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Research Article

A QUANTITATIVE STUDY OF THE URINARY EXCRETION OF HYPOPHYSEAL GONADOTROPIN, ESTROGEN, AND ANDROGEN OF NORMAL WOMEN

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Work upon the assay of hormones in blood and urine has been progressive in volume and quality since Loewe (1) and Frank (2) demonstrated the presence of estrogenic hormone in blood. Loewe (3) then revealed that this substance is excreted cyclically in the urine. Such assays were made practicable by the work of Kahnt and Doisy (4) who standardized the procedure of assay for this hormone, using the changes induced in the vaginal smear of castrated adult rats. The presence of gonadotropic substance in blood and urine was established shortly thereafter. The existence of such a gonadotropin and its production in the anterior hypophysis were made clear by the fundamental work of P. E. Smith (5) and Zondek and Aschheim (6). They also provided a method for determining its presence by finding that premature maturity is induced in the immature rat and mouse by this hormone (6, 7). Aschheim and Zondek (8, 9), using this test object, were able to show that a gonadotropin may be detected in the urine of pregnancy, although this gonadotropin is now known to be chorionic in origin. Fluhmann (10), Zondek (11), and others soon pointed out that the hypophyseal gonadotropin is similarly excreted after ovariectomy and the menopause. The same gonadotropin next was found to appear during the middle of the normal menstrual cycle (12). The demonstration of the excretion of androgenic substances soon followed (13, 14), and methods of assay were provided by the capon comb method (15) and the colorimetric reaction of Zimmermann (16). Finally, excretion products of progesterone were shown to appear in the urine (17, 18).

Despite the volume of work which has been published since these initial studies, further quantitative information is highly desirable. Recent improvements in methods of extraction and assay now aid in such a study. The tannic acid precipitation procedure of Levin and Tyndale (19), and their method of assay, using the immature mouse uterine weight (20), make possible the quantitative assay of hypophyseal gonadotropin in normal urine. In addition, it has been found that estrogen and androgen may be quantitatively recovered from the supernatant and the alcohol-acetone-ether washings of the tannic acid precipitate, as was observed by Freed and Hechter (21) after tungstic acid precipitation. Thus it is possible to assay the gonadotropin, estrogen and androgen in the same specimen of urine. Data are here presented from such a study of the complete urinary output of five normal women with regular menstrual cycles over a period of three to four months for each woman. An additional three cycles scattered throughout the year were examined in one of these cases. These assays have been directed towards determining the normal values and the pattern of hormone excretion in medically normal healthy women with regular cycles. This was thought to be essential for the later determination as to whether or not patients were excreting abnormal amounts of hormone. The interpretation of the results has suggested a possible mechanism affecting the hypophysis-ovary relation.

METHOD

All urine specimens were complete forty-eight-hour collections kept in tightly stoppered bottles containing 10 cc. of chloroform for a day's output. The specimens were kept in the cold during collection in most instances. The urine was then placed in the ice chest at 4° C. for twenty-four hours before beginning the extraction. It was then decanted from the chloroform, which was also saved, and measured and tested to litmus. No alkaline specimens have been received, except in a few collections unassociated with this study, in which the preservative was missing or evaporated.

Extraction. The procedure of Levin and Tyndale

[1] Aided by a grant for Teaching and Research in Neurology, Rockefeller Foundation.

(19) was followed for the gonadotropin. The urine was acidified with glacial acetic acid to the turning point of brom-cresol-green pH 3.8 to 5.4. Twelve to fourteen grams of tannic acid as a 20 per cent solution were then added and the precipitate was allowed to settle for half an hour in the ice chest. The precipitate was then collected by centrifugation, saving the supernatant, carefully washed with 95 per cent alcohol, three times with 80 per cent alcohol, left overnight in 95 per cent alcohol and then dried with acetone and ether. The washings were pooled to be treated as described below. The dried precipitate was extracted three times with slightly alkaline water, then neutralized and brought to a volume of 24 cc. per forty-eight hours' collection immediately before the assay. The dried tannate powder is stable for long periods without deterioration but the solution deteriorates slowly.

The pooled alcohol, acetone and ether washings of the tannic acid precipitate were combined with the chloroform preservative of the original urine. The volatile solvents were then removed over a water bath temperature of 90 to 100° C. under negative pressure. The watery fraction was added to the supernatant from the original tannic acid precipitate. This mixture was acidified with concentrated HCl (about 12N) to pH 1 to 2, the turning point of orange IV, and 3.3 cc. concentrated HCl per 100 cc. original urine were added to insure excess acid (22). The whole was boiled under a reflux condenser for half an hour, a time insuring maximum recovery of both estrogen and androgen (23, 24, 25). Acid hydrolysis split the combined estrogen and androgen, a necessary step before assay could be conducted.[2] The substance thus freed and the small fraction of the hormones in the urine not combined originally are soluble in ether. Accordingly, the hydrolysate was then cooled and extracted with ether in a four liter pyrex separatory funnel, using 200 cc. ether per liter of original urine for the first extraction, then three times more with 100 cc. per liter, rinsing with the ether the flask in which the urine was hydrolyzed. No emulsion formed during any of the extractions with the ether. The pooled ether extracts were then washed three times with 30 cc. H_2O each per liter of original urine.

The estrogenic fraction was then separated from the androgenic fraction by extracting the ether three times with 100 cc. each of 1 N NaOH per liter of original urine. The remaining ether, containing the androgen, was washed three times with water as before, and the washings were added to the NaOH extract containing the estrogen. The NaOH fraction was acidified to pH 4.5 to 5, the turning point of congo red, with concentrated HCl, and extracted immediately three times with 100 cc. of ether per liter of original urine each time. The ether, now containing the estrogen, was washed three times with water as above. The ether fractions of estrogen and androgen were placed in the ice box overnight to allow the water to drain from the walls of the separatory funnel, and the water was then removed. The androgenic ether fraction was evaporated to dryness under negative pressure on the water bath and the residue redissolved in 10 cc. of absolute alcohol per twenty-four hours of original collection when ready for assay. This fraction permitted colorimetric or biologic assay to be done. The estrogenic ether fraction was evaporated to a small volume, transferred to a small Erlenmeyer flask, extracted with half the desired volume of corn oil (Mazola), and the ether removed by heating over a water bath. The tarry residue was redissolved in a small volume of ether and re-extracted with oil and the ether removed as before. The oils were combined, a total of 12 cc. of oil being used per forty-eight hours' collection.

Assay. All assays have been conducted on six to eighteen animals, twelve being used for most of the specimens. Hypophyseal gonadotropin was assayed by the mouse uterine weight method of Levin and Tyndale (20), three mice per dose level being used. Unit levels of 5, 10, 20, 27, 40, 60 and 78 units per twenty-four hours were tested for, as necessary. A uterine weight of 11.0 mgm. or over in two of the three mice was considered positive if the next higher unit level was negative and the next lower unit level positive. No serious discrepancies have been noted between responses at these different dose levels. Toxicity of the extract was high at the time of the menses. Reprecipitation in acetone at this time generally permitted assay of 5 units per twenty-four hours in those instances where the animals were killed by the original extract. Ten units could practically always be assayed without reprecipitation.

Estrogen was assayed by the Kahnt-Doisy (4) procedure, using the castrated rat vaginal smear. The method was followed in exact detail. Three rats per dose level were used as above for the gonadotropic assay and the same unit levels tested. The rats have been repeatedly tested for sensitivity to crystalline estrone.[3] One rat unit has been found to be equivalent to 0.7 to 0.8 gamma of crystalline estrone.

Androgen, or 17-keto-steroid, as it will hereafter be referred to in this paper, was determined by the colorimetric reaction of Zimmermann (16), as detailed by Callow and Callow (26), except that aqueous KOH 2.45 N was substituted for alcoholic KOH (27). Every run was controlled by concurrent determinations on 5, 10, 15 and 20 mgm. of crystalline androsterone.[3] This was necessitated by the range of color produced in various tests for a given dose of standard material, probably caused by slight variation in the reagents. Since the mother liquor from the urine was colored, a blank of this solution was run as for the test but alcohol was substituted for m-dinitrobenzene. The value then obtained was subtracted from the total value with m-

[2] Talbot, J. Biol. Chem., 1940, 136, 365, states that hydrolysis destroys 30 per cent of dehydroisoandrosterone. This compound is a small fraction of the normal ketosteroids. These are not similarly affected.

[3] The author is indebted to Dr. Edward Henderson of the Schering Corporation, Bloomfield, N. J., for the generous supply of estrone and androsterone which was used.

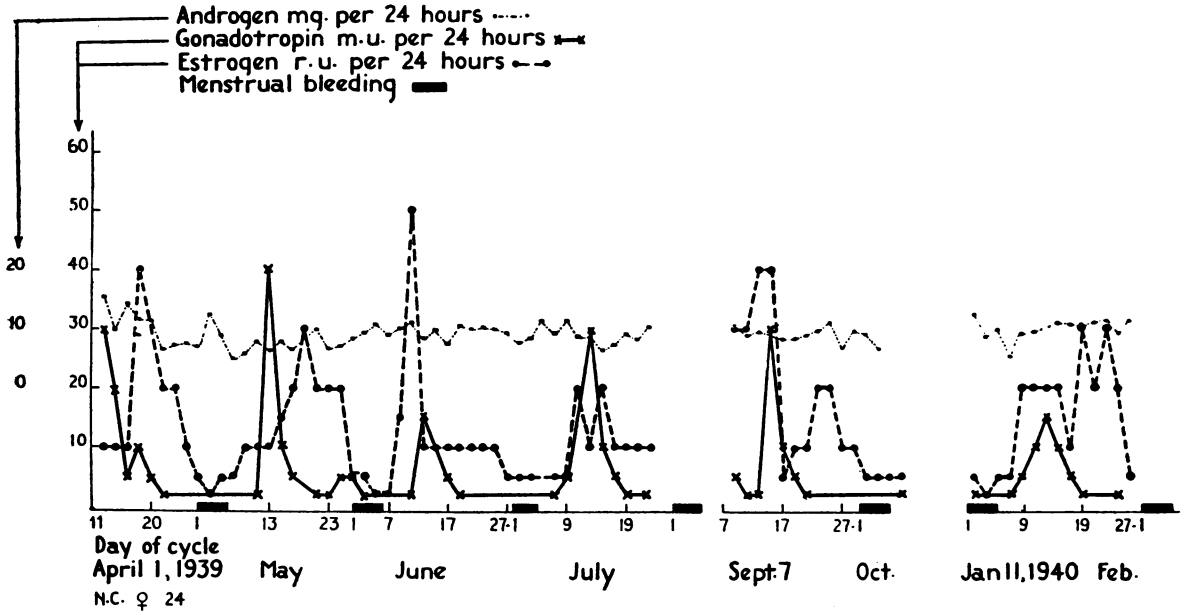

FIG. 1. FORTY-EIGHT-HOUR EXCRETION OF 17-KETO-STEROID, ESTROGEN, AND GONADOTROPIN IN RELATION TO THE MENSTRUAL CYCLE, INCLUDING THREE SINGLE CYCLES AT INTERVALS THROUGHOUT THE YEAR. SUBJECT N. C.

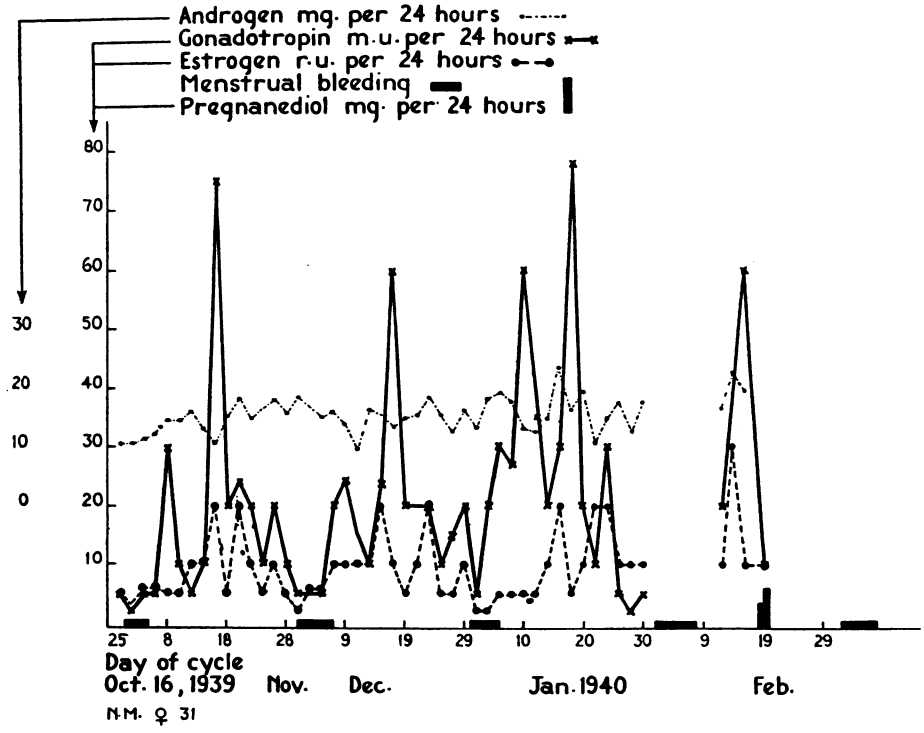

FIG. 2. FORTY-EIGHT-HOUR EXCRETION OF 17-KETO-STEROID, ESTROGEN, AND GONADOTROPIN IN RELATION TO THE MENSTRUAL CYCLE, INCLUDING PREGNANEDIOL GLUCURONIDATE EXCRETION IN THE MID-PERIOD OF AN ADDITIONAL CYCLE. SUBJECT N. M.

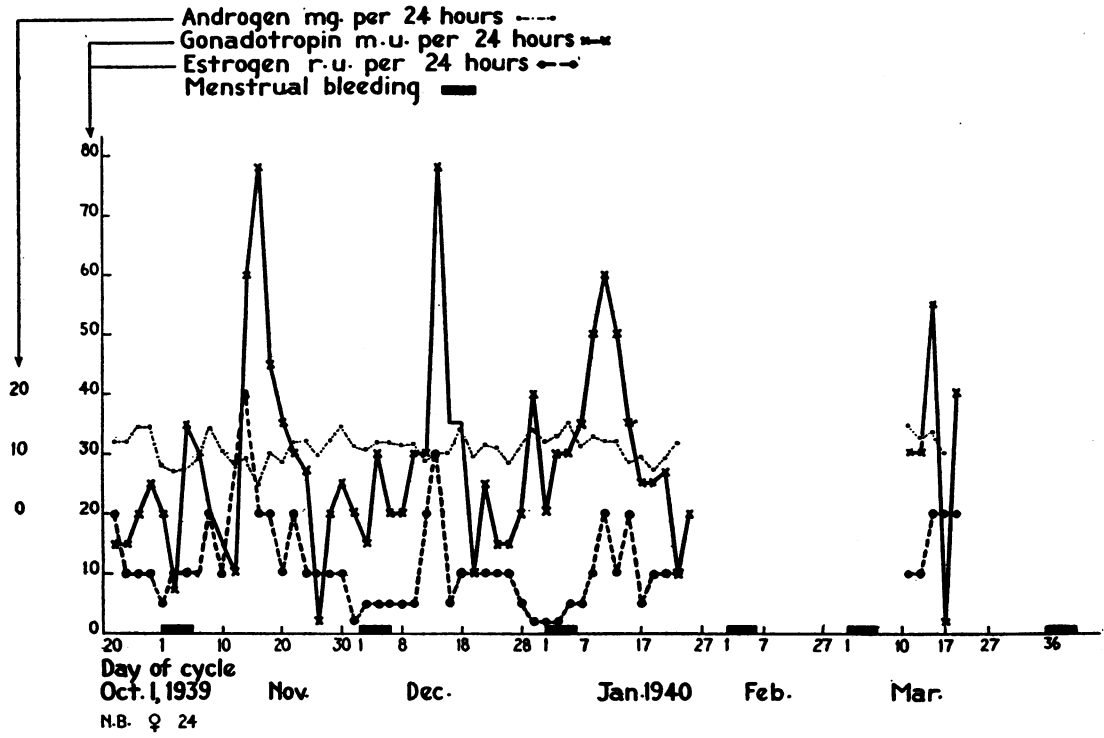

FIG. 3. FORTY-EIGHT-HOUR EXCRETION OF 17-KETO-STEROID, ESTROGEN, AND GONADOTROPIN IN RELATION TO THE MENSTRUAL CYCLE, WITH AN ADDITIONAL MID-CYCLE ILLUSTRATING FAILURE OF PREGNANEDIOL GLUCURONIDATE TO BE EXCRETED BY TWENTIETH DAY IN A THIRTY-SIX-DAY CYCLE. SUBJECT N. B.

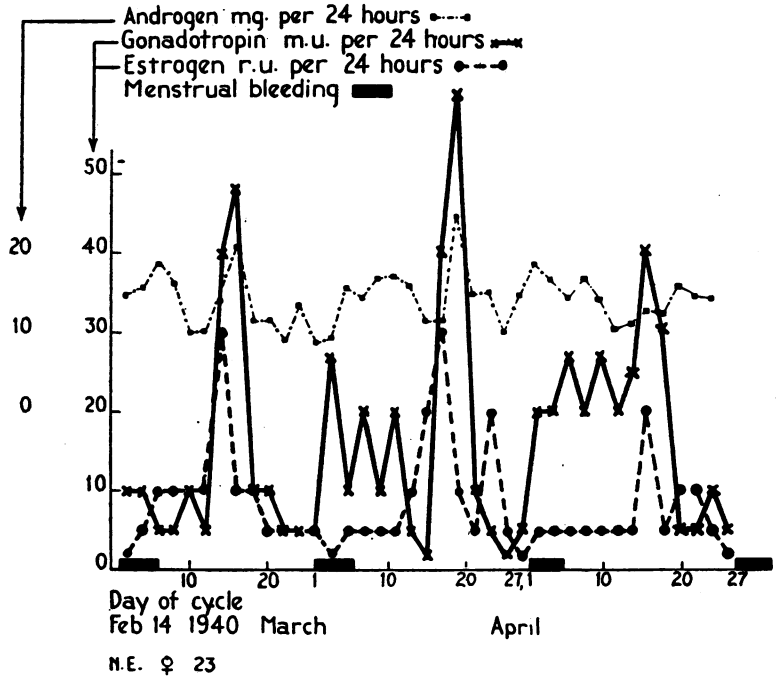

FIG. 4. FORTY-EIGHT-HOUR EXCRETION OF 17-KETO-STEROID, ESTROGEN, AND GONADOTROPIN IN RELATION TO THE MENSTRUAL CYCLE, AND ILLUSTRATING FAILURE OF PREGNANEDIOL TO BE EXCRETED THROUGHOUT ENTIRE LAST CYCLE. SUBJECT N. E.

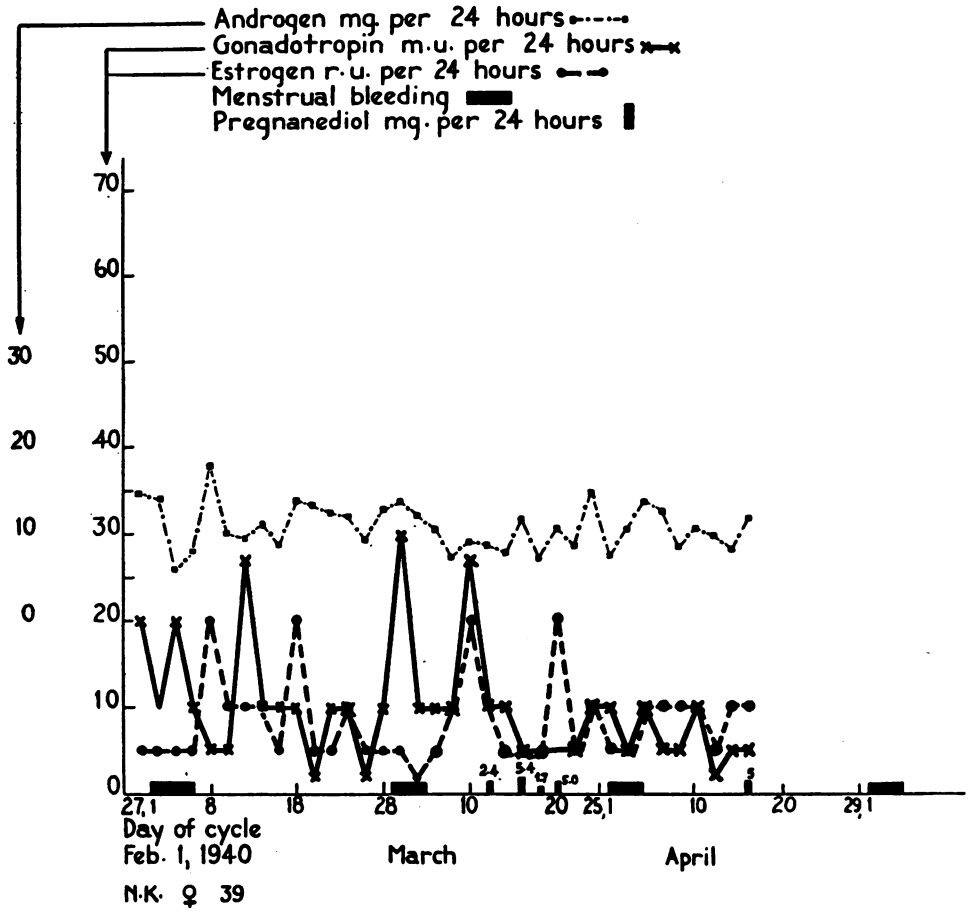

FIG. 5. FORTY-EIGHT-HOUR EXCRETION OF 17-KETO-STEROID, ESTROGEN, AND GONADOTROPIN IN RELATION TO THE MENSTRUAL CYCLE, AND ILLUSTRATING FAILURE OF PEAKS OF GONADOTROPIN AND ESTROGEN TO BE DEMONSTRATED IN LAST MENSTRUAL CYCLE, AND SHOWING APPEARANCE OF PREGNANEDIOL GLUCURONIDATE IN THE ABSENCE OF THESE PEAKS. SUBJECT N. K.

dinitrobenzene. A reagent blank was also run. Colorimetric determinations were read on a neutral wedge photometer (American Instrument Company), using two 0.999 inch cells, one containing the blank in one beam and the solution to be read in the other. Readings were taken with a filter having a centrum at 510 millimicra. Values for the 17-keto-steroid content of the unknown are expressed in mgm. equivalents of androsterone per twenty-four hours.

Animals. A colony of mice and rats was maintained solely for the assays. The mice were of the Klasek or Swiss strain. Rats were the Long-Evans strain. The mice were fed on a standard mouse biscuit, with water *ad lib.* The rats were given a standard diet, McCollum mixture number 1 with water *ad lib.* and lettuce supplements. Weaning weight for the mice was 8 to 13 grams at twenty-two days, averaging 10 to 11 grams. Only twenty-two to twenty-three-day old mice were used. The rats were castrated at four months of age and tested for responsitivity according to the method of Kahnt and Doisy.

Extracts. Tannate and estrogenic extracts were assayed immediately after preparation in almost every instance. The androgens were kept dry in stoppered flasks for one to two weeks, and then dissolved in alcohol on the day of the determination. All extracts were kept in the ice box when not being used.

EXPERIMENTAL

The urine which was assayed was collected from five healthy, normal women with regular menstrual cycles. The subjects carried on their daily routine during the course of the experiment. Their ages were: twenty-three, twenty-four (2 subjects), thirty-one and thirty-nine. Onset of menses occurred at ten and one-half to thirteen years of age. There were no menstrual abnormalities beyond slight dysmenorrhea the first day of bleeding. Menses were moderate in flow except for the first

two days of N. M.'s cycle when the flow was profuse. The patients were healthy throughout the study. Subjects N. K. and N. B. were married.

The output of hormones for each of the five subjects (N. C., N. M., N. B., N. E., and N. K.) is shown in Figures 1 to 5. The general pattern of all cycles except the last one of N. K. is similar in that there is a more or less constant 17-keto-steroid excretion, a central peak of gonadotropic and estrogen excretion, followed by a second peak of estrogen output. However, the pattern is not exactly reduplicated from cycle to cycle in the same individual or from subject to subject. The relationship between the peaks of estrogen and gonadotropin is not constant (Table I). The initial estrogen peak may precede, occur with, or come later than the gonadotropin peak. The time in the cycle that both these peaks occur is also variable, as is the actual length of the menstrual cycles. The peaks, however, tend to occur later or earlier as the cycle is longer or shorter (N. M., N. B., N. K.). The 17-keto-steroid level (Table II) fluctuates from day to day within relatively narrow limits for the individual, but varies between subjects.

TABLE I

The days of occurrence in the menstrual cycle of the peaks of gonadotropin and estrogen excretion and of first appearance of pregnanediol glucuronidate

Subject	Number of cycle	Duration of cycle	Peak of gonadotropin	Peak of estrogen	First appearance of pregnanediol
N.C.	1	27	11 & 12	17 & 18	
	2	26	12 & 13	18 & 19	
	3	27	12 & 13	10 & 11	No determinations
	4	27	13 & 14	11 & 12	
	5	29	13 & 14	11–14	
	6	27	13 & 14	9–12	
N.M.	1	29	15 & 16	15 & 16	
	2	29	16 & 17	14 & 15	
	3	30	17 & 18	15 & 16	
	4	32	15 & 16	13 & 14	18 & 19
N.B.	1	32	16 & 17	14 & 15	
	2	31	14 & 15	14 & 15	
	3	31	11 & 12	11 & 12	
	4	36	14 & 15 20 & 21?	14 & 15?	None
N.E.	1	25	15 & 16	13 & 14	
	2	26	18 & 19	16 & 17	
	3	28	16 & 17	16 & 17	None
N.K.	1	28	11 & 12	7 & 8	
	2	25	1 & 2 10 & 11	10 & 11	12 & 13
	3	27	None	None	17 & 18

TABLE II

Range of normal values for the excretion of androgen as 17-keto-steroid, estrogen and gonadotropin, excluding a few extreme values

Subject	Androgen 17-keto-steroid	Estrogen	Gonadotropin
	mgm. per 24 hours	*rat units per 24 hours*	*mouse units per 24 hours*
N.C.	5.4–12.5	<5–50	<5–40
Average	10.0		
N.M.	10.6–19.6	<5–30	<5–78
Average	15.0		
N.B.	7.4–14.6	<5–40	<5–78
Average	10.5		
N.E.	8.9–18.9	<5–30	<5–60
Average	14.0		
N.K.	5.7–14.7	<5–20	<5–30
Average	11.0		
Range for series	5.4–19.6	<5–50	<5–78

Gonadotropin. Contrary to previous work, gonadotropin was demonstrable throughout the cycles of all but one subject (N. C.). In this latter, less than 5 mouse units per twenty-four hours of gonadotropin were excreted, except during the middle of the cycle from the ninth to about the sixteenth day. The relatively constant amounts of gonadotropin throughout the cycles of the other subjects were also replaced by a sharp rise in output about the middle of the cycle. This peak of gonadotropin output reached its maximum at various times in the cycle, however, even in the same individual, varying from the tenth to the eighteenth day of the cycle in the series, or as with N. B., from the eleventh to possibly the twentieth day when the mensis was delayed. The height of the peak varied from 15 to 80 mouse units per twenty-four hours but, except for N. K.'s last cycle, it was relatively constant for the individual. The peak of gonadotropin was sometimes preceded by a lesser peak. Thus, in one cycle each of subjects N. M. and N. B. there was a preliminary rise in gonadotropin output to 60 units. This preliminary peak was not associated with a corresponding estrogen peak, but was followed later by a sharper gonadotropin rise associated with a peak of estrogen. Gonadotropin may be present in large amounts during menstrual bleeding (N. M., N. B., N. K.). There is a range in excretion of gonadotropin for this series

of from less than 5 mouse units to 78 mouse units per twenty-four hours. This broad range has been found in a single individual's output, depending on the time of the cycle.

Estrogen. Estrogen values represent the total of free and combined estrogen. No attempt has been made to separate the various estrogens as reported by Smith, Smith and Pincus (28). The double peak of excretion described by Gustavson *et al.* (29) was generally found in this series. However, the contour of the excretion pattern varied considerably, a double peak being absent (N. C., N. K.) or hard to differentiate (N. C.). Estrogen was present throughout the cycle except about the time of menstrual bleeding, though its disappearance has no constant relation to the time of onset of bleeding (N. C., etc.). Values of excretion varied considerably from cycle to cycle. Thus, less than 5 rat units per twenty-four hours were found for forty-eight hours at one point during the menses, although rarely for a longer time (N. B.). At other times, values from 5 rat units to 50 rat units per twenty-four hours have been found depending on the time of the cycle. There seems to be a tendency to a slow rise and fall in the amount of hormone excreted after and before the menses, with a spurt in excretion during the mid-cycle (N. C., etc.) and again after a week or so. The point at which these peaks of estrogen occur is quite variable. The time of appearance of the first peak of excretion may vary from cycle to cycle in a given individual—for example, from day 9 to day 18 in N. C. or from day 7 to day 18 in the series. The height of the peak, too, may vary from 20 to 50 rat units per twenty-four hours.

Androgen or 17-keto-steroid. The androgen excretion as determined colorimetrically is really a measure of 17-keto-steroid excretion (26). The ketonic fraction was not separated from the non-ketonic fraction (30) in this study. The error resulting is probably not significant in normal urine. It should be mentioned that the introduction of Aq. KOH instead of alcoholic KOH may be responsible for altered reactivity of the various keto-steroids, although the total values obtained for this series compare favorably with those obtained with alcoholic KOH.

A fairly constant mean level of 17-keto-steroid excretion for a given individual throughout the cycle was found. There was a day-to-day range of plus or minus 5 mgm., with an occasional more extreme value. The level of output varied from subject to subject but did not seem to show seasonal variation (N. C.). Normal values of excretion ranged from 5.4 to 19.6 mgm. if a few extremes were excluded.

Pregnanediol. Through the courtesy of Dr. E. T. Engle pregnanediol determinations were made during the cycles of all but N. C. (Table I) according to the method of Venning and Browne (18). In the case of N. M. and the first cycle of N. K. pregnanediol was present forty-eight to seventy-two hours after the peaks of estrogen and gonadotropic excretion. This is harmonious with the reports of Venning and Browne (18) that pregnanediol appeared shortly after the one point in the cycle at which they were able to demonstrate gonadotropin. However, in this series, no pregnanediol was demonstrable throughout the entire last cycle of N. E. and up to the nineteenth day in the last cycle of N. B. It should be mentioned, though, that the mensis was unusually late in appearing in this latter cycle. Pregnanediol appeared without an associated peak of gonadotropin or estrogen in the third cycle of N. K.

DISCUSSION

Data have been obtained concerning the quantitative excretion of gonadotropin, estrogen and 17-keto-steroid through eighteen cycles in five medically normal women. Except in one subject, these hormones were found throughout the entire menstrual cycle. There is, however, a cyclic influence on the excretion of gonadotropin and estrogen. A sudden peak of excretion of both hormones was generally found near the mid-interval with a reduplication of the estrogen peak later in the cycle. The 17-keto-steroid output, on the other hand, varied from day to day but showed no cyclic change. This relative constancy of output of 17-keto-steroid both for the individual and between individuals makes it possible to define the limits of normal excretion. Outside this range abnormality would be suspected.

The variation in pattern of excretion of gonadotropin and estrogen in different cycles makes the determination of the range of normality much more difficult. Thus the excretion curves of the subject N. K. are extremely variable, the last

cycle showing no peaks at the mid-interval. Had this cycle been assayed in a patient with some abnormality, this cycle might well have been interpreted as the result of a pathological state. The relatively normal contour of the remaining cycles, however, suggests the error of such an interpretation, although the possibility exists that there was some temporary abnormality. If the assay of the urinary output of an entire menstrual cycle may be inadequate, the significance of single twenty-four- or forty-eight-hour specimens is even more open to question. Single assays for estrogen and gonadotropin, as seen from this study, would be indicative of abnormality only if extremely high values were found, such as are found after castration and the menopause in the case of the gonadotropin. Very low values of less than 5 units per twenty-four hours may be significant also although, as seen in the case of subject N. C., extremely low values may be meaningless if found in only one specimen. However, the repeated finding of very low values, at weekly intervals for example, would have significance in view of the normally cyclic character of excretion.

The appearance of peaks of excretion of estrogen and gonadotropin near the middle of the menstrual cycle raises the speculation as to whether these peaks are related to ovulation. Kurzrok (31), using a less sensitive method of assay, obtained a positive reaction for gonadotropin only for one day in the cycle and interpreted this as indicating ovulation. Venning and Browne later pointed out that pregnanediol appeared shortly after this peak, indicating an association with corpus luteum formation. D'Amour (32) found several days in the cycle on which gonadotropin was present and so postulated several ovulations in a cycle.

The present study reveals that gonadotropin and estrogen are generally excreted throughout the cycle but that there is only one definite and associated peak of excretion of both hormones near the middle of the cycle. This peak is undoubtedly the point at which the gonadotropin was found in the earlier studies. The gonadotropin peak may be reduplicated (subjects N. M. and N. B.) but there is still only one time in the cycle when the gonadotropin peak is associated with the estrogen peak, a point also noted in the cycles recently reported by Von Haam and Rothermich (33). This persistent relation at about the middle third of the cycle suggests that the phenomenon is associated with ovulation and, if so, indicates that there has been only one ovulation per cycle in these subjects. Further support for this view is seen in the appearance of pregnanediol shortly after this point in each of two subjects (N. M. and N. K.), revealing that a functional corpus luteum was formed about this time and implying that ovulation probably occurred as well. On the other hand, corpus luteum formation may be shown by the appearance of pregnanediol in the absence of the gonadotropin-estrogen peaks (last cycle of N. K.) and the latter peaks may occur without the subsequent excretion of pregnanediol (last cycle of N. E.). Thus the relation of the peaks of gonadotropin-estrogen excretion to ovulation remains presumptive pending further study.

The almost constant occurrence of the peaks of estrogen and gonadotropin output suggests a relationship between the two. However, the estrogen peak may appear before, with, or after the gonadotropin peak, and the shifting time relations involved make it difficult to explain either peak as the result of the other, as advanced by D'Amour (34). It would appear, rather, that the peaks may reflect two concurrent rhythms. One rhythm is the development of the ovarian follicle, reflected by the estrogen excretion, which takes place in the presence of the relatively constant output of hypophyseal gonadotropin that is found during the first part of the cycle preceding the peak of gonadotropin excretion. The other rhythm is the independent, though somehow related, one occurring in the anterior hypophysis. This results in a sudden release of an excess of gonadotropin at some time during the middle third of the cycle. As stated above, the association between the gonadotropin-estrogen peaks and the occurrence of ovulation has not been established. However, if this relationship should be demonstrated, it would appear likely that the gonadotropin peak results in ovulation if the gonadotropin spurt occurs at a time when the ovarian follicle has reached maturity, and does not produce ovulation if the follicle has not yet matured. Evidence for this supposition is found in the cycles of N. M. and N. B. in which early gonadotropin peaks, occurring before the estrogen peak, were reduplicated later at the time of the rise in estrogen excretion.

The mechanism coordinating the two rhythms is not clear.

There is one possibility which must be considered in interpreting any peak of hormonal output in the urine—the possibility of a temporary change in renal permeability which would permit excess excretion of the hormones. In this instance, the peak would be only an apparent one and not a true reflection of the rate of secretion of the hormones. Frank (35) found no evidence for such a change in permeability. Also, under such circumstances, one would expect the androgen output to rise cyclically in the same specimens in which the titer of gonadotropin and estrogen is rising.

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