EFFECTS OF ORAL CONTRACEPTIVE ENOVID ON THE QUANTITY AND COMPOSITION OF MILK OF RATS

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ABSTRACT

EFFECTS OF ORAL CONTRACEPTIVE ENOVID
ON THE QUANTITY AND COMPOSITION
OF MILK OF RATS

By

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Effects of Enovid, a combined type of oral contraceptive preparation containing norethynodrel and mestranol, on lactating rats and their pups were studied in three experiments.

In each experiement, the rats were divided into two groups after parturition and the number of pups was adjusted to 5 males and 5 females. The control group was pair-fed a basal grain diet to that of their counterpart in the treatment group that received on an ad libitum basis the basal diet containing Enovid. The concentration of the Enovid in the diet was adjusted so that mestranol was equivalent to 0.1 mg/kg body weight/day and norethynodrel 0.0015 mg/kg body weight/day.

The body weights of the lactating rats and their pups in both groups of each experiment were measured at weekly intervals until weaning.

In experiment 2, the mother rats were sacrificed on the day of weaning, their mammary glands were collected,

weighed, and analyzed for DNA and RNA. In the third experiment, the mother rats were milked on 16th and 18th days of lactation following a separation from their young for 12 hours. Protein, lipid, sodium and potassium of the milk were determined.

In all three experiments, the weight gain of the pups was higher in the control group than in the Enovid group although the differences were not statistically significant.

There was significantly more ($P<0.025$) mammary gland DNA expressed as mg/100 g of body weight in the Enovid group than in control. The gland RNA and RNA/DNA ratio were also higher in the treatment group than in the control group but the differences were not statistically significant.

Milk of the treatment group contained higher ($P<0.025$) concentrations of protein than milk from the control group on both 16th and 18th day of lactation. No significant difference was found in the sodium and potassium concentrations of milk between the two groups. Fat concentration of milk was significantly higher ($P<0.025$) in the Enovid group than in the control group.

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CHAPTER I

INTRODUCTION

Milk is the natural food of the new-born, and among some people it continues to be used for many months. The duration of lactation varies with a great many factors, both social and physiologic.

In certain cultures, such as the United States and some European countries, breast fed babies may be supplemented with other foods as early as three or four weeks of age. For some mothers, lactation is terminated when the amount of milk produced declines to a point where such extensive supplementary feeding is required that it is no longer feasible to continue breast feeding.

Persistency of lactation and spontaneous lactogenesis are rare conditions in modern civilized society. In western countries in the past, and even now in primitive cultures, it was an economic policy for mothers to prolong lactation for many years by repeated suckling, so as to assist in feeding their large numbers of children. Geschichten (1960) quotes a figure of one to four percent of women in England and the United States with persistent lactation.

In many other cultures, however, breast feeding is carried on for two to three years with milk being the sole source of food for the infant for the first year or more.

In Turkey, 7 to 48% of mothers breast feed their babies for six months, 12 to 29% for twelve months, 13 to 39% for eighteen months, and 6 to 56% for twenty-four months and longer. The variation in the range differs from urban to rural areas and from west to east. One of the reasons for this long period of breast feeding is to prevent a second pregnancy (Baysal, 1968).

The total average duration of lactation in Egypt is 17.1 months, 8.3 months of it being full lactation (Hefnawi, 1966).

According to a statistical study on 1000 Lybian women, 75% breast-fed for more than 12 months; 40% of them longer than 18 months (Semm, 1966).

Breast feeding of infants is widespread in Jordan; almost all infants are breast fed until three months of age. The proportion of those weaned increases thereafter, but even at 12 months only 25% are weaned, at 16 months 50%. Ten percent of children continue to feed at breast even at 20 months (Pharaon, H. M. et al., 1968).

In Lebanon 97% of the infants is breast fed in the first month after birth and about 4% is completely breast fed for at least 9 months (Patwardhan, 1972).

In India, lactation is continued for two to four and a half years (Newman, 1970).

In Indonesia breast feeding is usually prolonged for two years (Burgess and Dean, 1962).

Another study showed that (Bhiraleus, 1970) Thai women breast feed their babies, on the average, from 12 to 18 months.

Frequently, the lactation period is a sterile one. As a matter of fact, lactation appears as the natural birth-spacing method, but a significant percentage of fully breast feeding mothers nevertheless become pregnant, or at least show ovulatory signs (Ferin, 1964).

In any population, a substantial number of women can be found who repeatedly start one pregnancy very soon after finishing the previous one. They are highly fecund noncontraceptors, many of whom wish to avoid further childbearing. Completion of a pregnancy is the right time to suggest effective contraceptive methods before the women settle into carelessness and inferior methods of birth limitation.

During the past decade, there has been a rapid acceptance of intra uterine devices and of steroid oral contraceptives.

The wide use of oral contraceptives attests to their exceptional contraceptive effectiveness, demonstrably greater than that afforded by any other method of contraception (Pincus, 1965a).

The original reports on hormonal contraceptive agents were primarily concerned with efficacy of ovulation suppression, pregnancy protection, and systematic side effects. Relatively little attention has been paid to

the effect on lactation performance and on breast fed infants.

In many developing countries where per capita income is low and substitute foodstuffs are not easily available, the infant is practically dependent on human milk for its survival and normal growth, and failure of breast feeding may lead rapidly to an increase in the prevalance of protein-calorie malnutrition (Chopra, 1972).

Since the introduction of oral contraceptives, physicians have suspected an inhibitory effect on lactation. Several studies have been published on this subject but the results have been conflicting. If lactation is adversely affected by oral contraceptives, a major problem can arise in countries where breast milk is commonly used as a sole source of nutrients for infants.

Investigations into both reproductive functions and lactation in human are difficult. For instance, practical objective measures of milk output and also other factors responsible, especially psychologic influences, lead to varying degrees of motivation and assurance, with consequent effects on the key let-down reflex.

In addition, in most of the reports the basis for comparison has been the women's own recollection of their previous performance. Human experiments often lack the rigid control which the animal experiments provide. Thus, it is sometimes imperative to carry out controlled work on laboratory animals. Much of the studies of contraceptives

and lactation have been done on animals such as rats, rabbits, mice, goats, ewes, and cows.

In this study we chose rats as experimental subjects. Enovid, a combined type oral contraceptive, was administered to the treated group. The oral contraceptive was added to a basal diet, control rats received the basal diet without added contraceptives.

The objectives were: First, to determine the quantity of milk of nursing rats by measuring the body weight gain of the pups; the mammary gland weight of the dams; and the Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) of the mammary glands. Secondly, to determine the composition of milk by determining the concentration of protein, lipids, sodium and potassium.

CHAPTER II

REVIEW OF LITERATURE

Oral Contraceptive Steroids

The idea of an oral contraceptive is as old as the human race. Nomatic clans and tribes were forced to range widely for foods for their animals as well as themselves, especially in times of famine, hence pregnancy and offspring became a double burden that compounded their condition. Native herbs were sought to ward off conception during lean years. Contraceptive plants were found, but the preparations could not be standardized because of oxidative degradation of the active ingredients. Abortion and infanticide for population control thus became historical registers of ancient crimes against human life (Goldzieher and Rice-Wray, 1966).

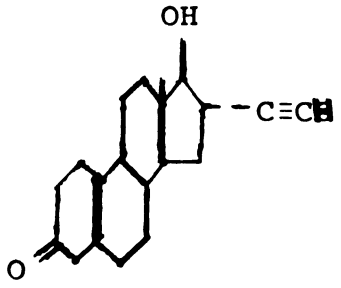
The first report on reproduction considered the corpus luteum responsible for ovulation suppression during pregnancy and this theory was confirmed by demonstrating prompt resumption of estrus following the experimental removal of the retained corpus luteum. In 1950, progesterone and stilbestrol was combined to suppress ovulation and in 1954 the effectiveness of cyclically administrated progesterone was demonstrated which led to the use of

19-norsteroids combined with estrogen to inhibit ovulation (Goldzieher and Rice-Wray, 1966).

Oral contraceptive "pills" are of two major types, combined and sequential. They both consist of estrogenic and progestational compounds.

"Enovid" contains Norethynodrel, 17-ethynyl-17-hydroxy-5(10)-estren-3-one, and Mestranol, 3-methyl ether of ethynylestradiol (Saunders and Drill, 1958). The structural formulas of these compounds are shown below.

Norethynodrel (progestin)



Mestranol (estrogen)

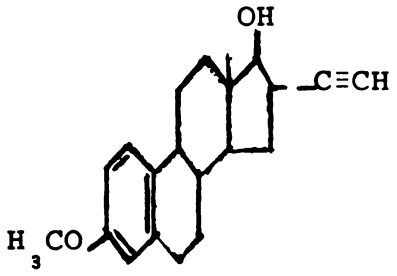


Figure 1. The structural formulas of Norethynodrel and Mestranol.

Mechanism of Action

Although hormonal preparations have been in use as oral contraceptives for more than a decade, their exact mechanism of action is still not known.

The principal levels at which contraceptive drugs may exert their primary action are as follows:

The Anterior Pituitary Gland or Source of Gonadotropins:

The oral contraceptives act to prevent ovulation by inhibiting the secretion of gonadotropins from the

pituitary gland. The pituitary gonadotropins stimulate the ovary to secrete estrogen and progesterone, and these hormones act in turn to inhibit the secretion of pituitary gonadotropins (Drill, 1966).

If ovulation is inhibited, this must be due either to a diminution in the responsiveness of the ovaries to gonadotropins or to changes in the secretion or release of one or more gonadotropins or other substances.

The effect of these preparations on the gonadotropic function of the pituitary gland is well documented. Paesi and Van Rees (1960) showed that estradiol inhibited the synthesis of the gonadotropins. More specifically Overbeck and Visser (1964) concluded that lynestrenol inhibits follicle stimulating hormone (FSH) secretion while 6-methyl-lynestrenol inhibits luteinizing hormone (LH) secretion in the rat. Brown (1964) found that norethynodrel with mestranol inhibits the secretion of LH; Taymor (1964) reported the same effect with norethisterone.

The Hypothalamus and Central Nervous System:

It seems reasonable to postulate that the reduction in gonadotropic secretion is due to the inhibition of the activity of the hypothalamus or associated parts of the central nervous system (CNS), since there is firm evidence that pituitary function is normally dependent upon the secretion of hypothalamic "releasers" (Fanard and Ferin, 1966). Oral contraceptives might affect the pituitary

by reducing or suppressing entirely the production of these releasers.

The Gonads:

The steroids in oral contraceptives suppress hypophyseal function, secondarily resulting in a decrease in the endogenous secretion of estrogens from the ovaries (Diczfalusy, 1965).

Further supporting evidence for the mechanism of action comes from reduction in urinary pregnanediol in the second half of treated cycles indicating absence of functioning corpora lutea. This is also supported by absence of the luteal thermogenic effect; this is a satisfactory indirect index of ovulation in untreated and estrogen treated cycles but not during administration of progestins as they may produce a thermogenic effect. Data from administration of norethynodrel (Swyer, 1969) for varying periods during the first half of the cycle suggest that the mechanism of action is related to the early phase of follicle development and not to specific events immediately preceding ovulation. This temporary arrest of follicle development resembles the situation which exists in normal pregnancy (Venning, 1962).

The Accessory Organs of Reproduction:

It is likely that estrogen-progesterone preparation can, at all events in the rat and the rabbit, affect the transport of the ovum through the Fallopian tubes, by

virtue of an effect on the secretions or motility of the tubes themselves.

It is also possible that the sperm cannot penetrate the very viscid cervical mucus produced by these compounds.

Changes in the uterus have also been claimed to be responsible for the effects of oral contraceptives. For example, the endometrium, responsive as it is to the administered hormones, would not accept a fertilized ovum (Shearman, 1965).

Especially the first three of these levels are so completely interdependent that an agent acting primarily at any one of these levels suppresses the other two.

In some species, higher centers are influenced by changes in the accessory organs or by mating but there is no evidence that this is so in humans.

General Effects

All of the various progestin-estrogen combinations have quite similar biological properties (Pincus, 1966a). These properties are divisible into three categories: actions on reproductive tract and allied organs; effects on other endocrine systems; and effects on systems and functions not ordinarily associated with sex hormone action.

Reporting on a 3-year period of cyclical administration of norethynodrel-mestranol (Enovid, 10 milligrams per 60 kilograms of body weight) to prepubertal female rhesus

monkeys, Kar et al. (1965) stated that Enovid did not cause any change in the ovary except a consistent increase in weight.

The effect of progestin-estrogen contraceptives on the uteri is characterized by a relative predominance of stromal edema and by a degree of glandular involution. There is a reduction in duration of menstruation and also a reduction in the quantity of menstrual fluid (Pincus, 1965b and Garcia, Satterthwaite and Pincus, 1964).

Young animals fed fairly high doses of progestin-estrogen combinations continuously for many months tend to have lower body weights than control animals. This is in large measure due to an inhibition of appetite (Husain and Pincus, 1965).

Two measures of thyroid function commonly used in human subjects have been applied to users and nonusers of Enovid. The first of these, the amount of protein-bound iodine in blood, exhibits an average increase during medication (Pincus, 1965a). The second measure is the uptake ot administered radioactive iodine. Pincus (1966b) found no difference in percentages of uptake between long-term users and controls. Employing much larger than normal dosages of medroxy-progesterone alone or in combination with estradiol, Maneschi et al. (1962) found a decrease in uptake of radio-iodine and other evidence of depressed thyroid function.

The hematocrit values and the white blood counts are fairly constant from year to year in any given woman, and no meaningful trends occur in long-term users (Pincus, 1966b).

Winter (1965) showed no significant increase in the risk of thromboembolic death from the users of Enovid.

Studies of the cardiovascular system have revealed no significant trends in blood pressures.

A tendency to lowered glucose tolerance has been reported in some users of Enovid, with a high frequency in women with a family history of diabetes (Gershberg, Javier and Halse, 1964).

Exceptional weight gain has been attributed to some but not all of the oral contraceptives.

The psychological effects have been also discussed. The users of the contraceptives have complained about headache, nervousness, and so on.

Both increases and decreases in libido have been reported.

At present there are a large number of reports which are concerned with the fact that synthetic progestogens have a favorable influence upon existing malignant tumors.

In endometrial carcinoma with lung and epithelial metastases, favorable influences have been observed during treatment with progesterone (Kelley, 1961 and Kennedy, 1963).

The occurrence of mammary carcinoma during treatment with steroid combinations has not been described so far (Haller, 1972), in spite of the fact that 913 women have been under constant medical supervision in San Juan, Puerto Rico, for the past 6-9 years. According to Pincus (1964), there is a significant reduction in tumor incidence during Enovid treatment.

One cannot yet answer the question of whether or not treatment with commercially available ovulation inhibitors increases the risk of contracting cancer. The American Food and Drug Administration, the British Council on Drugs and the German Health Commission have considered all the findings up to the present time but have not decreased the availability of ovulation inhibiting preparations because of any potential risk of cancer (Haller, 1972).

Lactational Effects

Lactation comprises two distinct processes, secretion of the milk into the alveoli and discharge of the milk via the milk ejection action of oxytocin.

Prolactin is a hormone which is necessary and specific for milk secretion. However, prolactin is not the only hormone which initiates and maintains milk secretion, a group of hormones are necessary. Secretion might also be stimulated by suction and various local stimuli. In all lactating females suckling or milking maintains secretion,

which stops at weaning or if milking ceases (Houssay, 1965).

The hormonal factors responsible for mammary development are well known for rodents such as the mouse and rat, but less completely known for other species.

An alveolar development like that of early pregnancy is obtained with a combination of three hormones: estrogen, progesterone, and prolactin. If growth hormone, corticosteroid and thyroxine are added, the development is greater. The suprarenal hormones are not essential, but they usually assist development and are necessary for secretion (Houssay, 1965).

Estrogens can evoke considerable development of the mammary gland, initiate milk secretion and alter milk composition. It is generally believed that estrogens act by stimulating the production of lactogenic hormones of the anterior pituitary. Whether the estrogens act through the hypothalamus or directly on the pituitary is uncertain (Cowie, 1961).

By itself, progesterone has no effect; but it does have a marked one if estrogen is injected beforehand or simultaneously.

Oral contraceptives, when given in high dosages, have been shown to inhibit lactogenesis, and may also inhibit established lactation. According to Venning (1966), during oral contraception, the stimulus of continued suckling will usually overcome hormonal suppression.

With lower dosage there is little, if any, effect (Swyer, 1969). For example, in a study reported by Pincus (1965b) with norethynodrel and mestranol at 20 mg per day 77% of the women alleged diminished lactation, 5% an increase; whereas at the 2.5 mg dosage the corresponding figures were 15% and 50% respectively.

Little is known about the possibility of the excretion of the hormones present in oral contraceptives or their metabolites into the milk.

Effects on the initiation of lactation (lactogenesis):

During pregnancy, the increased circulating levels of estrogen and progesterone as well as other hormones, result in an extensive ductal and lobulo-alveolar breast development which appears to be functionally complete during the mid-trimester (Morris, 1967).

Various theories have been proposed on the nature of the mechanism which ensures the correct timing of lactogenesis:

According to the hypothesis of Nelson (1936), the estrogenic hormone is the agent depressing the lactation in the second part of pregnancy. The high estrogenic level in the blood would inhibit the prolactin secretion of the pituitary and the sensitivity of the mammary gland to prolactin.

The theory of Meites and Turner (1942) is based on the determination of the prolactin content of the pituitary.

Estrogenic hormone increases the prolactin content of the pituitary in every dose and species they studied. Progesterone, at the same time, inhibits the effect of estrogenic hormone and the prolactin outflow from the pituitary. From the second part of the pregnancy the progesterone level decreases and it cannot be detected at birth. At this period prolactin secretion is gradually increased due to the lack of inhibitory effects of progesterone.

In the "two thresholds" theory, Folley and Malpress (1944) postulated that estrogenic hormone has two kinds of effect: Lower doses of estrogen stimulate and higher doses inhibit prolactin secretion.

Folley (1956) and Cowie (1961) emphasized the role of estrogen-progesterone combinations; and in general they agreed that a fall in progesterone at parturition allows estrogen to exert its lactogenic effect by stimulating secretion of prolactin or the lactogenic complex, at the maximum rate.

A widely held concept (Falconer, 1971) is that steroids from the ovaries and placenta, and possibly even from the mammary gland itself, can inhibit milk secretion by exerting a direct inhibitory effect on the pituitary and placental lactogenic hormones; in addition it has been shown that the high levels of estrogen acting at both hypothalamic and pituitary levels, depress the release of prolactin from the anterior pituitary. At parturition the fall in the blood levels of steroids permits the lactogenic

hormones to act on the alveolar cells while the lower levels of estrogen may allow increased output from the pituitary.

Effects on the Maintenance of Lactation (Galactopoiesis):

The two chief physiologic mechanisms involved in the maintenance of lactation are the secretion of prolactin from the anterior pituitary gland as the result of suckling stimulation, and the ejection reflex leading to the release of oxytocin from the posterior pituitary gland upon the stimulus of nipples.

Since small or moderate doses of estrogens can initiate lactation and increase pituitary prolactin content in a number of species, several workers have administered estrogens in an attempt to increase milk production.

In one study, five cows were fed 10 mg of diethylstilbestrol beginning 60 days after parturition and continuing for about eight months. Five identical twins served as controls. The estrogen-fed cows averaged 13% more fat-corrected milk during the treatment period than the controls, which was attributed to greater persistency (Meites, 1961).

On the contrary, some studies have shown that the effects of estrogens on established lactation, in dosages that produce discernible effects on milk flow, are inhibitory both in goats and in cows (Sykes, 1953).

Some human studies have indicated inhibition of galactopoiesis by estrogen-progesterone combinations.

Kamal et al. (1969) in a double-blind study administered different combinations of gestagen to 120 Egyptian women divided into four groups, 6 to 10 weeks postpartum; a fifth group (control) was given a placebo. The subjects and their infants were followed until weaning. The results showed that the small-dose lynestrenol had the least and the high-dose, Lyndiol 2.5 mg, or deladroxade injections had the greatest inhibitory effects on lactation.

The study of Bhiraleus et al. (1970) showed that 22 Thai women on Ovulen (1 mg ethynodiol diacetate and 0.1 mg mestranol) or C-Quens (80 µg mestranol plus 2 mg chlormadinone acetate), started 6 weeks postpartum, exhibited decreased milk volume.

Effects on the Quantity of Milk:

The available literature describing the use of oral contraceptives during lactation reports observation ranging between an inhibitory effect on milk production to an increase in milk output.

Decrease in quantity of milk:

With the introduction of pure crystalline estrogens, several studies carried out in laboratory animals found that the growth and mortality rates of the litters were adversely affected (Folley, 1952).

Joshi and Rao (1968) demonstrated in rats that both the dose of 50 µg per day and 500 µg per day Enovid

caused 10 to 20% inhibition in growth rate of the young. However, the inhibition was not proportional to the dose (Table 1).

Table 1. Growth of the rat litters whose mothers were fed Enovid (6 young per litter).

Compound Administered and Dose	Weight of the litter (g)				
	Day 2	Day 5	Day 10	Day 15	Day 20
Propylene glycol	35.5	49.2	72.3	108.3	143.3
S.E.	±2.1	±2.43	±3.7	±7.9	±8.1
50 µg Enovid	36.2	49.8	70.3	94.5	127.0
S.E.	±2.4	±3.18	±3.13	±9.2	±10.5
500 µg Enovid	35.9	50.0	70.0	92.3	123.6
S.E.	±2.72	±3.49	±2.77	±6.9	±8.5

Chinnatamby (1967) stated that lactation suppression was dose related to the progesterone component of the medication; the higher the progesterone content, the greater the amount of suppression.

The same observation was made by several other authors. They stated that, in general, the high dose of norethynodrel may reduce or inhibit lactation in women, but as dosages were reduced, lactation suppression was also reduced (Drill, 1966; Goldzieher and Rice-Wray, 1966).

Kaern (1967) fed placebos or 1 mg norethindrone with 0.05 mg mestranol to 451 lactating women starting from 1 to 8 days postpartum. Although cessation of lactation did not occur during this period, the infants in the treated

group required more supplementary feedings than the control group.

Frank and co-workers in Chicago studied 124 women started on 1 mg oral ethynodiol diacetate and 0.1 mg mestranol, 72 hours postpartum. Sixteen mothers were desirous of breast feeding. Only four mothers were able to do so for more than two months. In the 12 others, lactation gradually disappeared. No adverse effects on the infants were noted. The authors conclude that in the small number of patients studied, lactation is not well initiated or will soon be depressed in the majority of women on this regimen (Frank et al., 1966).

Rice-Wray and associates (1968) studied a low-dosage, totally synthetic progestogen norgestrel (0.5 mg) combined with ethinyl estradiol (0.05 mg) (Ovral). From a group of 300 women observed during a total of 3175 cycles, the authors were able to follow 20 women who began treatment while lactating. Eleven women had already noted a tendency toward a decrease in the amount of milk. Of the remaining nine, three reported diminished lactation after starting treatment. Four continued lactating as before for from five to nine cycles.

Miller and Hughes (1970) studied the effects on lactation of another low-dosage, oral, combined medication, 1 mg norethindrone and 0.8 mg mestranol, or a placebo, given daily for 21 days, starting 2 to 5 weeks postpartum and on a randomized double-blind basis. One hundred mothers

who wanted to nurse their infants for at least 3 months, one-half of whom wished to use an oral contraceptive while nursing and one-half of whom did not use any preparation, comprised the study cohort. During the second and third weeks after birth, infants of the mothers taking oral contraceptives exhibited a decrease in the mean weekly weight gain. Infants in the placebo group had better weight gain and required fewer supplementary feedings. The authors imply that the estrogen component is the lactation suppressing agent.

Im Bombay, Kora (1967) studied the effect of 1 mg oral ethynodiol diacetate combined with 0.1 mg mestranol (Ovulen) on a group of low-income Indian mothers who were lactating satisfactorily and were breast feeding their infants between 4 and 24 weeks of age. The infants from control group mothers received greater amounts of breast milk than the infants whose mothers were on Ovulen; the amount of milk obtained from control mothers increased weekly, whereas medicated mothers produced less milk than prior to taking Ovulen; and weight gain per week was significantly less in the experimental infants than in the control infants.

According to Garcia and Pincus (1964) the exact effect of Enovid on lactation when it is initiated during the puerperium probably depends upon how early it is started and how large a dosage is used. They stated that when the treatment, even at 10 mg per day, was initiated between

five and eight weeks postnatally, the patients did not complain of any alteration in lactation. However, in the series followed by Satterthwaite (1962) in Humacao, Puerto Rico, where medication was started by the third week postnatally, to better insure the continued inhibition of ovulation beyond the pregnant state, there is the suggestion that lactation may be diminished at the higher dosage. At lower doses, lactation does not appear to be significantly altered (Table 2).

Table 2. Effects of Enovid on lactating women (Humacao).

Dosage (mg/day)	Total No. Followed	Less than Previously	Same as Previously	More than Previously
		(Percent Lactating)		
20.0	22	77	18	5
10.0	37	38	57	5
5.0	84	45	45	10
2.5	34	15	70	15

Increase in quantity of milk:

Semm (1966) administered placebos or 2.5 mg lynestrenol combined with 0.075 mg mestranol to 100 lactating mothers from 1 to 10 days postpartum and found the daily production of milk to be identical in the treated and control mothers. He then administered the drug or placebos to a second group from 10 to 31 days postpartum and found higher milk production in the treated group. The estrogen used in both of these investigations was mestranol, the higher

doses being used in the study that showed a beneficial effect on milk production. Semm concludes that 2.5 mg Lyndiol taken daily has no inhibitory influence on lactation regardless of whether the administration was started immediately postpartum or 10 days later.

Kamal et al. (1970) used placebos, 0.1 mg ethinyl estradiol, 0.5 mg lynestrenol, or 1 mg lynestrenol with 0.1 mg mestranol on 40 subjects on the second day postpartum. The results showed greater milk secretion and weight gained by the babies whose mothers were receiving estrogen or progesterone or the combined oral contraceptives.

Karim et al. (1971) reported that norethisterone ethanate (200 mg every 84 days) and medroxy-progesterone acetate (150 mg every three months) were found to be completely effective in fertility control when started in the puerperium. Neither agent had any ill effects on the amount of milk nor on the duration of lactation. From the third month onward, the hourly available milk and the infant weight gain per month were statistically higher in the treated than in the controls (Table 3).

Table 3. Average weight gain per month in grams during the period of full lactation.

Month	Group (number of subjects)				
	1(100)	2a(57)	2b(55)	3a(68)	3b(51)
1	633.2	636.0	652.6	641.4	643.6
2	702.8	709.5	714.3	724.3	712.0
3	553.0	738.0	716.9	721.0	736.0
4	552.4	672.3	730.0	667.4	678.3
5	549.6	791.8	763.0	798.0	801.0
6	499.3	739.8	748.3	693.8	728.6

1 control group

2a norethisterone ethanate (NE) 200 mg every 84 days

2b medroxyprogesterone acetate (MPA) 150 mg every three months

3a on the seventh postpartum day received NE

3b on the seventh postpartum day received MPA

Effects on the Composition of Milk:

The biochemical composition of milk is equally as important as the total milk yield in contributing to the growth, vitality, and well-being of the infant. Abdel Kader and co-workers (1969a) administered placebo, Lyndiol 2.5 (lynestrenol 2.5 mg plus mestranol 0.075 mg), Lyndiol 1.0 (lynestrenol 1.0 mg plus mestranol 0.10 mg), lynestrenol 0.5 mg alone, and Deladroxate (hydroxyprogesterone acetophenide 150 mg plus estradiol enanthate 10.0 mg) to women and found that total proteins diminished gradually in the milk of all the lactating mothers. The

observed diminution was statistically significant in all the groups except the placebo, in which it was insignificant. Milk fats and lactose showed a similar behavior. The general trend of the inorganic constituents of milk in the different groups was toward diminution. They stated that the change in milk composition induced by gestagens might be through a direct effect on mammary tissue or indirectly by affecting blood components. According to the authors, gestagens may also exert a selective action on the permeability of the alveolar epithelium, leading to a decrease in the different milk electrolytes, Na, K, Ca, Mg, and P.

Kader et al. (1969b) also studied the effects of gestagens on the quantitative and qualitative changes in goat's milk. The results showed a decrease in total quantity per 24 hour as compared with the control group. The milk fats and proteins dropped gradually; lactose showed minor variation, whereas changes in minerals were inconsistent.

They indicated that estrogen facilitates the transfer of amino acids into estrogen-sensitive cells. This would include the mammary tissue and would affect, in the long run, protein biosynthesis. The authors concluded that gestagen administration adversely affects lactation as judged by the changes in milk yield and composition.

In contrast to the findings of Egyptian investigators, in Israel, Toaff et al. (1969) treated nursing mothers

orally with an estrogen (ethinyloestradiol, 0.3 mg daily) or a progestin (6-alpha-methyl-17-alpha-hydroxyprogesterone acetate, 30 mg daily) for 5 full days after birth. They found that a highly significant increase in the protein content of milk followed the use of relatively high doses of estrogen, while the milk yield remained unaffected (Table 4).

Table 4. Values of lactose, protein and fat (G/100 ml mean ± S.D.) in transitional milk of healthy nursing mothers.

Milk component	Control	Group 1-EO	Group 2-MAP
Lactose	5.73±0.11	5.54±0.12	5.62±0.14
Protein	1.76±0.07	2.20±0.09	1.88±0.06
Fat	3.55±0.28	3.77±0.19	3.06±0.14

Estrogens promote growth and increase the functional capacity of tissues which are usually considered target organs of estrogens, through the activation of a specific enzyme, an estrogen-dependent pyridine nucleotide transhydrogenase. This enzyme has been shown to be present in human endometrium, placenta, myometrium, pituitary and mammary gland. The increased activity of the enzyme resulting from the addition of estrogens, both natural and synthetic, leads to an increase in the supply of biologically useful energy and thereby to an increase in the biosynthesis of proteins, nucleic acids and fats which are fundamental for growth. The same steps may lead to the

biosynthesis of proteins and fat secreted by the mammary gland into the milk resulting with an "enrichment" (Toaff et al., 1969).

The effects of administered estrogen on milk composition of lactating cows has been noted by Folley et al. (1941) and by Spielman et al. (1941). Dosages of estrogen which caused no change or only transient decreases in milk yield increase the milk solids and fat percentage of milk. This effect was also seen with dosages which caused marked decreases in milk yield (Sykes, 1953).

Effects on the Infant:

It is frequently assumed that the depression of the growth of young is due to failure of milk secretion. However, there are observations showing that the mammary glands of estrogenized rats whose litters have died may contain considerable quantities of milk. Faulty milk ejection, unsatisfactory maternal behavior and the presence of estrogens or their toxic metabolites in the milk could equally well lead to failures in growth process (Cowie, 1961).

A single case of gynecomastia developing in an infant nursed by a mother receiving an oral contraceptive has drawn attention to possible effects on infant tissues if hormonal agents are transmitted through the milk (Curtis, 1964).

Two groups have attempted a direct study of the excretion in breast milk of the steroids of oral

contraceptives. Laumas et al. (1968) found that 1.1% of the radioactivity present in a single dose of tritiated norethynodrel was excreted in breast milk in the subsequent 5 days. On the other hand, Pincus et al. (1966) found only 0.004 to 0.13% of the radioactivity of orally administered tritiated norethynodrel and ethynodiol in the breast milk over the course of 4 days. The disadvantage of these studies is that they do not distinguish whether the radioactive label was still in the form of the original steroid or of an inactive metabolite.

In the Netherlands, Wijmenga and Van der Molen (1969) administered ^{14}C mestranol orally in a Lyndiol tablet to four women using Lyndiol during the lactation period shortly after delivery. They studied the concentration of radioactivity in the plasma and excretion in the urine and milk. During a collection period of 4 days after oral administration, 0.0002 - 0.013% of the administered dose was excreted in the milk.

Theories as to Why Contraceptive Steroids Decrease Lactation

According to Cowie (1961), in the intact rat, progesterone from the ovary is the essential hormonal contribution for the inhibitory effect of estrogen on lactation. Estrogen and progesterone together can act locally on the mammary gland rendering it insensitive to prolactin. In the cow and goat the mechanism of action is

uncertain, it may be a direct effect blocking the release of galactopoietic factors from the anterior pituitary or estrogen may act in conjunction with progesterone as in the rat.

It was also indicated by the same author that the ability of prolactin to initiate milk secretion in rabbits with well developed mammary glands could be effectively inhibited if mammary growth promoting doses of estrogen and progesterone were injected at the same time; the presence of either estrone or progesterone alone had no inhibitory effects. Antagonism between the estrogen plus progesterone combination and prolactin was relative and could be overcome by increasing the dose of prolactin or decreasing that of the steroids.

Masson (1948) stated that on the nursing female rat a ratio of $\frac{\text{estrogen}}{\text{progesterone}} = \frac{10}{10,000}$ stops the secretion of milk while each of the two hormones given separately produces no effect. The hormonal combination acts synergistically to cause proliferation of the acini of the mammary glands, and the secretory activity can no longer continue. This proliferation effect was attributed to Enovid by Joshi and Rao (1968).

Griffith and Turner (1962) studied the effects of estradiol benzoate and progesterone and found a depression in lactation. They suggested that these hormones influenced lactation by some action upon ovaries and secretion of some substance detrimental to lactation rather than hormones

acting directly upon secretion process. Since estradiol benzoate or estradiol benzoate plus progesterone may stimulate endogenous secretion of relaxin by ovaries, it appears that relaxin may be involved. Inhibitory substance appears to interfere with normal action of oxytocin on contraction of myoepithelial cells and milk removal. In normal rats receiving EB or EB+P mammary glands were still engorged with milk at the end of nursing period. Since exogenous oxytocin was administered at the beginning of nursing period, inhibitory substance could not have influenced secretion of oxytocin. This lack of contraction was not a vascular problem, because topical administration of oxytocin did not affect myoepithelial cells.

Theories as to Why Steroid Contraceptives Increase Lactation

Prolactin is an important factor in the induction of lactation and estrogen and progesterone are needed for the development of the glandular apparatus of the breast and to ensure the responsiveness of the gland to prolactin (Donovan, 1970).

Estrogen is one of the most potent stimulators of prolactin secretion by the pituitary. Low and moderate doses of estrogen are more effective in this respect than large doses, but even large doses have not been demonstrated to reduce prolactin secretion (Meites, 1970).

Donovan (1960) showed that estrogen can act directly on the anterior pituitary to stimulate prolactin secretion *in vitro*, and it seems probable that it also acts through the hypothalamus to decrease the chronic inhibition of prolactin secretion.

Meites (1967) has reported that many agents, including estrogen and progesterone, which are known to stimulate prolactin secretion and induce lactation experimentally act by depressing hypothalamic production of prolactin inhibiting factor (PIF), thereby increasing secretion of prolactin by the pituitary.

The effects of estrogen on PIF were determined by injecting estradiol benzoate into intact rats for 10 days, killing the animals on the eleventh day and assaying neutralized acid extracts of the hypothalamus *in vitro* (Ratner and Meites, 1964). Hypothalamic PIF was depleted.

In relation with the contraceptives, Meites (1967) has observed that injections of norethynodrel with mestranol (Enovid) into virgin female rats resulted in inhibition of hypothalamic PIF production, increased prolactin secretion by the pituitary, and mammary growth and lactation. At the same time, Enovid was found to decrease the content of luteinizing hormone-releasing factor (LH-RF) and follicle stimulating hormone-releasing factor (FSH-RF), in the hypothalamus, decrease pituitary LH and FSH concentrations, and inhibit follicle growth and ovulation in the ovaries. It appears, therefore, that Enovid acts primarily through

the hypothalamus to depress ovarian activity while at the same time stimulating mammary function.

Role of FSH and LH in the Induction of Lactation:

Because the follicle stimulating and luteinizing hormones act synergistically and because it is unlikely that either hormone is ever secreted alone, the role of these two gonadotrophins will be discussed together.

Early follicular development depends on FSH (follicle stimulating hormone), this hormone together with LH (luteinizing hormone) leads to follicular maturation and estrogen production in the ovary. Due to the increase in estrogen production, an inhibition of FSH secretion occurs, probably via the sexual center situated in the mid-brain, while there is also a simultaneous stimulation of LH secretion. Follicular rupture should, therefore, depend largely upon FSH/LH quotient. Following extrusion of the egg, prolactin is secreted which fosters the production of progesterone by the corpus luteum (Haller, 1972).

In essence, gonadal hormones estrogens and progestogens inhibit the secretion of pituitary hormones when present in high concentrations so that when the blood levels of steroids fall, the output of gonadotrophin rises. Estrogen and progesterone on certain time will evoke, not inhibit, gonadotrophin release. This is termed a positive feedback action. A single, suitably-timed injection of estrogen causes ovulation in rats, but is ineffective after

hypophysectomy, thus indicating that the steroid provokes gonadotrophin secretion (Donovan, 1970).

Donovan (1970) further suggested that a reciprocal relationship exists between the secretion of FSH and LH on the one hand, and prolactin on the other. Prolactin secretion is favored, and that of FSH and LH depressed by chronic estrogen treatment. The stimulus of suckling also tends to suppress gonadotrophin secretion and to raise that of prolactin.

Hormonal Development of the Mammary Gland:

The mammary gland represents a unique organ from a neuroendocrine point of view, since its development and secretory functions are regulated by a harmonious concord of most of the endocrine glands and the central nervous system. All of the anterior and posterior pituitary hormones may act on the gland, either directly or through hormones of target organs, and all of them are controlled by the hypothalamus.

Ovarian hormones:

The most vital peripheral hormones for mammary proliferation are the ovarian hormones, estrogen and progesterone. Normal cyclic changes in these two hormonal components govern the normal cyclic changes in mammary gland development associated with estrus or menstrual cycle. The same changes can be reproduced experimentally by exogenous hormonal treatment. Animals such as the

mouse, rat, rabbit or cat will respond to physiological amounts of estrogen by proliferation of the duct system, while for lobulo-alveolar growth progesterone is also required (Sulman, 1970).

Kohn and Baker (1964) observed that norethynodrel alone or combined with mestranol in high levels injected into virgin intact rats for 28 days produced mammary gland development comparable to glands of late pregnancy.

As to the question whether the ratio of progesterone to estrogen, or the absolute amounts of these hormones are the critical factors in mammagenesis, it seems clear, on the basis of thorough experiments carried out in the guinea pig (Benson et al., 1957), that the absolute amounts of estrogen and progesterone determine the growth response of the mammary gland.

Ovariectomy does not affect the growth responses during early lactation, however, it retards the prolonged growth response caused by intense nursing (Tucker, 1967). This observation suggests that the ovary is not the principal organ for mammary gland development during lactation.

Pituitary hormones:

Meites (1966) stated that the anterior pituitary hormones are the primary stimulators of mammary growth, even in normal physiological states. He indicated that part or all of the effect of estrogen on mammary growth might be explained by its relationship to prolactin

secretion, since it has been shown that estrogen stimulates prolactin secretion in the intact animal both by stimulating synthesis of the hormone in the pituitary and by acting on the hypothalamus to block release and synthesis of the prolactin inhibiting factor.

In some species, somatotrophin (growth hormone) may replace prolactin and in all it enhances its action. Human somatotrophin has the action of prolactin; the two effects have not been separated (Houssay, 1965).

Anterior pituitary hormones alone induce full lobulo-alveolar development in the absence of pituitaries, ovaries, and adrenals (Talwalker and Meites, 1961). However, prolactin from anterior pituitaries transplanted into ovariectomized rats did not produce as much total development of mammary gland as prolactin from pituitaries transplanted into intact animals (Sinha and Tucker, 1968). Therefore, the total development of the breast is believed to involve synergistic action of ovarian and pituitary hormones (Reid et al., 1972).

Posterior pituitary hormone oxytocin exerts its effects by maintaining the structural integrity (DNA), directly on the mammary gland (Tucker, 1969).

Other hormones:

Adrenocorticotrophic hormone (ACTH), and thyroid stimulating hormone (TSH) of the anterior pituitary gland,

adrenal steroids, thyroid hormones and placental hormones play a secondary role on mammary gland development (Figure 2).

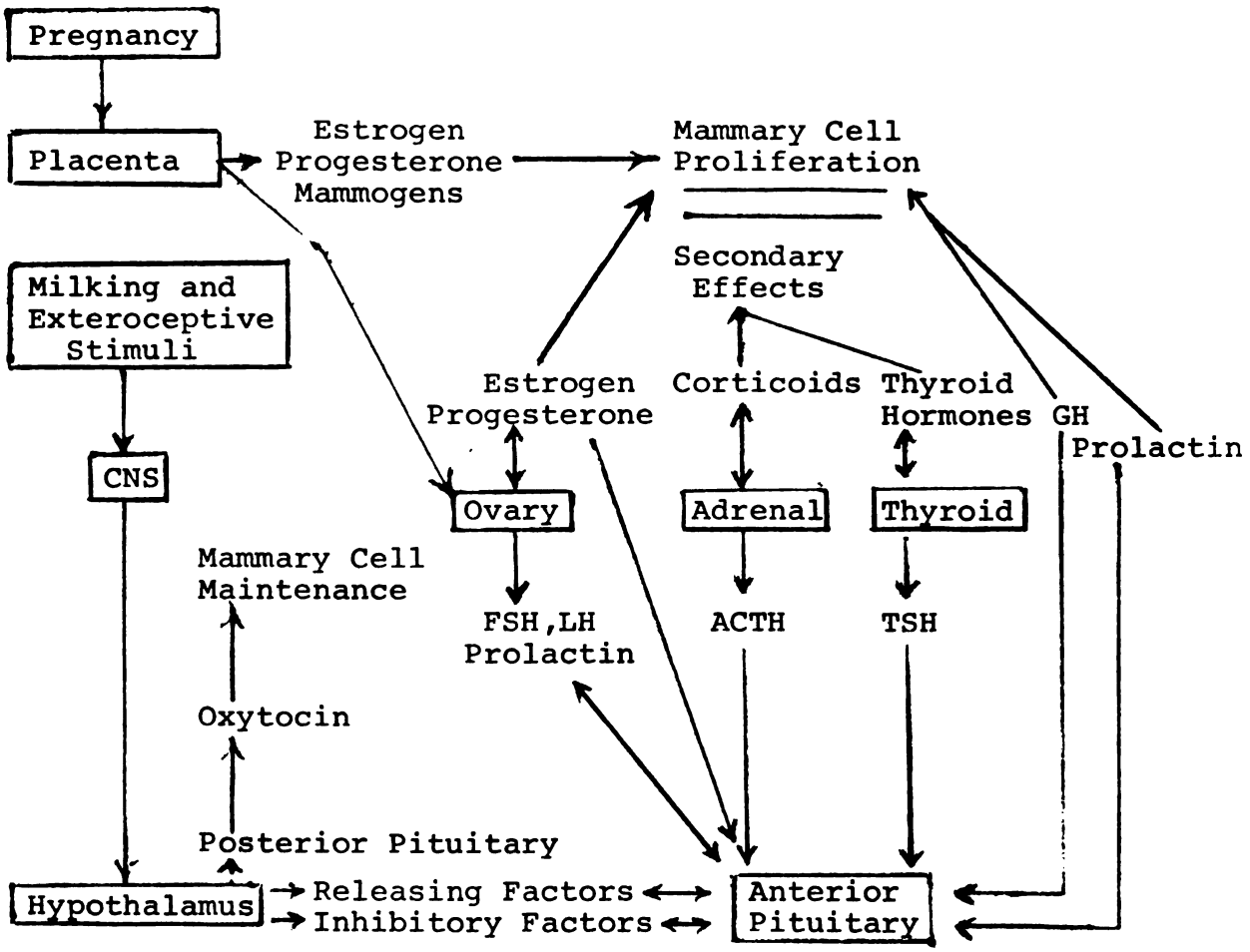


Figure 2: Hormonal factors affecting mammary cell proliferation (From H. A. Tucker, 1969).

CHAPTER III

MATERIALS AND METHODS

Animals

Eight-week-old pregnant Sprague-Dawley rats close to term were used in three experiments. The first experiment consisted of eight rats and the second and third experiments each sixteen rats. All three experiments were divided into treatment and control groups, each having equal number of animals. The rats were housed singly in metal cages and were given water and food, a natural grain ration of our laboratory, ad libitum until delivery. On the first day of lactation (the day of delivery), the number of pups was fixed at 10 by supplying those litters not having ten with pups of the same age. In order to control the differences in growth rate between sexes, each litter contained five males and five females.

The Trials:

In the first trial, the weights of the dams and the pups were recorded at the day of parturition, and at weekly intervals until weaning (3 weeks). The rats were carried to the scale in their home cages to avoid disturbing the animals.

In the second trial, body weights were also recorded every week until weaning. In this experiment rats were further divided into two sub-groups so that one sub-group each from the control and treated animals was used only for milk sampling. The other sub-groups were used for growth measurements. In the latter sub-groups, control rats were pair-fed to the treated rats. For those used in milk sampling, milk was obtained on the 16th and 18th days of lactation. However, the milk collected in this trial could not be used for analyses because of evaporation and the small quantity of milk obtained. On the 21st day, all the dams were sacrificed and their abdominal-inguinal mammary glands were quantitatively collected.

The third trial was conducted like the second one except the mammary glands were not removed. Furthermore, the mother and young rats in the growth group were weighed on the first, 15th and 21st days of lactation. More frequent weighing was not attempted because previous experiments showed that frequent handling of young rats resulted in high mortality, largely due to cannibalism.

The Cages:

To prevent the pup from eating the feed provided the mother, the 20" x 14" x 12" metal cages were separated into two parts (Figure 3). The litter was put in the back part and the food in front part so that the mothers could jump over to reach the food but the pups could not. The water bottles were also placed in the front.

In order to prevent excessive food spillage, the food cups were provided with food cup lids and food followers.

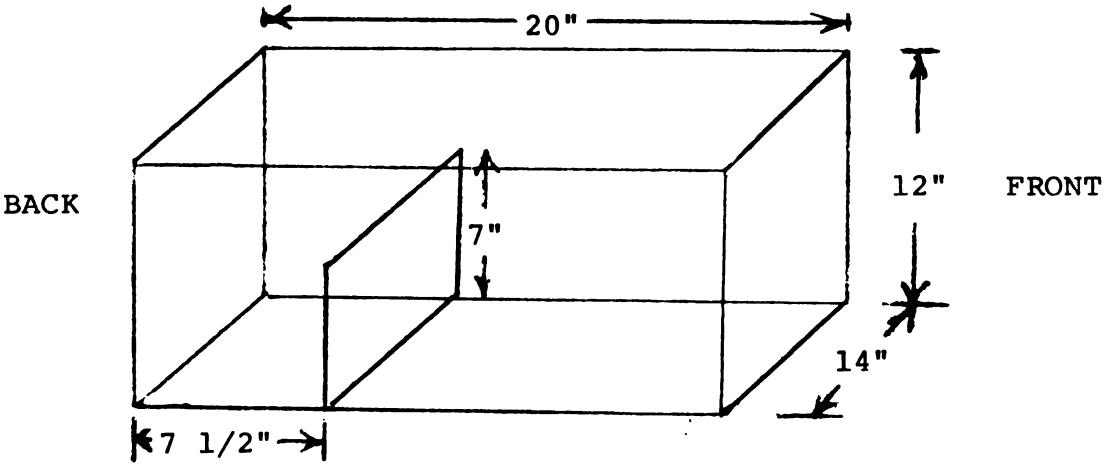


Figure 3: The diagram of the cages for lactating rats.

Method of Treatment and Dose of Enovid

At the first day of lactation the animals were distributed between the basal ration group and that supplemented with the synthetic steroids so as to equalize the body weights of the pups at birth in both groups.

The ration of treatment group was supplemented with mestranol and norethynodrel so that it will provide 0.1 mg mestranol per kg body weight and 0.0015 mg norethynodrel per kg body weight per day. These rats were permitted to eat on ad libitum basis. Everyday, each rat in the basal ration group received the same amount of feed as that consumed in the previous 24 hours by its counterpart in the supplemented group. This pair feeding regimen continued for 21 days until the pups were weaned.

Collection of Milk

Milking Apparatus:

The milking apparatus consisted of these interrelated components (Figure 4): a very fine and small teat tube, milk collector, vacuum system and monometer.

The teat tube was connected with the milk collecting vial which was covered with a rubber stopper. In operation, the nipple was placed into the teat tube.

The vacuum pressure was monitored by means of the monometer.

The pressure on the monometer was controlled by the operator while milking the rat. It was set to maintain the vacuum at approximately 6-8 cm of mercury in order to prevent the injury of nipples and to provide easier milk flow.

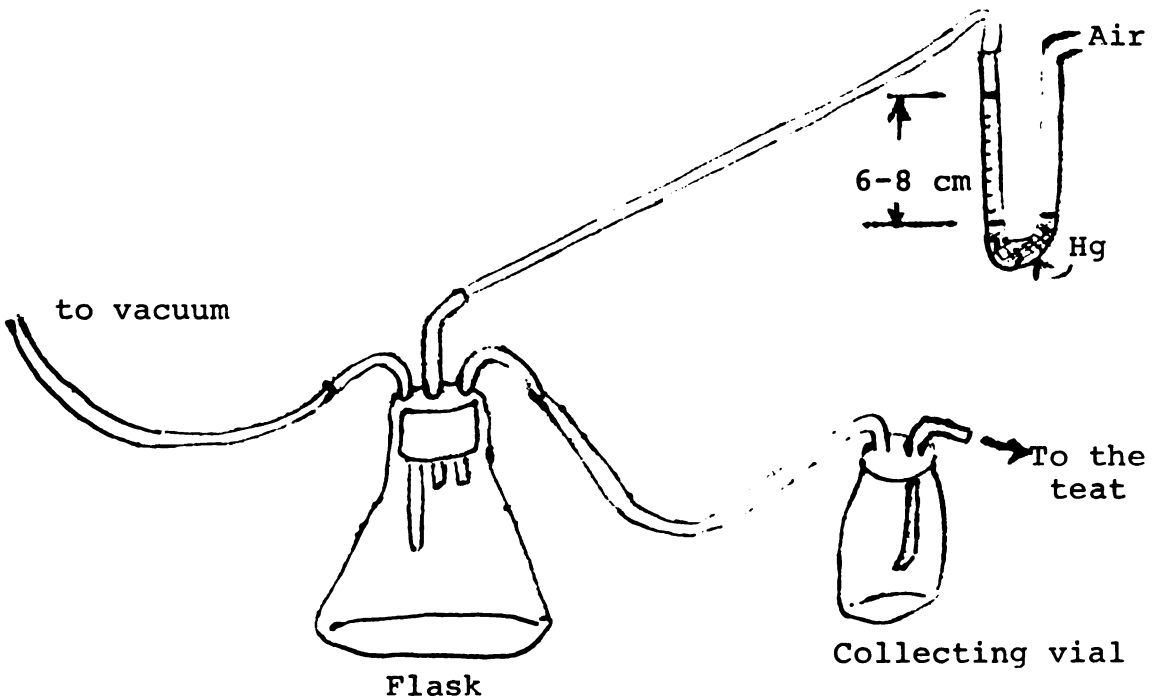


Figure 4: The diagram of milking apparatus.

Milking Procedure:

The lactating rats were separated from their young for 12 hours prior to milking. In order to immobilize the rats during the milking operation, they were lightly anesthetized.

0.1 units of oxytocin were given in 1/10 cc volume intraperitoneally to cause a milk ejection reflex to take place. Approximately 1 min after the injection of oxytocin, the nipples were moistened with sterile saline solution.

Each rat was milked for 15 minutes.

The teat tube was applied to each nipple one at a time. With the thumb and index finger of the left hand, the breasts were squeezed gently toward the nipples while the right hand was applying the tube.

After being milked, the rats were immediately returned to their young.

Collection of Mammary Glands

On the 21st day of lactation, the mother rats were sacrificed by cervical dislocation and six abdominal-inguinal mammary glands were removed. The weights of the glands were recorded and the glands were stored in the freezer until analyzed.

Chemical Methods

Protein:

The amount of protein in the milk was determined by Lowry's method (Lowry et al., 1951) (Appendix II).

Reagents;

Reagent A - 2% $NaCO_3$ in 0.1 N NaOH

Reagent B - 0.5% $CuSO_4.5H_20$ in 1% Na or K tartrate

Reagent C - Alkaline copper solution. Prepared on the day of use by mixing 50 ml of reagent A with 1 ml of reagent B.

Reagent D - Folin:ciocalteau reagent diluted to make it 1 N in acid.

After preparing the samples with the reagents, absorbance of the solutions were determined with a Beckman Spectrophotometer (Acta C III).

Concentrations of unknown was calculated from standard curves using casein as standards.

Lipids:

The amount of lipids was determined by methanol-chloroform extraction (modified method of Folch et al., 1957) (Appendix III).

Reagents:

Chloroform:methanol (2:1)

Chloroform

The lipids were dried by evaporation in a vacuum oven at 65°C from the chloroform-lipid solution obtained from the Ch:me extracts.

Calculation was done by substracting the weight of flasks from the weight of flask + lipid.

Sodium and Potassium:

Sodium and potassium in the milk were determined by atomic absorption using a Model 303 Perkin Elmer AA after diluting the samples with deionized water (0.1 ml of sample was diluted with 25 ml of water). Concentrations were calculated from the standard curve containing both Na and K.

RNA and DNA:

The nucleic acids in the mammary glands were determined by the method described in "Methods of Biochemical Analysis" (Munro and Fleck, 1966) (Appendix I).

The tissues were diluted to 20 times the weight with ice-cold water and homogenized in a Waring blender. Two ml of the homogenates were defatted by extracting with chloroform:methanol (2:1) and then with chloroform for 24 hours.

The RNA levels were estimated by the procedure mentioned above and DNA concentrations by the method of Ceriotti (1952).

Table 5. Operating conditions and instrument settings for Na and K determination.

Na		K	
Operating Conditions	Instrument Setting	Operating Conditions	Instrument Setting
Wavelength- 589 A°	Range- Visible	Wavelength- 7665 A°	Range- Visible
Slit	Slit- 4	Slit	Slit- 4
Source- Hallow cathode	Source- Current given on lamp	Source- Hallow cathode	Source- given on lamp
Fuel- Acetylene (oxidizing flame)	Flow- 9	Fuel- Acetylene	Flow- 9
Oxidizer- Air	Flow- 9	Oxidizer- Air	Flow- 9
Filter		Filter	Filter- in

Statistical Analyses

The data were analyzed on an electronic calculator.

Student's t-test was performed on the weight gain data for each trial.

A two-way analysis of variance was conducted on the combined weight gain data, and on the mammary gland and milk analyses.

CHAPTER IV

RESULTS AND DISCUSSION

Quantity of Milk

Weight Gain of the Pups:

In experiment 1, the average weight gain per pup was lower for the Enovid group than for the control group. The Enovid group gained 15% less weight than the control group. For experiment 2, the weight gain of the pups in the control group was again higher than the pill group. This time, the pups in the pill group gained 5% less weight than those in the control group. Experiment 3 showed a similar trend in which the control group gained 4% more weight than the pill group (Table 6).

On the average, body weights of the pups whose mothers were treated with oral contraceptive steroids were 8% lower than the body weights of the pups in the control group (Figure 5).

This result is consistent with the findings of Joshi and Rao (1968) who found 10 to 20% decrease in weight gain of rat pups in the treatment group, with the observation of Chinnatamby (1967) who showed an 8 to 10% reduction in lactation on women, and with the finding of Miller and Hughes (1970) who also observed a decrease in the mean

Table 6. Average body weight and weight gain of pups in three experiments (g/pup).

Experiment	Initial wt.		1st week		2nd week		3rd week		Gain	
	Enovid	Control	Enovid	Control	Enovid	Control	Enovid	Control	Enovid	Control
1	5.8 ±0.1	5.9 ±0.4	10.7 ±0.7	11.2 ±0.9	22.6 ±0.9	21.3 ±2.4	31.0 ±1.5	35.6 ±1.7	25.3 ±1.4	29.7 ±1.4
2	6.9 ±0.2	6.3 ±0.2	14.8 ±0.5	14.0 ±0.5	27.0 ±0.7	27.9 ±0.5	38.4 ±1.6	39.5 ±1.1	31.5 ±1.4	33.2 ±0.9
3	7.4 ±0.8	7.4 ±1.1	--	--	24.0 ±3.7	26.3 ±3.2	29.6 ±3.7	30.5 ±4.8	22.2 ±11.9	23.0 ±11.7
Average	6.7 ±0.5	6.5 ±0.4	12.8 ±2.1	12.6 ±1.4	24.6 ±1.3	25.2 ±1.9	33.0 ±2.7	35.2 ±2.6	26.3 ±2.7	28.6 ±2.9

± indicates standard errors

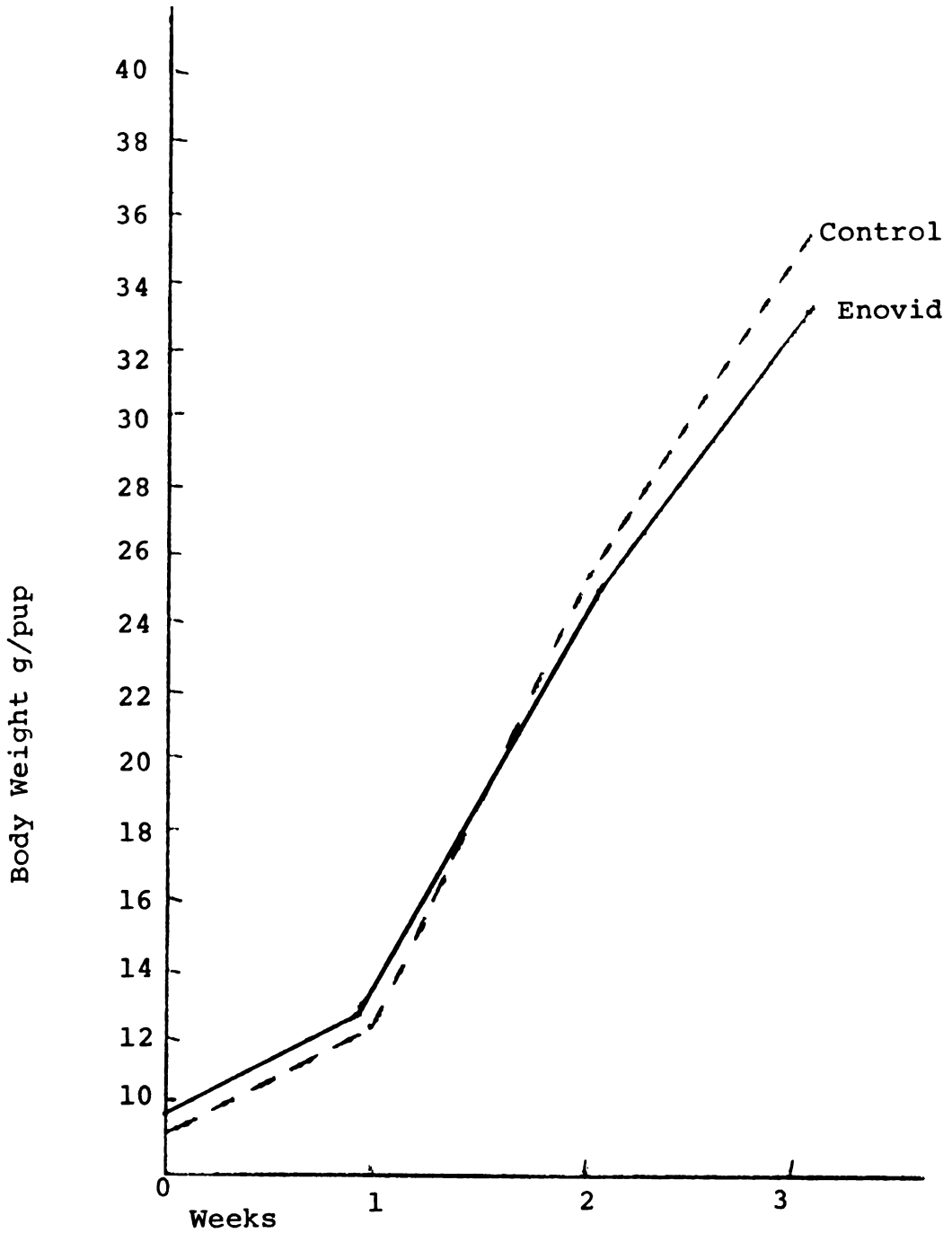


Figure 5: Body weight of pups (average of three experiments).

weekly weight gain in infants whose mothers were taking oral contraceptive pills.

Since rat pups ordinarily start to eat small quantities of solid food at about 14-16 days of age, a wall was provided in order to allow the mothers access to the food but not their pups. Occasionally, pups were observed in the compartment containing the food. This was considered of minor importance since the weight of the pups during the first two weeks of life wherein solid food was not consumed showed similar trend as that found for the third week. The pups of treatment group always weighed less than pups of control group.

There appear to be an inverse relationship between weight gain of mothers and that of the pups. In the three experiments, on the average, the dams in Enovid group gained and those in control group lost weight (Table 7). Whenever the lactating rats lost or gained little weight their pups gained weight.

Mammary Gland Weights of the Dams:

The mammary gland weights (g/100 g body weight) of the Enovid group was significantly higher than that of the control group (Table 8). According to Sulman (1970), estrogen and progesterone are the most important hormones for mammary tissue proliferation. Estrogen causes the proliferation of the duct system and progesterone lobulo-alveoli.

Table 7. Average body weight and weight gain of the dams in three experiments (g).

Experiment	Initial weight		1st week		2nd week		3rd week		Gain in 3 weeks	
	Enovid	Control	Enovid	Control	Enovid	Control	Enovid	Control	Enovid	Control
1	259.8 ±8.9	286.5 ±7.1	272.8 ±9.5	279.0 ±11.4	299.0 ±11.7	317.3 ±13.6	298.8 ±9.9	307.0 ±12.1	39.0 ±6.8	20.5 ±8.5
2	312.3 ±8.3	306.0 ±7.8	281.3 ±8.4	284.3 ±11.7	318.0 ±5.8	319.0 ±10.2	300.0 ±7.1	277.8 ±11.1	-12.3 ±10.2	-28.3 ±8.8
3	274.5 ±14.4	308.8 ±31.5	---	---	312.0 ±6.7	310.0 ±25.4	203.3 ±16.8	281.0 ±15.1	8.8 ±8.5	-27.8 ±8.9
Average	282.2 ±15.6	300.4 ±7.0	277.0 ±4.3	281.4 ±2.6	309.6 ±5.6	315.4 ±2.8	294.0 ±5.4	288.6 ±9.3	11.7 ±14.9	-11.8 ±11.0

± = standard errors

Kohn and Baker (1964) reported that development of the mammary gland occurred in virgin rats injected high levels of mestranol and norethynodrel. Thus, our finding is in agreement with this early finding that weights of mammary glands in rats increased when treated with these two steroids.

Table 8. Average mammary gland weights of control and steroid treated rats.

Rat No.	Total (Fresh) (g) Control	Enovid	g/100 g BW Control	Enovid
1	7.1	7.7	2.5	2.8
2	6.2	8.8	2.3	2.9
3	6.6	9.8	2.5	3.1
4	11.5	7.6	3.6	2.5
5	6.0	9.3	2.0	3.0
6	7.7	7.6	2.4	2.7
7	9.3	10.6	2.9	3.3
8	10.2	8.0	2.9	2.3
Average ± S.E.	8.1 ±0.68	8.7 ±0.40	2.7 ±0.27	2.8 (1) ±0.12

± S.E. - standard errors

(1) - significant difference, $p<0.05$

DNA and RNA of Mammary Glands:

In the mammary glands of lactating rats, DNA content, a measure of cell numbers was significantly higher, and RNA content, and RNA/DNA ratio, measures of protein

synthetic activity, were slightly higher in the Enovid group in comparison with the control group (Table 9).

The value of DNA and RNA for the control rats agree with the values for rats having 8 to 10 pups (Tucker, 1964).

Determination of DNA of the mammary gland has been proven to be most suitable for measurement of mammogenesis in the mouse, rat, and calf. The nuclei of the cells of mammary gland contain equal quantity of DNA. If there is a change in the quantity of DNA, it means that the number of cells has been changed (Kurcz, 1967). According to Tucker (1969), the number of milk synthetizing cells is one of the basic elements that limit milk production. However, a cell maintained during lactation does not necessarily mean that it can synthesize milk at a sustained maximal level. Therefore, in our experiment significantly higher DNA content found in the Enovid group does not necessarily indicate a higher amount of milk synthetized by these animals.

Composition of the Milk

Protein:

Milk protein concentration was significantly higher in the Enovid group than in the control group (Figure 6). This result is in agreement with the findings of Toaff et al. (1969) who found a highly significant increase in the protein content of human milk following the use of an

Table 9. Average DNA and RNA of mammary glands (based on 6 abdominal-inguinal glands or 3 pairs).

Treatment	DNA			RNA			RNA/DNA
	Total (mg)	mg / 100 g BW	mg/g gland	Total (mg)	mg / 100 g BW	mg/g gland	
Control	22.1 ±3.2	7.3 ±0.9	2.7 ±0.3	60.0 ±6.9	20.5 ±2.7	7.8 ±1.1	3.1 ±0.4
Enovid	23.1 ±3.3	7.5 (1) ±0.9	2.6 ±0.3	68.0 ±5.8	22.2 ±1.8	7.9 ±0.6	3.4 ±0.5

± indicates standard errors

(1) significant difference, $p<0.025$

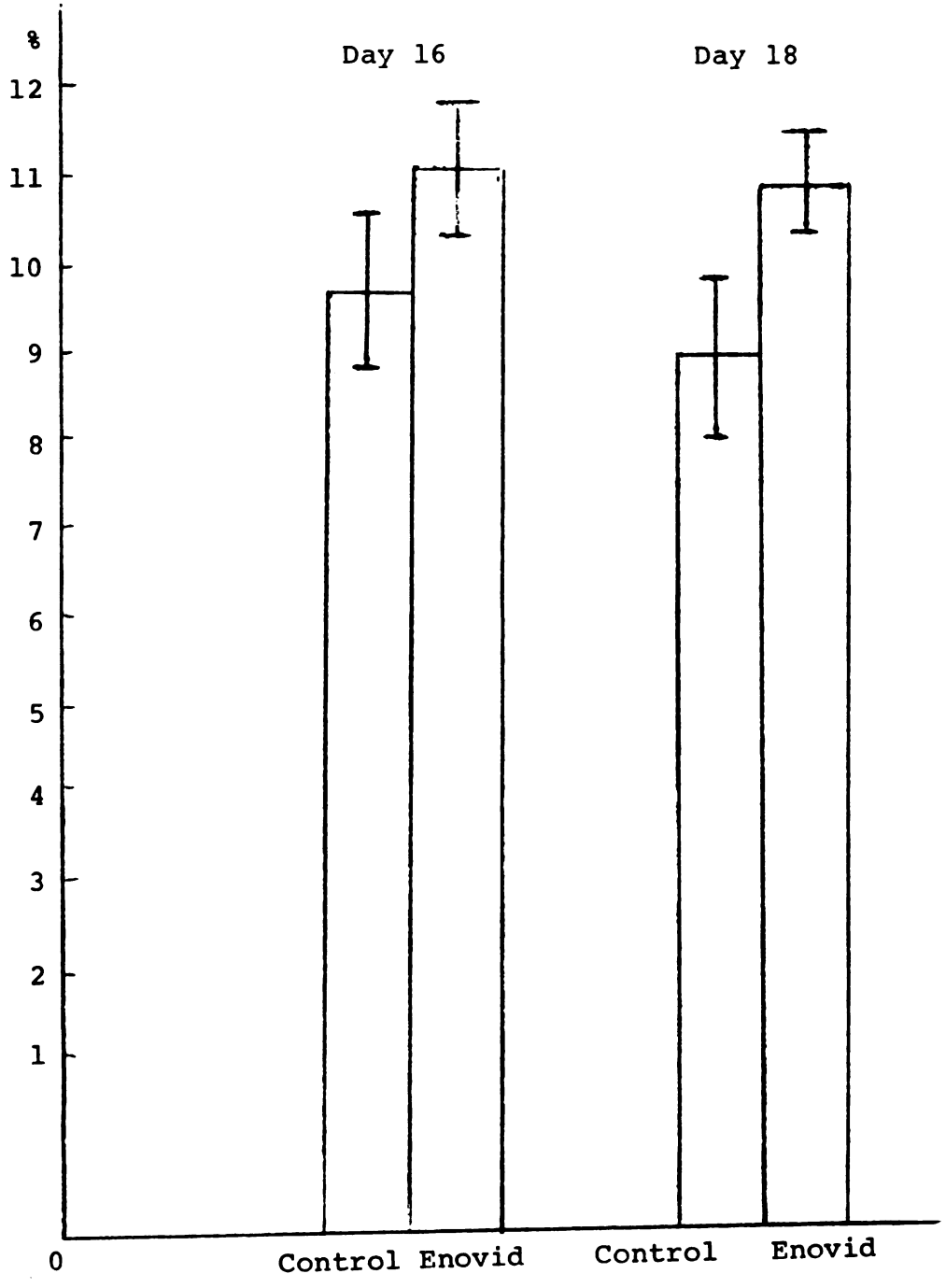


Figure 6. Protein concentration of milk in control and steroid treated rats (%). Vertical bars indicate ± standard errors.

estrogen or progesterone for five days after birth. However, our finding does not agree with the results obtained by Kamal et al. (1969, Abdel Kader et al. (1969), and Ramadan et al. (1972).

In the present study the concentration of protein in milk decreased from 16th to 18th days in both groups. Similar diminution with time of lactation was observed by Kader et al. (1969a and b) in women's and goat's milk. It is generally true in growing animals that, as growth proceeds, the protein requirement falls and the requirement for energy-yielding constituents rises (Kon and Cowie, 1961).

Sodium and Potassium:

Sodium and potassium concentrations were not different in the milk of control rats, in comparison with that of the steroid treated rats (Figures 7 and 8). Similar results were found by Kader et al. (1969) when women were given oral contraceptives.

The concentration of sodium was higher on the 18th day than on the 16th day in both control and Enovid groups. On the contrary, the concentration of potassium decreased toward the end of lactation period, that is it was higher on the 16th than on the 18th day in both groups.

Fisher et al. (1970) found that cows milk potassium dropped from the fifth to ninth month, while sodium increased from the sixth month of lactation and that

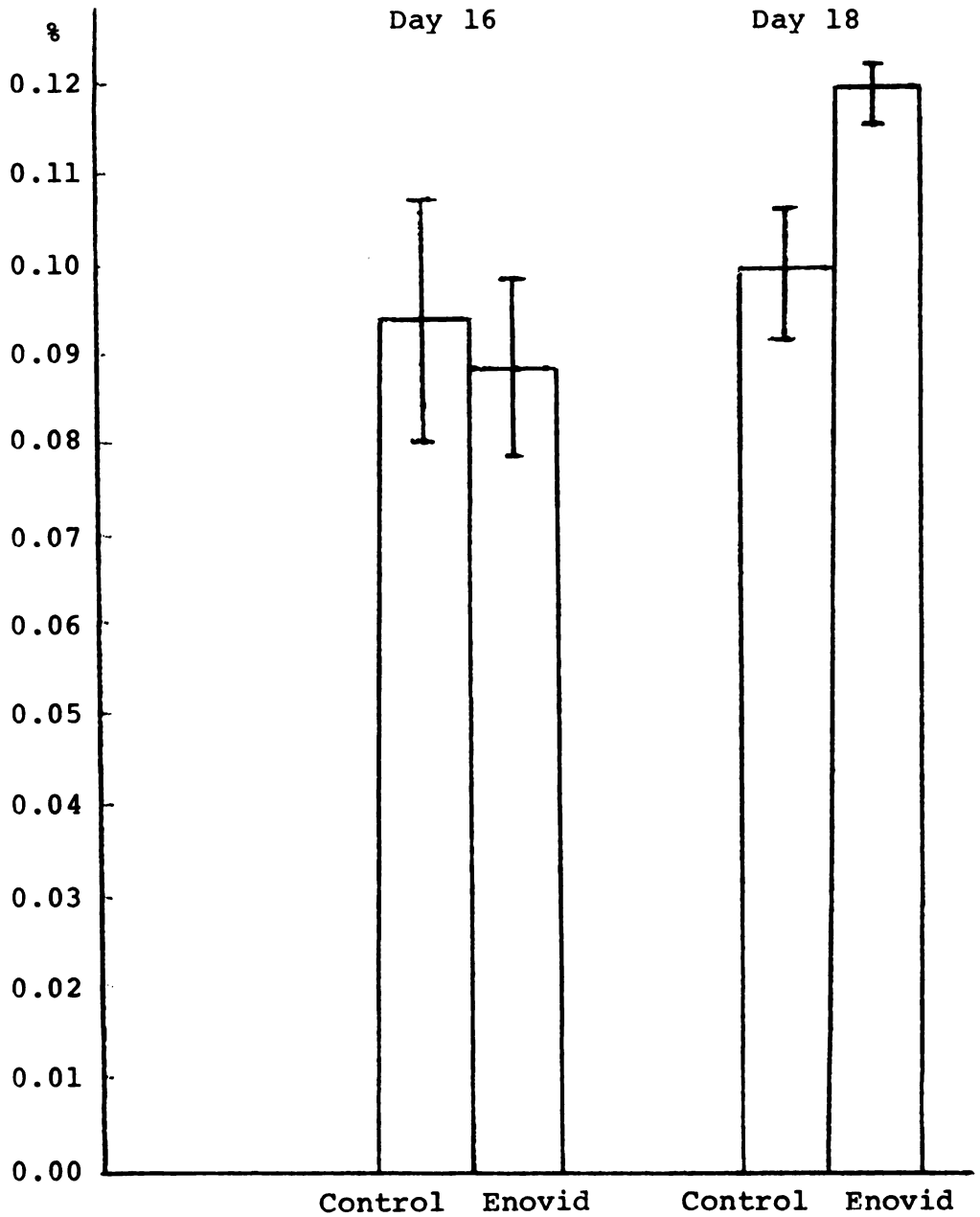


Figure 7: Sodium concentration of milk in control and steroid treated rats (%). Vertical bars indicate standard errors.

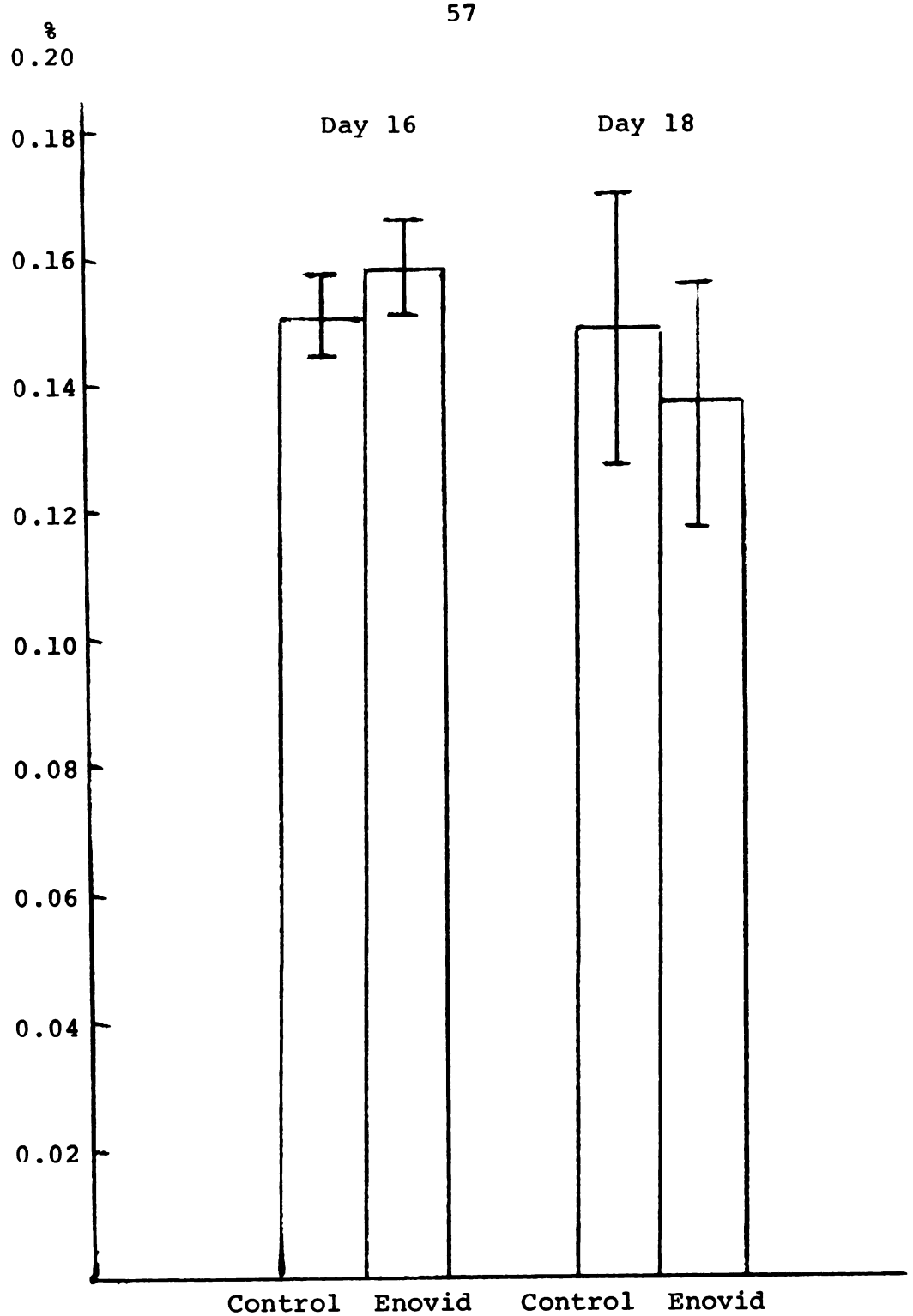


Figure 8: Potassium concentration of milk in control and steroid treated rats (%). Vertical bars indicate standard errors.

potassium concentration was correlated positively with milk production.

Similar changes during advancing lactation in sodium and potassium concentrations of milk were found by Kamal et al. (1961), and Rook and Campling (1965) which support our result.

Lipids:

The concentration of milk lipids was significantly higher ($P<0.025$) in the Enovid group than in the control group (Figure 9). There are several studies which support our finding: In the trial of Hutton (1958) it was found that injections of 12.5 mg of estradiol monobenzoate in lactating cows caused significant increases in milk fat. Karim et al. (1971) reported that human milk fat increased following injection of a progestogen. Ramadan et al. (1972) observed a marked increase in milk fat of women after administration of oral contraceptive Ovosiston.

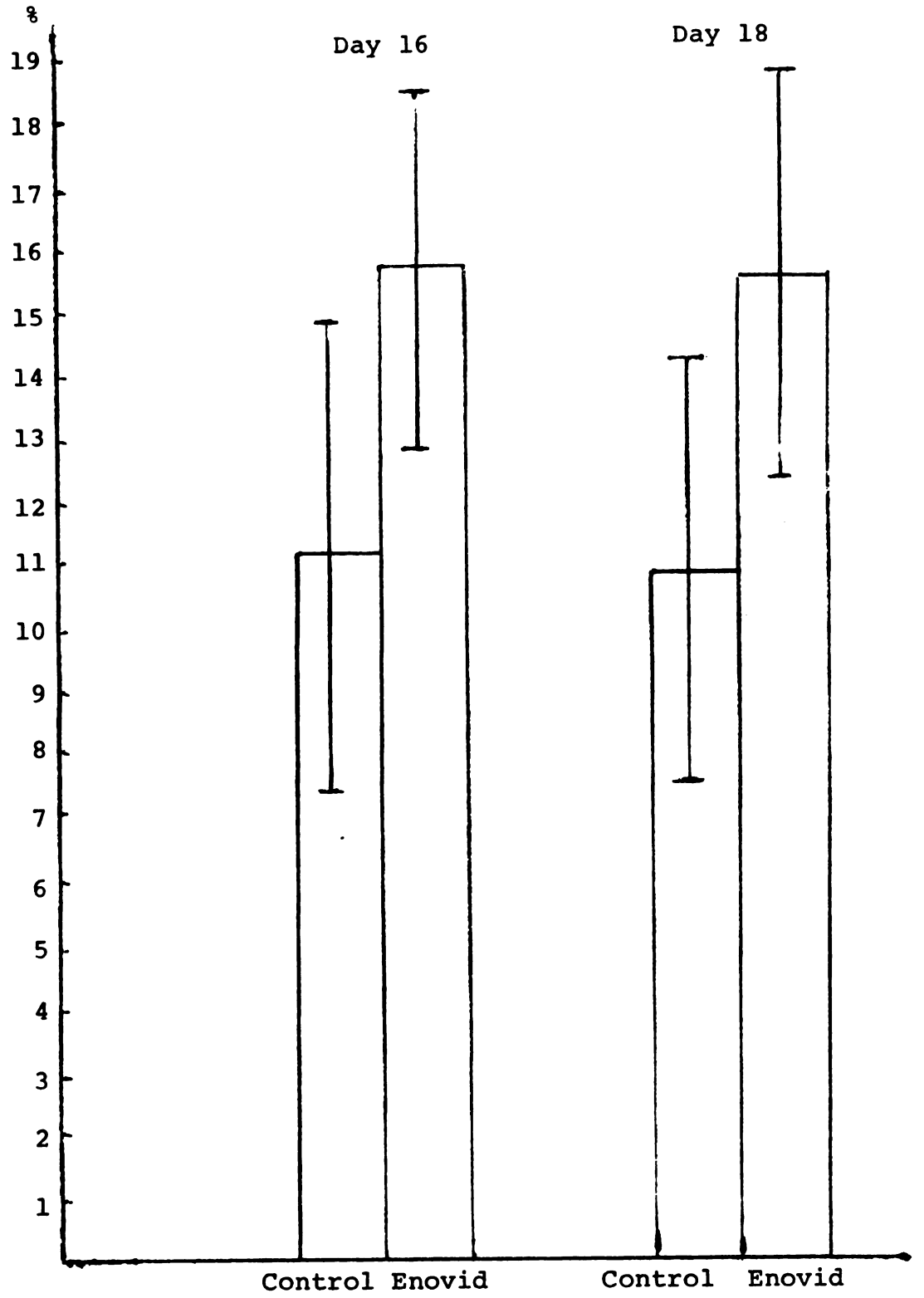


Figure 9: Lipid concentration of milk in control and treated rats (%). Vertical bars indicate ± standard errors.

CHAPTER V

CONCLUSION

In many countries, the number of women taking oral contraceptives is increasing rapidly. The lactational effect of these contraceptives is important for women who want to nurse their infants and who need to breast feed because of lack of adequate substitutes for maternal milk.

Studies with both humans and animals on the effects of oral contraceptives on lactation have yielded conflicting results. In the present study, the mammary glands of steroid treated rats weighed more and had more cells then the control rats. The protein synthetic activity of the glands was not different in the two groups. Protein and fat concentrations were higher in the milk of Enovid group. However, the pups of the treatment group tended to weigh less than the control pups in all three experiments. Usually, during lactation rats lose weight because they divert energy for milk production. In our study, on the average, dams of the control group lost weight but those of treatment group gained weight indicating that energy expenditure for milk was low. There also appear to be a negative correlation between milk production and fat concentration in milk (Folley, 1941; Spielman, 1941;

Sykes, 1953). In the present study, milk fat concentration was higher in the treatment group than in control group. These results might indicate that the rats in the Enovid group synthesized less milk. Another possibility is the impairment of milk ejection reflex by estrogen-progestin compounds indicated by Cowie (1961), and Joshi and Rao (1968). This, however, has yet to be determined for lactating rats treated with Enovid.

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APPENDICES

APPENDIX I

DETERMINATIONS OF MAMMARY GLAND DNA AND RNA

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DETERMINATIONS OF MAMMARY GLAND

DNA AND RNA

Preparation:

1. Make a 1:20 homogenate with ice-cold water in a blender.
2. Take 2 ml of homogenate, add 10 ml methanol:chloroform.
3. Shake for 12 hours, leave over-night, centrifuge. Transfer precipitate to polypropylene tubes.

Determination:

1. Add 2.5 ml of ice-cold 0.6 N $HClO_4$, mix, and stand 10 min at 0°C.
2. Centrifuge for 15 min at 18,000 x g, discard the supernatant fraction (acid-soluble), and wash the precipitate twice with 5 ml cold 0.2 N $HClO_4$, centrifuge.
3. Drain off excess acid by inverting the tube swiftly over filter paper.
4. Add 4 ml of 0.3 N KOH.
5. Incubate at 37°C for one hour (use force-air oven or water bath).
6. Cool in ice and precipitate protein and DNA by adding 2.5 ml of 1.2 N $HClO_4$.
7. Stand for 10 min in the cold, centrifuge, decand supernatant (RNA) fraction.
8. Wash the precipitate twice with 5 ml of 0.2 N $HClO_4$ and add washing to the RNA fraction.
9. Add 10 ml of 0.6 N $HClO_4$ to the RNA fraction and washings. This fraction is then diluted to 100 ml with water, giving a solution of ribonucleotides in 0.1 N $HClO_4$. The ultraviolet absorbtion of which is read at 260 mμ gives a measure of RNA content.

10. To determine DNA content, dissolve the precipitate (8) in 5 ml of 0.3 N KOH by warming briefly at 37°C if necessary. Transfer to a 50 ml volumetric and wash the tubes with 5 ml of 0.3 N KOH twice. Add these washes to the volumetric. Add an additional 2 ml of 0.3 N KOH to the volumetric. Dilute this to 50 ml with water to give a solution of DNA in 0.1 N KOH. Two ml of these solutions are used for DNA estimation by the method of G. Ceriotti (1952).

    Reagents: 0.04% indole solution in distilled water,
    concentrated HCl,
    standard DNA solution - 2.5 to 150 µg/ml

    The indole is dissolved in warm water. The solution is then cooled under running water and diluted to volume. It is stable when stored in the refrigerator.

    To a clean test tube add: 2 ml of solution to be tested, and 1 ml of concentrated HCl

    and shake well. Cover the test tubes with aluminum and place in boiling water for 10 min. Then cool them. Extract 3 times with 4 ml chloroform, each time centrifuging to give a completely clear water phase. Read the yellow color at 490 mµ on a Beckman.

APPENDIX II

PROTEIN DETERMINATION

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PROTEIN DETERMINATION
(Lowry et al., 1951)

1. To clean dry tubes add 1 ml of H_2O (blank), standard protein solution or unknown.
2. Add 5 ml of reagent C, mix.
3. After 10 min add 0.5 ml of reagent D, mix and let stand for 30 min.
4. Read at 750 mμ wavelength (if readings are too high read at 500 mμ).

APPENDIX III

LIPID EXTRACTION PROCEDURE

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LIPID EXTRACTION PROCEDURE
(Modified method of Folch *et al.*, 1957)

1. Put round bottom flasks in a dryer and weigh after drying.
2. Put 1 ml of sample in test tubes (15 ml tube w/cap). Add 10 ml of chloroform:methanol (2:1), shake.
3. Heat 30 min at 45°C, shaking every 10 min. Cool to room temperature. Centrifuge 15 min.
4. Remove bottom layer of mixture using a disposable pipette.
5. Add 2 ml chloroform to remaining mixture and repeat steps 3 and 4.
6. Let stand overnight.
7. Pour samples into flasks (washing with 3 ml chloroform), evaporate, dry overnight.
8. Weigh flasks and lipid, substract tair weight to find the lipid content.