Changes in Daily Urinary Excretion of Follicle-stimulating and Luteinizing Hormones Caused by Ethinyl-estradiol and Conjugated Equine Estrogens (Premarin) in Low Doses

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The changes in daily urinary excretion of follicle-stimulating and luteinizing hormones caused by ethinyl-estradiol and conjugated equine estrogens in low doses have been studied with urine samples collected from eight normal menstruating women, three normal men, and seven women with abnormal menstrual cycle.

Follicle-stimulating hormone (FSH) determinations were done with two separate twenty-four hour urine samples before ethinyl-estradiol (EE) was given; EE was administered in a daily dose of 0.05 mg for four consecutive days and urinary FSH levels were determined in daily urine samples. Two twenty-four-hour urine samples were collected after EE administration was discontinued, and urinary FSH levels were also determined. Conjugated equine estrogens (Premarin) was also given in a daily dose of 0.625 mg for six consecutive days immediately after the EE test; urine specimens were collected daily for luteinizing hormone (LH) determinations. Two twenty-four-hour urine samples were also collected before Premarin was given and basal LH values were obtained. In addition, three normal women received 0.625 mg per day of Premarin early in their menses for six consecutive days, without first applying the EE test for FSH, and LH determinations were performed on daily urine samples.

It was found that 0.05 mg of EE exerted a stimulatory effect on FSH excretion in the normal woman during the second and fourth to fifth days, and that this was statistically different from the response of the normal man and amenorrheal women. Premarin exerted its action principally in amenorrheal women and probably in normal men by increasing LH excretion values on the third to fifth days of its administration. No action was observed in normal women on LH excretion levels by Premarin administration.

As a conclusion, it is considered that there is a positive feed-back control mechanism of gonadotropin secretion by the action of estrogen, and the different response pattern of gonadotropin excretion between normal women and those with abnormal menstrual cycle, and between both sexes.

Albert¹ has suggested that in non-pregnant human subjects the excretion of urinary gonadotropins may reflect the secretory rate of these hormones from the anterior pituitary gland, and most investigators agree that the excretion of

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urinary gonadotropins varies widely when measured over a considerably long period. Werner² experienced daily fluctuations of urinary gonadotropin while studying five healthy men, in whom he frequently found negative values when assays were done daily for thirty days. Also, Albert¹ noted fluctuations of the hormone up to tenfold in daily output, and recently, Rosenberg³ reported a variation of as much as threefold in 'total' gonadotropin excretion from subject to subject.

The existence of an estrogen feedback control mechanism in gonadotropin excretion is generally accepted, and the theory is advanced that this feedback control of gonadotropin secretion may be exerted directly on the pituitary⁴ or indirectly on the hypothalamus.⁵

With the application of specific bioassays for follicle-stimulating hormone (FSH)⁶ and for luteinizing hormone (LH)⁷ in urine, the authors have established a clinical test for excretion responses of urinary follicle-stimulating hormone and luteinizing hormone to a given dose of estrogen.

METHODS AND MATERIALS

Daily 24-hour urine samples were collected from eight normal menstruating women and from three normal men for the entire period of estrogen administration. Two separate 24-hour urine samples were also collected both prior to the trial for basal measurement of gonadotropins and after the withdrawal of estrogen.

In order to ascertain that the female volunteers were normal ovulating subjects, basal body temperature (BBT) recordings were taken daily for one month prior to the beginning of experiment, during the period of estrogen intake, and after the test was completed. In all of the women steroid determinations were made on the first 24-hour urine. The urine samples were treated by Albert's kaolin-acetone method of gonadotropin determination.⁸ Gonadotropin was adsorbed from urine with kaolin, eluted with ammonium hydroxide and precipitated with acetone. Thereafter, FSH and LH were separately treated. The acetone precipitate was washed twice in absolute ethanol, dried and prepared for FSH assay. For LH determination the acetone precipitate was treated further to remove contaminants. Celite was added to the precipitate, which was then mixed, filtered and extracted with two volumes of 10% ammonium acetate in 70% ethanol. This extract was precipitated again with 10% ammonium acetate in absolute ethanol, dried and prepared for assay.

For FSH determination, Steelman and Pohley's⁶ assay method was used. Immature female rats of Wistar-Imamichi strain, aged 22-23 days, were used for the assay. A total dose equivalent to the content of 6-hour urine was injected into each rat together with 40 I.U. of human chorionic gonadotropin (HCG). Control animals received only HCG in the same dose as the rats under assay. 72 hours after the first injection, the animals were killed by dislocation of the neck; the ovaries were excised quickly, removed of the surrounding tissue, and weighed on a torsion balance to milligram. FSH values obtained were expressed in terms of NIH-FSH-S₁ μ g/24 hrs.

LH determination was made with a modification of the ovarian ascorbic acid depletion method of Parlow.⁷ Wistar-Imamichi female rats of 25 days of age were primed with 100 I.U. of pregnant mares' serum and 50 I.U. of HCG 60 $\pm$ 4 hours later, administered subcutaneously *in torso*. Seven days after HCG priming the rats were used for assay. The assay material dissolved in 1.0 ml of saline was injected at 13:00 hours under light ether anesthesia into the femoral vein or jugular vein. Control animals received only saline. Three hours later the animals were sacrificed by dislocation of the cervical vertebrae. The right ovary was quickly excised and weighed on a torsion balance. The tissue was homogenized in 2.5% metaphosphoric acid, and the ascorbic acid content was determined

by means of a modification of Mindlin and Butler's method.⁹ The equivalent of a six-hour urine specimen was used for each rat, and four rats were used for each assay. The values of LH were expressed as NIH-LH-S₆/μg 24 hr.

The estrogens used were ethinyl-estradiol (EE) (17α-ethinylestra-1, 3, 5 (10)-triene-3, 17β-diol) and Premarin (conjugated equine estrogens).

The clinical assay plan was as follows: FSH determinations were done in two separate 24-hour urine samples before EE was given. EE was administered for four consecutive days in a daily dose of 0.05 mg and urinary FSH levels were determined on daily urine samples. Two 24-hour urine samples were collected after EE was discontinued, and urinary FSH levels were also determined.

Premarin was also given for six consecutive days in a daily dose of 0.625 mg immediately following the EE test; urine specimens were collected daily for LH determinations. Two 24-hour urine samples had been collected before Premarin was given and basal LH values were obtained.

The same plan was followed in seven women with abnormal menstrual cycle most of whom presented secondary amenorrhea. In other women with abnormal menstrual cycle only LH determination with Premarin was done.

Some selected female cases of menstrual disorder are presented below.

Statistical analysis of the data was done by Student's *t*-test.

CASE REPORT

Case 1. R.K., a 22-year-old unmarried woman consulted us because of secondary amenorrhea of two years' duration. Her menarche took place at 14 years of age, but after a year her menses gradually became irregular. She was 163.5 cm in height and 55.5 kg in weight. No remarkable change was found on general physical examination and on pelvic examination, except for a slight hirsutism. Thyroid function was normal (+5% BMR; resin sponge uptake of 28%; 24 hours' ¹³¹I-uptake of 8%; and thyroid scan revealed normal size and position of the gland). Determinations of urinary steroids were performed and gave the following values: 55μg of total estrogens per 24 hours (estrone 13.2μg, estradiol 15.4μg, and estriol 26.4μg per 24 hours, normal values for our method being 40–60μg of total estrogens per 24 hours); 0.44 mg pregnanediol, normal values being 0.5–1.2 mg per 24 hours; 3.9 mg of 17-ketosteroids per 24 hours, normal values being 3–5 mg per 24 hours, and 0.4 mg of 17-OHCS per 24 hours, normal values being 0.5–2.5 mg per 24 hours.

She had once received the so-called Kaufman therapy in order to induce ovulation and gonadotropin (PMS-HCG), and cortisone therapy was also effective on one occasion to induce ovulation, but further use of the hormones was ineffective.

In our test the daily administration of 0.05 mg of EE elicited a first peak of increased urinary FSH excretion on the first day, and from the third to fourth days urinary FSH excretion continued to increase as in our normal female subjects on the same days. Unfortunately, we could not make further urinary FSH determination in this patient beyond the fourth day.

On the second day of administration of Premarin for the LH test in a daily dose of 0.623 mg, the urinary LH excretion increased to 32.8μg per 24 hours, suggesting spontaneous ovulation from the changes of the BBT chart. Thereafter the urinary LH excretion reached higher levels, attaining the maximum on the

Fig. 1. Case 1: R.K. This figure shows an increased level of FSH excretion on the first and the fourth days with EE, and an increase of urinary LH excretion on the second, third and fourth days while receiving 0.625 mg daily of Premarin. Values are given as NIH-FSH-S₁/μg 24 hours for FSH excretion and NIH-LH-S₆/μg 24 hours for LH excretion. Solid lines in the upper part mark the estrogen schedules, whereas those lines in the lower part indicate the minimal value of FSH in 24 hours as they differ from zero. The dotted line in the lower part also indicates the minimal confidence value for the LH method employed.

fourth day, with a sharp decrease on the fifth day as shown in Fig. 1. Subsequently, spontaneous ovulation was observed for further two menstrual cycles as monitored on her BBT charts.

Case 2. K.T., a 22-year-old unmarried woman had a chief complaint of primary amenorrhea. She was 143.2 cm in height and 44.5 kg in weight. She had poorly developed secondary sexual characteristics, and shallow vagina and uterus were palpable on pelvic examination. The ovaries could not be touched in this way, but their presence could not be excluded inasmuch as this patient once ovulated in response to gonadotropin (PMS+HGC) administered by the one-two-cyclic method. No remarkable congenital deformities were discovered on physical examination and the patient was found to be chromatin-positive. Thyroid function was normal, BMR being +12% and 24 hours' ¹³¹I-uptake being 28%, and the thyroidal scintigram revealing a normal size and position of the gland. The ACTH stimulation test and the Methopyrapone test were within normal limits. Urinary steroid determinations gave the following results: 32.5 μg total estrogens (estrone 7.8 μg, estradiol 9.1 μg, and 15.6 μg of estriol per 24 hours), 0.78 mg pregnanediol per 24 hours, 2.1 mg 17-ketosteroids per 24 hours, and 2.1 mg 17-OHCS per 24 hours.

As shown in Fig. 2, she did not show a reaction to a daily dose of 0.05 mg of EE for urinary FSH excretion, nor did she react to a daily dose of 0.625 mg of Premarin for LH excretion determination.

Fig. 2. Case 2: K.T. No reaction is observed for FSH excretion following ethinyl-estradiol administration, nor for LH excretion while on Premarin. Other explanations are the same as in Fig. 1.

Case 3. M.S., a 20-year-old unmarried woman, suffered from polymenorrhea and/or hypermenorrhea without ovulation since her menarche at the age of 14. She was 152 cm in height and 54.5 kg in weight. There was no hirsutism and she had normally developed secondary sexual characteristics. No remarkable changes were observed on general physical examination; nor was there any sign of hemorrhagic diathesis. The 17-OHCS values were within normal limits upon ACTH stimulation as well as during the Methopyrapon test. Urinary steroid included total estrogens of 22.5 μ g, 4.8 μ g of estrone, 8.1 μ g of estradiol and 9.6 μ g of estriol, 0.42 mg of pregnanediol 8.7 mg of 17-ketosteroids (slightly elevated over normal values), and 0.6 mg of 17-OHCS per 24 hours. The hysterosalpingograph revealed irregularity of uterine wall, and endometrial biopsy showed findings of cystic glandular hyperplasia. By culdoscopy polycystic ovaries were suspected. Consequently, wedge resection of bilateral ovaries and endometrial curettage were performed. At the time of operation, the ovaries were slightly enlarged and had a thick pearl-colored smooth capsule, under which cystic follicles could be seen. The microscopic findings also supported the diagnosis of Stein-Leventhal syndrome.

Ten days after the operation, the FSH-LH test was started according to the schedule as described above. She exhibited an initial increase of FSH excretion on the first day of EE administration, but from the third to fourth days, the FSH level decreased below the confidence level, suggesting an abnormal response of urinary FSH excretion to EE. As for LH excretion, high urinary LH excretion (40.0 μ g/24 hr) was observed only on the fourth day of Premarin administration in a daily dose of 0.625 mg; but, unfortunately, further LH determination was not done, because no more urine samples could be collected of this patient beyond the fourth day. The results obtained are shown in Fig. 3. During the experimental cycle her BBT was monophasic and during the following menstrual cycle she exhibited a biphasic BBT pattern suggesting that spontaneous ovulation had taken place.

Fig. 3. Case 3: M.S. Diagnosis: Stein-Leventhal syndrome. This figure shows also the response to estrogens. An increase on the first day with EE is observed, but on the fourth day the FSH value falls to beneath our confidence values. With Premarin we observed an increased value of LH on the fourth day. Otherwise the data are the same as in Fig. 1.

Case 4. N.S., a 19-year-old unmarried woman was hospitalized with a clinical diagnosis of diabetes insipidus. Her chief complaints were thirst and polyuria of over five liters in 24 hours over a six-year period. No preceding history of trauma, infections or hereditary disorders were found. There was no disturbance of the visual field. Menarche had not occurred and the development of secondary sexual characters was absent. She was only 148 cm in height and 26 kg in weight. The skull x-ray showed no change of the sella turcica either in size or in form; no calcifications of the pituitary or of any other organ appeared in the x-rays. Ophthalmological data were normal. Thyroid function was within normal values, (BMR, -3.5%, 24-hour ^{131}I -uptake, 22.3%, and resin sponge uptake test, 22%). The Methopyrapone test indicated exhaustion of reserve capacity for ACTH secretion. No ACTH stimulation test was done. Basal urinary steroid determinations showed the following values: 77 μg of total 24-hour estrogens (estrone, 22 μg , estradiol, 30 μg , and estriol, 25 μg , per 24 hours). Pregnanediol value was 1.4 mg/24 hours, 17-ketosteroids 0.9 mg/24 hours and 17-OHCS 1.6 mg/24 hours.

A Carter-Robbins test was performed in order to evaluate the severity of diabetes insipidus, but no increase of plasma osmolarity was found. Therefore, a water deprivation test was performed in the usual manner. In the early morning the patient was weighed and samples of plasma and urine were obtained for basal osmolarity test. The basal values were 300 milliosmols/kg H_2O , 60 milliosmols/kg H_2O and 25.8 kg for plasma, urine and body weight, respectively. A dramatic response was elicited six hours later with the body weight falling from the initial value to 24.5 kg, corresponding to a loss of 5.1% body weight, causing an increase of the plasma osmolarity to 330 milliosmols/kg H_2O , without changes in urinary osmolarity. At this time the water deprivation test was stopped because

of the risk to the patient, although there were no changes in arterial blood pressure, nor complaint of subjective symptoms. One hour later 2.5 I.U. of vasopressin tanate was intramuscularly injected and the response in plasma and urine osmolarity was observed for four hours. The urine osmolarity rose from 90 milliosmols/kg H_2O to 370 milliosmols/kg H_2O , while that of plasma decreased from 320 milliosmols/kg H_2O to 305 milliosmols/kg H_2O . The results of this test confirmed the diagnosis of diabetes insipidus.

Fig. 4. Case 4: N.S. Diagnosis: diabetes insipidus. Responses to EE and to Premarin are shown in addition to that of therapy with cortisone and hydrocortisone also given to the patient. There are no changes in the FSH and LH excretion with estrogens used. Other readings are the same as in Fig. 1.

During the second day of EE in the FSH test, the patient developed adrenal crisis and was treated with hydrocortisone injections and supportive therapy, but the FSH test with EE was not stopped. Cortisone tablets in a daily dose of 25 mg were given adjunctively and maintained during the entire estrogen trial. No important changes in the basal values were observed for urinary FSH excretion during EE administration or for urinary LH excretion during Premarin test. No effect on gonadotropin excretion was exerted by additional cortisone therapy. No further adrenal crisis occurred. The results of FSH-LH test on this patient are presented in Fig. 4.

RESULTS

Changes in daily urinary FSH excretion in normal women. The daily changes of urinary FSH excretion under the action of ethinyl-estradiol (EE) in a daily dose of 0.05 mg were studied in eight normal menstruating women. The subjects were given a daily dose of EE for four consecutive days starting early in their menstrual cycles and the daily changes in urinary FSH excretion were measured and graphed in the composite curve in Fig. 5. It was observed that on the second day of EE and from the fourth day, the curve tended to rise and the elevation

Fig. 5. The changes in FSH excretion due to EE administration during four days starting from their early menstrual period in eight normal women. It shows an increase of FSH excretion on the second, fourth and fifth days of EE administration. Interpretation of this figure is the same as for Fig. 1. The vertical lines represent standard error from the mean.

was statistically significant ($p < 0.05$) in both periods when compared with the basal values. On the fifth day, or the first day after withdrawal of the drug, the highest mean value was reached ($125.4 \pm 32.0 \mu\text{g}/24 \text{ hours}$), but the standard error of the mean was highest too. On the second and fourth days of EE test and on the fifth and sixth days or the first and second days after withdrawal of the drug, the differences of urinary FSH from the basal value were all statistically significant ($p < 0.05$), but the comparison between the two periods did not give a significant difference ($p > 0.05$).

Changes in daily urinary FSH excretion in women with abnormal menstrual cycle. Seven women with menstrual disorders of widely differing pathology were studied. There was one case of hypopituitarism with associated diabetes insipidus of unknown etiology; one case of Stein-Leventhal syndrome having been operated on shortly before the time of the study; four cases of secondary amenorrhea; and one case of primary amenorrhea of unknown etiology. Some of these cases were described in detail in case reports of the present paper. One case of secondary amenorrhea was excluded (case 1), because the patient had presumably ovulated during the test, as indicated by her BBT chart, and consequently her menstrual cycles were normalized as was observed during the following two months on her BBT charts of a normal biphasic pattern.

The results obtained in six women with menstrual disorders are shown as a composite curve in Fig. 6. Here it was possible to observe that for the most

Fig. 6. Legends are the same as Figs. 1 and 5. The results of six amenorrheal women under the action of EE 0.05 mg/day for four days. It can be seen that there are no changes on FSH excretion with EE administration.

part the mean values of daily urinary FSH excretion were below the lower confidence value of $30\mu\text{g}$ per 24 hours, and that on the first day of EE in a dose of 0.05 mg the mean value tended to be higher than on the other days, but this difference was not significant ($p > 0.05$) when compared with the basal values. No other changes were observed on the following days.

Changes of daily urinary FSH excretion in normal men. In order to observe whether our results of normal menstruating women were in agreement with the results of normal men, three normal men were examined with the same plan for daily changes in urinary FSH excretion. The small number of male subjects made it impossible to compile the results into a composite curve, but in two of the three subjects it was observed that the normal man had the same tendency as that of the normal woman on the second day of the examination. However, starting from the fourth day of EE, the curve tended to fall below the lower confidence level. The results of these normal men are presented as individual patterns in Fig. 7.

Fig. 7 The results obtained from three normal men in terms of individual patterns of FSH excretion. Increased values on the second day are observed in two cases, and from the fourth day on, the FSH values drop below the confidence values.

Comparison of the daily changes of the mean urinary FSH excretion between normal menstruating women and amenorrheal women. Table 1 shows the comparison between eight normal menstruating women and six amenorrheal women. From this table it was possible to conclude that the basal values of normal and abnormal women were not different ($p > 0.05$), and that, from the second day of EE on, statistically significant differences appeared especially on the second ($p < 0.05$) and fourth days ($p < 0.001$). The results on the fifth and the sixth days were not compared, because only 3 cases of women with menstrual disorders were examined and the limited number made a comparative study of little value.

TABLE 1. *Comparison of the daily mean changes of urinary FSH excretion between eight normal and six amenorrheal women under the action of ethinyl-estradiol 0.05 mg/day for four days*

Day	Ethinyl-estradiol dose in mg	Normal women (M $\pm$ SE) $\S$	Significance over own basal values ($p = < 0.05$)	Abnormal women (M $\pm$ SE) $\S$ $\ddagger$	Significance between normal and abnormal women ($p = < 0.05$)
Basal value		36.7 $\pm$ 10.3		27.6 $\pm$ 7.8	
Basal value		38.5 $\pm$ 7.3		12.6 $\pm$ 7.0	>0.1
1st day	0.05	31.5 $\pm$ 13.8		36.6 $\pm$ 13.3	
2nd day	0.05	*76.8 $\pm$ 15.5	<0.05	18.3 $\pm$ 7.5	>0.05
3rd day	0.05	34.5 $\pm$ 8.2		30.6 $\pm$ 12.6	
4th day	0.05	71.0 $\pm$ 6.5	<0.05	5.6 $\pm$ 5.6	>0.001
5th day		*125.4 $\pm$ 32.0	<0.05	0.0 $\dagger$	
6th day		*101.2 $\pm$ 22.5	<0.05	+29.3 $\pm$ 25.4	>0.05

* Seven cases

$\dagger$ Three cases

$\S$ Mean $\pm$ standard error

Values are given as NIH-FSH-S₁/μg 24 hrs.

$\ddagger$ Not a single mean was significantly higher than basal values.

Effect of Premarin in a daily dose of 0.625 mg on urinary LH excretion in normal women. Five of the subjects studied were suitable for computing the data obtained. Three cases were excluded because of incomplete urine samples (two subjects) and erroneous use of urine extracts (used for FSH assay) in the third subject. All the volunteers received 0.625 mg of Premarin daily for six consecutive days, starting immediately after the FSH study with ethinyl-estradiol had been finished. Two 24-hour urine samples of each subject were used for basal LH measurements, and six other consecutive samples under Premarin administration were used for studying the daily changes of urinary LH excretion. The computed data are presented in Fig. 8 as a composite curve of five cases. We observed that the mean values of NIH-LH-S₆ μg in 24 hours never exceeded 10 μg/24 hours, and even if some tendency to increase in LH excretion was observed on the third and fourth days of Premarin, these values were not significantly different

Fig. 8. The effect of 0.625 mg of Premarin per day in five normal women for six consecutive days starting immediately after the FSH study with EE had been finished. Explanation in text.

($p > 0.05$) from the basal values of LH excretion, whether individual or pooled values may be compared.

It should be noted that the Premarin test in our normal volunteers was performed during the late proliferative phase of the menstrual cycle, because the FSH response to ethinyl-estradiol could be elicited only after 8 days from the early days of menses. Also, as it is possible to observe in Table 2, Premarin in low doses had some effect on the length of menstrual cycles. Only one of the eight normal women showed no changes in the length of her menstrual cycle, whereas in the other seven the menstrual cycles as determined prior to the experiment were prolonged by two to twelve days, the mean prolongation being 5.8 days.

In order to ascertain whether the Premarin-induced response of urinary LH excretion was influenced by the plan of medication or the order of ethinyl-estradiol

TABLE 2. *Effect of the Premarin test on length of menstrual cycle in normal menstruating women*

Subject	Length of normal cycle (days)	Length of experimental cycle (days)	Prolongation (days)
A.E.	25	37	12
M.Ya.	31	36	5
K.M.	35	40	5
N.A.	32	34	2
M.F.	31	40	9
M.Yu.	33	33	0
K.O.	31	37	6
O.Y.	30	38	8
Mean	31	36.8	5.8

and Premarin, we used Premarin in three normal women with the following plan. Instead of first using ethinyl-estradiol, Premarin was given in a daily dose of 0.625 mg for six consecutive days starting early in menses, and the urinary LH excretion was measured in daily urine samples. The results obtained with this plan are presented in Fig. 9. Again Premarin had no significant action on urinary LH excretion in normal women. The pattern of urinary LH resembled those in normal women in the late proliferative phase of the menstrual cycle, but the apparent elevation lacked statistical significance ($p > 0.05$), when individual or pooled values were compared with the basal values. Pooled values from the third and fourth days did not differ ($p > 0.05$) from those from corresponding days in normal women in the late proliferative phase of the menstrual cycle.

Fig. 9. The effect of 0.625 mg of Premarin per day for six consecutive days starting from early in the menstrual periods of 3 normal women. No EE was given to these patients for FSH studies.

Changes in daily urinary LH excretion in women with menstrual disorders under the action of Premarin. Four of our six subjects with menstrual disorder reacted to a daily dose of 0.625 mg of Premarin given during six consecutive days. The seventh subject was examined only for FSH excretion. Two subjects did not show any reaction to Premarin (Cases two and four). The computed data of these four cases are presented in Fig. 10. The mean values as NIH-LH-S₆ μg 24 hours were higher than the values of normal women. The highest mean value for normal women in the late proliferative phase of the menstrual cycle never surpassed 10 μg per 24 hours, but in the women with menstrual disorders the highest mean value reached 64 μg per 24 hours on the fourth day under Premarin administration. When Student's *t*-test was applied to the daily mean values of the amenorrheal women, no statistical significance ($p > 0.05$) was found, but one case exhibited a single peak for LH excretion on the third day, two cases on the fourth day, and another case exhibited two peaks on the third and fourth days

Fig. 10. The effects of 0.625 mg of Premarin per day for six consecutive days in four amenorrheal women, starting immediately after the FSH study with EE had been finished. Explanation in text.

under the action of Premarin, while the other values for LH were close to the lower confidence level of $1.2\mu\text{g}$ per 24 hours in our method. This taken into consideration, pooled values of the third and fourth days of Premarin were compared with the corresponding basal values, and in this way we ascertained a statistically significant difference ($p < 0.05$).

Effect of LH excretion in normal men. Three healthy men were studied for the action of 0.625 mg of Premarin daily for six consecutive days, and the results are arranged in the form of the mean $\pm$ standard error of the mean and presented

Fig. 11. The effect of 0.625 mg per day of Premarin in three normal men. Explanation in text.

in Fig. 11. In this study ethinyl-estradiol was given first and Premarin later in the same way as in cases of normal and amenorrheal women. In normal men, there also appeared an LH peak on the fourth (two cases) or on the fifth (one case) day of Premarin administration. Because of the small number of cases, statistical treatment was not applied.

Comparison of the effects of Premarin in a daily dose of 0.625 mg on urinary LH excretion between normal women and non-menstruating women. Table 3 shows the results from eight normal menstruating women at the early and late proliferative phase of the menstrual cycle, and four cases of women with abnormal menstrual

TABLE 3. Comparison of the effects of 0.625 mg daily dose of Premarin in urinary LH excretion between eight normal menstruating women and four abnormal non-menstruating women who reacted to the test

Days	Premarin daily dose mg	Normal women* (M $\pm$ SE) $\S$	Abnormal women $\dagger$ (M $\pm$ SE) $\S$	Significance (p<0.05)
First basal + second basal		5.4 $\pm$ 2.9	3.64 $\pm$ 1.0	NS
First day + second day	0.625	3.6 $\pm$ 1.3	6.70 $\pm$ 3.8	
Third day + fourth day	0.625	9.1 $\pm$ 2.4 $\ddagger$	61.20 $\pm$ 23.5 $\#$	<0.01
Fifth day + sixth day	0.625	5.8 $\pm$ 1.0	10.00 $\pm$ 10.0	

* Eight cases; 16 experiments are represented in each value

$\dagger$ Four cases; eight experiments are represented in each value only values from the fifth day; four experiments

$\S$ Mean $\pm$ standard error

$\ddagger$ p>0.05 (comparison from own basal values)

$\#$ p<0.05 (comparison from own basal values)

Mean values are given as as NIH-LH-S₆/μg 24 hrs.

cycles who reacted to Premarin. The basal values of these two groups did not differ from each other (p>0.1), but pooled values of the third and fourth days showed a difference between women with abnormal menstrual cycles and normal women (p<0.01). The comparison of pooled values of other days between normal and amenorrheal women did not yield a significant difference (p>0.05).

DISCUSSION

Action of ethinyl-estradiol on FSH excretion. The results of the present study reveal that the response of normal women to 0.05 mg of ethinyl-estradiol per day for four days as indicated by urinary FSH excretion differs from that of women with abnormal menstrual cycle and normal men.

The composite curve for urinary FSH of our normal women shows a statistically significant increase (p<0.05) of FSH values compared with the basal values

on the second and on the fourth through fifth days of ethinyl-estradiol treatment. Such an increase was absent in women with menstrual disorders. Table 1 also shows that the difference in urinary FSH is statistically significant between cyclic and acyclic women on the second ($p < 0.05$) and fourth ($p < 0.001$) days of EE. These differences were also noted by McArthur *et al.*¹⁰ and Brown¹¹ who demonstrated that women with menstrual disorders did not exhibit such fluctuations of FSH excretion as seen in normal women. Moreover, Brown¹¹ could not find an increase in FSH excretion in women with menstrual abnormalities, even under a daily dose of 0.5 mg of stilbestrol for 3 to 5 days; but he demonstrated that in three out of nine women so treated the excretion did increase over control values, and confirmed his previous observation.¹²

Parlow,¹³ working with ovariectomized female rats treated with graded doses of estradiol, found that daily injections of $0.025\mu\text{g}$ and $0.100\mu\text{g}$ for 16 days increased the pituitary FSH content over that of the ovariectomized animals without estradiol. However, the difference lacks statistical significance. Smith and Albert¹⁴ studied the effect of graded doses of stilbestrol and Premarin on a 24-year-old parous castrated woman and found that 0.1 mg of stilbestrol per day appeared to stimulate the excretion of gonadotropins, while 1 mg of stilbestrol per day produced a slight decrease of gonadotropins, and that only 10 mg per day of stilbestrol reduced gonadotropin values to zero. These works clearly demonstrate that the effect of estrogen on gonadotropins is dose-dependent. Confirmatory results had been recently reported by Vorys *et al.*,¹⁵ who used graded doses of ethinyl-estradiol in normal women, and found that 0.02 mg and 0.05 mg daily stimulated FSH excretion without modification of LH excretion, and that 0.07 mg and 0.1 mg per day of ethinyl-estradiol suppressed FSH excretion.

That this effect of exogenous estrogens on FSH excretion is not mediated through the ovaries was well established by Greep and Chester Jones,¹⁶ who found that the presence of the pituitary was a necessary condition for the release of gonadotropins as demonstrated previously by Bradbury¹⁷ in rats, and by Albert¹⁴ in humans.

Our results seem to have established that the normal woman reacts to a small dose of ethinyl-estradiol with increase of FSH excretion which begins on the second day of administration. The explanation of this may be that a release at the pituitary level has occurred. This seems to be true inasmuch as Brown *et al.*¹⁸ found that in estrogen-administered female rats gonadotropin content of the pituitary decreased 48 to 96 hours later, and interpreted the results as indicating a release of gonadotropins at the pituitary level. Döcke and Dörner⁴ implanted estradiol benzoate stereotaxically into the brain or the anterior pituitary of prepubertal female rats and found that 1/25 of the subcutaneous effective dose into the anterior hypothalamus and 1/100 of the peripherally effective dose into the pituitary were sufficient to induce corpus luteum formation in most of the treated animals. They concluded that the anterior pituitary was the main site

of action of estrogen in the positive feed-back mechanism of estrogens, and they suggested too that pituitary sensitivity to the stimulatory effect of the gonadotropin-releasing factor is increased by estrogen. Similar conclusions were obtained by Attramadal¹⁹ using radioactive estradiol 17β -6, 7- H^3 . He also found that estrogen acted upon the cells of both the hypothalamus and the anterior pituitary gland.

The increase in FSH excretion on the second day of ethinyl-estradiol is almost the same in normal men, because two of our three subjects exhibited a remarkable increase in FSH values on the same day. The woman with abnormal menstruation did not exhibit such reaction on the second day, and the same was observed by Brown¹¹ in his studies of FSH and estrogen treatment; he tried to interpret this by suggesting that in the amenorrheic woman there might be a disturbance in the rhythm of gonadotropin secretion.

It is more difficult to explain the second peak of FSH excretion on the fourth day of ethinyl-estradiol in the normal woman. Neither normal men nor amenorrheal women exhibited such a peak on the fourth day, and all the three normal men showed a decrease in FSH values below the confidence level for the FSH method employed on the afore-mentioned day.

Both normal men and women have a certain FSH content at the pituitary level,²⁰ and by experimental studies²¹ it has been demonstrated that the anterior pituitary tissue does not show sexual differentiation, but remains pluripotent. If it is true, the difference in response to estrogens on the fourth day in the normal woman will require an explanation other than that with a FSH release at the pituitary level. There is presumably a difference between the two sexes which has been attributed to the effect of androgen on the hypothalamus during the first week of life, as revealed in experiments with androgen administration to young female rats,²¹ and the fixation of the brain in a 'male type' (acyclic) or 'female type' (cyclic) might be the main cause of different FSH excretion between the normal man and woman.

We suggest that in the normal woman increased FSH excretion on the fourth day may be a result of direct action of estrogen on the FSH-RF (FSH-releasing factor). The FSH-RF in the hypothalamus²² advocated by Igarashi and McCann seems to be well proved by studies both *in vitro* and *in vivo*.²³ Recently Mittler and Meites²⁴ studied the influence of androgens on FSH-RF in male rats and found that testosterone propionate injections in a daily dose of 2 mg reduced FSH-releasing activity of hypothalamic extracts; however, this depressant effect of testosterone can be also explained on the basis of excessive dosage, because Greep and Chester Jones¹⁶ examined the effect of androgens on the pituitary of intact adult female rats and found that within a specified dose range (0.1 to 0.5 mg of testosterone propionate per day) FSH potency was increased over that of untreated controls. Confirmatory results of the effect of estrogens on FSH-RF have been reported by David *et al.*,²⁵ who asserted that estrogen administration in a dose of 200 μ g/100 g to castrated female rats caused reduction of hypothalamic FSH-RF and decreased FSH content

of the pituitary, and they suggested that estrogens might control pituitary function through the suppression of hypothalamic FSH-RF. This apparent discrepancy between our results showing stimulatory effects of estrogens in low doses on the normal woman and depressant effects of estrogen in rats cited by David *et al.* can be explained by a too high dose of 200 $\mu\text{g}/100\text{ g}$ used by them.

Action of Premarin on LH excretion. The results of the present study show that women with menstrual disorders exhibit a different response of urinary LH excretion from that of normal women, when treated with 0.625 mg of conjugated equine estrogens (Preamarin) per day for six consecutive days.

The composite curve (Fig. 8) of our normal women shows that LH excretion was not significantly increased ($p > 0.05$) compared with basal values, even though some tendency toward increased LH excretion on the third and fourth days of Premarin was observed. On the other hand, in women with abnormal menstrual cycle a significant elevation ($p < 0.05$) of LH peak was observed on the third and/or fourth days of Premarin. Normal men showed the same tendency in their response as that of women with menstrual disorders.

The evidence for the release of LH by estrogen stimulation during the fourth and fifth days after estrogen implantation into the hypothalamus of rats has been presented in a recent work of Palka *et al.*²⁶ Smith and Albert¹⁴ studied the effect of graduated doses of Premarin on the urinary gonadotropin excreted from a castrated woman and found that 0.1 mg of Premarin per day stimulated gonadotropin excretion, and that when the Premarin dose was increased to 10 mg per day, a diminution but not an abolition of gonadotropin excretion occurred. Vorys *et al.*¹⁵ also reported that, when they used graduated doses of Premarin in a female prisoner with ovulatory cycles, her LH excretion reached a peak on the sixth and fourth days at 0.625 and 1.25 mg per day respectively, without any change in FSH excretion, and that 2.5 mg and 3.75 mg per day also seemed to exert a slight stimulatory effect on LH excretion.

It is difficult to explain the difference between normal and amenorrheal non-menstruating women in our results, but we suggest that there may be an altered sensitivity of the hypothalamic center(s) which stimulate(s) LH secretion in response to graduated dose and/or to different kinds of estrogen. This assertion seems to be supported by the studies of Brown^{11,12} in amenorrheic women using 0.5 mg per day of stilbestrol for three to five days, and by Funnel *et al.*²⁷ in menopausal women employing estradiol benzoate in doses somewhat larger than those we used; and these investigators also found increased LH following estrogen administration. Moreover, Brown¹¹ pointed out that the amenorrheic woman might be subjected to derangement in the rhythm of gonadotropin secretion.

There was no difference in the basal values for LH excretion between normal and amenorrheal women. This is the reason why this kind of LH test is necessary in the clinical diagnosis of any abnormalities of gonadotropin secretion.

We repeated the experiment with Premarin in the normal woman. Instead of

first using ethinyl-estradiol, 0.625 mg of Premarin per day was given to three normal women early in their menses. This plan of medication was to assess whether or not the response of urinary LH excretion to Premarin was influenced by FSH 'priming'. We found that normal women did not show a significant response irrespective of the FSH 'priming'. In four out of six women with menstrual disorders, LH was elevated significantly by Premarin. Among them, however, only Case 1 showed a good FSH excretion reaction to ethinyl-estradiol prior to the Premarin test which succeeded in inducing ovulation. The three others had no spontaneous ovulation as controlled by changes on their BBT charts, even though they did have LH release, and one of them could be diagnosed as presenting the Stein-Leventhal syndrome. These findings suggest a dissociation between ovulation and release of LH. In other words, ovulation follows LH release, but LH release does not always induce ovulation. Woman with secondary amenorrhea, whose ovulation was induced immediately after LH release, showed also a significant reaction of FSH excretion. The result suggests that not only ovarian, but also hypothalamic conditioning, *i.e.*, 'FSH priming' may be very important in the induction of ovulation after LH release.

Premarin in small doses affected the length of menstrual cycles in normal women. The mean prolongation in our experimental cycles was 5.8 days. This is similar to that reported by McArthur *et al.*²⁸ who regarded 4.6 days as the mean prolongation of their experimental cycles following a single high dose of Premarin. Greenblatt²⁹ assumed that this is due to prolongation of the luteal phase by hypersecretion of LH. In our data, however, the prolongation of the experimental cycle did not correspond to LH excretion.

We assume, therefore, that increased LH excretion three to five days after estrogen administration in women with menstrual abnormalities and in normal men may be the result of direct stimulation of the LH-RF by estrogen. The recent report by Palka and his associates²⁶ is highly encouraging to our hypothesis. That is to say, a dramatic rise in plasma LH was observed in adult Sprague-Dowley rats with normal cycles four to five days after estrogen implantation into the median eminence, whereas LH was not detectable in the plasma of the rats with estrogen introduction into the pituitary. Thus, they concluded that estrogen has a stimulatory action on the release of LH from the pituitary and the crucial site of this stimulatory action is the median eminence.

Our LH excretion patterns appear to have a single peak different from that of FSH excretion. This also suggests a different mechanism of secretion between LH and FSH, and an internal feedback control of LH secretion that may work at the hypothalamic level when the circulatory or portal LH level is rising. This seems to be true, because David *et al.*³⁰ implanted solid LH in the median eminence region of normal or castrated male and castrated female rats and found that pituitary LH store was significantly decreased after implantation. Plasma LH levels were also reduced in castrated female rats on LH implantation into the median eminence. They concluded that a 'short' feedback loop might link synthesis

and release of LH to the preexisting level of the hormone. Thus, if the excretion of LH is sustained at a high level for a few days, it might represent a kind of abnormal type of LH secretion.

We noticed that LH excretion response to the action of Premarin was different between normal women and normal men, as was also mentioned previously in the case of FSH excretion upon stimulation by ethinyl-estradiol. The paper of Harris,²¹ who proved a different pattern between the two sexes at the hypothalamic level, may also support our observation. Therefore, it seems reasonable to conclude that the abnormal non-menstruating woman tends to have a 'male type' (acyclic) in both LH response and FSH excretion as regards the feedback control mechanism of gonadotropin secretion.

Briefly we can state that 0.05 mg of ethinyl-estradiol exerts a stimulatory effect on FSH excretion in the normal woman during the second and fourth to fifth days, and that this response is statistically different from that of the normal man and the woman with menstrual disorders. We cannot yet state that different doses or different times of administration of the drug may have different actions, and further studies are necessary about this question.

We can also state that Premarin exerts its action principally on women with abnormal menstrual cycle and probably in the normal man by increasing LH excretion on the third to fifth days of its administration. We are not certain whether different doses, times or routes of Premarin administration might produce a different response. The problem of the action of Premarin on LH secretion awaits also further studies.

Our FSH-LH test with ethinyl-estradiol and Premarin in low doses seems to contribute to the solution of some clinical problems related to women with abnormal menstrual cycle and especially to those related to abnormalities of gonadotropin secretion mechanisms.

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