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Serum Lipid Components of University Women During the Menstrual Cycle

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SERUM LIPID COMPONENTS OF UNIVERSITY WOMEN DURING THE MENSTRUAL CYCLE

by

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Hih-min Sang

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ABSTRACT

Serum Lipid Components of University Women

During the Menstrual Cycle

by

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Four healthy university women students (age 21-26) while on self-selected diets and living under normal living conditions served as experimental subjects in a study of changes of serum total lipids, distribution of lipid classes, and the fatty acid composition of lipid fractions during the menstrual cycle. Effect of meal frequency (2 meals vs 3 meals with no breakfast) was also included.

Total lipids were extracted from serum. Chemical determinations of total lipids, total and free cholesterols, and lipid phosphorous were made on these extracts. Concentration of cholesterol esters and phospholipids were calculated and triglycerides plus free fatty acids were calculated by difference. The total lipids were separated into lipid classes by thin-layer chromatographic method while the methyl esters of fatty acids were analyzed by Gas-Liquid chromatographic method.

The variation between the individual subjects was quite great in this study. Further work using a greater number of subjects, analyses for more days to obtain maximum peaks, and selection of subjects who have menstrual cycles of the same length, such as 28 days, are recommended to clearly identify changes of serum lipid metabolism during the menstrual cycle. Relating these basic data to endocrine excretion values should give information needed for future studies relating abnormal metabolism of lipids to atherosclerosis and coronary heart disease.

(86 pages)

INTRODUCTION

Among the degenerative diseases of old age which appears to be related to abnormal lipid metabolism are atherosclerosis and coronary heart disease. The role of steroid hormones on lipid and cholesterol metabolism has been studied by a number of investigators. A decrease in serum cholesterol and change in the proportions between certain fatty acids has been found when estrogens are administered to rats. Serum cholesterol values in humans have been decreased by estrogen but androgen administration has been shown to increase the circulating cholesterol in humans. Women rarely have coronary heart disease until after the menopause. This is attributed to the decrease in the production of estrogens following the menopause.

Information from detailed studies of serum fatty acids changes in normal young adult women from day to day of the menstrual cycle is limited. Studies of these changes will give basic information of what is occurring in women during the menstrual cycle. As a part of a larger problem whose purpose was to determine relationships among serum cholesterol, serum fatty acids, and concentration of estrogens and degradations products of the androgens and corticosteroids in urine in healthy, young adult men and women, interest was directed to investigate in greater detail the influence of day of menstrual cycle of the women on these relationships. This type of basic information on individuals living under home conditions is needed as a base to help in the interpretation of the findings and enlarge our knowledge of

interrelationships between serum lipids and secretion of the steroid hormones and for future study of the role of abnormal metabolism of lipids in atherosclerosis and other degenerative diseases.

The purpose of this experiment was to determine the changes in cholesterol content, distribution of lipid classes, and fatty acid composition of cholesterol esters, triglycerides, and phospholipids in serum at different days of the menstrual cycle of university women on self-selected diets.

REVIEW OF LITERATURE

Lipids in Blood Serum

The lipids in serum occur mainly as cholesterol esters, free cholesterols, phospholipids, triglycerides, and free fatty acids. Under certain physiological conditions, such as disease and differences in hormone secretion, the value of total lipids and the individual constituents will change widely from the usual values (Cook, 1958; Deuel, 1955; Feldman, 1963; Waxler and Craig, 1964).

Albritton (1952) reported that the range of the serum total lipid values for humans was 397 to 722 mg per 100 ml serum. Slightly more than one-third of the total lipids were cholesterol esters, about one-third phospholipids, one-fifth triglycerides, one-tenth free cholesterol, and two to three percent free fatty acids. The total lipid values and the distribution of individual constituents have been reported by several investigators (Table 1). Most mean values for total lipid for men and women recorded over a thirty-year period ranged from 530 to 650 mg per 100 ml serum. Higher values also have been obtained, 735 mg (Page et al., 1935) and 836 mg (Kornerup, 1950).

The fatty acid contents of cholesterol esters, triglycerides, and phospholipids of serum reported by serveral investigators are presented in Table 2. The major fatty acids in serum are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic (20:4). In cholesterol esters, about half the fatty acids are linoleic, one fourth oleic, and a slightly more than

Table 1. Total lipids and the distribution of lipid classes in serum reported in the literature

Investigator	Year	Sex	Total lipid mg/100 ml	Lipid classes			
				Cholesterol esters	Free cholesterol	Triglycerides plus free fatty acids	Phospho-lipids
				Percent total lipid			
Man and Peters	1933	M W	659	--	--	--	34
Boyd	1935	M W	595	35	9	25	31
Page et al.	1935	M	735	34	11	31	25
Kornerup	1950	M W	836	32	7	--	21
West and Todd	1957	M W	530	34	9	27	31
Wilmot and Swank	1952	M W	591	37	10	18	36
Luddy et al.	1958	M	--	39	8	16	32
Hallgren et al.	1960	M W	588	44	12	16	34
Lund et al.	1961a	M W	635	47	4	--	--
Lund et al.	1961b	M W	568	62	5	--	--
Svanborg and Svennerholm	1961	M W	629	34	10	14	35
Waxler and Craig	1964	W	587	--	--	14	--

Table 2. Major fatty acids of lipid classes in serum of men and women as reported by several investigators

Lipid class	Percent fatty acids					Investigators
	16:0	18:0	18:1	18:2	20:4	
Cholesterol esters	11-17	tr-4	21-30	40-52	4-8	Hallgren et al., 1960; Hanahan et al., 1960; Luddy et al., 1958; Michaels et al., 1959; Scott et al., 1964; Swell et al., 1962; Tuna and Mangold, 1963; Tuna et al., 1958.
Triglycerides	25-31	4-7	36-45	10-14	tr-2	Hallgren et al., 1960; Luddy et al., 1964; Schrade, 1961; Swell et al., 1962; Tuna and Mangold, 1963.
Phospholipids	28-37	12-14	14-19	14-21	4-9	Dole et al., 1959; Hallgren et al., 1960; Schrade, 1961; Scott et al., 1964; Swell et al., 1962.

one-tenth palmitic. In triglycerides, most of the fatty acids are palmitic and oleic, with slightly higher concentrations of oleic, and only about one-tenth linoleic. In phospholipids, about one-third of the total fatty acids are palmitic acid, about half are divided between stearic, oleic, and linoleic acids in relatively comparable amounts.

The Influence of Steroid Hormones on Serum Lipids

Information concerning hormonal influences on lipid metabolism is limited, although evidence is accumulating that such mechanisms exist and probably are important (Adlersbert, 1958).

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 before the menopause is well known. Bilaterally oophorectomized women show a higher incidence of coronary artery disease than normal women of corresponding ages.

Considerable information is available as to the effects of gonadal hormones in man. Eilert reported in 1949 and 1953 (Adlersbert, 1958) on the effect of estrogens in pre- and post-menopausal women. A decided reduction in plasma cholesterol and the cholesterol: phospholipid ratio was observed. Adlersbert summarized the findings of several investigators (Oliver and Boyd, 1954, 1956; Robinson et al., 1956; Russ et al., 1951) from their extensive studies on the effects of estrogens and androgens in patients who survived myocardial infraction. Synthetic or naturally occurring estrogens lowered plasma cholesterol and raised the cholesterol concentration of the

alpha lipoproteins and correspondingly diminished the cholesterol concentration of the beta lipoproteins. It was found that estrogen can decrease the serum total cholesterol, and increase the phospholipid and the alpha lipoprotein cholesterol. Ethinyl estradiol can depress total plasma cholesterol and the cholesterol:phospholipid ratio. Depression of the plasma cholesterol, plasma cholesterol:phospholipid ratio (C:P ratio), and the beta lipoprotein cholesterol with elevation of the alpha lipoprotein cholesterol occurred regularly at ovulation and during menstruation in young, healthy women.

The opposite changes in the circulating lipids and lipoproteins developed during the luteal phase of the cycle. The secretion of estrogens also undergoes regular cyclical variation and is generally believed to rise to a maximum at or about the time of ovulation and some workers have observed a secondary peak just before menstruation (Aldersberg, 1958).

Progesterone does not cause any marked elevation of the circulating lipids and lipoproteins, but androgens can elevate serum lipids (Oliver and Boyd, 1958; Aftergood and Alfin-Slater, 1965).

Serum Lipid Levels and Eating Frequency

Cohn (1964) stated that a change in body metabolism associated with a difference in the frequency with which a day's allotment of food was ingested was first noted by Tepperman et al. (1942). These workers trained the laboratory rat, an animal that usually eats small quantities of food on many occasions over 24 hours, to consume its ration in a daily 1- to 2-hour period. The new eating habit resulted in an increase of the RQ sufficiently great to be

interpreted as indicating an increase in lipogenesis. Experiments accomplished since these original observations have made it clear that when meal size (quantity of absorbed nutrients to be handled per unit of time) is varied, the body adapts itself not only with respect to its handling of fat but also as regards overall body metabolism. Thus, measurements of certain aspects of carbohydrate and protein metabolism have yielded results suggesting that the disposition of these foodstuffs also are affected by the rate of ingestion of the diet (Cohn et al., 1959, 1960, and 1963; Febry et al., 1961; Hollifield 1962) as summarized by Cohn(1964). Some of the evidence suggesting feeding frequency to be a significant factor in the regulation of intermediary metabolism is based on the fact that the activities of a number of enzymatic pathways have been shown to be influenced by the load of nutrients (substrates) presented to them per unit of time. It follows, therefore, that the alternations in body metabolism seen with different eating patterns could conceivably result from the limited activities of some enzymatic pathways with consequent adaptation of alternate ones.

The animal experiments appear to have yielded clear-cut results in demonstrating that feeding frequency plays a significant role in the regulation of serum cholesterol levels and in the induction and regression of experimental atherosclerosis (Okey et al., 1960; Hollifield, 1962; Cohn et al. 1961). One can not deny the fact that a number of special conditions appear to be necessary for demonstrating these phenomena, such as the species of animal used, the sex of the animals (some species), and the presence or absence of added cholesterol in the diet. With respect to this latter condition, the results of

Gopalan et al. (1962) take on added significance. These workers not only observed that decreased feeding frequency was capable of raising serum cholesterol levels in a primate but, in addition, did so without added cholesterol in the food. Thus, one can see that the important fact emphasized by all the animal data is that meal frequency (size) plays a role in the experimental production of a disease to which man is prone.

Reports indicating any influence, positive or negative, of eating frequency on serum cholesterol concentrations of man are few and the results are not clear cut. Furthermore, some of the data are subject to multiple interpretations (Cohn, 1964). Subjects placed on a 6-times-a-day formula diet, after having been on a 3 meals a day hospital diet, responded with decreased serum cholesterol levels, regardless of the fat in the diet. The type of fat in the diet, however, did influence the magnitude of the fall.

The data (Cohn, 1964) indicated that chickens and rabbits restricted to eating a diet containing modest amounts of cholesterol, for 2 hours per day exhibit double the serum cholesterol levels and four to seven times the incidence of atherosclerotic lesions than animals consuming 30 percent more diet over a 24-hour period. The monkey and female rat likewise respond to a restriction of the time during which food is available by an increase in the levels of serum cholesterol. Studies on the manner in which man responds to different feeding frequencies have not yielded as well defined results as those from the experimental animal. The results now available are suggestive, however, in indicating that man reacts like other species. If the preliminary data are substantiated by additional experiments, a new factor in the pathogenesis of human atherosclerosis might be man's eating habits.

Cholesterol and Atherosclerosis

Atherosclerosis is not a new disease. Kritchevsky (1962) points out that this disease was discussed by Aristotle and his contemporaries about 400 B.C. and that it has been touched upon by many medical writers since then. The fatty nature of the atherosclerotic lesion was emphasized about a century ago by Vogel (1847) who identified cholesterol as the major constituent of atheromatous plaques, an observation later enlarged upon by Aschoff (1907) and by Virchow (1867), who described the progression of the fatty degenerative changes in the aorta (Kritchevsky, 1962). The name atherosclerosis was adopted on the suggestion of Marchand (1904). The word is derived from the Greek "athero" meaning mushy, and "sclerosis" meaning hardening. The term is now used to describe various phases of coronary heart disease.

Kritchevsky (1962) discussed a number of theories concerning the pathogenesis of atherosclerosis. Some workers picture an initial fatty infiltration (Virchow, 1856; Leary, 1941), others an initial arterial damage followed by fatty infiltration (Duff, 1936; Daguid, 1955; Moon and Rinehart, 1952), and still others believe that the metabolic role of the (aortic) tissue is also a contributing factor (Holman et al., 1957). Whatever the final definitive elucidation of the pathogenesis of coronary heart disease, it is now one of the most pressing medical problems of the western world; and this discussion is limited to one area, namely, cholesterol as it is related to the etiology of atherosclerosis.

There is a strong tendency in both the popular and scientific press to correlate dietary fat and especially dietary cholesterol with coronary heart disease. It is now believed that this relationship is purely circumstantial. Experimental data on the effects of dietary fat on serum cholesterol levels are now well documented; also other factors, elevation of blood pressure above normal, kidney damage, presence of diabetes, heredity, hypothyroidism, obesity, exercise, heavy cigarette smoking, age, and sex (Stamler et al., 1963), have been shown to influence this disease.

As the severity of the atherosclerosis increases, the phospholipid content is diminished with a concomitant rise in total cholesterol (Weinhouse et al., 1940; Mead et al., 1961; in Kritchevsky, 1962). Other sterols have been isolated (McArthur, 1942; Mead et al., 1961) but all save cholesterol are probably artifacts. The cholesterol ester fatty acids were initially observed to be oleic, stearic, palmitic, arachidonic, and linoleic (Schoenheimer, 1928; McArthur, 1942). Reinvestigation by a number of groups using newer analytical techniques (silicic acid chromatography, alkali isomerization and gas liquid chromatography) have indicated the presence of a few other acids such as palmitoleic and penta-and-hexaenoic acids. The newer results (Kritchevsky, 1962) differs among themselves in some aspects but are in general over-all agreement as indicated from the data of Mead et al., (1961); Luddy, et al., (1958); Tuna et al. (1958); Lewis (1958); Wright et al., (1959); Bottcher et al. (1959).

The data connecting elevated serum cholesterol levels with coronary disease are numerous. In diseases such as diabetes, myxedema, nephrosis

and xanthomatosis, in which serum cholesterol levels are uniformly elevated, the coronary death rate is also elevated.

Beta-lipoproteins have been shown to be directly implicated in coronary artery disease; the beta:alpha lipoprotein ratios are also implicated. These ratios have been shown to vary with age and sex. Several investigations have shown that the "resistant" species have a much lower beta:alpha lipoprotein ratio than do the susceptible ones (Kritchevsky, 1962). When it is considered that young women, who are substantially free of coronary artery disease, carry less cholesterol in their serum beta-lipoprotein fraction than do young men or older women or men, measurement of this blood fraction becomes of interest.

Albrink (1965) postulated that it is quite possible that serum cholesterol and serum triglycerides together operate to accelerate the atherosclerotic process and that the effects of one cannot be separated from the effects of the other. On the other hand, the value of serum cholesterol levels in predicting coronary artery disease could conceivably be due to its value in predicting triglyceride elevation.

METHOD AND PROCEDURE

Description of Subjects

In this study four university women living under their usual living conditions, were placed on self chosen diets, during two menstrual cycles of 28-34 days. Half of the girls went without breakfast in the first cycle while the others ate their regular three meals a day. At the beginning of the second cycle, each group reversed their eating pattern as shown in Table 3.

On the day prior to the blood sampling, the dietary records of all food eaten were kept. Diets were calculated for total calories, carbohydrates, proteins, total fats, total saturated fatty acids, unsaturated fatty acids, including oleic and linoleic acids using values found in the U. S. D. A. Agricultural Handbook No. 8 by Watts and Merrill (1963), except for the cholesterol values which were calculated from the tables by Okey (1945).

Body weights were taken before breakfast on each morning that blood samples were obtained. Data on age, height, and body weights are presented in Table 4.

All subjects received a physical examination by the University Health Service[1] before starting the experiment and were found to be in excellent condition.

---

[1]Doctor S. M. Budge

Table 3. Experimental design

Periods	Three meals with breakfast	Two meals without breakfast
I November 5 to December 19, 1964		D. W.
	A. M.	P. B.
	C. L.	
II January 2 to February 12 1965	D. W.	
	P. B.	A. M.
		C. L.

Table 4. Data on age, height, and weight of subjects

Subject	Age	Height		Weight
	year	feet	inches	pounds
A. M.	26	5	4.0	115.5
C. L.	21	5	9.0	145.5
D. W.	23	5	8.5	168.0
P. B.	22	5	6.7	131.0

Blood and urine samples were obtained from five subjects in the original large project during one menstrual cycle.

One of the subjects, J. B. became pregnant during the second cycle. As blood samples were not obtained for as many days as were urine samples after the 28th day in the second cycle, her data were not included in this study.

Preparation of Serum Samples

Blood was taken from the finger-tip of subjects in the morning before food was eaten for the following days: 1, 4, 7, 9, 11, 13, 15, 17, 20, 26 and at the first day of the next cycle. About 1 ml of blood on day 4, 7, 9, 11, 15, 17, 20, 26 (for cholesterol analysis only) and 5 ml of blood on days 1, 13 to 15 and 26 for distribution of lipid classes and composition of fatty acids) were drawn from each subject. Placed in a 10 x 100 mm tube, allowed to clot for at least a half hour and then centrifuged at 2500 rpm for 30 minutes. All serum was transferred to 10 x 75 mm tubes. The tube was stoppered and the sample was stored at -5 to -10 C until analysis. Hematocrit and hemoglobin content was determined, and all values were normal (Tables 17 and 18).

Determination of Lipids in Serum

The method used for extraction of lipids from serum was described by Smith (1965) and is given in Appendix B.

Total lipid concentrations were determined by the method of Bragdon (1951). Procedures were followed as outlined except for the adaption to use smaller volumes of sample and color reagent (Appendix C).

Lipid phosphorus was determined by the micromethod of Lowry et al. (1954) was modified by Smith (1961) is outlined in Appendix D.

The method used for the separation of total lipids into lipid classes was used by thin-layer chromatography of Maier and Mangold (1964). The method used for gas-liquid chromatographic analysis of methyl esters of fatty acids was a modification of the method of Stoffel, Chu and Ahrens (1959) as described by Smith (1965) (Appendix E).

The method used for cholesterol determination was the micromethod of Galloway et al. (1957) as modified by Smith (1961) (Appendix F).

The amounts of cholesterol esters were calculated as the difference between the total and free cholesterol (cholesterol oleate) multiplied by 1.68. The phospholipids were calculated by multiplying the grams of lipid phosphorus by 25. The percentages of cholesterol esters, free cholesterol, and phospholipids in total lipids were computed. The value of triglycerides plus free fatty acids were calculated by difference.

RESULTS AND DISCUSSION

Days 1, 13 to 15, and 26 were selected for obtaining the larger samples of blood for fatty acid analyses as representative days of the beginning of the menses, approximate time of ovulation, and at the end of the cycle or just before the menses start. Values for cholesterol, estrogens and other steroid hormones have been shown to be influenced by the menstrual cycle in studies in this laboratory (Yu, 1964; Chiang, 1966; Yam, 1966) and by other investigators (Adlersberg, 1958 and others). Patterns for these biochemical indices have shown high or low peaks in the values at the beginning, at time of ovulation, and at the end of the cycle. If fatty acid composition of the lipid fractions is influenced by the menstrual cycle, it is hypothesized that differences in patterns would be detectable and that selection of these three days or periods of the menstrual cycle would give data showing the patterns which would suggest where the greatest differences might occur in the cycle.

Serum Total Lipids

The overall mean for serum total lipids for the four women was 602 $\pm$ 29 mg per 100 ml (Table 5). Standard error of the means among the four subjects were large compared to standard errors among the six values for each subject (25 to 36 among subjects and 4 to 16 among values for individual subjects). This finding points up the variations from subject to subject in lipid metabolism. It also indicates that many individuals tend to maintain a definite

Table 5. Mean serum total lipid plus standard error on day 1, 13 to 15 and 26 of menstrual period

Subjects	day 1	day 13	day 26	mean
	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
A. M.	681±16	677±5	659±6	672±7
C. L.	609±15	643±3	621±4	624±10
D. W.	590±3	534±0.5	567±8	564±16
P. B.	550±3	536±2	548±8	545±4
Mean	607±27	598±36	599±25	602±29

Table 6. Mean serum total lipids and standard error determined on two meals and three meals per day

Subjects	2 meals	3 meals	mean
	mg/100 ml	mg/100 ml	mg/100 ml
A. M.	690±23	665±27	677±12
C. L.	608±18	635±8	621±13
D. W.	560±23	567±15	564±3
P. B.	553±19	536±9	544±8
Mean	603±31	601±29	602±29

pattern of lipid metabolism when other conditions remain fairly constant. The range of individual values was 533 to 714 mg per 100 mg (Table 19). This values are within the normal range cited by several investigators (Table 1).

Influence of menstrual cycle

Day of menstrual cycle appeared to have a slight influence on total serum lipid values (Table 5). The total lipid values obtained for the middle of the cycle (days 13 to 15) were somewhat lower than for day 1 or 26 (598 vs 607 and 600 mg per 100 ml, respectively). This finding is in agreement with that reported by Oliver and Boyd (1958).

The ranges of total serum lipid values were 550 to 681 mg per 100 ml for day 1 of the menstrual cycle; 534 to 667 mg for days 13 to 15; and 548 to 659 mg for day 26. The least variation in values occurred for day 26 while the other analyses showed similar but somewhat more variance.

Influence of two meals vs three meals per day

The range of values on the two meal regime for total lipids in serum was 553 to 690 mg per 100 ml; whereas the values for the three meals group was 536 mg to 665 mg (Table 6). The overall mean was 603 and 601 for the subjects on 2 or 3 meals per day, respectively. This indicated no real influence of meal frequency on the total lipids values when the three periods of the menstrual cycle were combined. Differences due to meal frequency were not as great as those reported by Cohn (1964).

Consideration of total lipid content of serum by day of menstrual cycle and meal frequency did not show any consistant trend (Table 7).

Table 7. Mean values of total lipid in serum of four subjects consuming two and three meals per day.

Days of menstrual cycle	2 meals	3 meals	Mean
	mg/100 ml serum	mg/100 ml serum	mg/100 ml serum
1	608 ± 35	607 ± 24	607 ± 27
13 to 15	598 ± 35	598 ± 38	598 ± 36
26	603 ± 14	598 ± 28	600 ± 25
Mean	603 ± 31	601 ± 29	602 ± 29

The menstrual cycle influenced total lipid levels to a greater degree than did the number of meals per day in this study. Individual variations in total levels were much greater than the variance in values for days of the menstrual cycle by meals.

Distribution of Lipid Classes

After total lipid and lipid fractions were determined in the serum values for the fractions were expressed as percentage of the total lipids. Individual data for the distribution of lipid classes are presented in Table 19. Lipid classes include phospholipids, diglycerides (also probably 1 and

and 2-isomers), free cholesterol, free fatty acids, triglycerides, cholesterol esters and long-chain hydrocarbons. Serum is characterized by a greater concentration of cholesterol esters and phospholipids, with a small amount of triglycerides plus free fatty acids, and the least amount of free cholesterols. Overall mean percentages show good agreement with values reported by several investigators (Table 1). Mean percentage for the women in this study by percentage of total lipid were as follows: cholesterol esters, 37; phospholipids, 34; triglyceride fraction, 22; and free cholesterol, 8.

Variation within lipid classes among subjects (differences between low and high values) was much greater for the triglyceride fraction (1.2 to 13.8 percent) than for any of the other classes (Table 9). Percentage differences for free cholesterols were 1.2 to 3; cholesterol esters, 1.3 to 7.8; and phsopholipids, 3.2 to 12.6.

The menstrual cycle did not appear to influence the distribution of serum lipid classes, at least not for the days sampled in this study (Table 9). Whether or not changes were missed by not analyzing blood taken at other times during the menstrual cycle can only be answered by further research.

The mean values of lipid classes in serum for the four subjects were similar for each test day on the two and three meals per day regime (Table 8). Differences between the overall values for the 2 meal and 3 meal test periods varied from -2 to +2 percent. As these variations between meal frequency were much less than between subjects, serum lipid classes in this study were not influenced by the number of meals eaten. This finding applies to each of the individual lipid classes. The triglyceride fraction variance was from -2 to +2 percent, while other fractions were only from 0 to +1 percent.

Table 8. Percentage of lipid classes for subject for two and three meals per day

Lipid classes	Meals per day	A. M.	C. L.	D. W.	P. B.	Mean Standard Error
Cholesterol ester	2	37.2	37.6	37.3	36.1	37.1±0.4
	3	37.1	39.6	36.6	34.4	36.9±1.0
Mean ± standard error		37.2±1.3	38.6±1.2	36.9±0.7	35.3±0.8	37.0±0.6
Free Cholesterol	2	8.1	7.3	8.3	7.4	7.8±0.3
	3	8.2	7.4	8.5	6.4	7.6±0.5
Mean ± standard error		8.2±0.3	7.4±0.6	8.4±0.3	6.9±0.4	7.7±0.4
Triglyceride and Free fatty acid	2	24.0	18.1	20.1	24.1	21.6±1.5
	3	23.8	17.9	20.7	26.5	22.2±2.8
Mean ± standard error		23.9±1.4	18.0±2.1	20.4±1.1	25.3±0.8	21.9±1.7
Phospholipid	2	29.5	36.9	34.3	32.4	33.3±1.6
	3	30.9	35.0	34.2	32.6	33.2±0.9
Mean ± standard error		30.2±1.0	35.9±1.0	34.3±0.3	32.5±0.4	33.3±1.2

Table 9. Percentage of lipid classes on day 1, 13 to 15, and 26 of menstrual cycle

Days	Meals per day	Cholesterol esters	Free cholesterol	Triglyceride plus free fatty acid	Phospho-lipids
1	2	37 ± 1.3	8 ± 0.3	20 ± 3.2	34 ± 2.6
	3	38 ± 1.6	8 ± 0.5	22 ± 2.2	33 ± 0.7
13 to 15	2	37 ± 0.7	8 ± 0.3	22 ± 1.0	34 ± 1.1
	3	38 ± 1.1	8 ± 0.5	21 ± 2.4	34 ± 1.2
26	2	37 ± 0.3	7 ± 0.3	22 ± 0.3	34 ± 0.8
	3	35 ± 1.8	7 ± 0.4	24 ± 1.4	33 ± 0.8

Fatty Acid Composition of Lipid Classes

The fatty acids composition of cholesterol esters, triglycerides, and phospholipids in serum were determined. The most common accepted short method of designating is by listing according to number of carbon atoms and double bonds for each fatty acid. The fatty acids found in serum and in the tables in this study as follows: caprylic, 8:0; capric, 10:0, lauric, 12:0; myristic, 14:0; palmitic, 16:0; palmitoleic, 16:1; stearic, 18:0; oleic, 18:1; linoleic, 18:2; linolenic, 18:3 and arachidic, 20:0. There were two acids which appeared after lauric and myristic acids, respectively, which were not identified and were listed in the table as ?.

The percentage of fatty acids combined in cholesterol esters, triglycerides and phospholipids in the serum fraction for four subjects by day of menstrual cycle and meal frequency shown in Tables 20-22. The mean values

of percentage of fatty acids consuming two and three meals per day were shown in Tables 10-12. The values were expressed as percentages of the total fatty acids which were resolved by gas-liquid chromatography.

The fatty acid which was present in the highest concentrations in cholesterol esters was linoleic acid with palmitic and oleic acids present in second highest concentration (Table 10). These values were quite similar to those reported by the several investigators (Hallgren et al., 1960; Hanahan et al., 1960; Michaels et al., 1957; Luddy et al., 1958; Scott et al., 1964; Swell et al., 1962; Tuna and Mangold, 1963; Tuna et al., 1958). The mean values of oleic acid tended to be high on day 1; myristic, palmitic, and stearic acids were high on day 13 to 15; and linoleic acid was high on day 26 of the menstrual cycle in this study.

Palmitic and oleic acids were present in high concentration in triglycerides followed by stearic acid; with lower amounts of linoleic acid (Table 11). These values were quite similar to values reported by other workers. (Hallgren et al., 1960; Swell et al., 1962; Tuna and Mangold, 1963). Triglycerides contains little or no acids with chain lengths greater than 18-carbons or more polyunsaturated than linoleic acid. Mean values of palmitic and linoleic acids were high on day 1; stearic acid was high on day 13 to 15 and oleic acid was high on day 26 of the menstrual cycle in this study.

In phospholipids, palmitic acid was present in highest concentration followed by stearic acid; oleic as well as linoleic acids were present in the lowest concentration (Table 12). These values agree with those reported by several investigators (Dole et al., 1959; Hallgren, 1960; Scott, 1964 and

Table 10. Mean values of percentage of fatty acids of cholesterol esters consumed two and three meals per day

Day of study	Meals per day	8:0	10:0	12:0	?	14:0	?:	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0
1	2	2.1	2.1	2.0	5.7	2.3	0.5	26.8	4.5		7.2	16.8	28.9		
	3	1.7	2.2	1.3	10.4	2.4	1.8	22.3	4.4		4.3	19.4	29.2	0.9	
	Mean	1.9	2.2	1.7	8.1	2.4	1.0	24.5	4.5		5.8	18.1	29.1	0.4	
13 to 15	2	1.7	3.1	1.7	2.5	4.0	0.6	28.4	3.6	1.3	7.0	16.8	29.8	1.7	
	3	1.3	1.9	1.7	3.9	1.8	0.9	30.0	3.2	0.4	6.9	17.2	29.0	1.0	0.6
	Mean	1.5	2.5	1.7	3.2	2.9	0.8	29.2	3.4	0.9	7.0	17.0	29.4	1.4	0.3
26	2	0.5	1.7	1.7	5.9	2.6	1.8	25.3	4.6	3.0	6.1	13.7	29.6	0.4	0.4
	3	2.9	1.6	1.6	6.9	2.2	0.3	25.2	3.0	1.0	6.7	14.5	32.8	2.2	
	Mean	1.7	1.7	1.7	6.4	2.4	1.1	25.3	3.8	2.0	6.4	14.1	31.2	1.3	0.2

Table 11. Mean values of percentage of fatty acids of triglycerides consumed two and three meals per day

Day of study	Meals per day	8:0	10:0	12:0	?	14:0	?	16:0	16:1	17:0	18:0	18:1	18:2
1	2	1.7	1.7	1.6	3.6	4.9	2.0	35.3	4.4	1.6	14.8	20.0	8.0
	3	1.6	2.7	2.0	3.2	3.3	2.7	35.6	6.7	0.8	14.0	19.2	8.3
	Mean	1.7	2.2	1.8	3.4	4.1	2.4	35.5	5.6	1.2	14.4	19.6	8.2
13 to 15	2	2.5	2.3	2.2	3.9	5.7	1.3	37.1	4.1	0.7	16.6	16.3	7.3
	3	2.5	3.7	2.5	2.0	5.7	2.5	36.9	5.1	1.3	14.5	16.5	5.6
	Mean	2.5	3.0	2.4	3.0	5.7	1.9	37.0	4.6	1.0	15.6	16.4	6.5
26	2	1.8	1.6	1.5	1.7	5.3	2.6	37.1	5.8	1.5	13.6	20.4	7.2
	3	2.2	2.7	2.1	2.7	5.5	2.5	36.5	3.8	1.3	15.0	18.3	7.5
	Mean	2.0	2.1	1.8	2.2	5.4	2.6	36.8	4.8	1.4	14.3	19.4	7.4

Table 12. Mean values of percentage of fatty acids of phospholipids consumed two and three meals per day

Day of study	Meals per day	8:0	10:0	12:0	14:0	?	16:0	16:1	17:0	18:0	18:1	18:2
1	2		0.5	0.7	1.9	0.8	46.2	2.8	1.9	20.2	12.0	13.1
	3	0.6	2.7	2.0	2.9	3.8	43.7	3.5	0.7	18.6	11.9	9.7
	Mean	0.3	1.6	1.4	2.4	2.3	45.0	3.2	1.3	19.4	12.0	11.4
13 to 15	2		0.5	1.5	2.5	1.7	46.6	2.4	0.8	18.6	11.3	14.3
	3	...	0.6	1.1	2,3	0.8	42.4	1.9	...	26.0	11.5	13.5
	Mean		0.6	1.3	2.4	1.3	44.5	2.2	0.4	22.3	11.4	13.9
26	2		0.7	1.0	1.3	2.1	42.7	2.9	1.1	22.3	11.7	14.3
	3	0.9	1.8	2.5	2.7	1.0	43.3	3.4	0.4	17.7	13.7	12.8
	Mean	0.5	1.3	1.8	2.0	1.6	43.0	3.2	0.8	20.0	12.7	13.6

Swell, 1962) except that no arachidonic acid was present in samples of this study. No fatty acids with chain lengths greater than 18-carbons was found in the pattern of phospholipids. Mean values of palmitic acid was high on day 1; oleic and linoleic acids were high on day 13 to 15 and stearic acid was high on day 26 of the menstrual cycle in this study.

Serum Cholesterol Values

Use of a micro-method for analysis of cholesterol required only 0.04 ml of serum (Galloway et al., 1957). Thus, samples could be obtained frequently by finger-tip puncture without causing discomfort to the subject. Hence, analyses for cholesterol were made more frequently during the menstrual cycle than was used for the lipid classes and fatty acid analyses. To cover the middle of the menstrual cycle which would show changes for pre-ovulation, ovulation, and post-ovulation samples were obtained every other day; at the beginning and end of the cycle samples were taken less frequently (see page 13).

Total cholesterol

The overall mean for the 81 analyses was 185 $\pm$ 2.6 mg per 100 ml serum. These results are in agreement with the values reported by other workers. Man and Peters (1933) observed that 12 subjects had total serum cholesterol values about 207 $\pm$ 29 mg. Observation on 118 subjects gave results of 152 $\pm$ 24 mg in Boyd's 1937 study. It was shown by Bohle and Biegler (1958) that serum cholesterol values for 513 men and women were 205 mg per

100 ml. The range of values for the 81 cholesterol determinations were 124 to 282 mg per 100 ml serum or a difference of 158 mg (Table 13). This range was somewhat wider than those found to be normal for adults by other investigators but this study involved many more determinations per subject.

Individual ranges were not as wide; differences between high and low levels varied from 37 to 97 mg (Table 14). One subject had presistently higher values than did the other three (Figure 1). The range of serum cholesterol levels among subjects was reported to be 150 to 280; 150 to 250; 138 to 296 mg per 100 ml by Krupp et al. (1960); West and Todd (1962); Galloway et al. (1957), respectively. Although there are great differences on so called normal serum cholesterol values, the generally accepted range in adults is between 160 and 250 mg per 100 ml (Wilcox et al., 1962). A more recent suggested desirable cholesterol value for persons with coronary disease risk, is well under 200 mg or less (Stamler et al., 1963).

Statistical analysis of the data for the four subjects (Tables 14 and 23) showed that differences between the sampling dates, meals, and the interaction of sampling dates by meals were not significant. Use of more subjects would give more valid statistical treatment and a better measure of the population of young adult women, however, other factors should also be considered. The maximum peak for any one subject may not have been observed because the length of menstrual cycle varied and blood samples were obtained only every second day of this study.

The percentage distribution of four subjects within various ranges of serum total cholesterol values is presented in Table 15. Most of the values (77 percent) were within the range of 140-219 mg per 100 ml of serum.

Table 13. The range of serum cholesterol values for individual subjects

Subjects	No. of meals	Total cholesterol	Free cholesterol	Free in total cholesterol
		mg/100 ml	mg/100 ml	percent
A. M.	2	185-282	46.2-74.6	24.2-28.7
	3	186-254	46.8-66.9	23.1-26.4
C. L.	2	170-187	37.5-50.2	21.8-27.2
	3	180-207	43.1-52.6	21.5-26.6
D. W.	2	158-190	40.8-50.8	25.5-28.7
	3	149-188	40.2-54.0	26.3-29.6
P. B.	2	124-173	28.3-44.2	22.8-26.6
	3	130-153	29.5-38.0	21.0-24.8

Trends are shown by the data from these four subjects but conclusions as to influence of menstrual cycle cannot be drawn from the findings. Total cholesterol levels were slightly lower at the beginning of the menses. No definite peaks were shown but values tended to be higher at days 4 and 11 (Table 16 and Figure 2). Some other workers (Okey et al., 1927, 1933; Kritchevsky, 1958) have reported that the menstrual cycle does affect the concentration of cholesterol in blood.

The subjects had similar ranges of cholesterol values when eating two or three meals a day (Table 16). Individual cholesterol values by meal and day of menstrual period are plotted in Figure 1.

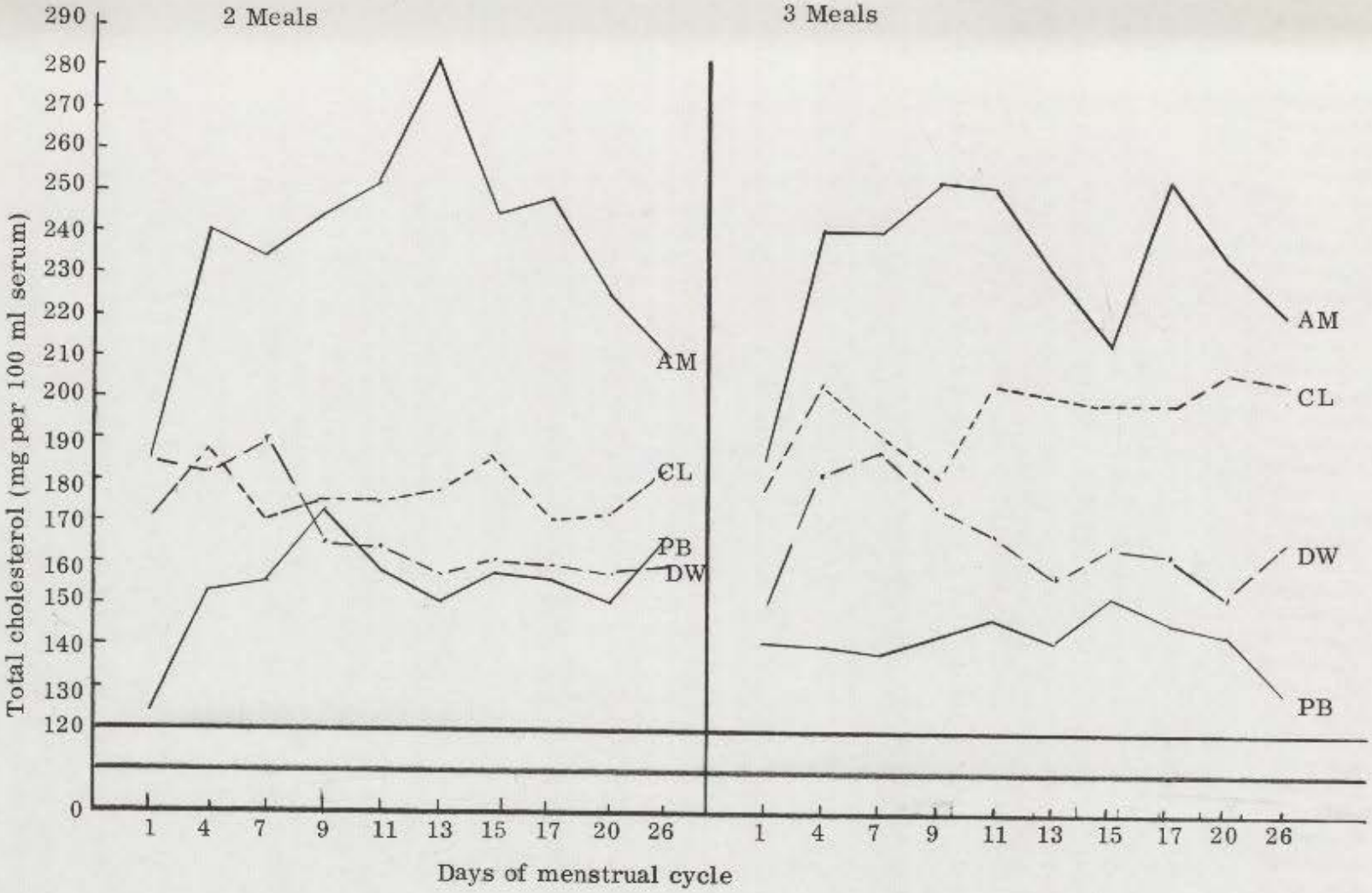

Figure 1. The effect of number of meals by days of menstrual cycle on total serum cholesterol values.

Table 14. Serum total cholesterol values for individual subjects eating two meals and three meals per day

Subjects	No. of meals	Period	Day of study										
			1	4	7	9	11	13	15	17	20	26	Mean
			mg/100 ml serum										
A. M.	2	2	185	240	234	244	253	282	245	249	225	211	237
	3	1	186	241	241	253	252	232	214	254	235	222	223
C. L.	2	2	170	187	170	175	175	178	186	171	172	183	177
	3	1	180	204	192	182	204	202	200	200	207	205	197
D. W.	2	1	185	182	190	165	164	158	161	160	158	160	168
	3	2	149	182	188	175	168	158	165	163	153	166	167
P. B.	2	1	124	153	156	173	159	151	158	156	151	167	155
	3	2	142	141	139	144	148	142	153	147	144	130	143

Table 15. Percentage distribution of four subjects within various ranges of serum total cholesterol values

Range of cholesterol	Number[a]	Percentage
mg/100 ml serum		
120-129	1	1.2
130-139	2	2.5
140-149	8	9.9
150-159	12	14.8
160-169	10	12.3
170-179	9	11.1
180-189	11	13.6
190-199	2	2.5
200-209	7	8.6
210-219	3	3.7
220-229	2	2.5
230-239	3	3.7
240-249	6	7.4
250-259	4	5.0
280-289	1	1.2

[a] Number of individual subjects whose cholesterol values for the different days of the study fell within each range (81 determinations for the four subjects.

Table 16. Mean values of total and free cholesterol in serum of subjects consuming two and three meals per day

Days of mens. cycle	Total cholesterol			Free cholesterol		
	2 meals	3 meals	mean	2 meals	3 meals	mean
	mg/100 ml			mg/100 ml		
1	166±14	164±11	165	41.9±4.6	41.5±3.0	41.7
4	190±18	192±21	191	49.8±4.8	48.6±5.8	49.2
7	188±17	190±20	189	49.1±4.9	49.7±6.1	49.4
9	189±18	189±23	189	50.6±4.5	48.8±5.5	49.7
11	188±22	193±22	191	48.5±4.5	50.0±6.1	49.3
13	192±19	184±20	188	49.5±8.2	43.4±5.2	46.4
15	188±20	184±14	186	47.3±5.8	47.4±4.0	47.4
17	184±21	191±23	188	46.4±6.2	47.3±6.7	46.9
20	177±17	185±21	181	44.1±4.2	43.9±4.5	44.0
26	180±9	181±20	181	46.4±4.6	43.6±4.7	45.0
Mean	184±2.6	185±2.9	185±2.6	47±0.9	46±1.0	47±0.9

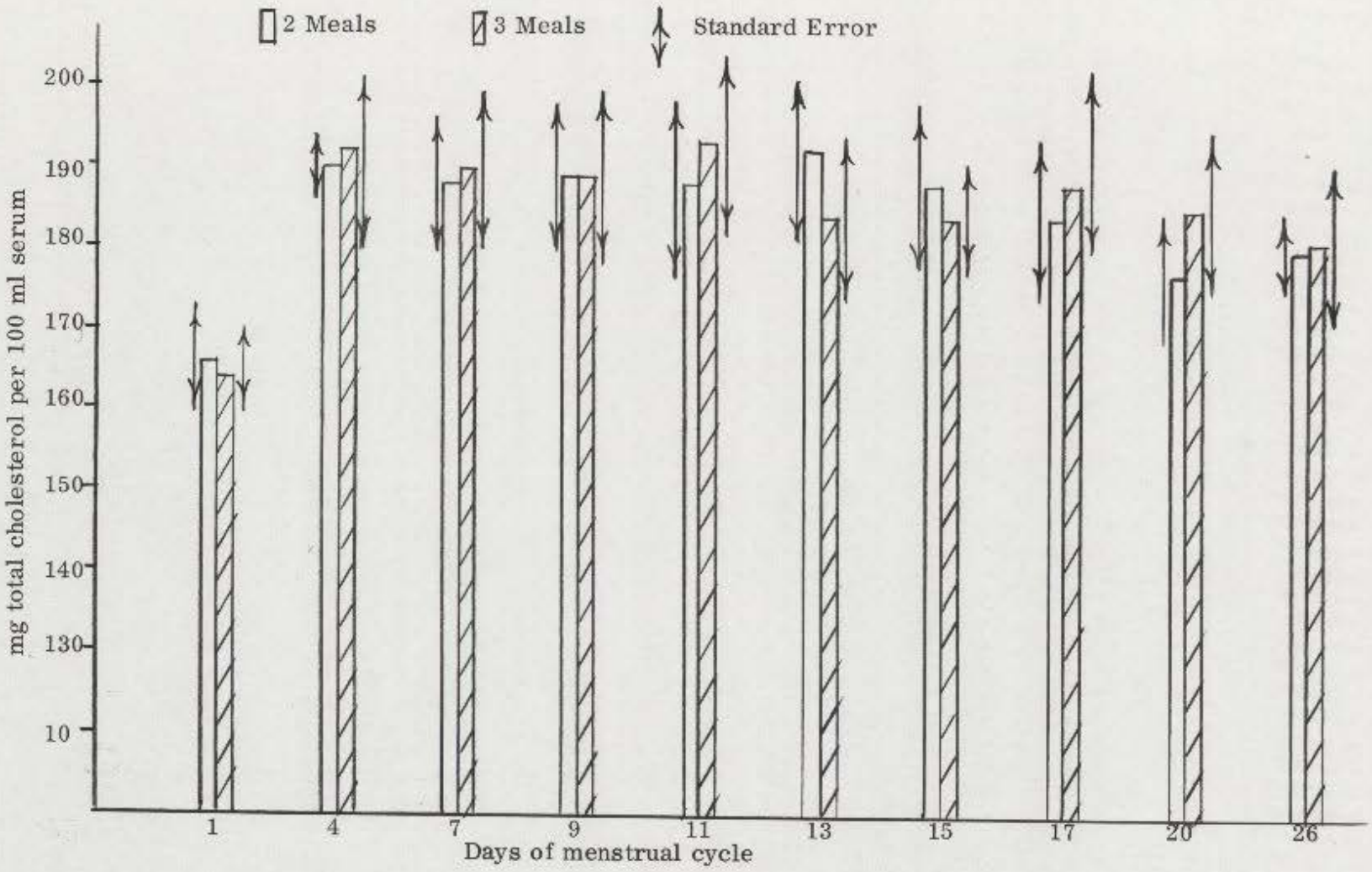

Figure 2. Mean total cholesterol values for four subjects with or without breakfast by days of menstrual cycle.

Free cholesterol

The mean free cholesterol value was 46.9 mg per 100 ml of serum. The data for serum free cholesterol levels were presented in Tables 16 and 24. The range for the percent of free in total cholesterol was found to be from 21.0-26.9 percent (Table 13). This percentage remained fairly constant, although the total and free cholesterol varied considerably. The mean percent of free in total cholesterol was 25.3.

Relationships Between Serum Cholesterol and Urinary Neutral 17-Ketosteroids and 17α, 21-Dihydroxy-20-Ketosteroids

Yam (1966) determined androgen and corticosteroid hormone degradation products in urine of the same subjects. These were measured as neutral 17-ketosteroids and 17α, 21-dihydroxy-20-ketosteroids. Patterns of excretion of these two components and cholesterol are shown in Figure 3 and Table 25. The cholesterol and neutral 17-ketosteroids tended to have a reciprocal relationship. When cholesterol values were highest then the neutral 17-ketosteroids tended to be lower. Cholesterol values were low on day 1 and highest on day 4 and 11 in this study while neutral 17-ketosteroids peaked at day 15. The 17α, 21-dihydroxy-20-ketosteroid values showed less variation and were somewhat lower on day 1.

Table 17. Hematocrit for four subjects

Subject	Day of menstrual cycle										Mean
	1	4	7	9	11	13	15	17	20	26	
A. M.	43.85	43.70	43.53	43.71	46.62	45.25	43.41	44.17	44.46	43.67	44.24
C. L.	44.58	46.51	44.01	46.09	45.60	46.53	46.23	46.94	44.63	43.58	45.47
D. W.	44.52	43.34	45.01	44.58	46.40	44.58	43.42	44.26	45.78	46.20	44.81
P. B.	44.96	43.01	44.56	44.22	43.21	43.27	44.19	45.95	46.30	45.13	44.48

Table 18. Hemoglobin content for four subjects

Subject	Hemoglobin gm/100 ml
A. M.	14.38
D. W.	14.03
C. L.	14.04
P. B.	14.04

Dietary Intake

Diet records were kept for all subjects on each day before blood sampling. In Table 26 is presented the daily intake as calculated of calories, proteins, carbohydrates, fats, saturated fatty acids, unsaturated fatty acids (oleic and linoleic acids), and cholesterol.

The mean caloric intake of the four subjects was 1885 calories per day; 1986 and 1781 calories for 3 meals-per-day and for 2 meals-per-day, respectively. Thus, the caloric intake was 205 calories or 10 percent less on 2 meals a day than on 3 meals. This difference was due to the high percentages of calories from protein (15.2 vs 14.7 percent) and fat (38.4 vs 37 percent) and a slightly increase from carbohydrate (46 vs 48 percent) on 3 meals vs 2 meals per day.

All the subjects had a lower caloric intake than the 2100 recommended by National Research Council (1964), except for one subject who was consuming

Table 19. Mean values of total lipid classes in serum fractions of four subjects of two and three meals on days 1, 13 to 15 and 26 of menstrual period.

Day	No. of Meals	Name	Total lipid	Cholesterol esters	Free cholesterol	Triglyceride plus free fatty acid	Phospho-lipid
			mg/100 ml	percent	percent	percent	percent
1	2	A. M.	713.9	35.2	7.6	26.3	26.1
		C. L.	577.5	40.0	8.7	12.5	38.7
		D. W.	583.7	39.0	8.5	17.1	35.4
		P. B.	556.4	34.6	7.1	24.4	33.9
Mean			608±35	37±1.3	8±0.3	20±3.2	34±2.6
13 to 15	2	A. M.	667.5	39.3	8.5	21.7	32.9
		C. L.	649.4	36.4	7.0	20.0	36.6
		D. W.	534.0	36.8	8.2	21.0	34.0
		P. B.	540.0	36.3	7.8	24.8	31.1
Mean			598±35	37±0.7	8±0.3	22±1.0	34±1.1
26	2	A. M.	676.0	--	--	--	--
		C. L.	612.9	36.5	6.1	21.9	35.4
		D. W.	563.4	36.0	8.1	22.3	33.6
		P. B.	564.0	37.3	7.4	23.1	32.2
Mean			603±14	37±0.3	7±0.3	22±0.3	34±0.8
1	3	A. M.	649.2	38.5	8.5	21.7	31.3
		C. L.	639.7	41.7	7.2	16.7	34.4
		D. W.	595.9	37.0	8.6	20.3	34.1
		P. B.	542.7	33.9	6.5	27.4	32.2
Mean			607±24	38±1.6	8±0.5	22±2.2	33±0.7
13 to 15	3	A. M.	685.7	39.1	8.2	21.7	31.1
		C. L.	637.0	40.0	8.3	15.0	36.7
		D. W.	534.6	37.0	9.2	19.7	34.1
		P. B.	532.8	34.8	6.4	26.6	32.2
Mean			598±38	38±1.1	8±0.5	21±2.4	34±1.2
26	3	A. M.	659.8	33.7	8.0	27.9	30.3
		C. L.	629.5	37.3	6.8	21.9	34.0
		D. W.	570.9	35.8	7.7	22.2	34.3
		P. B.	532.5	34.6	6.2	25.7	33.5
Mean			598±28	35±1.7	7±0.4	24±1.4	33±0.8

Table 20. Percentage of fatty acids of cholesterol esters

Subject	Mens. cycle	Day of cycle	Meals per day	8:0	10:0	12:0	?	14:0	?	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0
A. M.	1		2	4.0	1.1	4.0	3.3	1.9	1.0	25.0	6.2		6.2	15.1	27.8		
	13 to 15			3.1	5.6	2.1		3.6		25.2		2.6	7.5	18.3	26.8	5.3	
		26		0.6	0.8	2.1	6.1	1.3	4.4	24.0	6.2	11.4	3.5	12.9	25.4		
	1		3	3.7	5.6	3.3	5.1	4.7	3.4	21.7	4.9		2.0	16.5	28.3	0.8	
	13 to 15			4.4	5.1	2.9	6.8	3.8	0.7	18.0	1.3	1.5	5.2	16.7	27.3	3.8	2.5
		26		7.1	1.0	2.6	3.4	2.9	1.1	24.0	2.7	4.2	3.4	12.1	30.5	4.9	
C. L.	1		2	3.6	4.1	2.2	1.2	2.7		33.6	4.8		6.7	16.0	25.1		
	13 to 15				2.3	2.0	1.2	1.6		31.2	3.7		7.7	20.1	30.3		
		26			0.8	1.7	1.7	4.4		30.5	4.2		7.2	14.6	34.9		
	1		3	1.0	1.9	0.3	3.9	1.1	1.3	21.0	5.4		5.3	28.4	30.5		
	13 to 15				0.8	0.4	2.5	1.3		28.2	3.3		6.7	20.0	36.7		
		26		2.4	1.6	1.4	5.2	1.1		27.9	2.2		5.9	15.1	37.2		
D. W.	1		2		1.1	1.5	13.9	1.9		22.9	4.2		10.1	17.9	26.4		
	13 to 15			3.5	1.4	2.8	4.6	1.9	1.5	32.3	5.9	0.8	5.9	12.9	25.3	1.3	
		26			1.1	2.0	7.3	3.0	1.6	23.9	5.2	0.7	7.1	13.7	22.3	1.7	1.8
	1		3	1.0	0.6	1.0	20.1	2.1	0.9	22.6	4.7		4.1	16.9	23.3	2.9	
	13 to 15				0.5	2.7	3.1	1.9	0.6	37.0	4.5		7.5	16.1	25.3		
		26		2.1	2.9	1.8	15.1	3.5		22.8	4.2		9.7	13.1	24.7		
P. B.	1		2	0.6	2.1	0.4	4.3	2.6	1.2	25.6	2.6		5.8	18.3	36.5		
	13 to 15				3.0		4.2	1.1	0.8	24.8	4.8	1.6	6.9	15.7	36.9		
		26		1.3	4.2	1.1	8.6	1.8	1.4	22.7	2.9		6.6	13.5	35.9		
	1		3	0.9	0.8	0.4	12.7	1.6	1.1	24.0	2.5		5.6	15.7	34.9		
	13 to 15			0.9	1.2	0.6	3.1		2.5	36.8	3.8		8.1	16.0	26.9		
		26			1.0	0.6	3.9	1.2		25.9	3.0		7.7	17.8	38.9		

Table 21. Percentage of fatty acids of triglycerides

Subject	Day of menstrual cycle	Meals per day	8:0	10:0	12:0	?	14:0	?	16:0	16:1	17:0	18:0	18:1	18:2
A. M.	1	2	3.0	3.8	3.2	3.8	4.5	4.0	30.0	6.5		16.4	16.2	5.9
	13 to 15		3.1	2.2	1.6	2.8	6.6		31.0	2.4		27.4	14.1	8.9
	26		1.8	2.1	1.4	3.7	4.9	1.5	38.9	2.9	4.4	18.0	15.3	5.2
	1	3	0.7	0.7	1.1	7.0	2.8	2.6	30.2	7.8		17.6	20.6	8.8
	13 to 15		2.3	2.6	2.2	3.7	7.3	2.4	30.3	2.1	5.1	25.1	13.7	4.2
	26		1.2	2.9	1.5	6.5	8.6	2.0	32.8	2.1		21.9	13.6	6.8
C. L.	1	2	0.8	1.1	1.1	3.5	1.3		27.3	3.8		13.8	30.2	7.2
	13 to 15		0.9	0.5	0.7	7.2	2.8	0.7	37.8	4.4		9.9	28.0	7.1
	26		0.7	0.3	0.4	1.4	4.1	1.6	34.4	8.4		11.2	30.3	7.1
	1	3	1.1	1.3	1.6	3.6	2.6	2.5	36.8	6.3	3.3	9.0	25.8	6.1
	13 to 15		1.8	2.0	0.6		5.7	1.5	38.9	6.1		12.2	19.2	6.5
	26		3.0	3.1	1.8	1.2	4.3	1.6	34.9	5.7		10.7	25.6	8.2
D. W.	1	2	2.1	1.0	1.3	2.6	7.2	3.8	42.3	3.0	1.8	13.7	16.4	4.9
	13 to 15		3.5	2.9	2.9	1.8	7.9	4.5	43.3	4.8	2.6	12.6	8.7	4.3
	26		2.4	1.9	1.4	0.7	7.6	4.8	40.2	6.0		11.0	15.8	8.2
	1	3	1.1	3.9	1.7		4.5	2.9	38.4	8.3		14.2	18.4	6.7
	13 to 15		2.8	4.6	3.9	1.5	4.9	3.3	42.5	8.3		8.6	12.5	7.2
	26		2.7	3.0	2.8	2.2	6.3	2.5	41.4	2.1	2.8	13.2	12.9	8.2
P. B.	1	2	0.8	0.9	0.8	4.5	6.5		31.7	4.1	4.4	15.1	17.1	14.1
	13 to 15		2.5	3.4	3.6	3.7	5.4		36.4	4.9		16.6	14.5	8.9
	26		2.1	2.2	2.6	1.1	4.4	2.5	34.9	5.9	1.6	14.2	20.1	8.2
	1	3	3.4	4.9	3.6	2.0	3.4	2.9	36.8	4.5		15.1	11.9	11.4
	13 to 15		2.9	5.5	3.3	2.8	5.0	2.6	35.7	4.1		12.0	20.6	4.5
	26		1.7	1.8	2.1	0.9	2.7	3.9	37.0	5.4	2.7	14.0	21.0	6.8

Table 22. Percentage of fatty acids of phospholipids

Subject	Day of menstrual cycle	Meals per day	8:0	10:0	12:0	14:0	?	16:0	16:1	17:0	18:0	18:1	18:2
A. M.	1	2		0.7	0.7	2.0		43.0	1.5	4.4	20.2	17.1	10.5
	13 to 15				2.9	4.8	3.3	38.9	2.4		16.2	15.4	16.2
	26			1.0	1.0	1.9	3.2	40.0	1.7		23.0	14.6	13.6
	1	3		0.8	2.1	5.3	5.8	36.9	5.8		19.1	12.1	12.1
	13 to 15			1.2	1.8	3.6		47.2			20.6	12.7	12.9
	26		2.6	2.6	6.6	3.1		44.9	3.1		15.0	14.1	7.9
C. L.	1	2				0.8	1.3	46.8	2.0		20.8	10.0	18.4
	13 to 15			1.8	3.0	1.9		54.5	3.3		14.8	9.2	11.6
	26			1.3	3.1	2.2	3.7	48.6	4.1	1.8	16.6	8.9	9.7
	1	3	2.3	5.0	2.7	2.4		49.2	1.0		14.4	13.4	9.7
	13 to 15				1.4	2.0	1.3	41.8	1.0		26.1	13.0	13.3
	26				1.7	1.9		42.2	4.4		23.6	13.2	13.0
D. W.	1	2		1.4	1.1	1.6	0.7	49.5	5.8		16.9	12.3	10.7
	13 to 15					1.9	2.7	47.9	2.9	0.9	19.5	8.4	15.7
	26			0.3		0.7	0.7	42.5	2.6	2.1	20.7	13.4	17.0
	1	3		4.1	1.8	1.6	6.9	43.6	2.7	3.0	20.2	9.7	6.5
	13 to 15			1.1	1.1	1.9		42.2	4.4		29.3	12.2	7.8
	26		1.0	4.5	1.0	3.5	3.0	52.9	2.6		13.0	12.4	6.1
P. B.	1	2			0.8	3.1	1.3	45.5	1.9	3.1	22.9	8.5	12.9
	13 to 15					1.3	0.8	45.2	0.9	2.2	23.7	12.1	13.8
	26					0.4	0.9	39.5	3.0	0.6	29.0	9.9	16.7
	1	3		1.0	1.5	2.2	2.4	45.0	4.6		20.8	12.2	10.3
	13 to 15					1.8	1.8	38.4	2.0		28.2	7.9	20.0
	26				0.5	2.2	0.8	33.2	3.3	1.5	19.3	14.9	24.2

Table 23. Summary of mean squares obtained by analysis of variance of data on serum cholesterol

Source of variance	d. f.	Mean squares		Percentage of free in total cholesterol
		Total cholesterol	Free cholesterol	
Meals	1	332.11	0.0031	3.20
Experimental error	6	11,981.21	867.6520	20.46
Period	9	293.62	44.6945	2.94
Period vs. meals	9	248.83	47.3431	4.02
Sampling error	54	446.65	32.2767	3.50
Total	79	1,281.27	98.4455	4.78

2348 calories on 3 meals. However, this difference in the caloric intake is not great enough to effect the study.

The diets appear to be well balanced. Nutritionists recommend for a well balanced diet that the total calories be eaten as protein, should be 10 to 15 percent; as carbohydrates, 48 to 53 percent; and the rest as fat. When individual dietary intakes were considered, all subjects were consuming diets that were within the upper range for protein (13 to 17 percent of total calories as protein); adequate in carbohydrate for two subjects (48 to 56 percent), somewhat low carbohydrate intake for two subjects (43 and 44 percent); and medium to high fat intake (31 to 42 percent). The linoleic content of the diet was 5.9 and 5.7 percent of the total calories on 2 and 3 meals, respectively. The ratio of polyunsaturated fatty acids to saturated fatty acids was 0.45. The

Table 24. Serum free cholesterol values for individual subjects consuming two meals and three meals per day

Subjects	No. of meals	Period	Day of menstrual cycle 1	4	7	9	11	13	15	17	20	26	Mean
			mg/100 ml serum										
A. M.	2	2	46.2	62.4	62.3	62.9	61.3	74.6	61.0	65.0	56.6	60.5	61.2
	3	1	46.8	63.2	62.0	61.5	63.9	55.2	56.1	66.9	54.4	52.3	58.2
C. L.	2	2	43.4	50.2	44.3	47.7	43.6	44.2	45.4	37.5	37.5	42.6	43.6
	3	1	44.2	46.1	50.3	48.4	52.6	45.1	51.6	43.1	47.2	45.9	47.5
D. W.	2	1	49.8	47.8	50.8	47.6	46.9	44.0	44.0	44.0	43.5	40.8	45.9
	3	2	42.0	49.8	54.0	50.8	49.7	43.7	43.8	43.0	40.2	45.9	46.3
P. B.	2	1	28.3	38.9	38.9	44.2	42.3	35.3	38.9	38.9	38.7	41.5	38.6
	3	2	32.9	35.1	32.3	34.6	34.0	29.5	38.0	36.0	33.7	30.1	33.6

Table 25. The relationship between serum cholesterol and urinary neutral 17-ketosteroids and 17α,21-dihydroxy-20-ketosteroids for specified days of the menstrual cycle.

Day	Neutral 17-ketosteroids	17α,21-dihydroxy-20-ketosteroids	Cholesterol
	mg/24 hr	mg/24 hr	mg/100 ml
1	4.45	3.40	165
4	5.16	3.90	191
7	5.51	4.19	189
9	5.88	4.27	189
11	5.61	4.21	191
13	5.95	4.26	188
15	7.24	4.24	186
17	5.42	3.82	188
20	4.69	3.78	181
26	4.02	3.71	181

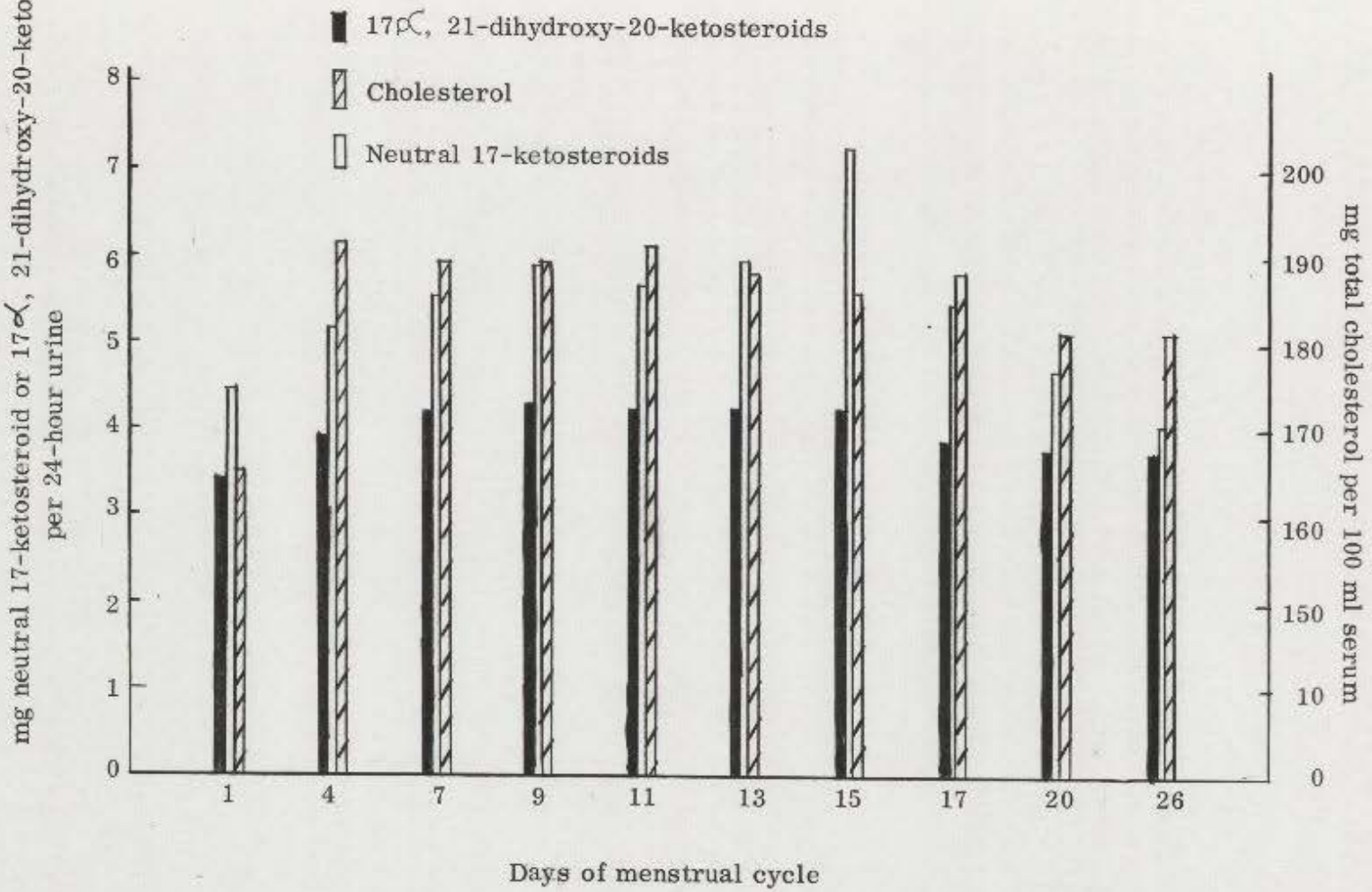

Figure 3. Serum cholesterol and urinary neutral 17-ketosteroids and 17α, 21-dihydroxy-20-ketosteroids by days of menstrual cycle

Table 26. Mean daily intake of certain nutrients of four subjects on self-selected diets

Name of subjects	Total calories	Carbohydrate	Protein	Total fat	Total saturated fatty acid	Unsaturated fatty acid		Dietary cholesterol
						Oleic	Linoleic	
			Two meals per day					
		Gm.	Gm.	Gm.	Gm.	Gm.	Gm.	Mg.
A. M.	1711	192	65	76	28	28	11.3	289
D. W.	1862	259	62	64	21	26	12.6	294
C. L.	1730	207	65	71	29	32	11.4	434
P. B.	1821	205	70	83	32	28	11.5	398
Mean	1781	216	65	74	27	29	11.7	354
Percentage of total calories		48.5	14.7	37	13.6	14.6	5.9	
			Three meals per day					
A. M.	2348	253	85	109	36	52	22.4	328
D. W.	1745	218	74	64	30	37	5.3	411
C. L.	2013	244	71	84	34	34	10.9	439
P. B.	1847	205	73	82	36	32	7.2	383
Mean	1986	230	76	85	34	34	11.5	390
Percentage of total calories		46.3	15	38.4	15.4	15.4	5.7	

dietary cholesterol intake ranged was 289 to 434 mg and 238 to 439 mg daily, on 2 and 3 meals per day, respectively.

The dietary pattern which is recommended by Stamler et al. (1963) to prevent altherosclerosis and coronary heart disease is to use diets that contain moderate intakes of the following nutrients, thus decreasing the quantity in the usual American diet:

1. Calories, 1700
2. Total fat, 30 instead of 45 percent
3. Saturated fatty acids, 20 instead of 44 g; 10 instead of 17 percent
4. Dietary cholesterol, 300 instead of 600 mg
5. P/S (polyunsaturated to saturated fatty acids) ratio, approaching 1.0 rather than 0.3 to 0.4

Also redommended are diets high in good quality protein, vitamins, and minerals.

In this study the number of meals was not free to influence body weight because the subjects were asked to maintain a constant weight variation in body weight was within five pounds.

SUMMARY AND CONCLUSIONS

Summary

Four university women students while on self-chosen diets and living under home living conditions served as subjects in a study of changes in serum fatty acid compositions during the menstrual cycle. Effect of meal frequency (3 meals vs 2 meals with no breakfast) was also included in this study.

The test periods consisted of two complete menstrual cycles for each subject (28 to 34 days). Finger-tip blood were taken on days 1, 4, 7, 9, 11, 13, 15, 17, 20, 26 and at day 1 of the next cycle for total and free cholesterol analyses. The concentrations of total lipids, the distributions of lipid classes, and the fatty acid compositions of cholesterol esters, triglycerides, and phospholipids were determined in larger samples of serum obtained from finger-tip puncture on days 1, 13 to 15, and 26.

A dietary record was kept by each subject on the day prior to blood sampling. Calculated dietary data showed that the mean caloric intake was 1885 calories per day. The percentages of calories consumed as protein was 12-17; carbohydrates, 48; and fat, 31-42.

Basic data on serum total lipids, distribution of lipid classes, and composition of fatty acids in lipid fractions during the menstrual cycle were obtained on healthy young women living under home conditions. Findings included the following:

1. The overall mean value for total lipids was 602 ± 29 mg per 100 ml which is within the normal range cited by others. The menstrual cycle influenced total lipid levels to a greater degree than did the number of meals per day in this study. Day of menstrual cycle appeared to have a slight influence on total serum lipid values. The values for the middle of the cycle (day 13 to 15) were somewhat lower than that for the other days.

2. The percentages obtained for the several lipid classes and for the fatty acid composition of each class or serum lipids were similar regardless of meal frequency.

3. The composition of the major fatty acids of lipid fractions but not percentages of lipid classes were influenced by day of menstrual cycle. In cholesterol esters, oleic acid tended to be high on day 1; myristic, palmitic, and stearic acids, on day 13 to 15; and linoleic acid, on day 26 of the cycle. In triglycerides, palmitic and linoleic acids were high on day 1; stearic acid, on day 13 to 15; and oleic acid, on day 26 of the cycle. In phospolipids, palmitic acid was high on day 1; oleic and linoleic acids, on day 13 to 15; and stearic acid, on day 26 of the cycle.

4. Statistical analysis of the cholesterol data for the four subjects showed that differences between the day of menstrual cycle, meal frequency, and the interaction of sampling dates by meals were not significant. Use of more subjects would give more valid statistical treatment and a better measure of the population of young adult women.

Conclusions and Comments

Basic information was obtained on changes in the composition of fatty acids in serum during the menstrual cycle of young healthy women living under home living conditions and consuming self-chosen diets.

The menstrual cycle appeared to have no influence on lipid patterns but changes were noted in fatty acid patterns for the major fatty acids at different times during the menstrual cycle. The finding of different fatty acid patterns for the major fatty acids on the days of the menstrual cycle that were sampled is of interest. Although the differences were not great, further work should be done to establish whether these variations normally accompany the beginning of the menses, ovulation, and at the end of the cycle. Additional subjects plus additional days of the cycle are needed. Variations in fatty acid patterns in this study included the following: on day one of the cycle oleic was high in the cholesterol esters (CE) fraction, palmitic and linoleic in the triglycerides (TG), and palmitic in the phospholipids (PL); on days 13 to 15, myristic, palmitic and stearic in CE, stearic in TG, oleic and linoleic in PL; on day 26, linoleic in CE, oleic in TG, and stearic in PL.

Cholesterol data did not show statistical differences between the day of menstrual cycle, meal frequency, and the interaction of sampling dates by meals.

The variation between the individual subjects was quite great in this study. Further work using a greater number of subjects, analyses for more days to obtain maximum peaks, and selection of subjects who have menstrual cycles of the same length, such as 28 days, are recommended to clearly

identify changes of serum lipid metabolism during the menstrual cycle. Relating these basic data to endocrine excretion values should give information needed for future studies relating abnormal metabolism of lipids to atherosclerosis and coronary heart disease.

LITERATURE CITED

Adlersberg, D. 1958. Influences of hormones on circulating lipids. p. 301-313. In G. Pincus (ed.). Hormones and atherosclerosis. 1959. Academic Press, New York.

Aftergood, L., and R. B. Alfin-Slater. 1965. Dietary and gonadal hormone effects on lipid metabolism in the rat. Journal of Lipid Research 6:287-294.

Albrink, M. J. 1965. Diet and cardiovascular disease. Journal of the American Dietetic Association 46:26-29.

Albritton, E. C. 1952. Standard values in blood. W. B. Saunders Company, Philadelphia, Pennsylvania. 199 p.

Bohle, E., and R. Biegler. 1958. The relation between increased lipids, constitution, and age in arteriosclerosis. Medixinsche 664:70.

Boyd, E. M. 1935. Diurnal variations in plasma lipids. Journal of Biological Chemistry 110:61-67.

Boyd, E. M. 1937. The interaction of blood lipids. Canadian Journal of Research 15(D):1-25.

Bragdon, J. H. 1951. Colorimetric determination of blood lipids. Journal of Biological Chemistry 196:513-517.

Cohn, C. 1961. Meal eating, nibbling and body metabolism. Journal of the American Dietetic Association 38:433.

Cohn, C. 1964. Feeding patterns and some aspects of cholesterol metabolism. Federation Proceedings 23:76-81.

Cook, R. P. (ed.). 1958. Cholesterol chemistry, biochemistry, and pathology. Academic Press, Incorporated, Publishers, New York. 542 p.

Deuel, H. J., Jr. 1955. The lipids, their chemistry and biochemistry. Vol. 2. Interscience Publishers, Incorporated, New York. 919 p.

Dole, V. P., A. T. James, J. P. W. Webb, M. A. Rizack, and M. F. Sturman. 1959. The fatty acid patterns of plasma lipids during alimentary lipemia. Journal of Clinical Investigation 38:1544-1554.

Feldman, E. B., P. Bendel and R. V. Nayak. 1963. Physiologic factors influencing circulating triglyceride concentration in women: age, weight gain, and ovarian function. Journal of Laboratory and Clinical Medicine 62:437-448.

Galloway, L. S., P. W. Nielson, E. B. Wilcox, and E. M. Lantz. 1957. Microdetermination of cholesterol by use of 0.04 ml of blood serum. Clinical Chemistry 3(4):226-232.

Gertler, M. M., and S. M. Garn. 1950. Lipid interrelationship in health and in coronary artery disease. Science 112(1):14-16.

Hallgren, B. O., S. Stenhagen, A. Svanberg, and L. Svennerholm. 1960. Gas chromatographic analysis of the fatty acid composition of the plasma lipids in normal and diabetic subjects. Journal of Clinical Investigation 39:1424-1434.

Hanahan, D. J., R. M. Watts and D. Pappajohn. 1960. Some chemical characteristics of the lipids of human and bovine erythrocytes and plasma. Journal of Lipid Research 1:421-432.

Hollifield G., and W. Parson. 1962. Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. Journal of Clinical Investigation 41:245-249.

Kornerup, V. 1950. Concentrations of cholesterol, total fat and phospholipid in serum of normal man. Archives of Internal Medicine A.M.A. 85:398-415.

Kritchevsky, D. 1958. Cholesterol. John Wiley & Sons Co., Incorporated. New York and London. 291 p.

Kritchevsky D. 1962. Cholesterol and Atherosclerosis. American Journal of Clinical Nutrition 10:269-276.

Krupp, S. 1960. Physician's Handbook. Eleventh edition. Lange. (Original not seen; Abstracted in Harper, H. A. 1961. Review of physiological chemistry eighth edition. Lange Medical Publications, Los Altos, California. 132 p.).

Lowry, O. H., N. R. Robert, K. Y. Kevner, M. L. Wu, and A. L. Farr. 1954. The quantitive histochemistry of brain. I. Chemical methods. Journal of Biological Chemistry 207:1-17.

Luddy, F. E., R. A. Barford and R. W. Riemenschneider. 1958. Fatty acid composition of component lipids from human plasma and atheromas. Journal of Biological Chemistry 232:843-851.

Lund, J. C., E. Sivertssen and H. C. Godal. 1961a. Studies on serum lipids. I. Healthy individuals. Acta Medica Scandinavica 169:623-627.

Lund, J. C., E. Sivertssen and H. C. Godal. 1961b. Studies on serum lipids. II. Patients with myocardial infraction. Acta Medica Scandinavica 169:629-635.

Maier, R. and H. L. Mangold. 1964. Thin-layer chromatography. 369 p. In: C. N. Reilley (ed.). Advances in Analytical Chemistry and Instrumentation. New York, Wiley.

Man, E. B. and J. P. Peters. 1933. Gravimetric determination of serum cholesterol adapted to the Man and Gildea fatty acid method, with a note on the estimation of lipid phsophorus. Journal of Biological Chemistry 101:685-695.

Micheals, G. D., P. Wheeler, G. Fukeyama, and L. W. Kinsell. 1958. Plasma cholesterol fatty acids in human subjects as determined by alkaline isomerization and by gas chromatography. Annals of the New York Academy of Sciences 72:633-640.

Okey, R., and R. E. Boyden. 1927. Studies of the metabolism of women. III. Variations in the lipid content of blood in relation to the menstrual cycle. Journal of Biological Chemistry 72:261-281.

Okey, R. and D. Stewart. 1933. Diet and blood cholesterol in normal women. Journal of Biological Chemistry 99:717-727.

Okey, R., G. Sheier, and R. Reid. 1960. Food restriction and cholesterol metabolism. Journal of American Dietetic Association 36:441-446.

Oliver, M. F., and G. S. Boyd. 1958. Hormonal aspects of coronary artery disease. Vitamins & Hormones 16:148-178.

Page, I. H., E. Kirk, W. H. Lewis, Jr., W. R. Thompson, and D. D. Van Slyke. 1935. Plasma lipids of normal men at different ages. Journal of Biological Chemistry 111:613-639.

Scott, R. F., K. T. Lee, D. N. Kim, E. S. Merrison, and F. Goodale. 1964. Fatty acids of serum and adipose tissue in six groups eating natural diets containing 7 to 40 per cent fat. American Journal of Clinical Nutrition 14:280-290.

Smith, E. 1961. Concentration of free and total cholesterol in human blood fractions. Master's thesis. Corvallis, Oregon State University. 73 numbered leaves.

Smith E. 1965. The lipids in human blood fractions. Ph. D. thesis. Corvallis, Oregon State University. 120 numbered leaves.

Stamler, J., D. M. Berkson, Q. D. Young, H. A. Lindberg, Y. Hall, L. Mojonnier, S. L. Andelman. 1963. Diet and serum lipids in atherosclerotic coronary heart disease. The Medican Clinics of North American. 47:3.

Stoffel, W., F. Chu and E. H. Ahrens, Jr. 1959. Analysis of long chain fatty acids by gas-liquid chromatography. Analytical Chemistry 31:307-308.

Svanborg, A. and L. Svennerholm. 1961. Plasma total lipids, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. Acta Medica Scandinavica 169:43-49.

Swell, L., P. E. Schools, Jr. and C. R. Treadwell. 1962. Correlation of diet and serum cholesterol level with serum lipids fatty acid composition. Society for Experimental Biology and Medicine, Proceedings 109:682-685.

Tuna, N., L. Reckers and I. D. Frantz, Jr. 1958. The fatty acids of total lipids and cholesterol esters from normal plasma and atheromatous plaques. Journal of Clinical Investigation 37:1153-1165.

Tuna, N. and H. K. Mangold. 1963. Fatty acids of the atheromatous plaque. In: Evoluation of the atherosclerotic plaque. R. J. Jones (ed.). Chicago, University of Chicago Press.

Watt, B. K. and A. L. Merrill. 1963. Composition of foods--raw, processed, prepared. U. S. Department of Agriculture. Agricultural Handbook. No. 8 (Revised Edition).

Waxler, S. H. and L. S. Craig. 1964. Lipid, cholesterol and triglyceride levels in obese women. American Journal of Clinical Nutrition 14:128-132.

West, E. S. and W. R. Todd. 1957. Textbook of Biochemistry. The Macmillan Company, New York. 884 p.

Wilcox, E. B., B. Howthorne, R. Okey. 1962. Cholesterol problems. California Agricultural Experiment Station. Bulletin 785. 4 p.

Wilmot, V. C. and R. L. Swank. 1952. The influence of low-fat diet on blood lipid levels in health and in multiple sclerosis. American Journal of the Medical Sciences 223:25-34.

Yam, S. S. 1966. Neutral 17-ketosteroid and 17α, 21-dihydroxy-20-ketosteroid excretion in urine of university women during the menstrual cycle. Unpublished MS thesis. Utah State University Library, Logan. 32 p.

APPENDIXES

Appendix A

Extraction of Total Lipids From Serum

Total lipids were extracted from the serum by the method of Smith (1965). The frozen serum samples were incubated at 37 C in a water bath for 30 minutes, cooled to room temperature and mixed gently with a buzzer. One volume of serum was added dropwise to 20 volumes of chloroform-methanol (2:1 v/v) in a beaker while the solvent was kept in motion by use of a magnetic stirrer. The lipid extract was filtered through fat-free glass wool into an Erlenmeyer flask. The precipitate was washed into the beaker with 20 volumes of fresh solvent, boiled, cooled, and filtered into the original filtrate. Twenty percent by volume of redistilled water was added to the lipid extract and mixed thoroughly. The flask was stoppered and allowed to stand until two distinct layers clearly separated. The water layer on the top was removed by aspiration and discarded, and the solvent was dried by adding amhydrous sodium sulfate to the lipid extract. The flask was flushed with nitrogen and stoppered with a cork stopper and was then held overnight at -5 C.

Next day, the lipid extract was filtered through fat-free glass wool into a 25 x 175 mm tube to remove the sodium sulfate. The solvent was evaporated to dryness under a stream of nitrogen gas. The lipid was immediately dissolved in a small volume of petroleum ether and was quantitatively

transferred to a mone-milliliter volumetric flask. The total lipid extracts were used for following analysis:

1. Two hundred microliters were transferred into a 10 ml volumetric flask for total lipid determination.
2. Duplicate 40 microliters were transferred into two 1 ml volumetric flasks for cholesterol analysis.
3. Twenty microliters were transferred into a 1 ml volumetric flask for lipid phosphorus analysis.
4. The remaining were stored in freezer for fatty acid analysis.

Appendix B

Determination of Total Lipids

The method of Bragdon (1951) was used for total lipid determination.

Reagent

Twenty grams of potassium dichromate crystals, c. p. was powdered in a mortar and added slowly with shaking to 1000 ml of sulfuric acid, c. p. (sp. ge. 1.84) maintained at a temperature not exceeding 100 C. There should be no dissolved residue. If the reagent is protected from contamination and from exposure to direct sunlight, itdarkens only very slowly with age.

Procedure

Two hundred microliters of lipid extracts in the 10 ml volumetric flasks were evaporated under nitrogen (water pumped) at a temperature under 60 C, and 4 ml of the reagent were added rather promptly. The flasks were then stoppered, sealed with a drop of reagent, and placed in boiling water for 30 minutes. An appropriate blank was similarly prepared. The contents of the flask were cooled in water and about 5 ml of redistilled water was added. The flasks were restoppered, recooled, and brought to final volume in a water bath at 25 C.

Colorimetry

Colorimetry was performed with filtered light of predominantly 580 mu using a Junior Coleman Model 6 B to measure the amount of dicromate reduced. Four ml of color reagent, similarly heated and diluted, was used as the reference blank. The color has been shown to be stable between 15 and 90 minutes after dilution. After checking color development at six time periods, 30 minutes was selected to use in this study. Oleic acid was used as the standard and treated the same as unknown. A standard curve was prepared by using different concentrations of oleic acid, and the slope of the curve was determined.

Calculations

Mg of total lipid per 100 ml of serum =

$$\frac{D_u}{D_s} \times \text{factor} \times \frac{100}{\text{volume of unknown}}$$

D_u : corrected optical density of unknown

D_s : optical denisty of the standard

factor: slope of standard curve

Appendix C

Determination of Lipid Phosphorous

The method of Lowry et al. (1954) as modified by Smith (1961) was used for lipid phosphorous determination.

Reagents

1. Ashing mixture : 6.5 ml of 70 percent percholric acid was mixed with 12.5 ml of 20 N sulfuric acid and diluted to 50 ml with redistilled water.
2. Phosphate color reagent : 0.25 percent ammonium molybdate in in 0.1 N sodium acetate, to which was added just before use 1 ml of 10 percent ascorbic acid per 10 ml of solution. The ascorbic acid was dissolved in redistilled water just before use. After addition of ascorbic acid, the solution was kept in ice water and used within an hour.
3. Stock standard solution of phosphate in water, 10 mM stock solution: 680 mg KH_2PO_4 dissolved and made up to 500 ml with water.
4. Working standard solution of phosphate, 0.2 mM solution: 2 ml of stock solution diluted to 100 ml with water.

Procedure

Twenty microliters lipid extract in the 1 mililiter volumetric flask was made up to volume by petroleum ether. Triplicate 100 microliters of this diluted sample were pipetted into the 7 x 70 mm tubes. The tubes were placed in order in the racks with one space between each tube to assure more uniform heating and the solvent was evaporated . Eighty microliters of ashing mixture was added to each tube, mixed by gentle tapping, and dried for 2 hours in an oven maintained at 95 C measured near the rack of tubes. After 1 hour the tubes were removed and tapped gently to wash down any adhering predipitate on the sides of the tubes and returned the tubes to the oven for the remainder of the drying period. At the end of the 2 hour drying period, the butes were held over-night in a desiccator. The samples were ashed for 2 hours in a 165 C oven, cooled to room temperature, 400 microliters of ice-cold phosphate color reagent were added to each tube while the tube was held in an ice bath. The tube was buzzed immediately and thoroughly, tapped with parafilm and incubated at 37 C for 2 hours. At the end of color development period, the tubes were buzzed and transferred to microcuvettes. Optical densities were measured at a wave length of 820 mμ (without filter) on the Beckman DU Spectrophotometer against a reference solution of 0.1 N sodium acetate. Ten microliters of 0.2 mM phosphate solution was used as the standard. Triplicate 10 microliters of redistilled water, which served as the standard blank, were placed at the beginning of sample tubes.

Calculation

Mg of lipid phosphorous per 100 ml of serum =

$$\frac{\text{density of unknown} - \text{density of blank}}{\text{density of standard}} \times \text{mg phosphorous in standard} \times \frac{100}{\text{volume of unknown}}$$

Mg of phospholipid per 100 ml serum = mg lipid phosphorous x 25

Appendix D

Determination of Fatty Acids in Serum Lipid Classes

Reagents

1. Silica Gel H[1]
2. Iodine
3. Acetone, reagent grade; redistilled
4. Glacial acetic acid
5. Skelly Solve B (petroleum ether), distilled and the fraction distilling between 66-69 C was collected.
6. Diethyl ether.
7. Developing solvent: petroleum ether-diethyl ether glacial acetic acid (80/20/1, v/v/v).
8. Methanol, absolute. Methanol was distilled over potassium hydroxide.
9. Chloroform, reagent grade.
10. Nitrogen gas.
11. Lipid standards for thin-layer chromatography.[2]

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[1]Brinkman Instruments Inc., Cantiague Road, Westbury, New York, 11590.

[2]The Hormel Institute, University of Minnesota, 801, 16th Ave., N. E. Austin, Minnesota.

Procedure

The method used for the separation of total lipids into lipid classes in this study was by thin-layer chromatography of Maier and Mangold (1964). The glass plates were washed with acetone to remove any dust or contaminating materials just before use. Silica Gel H was used as the absorbent for coating the glass plates, and the volume of slurry required to coat five 20 x 20 cm plates was 27 gm Silica Gel H and 72 ml redistilled water. The coated plates were activated by heating in an oven at 110 C for two hours. The coated plates were developed with chloroform before using. The sample of total lipid, dissolved in petroleum ether, was applied to coated plate with a micro pipet at a point approximately two cm from the edge of the plate at right angles to the direction in which the plates were coated. A lipid standard prepared for thin-layer chromatography was applied to coated plate layer as a marker for identifying the lipid classes. Immediately after the sample was applied, the plate was placed in a developing tank which previously had been saturated by adding 100 ml of developing solvent. Saturation was accomplished by lining the tank with a filter paper wick.

When the solvent front had reached a pre-determined mark, the plate was removed from the chamber, air dried, and quickly placed in an iodine atmosphere until the lipid components became visible (yellow spots). The plate was removed from the iodine atmosphere, the areas of particular lipid classes were located, and the portion of Silica Gel H containing the respective lipid component was immediately scraped off into an Erlenmeyer flask containing eluting solvent. Diethyl ether was used for the elution of cholesterol esters and triglycerides. Methanol was used for the elution of phospholipids.

The Silica Gel H lipid mixture was filtered through a sintered glass filter into a 20 x 150 mm tube. The flask was rinsed several times with solvent to insure quantitative transfer of the lipid. The solvent was evaporated just to dryness in a nitrogen atmosphere while the tube was held in a warm water bath. The lipid was dissolved in petroleum ether and quantitatively transferred to a 12 x 100 mm tube, and the volume of solvent was concentrated to less than 0.5 ml. The individual lipid components were re-chromatographed on activated and washed plates, developed, isolated, and eluted from the Silica Gel H as previously described. The sample was filtered and the solvent was evaporated. The lipid was dissoved in petroleum ether and quantitatively transferred to a 12 x 100 mm tube which was stoppered with a cork stopper, and the sample was held at -5 C until the time of preparation for gas-liquid chromatographic analysis.

Gas-Liqyid Chromatographic Analysis of Methyl Esters of Fatty Acids

Reagents

1. Methanol, absolute. Methanol was distilled over potassium hydroxide.
2. Hydrochloric acid in superdry methanol, 5 percent solution. A weighed amount of hydrochloric acid gas was bubbled into a weighed amount of methanol.
3. Benzene, distilled.
4. Skelly Solve B (petroleum ether) was distilled and the fraction distilling between 66-69 C was collected.

5. Chloroform, distilled. Five-tenths to one percent ethanol was added as a preservative. Redistilled chloroform was stored in the cold.
6. Nitrogen gas.
7. Sodium sulfate, anydrous.
8. Lipid standards for gas-lipid chromatography.[3]

Procedure

The method used in this study was a modification of the method of Stoffel, Chu and Ahrens (1959) was described by Smith (1965).

The lipid samples to be methylated, 1 to 3 mg, were dissolved in a mixture of 4 ml of 5 percent hydrochloric acid in superdry methanol and 2 ml of dry benzene in a microsublimation tube to which a condenser was connected. Boiling chips were used to prevent bumping of the solvents. The tubes were placed in a heating block and the mixture was refluxed at 90-100 C in a nitrogen atmosphere for two hours for the interesterification of triglycerides and six hours for the interesterification of cholesterol esters and phospholipids. The samples were shaken frequently at the start of the refluxing period to dissolve the lipid mixture. At the end of the refluxing, the hydrochloric acid, methanol, and benzene were evaporated under a stream of nitrogen while the tubes were held in a warm water bath. To remove any water in the sample,

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[3]The Hormel Institute, University of Minnesota, 801 1th Ave., N.E. Austin, Innesota.

a small amount of benzene was added which was then blown off with nitrogen. Drying the sample with benzene was repeated twice if necessary. The esters were dissolved in a small volume of petroleum ether (phospholipids were dissolved in a small volume of methanol) and quantitatively transferred to a 12 x 100 mm tube. The samples were further dried with anhydrous sodium sulfate and then centrifuged for ease in concentrating the water and sodium sulfate in the bottom of the tube. The solvent was evaporated in preparation for injection of the sample on the gas-liquid chromatographic column. The methyl ester sample was dissolved in 10 microliter of chloroform and injected into the column using a microliter syringe. The gas-liquid chromatograph was an Aerograph Hi-Fi Model 600 C. equipped with a flame ionization detector. A stainless steel column of one-eight inch inner diameter was 7.5 feet long and packed with 20 percent carbowax 20M : KOH (4:1 ratio) on 80 to 100 mesh acid washed chromsorb W. The column was coiled in 2 1/4 cm diameter coils and conditioned for 24 hours before samples were injected. The temperature of the column was 180 to 185 C. The rate of flow of the nitrogen carrier gas was adjusted to give good resolution of the methyl esters of the fatty acids. Standard mixtures of methyl esters of fatty acids were analyzed at frequent intervals for identification of the various fatty esters and to determine the degree of quantitative resolution obtained with the specific column and instrument in use.

Calculations

The proportional composition of the fatty acids in the total sample was obtained by a triangulation of the area associated with each component and calculation of the percentage of the total area. The amounts of fatty acids calculated as components of several standard mixtures varied no more than 5 percent of the actual values.

Appendix E

Determination of Cholesterol

The method of Galloway et al. (1959) as modified by Smith (1961) was used for cholesterol determinations.

Reagents

1. Solvents: Acetone-absolute ethanol (1:1); acetone-ether (1:2). Ether, tested peroxide-free. Acetone and ethanol redistilled.
2. Acetic acid, glacial (A. C. S.).
3. Acetic acid solution, 10 percent in alcohol.
4. Acetic anhydride (A. C. S.). Refrigerated after opening.
5. Sulfuric acid, concentrated, c. p.
6. Digitonin solution, 0. 5 percent in 50 percent alcohol.
7. Potassium hydroxide solution, approximately 16. 5 percent. Apprdximately 33 percent potassium hydroxide solution was made in dissolving 5 gm of pure potassiun hydroxide in 10 ml of freshly boiled and cooled redistilled water. Three ml of approximately 33 percent potassium hydroxide solution was mixed with 3 ml freshly boiled and cooled redistilled water. This solution was made every two weeks.
8. Phenolphthalein solution, 1 percent in alcohol.

9. Stock standard solution of cholesterol in glacial acetic acid, 100 mg per 100 ml.

10. Working standard solution of cholesterol.

0.05 mg per ml: 5 ml of stock solution made up to 100 ml with glacial acetic acid.

0.10 mg per ml: 5 ml of stock solution made up to 50 ml with glacial acetic acid.

Procedure

Extraction and sampling. The frozen serum sample was warmed to room temperature and mixed gently to insure a uniform sample. Forty microliters of serum were delivered with a 40 microliter pipette into a 1 ml volumetric flask containing 0.4 ml of acetone-ethanol (1:1). Each flask was buzzed immediately until the precipitate was divided into fine particles. The solvent was brought to a boil by placing the flask in a boiling water bath for 6 seconds, immediately cooled to room temperature, and the contents of the flask made up to exactly 1 ml volume with acetone-ethanol. The flask was stoppered and buzzed. The extraction mixtures were centrifuged at 0 C for 30 minutes at 2500 rpm; the precipitate was packed sufficiently to allow aliquots to be measured directly from the flask.

Triplicate aliquots of 100 microliters were pipetted into 6 x 50 mm tubes for the determination of total cholesterol and duplicate 200 microliter aliquots were similarly taken for free cholesterol determinations.

Precipitation of total cholesterol. Five microliters of 16. 5 percent potassium hydroxide solution were added to the 100 microliter aliquots in the 6 x 50 mm tubes. The tubes were carefully buzzed until the potassium hydroxide was finely dispersed and then were placed in a 38 C water bath for 30 minutes. After the tubes were removed from the water bath and cooled to room temperature, 110 microliters of acetone-ethanol were added to bring the total volume to 200 microliters.

Five microliters of phenophthalein were added as an indicator and approximately 15 microliters of alcoholic 10 percent acetic acid were added to bring the contents to excess acidity. If a slight color remained after mixing, an additional 5 microliters of the acid were added to insure excess acidity. Following the addition of 100 microliters of digitonin solution, the tubes were mixed well by buzzing, closed tightly with rubber stoppers, and stored over-night at room temperature. Triplicate blanks of 100 microliters of acetone-ethanol were treated in the same manner.

Precipitation of free cholesterol. Five microliters of 10 percent acetic acid and 100 microliters of digitonin were added to the 200 microliter aliquots in the 6 x 50 tubes. After buzzing, the tubes were closed tightly with rubber stoppers and allowed to stand over-night at room temperature. Triplicate blanks of 200 microliters of acetone-ethanol were treated in a similar manner.

Washing of the cholesterol digitonide precipitates. The next day the rubber stoppers were removed, each tube was buzzed gently to free any precipitate clinging to the sides, and the tubes were centrifuged at 2500 rpm

for 30 minutes at 0 C. The supernatant was drawn off with a fine tipped transfer pipette by careful aspiration. The walls of the tube were washed well with 200 microliters of acetone-ether (1:2) which was delivered by a syringe pipette adjusted for 2 ml. The tubes were buzzed well to wash the precipitate and then centrifuged for 30 minutes. The supernatant was removed as above.

The precipitates for total cholesterol determinations were washed once more with ether in the same manner. The precipitates for free cholesterol determinations were washed twice more with ether. The tubes were placed in a 60 C water bath for the evaporation of all remianing ether from the precipitates. The samples may be stored several days at this stage.

Drying of the cholesterol digitonide precipitates. Sample tubes were placed in order in a rack in a shallow pan of preheated sand. The contents of the tubes were dried in an oven at 105-110 C for 30 minutes.

Color development. The pan, containing the sample tubes, was removed from the oven and to each sample tube, was added 50 microliters of glacial acetic acid, while the tube was rotated allowing the acid to wash down the walls of the tube. The tube was tapped to mix and replaced in the hot sand for approximately 30 seconds while acid was added to three more tubes, which in turn, were placed in sand. The tubes were transferred, one at a time, to a rack at room temperature. This procedure was continued until all the tubes had been similarly treated. The reagent blanks were handled in the same manner. Triplicate aliquots of 50 microliters of working standard, which contained 5 mcg of cholesterol, were placed at the beginning and at the end of the sample tubes.

Triplicate 50 microliters of glacial acetic acid, which served as standard blank, were placed at the beginning of the sample tubes.

The Liebermann-Burchard reagent was prepared as follows: 0.5 ml of concentrated sulfuric acid was added drop by drop to the 10 ml ice-cold acetic anhydride and shaken vigorously for a few moments and allowed to develop in an ice bath for 10 minutes before being used. To each tube was added 100 microliters of this reagent in a group of three tubes every three minutes. The tubes were buzzed and placed in a 28 C water bath in a dark cabinet for color development. At exactly 30 minutes after the addition of color reagent, optical densities were measured at a wave length of 635 mμ (without filter) on the Bechman D U Spectrophotometer against a reference solution of glacial acetic acid. Liquid was drawn out with a transfer pipet by the use of aspiration; the cuvettes were not washed between readings.

Calculations

Total or free cholesterol in serum:

Mg cholesterol per 100 ml serum =

$$\frac{\text{Density of sample} - \text{Density of Blank}}{\text{Density of standard}} \times \text{cholesterol in standard (mg)} \times \frac{100}{\text{Volume of serum in aliquot}}$$

VITA

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