SERUM CHOLESTEROL CONCENTRATIONS OF WOMEN ON A CONTROLLED DIET

by

ANN STIRNIMAN

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1957

APPROVED:

[redacted]

Associate Professor of Foods and Nutrition

In Charge of Major

[redacted]

Head of Department of Foods and Nutrition

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

Date thesis is presented February 5, 1957

Typed by Jean B. Proctor

ACKNOWLEDGMENT

The author wishes to express her thanks to Betty E. Hawthorne, Associate Professor of Foods and Nutrition, for her direction and encouragement during the course of this investigation.

She also wishes to thank Dr. Clara A. Storvick and Dr. Margaret L. Fincke for their advice and interest in this work, and to acknowledge the assistance and co-operation rendered by the subjects of the experiment and other laboratory workers.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
METHOD FOR THE DETERMINATION OF FREE AND TOTAL CHOLESTEROL IN 40 LAMBDA OF SERUM	3
METHODOLOGY STUDIES	5
Use of Alcholic Acetic Acid	6
Reduction of Amount and Concentration of Potassium Hydroxide	6
Effect of Acidity in the Free Cholesterol Determination.	9
Use of a Reagent Blank.	10
METHOD	11
Reagents	11
Procedure	12
Precipitation of Free Cholesterol	12
Washing of Precipitate.	13
Precipitation of Total Cholesterol.	13
Drying of Cholesterol-Digitonide	14
Color Development	14
Calculations.	16
RELIABILITY OF METHOD	16
THE EFFECT OF FREEZING UPON SERUM SAMPLES	22
SERUM CHOLESTEROL CONCENTRATIONS OF FOUR WOMEN ON A CONTROLLED DIET.	23
REVIEW OF FACTORS INFLUENCING SERUM CHOLESTEROL CONCENTRATIONS IN WOMEN	23
Influence of Age.	23
Influence of Weight and Diet Composition.	25

TABLE OF CONTENTS (continued)

Page

Relative Body Weight 25

Changes in Body Weight 26

Fat Intakes 27

Other Diet Components. 28

Cholesterol 29

Ascorbic Acid 30

Hormonal Regulation and Cyclic Fluctuations 31

EXPERIMENTAL PROCEDURE 34

Subjects 34

Diet 34

Blood Samples 35

Determination of Free and Total Cholesterol 35

RESULTS AND DISCUSSION 38

SUMMARY AND CONCLUSIONS 46

BIBLIOGRAPHY. 47

LIST OF TABLES AND FIGURES

Table		Page
1	Total Cholesterol Recoveries with Decreasing Amounts and Concentrations of KOH Used for Saponification . . .	8
2	Consecutive Cholesterol Concentrations Determined in Duplicate Extractions of Control Serum Samples	18
3	Mean Serum Total Cholesterol Concentrations of Women at Various Ages	24
4	Age, Height, and Weight of the Subjects	34
5	Composition of Diet	36
6	Daily Serum Total and Free Cholesterol Concentrations For Four Women on a Controlled Diet	39

Figure		
1	Daily Cholesterol Concentrations of Four Women on Controlled Diet	41

SERUM CHOLESTEROL CONCENTRATIONS OF WOMEN ON A CONTROLLED DIET

INTRODUCTION

Extensive research has been undertaken in recent years in the United States seeking to uncover reasons for the growing incidence of heart disease. Atherosclerosis, a disease in which lipid deposits are built up within the intima of the artery, has been cited as a predominant cause. This disease, though diagnosed rarely in women before the menopause and seemingly occurring as a sexual difference, has been widely associated with dietary pattern (19, p.39) and lipid metabolism (13, p.691).

Elevated serum cholesterol concentrations accompany atherosclerosis in experimental animals and the present trend has been to affix an index of the disease on cholesterol-containing substances in human blood (13, p.691). However, not all humans with elevated serum cholesterol concentrations suffer from atherosclerosis nor do all patients with this disease experience high serum cholesterol levels.

Since all factors influencing human serum cholesterol concentrations are not completely understood, further research on human cholesterol metabolism is needed. No studies of daily determinations of human serum cholesterol concentrations have been reported; previous data on serial determinations of serum cholesterol have been based on biweekly, weekly, monthly or irregular periods of time. Further, only a very few studies of serum cholesterol concentrations of humans on controlled diets for extended periods of time have been reported.

This study was undertaken to ascertain the extent of daily variations of serum cholesterol concentrations of four women on a constant diet for thirty days. The research was carried out in the Nutrition Research Laboratory, School of Home Economics, Oregon State College, as a part of a project determining riboflavin and diphosphopyridine nucleotide concentrations of blood samples from the same four subjects. The relationships of the serum cholesterol concentrations to concentrations of the other determined serum constituents, i.e. riboflavin, diphosphopyridine nucleotide, ascorbic acid and phospholipids, will be analyzed in a subsequent report.

An unpublished micromethod requiring only 40 lambda of serum for the determinations of both free and total cholesterol was made available to this laboratory for modification and use. Following extensive methodology studies, the modified method was satisfactory for the analysis of the serum samples. The micro amount of serum required by the method made it possible to analyze the daily serum samples for free and total cholesterol concurrently with analyses of other serum constituents.

METHOD FOR THE DETERMINATION OF FREE AND TOTAL CHOLESTEROL IN 40 LAMBDA OF SERUM

The evolution of methods for the determination of cholesterol in blood has continued for almost fifty years. Since Windaus first applied digitonin to the macrogravimetric determination of cholesterol in 1909 and Grigaut devised a method employing the Liebermann-Burchard reagent for visual determinations in 1910, more than fifty original or modified cholesterol methods have been published. The development of cholesterol methods and cholesterol methodology have been critically reviewed by Zak and Ressler (58, p.433-446).

Cholesterol methods have evolved with variations along three main pathways: the means of separating cholesterol from its protein complexes; the isolation of the cholesterol; and the final means of measurement. Counter-current separation, absorption in plaster of Paris, column chromatography and extraction into various organic solvents have been used to separate cholesterol from blood protein fractions. Methods of isolation have included chromatography and precipitation. Final measurements have been made by various gravimetric, titrimetric, gasometric and spectrophotometric procedures.

Although various abridged methods have been proposed, precise methods for the determination of total cholesterol in serum have involved four steps: 1) extraction of cholesterol from protein complexes; 2) saponification of cholesterol esters to free cholesterol; 3) isolation of the free cholesterol as the cholesterol-digitonide precipitate; and 4) final measurement.

Extraction by acetone-ethanol, first reported by Schoenheimer and Sperry (43, p.745-760), has been accepted widely as a simple procedure and yet it facilitates complete liberation of cholesterol from its accompanying protein. Mild saponification of esterified cholesterol, although time consuming, generally has been accepted as essential for accurate analysis because of the varied absorbancy characteristics of the free and esterified forms of cholesterol and because it is essential to the complete isolation of cholesterol. Digitonin of high quality, added in excess, has been basic to most isolation procedures. Of all final measurement procedures used, the Liebermann-Burchard color reaction has remained the most important, although its mechanism is still unknown. The color reaction with cholesterol is sensitive to temperature, time, light and the presence of water; carefully controlled conditions must be followed.

The Schoenheimer and Sperry method (43, p.745-760), which was published in 1934, was a classic in the development of cholesterol methods. The method allowed accurate analyses of both free and total cholesterol on only 200 lambda of serum and greatly facilitated serum cholesterol analyses in both animals and humans. Schoenheimer and Sperry extracted the cholesterol by acetone-ethanol, saponified the esterified cholesterol with potassium hydroxide, precipitated the free cholesterol with high quality digitonin and measured the cholesterol present by the Liebermann-Burchard reaction under specified conditions. The original method and its subsequent modifications have been standard references in cholesterol methodology studies.

The Sperry and Webb modification of the Schoenheimer and Sperry method was published in 1950 (48, p.97-106). Isolation with an alcoholic rather than an aqueous solution of digitonin and slight changes in the conditions for development of the Liebermann-Burchard color reaction were the principal modifications. For each analysis 200 lambda of serum was required.

The need for using single samples of finger-tip blood of humans for analyses of several serum constituents stimulated interest in developing an accurate cholesterol method requiring only a fraction of the serum of existing methods. Because of its accuracy, the Sperry and Webb method was selected for adaptation.

METHODOLOGY STUDIES

The Sperry and Webb cholesterol method (48, p.97-106) was adapted to a micromethod requiring only 40 lambda of serum for each analysis by workers at the New Mexico and Utah State Agricultural Experiment Stations. This unpublished method (8), made available to this laboratory by Dr. Ethelwyn Wilcox[1], has been further modified in certain details as a result of extensive methodology studies.

Certain techniques and procedures of the method were modified in this laboratory for greater precision or convenience: 1) times of centrifuging at all separation steps were increased 10 minutes;

---

1 Appreciation is expressed to Dr. Ethelwyn Wilcox, Utah State Agricultural College, and Miss Edith Lantz, Professor of Nutrition, New Mexico State College of Agriculture and Mechanic Arts, for their generous permission to modify and use this unpublished method.

2) samplings of the acetone-ethanol lipid extracts were measured directly from the supernatant in the centrifuge tubes rather than after transfer; and 3) the use of a Bausch and Lomb lighted magnifier was introduced to increase visibility and to allow more accurate separations of washing solvents from the cholesterol digitonide precipitates. Major modifications made in the method were: 1) the use of alcoholic 10 per cent acetic acid instead of aqueous 10 per cent acetic acid for acidification of acetone-ethanol extracts prior to digitonin precipitation; 2) reduction of the amount and concentration of the potassium hydroxide used in saponification of the esterified cholesterol, with a consequent reduction in the amount of acetic acid used for neutralization; 3) reduction of the amount of acidification of acetone-ethanol extracts prior to digitonin precipitation in free cholesterol determination; and 4) inclusion of a reagent blank for all series of analyses.

Use of Alcoholic Acetic Acid

The 10 per cent acetic acid solution was prepared with absolute alcohol rather than distilled water as this alcoholic preparation had been recommended by Sperry and Brand (47, p.315). This modification had been made in the use of the Sperry and Webb method in this laboratory. The use of alcoholic rather than aqueous 10 per cent acetic acid reduced the introduction of water in the procedure.

Reduction of Amount and Concentration of Potassium Hydroxide

Recoveries of total cholesterol were low when, as originally

recommended in the Utah method, 10 lambda of 33 per cent potassium hydroxide was used for saponification and 60 lambda of 10 per cent acetic acid was used for neutralization. Repeated determinations were made on standard solutions of cholesterol and cholesterol acetate and on serum with known amounts of added cholesterol and cholesterol acetate. Methods of washing, times of centrifugation, mixing techniques, lengths of time for saponification and amounts and concentrations of potassium hydroxide were varied. When reductions were made in the amount and concentration of potassium hydroxide and, consequently, a reduction in the amount of acetic acid required for neutralization, recoveries were satisfactory, table 1. These data resulted in the adoption of 5 lambda of 16.5 per cent potassium hydroxide for saponification in total cholesterol determinations; approximately 15 lambda of 10 per cent acetic acid in absolute alcohol were required for neutralization. It was found also that aliquot variations were decreased with the lesser amounts and concentration of potassium hydroxide.

The possibility that the use of alcoholic rather than aqueous acetic acid for neutralization might have been a factor in the per cent recoveries was investigated. Determinations of the total cholesterol concentration of a sample of rat serum were made using the micro-adaptation to compare the effects of neutralizing with alcoholic and with aqueous acetic acid following saponification with varying amounts and concentrations of potassium hydroxide. Upon neutralization with alcoholic 10 per cent acetic acid following saponification with 10 lambda

TABLE 1

Total Cholesterol Recoveries with Decreasing Amounts and Concentrations of KOH Used for Saponification

Substrate	KOH Concentration	Amount	Cholesterol Concentration Theoretical	Determined	Recovery
	%	ml	mg/100 ml	mg/100 ml	%
Cholesterol (Eastman) in Acetone-Ethanol	33	10	100.0	77.4	77.4
	33	10	100.0	72.0	72.0
	33	7.5	100.0	84.7	84.7
	33	5	100.0	92.0	92.0
	33	5	100.0	94.1	94.1
	16.5	5	100.0	101.3	101.3
	16.5	5	100.0	97.3	97.3
	16.5	5	100.0	100.6	100.6
	16.5	5	100.0	99.0	99.0
	16.5	5	100.0	100.0	100.0
	8.25	5	100.0	98.0	98.0
Cholesterol-Acetate (Eastman) in Acetone-Ethanol	33	5	90.2	77.1	86.0
	16.5	5	90.2	91.0	100.9
	16.5	5	90.2	86.5	95.9
Serum I	33	7.5		127.3	
	33	5		144.4	
	33	5		149.7	
	33	5		142.8	
	16.5	5		157.6	
Serum I plus Cholesterol Acetate	16.5	5	247.8	252.1	101.7
	8.25	5	247.8	245.0	98.9

of 33 per cent, 5 lambda of 33 per cent and 5 lambda of 16.5 per cent potassium hydroxide, the values obtained for total cholesterol for that serum sample were 77.8, 96.6 and 112.5 mg per 100 ml, respectively; upon neutralization with aqueous 10 per cent acetic acid following saponification with the same amounts and concentrations of potassium hydroxide the values obtained for the total cholesterol for the serum were 106.8, 105.1 and 110.6 mg per 100 ml. When this same sample was analyzed for total cholesterol by the Sperry and Webb procedure a value of 111.6 mg per 100 ml was obtained. A difference in reactions is apparent; the explanation for this difference has not been discovered.

Effect of Acidity in the Free Cholesterol Determinations

In the determination of free cholesterol in the Utah method, 5 lambda of aqueous 10 per cent acetic acid was added to the aliquots of acetone-ethanol extract prior to the addition of digitonin for the precipitation of the free cholesterol. This addition of acid was designed to yield comparable degrees of acidity for the total and free cholesterol determinations. In the free cholesterol determinations the cholesterol digitonide precipitates had a tendency to be flocculent; packing of the precipitate by centrifigation was particularly difficult following the second ether wash. It was found that omission of the addition of acetic acid at this stage reduced the flocculence and made it much easier to wash the free cholesterol precipitates. An addition of an intermediate amount of acid, approximately 2 lambda, was found to give the most consistent values among aliquots.

This modification was not introduced until after the original analyses for the serum samples of the four women subjects in this study had been completed. It was used in repeat analyses on some of the serum samples.

Use of a Reagent Blank

The development of a greenish-blue tint in sample tubes as compared to a true blue tint in standard tubes in the Liebermann-Burchard reaction indicated the need for analysis of a reagent blank. Tubes containing aliquots of acetone-ethanol were identical to sample tubes throughout the entire procedure. In 63 series of determinations, reagent blanks exhibited variations in density readings of 0.002 to 0.010 with an average reading of 0.004, which is equivalent to approximately 3.7 mg cholesterol per 100 ml of serum. This reading was determined to be caused partially by the digitonin since acetone-ethanol aliquots analyzed without the addition of digitonin had density readings of only 0.001 to 0.002.

Density readings for the serum samples were corrected using the average of the appropriate reagent blanks, three of which were included with each series of total cholesterol determinations and three which were included with each series of free cholesterol determinations.

The blank employed in the Sperry and Webb method, consisting of glacial acetic acid and the Liebermann-Burchard reagent only, was the blank originally designated for the method. This blank had a density reading of 0.000 against glacial acetic acid in the reference cuvette.

METHOD

Reagents

The reagents used were identical to those of Sperry and Webb (48, p.100) except for the modifications introduced for preparation of the 10 per cent acetic acid and the potassium hydroxide, which have been discussed.

1. Solvents: Acetone-absolute ethanol (1:1); acetone-ether (1:2). Ether, tested peroxide-free. Acetone and ethanol, redistilled.
2. Digitonin solution, 0.5 per cent in 50 per cent alcohol. 500 mg of digitonin made up to 100 ml with 50 per cent alcohol (55 ml of 95 per cent alcohol and 45 ml of redistilled water).
3. Potassium hydroxide solution, 16.5 per cent. 5 grams of pure potassium hydroxide was dissolved in 10 ml of freshly boiled redistilled water. 3 ml of 33 per cent potassium hydroxide solution was mixed with 3 ml of freshly boiled redistilled water. This solution was made every two weeks.
4. Phenolphthalein solution, 1 per cent in alcohol. 0.3 gm of phenolphthalein was dissolved in 30 ml of 95 per cent ethyl alcohol.
5. Acetic acid solution, 10 per cent in alcohol. 10 ml of glacial acetic acid was made up to 100 ml with absolute alcohol.
6. Acetic acid, glacial (A.C.S.).
7. Acetic anydride (A.C.S.). Refrigerated after opening.

8. Sulfuric acid, concentrated, C.P.

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

   100.0 mg cholesterol (Eastman) made up to 100 ml with glacial acetic acid.

10. Working standard solution of cholesterol, 10 mg/100 ml.

    1 ml of stock standard solution of cholesterol made up to 10 ml with glacial acetic acid.

Procedure

With a constriction pipette 40 lambda of blood serum was delivered into a 1 ml volumetric flask containing 0.4 ml of acetone-ethanol (1:1), while the flask was held against the rotating bar of a mechanical agitator (referred to as the buzzer) to set the solvent swirling. The flask was buzzed again; the solvent was brought to a boil by placing the flask in a boiling water bath for 6 seconds. Cooled immediately to room temperature, the contents of the flask were made up to 1 ml volume with acetone-ethanol. The flask was stoppered and buzzed. Following 30 minutes of refrigerated[2] centrifugation (0°C.) at 2800 rpm, the precipitate was packed sufficiently to allow aliquots to be removed directly from the flask.

Precipitation of Free Cholesterol. Duplicate 200 lambda aliquots of each extraction filtrate were pipetted into 6 x 50 mm test tubes. Five lambda of alcoholic 10 per cent acetic acid and 100 lambda of

2 International Portable Refrigerated Centrifuge, Model PR-2, International Equipment Company, Boston, Massachusetts.

digitonin were added to all tubes. (Reduction of acetic acid is recommended as per discussion on page 9). After buzzing, the tubes were covered with rubber caps and placed in a preserving jar containing 1½ inches of sand to support the tubes. This jar, tightly covered, was allowed to stand over-night at room temperature.

Washing of Precipitate. The next day the rubber caps were removed, each tube was vigorously tapped to free any precipitate clinging to the sides and the tubes were centrifuged at 2800 rpm for 30 minutes in the refrigerated centrifuge. The supernatant was drawn off as close to the precipitate as possible without disturbing the packed precipitate. The use of a magnifying lens[3] with light attached and the use of a fine tipped transfer pipette connected to very slow suction facilitated this transfer. The walls of the tubes were washed with 200 lambda of freshly made acetone-ether (1:2) using a 2 ml syringe pipette adjusted for delivery; a needle was attached for direct force. The tubes were buzzed and then centrifuged for 30 minutes; the supernatant was removed as above.

The precipitate was treated twice more in the same manner using an ether wash. Then the precipitate was dried by placing the tubes in warm water for the removal of all remaining ether. The dried sample could be stored several days at this stage.

Precipitation of Total Cholesterol. Triplicate 100 lambda aliquots of

---

3 Bausch and Lomb magnifying lens with light, American Optical Company, Buffalo, New York.

each extraction filtrate were pipetted into 6 x 50 mm tubes. After the addition of 5 lambda of 16.5 per cent potassium hydroxide, the tubes were carefully buzzed until the potassium hydroxide was finely dispersed. Capped tubes were placed in a 40°C. water bath for 30 minutes. After the tubes were removed from the water bath and cooled to room temperature, 120 lambda of acetone-ethanol (1:1) was added to compensate for evaporation and to bring the total volume to 200 lambda.

Using 5 lambda of phenolphthalein as an end point indicator, approximately 15 lambda of alcoholic 10 per cent acetic acid was needed to bring the contents just to excess acidity. Fifteen lambda of alcoholic 10 per cent acetic acid was added; complete disappearance of the indicator color was checked after vigorous buzzing. If slight color remained, an additional 5 lambda of acid was added to insure excess acidity. Following the addition of 100 lambda of digitonin solution, the tubes were buzzed, capped, and stored over-night at room temperature.

The next day the samples were treated as described for free cholesterol determinations except that the precipitate was washed with ether once only.

Drying of Cholesterol-Digitonide. Sample tubes were placed in order in a rack in a shallow pan of preheated sand. The tube contents were dried for 30 minutes in sand kept at 110° - 115°C. An oven was used to maintain constant temperature.

Color Development. The pan containing sample tubes was removed from the oven and to each sample tube removed in turn from the sand, 50

lambda of glacial acetic acid was added 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; these tubes were placed in sand in turn. The tubes were removed three at a time to a rack at room temperature. This procedure was continued until all tubes had been similarly treated. The reagent blank was handled in the same manner. Triplicate aliquots of 50 lambda of working standard, which contained 5 gamma of cholesterol, were placed at the beginning and at the end of the sample tubes. The Liebermann-Burchard reagent, acetic anhydride and concentrated sulfuric acid (20:1), was prepared and held in an ice bath; the solution was agitated as the acid was added drop by drop to the ice-cold acetic anhydride. The Liebermann-Burchard reagent was allowed to develop 10 minutes. One hundred lambda of the Liebermann-Burchard reagent was added to each tube; the color reagent was added to 3 tubes every three minutes. The samples were buzzed, placed in a 28°C. water bath in a dark cabinet and allowed to develop for 32 minutes. Triplicate aliquots were transferred to the microcuvettes by pipette. Their densities were read at 635 mn (slit width 0.5, without filter) in the spectrophotometer[4] against a blank of glacial acetic acid. Solutions were drawn out of the cuvettes with suction facilitated by the use of polyethylene tubing of fine diameter; the

4 Beckman Spectrophotometer, Model DU, Beckman Instruments, Inc., Fullerton, California. Equipped with micro attachment, Pyrocell Manufacturing Company, 207 East 84th Street, New York, New York.

cuvettes were not washed between readings.

Recommended use of the Liebermann-Burchard reagent for addition to sample tubes was limited to a thirty minute period. Hence the number of sample tubes which could be determined in a single series was limited.

Calculations

mg cholesterol per 100 ml serum =

$$\frac{D_S - D_B}{D_{Std}} \times \text{mg cholesterol in Std.} \times \frac{100}{\text{Volume of serum in aliquot}}$$

where: D_S = density of the serum aliquot

D_B = density of the blank

D_{Std} = density of the standard

RELIABILITY OF METHOD

The basic merit of the modified micro-adaptation of the Sperry and Webb method for total cholesterol has been indicated by the recovery data previously presented in table 1.

The reliability of the method was further tested through the analysis of an alcohol extract of rat serum sent from the University of California[5] and by repeated determinations on a control serum sample over a 6 month period. The rat serum, analyzed in the California laboratory by the Sperry and Webb method, was reported to contain

---

5 Appreciation is expressed to Mrs. Marian Lyman and Dr. Ruth Okey for preparing the referee sample.

115 mg cholesterol per 100 ml of serum. The rat serum was analyzed in this laboratory by the Sperry and Webb method to contain 111.6 mg cholesterol per 100 ml. By analyses with the modified micro procedure an average concentration of 111.6 mg cholesterol per 100 ml of serum was found; the range was 107.5 to 120.0 mg per 100 ml of serum.

During the period when serum samples of the four subjects in this study were being analyzed for free and total cholesterol, analyses of control serum samples were made concurrently with each series. Although it was planned to use one control serum throughout the analyses, it became necessary after seven series to obtain a new control serum. The control serum samples were divided into 100 lambda aliquots and held in frozen storage. The total and free cholesterol concentrations determined for the control serum samples in consecutive analyses of duplicate extractions are tabulated in table 2.

In the first control sample the mean of seven duplicate extractions was 170.3 mg total cholesterol per 100 ml of serum with a range of 161.2 to 182.9 mg per 100 ml. The standard deviation was calculated to be 7.7 and the standard error of the mean, 3.0 mg. For the second control serum the mean of fifty-six extractions was 359.5 mg total cholesterol per 100 ml serum with a range of 309.5 to 400.2 mg per 100 ml. The standard deviation for the total cholesterol data was 19.4 and the standard error of the mean, 2.6 mg. Determinations of free cholesterol in the second control serum averaged 91.5 mg cholesterol per 100 ml serum with a range of 80.0 to 100.7 mg per 100 ml. The standard deviation for the free cholesterol data was 4.3 and the

TABLE 2

Consecutive Cholesterol Concentrations Determined in Duplicate Extractions of Control Serum Samples

Serum	Cholesterol Concentrations			
	Total		Free	
	1st Extraction	2nd Extraction	1st Extraction	2nd Extraction
	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
Control I	175.0			
	176.0			
	161.9			
	163.2			
	166.4			
	166.6			
	182.9			
Control II	-----	-----	89.2	88.3
	352.9	351.3	97.4	----
	376.4	-----	80.0	89.9
	352.9	365.9	98.1	91.5
	315.8	336.1	96.9	94.9
	309.5	329.9	81.5	82.2
	322.6	342.0	92.0	97.1
	365.8	334.4	93.1	94.8
	343.6	330.2	96.1	93.1
	334.2	356.9	95.0	93.1
	400.2	386.8	93.0	92.4
	372.1	375.7	89.2	94.0
	366.6	357.6	85.4	92.8
	370.1	387.7	93.6	88.3
	363.3	376.7	81.8	89.0
	379.7	387.1	91.2	85.9
	399.7	375.0	90.9	79.2
	360.7	356.7	87.8	84.5
	358.9	348.0	88.2	88.4
	366.4	366.7	94.1	94.8
	353.9	357.5	----	----
	352.6	358.5	----	----
	362.4	271.1	91.8	94.1
	345.1	325.4	91.6	93.9
	364.6	343.1	80.9	92.7
	363.0	-----	93.7	100.7
	341.5	376.4	92.1	100.1
	368.4	376.7	99.3	96.7
	371.5	380.1	97.9	93.4
	361.9	367.4	----	----
	374.6	-----	----	----

TABLE 2 (continued)

Serum	Cholesterol Concentrations: Total, 1st Extraction	Total, 2nd Extraction	Free, 1st Extraction	Free, 2nd Extraction
	Total mg/100 ml		Free mg/100 ml	
Control I:				
Mean	170.3			
Range	161.2-182.9			
Standard Deviation[1]	7.7			
Standard Error of Mean[2]	3.0			
Control II:				
Mean	359.5		91.5	
Range	309.5-400.2		80.0-100.7	
Standard Deviation	19.4		4.3	
Standard Error of Mean	2.6		0.6	

1 Standard Deviation $= \sqrt{\frac{\Sigma(x-\bar{x})^2}{N-1}}$

2 Standard Error of Mean $= \frac{\text{Standard Deviation}}{\sqrt{N}}$

standard error of the mean, 0.6 mg. The standard deviations calculated as per cents of the mean total cholesterol concentrations for both control serums are similar: 5.3 and 5.4 per cent for control serum I and control serum II, respectively. The standard deviation of the free cholesterol of control serum II was 5.0 per cent of the mean value. However, the extremes of the range of determined total cholesterol concentrations were much greater proportionally for control serum II than control serum I.

Sperry and Webb (48, p.99) have demonstrated that errors of method can be reduced to as small an amount as that represented by standard deviations of 0.77 and 1.81 mg per 100 ml serum for free and total cholesterol, respectively.

Typical ranges of determinations for total cholesterol concentrations on replicate aliquots, varying in number, of serum samples reported by Sperry (46, p.387) were: 162.3 to 168.4, 192.0 to 213.3 and 199.8 to 207.1 mg per 100 ml of serum. Ranges of determinations for free cholesterol on replicates of the same serum samples, in mg per 100 ml serum, were: 50.0 to 53.9, 57.9 to 61.0 and 57.0 to 63.6. Sperry and Brand (47, p.317) reported similar ranges of total cholesterol with eight replications in mg per 100 ml of serum as follows: 228 to 234, 230 to 234, 226 to 235 and 229 to 242; typical ranges of four replications were, similarly; 213 to 226, 183 to 185, 240 to 256, and 216 to 221.

The range of replicate determinations on the control serums by this micromethod are somewhat greater than the replicate determinations

by macromethods which have been cited. The ranges of control serum II are particularly wide, although two-thirds of the values are within 10 per cent of the mean value.

Cholesterol determinations are subject to error through variations of reagents as well as technique. Schoenheimer and Dam (42, p.59-63) have stated that the usual digitonin method is susceptible to an error up to 10 per cent. This error could be the result of an accumulation of errors in any one series of determinations. Schoenheimer and Dam referred specifically to the quality of digitonin which will affect the completeness of the cholesterol-digitonide union and to the absorption of cholesterol by alcohol. Errors may also follow from poor techniques such as inaccurate pipetting, excess acidification, or careless washing of the precipitate. The Liebermann-Burchard color reaction, sensitive to light, time, temperature and moisture, has stipulations for satisfactory results which must be followed consistently. Pipetting organic solvents is difficult and changes in environmental temperature affect concentrations. The cholesterol standard in glacial acetic acid and the accuracy of measurement will influence an entire series as the density reading of the standard is used in all calculations. Excessive acidity or alkalinity in the sample tubes treated and stored over-night with digitonin will reduce the cholesterol-digitonide yield. Another loss of cholesterol may occur during the repeated washings with solvents through the loss of precipitate when the filtrate is removed by slow suction.

THE EFFECT OF FREEZING UPON SERUM SAMPLES

The current analysis for free and total cholesterol of the daily serum samples from the subjects was not possible and the serum was stored in 6 x 50 mm capped tubes at -5°C. for four to seven months. Therefore the effect of freezing upon serum samples was investigated.

Aliquots of a serum sample were analyzed both fresh and after being held at -5°C. No change in the total cholesterol content between fresh or frozen samples was apparent. The mean total cholesterol concentrations per 100 ml of serum determined at various times were as follows: fresh serum, 189.4 mg; frozen four days, 190.4 mg; frozen one week, 185.6 mg; frozen one month, 197.0 mg; frozen two months, 185.5 mg; frozen five months, 182.9 mg. Butler, et al. (5, p.470) have recently reported no effect of freezing or thawing on the total and free cholesterol concentration of serum stored up to 22 weeks.

Further evidence of cholesterol stability in frozen serum samples was observed in the control serum samples which were stored up to six months. No trend to increase or decrease in concentrationsof free or total cholesterol occurred, table 2.

SERUM CHOLESTEROL CONCENTRATIONS OF FOUR WOMEN ON A CONTROLLED DIET

REVIEW OF FACTORS INFLUENCING SERUM CHOLESTEROL CONCENTRATIONS IN WOMEN

The number of factors which have been implicated, through animal and human studies, as influencing blood cholesterol concentration is extensive. These have been reviewed by Deuel (7, p.395-486) and include: age, sex, species, race, heredity, environment, ingestion of fat, ingestion of cholesterol, quantity and quality of protein intake, essential fatty acid intake, vitamin deficiencies, disturbances of the endocrine glands, diabetes mellitus, diseases of the thyroid gland, anemia, diseases of the gastrointestinal tract, hepatic and kidney diseases, arteriosclerosis, ingestion of certain drugs, pregnancy, lactation and menstruation. Because of the higher incidence of atherosclerosis in the male, the greater proportion of studies of factors influencing serum cholesterol concentrations in humans have been on men; only limited data have been published on women. The influence of age, weight, diet composition, hormonal regulation and cyclic fluctuations on serum cholesterol concentrations have been observed in studies on women.

Influence of Age

Increased serum cholesterol concentrations with increasing age have been reported in survey studies on women, but evidence of this influence of increasing age was less pronounced when smaller groups of women were studied before and after a span of years. Butler, et al.

(5, p.469-478), Garcia, et al. (9, p.601-609), Gram and Leverton (14, p.384), and Swanson, et al. (51, p.41-47) surveyed groups of women, predominantly homemakers, living in the Midwest. Gillum, et al. (12, p.449-467) surveyed men and women over 50 years of age who lived at home in California. Serum total cholesterol concentrations of women surveyed in these studies are summarized in table 3.

TABLE 3

Mean Serum Total Cholesterol Concentrations of Women at Various Ages

	Survey Study									
	Butler, et al. (5, p.471)		Garcia, et al. (9, p.608)		Gillum, et al. (12, p.451)		Swanson, et al. (51, p.44)		Gram and Leverton (14, p.384)	
Age	No.	mg/ 100 ml	No.	mg/ 100 ml	No.	mg/ 100 ml	No.	mg/ 100 ml	No.	mg/ 100 ml
Years										
30	6	207								
30-39	36	211	10	246			10	176	20	200
40-49	29	250	12	236			16	190	28	207
50-59	13	293	13	284	105	262	13	219	32	250
60-69	20	302	10	320	114	275	6	250	20	257
70-79	9	239	10	285	65	265	8	251		
80-89			3	275	12	236	1	154		

A gradual trend upward in serum cholesterol concentrations continued until a maximum peak was reached in the seventh decade. This peak was followed by a reduction in serum cholesterol concentrations through

the eighth and ninth decades.

On the other hand, when Sperry and Webb (49, p.107-110) compared serum cholesterol concentrations of 14 men and 9 women with values determined 13 to 15 years before, it was found that serum cholesterol concentrations had not increased with age in all subjects. A mean 22.7 per cent increase had occured in the women subjects; one woman had no appreciable change, while six women had increases within the range of 15 to 30 per cent. The authors indicate, however, that part of the apparent increase may have been influenced by changes made in the analytical procedure between the two periods. In another study Man (24, p.738-744) remeasured the serum of 7 men and 9 women after an interval of 10 to 20 years. A tendency toward a rise in cholesterol concentrations was more frequent than a fall but "not significantly preponderant to warrant the deduction that it is a characteristic of the aging process".

Influence of Weight and Diet Composition

Relative Body Weight. A general inference that obesity implies high serum cholesterol concentrations is found frequently in the literature, e.g. (11, p.1259), (22, p.415). However, the association of serum cholesterol concentration with relative body weight in women has been limited. Butler and associates (5, p.474) reported that only 4 per cent of the variation in serum cholesterol concentration of their 113 subjects was related to relative body weight when the data were treated statistically. Negligible correlations between body weight and serum

cholesterol values for women were reported by Gillum, et al. (12, p.463) and Swanson, et al. (51, p.46). Similarily, after extensive surveys on men, Keys and co-workers (18, p.335) could find no important relationship between serum cholesterol concentration and relative obesity as measured by weight and skin folds.

Changes in Body Weight. Effects of changes in body weight on serum cholesterol concentrations have been reported, but primarily on men. Most studies, however, are complicated by changes in diet pattern; further, some of the subjects used in such studies have been suspected or diagnosed atherosclerotic patients. Keys, et al. (19, p.39-56) observed that serum cholesterol was decreased in severe calorie undernutrition at all ages but was high in starvation. Keys further reported increased serum cholesterol concentrations that were not dependent on fat intake in active weight gain in men. In 1955 Mann (25, p.442) reported the effect of changes in calorie disposition on serum cholesterol in three healthy men on constant high levels of fat intake, 150 to 175 gm daily. It was found that doubling the calorie intake had no effect on serum cholesterol concentrations when the excess calories were used for exercise. When exercise was restricted and weight gain occurred, there was a significant increase of serum cholesterol levels. Subsequent weight reduction by calorie restriction resulted in the prompt return of cholesterol levels to their original concentrations. No similar studies have been reported for women.

Serum cholesterol concentrations were reported by Walker (54, p.705-716) for eleven women and twenty-eight men following weight

reduction on a 1000 calorie diet containing only 20 gm of fat. Marked individual variations in changes of serum cholesterol concentrations occurred and no relation of degree of change of serum cholesterol concentration to amount of weight loss was found. Subjects, whose initial serum concentration of lipoproteins of the S_f 12-20[6] class were high, experienced the greatest changes in cholesterol concentrations, a mean loss of 40 mg cholesterol per 100 ml of serum. It was noted further that lowering of serum lipid components generally was accomplished early in the weight-losing phase.

Fat Intakes. Pomeranze, et al. (39, p.742-746) reported the effect on total cholesterol concentrations of fat levels of 20 gm, 60 gm and 60 gm or more, with or without caloric restriction, on five groups of normal, geriatric and obese men and women; 27 men and 10 women were studied for periods of five to fifty weeks. Weight reduction without fat restriction appeared to exert no influence on serum cholesterol concentrations in these subjects, but with fat restriction alone and no evidence of weight loss, hypercholesterolemias when present were reduced.

Other reports of effects of prolonged and severe fat restriction have been similar. The effect of controlled eucaloric diet intakes on three men for 3 to 8 month periods supported this influence of drastic fat restriction; drastic fat restrictions reduced serum

---

6 A Svedberg unit (S) equals 10^{-13}cm/sec/dyne/g. Compounds described as S_f 12 to 20 are those which have flotation rates consistent with 12 to 20 S units.

cholesterol concentrations while moderate fat restrictions exerted no effect (16, p.641-646). Morrison, et al. (31, p.476) and Swank and Wilmot (50, p.33) applied fat restriction therapeutically and placed patients on diets low in fat. They have reported that adherence to a low fat intake over an extended period maintained the plasma cholesterol at a lowered concentration in both men and women.

Conversely, when fat in the diet has been increased appreciably, a rise in serum cholesterol has resulted (27, p.316-322). An increase of fat from 10 per cent to 34 to 46 per cent of the calories in the diet through the addition of vegetable fat resulted in a significant increase of plasma cholesterol in 5 male subjects. A substitution of animal fat did not further increase the plasma cholesterol concentrations.

Although controversial data exist relative to effects of animal versus vegetable fat, the "plasma-lipid-raising-potentials" of fats in man have been shown to be similar when regular mixed diets were employed rather than simplified formula diets (3, p.311-320; 17, p.80). Recent animal studies suggest that an understanding of the metabolic roles of essential unsaturated fatty acids may be the key to the elucidation of the role of dietary fats in influencing serum cholesterol concentrations (28, p.401; 52, p.126; 1, p.185).

Other Diet Components. The influence on serum cholesterol concentrations of other diet components in addition to fat has been reported.

A comparison of adult vegetarians and non-vegetarians showed a lower mean serum cholesterol for the vegetarians (15, p.87). Hardinge

and Stare reported a mean cholesterol concentration of 206 mg per 100 ml for the 11 women and for the 14 men who were pure vegetarians. For the 15 women on lacto-ovo-vegetarian diets a mean of 269 mg per 100 ml was reported and for the 15 men, a mean of 243 mg per 100 ml. The mean serum cholesterol concentration for 15 women following non-vegetarian diets was 295 and for the men 288 mg per 100 ml. Findings on 6 men who had followed a low fat, lacto-ovo-vegetarian diet for 14 to 22 years were similar to those on men who consumed meat and unlimited fats (30, p.246-251); blood cholesterol concentrations ranged from 190 to 235 mg per 100 ml.

No demonstrable relationship between the calculated amounts of carbohydrate, fat, protein or cholesterol in the diet and the level of total blood cholesterol were found by Reimer, et al. (41, p.842-844) studying a kindred group in which essential familial hypercholesteremia was present. Similarly, weekly protein and fat intakes of a group of 184 Midwestern women showed no correlation to concentrations of total serum cholesterol (51, p.46). Gillum and coworkers (12, p.449-467), on the other hand, reported a significant positive effect on serum cholesterol concentrations of dietary cholesterol and fat and a slight effect of dietary protein among the 296 women and 234 men of their survey study.

Cholesterol. Dwindling emphasis on the amount of dietary cholesterol as an influence on serum cholesterol concentrations has resulted from survey and controlled experimental data reported by Keys, et al. (17, p.79-81). Keys and co-workers have concluded from their studies that,

"Serum cholesterol concentrations in adult men are essentially independent of cholesterol intakes over the whole range of natural human diets". They state, "It is probable that infants, children and women are similar" (19, p.54).

However, excessive cholesterol additions to the diet can produce slight but transient increases in serum cholesterol. A slight increase was observed in the mean serum cholesterol values when four egg yolks were fed daily to 4 women for a five week period (36, p.717-727). On a one day special diet of 20 eggs, a male subject exhibited an increase in serum cholesterol concentration (6, p.225-234); however, serum analyses on the next day and the fourth day showed a return to the former cholesterol level. The majority of 24 subjects fed 100 or 150 gm of egg yolk powder containing 2.50 to 3.75 gm cholesterol in addition to their regular diet each day for 48 days or more exhibited increases in their serum cholesterol values (29, p.189-195). These drastic measures would not seem comparable, however, with the estimated usual cholesterol intakes of 250 to 800 mg per day (17, p.79).

Ascorbic Acid. Gillum and co-workers (12, p.467) reported a slight parallelism between serum ascorbic acid and serum cholesterol concentrations among their women subjects; no such relationship was found among the men. All of the subjects of the study were fifty years or older. Recently Walker and co-workers (53, p.517-525) have reported a difference in serum cholesterol response to increasing ascorbic acid intakes between a younger and an older age group of women. Twenty-nine mentally retarded women served as subjects; fifteen subjects

ranged in age from 28 to 34 years and fourteen were 56 to 77 years of age. Increasing ascorbic acid intakes resulted in elevated serum ascorbic acid concentrations in both groups. The rise in serum ascorbic acid concentrations was accompanied by a rise in mean serum cholesterol concentration from 219 ± 9[7] to 238 ± 10 mg per 100 ml among the older group; no significant change in serum cholesterol concentrations occurred in the younger group. Gillum, et al. (12, p.454) have suggested that the higher serum cholesterol concentrations generally found in older women might be influenced by the accompanying decrease in steroid hormone production with aging. Walker, et al. (53, p.523) concluded that this same mechanism might explain the difference in serum cholesterols among their two groups in response to increased intakes of ascorbic acid.

Hormonal Regulation and Cyclic Fluctuations

The triangular relationship existing among adrenocortical hormones, ascorbic acid and cholesterol metabolism (4, p.377) is but one hormonal influence in lipid metabolism. The hormones produced by the thyroid gland, by the islet tissue of the pancreas, by the posterior and anterior portions of the pituitary, by the adrenal cortex and by the sex glands all influence lipid metabolism and the activity of any one endocrine gland affects the behavior of the other glands (7, p.436). Hormonal secretions from the thyroid gland and the islet tissue of the pancreas have the most potent effect on blood lipids,

---

7 Standard deviation

particularly cholesterol. Excessive secretions of thyroid hormone cause a marked reduction in blood cholesterol concentrations while hypothyroidism increases cholesterol considerably above normal levels (7, p.436). Removal of the pancreas will precipitate hyperlipemia and hypercholesteremia in experimental animals (7, p.444). Diabetes in humans is usually accompanied by high blood lipid levels.

Deuel (7, p.406) reported a general agreement among investigators that the cholesterol of blood increases markedly during early pregnancy. The maximum values are 50 to 100 per cent higher than the normal. The increase in cholesterol begins after the second month and continues rising until the thirtieth week after which it decreases until delivery. As lactation begins the mother has a high level of blood lipids and the child has a low level. This difference is moderated as lactation proceeds and the mother's blood lipids are lowered and the child's lipids are raised (7, p.409).

Two hormones, estrone and progesterone, secreted by the ovaries and products of the female sex cycle have been associated with the decrease of esterified cholesterol at the onset of menstruation and time of ovulation (37, p.217-222). Okey and Boyden (35, p.261-281) also reported a decrease in plasma cholesterol within a few days of menstruation followed by an increase. Offenkrantz (32, p.536-546) reported similar findings. Man and Gildea (23, p.777) and Wagner and Poindexter (55, p.335), however, could find no influence of menses on serum cholesterol concentrations in their subjects.

Cyclic fluctuations of serum cholesterol, while frequently associated with the menstrual cycle, also have been reported in studies on serum cholesterol concentrations for both men and women by Man and Gildea (23, p.776), Watkin, et al. (56, p.880) and Sperry (45, p.393). Man and Gildea concluded that the variations of serum cholesterol were as great in men as in women during the one to four year period they studied their subjects. Sperry found the average standard deviation in an individual, when 2 to 10 determinations on 25 subjects were carried out over a period of months, was 12.1 mg cholesterol per 100 ml blood serum. Watkin, et al. (56, p.777) reported no definite influence of seasonal pattern, relative body weight, food, or menses on cholesterol concentrations which they observed in 6 women and 4 men subjects.

EXPERIMENTAL PROCEDURE

Subjects

Serum cholesterol concentrations were determined for four women subjects who were on a weighed constant diet for 30 days. The subjects were of apparent normal health and were all on the staff of the Nutrition Research Laboratory, Oregon State College.

Data on age, height, and weight of the experimental subjects are tabulated in table 4.

TABLE 4

Age, Height, and Weight of the Subjects

Subject	Age	Height	Weight 2-9-56	Weight 3-10-56
		in	lb	lb
CAS	49	68½	154.6	153.6
FD	23	69	245.6	239
NM	46	66	121	120
AS	28	70	137	136

Diet

The constant weighed diet, a modification of that of Gifft and Hauck (10, p.637), contained approximately 2000 calories of which 12 per cent was protein, 46 per cent was carbohydrate, and 42 per cent was fat. The calorie, protein, fat, carbohydrate, and cholesterol contents of the diet are tabulated in table 5. The menu was divided

into these meals:

Breakfast	Lunch	Dinner
Orange juice	American cheese	Beef, ground round
Egg	String beans	Carrots
Cream of wheat	Prunes	Peaches
Coffee	Biscuits	Biscuits
Sugar, brown	Butter	Butter
Milk, evaporated	Cookies	Cookies
Wheat germ	Coffee	

A riboflavin supplement of 100 mcg and a thiamine supplement of 500 mcg consumed with breakfast brought the daily intake of these nutrients to 1.37 mg and 1.09 mg respectively. The diet was adequate to meet the nutritional allowances set up for sedentary women by the Food and Nutrition Board of the National Research Council.

Blood Samples

Fasting blood was drawn each morning from the finger tip with a Bard-Parker blade. The whole blood was centrifuged under refrigeration after allowing an half hour for clotting. Serum was sampled and frozen in 6 x 50 mm tubes with rubber caps and held at -5°C. until analyzed.

Determination of Free and Total Cholesterol

To assure a uniform initial sampling of the serum aliquots, the frozen serum, when removed from cold storage, was incubated at 28°C. for 30 minutes. Each serum sample in turn was removed from incubation for pipetting and buzzed gently to mix without foaming.

Serum free and total cholesterol were determined according to the micromethod outlined on pages 11 to 16.

TABLE 5

Composition of Diet[1]

Food	Amount	Calories	Protein	Fat	Carbo-hydrate	Choles-terol[2]
	gm		gm	gm	gm	gm
Milk, evaporated	100	139	7.0	7.9	9.9	44
Carrots, canned	100	30	0.5	0.4	6.1	--
Beef, round, raw wt.	100	177	19.5	11.0	---	95
Wheat germ	6	24	1.5	0.6	3.1	--
Prunes, pitted	100	165	1.0	0.2	43.2	--
Peaches, canned	100	75	0.4	0.1	18.2	--
Green beans, canned	100	22	1.0	0.1	4.2	--
Orange juice, canned	166	80	1.0	0.3	18.4	--
Cream of Wheat, dry wt.	30	108	3.3	0.2	23.2	--
Egg, E.P.	54	96	7.8	7.0	----	383
Cheese, American	30	120	7.5	9.7	0.6	48
Butter	30	220	0.2	24.4	---	84
Sugar, brown	10	40	---	---	10.0	--
Biscuits[3]	110	332	6.0	15.6	42.0	--
Cookies,[3] brown sugar	96	430	4.8	18.6	60.8	128
Total		2058	61.5	96.1	239.7	782

1. Values were calculated from Watt and Merrill (57).
2. Values were calculated from Okey (33, p.342).
3. Values were calculated from ingredients.

Under the conditions of this procedure it was possible for one worker to determine the free and total cholesterol concentrations of duplicate extractions of serum for 4 subjects and 1 control serum sample in a single series. From each duplicate extraction, three aliquots were pipetted for total cholesterol and two aliquots for free cholesterol making a total of 50 sample tubes in a series. Six additional tubes were necessary for the reagent blanks. After the final washing of digitonin precipitates in the procedure, the sample tubes were separated and analyses of total cholesterol were completed. The free cholesterol sample tubes were stored together with 3 tubes of the reagent blanks. As soon as time permitted, the remaining analyses were completed.

Analyses were repeated until duplicate extractions for total cholesterol concentrations were within a maximum difference of 5 per cent and for free cholesterol concentrations within a maximum difference of 10 per cent with a few exceptions. More than duplicate extractions were necessary for 33 of the serum samples before values of extraction duplicates were within these limits. In a few cases the results of all extractions were averaged; the range among these values was sometimes in excess of the 5 per cent or 10 per cent limit. Determinations of free cholesterol for 6 serum samples are not recorded in table 6; the data have been omitted as the results appeared to be unreliable.

RESULTS AND DISCUSSION

The free and total cholesterol concentrations in the serum of the four women were determined for thirty days on a controlled diet and are presented in table 6 and figure 1. During the first portion of this study, subject NM became ill and was unable to eat her meals on the eighth and ninth days or to report for blood sampling on the ninth day. This physical upset may have affected the reliability of the determined results during this period of time. No data are presented for subject AS for the fifth day. The mean of duplicate serum extractions, 126.3 mg total cholesterol per 100 ml and 33.2 mg free cholesterol per 100 ml, were within the 5 and 10 per cent limits, respectively, but the values are questionable. Repeat determinations were indicated for serum samples of all subjects on this day because of the extremely low cholesterol concentration, 309.5 mg per 100 ml, determined for the control serum with this series. Additional serum was not available to repeat these determinations for subject AS.

The mean total cholesterol concentrations per 100 ml of serum for these four women, CAS, 145 ± 1.4[8], FD, 184.2 ± 2.8, NM, 147.6 ± 2.2 and AS, 158.0 ± 1.7 mg, were low compared with the means of their respective age groups reported in table 3 but are within reported ranges. Among the four subjects, FD, who was overweight, had the highest cholesterol concentration but it was still in the lower range of the accepted normal. No effect of age on cholesterol concentrations

---

8 Standard error of mean

TABLE 6

Daily Serum Total and Free Cholesterol Concentrations
For Four Women on a Controlled Diet

Subjects	CAS		FD		NM		AS	
Cholesterol	Total	Free	Total	Free	Total	Free	Total	Free
Day on diet	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml
0	156.5	43.1	209.5	54.1	176.2	46.1	173.0	48.9[1]
1	154.7	40.5	208.8	50.7	173.4	44.4	175.5	45.3[1]
2	142.7	37.1[1]	209.8	53.4	162.1	41.3	170.7	43.2
3	156.2	38.3	203.5[1]	49.4	148.2	36.1	158.1	38.4
4	136.6[1]	37.7	208.7	51.7[1]	136.2	40.4	-----[3]	----[3]
5	136.7[1]	40.1	205.8	49.9	140.2	39.9	156.21	41.9
6	144.8	36.9	203.6	50.7	141.7[1]	35.8[1]	149.7	37.1
7	146.7	42.3	195.8	51.6	158.6	39.2	174.7	44.6
8	136.3	38.0	192.5	48.1	157.3	39.9	159.7	40.2
9	140.2	39.0	192.2	50.1	-----[3]	----[3]	149.3	37.3
10	149.5	42.1	165.1	48.0[1]	146.8	38.4	150.7	40.2
11	148.4	44.0	177.1	47.1	132.0[1]	36.8	152.7	39.7
12	125.3[1]	38.6	168.6	46.9[1]	126.1	36.4	M 139.4[2]	39.9
13	143.1	42.1	162.2[1]	44.2	M 129.7[2]	37.5	148.2[1]	42.4
14	143.6	40.4[1]	182.8	46.3	124.4	34.6	153.9[1]	42.0
15	147.3	38.9	186.6	----[4]	132.9	34.8	150.0	37.7
16	141.3[1]	34.0	178.3	43.5	148.0	39.0	156.7	44.5
17	152.5	38.3	173.6	43.5[1]	149.4	37.3	163.9	40.2
18	151.5	40.5	180.6	44.7	153.1	40.7	165.5	42.1
19	144.0	38.4	179.5	43.3	150.2	----[4]	164.1	41.2
20	152.0	38.9	175.9	45.4[1]	153.8[1]	37.7	166.1	40.7
21	143.6	40.9	176.4[1]	----[4]	157.3	41.8[1]	165.2	39.6
22	M 144.3[2]	38.8	183.5	45.8	159.6[1]	40.9	160.4[1]	40.2
23	144.8	37.2	M 168.4[2]	46.0	151.9	40.1	158.2	39.7
24	144.5	40.6	149.6	42.5	147.7	39.9	158.2[1]	39.9
25	140.2	34.3	172.3	48.2	139.4	34.3	151.2	----[4]
26	138.9	42.3	167.8	46.9	142.8	41.1	146.0	38.9
27	148.2	38.5	178.9	----[4]	153.3	38.3	150.5	----[4]
28	145.1	40.9	170.7	40.5	142.1	39.4	144.7	39.9[1]
29	133.5	38.3[1]	190.1	45.7[1]	147.9	39.5[1]	169.3	42.2
30	165.3	40.4	192.5	50.7	146.6	36.0[1]	158.1	40.9

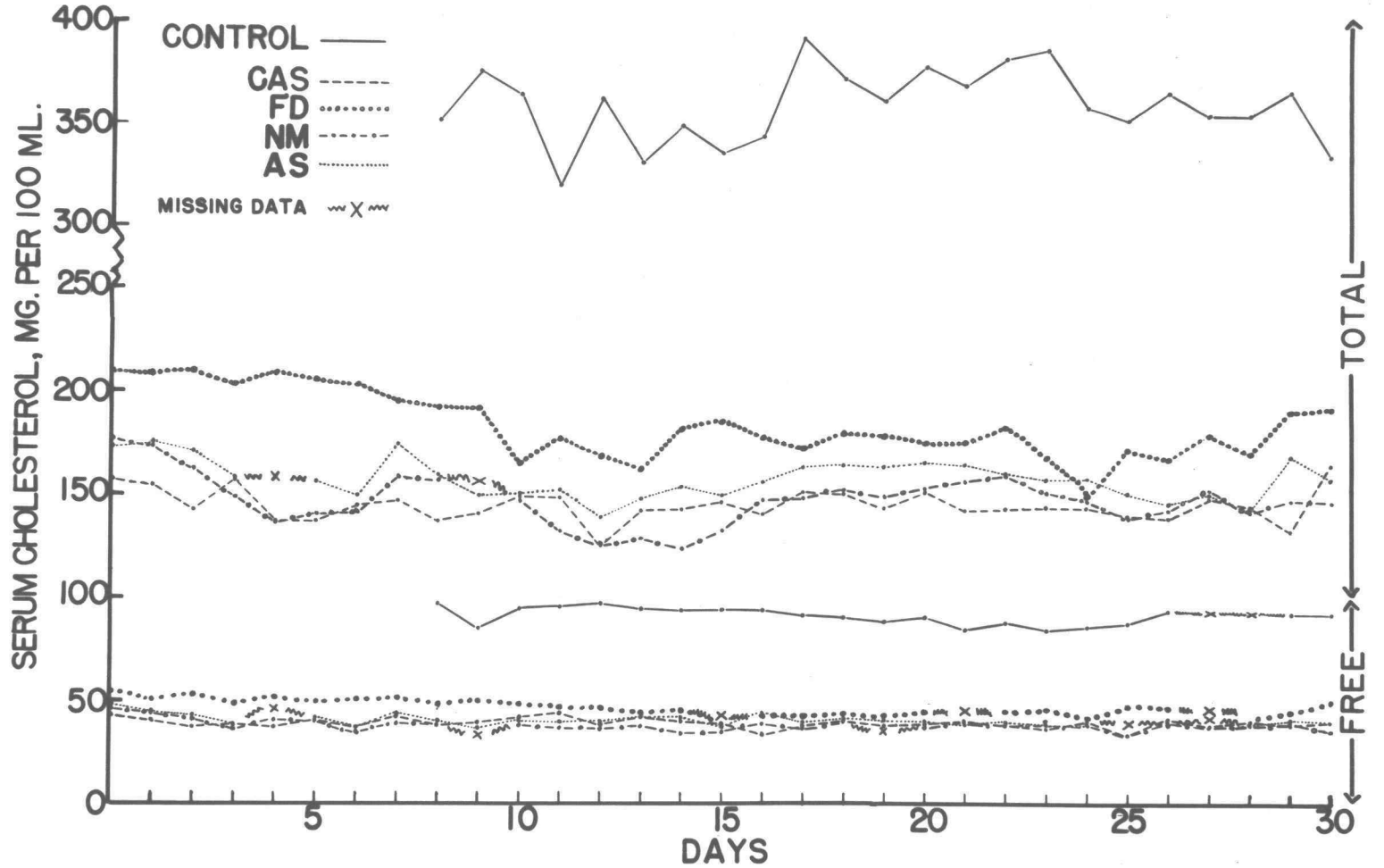
TABLE 6 (continued)

Subjects	CAS		FD		NM		AS	
Cholesterol	Total	Free	Total	Free	Total	Free	Total	Free
	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml	mg/ 100 ml
Mean	145.1	39.4	184.2	47.5	147.6	38.9	158.0	41.0
Range	125.3-165.3	34.0-44.0	149.6-209.5	40.5-54.1	124.4-176.2	34.3-46.1	139.4-175.5	37.1-48.9
Standard Deviation	7.7	2.3	15.5	3.4	12.1	2.7	9.3	2.5
Standard Error of Mean	1.4	0.4	2.8	0.6	2.2	0.5	1.7	0.5

1 More than 5% or 10% difference between replicate extractions for total or free cholesterol, respectively.
2 M: Onset of menstruation.
3 Serum not available.
4 No checks obtained on duplicates.

Figure 1

Daily Cholesterol Concentrations of Four Women on Controlled Diet



was apparent among these subjects; subjects CAS and NM had the lower mean concentrations of total cholesterol in this group.

Absolute free cholesterol concentrations changed little throughout the 30 day period. Mean free cholesterol concentrations for the subjects were: CAS, 39.4 ± 0.48, FD, 47.5 ± 0.6, NM, 38.9 ± 0.5 and AS, 41.0 ± 0.4 mg per 100 ml of serum. The ratios of free to total cholesterol for each subject were within the accepted range of 0.24 to 0.30 (44, p.263); the respective mean ratios were: CAS, 0.27, FD, 0.26, NM, 0.26 and AS, 0.26.

The ranges of serum total cholesterol concentration determined for the thirty day period were: CAS, 125.3 to 165.3; FD, 149.6 to 209.5; NM, 124.4 to 176.2; and AS, 139.4 to 175.5 mg per 100 ml, figure 1. These ranges, expressed as percentages of their respective means, are: CAS, 27.6; FD, 32.5; NM, 35.1; and AS, 22.8 per cent. These percentages of maximum difference for a month's period are generally greater than those reported by Man and Gildea (23, p.776) and Sperry (45, p.393) over longer periods of time. Man reported maximum differences of 31, 20, 19 and 18 per cent for four men and 24, 19, 13, 9, and 1 per cent for six women from determinations at irregular intervals, not exceeding two months, over one to four year periods. Sample ranges of total cholesterol reported by Sperry were 16.1, 19.6 and 24.6 per cent of their mean values for three men and 19.4, 12.2 and 18.8 per cent, for three women; however, these represented only two or three samplings over a period of one and one-half years. The somewhat higher ranges found in this study may be due to daily sampling giving

a more complete record of variation, the variation pattern of these particular individuals, or to a wider method variation.

The lowest level of these serum cholesterol ranges was recorded about the time of menses in subjects FD, NM, and AS, table 6. A low cholesterol concentration at the onset of menstruation has been reported Okey and Boyden (35, p.261-281), Offenkrantz (32, p.536-546) and Oliver and Boyd (37, p.217-222). However, lower cholesterol concentrations at menses were not found by Man and Gildea (23, p.777), Sperry (45, p.393) and Wagner and Poindexter (55, p.335-339); the infrequency of subject sampling in these studies may limit the validity of their observations.

Variations of total serum cholesterol concentrations for the four subjects were compared to each other and to the controls by calculations of standard deviations. The calculated standard deviations from the mean for data of these subjects were as follows: CAS, 7.7; FD, 15.5; NM, 12.1; and AS, 9.3 mg per 100 ml. These standard deviations are similar to those reported by Wagner and Poindexter (55, p.338) on 19 student nurses whose serum cholesterol was determined 4 to 6 times during the course of thirteen months; standard deviations from the individual means for theirsubjects ranged from 4.9 to 30.0 mg per 100 ml.

The standard deviations from these daily determinations, expressed as a per cent of their respective means, would indicate a variation of cholesterol concentration beyond that due to method variation for two subjects. The standard deviations for FD and NM were 8.3 and 8.0

per cent of their means whereas the standard deviations for the control serums were 5.3 and 5.4 per cent of their respective means. It would appear that the variations observed for subjects CAS and AS were influenced largely by method error; their standard deviations were 5.3 and 5.8 per cent of their means, respectively. The maximum per cent differences for subjects FD and NM, 32.5 and 35.1 per cent, also are significantly greater than would be expected within limits of errors of this micromethod. Analyses of the control serum indicated that a range of approximately 22 per cent (plus and minus two standard deviations expressed as percentage) of the mean concentration could be expected from method variation.

For FD and NM there was a reduction of approximately 30 mg total cholesterol per 100 ml between the inital serum cholesterol concentrations and the final concentrations measured. This change may be a result of the constant diet with respect to the fat content and/or calorie intake. It is possible that either subject was accustomed to a higher daily fat intake than the 96 gm the diet provided. However, to effect a significant reduction of cholesterol concentration in most persons, an extreme change in fat intake must be inflicted. Hildreth, et al. (16, p.641-646) used a 9 gm fat diet to show a drop of 47 mg cholesterol per 100 ml serum in 5 days, whereas diets with fat intakes similar to the subjects' usual fat intakes caused no change.

Of the four subjects on this diet, FD was the only one to show a change in weight (table 4) and this change was only a 4 per cent loss. This indication of calorie restriction might explain the

fluctuation in her total cholesterol concentration. However, the effect of calorie restriction without fat restriction has been reported previously as exerting no influence on serum cholesterol concentration (39, p.746). The influence of diet is further discounted by the reports of subjects on self-selected diets who exhibited cholesterol variations of 40 to 60 mg per 100 ml of serum (56, p.880) or plasma (35, p.261) during extended observation periods. The length of this study was insufficient to show if the lowered cholesterol concentrations of subjects FD and NM would be maintained by the diet used or whether their cholesterol concentrations would repeat some similar pattern in a subsequent period.

SUMMARY AND CONCLUSIONS

1. An unpublished micromethod for the determination of free and total cholesterol, which had been adapted to 40 lambda of serum from the Sperry and Webb method, was modified and proved to be practical and reliable.
2. Analyses of fasting serum samples for free and total cholesterol of four women on a controlled diet for thirty days showed significant variations in daily total cholesterol concentration, for two subjects beyond the variations due to method.
3. The lowest serum total cholesterol concentrations for the thirty day period occurred about the time of menses in three subjects.
4. Failure of two subjects to return to their initial higher serum total cholesterol concentrations may be a result of diet. However, unknown and unrecognized individual characteristics could account for these trends.

BIBLIOGRAPHY

1. Alfin-Slater, R. B., et al. The effect of essential fatty acid deficiency on the distribution of endogenous cholesterol in the plasma and liver of the rat. Archives of Biochemistry and Biophysics 52:180-185. 1954.

2. Anfinsen, Christian B., Jr. Biochemical aspects of atherosclerosis. Federation Proceedings of American Societies for Experimental Biology 15:894-899. 1956.

3. Beveridge, J. M. R., et al. The effects of certain vegetable and animal fats on the plasma lipids of humans. Journal of Nutrition 56:311-320. 1955.

4. Booker, Walter M., et al. Experimental studies on ascorbic acid metabolism. American Journal of Physiology 166:374-379. 1951.

5. Butler, Lillian C., Marian Tolbert Childs and Alice J. Forsythe. The relation of serum cholesterol to the physical measurements and diet of women. Journal of Nutrition 59:469-478. 1956.

6. Cook, R. P., D. C. Edwards and Christine Riddell. Cholesterol metabolism. 7. Cholesterol absorption and excretion in man. Biochemical Journal 62:225-234. 1956.

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

8. Galloway, Leora S. and Ethelwyn Wilcox, Professor of nutrition. Utah State Agricultural College, Logan, Utah. Personal communications. 1955-1956.

9. Garcia, Pilar, Charlotte Roderuck and Pearl Swanson. Age and fat absorption. Journal of Nutrition 55:601-609. 1955.

10. Gifft, Helen H. and Hazel M. Hauck. A comparison of four methods for studying the urinary excretion of thiamine. Journal of Nutrition 31:635-646. 1946.

11. Gildea, Edwin F., Eugen Kahn and Evelyn B. Man. The relationship between body build and serum lipoids and a discussion of these qualities as pyknophilic and leptophilic factors in the structure of the personality. American Journal of Psychiatry 92:1247-1260. 1936.

12. Gillum, Helen L., Agnes Fay Morgan and Dorothy Jerome. Nutritional status of the aging. IV. Serum cholesterol and diet. Journal of Nutrition 55:449-467. 1955.

13. Gofman, John W., et al. Evaluation of serum lipoprotein and cholesterol measurements as predictors of clinical complications of atherosclerosis. Circulation 14:691-725. 1956.

14. Gram, Mary R. and Ruth M. Leverton. Interrelation of age, serum cholesterol and basal metabolism. Federation Proceedings of American Societies for Experimental Biology 8:384. 1949.

15. Hardinge, Mervyn G. and Fredrick J. Stare. Nutritional studies of vegetarians. 2. Dietary and serum levels of cholesterol. American Journal of Clinical Nutrition 2:83-88. 1954.

16. Hildreth, E. A., et al. The effect of vegetable fat ingestion on human serum cholesterol concentration. Circulation 3:641-646. 1951.

17. Keys, Ancel, et al. The relation in man between cholesterol levels in the diet and in the blood. Science 112:79-81. 1950.

18. Keys, Ancel, et al. Studies on serum cholesterol and other characteristics of clinically healthy men in Naples. Archives of Internal Medicine 93:328-336. 1954.

19. Keys, Ancel, et al. Diet and serum cholesterol in man: lack of effect of dietary cholesterol. Journal of Nutrition 59:39-56. 1956.

20. Keys, Ancel and Margaret Haney Keys. Serum cholesterol and the diet in clinically healthy men at Slough near London. British Journal of Nutrition 8:138-147. 1954.

21. Kinsell, Laurance W., et al. Effect of vegetable fat on hypercholesterolemia and hyperphospholipidemia. Diabetes 3:113-119. 1954.

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

23. Man, Evelyn B. and Edwin F. Gildea. Variations in lipemia of normal subjects. Journal of Biological Chemistry 119:769-780. 1937.

24. Man, Evelyn B. and John P. Peters. Variations of serum lipids with age. Journal of Laboratory and Clinical Medicine 41:738-744. 1953.

25. Mann, George V. Importance of calorie disposition in cholesterol and lipoprotein metabolism of human subjects. Federation Proceedings of American Societies for Experimental Biology 14:442. 1955.

26. Mann, George V. and Hilda S. White. The influence of stress on plasma cholesterol levels. Metabolism 2:47-58. 1953.

27. Mayer, G. A., et al. Diet and plasma cholesterol levels. American Journal of Clinical Nutrition 2:316-322. 1954.

28. Mead, James F., William H. Slaton, Jr. and Arthur B. Decker. Metabolism of essential fatty acids. II. The metabolism of stearate, oleate, and linoleate by fat-deficient and normal mice. Journal of Biological Chemistry 218:401-407. 1956.

29. Messinger, William J., Yetta Porosowska and Murray J. Steele. Effect of feeding egg yolk and cholesterol on serum cholesterol levels. Archives of Internal Medicine 86:189-195. 1950.

30. Mirone, Leonora. Nutrient intake and blood findings of men on a diet devoid of meat. American Journal of Clinical Nutrition 2: 246-251. 1954.

31. Morrison, Lester M., M. Zwierlein and E. Wolfson. The effects of low fat-low cholesterol diets on serum lipids. Circulation 2: 475-476. 1950.

32. Offenkrantz, Frederick M. Serum cholesterol fluctuations during the menstrual cycle. American Journal of Clinical Pathology 8:536-546. 1938.

33. Okey, Ruth. Cholesterol content of foods. Journal of American Dietetic Association 21:341344. 1945.

34. Okey, Ruth. Use of food cholesterol in the animal body. Relation of other dietary constituents. Journal of American Dietetic Association 30:231-235. 1954.

35. Okey, Ruth and Ruth E. Boyden. Studies on 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. 1927.

36. Okey, Ruth and Dorothy Stewart. Blood cholesterol in normal women. Journal of Biological Chemistry 99:717-727. 1933.

37. Oliver, M. F. and G. S. Boyd. Changes in the plasma lipids during the menstrual cycle. Clinical Science 12:217-222. 1953.

38. Page, Irvine H., et al. Plasma lipids of normal men at different ages. Journal of Biological Chemistry 111:613-639. 1935.

39. Pomeranze, Julius, William H. Beinfield and Max Chessin. Serum lipid and fat tolerance studies in normal, obese, and atherosclerotic subjects. Circulation 10:742-746. 1954.

40. Pomeranze, Julius and Max Chessin. Decholesterolizing agents. American Heart Journal 49:262-266. 1955.

41. Reimer, Ann, Elmira Blecha and Charles F. Wilkinson, Jr. Study of essential familial hypercholesterolemia. Relationship of diet and blood cholesterol level. Journal of American Dietetic Association 25:842-844. 1949.

42. Schönheimer, R. and H. Dam. Über die spaltbarkeit und löslichkeit von sterindigitoniden. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 215:59-63. 1933.

43. Schoenheimer, Rudolf and Warren M. Sperry. A micromethod for the determination of free and combined cholesterol. Journal of Biological Chemistry 106:745-760. 1934.

44. Sobel, Albert E. and Margot A. Mayer. Improvements in the Schoenheimer-Sperry method for the determination of free cholesterol. Journal of Biological Chemistry 157:255-265. 1945.

45. Sperry, Warren M. The concentration of total cholesterol in the blood serum. Journal of Biological Chemistry 117:391-395. 1937.

46. Sperry, Warren M. The determination of cholesterol. Journal of Biological Chemistry 118:377-389. 1937.

47. Sperry, Warren M., and Florence C. Brand. The colorimetric determination of cholesterol. Journal of Biological Chemistry 150: 315-324. 1943.

48. Sperry, Warren M. and Merrill Webb. A revision of the Schoenheimer-Sperry method for cholesterol determination. Journal of Biological Chemistry 187:97-106. 1950.

49. Sperry, Warren M. and Merrill Webb. The effect of increasing age on serum cholesterol concentration. Journal of Biological Chemistry 187:107-110. 1950.

50. Swank, Roy L. and Valerie C. Wilmot. The influence of low-fat diet on blood lipid levels in health and in multiple sclerosis. American Journal of Medical Science 223:25-34. 1952.

51. Swanson, Pearl, et al. Blood values of women: cholesterol. Journal of Gerontology 10:41-47. 1955.

52. Swell, Leon T., et al. Role of fat and fatty acid in absorption of dietary cholesterol. American Journal of Physiology 180:124-128. 1955.

53. Walker, Georgianna R., Ellen H. Morse and Martha Potgieter. Serum cholesterol levels of young and of elderly women consuming an institutional diet. Journal of Nutrition 60:517-525. 1956.

54. Walker, Weldon J. Relationship of adiposity to serum cholesterol and lipoprotein levels and their modification by dietary means. Annals of Internal Medicine 39:705-716. 1953.

55. Wagner, Arnold L. and Charles A. Poindexter. Serial cholesterol determinations in healthy young women. Northwestern University Medical School Quarterly Bulletin 26:335-339. 1952.

56. Watkin, Donald M., et al. A study of serum beta lipoprotein and total cholesterol variability and its relation to age and serum level in adult human subjects. Journal of Clinical Investigation 33:874-883. 1954.

57. Watt, Bernice K. and Annabel L. Merrill. Composition of foods-raw, processed, prepared. U. S. Department of Agriculture Handbook no. 8. Washington, U. S. Government Printing Office, 1950. 147p.

58. Zak, B. and N. Ressler. Methodology in determination of cholesterol. American Journal of Clinical Pathology 25:433-446. 1955.