ASCORBIC ACID AND THE REPRODUCTIVE CYCLE: ASCORBIC ACID AND THE ESTROUS CYCLE

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ABSTRACT

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By

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Serum ascorbic acid has been reported to increase about the middle of estrus in Cows (Phillips et al., 1941). This suggests that ascorbic acid might have an important role in the ovarian cycle, and may be related to ovulation. The purpose of this study was threefold: (1) to evulate serum ascorbic acid changes around the time of ovulation in cows, rats, rabbits and sows. (2) to determine serum ascorbic acid changes after hormone treatment with human chorionic gonadotropin, follicle stimulating hormone and prostaglandin $F_2\alpha$. (3) to determine whether there is any relationship between serum ascorbic acid changes and ovarian ascorbic acid.

Serum ascorbic acid in all species studied did not show a significant difference around the time of ovulation, compared to other stages of the estrous cycle. There was some specie variation, however serum ascorbic acid concentration in all animals was very constant throughout the estrous cycle. This suggests that serum ascorbic acid is not a good indicator of time of ovulation, and probably is not directly related to ovulation.

Treatment of rabbits with follicle stimulating hormone did not affect serum ascorbic acid changes, however ovarian ascorbic acid decreased. Prostaglandin $F_2\alpha$ at levels of 30 mg injected into hysterectomized cows increased serum ascorbic acid levels within 10 hours. Serum progesterone changes were not correlated with the ascorbic acid changes. The significance of this mechanism is not understood, however, ascorbic acid may be related to the steroid metabolism, possibly, estrogen. Ascorbic acid in follicular fluid of the cow ovary was measured. Follicles which were in the developing stages (diameter $\leqq$ 1.0 cm) had higher ascorbic acid concentration than mature follicles (diameter $\geqq$ 1.0 cm). Although follicular fluid had relatively high concentrations of ascorbic acid that did not influence serum ascorbic acid on a quantitative basis.

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TABLE OF CONTENTS

Chapter		Page
I	INTRODUCTION	1
II	REVIEW OF LITERATURE	3
	Ascorbic Acid and Ovarian Cycle	3
	Ascorbic Acid and Gonadotropic Hormone	5
	Ascorbic Acid and Ascorbic Acid Deficiency Related to the Reproductive Process	6
	Ascorbic Acid and Steroidogenesis	9
	Gonadotropic Hormone and the Estrous Cycle	11
	Objectives of the Present Study	15
III	MATERIALS AND METHODS	16
	First Trial: Rats	16
	Second Trial: Rabbits	16
	Third Trial: Sows	17
	Fourth Trial: Cows (Holstein)	19
	Fifth Trial: Ascorbic Acid and Follicular Fluids of Cow Ovaries (Holstein)	20
	Methods for Detecting Estrus	20
	Chemical Analysis of Ascorbic Acid	23
	Radioimmuno Assay of Progesterone	24
IV	RESULTS AND DISCUSSION	25
	Serum Ascorbic Acid and Estrous Cycle in Rats	25
	Ascorbic Acid Levels in Serum and Ovaries After Gonadotropin Treatment	28
	Serum Ascorbic Acid During Estrus in Sows	29
	Ascorbic Acid Changes in Cows	32
	Relationship Between Serum Ascorbic Acid and Ovarian Ascorbic Acid	38
	Calculation	39
V	SUMMARY	42
	BIBLIOGRAPHY	43

LISTS OF TABLES

Table		Page
1	Serum ascorbic acid levels in rats during the estrous cycle	26
2	Serum ascorbic acid levels in rats during estrus.	27
3	Ascorbic acid levels of the serum and ovaries of rabbits.	30
4	Serum ascorbic acid levels in sows.	31
5	Ascorbic acid concentration in follicular fluid of cows (Holstein).	40
6	Ascorbic acid concentration in large and small follicles	40

LIST OF FIGURES

Figure		Page
1	L-ascorbic acid and L-dehydroascorbic acid	7
2	Interrelationship between the pituitary, the corpus luteum, the uterus and hypothalamus . .	14
3	Protocol of rabbit experiment.	18
4	Changes in the vaginal cell types found in vaginal smears during the estrous cycle in the rat.	21
5	Serum ascorbic acid levels in cows just before and after estrus.	33
6	Serum progesterone levels in cows before and after estrus	34
7	Serum ascorbic acid level after $PGF_2\alpha$ injection into hysterectomized cows.	36

CHAPTER I

INTRODUCTION

Estrus and ovulation are the major events of the ovarian cycle. Furthermore, study of the ovarian cycle can lead to a better understanding of reproduction and thus may be useful in the areas of population control and treatment of infertility.

Interest in the role of ascorbic acid in the reproductive process was primarily brought about by the low or trace amount of ascorbic acid in fresh semen of low fertility bulls (Phillips et al., 1940). It was shown that ascorbic acid injections to these animals improved the reproductive success of sterile and partially sterile bulls. Study of the ascorbic acid role in the female reproductive cycle was initiated. According to Phillips et al. (1941), plasma ascorbic acid levels were higher during estrus than at other stages of the cycle in cows. These investigators stated that it is possible to detect poor breeding cows because of their abnormally low plasma ascorbic acid levels. Subcutaneous ascorbic acid therapy to these sterile cows resulted in 60% becoming fertile. Recently, Valjuškin (1971) reported that injection of 1.6 gm of vitamin C into Swedish cows increased two-fold the number of animals which came into heat.

The ascorbic acid changes related to the ovarian cycle have been reported not only for domestic animals, but also in humans (Mickelsen et al., 1943) and other laboratory animals (Hoch-Ligeti and Bourne, 1948 and Parlow, 1958). In rats, the ovary shows a significantly lower level of ascorbic acid at estrus than at diestrus (Hoch-Ligeti and Bourne, 1948). Other suggestive evidence that the ascorbic acid was involved in the ovarian cycle is that ascorbic acid is also related to the gonadotropic hormones which control the ovarian cycle. According to Parlow (1958) luteinizing hormone causes a significant decrease of ovarian ascorbic acid in gonadotropin pretreated rats. Although the physiological significance of this reaction is not well understood, ascorbic acid might be an important factor in steroid metabolism.

CHAPTER II

LITERATURE REVIEW

Ascorbic Acid and Ovarian Cycle

It has long been recognized that females show regular cyclic sexual changes that are under endocrine control. The cyclic alteration consists of many biological changes such as hormone release and other biochemical variance. The serum ascorbic acid changes related to this ovarian cycle were reported by several investigators in rats (Astrada and Laura, 1966 and Deane, 1952). The changes in serum ascorbic acid during the estrous or menstrual cycle perhaps affect puberty since females older than thirteen years of age have higher levels of serum ascorbic acid than males at the same dietary intake (Dodd, 1969). This sex difference suggests that there may be some relationship between ascorbic acid and the ovarian cycle. Evidence suggesting a relation between ascorbic acid and the ovarian cycle was reported in the 1940's by Phillips et al. (1941) who observed that during estrus, plasma ascorbic acid level in cows was increased. The data presented by Phillips et al. (1941) also suggested an increase in the serum level of ascorbic acid when ovulation was thought to occur. Mickelsen et al. (1943) observed

that some women had increased fasting plasma ascorbic acid levels at the midpoint of the menstrual cycle. More recently Rivers and Devine (1972) reported that plasma ascorbic acid levels increased the estrogen phases of the cycle in women. In humans, ovulation occurs in the middle of the cycle with the peak in plasma vitamin C levels appearing at that time. Mickelsen et al. (1943) and Loh and Wilson (1971) indicated that urinary excretion of ascorbic acid reached a peak when ovulation occurred in women. These observations suggest that serum ascorbic acid may be related to ovulation, although the exact mechanism is not understood.

In rats, ovarian ascorbic acid concentration is significantly lower during estrus than during diestrus or metestrus (Hoch-Ligeti and Bourne, 1948 and Mills and Schwartz, 1959). In 1966, changes of ovarian ascorbic acid were quantified by Astrada and Laura who reported that the minimal ascorbic acid level in the ovary at estrus was 56±0.6 mg per 100 g of tissue, while the diestrus ovary contained 69.3±1.1 mg per 100 g of tissue. The ovary is known to have a very high concentration of ascorbic acid and the ovarian cycle may reflect the effect of this cyclic variation of ovarian ascorbic acid. Although the significance of these cyclic changes and the exact role of ascorbic acid on the ovarian cycle is not fully understood, this evidence suggests that the ovarian cycle has a definite effect on the ascorbic acid.

Ascorbic Acid and Gonadotropic Hormone

A number of observations suggest a possible relationship between ascorbic acid and the hormones involved in the regulation of the estrous cycle. In 1949, Claesson et al. described that injection of pregnant mare's serum (PMS) to rabbits resulted in a decrease in ovarian ascorbic acid concentration. Ovarian ascorbic acid depletion by luteinizing hormone has been found in several laboratory animals such as rats (Deane, 1952 and Mills and Schwartz, 1959), and guinea pigs (Reid and Sykers, 1945). Parlow (1958) pointed out that the significant depletion of ovarian ascorbic acid in rats was due to luteinizing hormone and utilized this reaction as a bioassay technique for that hormone. Goldstein and Sturgis (1961), using histochemical methods, observed that ovarian ascorbic acid depletion by luteinizing hormone seemed to involve the corpus luteum of the rat's ovary. Ascorbic acid is widely distributed in the ovary, but the corpus luteum concentration is relatively high. By in vitro techniques, inhibition of ascorbic acid uptake by gonadotropin was examined by Stansfield and Flint (1967). He found that luteinizing hormone significantly decreased the uptake of ascorbic acid from the incubation medium. Addition of pregnenolone and progesterone also significantly inhibited ascorbic acid uptake by the tissue. He suggested that the very low level of ascorbic acid concentration in the ovary might be due to the inhibition of ascorbic acid uptake

rather than depletion of ascorbic acid from the ovary. Recently, Loh and Wilson (1971) observed that urinary secretion of ascorbic acid was increased in the middle of the menstrual cycle in women. This urinary secretion of ascorbic acid had the same pattern as that of luteinizing hormone secretion. Evidence suggests that ascorbic acid may be related to the steroids.

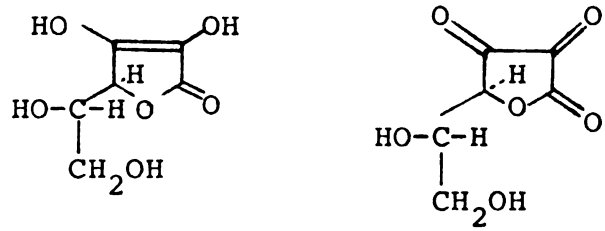
Observations suggest that ascorbic acid has an augmentative effect on gonadotropic hormone. Di Ció and Schteingart (1942) noted that the combination of gonadotropic hormone and ascorbic acid produced heavier female genital organs compared to injection of the hormone alone in rats. Reid and Sykers (1945) reported that the combination of ascorbic acid and gonadotropin injected into guinea pigs produced higher ovarian weights than when gonadotropic hormone alone was injected. This augmentative effect of ascorbic acid on the gonadotrpic hormone appears to further suggest that some relationship exists between ascorbic acid and gonadotropic hormones.

Ascorbic Acid and Ascorbic Acid Deficiency Related to the Reproductive Process

Zilva in 1923, did much of the early work on the isolation of L-ascorbic acid and established the general chemical properties of this vitamin. Dehydroascorbic acid (Figure 1) is formed from the L-ascorbic acid by the oxidative removal of two equivalents of hydrogen (Sebrell

and Harris, 1967). Although the distribution of this form in the body is different from L-ascorbic acid, it has the same biological activity.

Figure 1. L-ascorbic acid L-dehydroascorbic acid



A recent study by Horning et al. (1972) suggests that L-ascorbic acid uptake by the adrenals and ovaries is faster than that of dehydroascorbic acid. The reason is that L-ascorbic acid transport is an energy dependent process while the dehydroascorbic acid is transported by passive diffusion. The energy dependent uptake is mainly due to the structural specificity of the enzymes involved in this mechanism. The outstanding property of ascorbic acid is largely due to the ease with which it may be oxidized and reversibly reduced.

Ascorbic acid metabolism differs slightly depending on the species involved. For those animals that are not dependent on dietary sources, L-ascorbic acid is synthesized from six carbon precursors such as glucose, fructose, galactose and mannose (Sebrell and Harris, 1967). The synthesis of ascorbic acid differs in species, but most

of the synthesis takes place in the liver. In animals including men, monkeys and guinea pigs, there is no oxidative enzyme which converts L-gulonolactone to L-ascorbic acid. Therefore these animals must depend on an exogenous source of ascorbic acid (King, 1967). Burns et al. (1958) and Martin and Mecca (1961) found that only very small amounts of ascorbic acid are absorbed from the upper part of the small intestine of rats. Guinea pigs, on the other hand, exhibited active transport of the ascorbic acid from the duodenum to the blood. Another difference occurred in the potential of the kidney to oxidize ascorbic acid to carbon dioxide. The kidney homogenates of rats, rapidly oxidized ascorbic acid to carbon dioxide in vitro, whereas, the kidneys of guinea pigs were much less active in this mechanism.

The most important functions of ascorbic acid in the body is its role in hydroxylation and oxidation-reduction reactions. In the biochemical system, ascorbic acid plays a role in electron transport, the metabolism of tyrosine, collagen formation and perhaps in the reproductive process.

Many workers reported that a deficiency of ascorbic acid affects the female reproductive system. Greenblatt (1953) and Peña (1954) reported that ascorbic acid deficiency caused habitual abortion or still birth and frequently premature birth in women. They suggest that this might be due to capillary fragility of the placenta or vascular disturbances throughout the body because of the

abnormality of collagen hydroxylation. Another possibility involves the abnormality of steroid hormone synthesis, which might be related to ascorbic acid. Javet (1954) reported that ascorbic acid deficiency in pregnant women resulted in frequent signs of decidual hemorrhage and spontaneous abortion and these patients had very low serum ascorbic acid levels.

Kraemmer et al. (1933) showed that female guinea pigs receiving a scorbutigenic diet supplemented with less than 3 cc of orange juice per animal per day failed to give birth to live young. The ovaries from these animals showed degeneration of graafian follicles and a lack of normal development of new follicles. Ovaries of guinea pigs dying from scurvy contained many degenerating follicles and there were no new corpora lutea or no new follicles.

Ascorbic Acid and Steroidogenesis

Since the original description by Szent-György of the presence of a strong reducing substance in the adrenal cortex and pituitary, ascorbic acid was thought to be involved in steroidogenesis. Sayers et al. (1944) described changes of ascorbic acid and cholesterol concentrations in the adrenal cortex after ACTH treatment in rats; this was related to the secretion of steroids in the adrenal cortex. Nathani and Nath (1972) also observed that ACTH significantly increased the total blood ascorbic acid. In spite of these suggestions that ascorbic acid may play a primary

role in adrenal steroidogenesis, the direct mechanism whereby ascorbic acid is involved in these reactions has not been established. ACTH added to an in vitro system caused significant decreases in the ascorbic acid and cholesterol concentration in the adrenal glands and increased adrenal steroid synthesis (Kitabchi, 1967a). This suggests that the depletion of ascorbic acid from the adrenals removes hydroxylase inhibition thus permitting increased adrenal steroidogenesis. In 1951, Banerjee and Deb found that in scorbutic guinia pigs, the adrenal ascorbic acid and cholesterol content were decreased and the total cholesterol in the body of guinea pigs was much increased (Banerjee and Singh, 1958). Cholesterol is the most important precursor in steroid synthesis.

The ovary synthesized a class of steroids like the adrenals which have a common basic structure. The biosynthetic step, via cholesterol to pregnenolone are common for all steroids. In 1968, DeNicola et al. studied the effect of ACTH on adrenal ascorbic acid and its relation to steroid synthesis in rats. He observed that 17-β-estradiol and progesterone also inhibited the transport of ascorbic acid into the adrenal in vitro. In the presence of progesterone and estrogen in the incubation medium, adrenal corticoid synthesis was increased. In manners to that adrenal ascorbic acid was depleted by ACTH, ovarian ascorbic acid in a number of species, i.e., rats (Parlow, 1958), guinea pigs (Reid and Sykers, 1945) and rabbits

(Claesson et al., 1949) was reported to be depleted by LH. A specific effect of LH on the ovary seems to increase steroid hormone, especially production of progesterone. However, it is not clearly understood whether ascorbic acid depletion by LH is also related to sex steroid synthesis.

In 1970, Khatamov studied the effect of sex steroids on the content of ascorbic acid in the internal organs. With progesterone injection ascorbic acid content in all internal organs decreased significantly, and estrogen administration further decreased ascorbic acid in these organs. The interrelationship between adrenal steroidogenesis and gonadal steroidogenesis is not fully understood. However, there may be some mechanism for maintaining equilibrium between these two sites of steroid synthesis.

Gonadotropic Hormone and the Estrous Cycle

The estrous cycle is a series of physiological events involving the pituitary and ovary which provide for follicular growth and ovulation. Either fertilization and zygote maintenance occurs or the process starts over again.

The estrous cycle in cows, as in other species, is controlled by pituitary gonadotropic hormones. The ovarian-pituitary relationship has long been recognized. The luteinizing hormone, seems to be the major hormone responsible for triggering ovulation, corpus luteum growth and progesterone secretion (Baird, 1972). The onset of

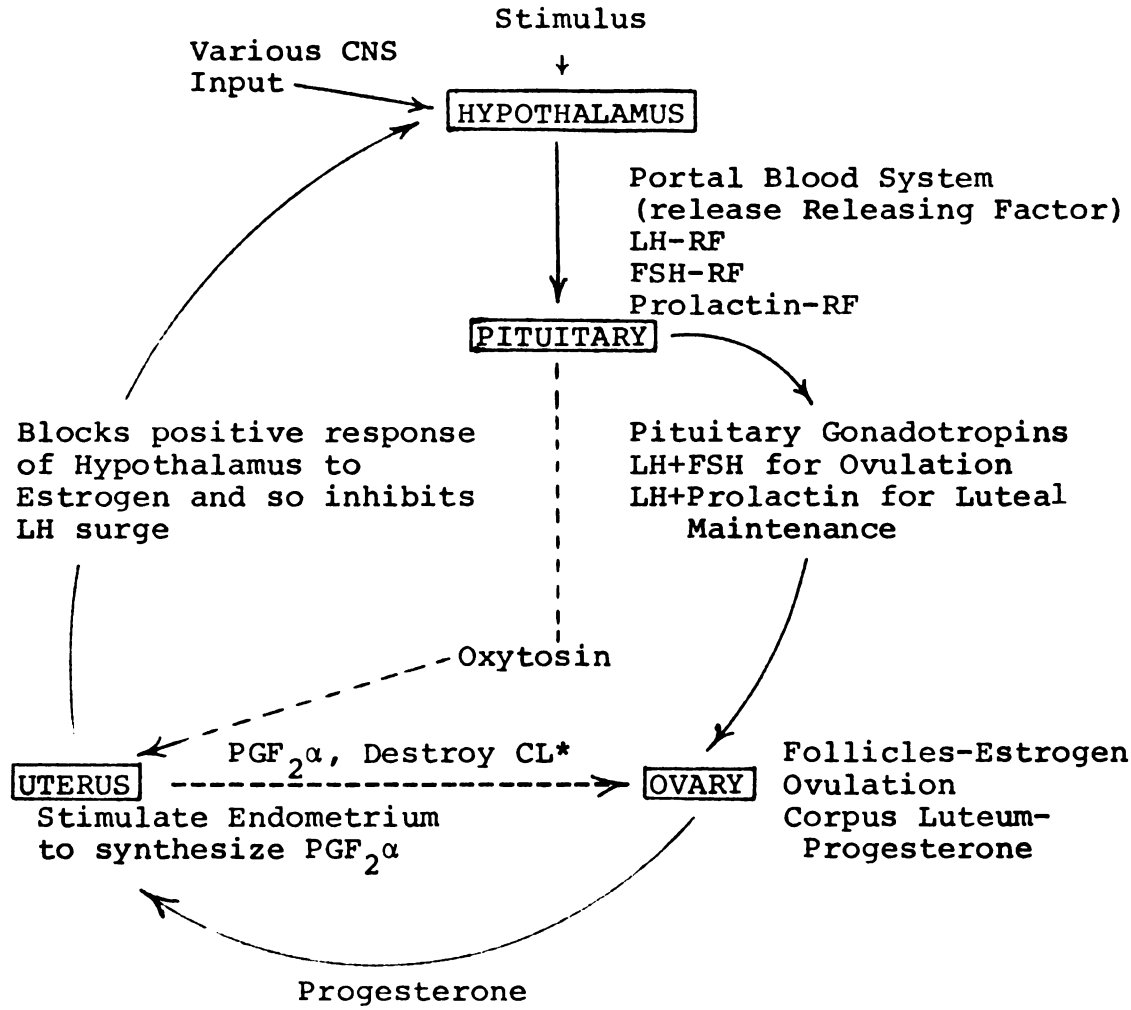
preovulatory maturation of the follicle is marked by the sudden and dramatic rise in the release of LH from the pituitary, the so-called LH surge. In cows LH surges occurred 3-6 hours after the onset of estrus (Henricks and Dickey, 1970). Schams and Karg (1969) using a radio-immunoassay reported that serum LH reached peak 15-20 hours before ovulation. In the cow, ovulation occurs 25-27 hours after the onset of estrus. This LH seems to be produced by the positive feed back of estradiol on the hypothalamus, causing a discharge of LH releasing factor. This releasing factor is transported to the anterior pituitary via the portal blood system.

Cows are unique, among all species, in that the pituitary contains relatively high levels of LH and low levels of FSH, however FSH also contributes to the growth and development of follicles from the antral to the preovulatory stage. A significant drop in FSH and LH content of the pituitary occurred in cows in 1-8 hours after the onset of estrus (Rakha and Robertson, 1965). This indicated that FSH as well as LH plays a role in the initiation of ovulation in cattle. Progesterone is one of the most important gonadotropic hormons controlling estrous cycle. Plasma progesterone levels declined precipitously between day 16-19 of the estrous cycle, while plasma LH levels rose rapidly in cows. After serum progesterone levels decreased to levels of 1 ng or less, ovulation occurred (Hansel and Trimberger, 1952).

Following the growth of the corpus luteum, progesterone secretion begins to increase, and remains relatively high during the luteal phase until the next ovulation and estrus stage occurs.

Recently, $PGF_2\alpha$ showed a luteolytic effect on the corpus luteum regression which resulted in a decreased progesterone synthesis. Progesterone stimulates the endometrium of the uterus to produce $PGF_2\alpha$. This $PGF_2\alpha$ is transported to the ovary and destroys the corpus luteum thereby decreasing progesterone synthesis. As a result of the falling progesterone levels, a graafian follicle begins to secrete a large amount of estrogen, which triggers a surge of LH from the pituitary, and another cycle begins.

Figure 2. Interrelationship between the pituitary, the corpus luteum, the uterus and hypothalamus. (From: Reproductive Hormones, IN Hormones in Reproduction, Baird, D. T., 1972, p. 56)



*CL - Corpus luteum

Objectives of the Present Study

The objectives of this study are:

1. To determine whether serum ascorbic acid changes around the time of ovulation in rats, sows, rabbits and cows.
2. To determine whether ovarian ascorbic acid depletion at estrus may directly influence the serum ascorbic acid.
3. To determine whether there is any relationship between the changes of serum ascorbic acid and progesterone in cows.

With the above mentioned objectives in mind, we chose rats, rabbits, sows and cows as our experimental subjects. These animals can synthesize ascorbic acid. Thus, serum ascorbic acid levels are less influenced by dietary sources than is true of guinea pigs and other animals dependent on the diet for this vitamin. Since measurement of the concentration of ascorbic acid in blood is a good tool that can be easily used in endocrinology, the experiments were mainly designed to determine the changes in the serum ascorbic acid.

The experiments were designed to:

1. Determine the serum ascorbic acid levels during estrus including that immediately before and after estrus.
2. Determine ovarian ascorbic acid concentrations and the follicular fluid ascorbic acid concentrations.

CHAPTER III

MATERIALS AND METHODS

First Trial: Rats

Twenty Osborne Mendel female rats weighing about 200 g were housed singly in metal cages, and were given water and fed a natural grain ration ad libitum. Stages of the estrous cycle were determined three times a day for each rat, by vaginal smear and chloride content of vaginal washings. About 30 µl of deionized water was put into the vagina of the rats, left for approximately two seconds and then sucked out with an eyedropper. This material was placed on silver chromate paper, which developed a white spot. A blood sample was secured each day; the rats were anesthetized with ether and blood was collected by heart puncture. As soon as the blood was collected, the tubes were placed in ice and brought to the laboratory. Serum ascorbic acid were analyzed by a slightly modified Schwartz and Williams method (1955) for total ascorbic acid. Hematocrits were also determined for each animal.

Second Trial: Rabbits

Twenty-five New Zealand White rabbits weighing about 3.5 Kg were separately caged. Water was always available

and laboratory chow diet was fed ad libitum. Experiments were designed following the protocol shown in Figure 3. Rabbits were divided into five groups of five animals. The rabbits in four groups received follicle stimulating hormone and human chorionic gonadotropin treatment while the rabbits in the fifth group were controls. At the beginning of the experiment, the animals in the first group were sacrificed immediately by cervical dislocation, blood was collected by heart puncture and the ovaries removed. The remaining animals were injected intramuscularly twice a day, with FSH at levels that were about 0.4 mg per rabbit per day. Seventy-two hours after the first FSH injection, 100 i.u. of HCG was given intravenously, and rabbits were killed at different time intervals after HCG injection; 30 minutes, 2 hours and 4 hours. All ovaries were trimmed and weighed separately. Each pair of ovaries was ground in a glass homogenizer with 10 ml of 5% of trichloroacetic acid solution and the homogenates were used for ascorbic acid determinations.

Third Trial: Sows

Five sows with normal estrous cycles were selected and estrus was detected by the behavior of the animals. During estrus and immediately before and after estrus, blood was collected from the anterior vena cava. Blood samples were usually taken about 8 o'clock in the morning. Blood samples continued to be taken after estrus every 24

Figure 3. Protocol of Rabbit Experiment.

Date	Time intervals for injection (Hours)	Time of injection	Hormone injected	Rabbits Nos of animals	
				Injected	Autopsied
5-29-73	0	8 A.M.	FSH	6 to 25	1 to 5
	12	8 P.M.	FSH	6 to 25	none
5-30-73	24	8 A.M.	FSH	6 to 25	none
	36	8 P.M.	FSH	6 to 25	none
5-31-73	48	8 A.M.	FSH	11 to 25	6 to 10
	60	8 P.M.	FSH	11 to 25	none
6- 1-73	72	8 A.M.	HCG	11 to 25	
		8:30 A.M.			11 to 15
		10:00 A.M.			16 to 20
		12:00 A.M.			21 to 25

FSH (Follicular Stimulating Hormone) 0.2 mg/1 dose injected subcutaneously
HCG (Human Chorionic Gonadotropin) 100 iu/1 dose injected intravenously

hours for one week. On the day when ovulation was scheduled to occur, a second sample of blood was collected about one o'clock in the afternoon. The tubes containing the blood were placed in an ice chamber and brought to the laboratory for immediate analysis.

Fourth Trial: Cows (Holstein)

Five cows with normal cycles were selected and fed a grain, hay and corn silage mixture ad libitum. Estrus was detected by ovarian palpation and behavior of the cows. Blood progesterone was analyzed to more accurately detect the time of ovulation.

Around estrus a series of blood samples were collected from these cows by jugular vena puncture at 12 hour intervals for a period of one week. Blood samples were usually taken at 7:00 a.m. and 5:00 p.m.

Three cows were hysterectomized and 30 mg of $PGF_2\alpha$ was injected intramuscularly in order to destroy the corpora lutea. A series of blood samples were taken by jugular vena puncture at different time intervals for five days; blood samples were taken at 10 minute intervals for the first hour after $PGF_2\alpha$ injection, at 30 minute intervals for the following three hours, at 6 hour intervals for the remaining 24 hours and at 2 hour intervals for the next 3 days. Blood serum from these cows was used for ascorbic acid and progesterone analysis.

Fifth Trial: Ascorbic Acid and Follicular Fluids of Cow Ovaries (Holstein)

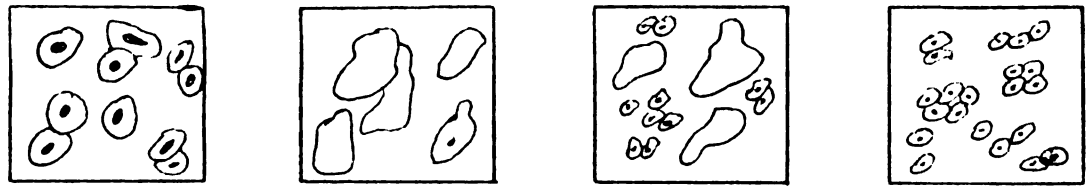
Fresh ovaries were collected from cows at slaughter house and the approximate stage of the cycle was predicted by the ovarian morphology and stage of pregnancy. Follicular fluid from each ovary was mixed with 10% trichloroacetic acid (Follicular fluid:TCA = 5:3 ratio) to stabilize the ascorbic acid and precipitate any protein present. Follicles from another group of similar ovaries were divided into two groups; small follicles (diameter $\leq$ 1.0 cm) and large ones (diameter $\geq$ 1.0 cm). The follicular fluid from each group of follicles in one ovary was combined and mixed with 10% of TCA as previously described. This solution of follicular fluid and TCA was used for the ascorbic acid analysis.

Methods for Detecting Estrus

Epithelial changes in the vagina were used to determine the time of ovulation in rats. The vaginal smear exhibits cyclic changes during the estrous cycle which can be differentiated on the basis of strong staining reactions (Erikson, 1963). These changes are influenced by estrogen and progesterone. This method is based upon the fact that under the influence of estrogen, cornification is induced in the outer epithelial layer of the vagina and can be observed in a smear. During proestrus, the epithelial cells are large with round nuclei; later in

estrus the cells consist only of large squamous epithelia without nuclei. In metestrus, these cells become infiltrated with leucocytes, and in diestrus, only leucocytes can be seen in the smear (Figure 4).

Figure 4. Changes in the vaginal cell types found in vaginal smears during the estrous cycle in the rat.



Ovulation can be estimated by the changes that occur in the cervix. Cervical mucus undergoes cyclic changes in physical and chemical properties. Characteristics physicochemical changes are induced by estrogen and progesterone (Cohen, 1966). One of these involves changes in the chloride concentration. The latter can be detected by silver chromate paper. A simple spot test for detecting ovulation by the chloride content of cervical mucus was studied by McSweeney and Sbarra (1964) and modified by Hardy et al. (1970). Silver chromate paper for this purpose was made from Whatman filterpaper immersed serially in solutions of silver nitrate (0.275 M) and potassium chromate (0.175 M); when dried at room temperature avoiding the sun light, the paper was brown in color. Vaginal mucus

from a series of rats was put on this paper. During ovulation or estrus, the mucus produces a white precipitate (white spot) in this paper the size of the spot being proportional to the chloride concentration of the cervical mucus. This high chloride concentration of mucus (0.5% and above) could easily be differentiated from low concentration (0.4% and less). On the other hand, during the anovulatory cycle, no positive spot was seen on this silver chromate paper. For the rat, the combination of the phenomena, vaginal smear and positive chloride test on the silver chromate paper, was regarded as the time of estrus and ovulation.

Rabbits do not have a cycle, but require a mating stimulus for ovulation and corpus luteum formation. Estrus usually occurs 9-12 hours after stimulation produced by copulation or gonadotropic hormone. This is due to stimulatory release of a hypothalamic neuro hormone which stimulates the anterior pituitary to release ovulatory gonadotropic hormone (Desjardins et al., 1967). For this reason FSH or PMS and HCG or LH are widely used for inducing ovulation in rabbits. For this study, FSH and HCG injections were used for inducing ovulation in rabbits.

For cows, estrus was detected by ovarian palpation and estrus behavior. This phenomena occurs under the influence of steroids, especially estrogen. The plasma progesterone levels begin to drop very rapidly on day 17 of the estrous cycle (21 day cycles in cows). During estrus, serum

progesterone levels are so low, they are almost non-detectable. After ovulation, progesterone starts to increase and high levels are maintained during the luteal phase. In cows, $PGF_2\alpha$ induced the regression of corpus luteum and the synchronization of estrus. Hafs et al. (1972) observed that about 74±3 hours after the $PGF_2\alpha$ injection, estrus begins to occur and a plasma LH peak appeared 60±15 hours later. He suggested that intravenous administration of $PGF_2\alpha$ would appear to be an extremely efficient method of inducing ovulation or synchronizing estrus in cows.

Chemical Analysis of Ascorbic Acid

The amount of ascorbic acid in serum and ovaries was determined by a slightly modified Schwartz and Williams method (1955) for total ascorbic acid.

One ml of each sample (serum or homogenates of ovaries) was placed in a centrifuge tube, to which was added 0.6 ml of 5% TCA solution in order to precipitate serum protein. The mixture was centrifuged at 3000 rpm for 10 minutes; 0.3 ml supernate was used for ascorbic acid analysis.

Reagents: Ascorbic acid (Merck) 1 mg% solution

5% Trichloroacetic acid (TCA)

0.2% 2,6,-Dichlorophenolindophenol

5g Thiourea dissolved in 95 g of 5% TCA

2g 2,4-Dinitrophenyl hydrazine dissolved in 98 g

of 65% H_2SO_4

Phospho-hydrochloric acid (85.8% H_3PO_4:37% HCl = 1:3)

Instead of metaphosphoric acid, we used the 5% TCA solution. The optical density of each sample was determined by means of a Beckman Spectrophotometer (DB), with wave length set for 530 mu.

Radioimmuno Assay of Progesterone

Progesterone of cow's serum was analysized by radio-immunoassay, which is described by Kittok and Louis (1973).

CHAPTER IV

RESULTS AND DISCUSSION

Serum Ascorbic Acid and Estrous Cycle in Rats

Serum ascorbic acid levels at estrus when compared to the other stages of the cycle indicated no significant changes of ascorbic acid during estrus in rats ($p<0.05$, Table 1). Serum ascorbic acid levels at the time of ovulation are shown in Table 2 as compared to the rest of the estrus stage. According to Yassman and Taymor (1970), ovulation occurs in rats around 2 A.M. early in estrus. For that reason blood samples were taken at specified intervals during the 24 hours following the appearance of proestrus in rats. Using Yassman's criteria, it would appear that the serum ascorbic acid levels at the time of ovulation were not different from those at the estrus stage. Serum ascorbic acid levels in estrus also showed no significant changes from those in the diestrus stage ($p<0.05$).

There was a certain amount of variation in the serum ascorbic acid level of the rats throughout the entire series. This slightly complicates the interpretation of the data. Part of that fluctuation may be associated with the stress imposed on the animals by collecting blood from

Table 1. Serum ascorbic acid levels in rats during the estrous cycle. All values are in mg per 100 ml.

Rat No.	Estrus	Metestrus	Diestrus	Proestrus
4	0.528±0.013	0.550±0.047	0.560±0.014	0.525±0.051
5	0.570±0.026	0.442±0.047	0.495±0.013	0.490±0.030
6	0.552±0.017	0.592±0.011	0.520±0.041	0.654±0.041
7	0.489±0.036	0.530±0.057	0.605±0.026	0.430±0.049
8	0.538±0.077	0.553±0.029	0.595±0.010	0.492±0.020
9	0.503±0.030	0.435±0.025	0.413±0.015	0.560±0.051
10	0.577±0.050	*0.570±0.030	0.520±0.041	0.500±0.028
11	0.500±0.033	0.400±0.028	0.440±0.045	0.500±0.051
13	0.510±0.070	0.400±0.066	*0.400±0.046	0.490±0.038
14	0.510±0.050	0.440±0.028	0.430±0.016	0.480±0.049
15	0.480±0.059	0.450±0.042	*0.420±0.026	0.455±0.021
16	0.537±0.025	*0.400±0.066	0.480±0.037	0.440±0.057
17	0.470±0.020	0.400±0.032	0.480±0.026	0.450±0.044
18	0.583±0.040	0.550±0.036	0.500±0.057	0.500±0.065
19	0.450±0.053	0.440±0.010	*0.450±0.046	0.470±0.082
20	*0.500±0.036	*0.500±0.010	0.510±0.114	*0.530±0.028
21	0.450±0.061	0.400±0.036	0.470±0.018	0.460±0.030
24	0.490±0.028	0.500±0.048	0.580±0.053	0.450±0.042
Average + S.E.	0.513±0.032	0.474±0.069	0.492±0.062	0.493±0.053

S.E.: standard error

Ascorbic acid values are the mean of four values except those marked with an * which is the average of three values.

Female Osborne Mendel rats weighing about 200 g were used.

Table 2. Serum ascorbic acid levels in rats during estrus.

Date Rat No.	3-27, 10 p.m. Late Proestrus	3-28, 2 a.m. Early estrus (Ovulation)	3-28, 6 a.m. Estrus	3-28, 10 a.m. Estrus
1	0.425	0.524	0.526	0.443
2	0.430	0.408	0.438	0.439
3	0.505	0.553	0.487	0.495
4	0.534	0.607	0.498	0.550
5	0.524	0.560	0.489	0.522
11	0.600	0.580	0.510	0.600
16	0.440	0.725	0.510	0.450
20	0.630	0.725	0.370	0.600
Average	0.511±0.077	0.585±0.104	0.495±0.051	0.512±0.07

(mg ascorbic acid/100 ml serum)

the heart following general anaesthesia. That stress may be involved in this procedure is suggested by the fact that when a series of blood samples were collected within a period of a day, the serum ascorbic acid level tended to increase serially. However, 12 hours after the first sample was collected, the serum ascorbic acid tended to return to the level existing prior to the start of the blood collection period.

Although there are no statistically significant differences in serum ascorbic acid levels at various stages of the estrous cycle, the highest levels occurred more frequently on the day when the rat was in estrus. The final answers to the question of possible variations in the ascorbic acid levels of rats' blood during the estrus cycle will have to await the development of a technique for securing blood which impose no trauma on the animal.

The ovarian ascorbic acid levels in estrus showed significantly lower than diestrus. This significant depletion of ovarian ascorbic acid in estrus confirmed the previous report (Hoch-Ligeti and Bourne, 1948).

Ascorbic Acid Levels in Serum and Ovaries After Gonadotropin Treatment

Rabbits do not have a regular cycle, but require a mating stimulus for ovulation and corpus luteum formation resulting in either pseudopregnancy, depending on whether

the mating was fertile. This is called induced ovulation. Usually 10 hours after the gonadotropin stimulus, ovulation occurs, induced by LH released from the anterior pituitary.

Gonadotropin treatment of the rabbits did not significantly change the serum ascorbic acid levels compared to the control group ($p<0.05$). Furthermore, serum ascorbic acid levels about the time of ovulation did not differ from those in the other stages of the cycle. Ovarian weight increased after HCG injection compared to control rabbits (Table 3).

In rabbits, ovarian ascorbic acid was also significantly decreased after FSH and HCG injection, these data agree with that from rats, which suggest LH causes the ovarian ascorbic acid depletion (Parlow, 1958). In rabbits, serum ascorbic acid level did not change at the time of ovulation.

Serum Ascorbic Acid During Estrus in Sows

Table 4 shows the serum ascorbic acid levels during the estrus cycle in five sows.

During estrus, serum ascorbic acid levels did not change significantly as compared to the other stages of the cycle ($p<0.05$).

In all of these animals including rats, rabbits and sows no significant changes of serum ascorbic acid at the time of ovulation have been observed. This may be due to species differences in ascorbic acid metabolism or serum

Table 3. Ascorbic acid levels of the serum and ovaries of rabbits.

	Serum As A. mg/100 ml	Wt. of Ovaries (g)	Ovarian AsA mg/g	Ovarian AsA mg/whole ovary
Control group	1.174±0.052	0.586±0.284	0.568±0.086	0.303±0.124
48 H after FSH injection	1.525±0.013	0.463±0.077	0.483±0.151	0.220±0.069
30 min. after HCG injection	1.283±0.393	0.869±0.378	0.436±0.076	0.377±0.117
2 H after HCG injection	0.910±0.565	0.646±0.311	0.324±0.041	0.248±0.129
4 H after HCG injection	1.044±0.197*	0.745±0.489*	0.302±0.042*	0.220±0.131*

Ascorbic acid values; average ± S.E.

Ascorbic acid values are the mean of 5 values except those marked with an * which is the average of 4 values.

New Zealand White rabbits weighing about 3.5 Kg were used.

Table 4. Serum ascorbic acid levels in sows.
All values are mg per 100 ml of serum.

Sow Nos	4-11 8 a.m.	**4-12 8 a.m.	**4-12 1 p.m.	4-13 8 a.m.	4-25 8 a.m.	4-26 8 a.m.	4-27 8 a.m.
Y-4	1.560	0.960	1.410	0.990	0.750	1.010	1.040
Y-14	0.920	1.030	0.990	1.030	0.940	0.960	0.980
Y-8	1.240	1.070	0.910	1.030	1.040	1.010	1.090
X-107	1.200	0.750	1.170	0.830	0.720	0.720	0.770
H-27-8	0.620	0.630	0.660	0.580	---	---	---
Average ± S.E.	1.108 ±0.355	0.888 ±0.170	1.028 ±0.283	0.892 ±0.193	0.863 ±0.153	0.925 ±0.139	0.970 ±0.141

**Estrus stage

S.E. = standard error

ascorbic acid may not be directly related to ovulation. At any rate, it does not appear to be a good parameter for measuring the time of ovulation.

Serum Ascorbic Acid Changes in Cows

Serum ascorbic acid levels at the beginning of estrus were significantly higher than later in this period. However serum ascorbic acid levels throughout estrus were not significantly increased above the other stages of the cycle. Phillips et al. (1941) reported that the plasma ascorbic acid levels showed a peak in the middle of estrus even though the increase was small.

Results of the present study showed that serum ascorbic acid did not change significantly ($p<0.05$), during the estrous cycle. Actually, the trend was toward a decrease at the middle of estrus. Our data (Figure 5) show that there are marked individual variations in plasma ascorbic acid levels. For example, cow No. 935 showed very high levels of serum ascorbic acid (about 6 times the "normal level") about 40 hours before estrus.

Serum progesterone levels for most of the cows were not highly related to serum ascorbic acid levels except in cow No. 935. The progesterone levels of this cow tended to be related to the changes in ascorbic acid concentration.

These results suggest that serum ascorbic acid levels do not relate to ovulation. The observations from other species including rats, rabbits and sows also confirmed

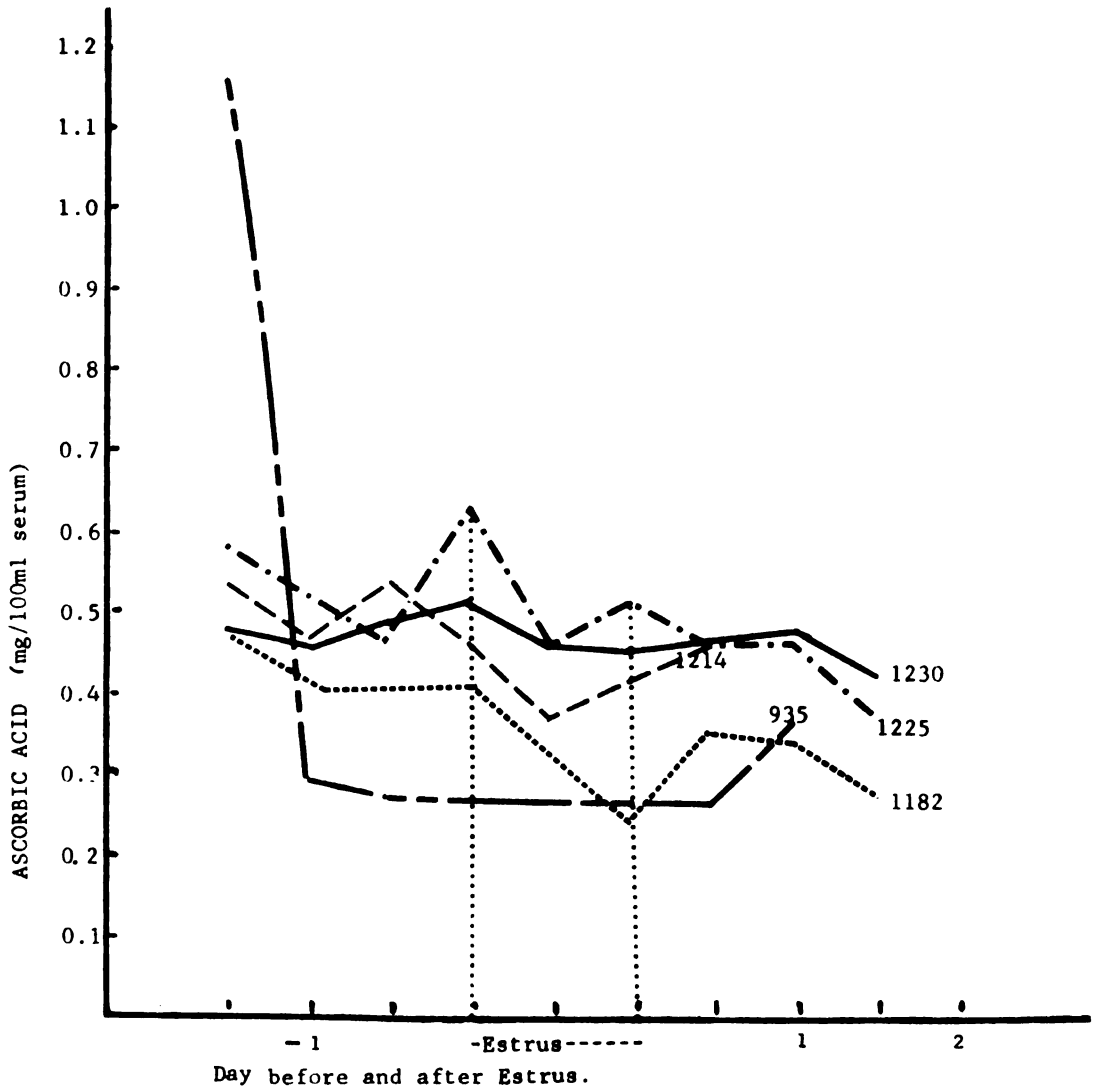
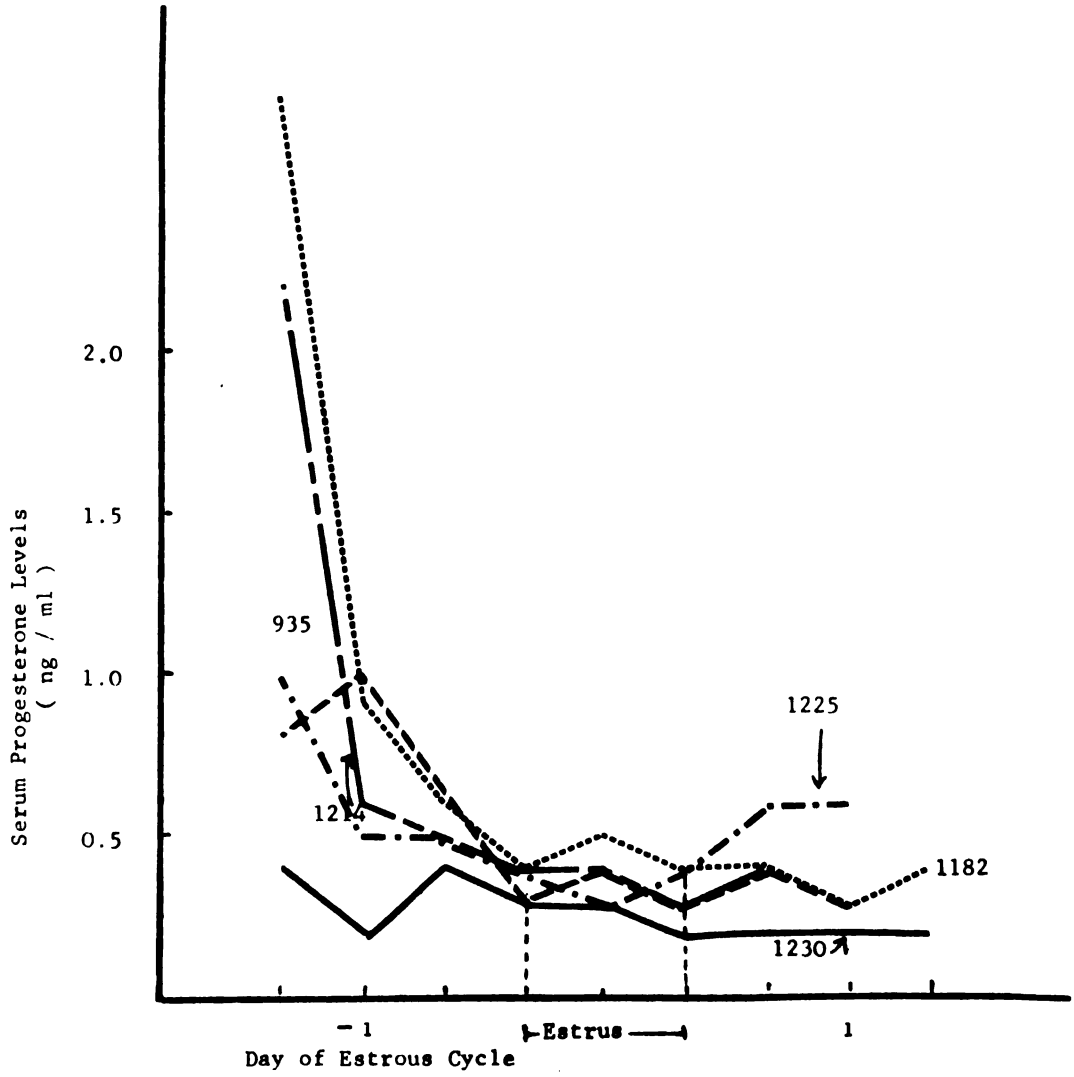


FIGURE 5 SERUM ASCORBIC ACID LEVELS IN COWS JUST BEFORE AND AFTER ESTRUS

FIGURE 6 SERUM PROGESTERONE LEVELS IN COWS BEFORE AND AFTER ESTRUS



the fact that ascorbic acid does not change at the time of ovulation. In other words, serum ascorbic acid is not a good indicator of the ascorbic acid role in the reproductive process.

On the other hand, there appears to be some indication of a relationship between serum ascorbic acid levels and prostaglandin injection in cows. Within 10 hours after $PGF_2\alpha$ injection, serum ascorbic acid starts to increase, reaching a peak which remains for only several hours (Figure 7). With one exception, cow No. 801, the cows showed secondary peaks of serum ascorbic acid 28 hours following $PGF_2\alpha$ treatment. According to Hafs et al. (1972) ovulation occurs about 74 hours after $PGF_2\alpha$ injection into cows. During this period of time in which ovulation should occur, serum ascorbic acid levels were very constant. Serum progesterone levels were also measured and within one hour after $PGF_2\alpha$ injection, progesterone levels decreased very rapidly and remained low for the three days following estrus which was as long as that parameter was measured (Figure 7). In cow No. 1188, serum ascorbic acid levels were low throughout the experimental period, although she also showed a second peak of ascorbic acid after $PGF_2\alpha$ treatment. She was very sick throughout the experimental periods, which might explain the low serum ascorbic acid. She also showed very low levels of progesterone throughout this period.

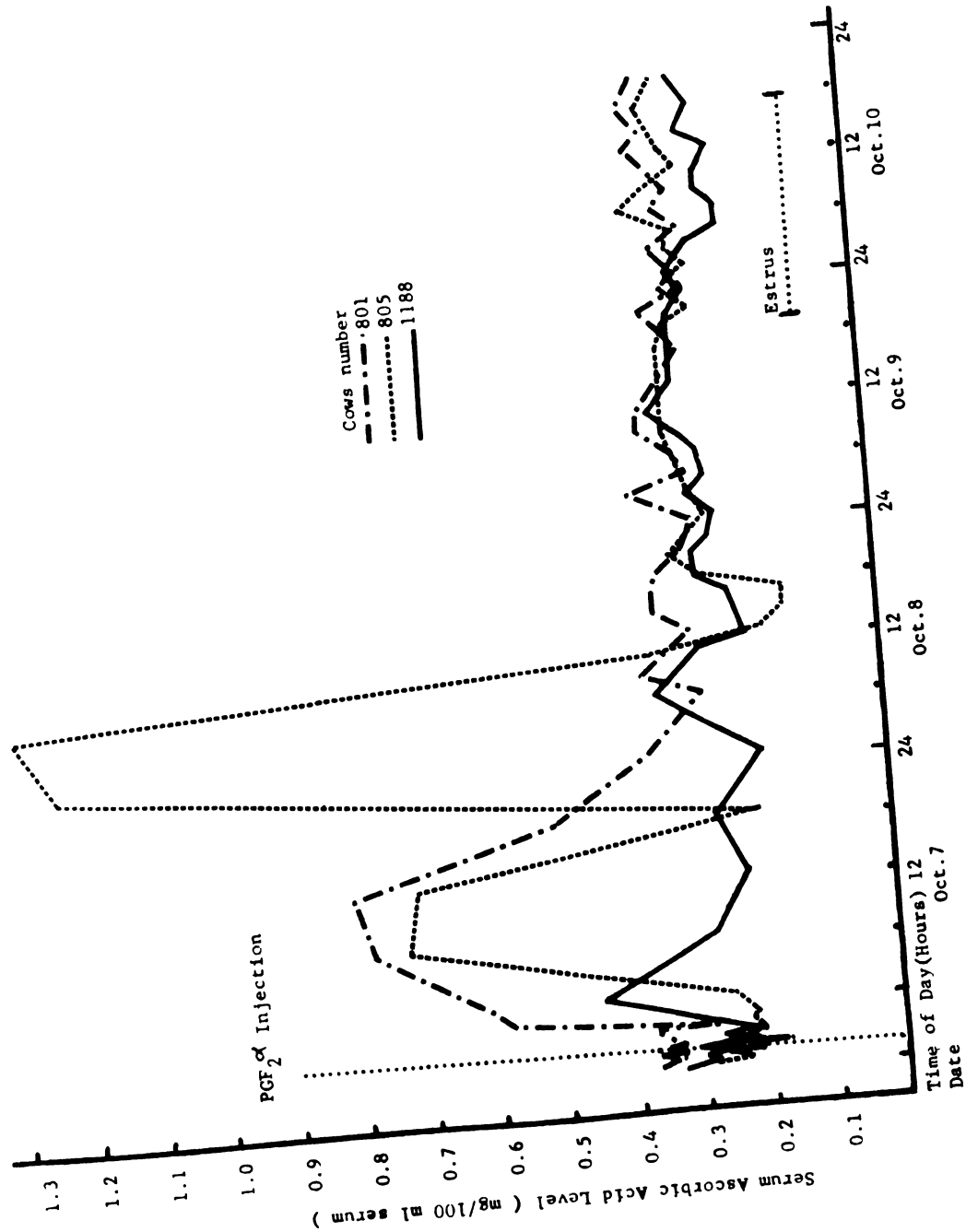


Figure.7 Serum ASCORBIC ACID LEVEL AFTER $PGF_2\alpha$ INJECTION INTO HYSTERECTOMIZED COWS

Prostaglandin is known to have luteolytic effects on the corpus luteum and to decrease progesterone synthesis. The significance of this phenomena and the exact mechanism of the $PGF_2\alpha$ and ascorbic acid is not clearly understood. One possibility is that ascorbic acid may be related to steroid synthesis. The role of ascorbic acid in sex steroid synthesis is not clear; it is not known whether ascorbic acid has the same effect on sex steroid synthesis as it does on adrenal steroidogenesis. However, all steroids share the same common pathway, i.e., the intermediate from acetate to cholesterol, is pregnenolone. Ascorbic acid is known to have an inhibitory affect on the hydroxylase system in adrenal steroidogenesis. A decreasing concentration of ascorbic acid in the adrenal cortex is associated with an increased adrenal steroidogenesis (Kitabchi, 1967b).

Our results showing increasing serum ascorbic acid after $PGF_2\alpha$ may be related to steroid synthesis possibly of estrogen. If ascorbic acid inhibits the hydroxylase system which appears to be an essential step in estrogen synthesis, then $PGF_2\alpha$ injection may possibly result in an increased release of ascorbic acid from the ovaries producing a transient increase in serum ascorbic acid. One other possibility is that $PGF_2\alpha$ has stimulated the anterior pituitary to release hormones especially ACTH. It may be that under these circumstances, ACTH acts on both the adrenal cortex and ovaries to release ascorbic

acid in manners analogous to a stress reaction. $PGF_2\alpha$ may also be related to the release of other anterior pituitary hormones, e.g. thyroid and growth hormone. The hormones might increase basal metabolic rate and carbohydrate metabolism. So overall metabolic rate may increase with a concomitant increase in ascorbic acid synthesis.

Relationship Between Serum Ascorbic Acid and Ovarian Ascorbic Acid

Cows, like other mammals, have high concentrations of ascorbic acid in the ovary. The most prominant changes in the ovary before ovulation is follicular development and the production of hormones that play a role in the growth of the follicles. On day 17 of the cycle in cows, progesterone levels start to decrease and maximum follicular growth occurs at this time with increasing estrogen synthesis. The significant increase of serum ascorbic acid after $PGF_2\alpha$ injection may be related to these hormonal changes before ovulation. These suggest that the ovary may be one of the possible sources of increased serum ascorbic acid. To directly test this hypothesis, ascorbic acid levels of the follicular fluid were measured. Table 5 shows the results of the ascorbic acid levels in follicular fluid of cows. During the stages of most rapid follicular fluid formation, there was a higher concentration of ascorbic acid than in any other phase of the cycle; this occurred about day 17 of the cycle. Such evidence

indirectly supports the hypothesis that ascorbic acid may be involved in estrogen formation. Small follicles, those in the growing stage, have higher ascorbic acid concentrations in their fluid than the large ones; the difference being about two-fold (Table 6).

Whether or not this high concentration of ascorbic acid in follicular fluid can be a possible source of increased serum ascorbic acid has been examined. Suppose a cow's weight is about 1000 pounds, and that the gut fill is about 75 pounds. Therefore the actual body weight of cow is 925 pounds or 412 Kg. The plasma volume of cows is about 3% of body weight or 12.36 Kg. The average serum ascorbic acid level in cows during anestrus is 0.3 mg%. Therefore, the total circulating plasma ascorbic acid is 37.1 mg. However the follicular fluid has only 2 mg% of ascorbic acid. Consequently the ascorbic acid in follicular fluid cannot influence the serum ascorbic acid on a quantitative basis. This stems from the fact that although follicular fluid has very high concentrations of ascorbic acid, the organ itself is relatively small.

Calculation

Body weight : 1000 pounds or 450 Kg

Gut fill : 75 pounds or 38 Kg

Corrected body weight : 450 Kg - 38 Kg = 412 Kg

Plasma volume : 3% of body weight or

412 x 0.03 = 12.36 Kg (12360 g)

Table 5. Ascorbic acid concentration in follicular fluid of cows (Holstein). All values in mg per 100 ml of follicular fluid.

Physiological state	Ascorbic acid level mg/100 ml
Mid cycle: Day 10-12	1.58
Mid to late cycle: Day 17	3.27
Midcycle	0.70
50 days of pregnancy	1.33
8.5 months of pregnancy	2.89

Table 6. Ascorbic acid concentration in large and small follicles. The ovaries were secured from cows immediately after slaughter. All values in mg ascorbic acid per 100 ml follicular fluid.

Physiological state	Large follicles $\geqq$ 1.0 cm	Small follicles $\leqq$ 1.0 cm
Day 6-8 of cycle	1.04	1.84
Day 10-12 of cycle	1.25	2.50
50-60 days of pregnancy	1.22	2.51
4-5 months of pregnancy	1.43	1.75
7-8 months of pregnancy	--	2.85

Plasma ascorbic acid : 0.3 mg%

$$\frac{0.3 \times 12360}{100} = 37.1 \text{ mg}$$

Follicular fluid : 2 mg% of ascorbic acid

CHAPTER V

SUMMARY

Serum ascorbic acid did not show significant differences at the time of ovulation from the other stages of the estrous cycle. Although there was some species variation, serum ascorbic acid levels were very constant during the estrous cycle. The result suggests that serum ascorbic acid may not be directly related to ovulation.

$PGF_2\alpha$ seems to be related to the ascorbic acid metabolism in cows. Intramuscular injection of 30 mg of $PGF_2\alpha$ to hysterectomized cows was associated with a significant increase in serum ascorbic acid may be related to steroid synthesis, possibly estrogen.

Ascorbic acid concentration in follicular fluid is high during the developing stages of the cycle which is also the estrogen phases of the cycle. Although the follicle has a relatively high concentration of ascorbic acid, there appears to be no relationship between serum ascorbic acid changes and ascorbic acid in follicular fluid.

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