy in Macaca mulatta. Am. J. Physiol. 123:208, 1938.

van Wagenen, G., and M.E. Simpson. Induction of multiple ovulation in the rhesus monkey (Macaca mulatta). Endocrinol. 61:316-318, 1957.

van Wagenen, G., and M.E. Simpson. Postnatal Development of the Ovary in Homo sapiens and Macaca mulatta and Induction of Ovulation in the Macaque, Yale University Press, New Haven and London, 1973.

Virutamasen, P., R.L. Hickok, and E.E. Wallach. Local ovarian effects of catecholamines on human chorionic gonadotropin-induced ovulation in the rabbit. Fert. Ster. 22:235, 1971.

Virutamasen, P., K.H. Wright, and E.E. Wallach. Effects of prostaglandins E_2 and $F_{2\alpha}$ on ovarian contractility in the rabbit. Fert. Ster. 23:675-682, 1972.

Wan, L.S., and H. Balin. Induction of ovulation in rhesus monkeys: A comparative study. Fert. Ster. 20:111-126, 1969.

Wan, L.S., and H. Balin. The physiological implication of induction of ovulation in the rhesus monkey. Med. Primat. 1970:504-514, 1971.

Wilks, J.W., K.K. Forbes, and J.F. Norland. Synthesis of prostaglandin $F_{2\alpha}$ by the ovary and uterus. J. Reprod. Med. 9:271-276, 1972.

Yang, N.S.T., J.M. Marsh, and W.J. LeMaire. Prostaglandin changes induced by ovulatory stimuli in rabbit Graafian follicles. The effect of indomethacin. Prostaglandins 4:395-404, 1973.

Yang, N.S.T., J.M. Marsh, and W.J. LeMaire. Post ovulatory changes in the concentration of prostaglandins in rabbit Graafian follicles. Prostaglandins 6:27-44, 1974.

Zachariae, F. Studies on the mechanism of ovulation. Permeability of the blood-liquor barrier. ACTA Endocrinologica 27:339-342, 1958.

Zeleznik, A.J., A.R. Midgley, Jr., and L.E. Reichert, Jr. Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. Endocrinol. 95:818-825, 1974.

Zor, U., S. Bauminger, S.A. Lamprecht, Y. Knoch, P. Chobsient, and H.R. Lindner. Stimulation of cyclic AMP production in the rat ovary by luteinizing hormone: Independence of prostaglandin mediation. Prostaglandins 4:499-507, 1973.

APPENDICES

APPENDIX I

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PORCINE FOLLICLE CULTURE IN VITRO

Following the observed lack of effects of gonadotropin saturated filter paper disks on the ovarian follicle in the macaque, it was decided to investigate the possibility that this may have been due to the short exposure interval necessitated by the anesthetic limitations. Since oocyte maturation had been shown to be a more sensitive index of gonadotropin stimulation than either ovulation or corpus luteum formation (Vermeiden & Zeilmaker, 1974), experiments were designed using porcine follicles in culture for various intervals with the LH saturated disks. The maturation of the oocyte was used as a sign of gonadotropin activity.

The ovaries were obtained at slaughter; individual follicles were dissected free and placed on stainless steel wire grids in sterile petri dishes. Only those follicles protruding from the ovarian surface and greater than three mm in diameter were used. Follicles were randomly assigned to one of four groups as follows:

E1 - Follicles in contact with an LH saturated ovarian disk for one hour

C1 - Follicles in contact with saline-saturated disk for 1 hour

E24 - Follicles in contact with LH-saturated ovarian disk for 24 hours.

C24 - Follicles in contact with saline-saturated ovarian disk for 24 hours.

All four of the groups were cultured for 24 hours in media consisting of 85% Hams F10 and 15% fetal calf serum, with 100 IU of penicillin per milliliter of medium. The environment was maintained at 37 C and contained a mixture of 5% CO_2 in the air.

Following culture, the follicles were opened, the oocytes recovered and placed on clean slides. The oocytes were then fixed with 10% formalin and stained with either aceto-orecin or lacmoid. The slides were scored under a light microscope on a scorer-blind basis. Oocytes were considered to be immature if there were no distinct developmental characteristics, or if a germinal vessicle was discernible. Only those containing mitotic figures or polar bodies were classified as undergoing maturation. Any estimate of oocyte maturation obtained by this technique will be conservative and will, therefore, have a greater chance of reflecting real differences.

The percentages of oocytes that did not exhibit signs of maturation were as follows: C1, 100% (N = 10); C24, 100%

(N = 10); E1, 74% (N = 19); E24, 18% (N = 17). Analysis reveals that the group cultured with the LH disk for 1 hour had a significantly greater number of follicle-enclosed oocytes revealing no signs of maturation than did the follicles cultured with the LH disk present for 24 hours. These data suggest that the follicle wall may impose a slowly permeable barrier to LH and that 1 hour exposure to LH in the foregoing manner is not sufficient to induce ovum maturation.

APPENDIX II

APPENDIX II

LAPAROSCOPY IN THE RABBIT

During the course of these studies, the technique of laparoscopy was developed to allow frequent examination and manipulation of the reproductive tract of the rabbit. This technique allows the serial observation of ovarian, oviductal and uterine phenomena in the same rabbit. A description of the technique and an example of its use is illustrated in the following paper.

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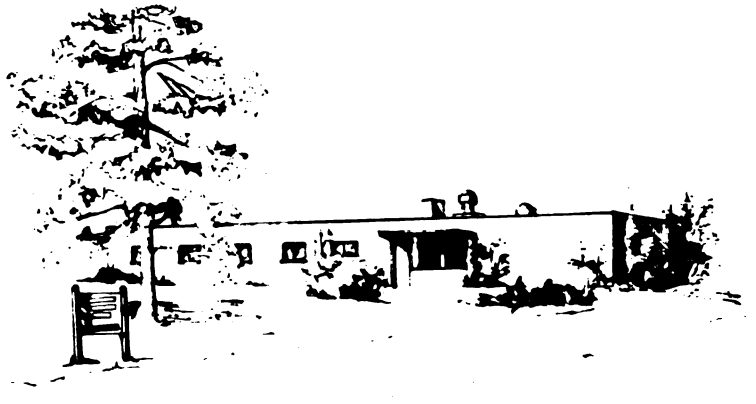
HORMONAL INFLUENCES ON THE TIME OF OVULATION IN THE RABBIT AS DETERMINED BY LAPAROSCOPY

By

S. Fujimoto, J. M. R. Rawson & W. R. Dukelow



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HORMONAL INFLUENCES ON THE TIME OF OVULATION IN THE RABBIT AS DETERMINED BY LAPAROSCOPY

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Summary. The technique of laparoscopy was modified for use in the rabbit and was found to facilitate continuous observation of follicular development and ovulation. The timing of ovulation was studied and suggests that 100 i.u. HCG, whether used in a superovulation regimen or solely as an ovulatory stimulus, causes a greater ovulation rate than does a mating stimulus. Follicular rupture was observed and frequently occurred in the smaller follicles first. Laparoscopy in pregnant rabbits suggests that quantification of preimplantation blastocysts is not only possible but also very accurate and is without deleterious effects on the developing embryos. These results suggest that the laparoscopic technique may be a valuable asset to the study of reproductive phenomena in the rabbit.

INTRODUCTION

The recent application of laparoscopy to ovulation examination, ovarian biopsy, oviducal ligation and clinical diagnosis of various reproductive disorders reflects the pioneering efforts of Semm (1969) and Balin, Wan & Rajan (1969). Previous reports have shown that ovulation in the rabbit occurs as early as 8 hr (Hill, Allen & Kramer, 1935) and as late as 13½ hr (Walton & Hammond, 1929) after mating. By contrast, Harper (1963) reported that no ovulation occurred by 10 hr but that 50% of the ovulations occurred between 10½ and 10¾ hr and 100% by 14 hr after an intravenous injection of 25 to 50 i.u. LH.

Laparoscopic observation of ovulation in rabbits has not been reported and, with the exception of the excellent cinematographic observations by Blandau (1955), few have been able to predict ovulation accurately and perform serial observation of follicular morphology near ovulation.

The present paper describes the adaptation of laparoscopy to the observation and timing of follicular development and ovulation in the rabbit.

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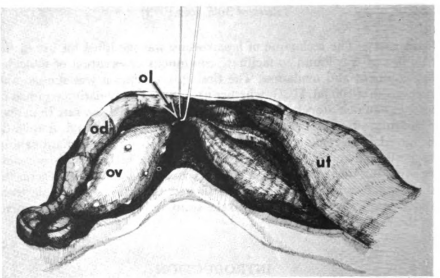
MATERIALS AND METHODS

Twenty-one, virgin, adult, female rabbits of mixed breeding and 3·4 to 4·6 kg in body weight were used. They were separately caged for at least 3 weeks before use to preclude the development of pseudopregnancy.

All rabbits were assigned to one of three treatments: *Group I, natural mating.* Six does were mated two or three times to males of proven fertility. No exogenous gonadotrophins were given; *Group II, HCG-induced ovulation.* Five does were injected intravenously with 100 i.u. HCG (A.P.L., Ayerst Laboratories); *Group III, PMSG-HCG induced ovulation.* Ten does were injected intravenously



TEXT-FIG. 1. Diagrammatic representation of the ovarian suspension technique used to facilitate ovarian observation during laparoscopy in the rabbit (ol = ovarian ligament; od = oviduct; ov = ovary; ut = uterus).

with 100 i.u. HCG 4 days after priming with 100 i.u. PMSG (Lilly Laboratories) intramuscularly.

The first laparoscopic examinations were made 8 to 9 hr after the ovulatory stimulus (mating or HCG) under sodium pentobarbitone anaesthesia.

The laparoscope was a Wolf paediatric 135° model. The laparoscopic equipment and its use in other species has been previously reported (Dukelow, Jarosz, Jewett & Harrison, 1971; Jewett & Dukelow, 1972, 1973; Rawson & Dukelow, 1973a, b). Abdominal distension by 5% CO_2/air was required in the rabbit.

For assistance in locating the ovaries, 3-0 gut sutures were threaded through the abdominal wall around the ovarian ligament and back through the wall where they were loosely tied (Text-fig. 1). Manual elevation of the external portion of the sutures made suspension and observation of the ovaries a simple procedure (Pl. 1, Fig. 1).

Serial observations were continued at intervals of 1 or 2 hr. Photographs were taken with a Canon-TL, 35 mm camera/Ektachrome EHB film at $\frac{1}{8}$ to $\frac{1}{4}$ sec exposure.

The laparoscopic technique described herein was subsequently used in four does from Group I for the counting of the potential implantation sites on Day 6 as well as the observation of the uterine vascularization associated with the site of implantation.

For ease of classification of developing follicles, five types were assigned to an alphabetical system: Type A follicles were large dome-shaped structures with a haemorrhagic appearance at the apex. Type B referred to a large dome-shaped structure without the haemorrhagic appearance. Type C was smaller, but still elevated above the ovarian surface while Type D was not elevated from the ovarian surface. Type E was used to refer to a follicle not present at the previous observation.

RESULTS

The total number of follicles, postovulatory follicles and percentage ovulation for the three groups are shown in Tables 1, 2 and 3, respectively. They are classified according to the time interval after ovulatory stimulus.

In Group I (Table 1), the initiation of ovulation was delayed and the

Table 1. Ovulation in rabbits* subjected to natural mating

Time between mating and laparoscopy (hr)	*Total no. of follicles*	*No. of postovulatory follicles*	*% ovulation*
8 to 8½	74	1	1·4
10 to 10½	86	6	7·0
12 to 12½	94	14	14·9
14 to 14½	99	25	25·3

* Group I, six does.

percentage ovulation at 14 to 14½ hr was 25·3%, that is, one half that of the other two groups (Text-fig. 2). The mean number of ovulation points per animal, 4·2, was also low.

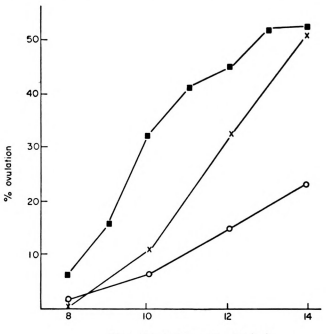
In Group II, twenty laparoscopic examinations were carried out in five females between 8 and 14½ hr after the HCG injection (Table 2). In this group, ovulation started later than in Group III, but as illustrated in Text-fig. 2, both groups ovulated at a similar rate at 14 to 14½ hr. The number of ovulation points per animal at 8 to 8½ hr and 14 to 14½ hr were, 0 and 4·8, respectively.

In Group III, forty-three laparoscopic examinations were performed in ten does between 8 and 14½ hr after the HCG injection (Table 3). Ovulations were observed at 8 to 8½ hr in four out of nine does (44%) and at 9 to 9½ hr in six out of ten (60%). At 10 to 10½ hr, ovulation had occurred in all ten does. Ovulation rates increased from 6·9% at 8 to 8½ hr, to 52·9% at 14 to 14½ hr. The mean

numbers of ovulation points per animal increased from 0·9 at 8 to 8½ hr to 10·8 at 14 to 14½ hr. Superovulation occurred in two animals with nineteen and twenty-three ovulations, respectively.

The results shown above demonstrate a large variation in the time of onset, the duration of ovulation and ovulation rates between groups.

We also observed the moment of rupture of ten follicles in two animals of Group I and three animals of Group III. Preovulatory follicles representing



TEXT-FIG. 2. Ovulation rate at various times after an ovulatory stimulus of mating (○), HCG alone (×) or PMSG and HCG (■) in rabbits.

various stages of development are illustrated in Pl. 1, Figs 2 and 3(a). Post-ovulatory sites are visible in Pl. 1, Figs 3(b) and 4. Occasionally haemorrhagic follicles attained a greater volume than the normally developing mature follicles. Plate 1, Fig. 5 illustrates a cumulus mass adhering to the ovarian surface shortly following follicular rupture. Later (Pl. 1, Fig. 6), the cumulus mass could be seen on the fimbria.

Although a small non-protruding follicle was not defined as a mature follicle

EXPLANATION OF PLATE 1

FIG. 1. Elevation of the external portion of sutures laparoscopically placed around the ovarian ligaments to facilitate suspension and observation of the ovaries in a rabbit.

FIG. 2. Preovulatory (Type A) follicle. Note the ease of observation of the entire ovarian surface made possible by the suspension procedure.

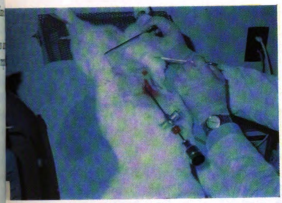
FIG. 3. Several preovulatory (a) and postovulatory (b) follicles present on the same ovary.

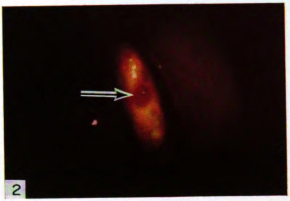
FIG. 4. Large immediately postovulatory follicle.

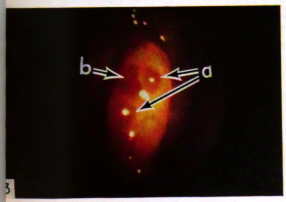
FIG. 5. Cumulus mass adhering to a postovulatory site.

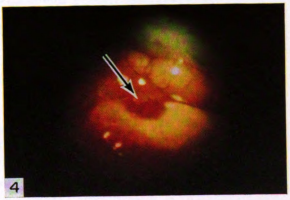
FIG. 6. Cumulus mass on fimbria.

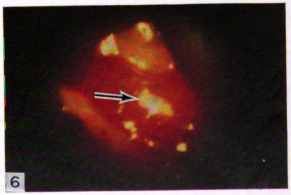
PLATE 1











(*Facing p.* 100)

Table 2. Ovulation in rabbits* subjected to induced ovulation with 100 i.u. HCG

Time between HCG and laparoscopy (hr)	*Total no. of follicles*	*No. of postovulatory follicles*	*% ovulation*
8 to 8½	34	0	0
10 to 10½	43	5	11·7
12 to 12½	44	15	34·1
14 to 14½	46	24	52·2

* Group II, five does.

by Walton & Hammond (1929), we observed laparoscopically that in many animals this type of follicle had developed into a large mature follicle during the serial examinations ranging from 8 to 14½ hr after the induction of ovulation.

As previously described by Walton & Hammond (1929), and Hill *et al.* (1935), the major developments of follicles about to ovulate take place about 2 hr before rupture. In the present studies, the blood supply increased remarkably on the follicular surface and extravasation of blood was observed during the last 30 min before rupture.

Laparoscopy of four pregnant rabbits on Day 6 revealed a total of twenty-two visible blastocysts, equally distributed in the uterine horns. Immediately after laparoscopy, the animals were killed and the blastocysts flushed from the horns. Twenty-three blastocysts were recovered, verifying the applicability of laparoscopy to achieving a close estimate of the number of Day 6 blastocysts.

Table 3. Ovulation in rabbits* subjected to induced ovulation with 100 i.u. PMSG and 100 i.u. HCG

Time between HCG and laparoscopy (hr)	*No. of does*	*Total no. of follicles*	*No. of postovulatory follicles*	*% ovulation*
8 to 8½	9	116	8	6·9
9 to 9½	5	66	10	15·2
10 to 10½	9	128	43	33·7
11 to 11½	4	61	25	41·0
12 to 12½	7	110	49	44·6
13 to 13½	4	65	34	52·3
14 to 14½	5	102	54	52·9

* Group III.

DISCUSSION

The present findings are in general agreement with the laparotomy–autopsy data of Walton & Hammond (1929) and Harper (1961, 1963), and the cinemicrographic observations of Hill *et al.* (1935).

Harper (1963) reported that no ovulation was observed by 10 hr, 50% ovula-

tion was observed between 10½ and 10¾ hr and 100% by 14 hr after the HCG injection.

The earlier onset of ovulation observed at 8 to 8½ hr after HCG injection in the present Group III was probably due to the regimen of ovulation induction (PMSG 100 i.u., HCG 100 i.u.).

The ovulation rates in the present studies were lower than the percentage ovulation in the time periods between injection of LH and autopsy reported by Harper (1963). The mean number of total follicles in Group I and Group II, however, did not differ from his data. Our data do indicate an increase in the slope of the ovulation curve (Text-fig. 2) for animals receiving HCG, indicating earlier ovulation. This was of greatest magnitude in animals subjected to a superovulation regimen, a factor to be considered in reproduction assay techniques involving timed ovulations.

An interesting observation on the development of the follicle towards ovulation was that often the Type B and C follicles at one examination period had ovulated before those follicles with a Type A appearance at the initial examination. Whether this is due to follicular atresia of the Type A follicles is not known.

The results of the laparoscopic examination of the pregnant rabbits reveal a high degree of accuracy in the quantification of potential implantation sites. These observations also suggest that the anaesthesia and laparoscopic technique had no effect on the fertilizability of the ovum since the pregnant animals had been previously subjected to laparoscopy at the time of ovulation. These results are similar to those reported after laparoscopy in non-human primates (Rawson & Dukelow, 1973a).

A significant finding of this study relates to the relative ease of the technical manipulation required for laparoscopy in the rabbit. With the suspension of the ovarian ligament, the entire ovary easily came into view and could be quickly and easily observed. The results serve to emphasize the usefulness of the laparoscopic techniques for studies necessitating ovulation detection and timing, diagnosis of pregnancy and enumeration of CL and for studies in which oviducal and uterine manipulations such as ligations or sampling are indicated.

ACKNOWLEDGMENTS

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REFERENCES

Balin, H., Wan, L. S. & Rajan, R. (1969) Experimental application of endoscopy. *Clin. Obstet. Gynec.* **12**, 534.

Blandau, R. J. (1955) Ovulation in the living albino rat. *Fert. Steril.* **6**, 391.

Dukelow, W. R., Jarosz, S. J., Jewett, D. A. & Harrison, R. M. (1971) Laparoscopic examination of the ovaries in goats and primates. *Lab. Anim. Sci.* **21**, 594.

Harper, M. J. K. (1961) The time of ovulation in the rabbit following the injection of luteinizing hormone. *J. Endocr.* **22**, 147.

HARPER, M. J. K. (1963) Ovulation in the rabbit: the time of follicular rupture and expulsion of the eggs, in relating to injection of luteinizing hormone. *J. Endocr.* **26,** 307.

HILL, R. T., ALLEN, E. & KRAMER, T. C. (1935) Cinematographic studies of rabbit ovulation. *Anat. Rec.* **63,** 239.

JEWETT, D. A. & DUKELOW, W. R. (1972) Serial observations of follicular morphology near ovulation in *Macaca fascicularis*. *J. Reprod. Fert.* **31,** 287.

JEWETT, D. A. & DUKELOW, W. R. (1973) Follicular observation and laparoscopic aspiration techniques in *Macaca fascicularis*. *J. med. Primatol.* **2,** 108.

RAWSON, J. M. R. & DUKELOW, W. R. (1973a) Effect of laparoscopy and anesthesia on ovulation, conception, gestation and lactation in a *Macaca fascicularis*. *Lab. Primate Newsl.* **12,** 4.

RAWSON, J. M. R. & DUKELOW, W. R. (1973b) Observation of ovulation in *Macaca fascicularis*. *J. Reprod. Fert.* **34,** 187.

SEMM, K. (1969) Gynaecological pelviscopy and its instrumentarium. *Acta europ. fertil.* **1,** 81.

WALTON, A. & HAMMOND, J. (1929) Observations on ovulation in the rabbit. *Br. J. exp. Biol.* **6,** 190.

Subsequently, the technique was employed to follow the complete pregnancy in this species. The animal involved had been mated for the recovery of capacitated spermatozoa and ovulation was confirmed by laparotomy on day 1 of pregnancy. On day 5 the sutures were placed and eight corpora lutea were observed on the ovaries. By day 15 the sutures were gone, probably due to the animal's metal cage, and 2 large corpora lutea were observed on the left ovary. The right uterine horn contained two large vascular fetal swellings. Similar observations were made on day 25 of pregnancy except the degree of swelling and uterine vascularization had increased. On day 32 (found on the morning of day 33), the animal delivered two normal-appearing infants which were dead when found.

This observation suggests that laparoscopy had little effect on pregnancy in the rabbit even though performed at several stages in the gestation. This further illustrates the utility of laparoscopy as a research tool.

APPENDIX III

APPENDIX III

INDUCTION OF FOLLICLE GROWTH IN AN IMMATURE SAIMIRI SCIUREUS

In 1974, a female Saimiri sciureus was brought into the primate colony at the Endocrine Research Unit and immediately began to exhibit traits frequently observed in immature animals. She was considerably smaller than usual and spent a great deal of time riding the other females. Laparoscopic examinations revealed the presence of an extremely small reproductive tract with ovaries appearing devoid of follicular growth. Such characteristics suggested that this animal was pre-pubertal. Four days of intramuscular FSH treatments (1 mg/day) produced negligible follicular development in this animal. Following a three month interval, this FSH regime was repeated with the addition of 500 IU of HCG on the fifth day. The follicular response was markedly increased with large, vascular follicles being present on both ovaries, although no ovulations were seen. Four weeks later, the initial FSH only regime was again administered without observable effects.

This was considered to suggest the possibility of a role of LH in the growth and development of follicles in the immature monkey.

APPENDIX IV

APPENDIX IV

PUBLICATIONS BY THE AUTHOR

Papers:

Natural and artificial control of ovulation in nonhuman primates. W.R. Dukelow, R.M. Harrison, Jon M.R. Rawson and M.P. Johnson. Medical Primatology, 1972.

Follicular development and ovulation in Macaca fascicularis, Saimiri sciureus and Galago senegalensis. W.R. Dukelow, D.A. Jewett and Jon M.R. Rawson, American Journal of Physical Anthropology, 38:207-209, 1973.

Effect of laparoscopy and anesthesia on ovulation, conception, gestation and lactation in a Macaca fascicularis. Jon M.R. Rawson and W.R. Dukelow. Laboratory Primate Newsletter, 12:4-5, 1973.

Observation of ovulation in the Macaca fascicularis. Jon M.R. Rawson and W.R. Dukelow. Journal of Reproduction and Fertility, 34: 187-191, 1973.

Ovulation in Macaca fascicularis. Jon M.R. Rawson. M.S. Thesis, Michigan State University, 1973.

Megestrol Acetate: II. Effects on ovulation in nonhuman primates as determined by laparoscopy. R.H. Harrison, Jon M.R. Rawson and W.R. Dukelow. Fertility and Sterility, 25: 51-56, 1974.

Hormonal influences on the time of ovulation in the rabbit as determined by laparoscopy. S. Fujimoto, Jon M.R. Rawson and W.R. Dukelow. Journal of Reproduction and Fertility, 38: 97-103, 1974.

Effects of intrafollicular administration of luteinizing hormone in the nonhuman primate using laparoscopy. Jon M.R. Rawson and W.R. Dukelow. Advances in Medical Primatology, 1975 (accepted).

Abstracts: The environmental enemy -- US. Jon M.R. Rawson; presented at First National Biological Congress, November 8, 1970, Detroit, Michigan.

The effect of bone marrow depletion on blood volume of the rat. Jon M.R. Rawson and J.R. Toepfer; presented at Regional Meeting of American Institute of Biological Sciences, 1971, Erie, Pennsylvania.

Ovulation morphology in nonhuman primates. Jon M.R. Rawson and W.R. Dukelow; presented at Michigan Academy of Science, Art and Letters, March 24, 1972, East Lansing, Michigan.

Comparative ovulation in nonhuman primates. W.R. Dukelow and Jon M.R. Rawson; presented at Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1972 (Fed. Proc. 31:277).

Ovulation-menstrual cycle relationships in Macaca fascicularis. Jon M.R. Rawson, W.R. Dukelow and T.J. Kuehl; presented at Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1974 (Fed. Proc. 32:283).

Clomiphene citrate: Effects on ovulation and menstrual cyclicity on the nonhuman primate. Jon M.R. Rawson and W.R. Dukelow; presented at the American Society for Pharmacology and Experimental Therapeutics, East Lansing, Michigan, Fall 1973 (The Pharmacologist 15:256).

Laparoscopic techniques for reproductive physiology studies in nonhuman primates and other species. Jon M.R. Rawson and W.R. Dukelow; presented at Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1974 (Fed. Proc. 33:281).

Lack of a species-specific response of Saimiri sciureus to gonadotropins at the follicular level. Jon M.R. Rawson, James P. Mahone, and W.R. Dukelow; presented at Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1975 (Fed. Proc. 34:544).

APPENDIX V

APPENDIX V

VITA

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Outstanding Graduating Biology Major
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International Primatological Society