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Determination of Estrone, 17B-Estradiol and Estriol in Urine of Young Adults

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DETERMINATION OF ESTRONE, 17β-ESTRADIOL AND ESTRIOL IN URINE OF YOUNG ADULTS

by

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Shiao-fan Lee

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and Estriol in Urine of Young Adults

by

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A study was made of the urinary excretion of estrogens of healthy university students consuming self-selected diets living under home conditions (4 men and 5 women). Two 31-day test periods were used. During the two test periods, two meal patterns were used; three meals a day or two meals a day with no lunch. Weight was kept constant. Calories, protein, carbohydrate, and fat of their diets were calculated.

Urine specimens were collected twice a week, and additional samples were also collected on specified days for female subjects. Urinary estrogens were determined by using Brown's method (1955, 1957) with some modifications.

The excretion values of the three individual estrogens, by both men and women showed that 17 β-estradiol was usually present in least amounts and estriol in greatest amounts. The total estrogen value for the women was much higher than that for the men. The mean values were 33.5 and 15.4 mcg per day for women and men, respectively.

The menstrual cycle did affect the excretion of estrogen which showed higher estrogen values during the middle of the cycle and again between the third and fourth week. However, this increase was less than the first time.

There was no definite evidence that the meal frequency had any effect on the excretion of estrogens in human urine.

Further work including a greater number of subjects, daily analyses during the menstrual cycle, and increasing the meal frequency is desirable and is recommended to obtain more reliable basic data on young adults maintained in their usual home living conditions.

(81 pages)

INTRODUCTION

Hormonal influence on carbohydrates and protein metabolism have been extensively studied. In contrast, information concerning hormonal influences on lipid metabolism is limited, although evidence is accumulating that such mechanisms exist and probably are important.

The concept that atherosclerosis was a metabolic disorder (Adlersberg, 1951) involving chiefly lipis and lipoproteins stimulated extensive investigation of the endocrine influences on circulating lipids and on lipid metabolism. Even in countries with low dietary fat consumption, there is still an individual susceptibility to atherosclerosis (Oliver, et al., 1956) which cannot be explained by environmental factors only. There is evidence that hereditary influences determine the individual susceptibility to atherogenesis, presumably through metabolic mechanisms and through genetically controlled enzymatic reactions (Adlersberg, 1957). The endocrine system may play an important regulatory or medicating role in these mechanisms.

Evidence is accumulating that the gonads exert an important influence on the level of circulating lipids and lipoproteins. The comparatively rare occurrence of coronary artery disease in women during the reproductive-phase is well known. The control of hypercholesterolemia

in men and in women after the menopause of by estrogen administration has been recognized (Hess, 1964; Scanu, 1965).

Sex differences in the distribution of serum lipoproteins have been reported by various laboratories. Adlersberg, et al. (1956) demonstrated that the male has the greatest increase in the early thirties, whereas the female maintains her youthful lipid level longer and does not reach her peak serum cholesterol level until the age of fifty or later.

The response of cholesterol levels to the menstrual cycle has been studied by several investigators with contradictory results. The decrease in cholesterol levels corresponded to the reported maxima for estrogen secretion (Kritchevsky, 1958; Boyd and Oliver, 1958). Further investigations of the relationships of estrogens and lipid metabolism in young adults while living under normal living conditions are needed.

This study is part of a larger project on lipid-steroid hormone relationships in young adults living under home conditions in which analyses will be made for serum cholesterol, total lipid, lipid phosphorous, urinary estrogens, androgens and adrenal steroids as influenced by dietary factors. The basic information on individuals living under home conditions is needed as a base for interpretation of findings when studying interrelationships of serum lipids and secretion of the steroid hormones to delineate their in role in abnormal metabolism of lipids in atherosclerosis and coronary heart diseases.

The present study was designed to obtain basic information on the excretion of urinary estrogens by normal healthy university students on self-chosen diets.

REVIEW OF LITERATURE

Biochemistry of Estrogens

The predominant natural estrogens of the human are 17β-estradiol estrone, and estriol. Several other estrogens representing estrogen metabolities, have been isolated in significant amounts from human urine and identified. These are listed in Table 1. All of the estrogens are C_{18} compounds and, for terminological purposes, may be considered as derivatives of the theoretical parent substance, "estrane." In the naturally occurring estrogens, they are characterized by the aromatic nature of ring A, the absence of a methyl group at C-10, and the presence of a phenolic hydroxyl group at C-3. They differ from one another only in the degree of unsaturation and in the nature and position of the substituents of the cyclopentano ring.

Sources of Estrogen

The origin of estrogen in the organism seems to be in the ovaries, the testes, the placenta and possibly the adrenals as well. Some estrogens are also of exogenous origin. These cells and the human corpus luteum are thought to secrete estrogen, but granulosa cells and ovaria stroma cells may also secrete estrogen, at least under abnormal circumstances. The placenta is a rich source of estrogen. In both males and females, the adrenal

Table 1. Estrogen isolated from human urine[a]

Name	Investigator	Date
Estrone	Doisy et al.	1929
	Buntenandt	1929
Estriol	Marrian	1930
	Doisy et al.	1930
	Doisy and Thayer	1931
17 β-Estradiol	Smith et al.	1939
	Huffman et al.	1940
16-Oxo-estrone	Serchi	1953
16-epi-Estriol	Marrian and Bauld	1955
6-Hydroxy-estrone	Loke et al.	1957
16 α-Hydroxy-estrone	Marrian et al.	1957
2-Methoxy-estriol	Fishman et al.	1958
2-Methoxy-estrone	Loke and Marrian	1958
16 β-Hydroxy-estrone	Layne and Marrian	1958
16-Oxo-17 β-estradiol	Layne and Marrian	1958
18-Hydroxy-estrone	Loke et al.	1958, 1959
16,17-epi-Estriol	Breuer and Pangels	1959
2-Methoxy-17 β-estradiol	Frandsen	1959
17-epi-Estriol	Breuer	1960
2-Hydroxy-estrone	Notchev and Stimmel	1962

[a]Adlecreutz, H., 1963. Studies on oestrogen excretion in human bile. Acta Endocrnologica. 42: supplementum 72.

cortex forms estrogen and in the male the interstitial cells of the testes secreted estrogen. Estrogen also occurs naturally in various plants. Pussy willows contain estriol; date seeds, estrone (Heftmann, 1965); and alfalfa, subterranean clover, and sprouting corn also, the estrogens (Lloyd, 1964). Mirestrol, a potent estrogen, has been isolated from Pueraria mirifica (Pope, et al., 1958). There are numerous synthetic substances, steroidal and non-steroidal, that are estrogenic. The most important of the non-steroidal substances belong in the stilbene group of which diethylstilbestrol is commercially and clinically important example. The various estrogens differ considerably in potency. The possibility of qualitative differences in their actions is not unlikely, but such differences have not been defined (Lloyd, 1964).

Synthesis and Metabolism of Estrogens

The biosynthesis of the estrogenic hormones has recently been reviewed by Adlercreutz (1963). The exact precursor of estrogens is not known, but both acetate and cholesterol may serve this purpose. Furthermore, testosterone, androsteredione and progesterone can be converted to estrogens. The ovary secretes estradiol and estrone. The rate of conversion of various substances by the human ovary is great when progesterone is the substrate but other precursors are not efficiently converted (Table 2).

Administration of isotopically labeled testosterone to an adrenalectomized woman has yielded labeled estrone in the urine. The pathway for the conversion

Table 2.[a] Incubations of FSH[b] stimulated ovaries

Substrate	Products	Percent of yield
Acetate	Estrone Estradiol	0.02
Cholesterol	Estrone	0.1
Acetate	Cholesterol	0.3
Progesterone	Estrone Estradiol	10.0

[a]See Lloyd p. 82
[b]Follicle-stimulating hormone

appears to proceed through the 19-hydroxylated intermediates of 4-androste ione and testosterone, followed probably by elimination of the C_{19} side chain and aromatization of the A ring (Ryan, 1960). The most estrogens biologically are estradiol and estrone. These are interchangeable in the body although the equilibrium lies in the direction of estrone.

The main pathway for the biosynthesis of estrogens, as judged from the data available at the present time is depicted in Figure 1. The metabolic pathways for the estrogens are very complex, as is best shown by the great number of different metabolites isolated from pregnancy urine. Moreover, there are still many unknown estrogenic and perhaps nonestrogenic products of this metabolism, as has been shown from experiments with ^{14}C-labeled estrogens (Beer and Gallagher, 1955 a, b; Hathaway, 1964).

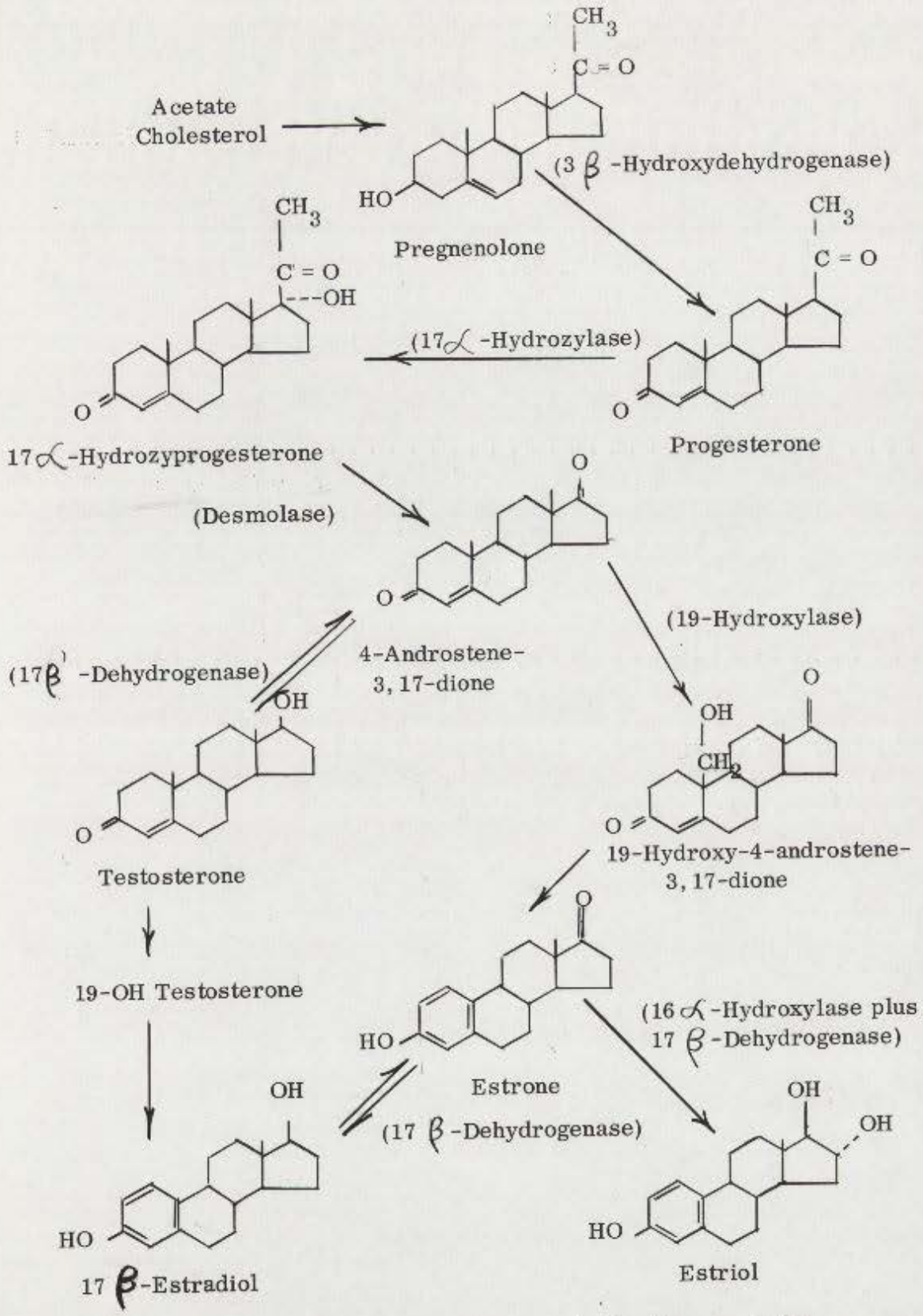

Figure 1. Probable pathways of estrogen biosynthesis (Turner, 1963).

The known metabolic transformations of the estrogen molecule are hydroxylation, oxidation, reduction, and methylation. Other possibilities are epimerization, epoxidation (Breuer and Knuppen, 1961), and the formation of p-quinols (Hecker, 1958). The conjugation with glucosiduronic acid and/or sulphate and the binding of estrogens to proteins also seems to be of great physiological importance in the metabolism of estrogens (Musa, 1965). Studies involving incubation of polycystic ovaries have suggested the possibility of two defects in estrogen synthesis, a deficiency in the 3β-hydroxysteroid dehydrogenase and a deficiency of the 19-hydroxylating system with a subsequent failure of aromatization. Either of these defects could increase the production of the androgen precursors and result in virilization which may be seen in the polycystic ovary syndrome (Segre and Lobosky, 1964).

It has been established beyond doubt that the liver plays the most important role in all metabolic processes relating to the estrogen molecule. On the other hand, there are certainly other tissues sharing in this activity, the intestine, the kidneys, the lungs, and the blood being the most important of these.

Estradiol, estrone, and estroil have been detected in the blood, but concentrations are very low (Svendsen et al., 1964a,b). The circulation estrogens are protein bound and 50 percent conjugated. Conjugation, primarily with glucuronide and secondarily with sulfate, occurs in the liver and estrogens are excreted in the urine in these forms. This process is illustrated in Figure 2.

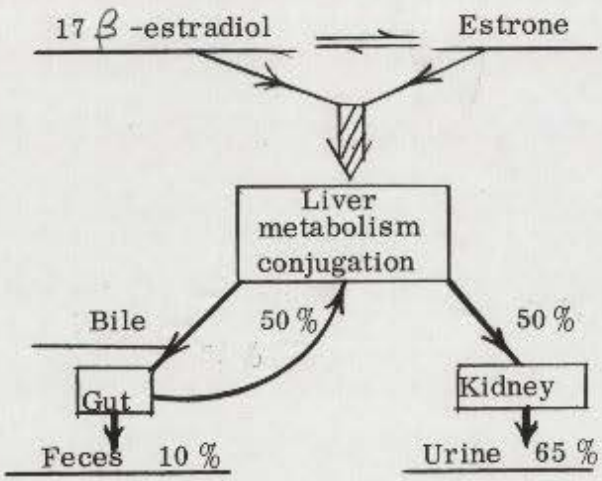

Figure 2. The metabolism and excretion of 17 β-estradiol and estrone (Brown, 1960)

Enterohepatic circulation occurs and, in contrast to man, the rat excretes estrogens primarily via the feces.

A difference in the behavior of the liver toward natural and synthetic estrogens has been observed. The inactivation was less rapid for the artificial estrogens and toxic effects of these were observed more often in experiments in vivo (Adlercreutz, 1963).

Further information concerning the metabolism of steriods by the body is gained by an attempted recovery of the metabolic products following administration of a known dose. It has been observed that only a small proportion of parentarally administered estrogens was excreted in the urine of human subjects in biologically active form. Injection of estradiol is followed by an increase in the excretion of estradiol, but more prominently of estrone and estriol. However, only 23 percent of the injected dose is excreted. On the other hand, injecting estriol permits an 80 percent recovery of the administered dose, and all as estriol. Figure 3 summarizes the present state of our knowledge concerning the intermediary metabolism of 17β-estradiol and estrone. According to recent papers by Fishman, et al. (1960), 17β-estradiol is rapidly oxidized to estrone which then serves as the precursor of estriol. One should perhaps not yet abandon the classical view that 17β-estradiol can also serve as an immediate precursor of estriol (Pangels and Breur, 1962).

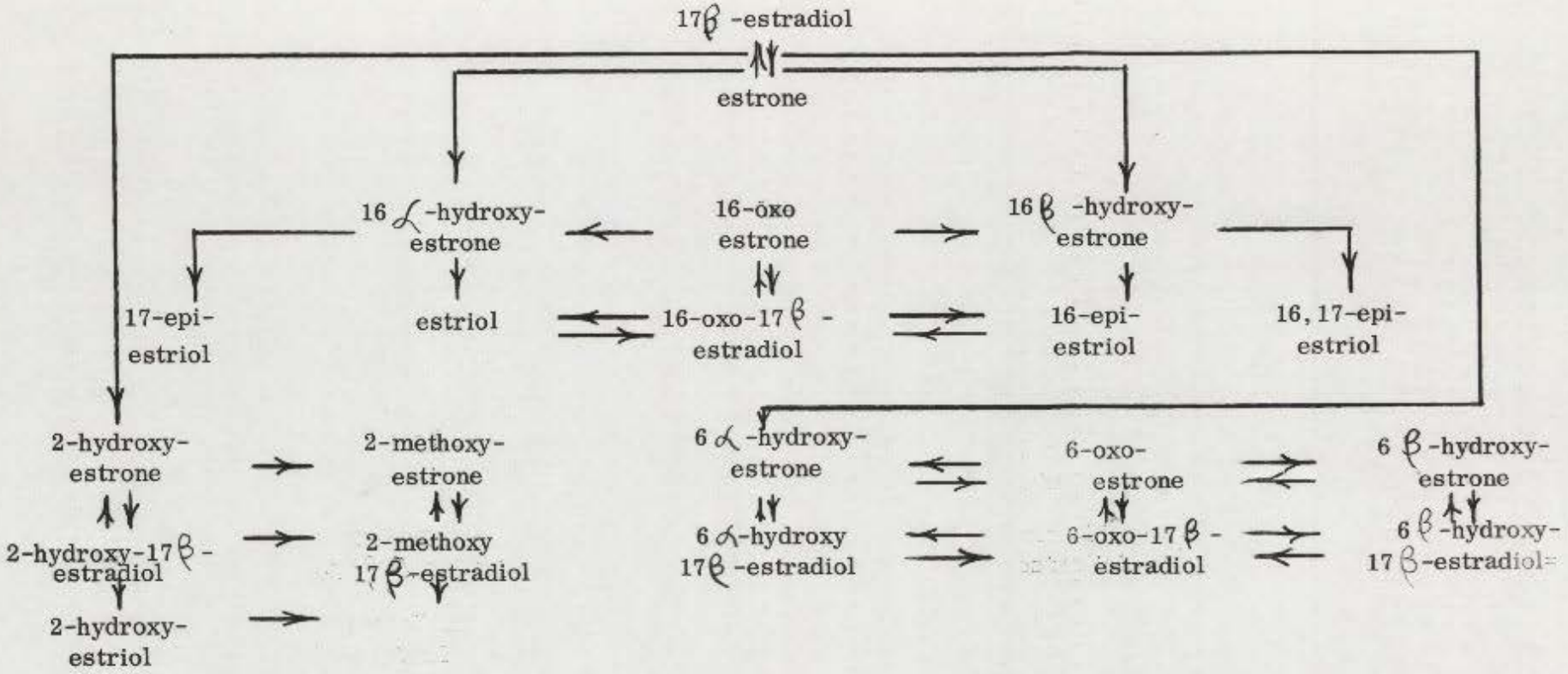

Figure 3. The metabolism of 17 β-estradiol and estrone in the human organism as evidenced by experiments in vivo and in vitro (Adlercreutz, 1963)

Some Biologic Effects of Estrogens

The estrogens act directly, or in cooperation with other hormones, to produce a great variety of effects on specific target organs and on the chemistry of the body as a whole. Estrogen secretion by the follicle is largely responsible for the development of the sex organs at puberty. It is also responsible for the development of the secondary sex characteristics: texture and distribution of hair, texture of the skin, character of the voice, and distribution of body fat. These effects, under control of follicular secretions, correspond to those induced in the male by testosterone. After ovariectomy the oviduct, uterus, vagina and mammary glands atrophy. They may be largely restored through adequate estrogen therapy. The menstrual cycle of the adult human female is interrupted in the absence of estrogenic hormones. The presence of circulating estrogen is responsible to a large extent for the rebuilding of the uterine endometrium after menstruation.

Proliferation of the uterine mucosa to the premenstrual stage can be accomplished by estradiol, estrone, and estriol, but potency of these compounds in causing this proliferation varies (White, 1964; Kleiner, 1966).

Excretion of Estrogens

Estrogens in non-pregnant women urine increase twice during the menstrual cycle. The first coincides with ovulation and the rise in basal body temperature, the second occurs during the luteal phase of the cycle (Turner,

1963; Loraine and Bell, 1963; Loraine and Bell, 1965). The estrogens of human urine are present as water-soluble conjugates of sulfuric or glucuronic acid of which the latter is most abundant. The relative amounts of the three estrogens excreted in the urine during the follicular and luteal phases of their menstrual cycle, the ratios of 17β-estradiol to estrone to estroil are 15:40:45 and 14:38:48, respectively (Brown, 1960).

During human pregnancy all of the estrogens increase rapidly in the urine. Just before parturition estrone and estradiol increase a hundred fold and estriol a thousand fold. The estrone and estradiol are usually excreted in a constant ratio of about 3:1. The urinary estrogens diminish rapidly after parturition and the loss of the placenta (Turner, 1963).

Surprisingly, an eight to ten day cycle of urinary estrone was excreted by normal men was recently reported by Exley (1966).

The absolute values of estrogen levels found vary depending upon the method used and the laboratory performing the procedure. The values reported by Segre et al (1964) using Brown's method (1955a, 1957), were 4 to 25 and 4 to 60 mcg per 24-hour urine for men and women, respectively.

Methods Used for the Determination of Estrogens

The methods described here are not suitable for use in a hospital routine laboratory; they were designed for use in specially equipped and staffed laboratories, and the precision and accuracy of which they are capable can only be attained under these conditions.

Many different methods are available for the determination of estrogens in urine and other body fluids and tissues. In recent surveys Bauld and Greenway (1957), Brown (1960), Salokangas et al. (1961), O'Donnell et al. (1961), Preedy (1962), Beling (1963), Wotiz and Chattoraj (1964), Hahnel (1965), Eechaute and Demeester (1965) and Scholler et al. (1966) have discussed at length all the important methods in this field. The following different types of methods have been used for the estimation of estrogens: biological, physical (involving gas chromatography), radio-chemical, biochemical, and chemical methods. However, only chemical methods will be considered here.

The most difficult problem in the chemical assay of urinary estrogens is the elimination of pigments and other extraneous materials which are fluorogenic or increase the background material in the color reaction. These non-specific fluorogens and chromogens are more abundant in extracts of urine than in extracts of blood or tissue. Urine of men and non-pregnant women, being of low estrogen titer, require more complete purification than urine of pregnant women or patients given estrogen.

There are different types of chemical methods available, the most important ones being the fluorimetric and colorimetric methods.

Fluorimetric methods

Fluorimetric estrogen assays were introduced by Jailer (1947, 1948), Bates and Cohen (1947, 1950) and Finkelstein et al. (1947). The two former methods use suphuric acid, the latter one phosphoric acid. The sensitivity of the fluorimetric estimations is very high and, according to Preedy and Aieken

(1961), 0.005 mcg of estrone can be measured with sufficient accuracy. This value was also reported by Ittrich (1958) for fluorimetric analyses of estrogens in biological material. Many problems are involved in the use of fluorimetry (Diczfalusy, 1953, Bauld and Greenway, 1957). The specificity of the reaction often leaves room for doubt in work with biological material. A number of investigators have shifted from fluorimetry to colorimetry. The methods of Ittrich (1958) and of Preedy and Aitken (1961) may be more specific than other previously published methods. The very time-consuming procedure is perhaps the greatest disadvantage of the method, as has been pointed out by the authors themselves.

Colorimetric methods

Of the known color reactions for estrogens the Kober (1931) reaction seems to be superior to the others as far as sensitivity and specificity are concerned. The original procedure was modified by Bauld (1954, 1956) and used by Brown and Bauld in the modified form for assaying urinary estrogens (Brown, 1955; Bauld, 1956). With these two methods it was possible to study the excretion of estrone, 17 β-estradiol and estriol in the urine in non-pregnant conditions also. Later, Brown et al. (1957) included an additional step in the original method, consisting of a boiling procedure in N NaOH. This additional procedure, first suggested by Bauld (1956) has been developed further by Givner et al. (1960a, b) and in its modified form includes the estimation of 2-methoxy-estrone, ring-D α-ketolic estrogens and 16-epiestriol, in addition to the three classical estrogens.

The method of Brown has been applied in modified form to human tissues (Diczfalusy and Magnusson 1958) and to human blood (Roy and Brown 1960).

A sensitive modification for the estimation of minute amounts of estrone in the urine was also developed from this method (Brown and Blair, 1960). It is obvious that the only real difference between a fluorimetric and colorimetric method is in the final measurement; both types of methods require extensive purification of the extracts before any reliable final estimations can be achieved.

The sensitivity of fluorimetric methods is better than that of colorimetric ones. But the sensitivity of the colorimetry is greatly enhanced if the final fractions are pure. For instance, in a modification of Brown's (1955) method for tissue estrogens, Diczfalusy and Magnusson (1958) reported an approximate sensitivity for blood determinations of 0.06 mcg estrogens per sample (2.0 mcg/kg blood, samples of 30 g), when they used 50 mm cells for measurement of the color. Therefore the sensitivity of the colorimetric determinations seemed to be satisfactory even for urine obtained from non-pregnant women and normal men. It should be also pointed out that in the opinion of most investigators fluorimetry in its present stage of development is less specific than colorimetry based on the well-developed Kober reaction.

Of the known chemical methods, only one (Brown, 1955; Brown et al., 1957) has been widely used and also modified for different purposes. More important, the method has been thoroughly investigated for reliability (Brown, 1955; Biczfalusy and Westman, 1956; Breuer et al., 1957; Brown et al., 1957; Bulbrook et al., 1957, 1958, 1960; Young et al., 1957; Gallagher et al., 1958; Diczfalusy et al., 1959; Brown and Blair, 1960; Adlercreutz et al., 1960; Fishmann and Brown, 1962; Siegel and Dorfman, 1963; Nakamura and Kushinsky, 1963; Brown and Blair, 1965). Both

acid and enzyme hydrolysis of the conjugated estrogens can be used. The method also includes the separation of estrone, 17 β-estradiol, and estriol. Considerable purification of the fractions is obtained as a result of a phase-change purification step involving methylation of the estrogens followed by chromatography of the methyl ethers on partially deactivated alumina.

It was therefore concluded that the method of Brown had many advantages, and it seemed justifiable to adopt it as a basic method of developing an estrogen method applicable to urine of non-pregnant women and normal men.

Influences of Sex Hormones on Serum Lipids and Lipoproteins

It is now recognized that sex and gonadal steroids may affect the distribution of plasma lipoproteins. Studies conducted largely in human subjects have indicated that plasma concentration of alpha-lipoprotein or cholesterol is higher in young women than in men of comparable age. This difference is not apparent after the menopause. Bilaterally oophorectomized women shown a higher incidence of coronary artery disease than normal women of corresponding ages. It was concluded that normal endogenous estrogen may protect humen being from coronary disease (Scanu, 1965). However, observations by Ritterband et al. (1963) have failed to confirm this.

Administration of estrogens to men and to postmenopausal women results in a decrease in the level of plasma cholesterol, and reduction in cholesterol to phospholipid ratios, and a relative increase in the high-density (alpha-) lipoproteins. Although there is no doubt about the ability of extraneous estrogen hormones to influence plasma lipid patterns in man (Furman et al., 1958; Moses, 1963; Musa

et al., (1965), the therapeutic value of long-term estrogen administration for coronary atherosclerosis is still controversial (Marmorston et al., 1960; Stamler et al., 1959, 1960; Hess, 1964). Androgens, on the other hand, promoted and increased plasma level of the beta-lipoprotein (Scanu, 1965).

Block et al. (1951) reported that, following a standard fat meal, men develop a significantly greater plasma lactescence than women of the same age group. They suggested that this may be of importance in the development of atherosclerosis.

The mechanism by which gonadal hormones affect lipid and lipoprotein metabolism is not known. Surprisingly, the literature on the subject is only in its earliest stages although a rapid increase is to be expected.

Effect of Meal Frequency on Body Metabolism

The periodic intake of large amounts of food with long time intervals between meals in laboratory rats leads to a number of metabolic changes (Tepperman et al., 1958; Cohn et al., 1960) among which enhanced lipogenesis is the most marked (Fabry et al., 1962; Hollifield et al., 1962; Cohn, 1963; Stevenson et al., 1964; Tepperman et al., 1964). Apparently the time distribution of food intake plays a role also in man. From observations in populations (Fabry et al., 1964, 1966) and volunteer (Cohn, 1963; Gwinup et al., 1963a,b) groups, it appears that the intake of a small number of large meals tends to promote the deposition of fat and overweight (Febry et al., 1964, 1966), hypercholesterolemia (Fabry et al., 1964; Coh, 1964; Gwinup et al., 1963a) and a reduced glucose tolerance (Fabry et al., 1964; Gwinup et al., 1963b).

METHOD AND PROCEDURE

Experimental Design

Nine healthy university students were selected to cooperate in this experiment; five women and four men. The University Health Service[1] gave all the subjects a physical examination before starting the experiment and found them to be in excellent condition. Blood pressure and pulse rate before and after exercise were checked at two week intervals and found to be normal. All subjects were maintained on self-chosen diets while eating two meals a day with no lunch or three meals a day. In order to minimize the effect of environmental factors such as weather, examination in classwork, and other factors, half the men and women were placed on each diet for period one as shown in the experimental design in Table 3. Eating patterns were reversed for each subject in the second period. Subjects were asked to maintain constant weight on their self-selected diets and to keep a record of all food consumed on the day before urine was collected. The content of calories, protein, fat, carbohydrate, saturated and unsaturated fatty acids (oleic, and linoleic), and cholesterol was then calculated from the food tables in the U.S.D.A. Handbook number 8 (1963), and in Church and Church (1963).

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[1]Dr. G. W. Neece gave the physical examinations.

Table 3. Experimental design

Subject	Two meals with no lunch		Three meals with lunch	
Period[a]	Male	Female	Male	Female
1	B. W.	T. D.	H. A.	B. N.
	D. P.	J. A.	B. E.	S. R.
		M. J.		
2	H. A.	B. N.	B. W.	T. D.
	B. E.	S. R.	D. P.	J. A.
				M. J.

[a]Period 1 refers to the first 31 days.
Period 2 refers to the second 31 days.

Subjects came to the Food and Nutrition Laboratory twice a week in the morning and finger-tip blood was taken for cholesterol determination. After weighing, the subjects were then served breakfast. The age, weight and height of subjects are presented in Table 4.

Two 24-hour urine samples were collected each week. Additional samples also were collected on specified days between menstrual periods for the female subjects, that is on days 9, 11, 13, 15 and 17 after the first day of the menstrual period. The additional urine samples were taken especially for estrogen analysis. The specific days, 9 to 17 were chosen to observe the effect of ovulation which occurs usually between the thirteenth and seventeenth day of the menstrual period (Best and Taylor, 1961).

Table 4. Age, height, weight of subjects

Name	Sex	Age	Height		Weight(mean)
			feet	inches	pounds
B. E.	M	21	5	9	204
B. W.	M	22	6	0	165
D. P.	M	20	6	1	167
H. A.	M	22	5	10	150
B. N.	F	21	5	6.5	104
J. A.	F	21	5	3	140
M. J.	F	21	5	5	146
S. R.	F	20	5	4.5	133
T. D.	F	21	5	6.5	136

Collection and Storage of Urine Specimens

Collection

Instructions for collecting 24-hour urine specimens were given to each subject, that is, to discard the first morning specimen on the day of collection and to collect all urine voided up to and including the first morning specimen of the next day. Container of adequate size (two one-quart bottles) labeled with name and date were given to the subjects for the 24-hour collection.

Creatinine values were used to check the accuracy to the completeness of the total urine for the 24-hour collection since these values have been shown to be nearly constant for a given individual. Folin's method as outlined by Hawk, et al. (1954) was used for the analysis. Results were all within the range of creatinine values for normal subjects.

Storage

Although all of the urinary steroids are relatively stable compounds, a preservative was added to each bottle in which urine was to be collected. A mixture of penicillin and streptomycin (1,000 units of penicillin and 5 mg of streptomycin for each 24-hour collection) was chosen as the preservative to prevent both bacterial contamination and deterioration upon frozen storage. All the specimens were than stored at -10° C.

Determination of Urinary Estrone, 17 β-Estradiol, and Estriol

The urinary estrogens were determined using the method developed by Brown (1955, 1957) with some modifications (Appendix C).

RESULTS AND DISCUSSION

Urinary Estrogen Levels

Total estrogens

The data on the excretion of urinary estrogen are presented in Tables 5, 8 and 9. Values for the men did not vary as much from day to day as the excretion of women. The overall means in the study for total urinary estrogen per day were found to be 15.6 and 29.9 mcg for men and women respectively. The mean value for women was approximately double that found for men. Excretion values for women ranged from 9.3 to 80.4 and for men, 3.3 to 27.2 mcg. These results were within the ranges of those reported by several investigators (Table 6).

Individual estrogens

Other studies have indicated that the measurement of the three estrogens excreted by men and women usually have followed a definite pattern with 17β-estradiol (estradiol or E_2) being present in the least amounts and estriol (E_3) in greatest amounts. Findings in this study followed this pattern. The mean values of estrone (E_1), estradiol, and estriol for the men during the entire study were as follows: 5.3, 2.3, and 7.6 mcg, respectively. These results were within the acceptable range for normal adults given by other investigators (Table 6). The mean excretion values of the three estrogens for the entire menstrual cycle

Table 5. Mean values of urinary estrone (E_1), 17 β-estradiol (E_2), estriol (E_3) and total estrogen (E_t) of subjects consuming two and three meals per day

Sex	Day of study	Estrone		17 -estradiol		Estriol		Total estrogen		Mean of E_t
	Meal:	2	3	2	3	2	3	2	3	2 and 3
		mcg per 24 hour urine								
M	3-4	5.08	6.21	1.09	2.76	12.43	4.63	18.60	13.60	16.10
	10-11	6.40	6.06	2.39	2.77	8.62	11.66	17.41	20.49	18.95
	17-18	8.20	3.30	1.87	3.78	8.02	8.53	18.09	15.61	16.85
	24-25	5.24	5.78	2.60	2.64	5.92	7.92	13.76	16.34	15.05
	30-31	3.63	3.43	0.50	2.00	8.75	3.46	12.87	8.89	10.88
	Mean	5.71	4.96	1.69	2.79	8.75	7.24	16.14	14.99	15.57
F	3-4	6.16	6.55	3.67	3.95	11.60	16.45	21.43	26.95	24.19
	10-11	7.09	7.07	3.43	4.67	24.34	20.64	34.86	32.38	33.62
	17-18	4.63	6.48	3.10	5.32	12.66	23.65	20.39	35.45	27.92
	24-25	10.40	6.19	3.31	2.68	18.01	16.92	31.72	25.79	28.76
	30-31	6.08	6.38	3.32	3.67	22.38	19.32	31.78	29.37	30.58
	Mean	6.87	6.53	3.37	4.06	17.80	19.40	28.04	29.98	29.01

Table 6. Urinary estrogens values of normal humans as reported in the literature

No. of Subjects	Age	Estrone	17 β-estradiol	Estriol	Total estrogen	Investigator	Mark
		Range (Mean) mcg/24- hour urine					
Female							
16	18-41	4-7 (5)	0-3 (2)	0-15 (6)	4-30 (14)	Brown	Menses, week 1
16	18-41	11-31(20)	4-14(9)	15-34(27)	35-100(57)	1955	Menses, Ovulation
16	18-41	10-23(14)	4-10(7)	8-72(22)	5-82 (33)		Menses, week 3-4
			(7.8)	(3.8)	(27.0)	Bauld 1956	Day 9 of cycle
10	18-37	1.2-11.0(4.5)	0-7.7 (1.7)	2.1-12.4(5.4)		Loraine	menstration
10	18-37	1.4-18.0(5.7)	0-13.0(2.9)	1.5-17.6(6.8)		and	follicular phase
10	18-37	2.5-24.6(12.6)	1.0-23(5.2)	2.7-73.8(17.8)		Bell	ovulatory phase
10	18-37	2.2-23.6(9.4)	0-13.2(3.6)	4.0-58.7(15.0)		1963	luteal phase
Male					4-60	Segre et al. 1964	
		3-21				Engel 1950	
30	25-55	1-11(6)	0-7 (2)	1-12(6)	9-25 (14)	Cameron 1957	
					4-25	Segre et al. 1964	

for the five women were 6.9, 3.7, and 18.5 mcg; means for the first week of the cycle were 4.5, 2.8, and 9.6; for the approximate time of ovulation (days 11 and 13), 9.2, 3.8, and 24.4, and for the third and fourth weeks, 6.6, 2.7, and 22.2 mcg per 24 hours of urine. These results were similar to those reported by Brown (1955) whose mean data are shown in Table 6.

Effect of menstrual cycle

The true significance of changes in excretion values for women can only be observed when day of menstrual cycle is considered.

The mean total estrogen excreted by the 5 women, from the beginning of the menses to the approximate time of ovulation (days 11 and 13) showed an increase of 23.7 mcg or 63 percent (Table 7). A second and lower peak occurred during the luteal phase of second half of the cycle, which was 53 percent (16 mcg) above the value at the start of the cycle. This was a decrease of 10 percent from the highest peak. Total estrogen values continued to decrease during the last few days of the cycle. Because of the large variation among subjects, it would be highly desirable to obtain daily estrogen data on more subjects to observe the significance of the effect of the menstrual cycle on this metabolite.

Individual data of the excretion of total estrogens in the urine of women on specified dates of the menstrual cycle are presented in Figure 4. The excretion of estrogen rose in the expected fashion during the ovulation (on or about day 13 of the cycle), except for one subject (TD) whose cycle was

Table 7. Mean values of urinary estrone, estradiol, estriol, and total estrogen (E_t) of female subjects consuming two and three meals per day during menstrual cycle

No. of subjects on 2 or 3 meals		Day of menstrual cycle	Estrone		Estradiol		Estriol		Total estrogen		Mean of E_t
2	3		2	3	2	3	2	3	2	3	2 and 3 (meal)
			mcg per 24 hour urine								
3	1	1-3	5.08	5.16	2.65	0.00	4.97	9.57	14.70	14.73	14.71
1	5	4-6	3.28	4.08	1.01	4.20	5.15	13.33	9.44	21.61	15.53
5	4	9	6.08	7.11	3.65	3.12	15.02	12.69	24.75	22.92	23.84
4	5	11	7.93	8.20	5.47	3.73	27.60	25.03	41.00	36.96	38.98
5	4	13	10.65	9.70	1.77	4.74	25.00	19.84	37.42	34.28	35.85
5	5	15	8.36	7.12	5.05	5.25	14.65	21.19	28.06	33.56	30.81
4	5	17	5.36	4.71	2.92	4.79	13.11	24.84	21.39	34.34	27.87
1	2	19-20	8.98	8.37	4.55	3.53	24.14	13.49	37.67	25.39	31.53
4	5	23-25	6.65	6.93	2.83	2.55	21.23	23.02	30.71	32.50	31.61
3	2	26-28	6.75	8.21	3.46	5.66	12.50	21.51	22.71	26.38	24.55

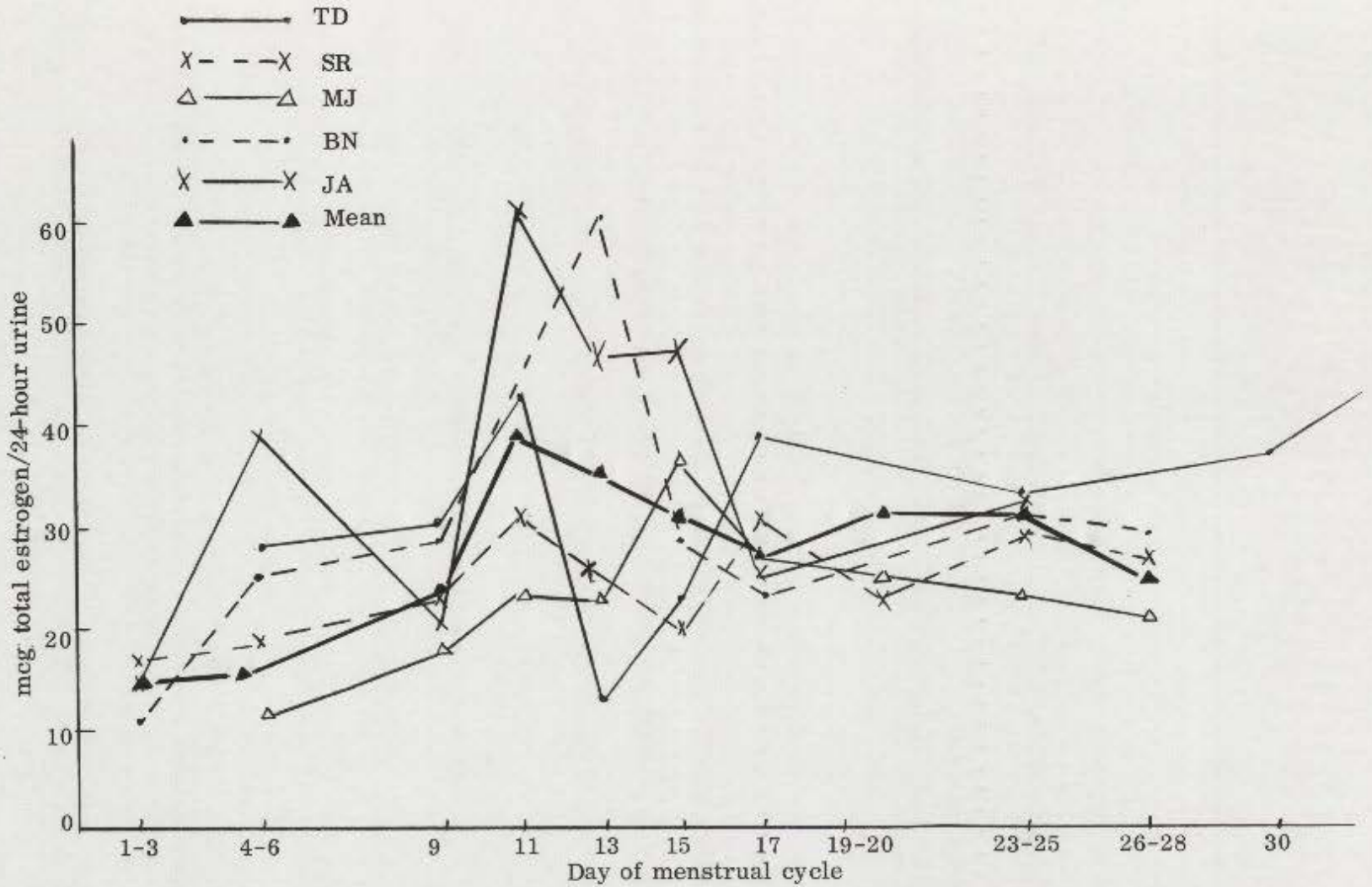

Figure 4. The effect of day of menstrual cycle on mean excretion values of total estrogen of 5 subjects.

much longer (40 days) than that of any other subject. Her value precipitously fell on day 13. Since her data did not follow the pattern of the other subjects and no definite peak was observed on the days of analyses, the time of ovulation may have been at a time when no sample was collected. Bell and Loraine (1965) have reported that the day on which ovulation occurs has been shown to be variable ranging from 4 days before to 6 days after midcycle.

Effect of two Meals Versus Three Meals Per Day

The range of values for total estrogen excreted was 12.9 to 18.6 mcg for the male subjects on two meals a day and 8.9 to 20.5 mcg for those on the three meals (Table 5 and Figure 5). Estrogen values were highest during the first and second week when subjects were on two and three meals per day, respectively, and lowest during the last week of the study for both groups. Means for the periods were almost the same. The fact that two changes on the two regimes of eating did not follow the same pattern and that the changes were small indicated that the number of meals eaten per day had not influenced excretion of total estrogen in males in this study. Also, the wide variation shown by one subject of each group probably influenced the means (DP on three meals and HA on two meals).

The changes in excretion of total estrogen on two or three meals per day showed the same pattern as was observed for changes by day of the menstrual cycle (Table 7 and Figure 6). However, the means for the entire period were almost identical. Hence, frequency of meals did not influence the excretion of estrogens

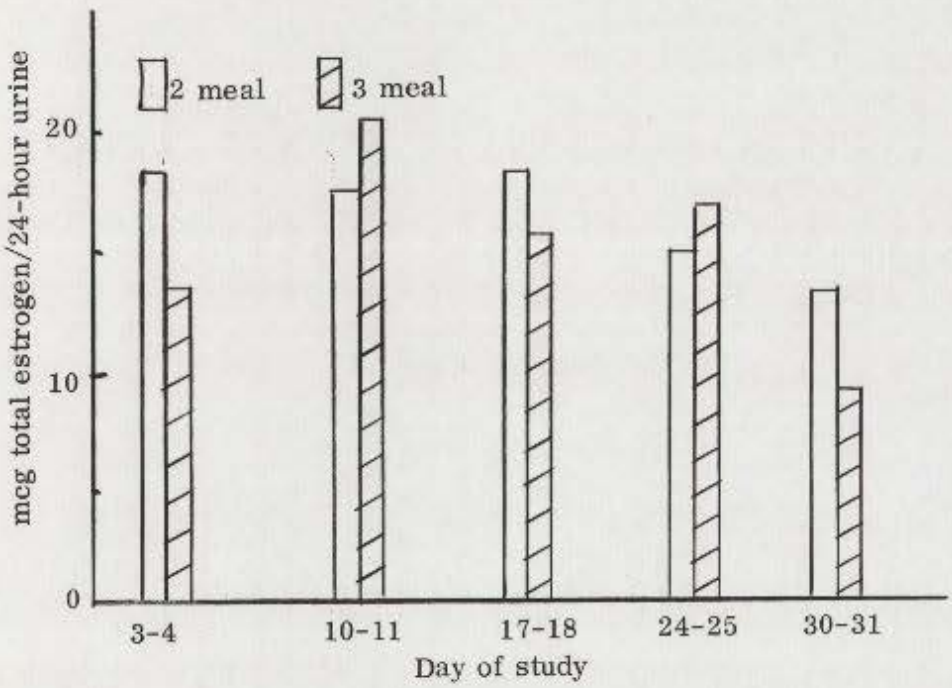

Figure 5. The effect of number of meals on mean excretion values of urinary total estrogen for male subjects.

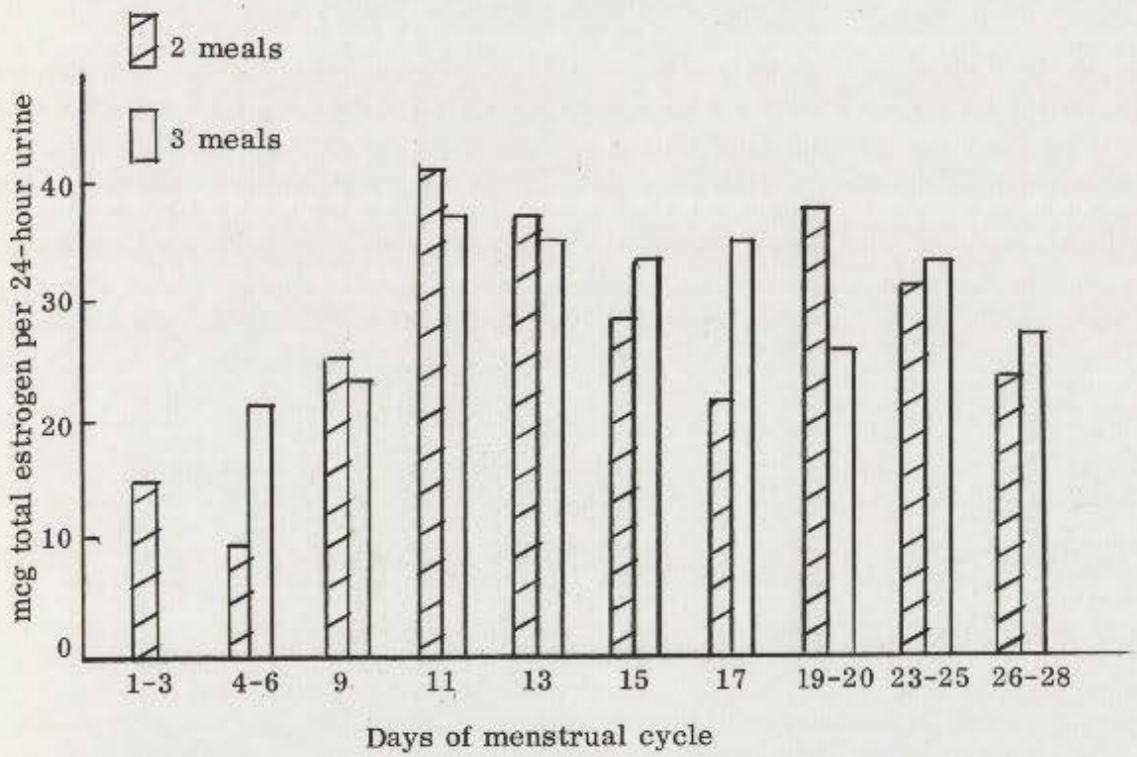

Figure 6. The effect of number of meals on mean excretion value of urinary total estrogen for female subjects

in the women of this study. More subjects would be needed to determine whether the number of meals really influence estrogen excretion.

The variations among individual excretion, values of total estrogen for men and women by number of meals and day of test period or day of menstrual period are shown in Figures 9 and 10.

The excretion of individual estrogens were not influenced by the meal frequency in this study (Table 5).

Effect of dietary intake

During the experimental periods, all of the subjects were asked to record exactly the amount of food that they had consumed the day before urine samples were to be taken. In Table 10 the daily intake is presented as calculated of calories, protein, carbohydrate, fat, fatty acids (unsaturated and saturated), and cholesterol. The male subjects were consuming almost the same calories when they had two or three meals a day (1.4 percent less). In case of female subjects, caloric intake was 6.5 percent higher when they had three meals a day. However, this difference in the caloric intake is not great enough to effect this study.

Body weight of subjects varied only 0 to 3 pounds for this study. Mean weights were within 1 pound for each period. Hence, all of the subjects had maintained constant weight regardless of the number of meals per day. Normally a 6.5 percent increase in calories would result in a gain in weight. At least two possibilities might explain the fact that the women did not gain on three meals

per day. No record was kept of the exercise of the subject, hence, the women could have been more active during the three meal period than when on two meals a day. The second possibility would be the effect of the meal pattern. This does not rule out other possibilities.

The total caloric intakes of the male subjects compared favorably with the NRC Recommended Daily Dietery Allowance for men aged 25, while the women ranked a little lower than the recommended allowance (2107 vs 2300). Proteins, carbohydrates, and fats were used in a well balanced proportion to each other from the nutritional view point. The mean percentage of calories consumed by all subjects as protein was 13; fat, 45; and carbohydrate, 42. The women were consuming 2 to 4 percent less protein and fats and 5 percent more carbohydrates than the men. These values were almost the same for two or three meals. A medium level of fat intake is considered to include approximately 35 percent of the calories as fat, these subjects were consuming 44 to 46 percent which might be designated as a high intake of fat.

There was no marked difference of saturated, unsaturated fatty acids and cholesterol intake by both male and female subjects between two and three meals a day. The ratio of polyunsaturated to saturated fatty acids was 0.23 which indicated a high percentage of saturated fat. The intake of cholesterol, saturated, and polyunsaturated fatty acids were slightly higher than the amount recommended by Stamler et al. (1963).

Relationships Among Urinary Estrogens, Pregnanediol, Neutral 17-Ketosteroids and Serum Cholesterol

Other biochemical indices, serum cholesterol, the urinary metabolites for progesterone (pregnanediol) and androgens (neutral 17-ketosteroids) have been reported previously for the subjects in this study by Chiang, 1966; and Yu, 1964). A comparison of the patterns shown for the excretion of estrogens and these other biochemical indices are presented in Figures 7, 8, and Table 11.

The cholesterol pattern for men showed higher values at the middle of the study and were almost the opposite of that shown by the neutral 17-ketosteroids. Estrogens and pregnanediol values were similar throughout the study.

When the serum cholesterol values of the women increased during the menstrual cycle, the pregnanediol levels tended to decrease. The pattern shown by total urinary estrogen was similar to that for cholesterol. However, the peaks for the two metabolites did not occur on the same day of the menstrual cycle.

The limited number of subjects make it impossible to draw definite conclusions from these data.

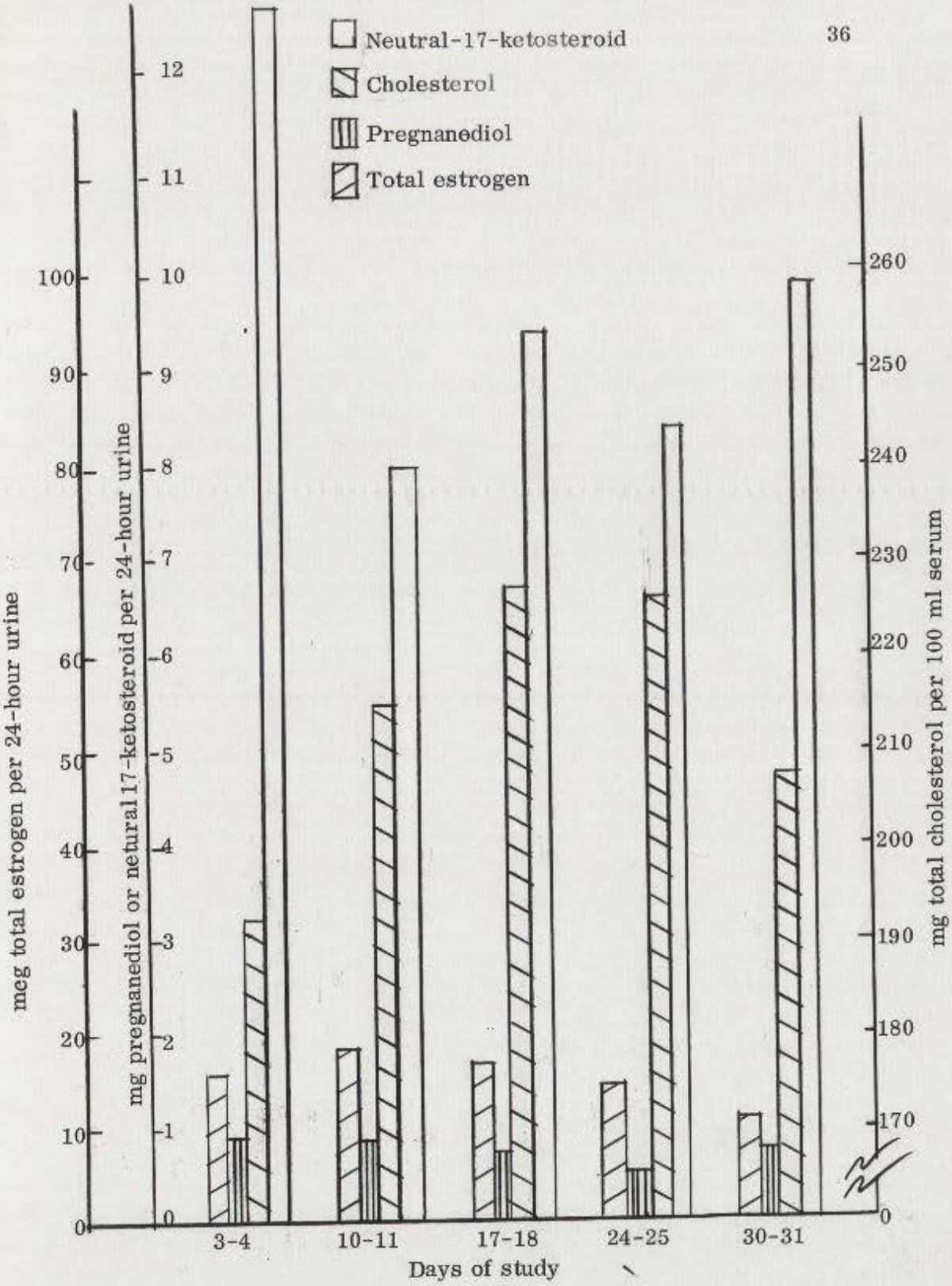

Figure 7. Serum cholesterol and urinary neutral 17-ketosteroid, pregnanediol and total estrogen values of study for male subjects

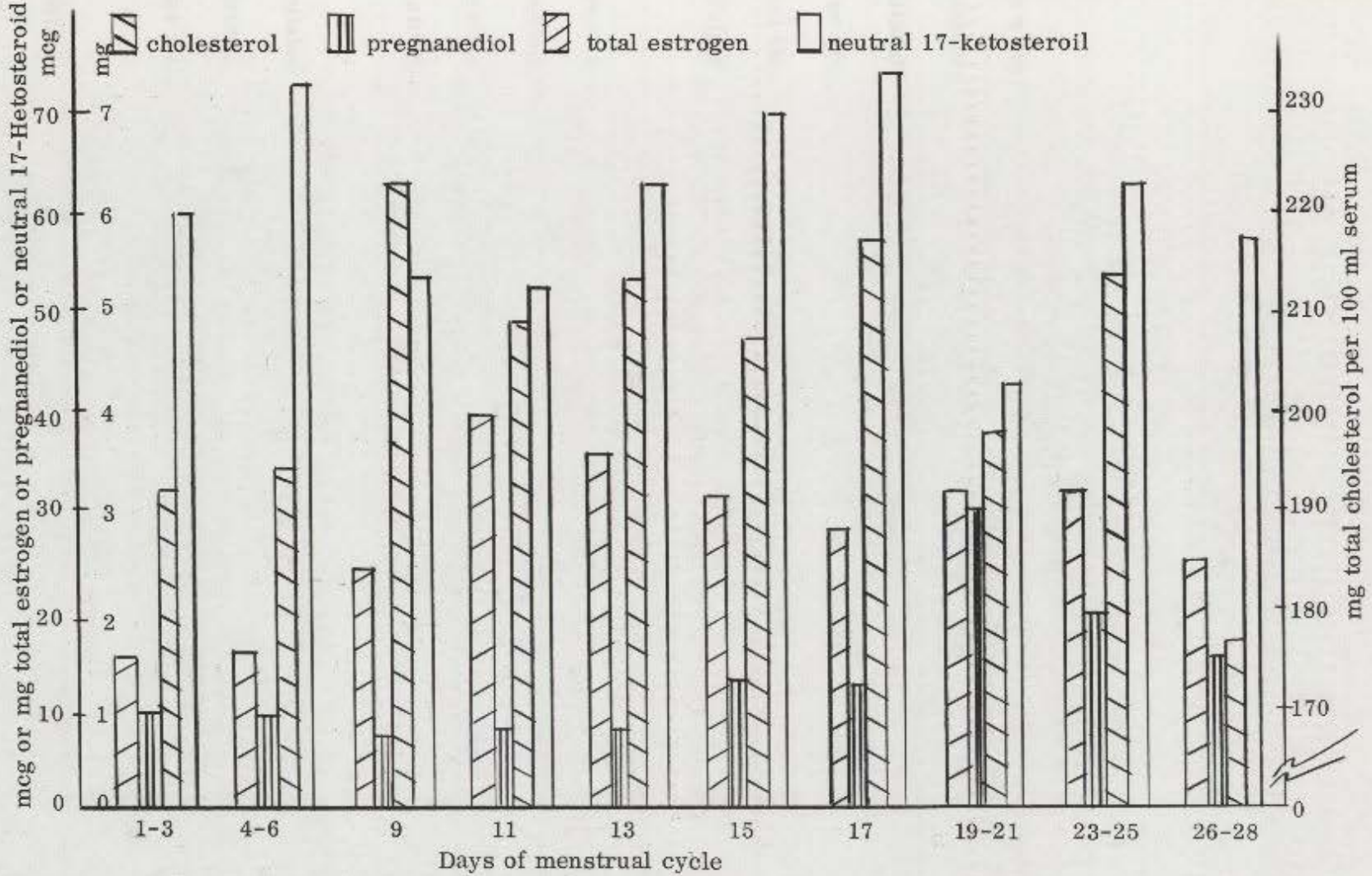

Figure 8. Mean serum cholesterol, and urinary neutral 17-ketosteroid, pregnanediol and total estrogen values by days of menstrual cycle for female subjects

SUMMARY AND CONCLUSIONS

Summary

Nine university students (four men and five women) served as subjects in a study of the urinary excretion of estrogens of healthy young adults (university students) consuming self-selected diets living under home conditions. Two 31-day test periods were used. During the first test period, one group of subjects had two meals a day with no lunch and the other group had three meals a day; their food pattern was reversed for the second period. Weight was kept constant.

Urine specimens were collected twice a week, and additional samples were also collected on specified days during the menstrual cycle for female subjects. Chemical analysis of urinary estrogens were made. Brown's method (1955, 1957) was used for determination of urinary estrone, 17 β - estradiol (estradiol), and estriol.

Dietary records were kept each day before urine was collected. Calculated dietary data showed that the percentages of calories consumed as protein was 13; fat, 45; and carbohydrate, 42. Total calories were similar for either the two-day or three-day regime.

Basic data on excretion of total estrogen and individual estrogens were obtained on healthy young adults living under home conditions. Findings included the following:

1. The urinary estrogen values of the women were much higher than that of the men. The excretion values of the three individual estrogens, by both men and women, showed that estradiol was usually present in least amounts and estriol in greatest amount. The mean values of estrone, estradiol, and estriol and total estrogen for the entire study were as follows: 5.3, 2.3, 7.6 and 15.4 mcg per day for men; 6.9, 3.7, 18.5 and 33.5 mcg per day for women.

2. The menstrual cycle did effect the excretion of estrogen which showed the lowest values (15.1 mcg per day) during the first week, and then rose to a peak (37.4 mcg per day) which occurred on or about the middle of the cycle. The estrogen excretion then fell and rose (31.5 mcg per day) again between the third and fourth week of the cycle. This peak was usually lower than the first one.

3. The number of meals per day did not appear to have any effect on the excretion of estrogens in human urine in these subjects.

According to the data obtained in this study, the variation from subject to subject was great. Further study on a larger number of subjects would be desirable to contribute more basic information on estrogen excretion by young adults living under home conditions. Daily analysis during the menstrual cycle would also be desirable.

Conclusions and Comments

The results herein reported are based upon the following assumptions: the subjects kept a complete record of the food consumed for the specified days, ate the number of meals exactly as they should, and collected specimens as outlined in the experimental design.

Basic information on estrogen excretion has been obtained on young adults living under home conditions who were consuming self-selected diets. The findings in this study showed that the exretion of estrogen appeared to be affected by ovulation and the luteal phase of the menstrual cycle. Values were lowest during the first week of the menstrual cycle, and then rose to a peak, which occurred on or about the middle of the cycle. Second but lesser increase in estrogen excretion occurred between the third and fourth week. As specimens were only kept at two day intervals the true "ovulatory peak" and "luteal maximum" may have missed for one or more subjects.

No definite relationship was found between the number of meals, with and without lunch and urinary estrogen excretion. As only a small number of subjects were studied the possibility can not be ruled out that with larger numbers of subjects and controlled diets, greater differences might have been observed and the effect of individual differences on means would not have been so pronounced. It might be valuable if the number of meals were increased to five or six.

However, the results in this study might be explained by enviromental factors. Exercise and emotional stress would be two such factors.

The variation among individual subjects of the same sex was great. Thus, a larger number of subjects is needed to give sound basic data for these biochemical indices under the conditions of this study. Since only nine subjects were observed, one high or one low value could skew the data. A larger population is recommended.

As a part of the larger problem under investigation, relationships between lipid metabolism and steriod hormone excretion, it would be desirable to determine the ratios of alpha-lipoprotein and beta-lipoprotein as well as fatty acids content of serum in addition to the cholesterol content. This is also recommended.

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APPENDIXES

Appendix A

Tables

Table 8. Urinary estrone, estradiol, estriol, and total estrogen values for individual male subjects consuming two or three meals per day.

Subjects	Meals	Day of study 3-4	10-11	17-18	24-25	30-31	Mean
		Estrone mcg/24 hr					
BE	2	5.73	7.45	4.33	2.35	0.00	3.97
	3	13.39	7.95	3.40	9.88	5.27	7.98
BW	2	7.12	0.00	13.04	2.19	12.67	7.00
	3	1.53	0.00	2.45	5.10	0.00	1.81
DP	2	3.22	12.83	4.58	2.53	1.79	5.01
	3	4.48	12.37	0.00	1.05	6.32	5.20
HA	2	4.15	5.30	10.85	13.90	0.00	6.84
	3	5.42	3.93	7.35	7.08	2.13	5.18
Mean	2	5.08	6.40	8.20	5.24	3.62	5.71
	3	6.21	6.06	3.30	5.78	3.43	4.96
		Estradiol mcg/24-hr					
BE	2	0.00	0.00	0.00	1.50	0.00	0.30
	3	3.36	0.00	0.00	2.09	1.70	1.43
BW	2	0.00	3.41	2.18	6.27	0.00	2.37
	3	3.82	0.00	4.89	0.00	0.00	1.74
DP	2	2.14	3.41	4.35	2.62	2.00	2.90
	3	1.00	5.86	3.35	2.09	5.28	3.91
HA	2	2.22	2.75	0.96	0.00	0.00	1.18
	3	2.85	5.21	6.89	6.39	1.02	4.47
Mean	2	1.09	2.39	1.87	2.60	0.50	1.69
	3	2.76	2.77	3.78	2.64	2.00	2.79

Table 8. Continued.

Subjects	Meals	Day of study 3-4	10-11	17-18	24-25	30-31	Mean
		Estriol mcg/24-hr					
BE	2	4.69	2.43	4.96	6.60	6.95	5.12
	3	3.53	10.37	8.55	12.60	3.12	7.63
BW	2	18.23	0.00	5.22	2.01	6.27	6.34
	3	5.54	11.30	2.49	6.00	4.17	5.90
DP	2	10.15	12.83	6.87	4.99	18.46	9.87
	3	6.89	16.16	15.58	8.57	1.76	13.48
HA	2	2.57	8.80	7.51	4.49	4.78	5.63
	3	16.63	19.21	15.03	10.09	3.33	12.86
Mean	2	12.43	8.62	8.02	5.92	8.75	8.75
	3	4.63	11.66	8.53	7.92	3.46	7.24
		Total estrogen mcg/24-hr					
BE	2	10.42	9.88	9.28	10.45	6.95	9.39
	3	20.27	18.32	11.95	24.61	10.09	17.04
BW	2	25.12	3.41	20.43	10.46	18.94	15.71
	3	10.89	11.30	9.83	11.09	4.17	9.45
DP	2	15.61	25.15	15.79	10.12	22.25	17.78
	3	12.37	34.38	18.93	11.70	13.35	22.59
HA	2	22.99	27.25	27.83	23.99	3.33	20.88
	3	10.83	17.94	21.74	17.95	7.92	15.28
Mean	2	18.60	17.41	18.09	13.76	12.87	16.14
	3	13.00	20.49	15.61	16.34	8.89	14.99
Overall mean		16.10	18.95	16.85	15.05	10.88	15.57

Table 9. Urinary estrone, estradiol, estriol, and total estrogen values for female subjects by meals and by menstrual cycle.

Subject	Meals	Days of menstraul cycle 1-3	4-6	9	11	13	15	17	19-20	23-25	26-28	30	Mean
						Estrone mcg/24-hr							
BN	2	4.10	----	8.96	7.57	16.41	8.33	3.56	----	5.42	7.18	----	7.69
	3	----	6.20	7.40	11.01	9.26	7.64	1.01	----	7.55	10.94	----	7.63
JA	2	4.47	----	6.63	10.58	13.72	9.42	7.07	----	10.15	----	----	8.86
	3	5.16	6.39	7.31	10.05	13.42	12.45	7.95	----	7.70	----	----	8.80
MJ	2	----	3.28	5.51	6.39	11.39	13.33	----	8.98	----	----	----	8.28
	3	----	5.72	7.48	8.56	7.95	5.27	7.75	11.23	6.45	6.95	----	7.48
SR	2	7.27	----	5.48	7.18	6.86	7.00	7.35	----	9.12	4.74	----	6.87
	3	----	3.63	----	7.13	8.15	5.77	6.75	7.14	6.75	----	----	6.47
TD[a]	2	----	----	3.80	----	4.85	3.73	3.47	----	1.89	----	15.91	3.49
	3	----	0.78	6.24	4.32	-----	4.45	2.12	----	5.42	----	6.75	3.87
Mean	2	5.28	2.03	6.08	7.93	10.65	8.36	5.36	8.98	6.65	6.75	----	6.81
	3	5.16	4.54	7.11	7.85	9.70	7.12	5.12	9.17	6.77	8.95	----	7.15
						Estradiol mcg/24-hr							
BN	2	0.00	----	7.22	5.47	4.14	2.33	1.12	----	1.81	3.59	----	3.21
	3	----	4.96	5.93	7.34	6.67	2.99	1.94	----	3.06	9.43	----	5.29
JA	2	3.25	----	3.68	5.99	1.08	12.66	4.34	----	1.37	----	----	4.62
	3	0.00	4.55	1.22	3.35	5.15	8.92	2.21	----	1.68	----	----	3.39
MJ	2	----	1.01	2.76	4.55	1.24	5.33	----	4.55	----	4.30	----	3.39
	3	----	3.12	2.14	2.51	2.01	7.38	4.90	4.15	2.78	4.17	----	3.68
SR	2	4.09	----	1.43	5.87	2.38	2.11	6.23	----	5.32	2.50	----	3.74
	3	----	5.45	----	2.50	5.12	3.68	1.69	4.76	3.37	----	----	3.80

Table 9. Continued.

Subject	Meal	Day of menstrual cycle 1-3	4-6	9	11	13	15	17	19-20	23-25	26-28	30	Mean
						Estradiol mcg/24-hr							
TD[a]	2	-----	-----	3.16	-----	0.00	2.80	0.00	-----	2.80	-----	6.79	1.75
	3	-----	3.25	3.15	2.96	-----	3.27	10.12	-----	2.17	-----	2.50	4.15
Mean	2	2.45	2.13	3.65	5.47	1.76	5.05	2.92	4.55	2.83	3.46	-----	3.43
	3	0.00	4.27	3.11	3.73	4.74	5.25	4.17	4.47	2.61	6.80	-----	3.91
						Estriol mcg/24-hr							
BN	2	6.83	-----	15.86	18.22	50.45	12.35	15.86	-----	30.69	15.24	-----	20.69
	3	-----	14.47	11.50	34.86	35.24	22.82	23.78	-----	21.30	11.48	-----	21.87
JA	2	7.92	-----	10.31	63.83	40.35	20.62	7.36	-----	16.93	-----	-----	23.90
	3	9.57	27.79	11.18	29.47	18.55	31.12	22.53	-----	26.79	-----	-----	22.13
MJ	2	-----	5.15	9.64	12.39	14.10	19.88	----	24.14	-----	8.77	-----	13.44
	3	-----	4.79	8.24	12.37	8.81	21.09	14.86	3.16	13.90	9.18	-----	10.71
SR	2	5.21	-----	15.26	15.96	12.02	11.09	13.60	-----	15.96	13.48	-----	12.82
	3	-----	9.09	-----	12.51	16.76	10.16	25.42	11.89	16.87	-----	-----	14.67
TD[a]	2	-----	-----	24.04	-----	8.08	9.32	15.61	-----	21.32	-----	12.35	15.67
	3	-----	24.98	19.84	35.94	-----	20.78	38.70	-----	31.83	-----	28.67	28.68
Mean	2	6.65	15.07	15.02	27.60	25.00	14.65	13.11	24.14	21.23	12.50	-----	17.50
	3	9.57	16.22	12.69	25.03	19.84	21.19	24.96	7.53	22.14	10.03	-----	16.95
						Total estrogen mcg/24-hr							
BN	2	10.93	-----	32.04	31.25	70.50	22.96	20.31	-----	37.90	27.01	-----	31.60
	3	-----	25.33	24.83	53.21	51.17	33.45	26.22	-----	31.92	31.85	-----	34.75

Table 9. Continued.

Subject	Mean	Days of menstrual cycle 1-3	4-6	9	11	13	15	17	19-20	23-25	26-28	30	Mean
		Total estrogen mcg/24-hr											
JA	2	15.51	----	20.62	80.40	55.15	42.71	18.77	----	28.45	----	----	37.37
	3	14.73	38.73	19.71	42.87	37.62	52.48	32.91	----	36.18	----	----	34.40
MJ	2	----	9.39	17.91	23.33	26.72	38.63	----	37.66	----	21.42	----	25.01
	3	----	13.63	17.86	23.44	18.77	33.99	27.01	14.64	23.13	20.30	----	21.42
SR	2	16.57	----	22.36	29.01	21.26	20.20	27.18	----	30.40	27.22	----	24.34
	3	----	18.17	----	22.14	30.03	19.66	33.80	23.18	27.04	----	----	26.44
TD[a]	2	----	----	31.00	----	12.93	16.12	19.08	----	26.00	----	35.05	21.03
	3	----	29.07	29.23	43.13	----	28.50	55.24	----	39.42	----	37.89	37.43
Mean	2	14.34	99.39	24.75	41.00	37.42	28.06	21.34	37.66	30.71	25.38	----	27.00
	3	14.73	24.99	22.92	36.96	34.28	33.56	35.04	18.91	32.50	26.07	----	27.88

[a]TD: Day 36 on 2 meals: E_1, 7.23; E_2, 6.03; E_3, 40.97; E_t, 54.23.

Table 10. Mean food intake of the subjects

Subject	No. of meals	Order of diet	Weight	Calories	Proteins	Fats	Carbohydrates	Fatty acids Satu.	Unsaturated Oleic	Unsaturated lino.	Cholesterol
Women		period	lbs.		gm	gm	gm	gm	gm	gm	mg
B. N.	2	2nd	103	1824	58	87	203	38	32	5.0	557
J. A.	2	1st	139	2080	70	100	224	40	36	9.8	526
S. R.	2	2nd	132	1883	61	88	212	28	32	7.3	485
M. J.	2	1st	147	2016	73	105	194	40	36	9.2	638
T. D.	2	1st	136	2052	69	84	254	30	37	7.2	518
		mean	132	1971	66	93	217	35	35	7.7	548
percentage of calories					13	42	44	18	18	0.4	---
B. N.	3	1st	104	2148	72	100	239	37	41	9.0	563
J. A.	3	2nd	142	2158	76	99	241	37	38	9.6	438
S. R.	3	1st	134	1921	56	92	216	26	29	5.9	408
M. J.	3	2nd	146	2187	76	104	236	38	44	10.0	609
T. D.	3	2nd	137	2123	69	106	223	33	37	8.8	458
		mean	133	2107	70	100	231	34	38	8.7	495
percentage of calories					13	43	44	17	19	0.4	---
Men											
B. W.	2	1st	166	3361	109	168	353	73	63	11.1	1057
H. A.	2	2nd	150	2924	91	154	292	53	58	12.3	1150
D. P.	2	1st	166	3156	118	147	339	54	55	13.2	1069
B. E.	2	2nd	214	3380	122	187	300	80	70	9.5	1287
		mean	174	3205	110	164	321	65	61	11.5	1141
percentage of calories					14	46	40	20	19	0.4	----

Table 10. Continued.

Subjest	No. of meals	Order of diet	Weight	Calories	Proteins	Fats	Carbohydrates	Fatty acids			Cholesterol
								Satu.	Unsaturated		
									Oleic	lino.	
Men		period	lbs.		gm	gm	gm	gm	gm	gm	mg
B. W.	3	2nd	164	3216	121	168	306	63	70	16.4	1257
H. A.	3	1st	150	3266	106	170	327	61	68	11.7	1090
D. P.	3	2nd	167	2945	110	147	295	62	60	11.8	1853
B. E.	3	1st	213	3574	134	191	330	76	71	14.3	1249
		mean	174	3205	118	269	314	66	67	13.5	1112
	percentage of calories				15	47	39	20.2	19.4	0.4	----

Table 11. The relationship among serum cholesterol and urinary neutral 17-ketosteroid, pregnanediol and total estrogen by sex.

Sex	Days of study	Cholesterol	Pregnanediol	Neutral 17-ketosteroid	Total estrogen
		mg/100 ml	mg/24 hr	mg/ 24 hr	mcg / 24 hr
M	3-4	192	0.918	12.84	16.10
	10-11	215	0.837	8.13	18.95
	17-18	227	0.786	9.41	16.85
	24-25	226	0.519	8.36	15.05
	30-31	207	0.810	9.89	10.88
F	Days of menstrual cycle				
	1-3	192	0.92	6.17	14.70
	4-6	194	0.91	7.26	15.53
	9	223	0.72	5.37	23.84
	11	209	0.75	5.26	38.98
	13	213	0.78	6.32	35.85
	15	207	1.25	7.00	30.81
	17	217	1.18	7.37	27.87
	19-20	198	2.98	4.25	31.53
	23-25	214	1.93	6.33	31.61
	26-28	177	1.53	5.75	24.55

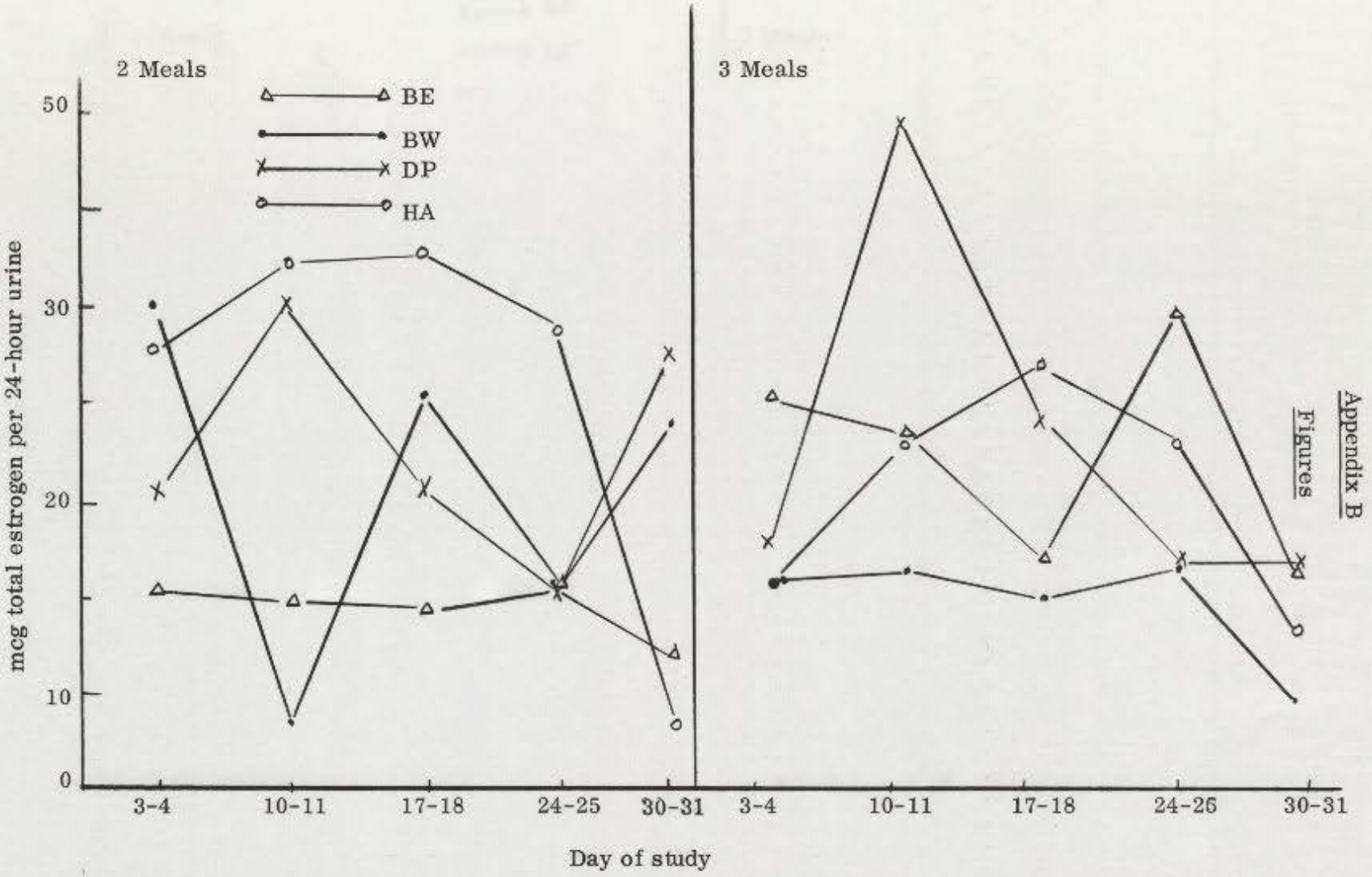

Figure 9. The effect of number of meals by day of test period on total estrogen excretion values for male subjects.

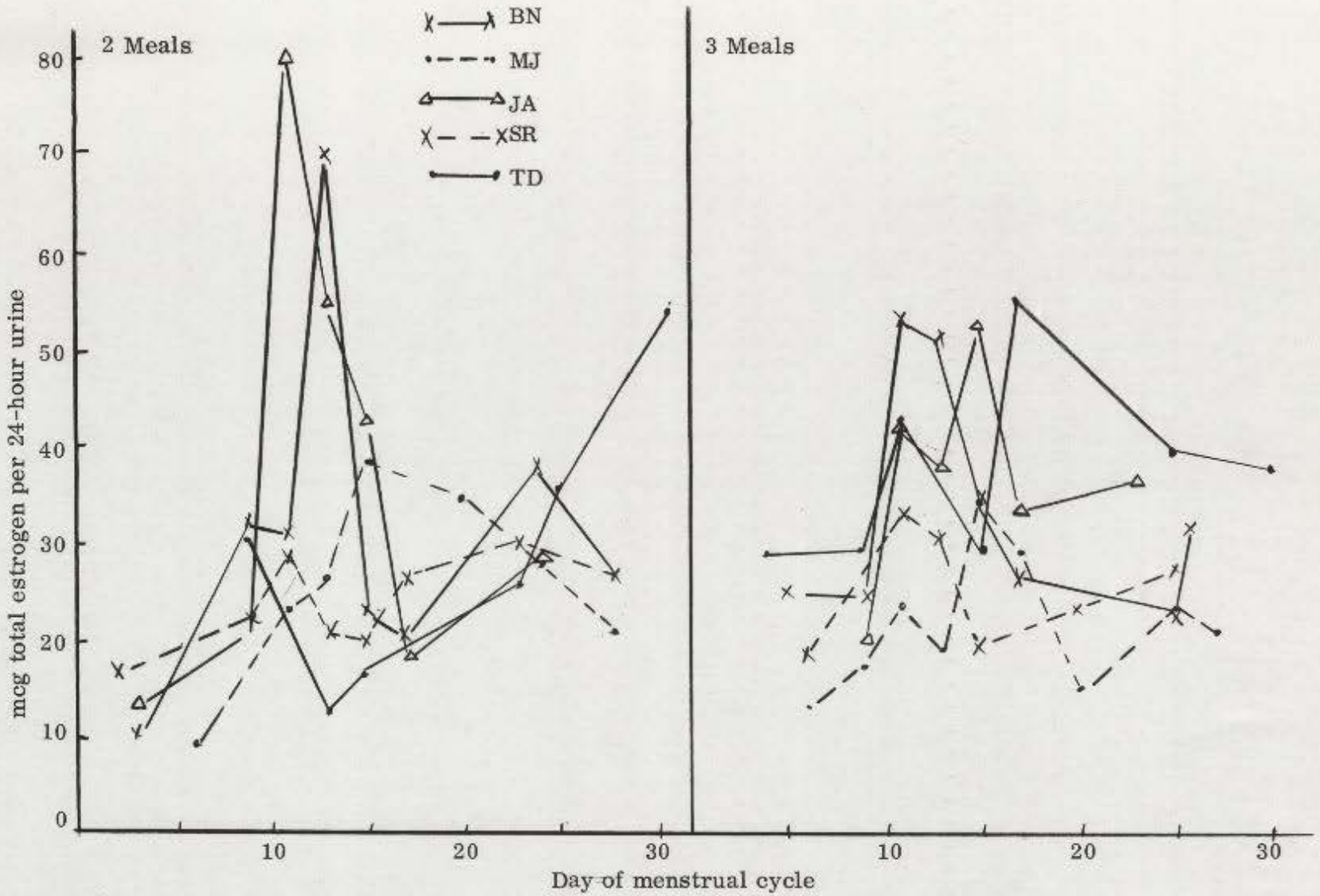

Figure 10. The effect of number of meals by day of menstral cycle on urinary total estrogen excretion values.

Appendix C

Method Used for Estrogens Determination

Material

The method for the determination of estriol, estrone, and 17 β-estradiol by absorption chromatography of the methyl ethers and colorimetric determination (Brown, 1955). Two or three determinations, each in duplicate, are usually performed at one time.

Diethyl ether (reagent grade). This is freed from peroxides by shaking with saturated $FeSO_4$ solution, washed with water, and distilled before use.

Light petroleum ether (b.p. 40-60 C, reagent grade). Shake with concentrated H_2SO_4 1/10-2/10 volume. Let stand 1-3 days then wash with water until pH 7 and dry with Na_2SO_4 over night before final distilling; saturate with water to use.

Benzene (reagent grade). Redistilled and treated as light petroleum before final distilling; saturate with water to use.

Ethanol (absolute). Redistill unless U.S.P.F.

H_3BO_3 powder was B.P. grade.

Dimethyl sulphate was redistilled.

Concentrated HCl.

NaOH pellets.

Solid $NaHCO_3$ powder.

30 % hydrogen peroxide.

Deactivated alumina. This is obtained by deactivating 100-150 mesh alumina of Brockman activity II-IV with 9-10 percent water and is standardized against estrogen methyl ether standards.

Concentrated carbonate buffer (pH 10.5, approximately). This is prepared by mixing 20 percent NaOH (150 ml) to 80 percent $NaHCO_3$ (1 liter).

8 % NaOH.

8 % $NaHCO_3$.

1.6 % NaOH--adjusted so that 50cc plus 1.2 gm H_3BO_3 gives a pH of 10.7.

20 % NaOH.

Column solutions:

25 % Benzene in light petroleum.

40 % Benzene in light petroleum.

1.4 % Ethanol in Benzene.

3 % Ethanol in Benezene.

Kober reagents. These are 2 percent solutions of hydroquinone (B.D.H. reagent) in 76, 66, and 60 percent (v/v) sulfuric acid (B.D.H. reagent) for the estimation of estroil, estrone, and estradiol-17 β, respectively. They are prepared by heating to dissolve the hydroquinone, and kept at least 24 hours before use. The estriol reagent was usually a light yellow, the estrone was light brown to pink, and the estradiol reagent was light pink in color. They were stable almost indefinitely at room temperature in the dark.

Standard estrogen solutions. Standard solutions of pure crystalline estrogens and their methyl ethers were prepared in ethanol (5 mg/100 ml). Solutions were stored at 4° and were stable indefinitely.

Apparatus. Glassware was rinsed after use with tap water, and then with distilled water unless visibly dirty. When necessary clean by: (a) soaking in a chromic-sulfuric acid mixture; washing with tap water; (b) soaking in an acid sulfite solution (Na_2SO_3, approximately 0.2 percent acidified with H_2SO_4) to destroy traces of chromic acid which would otherwise be harmful contaminant in this method, and (c) rinsing thoroughly with tap water, then distilled water, before being dried.

Optical densities were measured in a Beckman DU Spectrophotometer using 1cm glass cells.

Experimental C

Hydrolysis and extraction. Urine (20 ml) was heated to boiling under a reflux condenser. Concentrated HCl (12 N, 3 ml) was then added through the condenser and the urine-HCl mixture is boiled for 60 minutes and then cooled rapidly under running tap water. The cooled hydrolysed urine is extracted once with 20 ml and twice with 10 ml of ether. The ether is then extracted with concentrated carbonate solution, pf pH 10.5 (8 ml), and then shaken thoroughly with NaOH (2 ml of 8 percent). The NaOH layer is not discarded but is partly neutralized by adding 8 percent $NaHCO_3$ solution (2.0 ml) and then with water (1.0 ml). The water is drained off as completely as possible.

Extraction of the phenol fractions and methylation. The ether solution is poured into a flask and the ether was distilled just to dryness on a water bath. The flask was removed immediately from the water bath. Ethanol (1 ml) was added to dissolve the residue, since estriol is not easily soluble in benzene.

The flask was allowed to cool. Its contents are transferred with benzene (25 ml) to a separating funnel containing light petroleum (25 ml). The benzene-light petroleum solution is extracted with two 25 ml volumes of water and the aqueous layer (containing estriol) runs into 125 ml boiling flasks containing 2 gm NaOH pellets and a boiling chip. Boil under reflux for 30 minutes, and then cool under running water. The organic layer is then re-extracted twice with 1.6 percent NaOH (25 ml) and the alkaline solution (containing estrone and estradiol-17β) collected in 100 ml conical flasks (standard taper). To the estriol flask was added 6 gm solid $NaHCO_3$ powder and dissolved. The estriol fraction was transferred quantitatively to 125 ml separatory funnel using 50 ml ether for transfer. Shake well, then discard the aqueous layer. Extract ether twice using two 25 ml volume of 1.6 percent NaOH and the alkaline solution (containing estriol) collected in similar flasks. To both sets of flasks were added 1.2 gm H_3BO_3. The mixture was shaken well and then placed in a water bath at 47 C. Dimethyl sulfate (1 ml) added to each by means of a safety pipette. The flasks were closed with glass stoppers and shaken periodically until the boric acid and dimethyl sulfate have dissolved. After keeping at 37 C for a further 10-30 minutes, more dimethyl sulfate (1 ml) and 20 percent NaOH (2 ml) were added to each flask and the shaking at 37 C repeated. After the second portion of diemtnyl sulfate had dissolved, the flasks were allowed to stand at room temperature overnight or for 20-30 minutes at 37 C.

Oxidation of contaminants and extraction of methylated estrogens. Twenty percent NaOH (10 ml) and 30 percent H_2O_2 (2.5 ml) were added to each flask and the

contents transferred to 125 ml separating funnels. The "estriol" flasks were rinsed three times with benzene (a total of 25 ml) and the "estrone-estradiol" flasks with light petroleum (a total of 25 ml), rinsing being added to the corresponding separating funnels. The funnels were shaken, the aqueous layer discarded, and the organic layer washed twice with H_2O (5 ml). The water is drained off as completely as possible.

Chromatography. The chromatographic tubes used are 13 mm in diameter and 40 ml in volume with a centered glass support sealed near the bottom of the tube and a 19/36 standard taper cone for connection to the receiving tube, which is a 6 x 3/4 inch test tube with 19/36 socket. Slight suction from a mainfold may be applied to the bottom of the tube through a two-way stopcock which enables the suction to be applied to each column independently. The suction is adjusted so that the rate of flow of solvents is about 30 drops/minutes.

Column preparation. A chromatographic tube is filled with water-saturated benzene (for estriol) or water-saturated light petroleum (for estrone-estradiol) and 2 gm standardized deactivated alumina added in a thin stream so that it is freed from air as it settles. The surface of the alumina is leveled by tapping and a 5-6 mm layer of glass wool added to protect the surface from disturbances. Solvents are sucked to the surface of the glass wool before adding the next fraction, but no air is allowed to penetrate the alumina itself. All solvents are saturated with water before use.

Deactivation of alumina. Alumina of 100/150 mesh with Brockmann activity of II-III is deactivated by the addition of 9-10 percent water and shaken

until homogeneous. After cooling, a 2 g column in light petroleum is prepared from this alumina as described above and a solution of 10 mcg estrone methyl ether in 25 ml light petroleum applied. When the solvent level reaches the glass wool, benzene (25 percent) in light petroleum is added to the top of column, and the eluate is collected in 2 ml fractions and assayed for estrone methyl ether. This should begin to appear in the 16th to the 20th ml of eluate. If it appeared later than this, more water was added to the stock solution. Deactivated alumina is stored in an air tight container.

Standardization of alumina. When a new batch of active alumina is purchased, a portion was deactivated as described above and the elution patterns of all three estrogen methyl ethers from columns of this material determined. This was accomplished by eluting estrogen methyl ethers from test columns (each prepared from 2 gm deactivated alumina) as described below.

Column 1 was prepared in light petroleum. Estrone and 17β-estradiol methyl ethers (10 mcg each) were applied in light petroleum (25 ml) and the column eluted with: (a) 25 percent benzene in light petroleum (12 ml); (b) 40 percent benzene in light petroleum (collected in small fractions). Estrone methyl ether should not be eluted by (a) but should be completely eluted by the first 12 ml of (b). 17β-estradiol methyl ether should only appear after about 30 ml of (b) have been collected.

Column 2 is prepared in light petroleum. 17β-estradiol methyl ether (10 mcg) was applied in light petroleum (25 ml) and the column eluted with: (a) 25 percent benzene in light petroleum (12 ml); (b) 40 percent benzene in light

petroleum (27 ml); (c) benzene (collected in small fractions). 17β-estradiol methyl ether should not be eluted by (a) or (b) but should be completely eluted by the first 8-10 ml of (c).

Column 3 is prepared in benzene. Estriol methyl ether (10 mcg) was applied in benzene (25 ml) and the column eluted with: (a) 1.4 percent ethanol in benzene (collected in small fractions). Estriol methyl ether should only begin to appear in the 15th to 17th ml of (a).

Column 4 is prepared in benzene. Estriol methyl ether (10 mcg) was applied in benezene (25 ml) and the column eluted with: (a) 1.4 percent ethanol in benzene (12 ml); (b) 3 percent ethanol in benzene (collected in small fractions). Estriol methyl ether should not be eluted with (a) but should be completely eluted in the first 12 ml of (b).

If the elution patterns of the estrogen methyl ethers from test columns are consistently different from these described, the fractions collected in the chromatography of methylated urine extracts should be adjusted accordingly.

Columns are prepared from standardized, deactivated alumina (2 g) using the equipment and technique described above and solvents saturated with water (dry solvents would remove water from the alumina and alter its degree of activation). Methylated estriol fractions (in benzene) are applied to columns prepared in benzene and sucked down to the glass wool. The columns are first eluted with 1.4 percent ethanol in benzene (12 ml), which removed a pigment band and was discarded. It was then eluted with 3 percent ethanol in benzene (15 ml), which is collected in a standard taper test tubes (estriol methyl ether).

Methylated estrone-estradiol fractions were applied in light petroleum to columns prepared in this solvent and the columns eluted first with 25 percent benzene in light petroleum (12 ml). This fraction was discarded. Estrone methyl ether was then eluted with 40 percent benzene in light petroleum (15 ml) and collected. A further 12 ml of this mixture was passed through the column and discarded. Finally, 17β-estradiol methyl ether was eluted with benzene (12 ml) and collected.

Evaporation of solvents and color development. A 2 percent (W/V) solution of hydroquinone in ethanol (0.2 ml) was added to each test tube containing estrogen methyl ether. The tubes were heated in a water bath. A slightly reduced pressure was applied to each tube from a manifold (evacuated by a water-pump) to aid removal of the solvents. When all tubes were dry, the pressure was reduced further, the tubes removed from the water bath, and the vacuum released by admitting nitrogen to the system. The process of evacuation and admitting nitrogen is then repeated.

The appropriate color reagent (3 ml) was added to each tube, all tubes are placed in a boiling water bath. During the first 6 minutes the tubes were shaken twice. After 20 minutes they were removed and cooled in a bath of cold water. Water was then added to all tubes (1 ml to estriol fractions, 0.5 ml to the estrone fractions, and 0.2 ml to the 17β-estradiol fractions). The tubes are then shaken, heated in the water bath for 10 minutes, and cooled in cold water for about 10 minutes. The optical densities of the solution were then read in the spectrophotometer against the appropriate reagent blank, prepared

by evaporating 2 percent ethanolic hydroquinone (0.2 ml) in a tube and treating with color reagent in the same way as the samples. Estriol and estrone fractions are read at 480, 516, and 552 mμ and 17β-estradiol fractions at 480, 518, and 556 mμ. That part of the optical density (D) at the second wavelength due to estrogen ether was calculated from the formula (this was derived from Allen's correction formula multiplied by 2):

For estriol and estrone

$$D_{corrected} = 2D_{516} - (D_{480} + D_{552})$$

For 17β-estradiol

$$D_{corrected} = 2\,D_{518} - (D_{480} + D_{556})$$

The amount of estrogen methyl ether in each sample was obtained from the corrected value by means of a calibration curve prepared from the pure methyl ether. Final values were expressed as micrograms of free estrogen by multiplying by the ratio of the molecular weights in the whole 24 hour urine specimen.

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