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To the Graduate Council:

I am submitting herewith a dissertation written by Ellen Speiden Parham entitled "The Influence of Dietary Protein on Serum Lipid Levels of Premenopausal Women." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

Frances A. Schofield
Major Professor

We have read this dissertation
and recommend its acceptance:

Jane R. Savage

Ada Marie Campbell

John T. Smith

Samuel R. Tipton

Accepted for the Council:

Hilton A. Smith
Vice President for
Graduate Studies and Research

THE INFLUENCE OF DIETARY PROTEIN ON SERUM LIPID LEVELS OF PREMENOPAUSAL WOMEN

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Ellen Speiden Parham

December 1967

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CHAPTER I

INTRODUCTION

Serum lipid levels of young women are of particular interest because, in a population riddled with atherosclerosis, premenopausal women seem to have a relative immunity both to severe hyperlipidemia and to those diseases thought to be related to it. The diet of this heterogeneous population is characterized by high intakes of animal protein, animal fat, and sugars.

Research has shown that the type and quantity of fat and carbohydrate have varying degrees of influence on serum lipid levels. Various micronutrients are also implicated. Animal experiments using wide ranges of protein intake suggest that under some conditions increased protein consumption lowered serum lipid levels, while under other conditions similar protein intakes raised these levels. The critical conditions have yet to be defined. Metabolic studies with humans have produced contradictory results, causing some authors to declare dietary protein to be hypolipidemic, while others consider it hyperlipidemic, and still others report no effect at all. In several similar studies, young women have responded to lowered protein intakes with lowered serum lipids while young men were unaffected. Because most of these metabolic studies have employed only small groups of subjects, it is possible that their differing results were caused by undiagnosed differences in the health and dietary history of the subjects.

The present study was designed to allow observation of the subjects' non-experimental dietary intakes and serum lipid levels as well as those serum lipid levels occurring during periods of consumption of experimental diets of minimum-adequate to moderate protein content. This experimental design allowed comparison of the subjects' usual dietary intakes with that considered typical of North Americans. The experimental serum lipid levels could be interpreted in terms of the dietary change involved rather than as responses to an absolute dietary intake.

A series of three metabolic studies of premenopausal females consuming diets limited in protein has been conducted in the Department of Nutrition at The University of Tennessee. The protein content of the diets varied from 20 to 48 g. per day, 35 to 65 per cent of the protein coming from animal sources. Variations in fat, sterol, and carbohydrate intakes were kept to the minimum that is possible with ordinary foods. Measurements of serum cholesterol and total esterified fatty acids were made both while the subjects were consuming their freely-selected diets and during the thirty-day experimental periods, thereby permitting at least partial characterization of each individual's serum lipid response. Serum fatty acids were determined during some of the periods in order to observe any qualitative changes in fatty acid patterns when the subjects changed from their usual diets to the experimental fare.

CHAPTER II

REVIEW OF LITERATURE

Serum lipids can be classified from the standpoint of the separate lipid constituents, mainly triglycerides, phospholipids, sterols, and unesterified fatty acids, or as the actual circulating moieties, including chylomicrons, the various classes of lipoproteins, and protein complexes of the free fatty acids. In general, the analytical techniques for the former group are simpler than those for the latter. Therefore, the first classification received most of the early attention and has remained popular, particularly as a clinical approach. The relative ease of measuring serum cholesterol also at least partially accounts for the persistent attention given it.

Some discussion of the sophisticated methods of studying the giant lipid-protein complexes is essential to an understanding of the terminology and classifications employed by the various laboratories. There are four main methods of determining lipoproteins: plasma protein fractionation, ultracentrifugal analysis, electrophoresis, and nephelometric determination (1). Plasma protein fractionation usually is based on Cohn's method 10 (2), depending upon separation of plasma proteins under carefully controlled conditions of pH, ethanol concentration, ionic strength, protein concentration, and temperature. The resulting fractions are designated as numbered Cohn fractions.

Ultracentrifugal analysis has been refined largely by Gofman and his coworkers (3). This method allows separation of the lipoproteins into several categories, defined by Svedberg flotation units, S_f. It is thus a classification according to density. Electrophoresis results in the separation of lipoproteins into groups, widely designated as alpha and beta. The alpha group is sometimes subdivided to include alpha-one and -two in correspondence to fractions obtained by other methods (4).

Nephelometric determination relies on specific precipitation of lower density lipoproteins by anionic polysaccharides. The turbidity of the suspension containing the resulting complex provides a rapid and sensitive measure of these lipoproteins (1).

The result is a spectrum of lipid-protein complexes which may be described and characterized in a number of ways. Considering this spectrum from the standpoint of density, at the extremely light end would be the chylomicrons. These particles are usually considered to represent transport form of recently ingested fat (5). They are mainly triglyceride and only approximately 2 per cent protein (4). Although they are rarely described in terms of their flotation rate, this measure is approximately S_f 40,000 (4). Chylomicrons are visible under a dark field microscope and are often reported as percentages of the total numbers of particles visible. They also produce serum turbidity visible to the naked eye.

The next fraction would be the very low density lipoproteins, S_f 10-400, corresponding to the electrophoretic fraction alpha two.

These macromolecules are approximately 50 per cent triglyceride and 22 per cent cholesterol. Gofman et al. (3) postulate that the S_f 10-20 part of this fraction is indicative of abnormalities of lipid metabolism. This category is inconspicuous in normal serum.

The low density or beta lipoproteins, corresponding to Cohn's fraction III$_o$ and Gofman's S_f 0-10 grouping are predominant in normal serum. These complexes are 47 per cent cholesterol, 23 per cent phospholipid, and only 9 per cent triglyceride. They may contain as much as two-thirds of the plasma cholesterol (1).

By contrast, the high density lipoproteins contain 19 per cent cholesterol, the greatest part of the molecule being protein. This group is designated alpha, and corresponds to Cohn's fraction IV. It is not implicated in coronary heart disease.

At the extreme of the spectrum are the unesterified fatty acids which travel complexed with albumin. This category accounts for only about 5 per cent of the total fatty acids in serum, but it is currently considered to be of great physiological significance, probably as the transport form of fatty acids mobilized from adipose tissue (6).

The specific function of each lipoprotein or the physiological relationship of one class to another is far from clear (6). It is easy to postulate a relationship of a single "parent" particle, perhaps the chylomicron, from which triglycerides are removed for storage or utilization, leaving the small, more dense molecules. Apparently, this is true to a limited extent as witnessed by various studies of blood clearing by heparin (4-8). However, evidence of small differences

in the protein moieties of the various lipoproteins (4) suggests that the relationship is not simply one of decreasing size.

The various circulating lipid complexes differ mainly in their relative concentrations of triglyceride, phospholipid, and cholesterol. The proportion of these building blocks in each lipoprotein fraction is thought to be relatively stable (3, 9). Thus, the character of a given fraction will vary little, even though the quantity may vary greatly from individual to individual. Knowing the usual composition of each circulating lipid fraction, one can speculate on the quantities of these moieties from the relatively easily performed determinations of triglyceride, phospholipid, or cholesterol. For example, triglyceride content increases as density of the lipoprotein decreases. Therefore, a high serum triglyceride value might suggest a large quantity of the lower density lipoproteins or chylomicrons. A high cholesterol concentration might indicate more of the beta lipoproteins. The phospholipid relationship is more complicated since the approximate percentage in very low density (alpha 2), low density (beta), and high density lipoproteins is 18, 23, and 26 per cent, respectively. Since neither cholesterol, phospholipid, nor triglyceride is unique to any one lipid fraction, such an approach is far from specific. A high cholesterol value could mean an increase of any one fraction or an increase of all the cholesterol containing fractions. Also, determination of only one lipid gives no information about those fractions containing relatively little of that compound; Gofman et al. (3) stated that it is not necessarily any more logical to study serum cholesterol

level as a measure of certain cholesterol containing lipoproteins than it is to study serum alanine and glycine levels as a measure of serum albumin.

In recent years, there has been a growing distrust of measurements for research purposes of a single lipid component. Often the preferred procedure involves determination of one component believed to be critical, plus a determination indicative of the state of the total lipid spectrum. For example, phospholipids, being fairly equally distributed among the lipoproteins, may be expressed as a ratio to cholesterol, which is less evenly distributed. Other combinations employed include beta/alpha lipoprotein ratio and cholesterol determination along with any of the following: triglycerides, esterified fatty acids, chylomicrons, or lipoproteins.

Studies of the individual fatty acids have also received recent emphasis. This may take the form of measuring the fatty acid composition of the total serum lipids or of each of the fractions. Also of current interest are the unesterified fatty acids, particularly in studies of the utilization of adipose lipids in obesity and other states. One aspect of these studies involves observation of the relative persistence of postprandial lipemia (5).

Much of the work on determination of serum lipids has been directed toward finding a diagnostic or predictive tool for coronary heart disease. In 1956 an evaluation of serum lipoprotein and cholesterol measurements was made jointly by the Technical Group of the Committee on Lipoprotein and Atherosclerosis and the Committee on

Lipoprotein and Atherosclerosis of the National Advisory Heart Council (10). The study resulted in a clear divergence of opinion between the University of California group headed by J. W. Gofman and the eastern laboratories represented by I. H. Page (Cleveland Clinic), F. J. Stare (Harvard), and M. A. Lauffer (University of Pittsburgh). The latter group felt that no measurements of serum lipids had predictive value in individual cases, although such measures might have useful application in epidemiologic studies. For use in such studies, they suggested that determination of serum cholesterol was just as meaningful as the more complicated measures including various lipoproteins.

The Gofman group was of the opinion that elevation of serum lipids precedes clinical evidence of atherosclerosis and can be used to predict it. This group felt that measurement of serum lipoprotein could give very valuable information on the state of an individual's lipid metabolism.

In the ten years since these differences of opinion, there has been very little resolution of the question. Albrink has been vocal and persistent about the value of triglyceride measurement as a means of distinguishing between healthy individuals and those with impaired lipid metabolism (9, 11, 12). As a result, various measures of triglyceride concentration are numerous in current literature. However, the literature still contains many studies, particularly of an epidemiological nature, based on serum cholesterol determinations alone (13).

Even though the current interest in serum lipid levels owes its impetus and urgency to the alarmingly high rate of coronary heart

disease, it has been both necessary and desirable to study lipid metabolism in normal individuals, as well as those with clinical evidence of atherosclerosis. The problem which has arisen again and again is the selection of individuals who are normal in this respect (7, 14-17). In most studies, normal refers to individuals with no clinical evidence of atherosclerosis, as witnessed by a medical history clear of myocardial infarction. These individuals may or may not be suffering from other physical ailments, including obesity. Labecki (7) pointed out that evidence of atherosclerosis may be found as discrete, isolated intimal lesions in children and even in infants. The control subjects may differ from the diseased only in the more advantageous location of arterial lesions (16). In comparison with other mammals and newborn human infants, even healthy young women in the U. S. A. have elevated serum lipids (15). Unfortunately, under the limitations of our present knowledge, we have no means of selecting individuals or groups as certainly normal controls. It should be borne in mind that in discussing normal serum lipid levels what is usually meant is the average, the mode, or the most frequently occurring serum lipid levels among individuals who give no evidence of abnormality.

Barr (15) compiled data from several studies in order to derive approximations of the lipid composition of normal human plasma. According to his figures, total cholesterol and phospholipid levels are 200 and 250 mg./100 ml. serum, respectively. The beta lipoproteins contain about 70 per cent of the total cholesterol content in a 1.25 ratio to phospholipid. The alpha lipoprotein fraction has a cholesterol/

phospholipid ratio of 0.50. A normal S_f 10-20 lipoprotein level is approximately 12 mg./100 ml. serum. These figures do not reflect any individual data on particular groups, but rather the trend of many studies. In actual practice, one cannot compare serum lipid levels without considering age and sex, as will be discussed below.

There may be wide variations in the level of any serum lipid among individuals. However, each individual has his own range which his body maintains with remarkable constancy. Brown and Page (14) followed the serum cholesterol levels of eighty-six men yearly for four to seven years and of six women weekly for five months. They reported that the serum cholesterol of these normal people eating freely chosen foods did not vary more than 17 to 25 mg./100 ml. from each individual's typical mean. Turner and Steiner (18), measuring serum cholesterol every four hours in nine subjects, found variations of approximately 10 per cent, even after feeding a breakfast rich in cholesterol. There are indications of some seasonal changes in serum cholesterol levels (19), although the literature on the subject is not consistent.

Most serum lipid determinations are made on blood collected after an overnight fast. This is not always possible, nor is it desirable in studying the rate of clearing of alimentary lipemia. Consumption of food seems to have little or no immediate effect on serum cholesterol levels (18, 20).

Brown, Heslin, and Doyle (20) observed serum triglycerides in healthy young men and women at various intervals after consumption of

test meals containing 10 to 70 g. fat. Three to five hours after ingestion of the 30, 50, and 70 g. fat meals, there were significant, but not proportional, increases in the serum triglycerides of the seven to ten subjects eating each meal. After five hours, the levels began to recede. In order to produce a significant rise in triglyceride levels, the amount of fat consumed in the preceding meal must be 30 g. or more, as shown by Brown's study and by others (21). The authors concluded that both fasting and postprandial triglyceride determinations are meaningful and that normal values would be less than 5.2 mEq./liter in fasted blood or less than 9.0 mEq./liter postprandially.

Recently attempts have been made to define the fatty acid patterns in normal human serum. In one such study, Lawrie and others (22) measured plasma fatty acids in sixteen healthy men and women, ages sixteen to forty-five years. They found that each class of lipids, triglyceride, phospholipid, or cholesterol ester, has a characteristic pattern of fatty acid composition. The remarkable constancy of the composition of the phospholipid fraction led the workers to conclude that those lipids must be under the control of processes that permit relatively little deviation from their pattern in which palmitic acid predominates. The cholesterol esters included 35 per cent linoleic acid, in agreement with the statement that cholesterol is preferentially esterified with long chain fatty acids. The triglyceride fraction was characterized by a relatively large amount of oleic acid, usually exceeding that of linoleic. The arachidonic acid content of triglycerides was extremely variable. Two male subjects had 31 and 47 per

cent of C_{20} unsaturated fatty acids. The female subjects showed significantly lower levels of arachidonic acid and significantly higher levels of palmitic and linoleic acid in triglycerides than did the males.

Lindgren, Nichols, and Wells (23) have studied the fatty acid composition of the major lipoprotein classes of serum from three normal adults. There seemed to be distinct differences between the fatty acid pattern of the very low density lipoproteins and that of the other fractions. Those differences may be characterized by an elevated level of palmitic and oleic acid and a reduced level of linoleic acid relative to the two higher density lipoprotein classes. The very low density fraction also showed greater variability from subject to subject. Serum samples collected a month apart from the same individual showed marked differences in fatty acid composition.

A sex-related difference in serum lipid levels is a usual finding (3, 7, 13, 16, 24-26). The serum lipids of young women compare to those of young men in the following way: cholesterol levels equal, phospholipids higher, cholesterol/phospholipid ratio lower, amount total cholesterol in alpha lipoprotein higher, and amount of S_f 10-20 lipoprotein lower. Women also have a larger number of mast cells (16). Barr (15) described the human female in respect to her serum lipid pattern as superior to her brothers, but grossly inferior to the dog and not so well equipped as the rabbit and the rat.

After the age of forty-five, or so, the serum lipids of females begin to resemble those of males. In fact, in her later years, the

female may have a higher serum cholesterol level than males of the same age. Before menarche, the female often has higher serum cholesterol and phospholipid levels than males (24). This period of low serum lipids between menarche and menopause coincides with the low incidence of coronary heart disease among females during those years.

In the male at about age twenty-two, there begins a period of rapid increase in serum cholesterol which continues upward until age fifty to sixty. Then it reaches a plateau or may actually decrease during the remaining decades (13). This is in contrast to the female who shows only a slow gradual increase until forty-five to fifty years. In general, comparison of younger adults with older adults of the same sex shows lower serum cholesterol, slightly lower cholesterol/phospholipid ratio, greater percentage of total cholesterol in the alpha lipoproteins, and lower S_f 10-20 bodies in the younger group (15).

In response to concern over coronary heart disease, there has been a widespread attempt to elucidate any differences between atherosclerotic and normal individuals. Comparisons of serum lipid levels, particularly cholesterol, usually show elevations among the coronary groups (27, 28). However, it has not been possible to prove a direct cause and effect relationship between elevated serum lipids and atherosclerosis. No one criterion will perfectly discriminate between individuals who have suffered myocardial infarction and those who have not. Barr (15) compared the overlap between the serum lipid levels of the two groups as determined by various laboratories. When total cholesterol was measured, 30 per cent of the heart patients had levels falling below

the mean of the controls, and 20 per cent of the normal group had levels above the mean of the patients. Cholesterol/phospholipid ratio gave little better discrimination. Use of Gofman's S_f 10-20 lipoprotein measurement reduced overlap to about 15 per cent for both groups. Determination of the per cent of total cholesterol found in beta lipoprotein gave the best discrimination, with only 2 per cent of the infarction survivors having values below the mean of the controls. However, 13 per cent of the controls had values above the average in the coronary group. It should be noted that the mean serum cholesterol value for the controls was 242 mg./100 ml., a value considerably above "normal" and probably reflecting the fact that the controls were matched in age with the coronary patients. Therefore, both groups showed the typical elevation of serum lipid with age. Rosenfeld (16) stated that the difficulty of demonstrating an association of serum lipids with atherogenesis may be related to the use of unrecognized atherosclerotic individuals as controls.

The difficulty probably is related also to the assumption that one measurement will identify all occurrences of elevated serum lipid levels. There is growing evidence of a number of different types of hyperlipidemia (9, 14). A simple classification presents three types: hyperglyceridemia, hypercholesteremia, and mixed hyperlipidemia. Mixed hyperlipidemia is a condition in which all serum lipid levels are elevated, but in normal proportions to each other. Brown and Page (14) found that this syndrome, in ten patients, readily responded to a low fat diet. This was in contrast to the other two types which showed

some response but still retained their distinctively distorted lipid patterns.

Albrink supports the idea that hyperglyceridemia is the common form of hyperlipidemia and the one most closely related to coronary heart disease. In a review (9) of epidemiological studies of healthy and atherosclerotic individuals in which both cholesterol and triglycerides were measured she points out that in each instance the triglycerides were more frequently elevated than the cholesterol. Such findings concur with the hypothesis supported by some authors (5, 7, 9, 17) that delayed removal of either endogenously or exogenously derived fat is a significant characteristic of patients with coronary artery disease. Brown and Page (14) state that in hyperglyceridemic individuals serum cholesterol levels are highly variable. Therefore, measurements on a single serum sample from an individual are not sufficient to classify his possible hyperlipidemia.

It may be that careful determination of several serum lipids in serum from both healthy and diseased individuals will bring to light significant subgroups of the three types of hyperlipidemia named above.

Serum lipid levels of groups similar in age and sex differ greatly in various parts of the world, usually with lower levels being found in the less developed countries (29). The possible causes of these differences have been investigated in numerous studies, a detailed discussion of which is not pertinent to this review. Suffice it to say that diet is most often implicated. In an effort to rule out influences of heredity, climate, amount of exercise, and other non-dietary

differences between populations, some investigators (30-32) have observed the effects of modifying the native diet, usually toward a typical Western diet.

In one such study (30) the serum lipid responses were observed when Korean soldiers were changed from their native fare to the U. S. Army diet. The U. S. ration was higher in calories and in animal fat and lower in carbohydrate than the Korean diet. The per cent of calories from protein was similar, but the protein sources differed greatly. Initially, Korean soldiers consuming their usual diet had lower total cholesterol, lower lipid phosphorus, and lower total esterified fatty acids than U. S. soldiers fed U. S. Army diets. Triglyceride levels were similar in both groups. All lipid indices except triglyceride had increased in the Koreans after they had consumed the U. S. Army diet for one month. After six to eighteen months, all serum lipids were elevated in the twenty-five Korean soldiers, but they were still well below the serum values of U. S. soldiers eating the same diet. The various lipid fractions did not behave uniformly; serum cholesterol responded to the dietary change more drastically and more rapidly than did the other lipids. By six months, it has actually decreased somewhat from the level reached after one month of the U. S. diet. Thus, diet does play a significant role in regulating serum lipid levels, but even a dietary change of six to eighteen months duration cannot completely override the cumulative influences of a diet consumed for a lifetime. Other workers (31, 32) have found this same type of response to dietary change.

Although indicating that diet is a factor in determining serum lipid levels, most studies of population groups can give no real evidence as to the role of the various dietary constituents. Keys et al. (29) plotted dietary fat against incidence of degenerative heart disease in six countries and have produced a convincing "dose-response" curve. Other workers (33) have shown that data from the same six countries can be used to construct an almost identical curve relating animal protein intake and incidence of heart disease. Lew (34) has emphasized the need for experimental and clinical testing of hypotheses drawn from epidemiological observation.

In considering the possible influence of diet on the serum lipid levels, the most obvious point of departure is the effect of quantity of food as determined by calorie intake. Various workers have followed the effects of weight loss on serum cholesterol levels and report rather disappointing responses (35-37). If the diet is the usual low fat, low calorie reducing regimen, there may be an initial lowering of cholesterol levels during weight loss, but as soon as the individual achieves calorie balance, serum cholesterol returns to the original level. Caldwell and others (35), studying coronary heart patients, concluded that correction of obesity alone appears to have no significant effect on serum cholesterol.

Other work (38, 39) supported the hypothesis that calorie balance is more significant than calorie intake. Mann and others (38) observed the serum lipids of three medical students who were consuming their usual intakes of approximately 3,000 Calories daily. During a subsequent

three-week period, the students were required to expend sufficient calories by exercise to prevent weight gain while ingesting diets of twice their usual calorie intake. So long as surplus energy was expended as heat and muscular activity, there was no increase in serum lipoprotein or cholesterol. During a three-week period of the high calorie diet with only the usual amount of exercise, the subjects gained weight and two of the three had elevated serum lipid levels.

A later study by Anderson, Lawler, and Keys (39) in part substantiated the results of Mann's group. Twenty schizophrenic men increased their calorie intake without increasing physical activity, resulting in an average weight gain of 0.5 kg. per week. Serum cholesterol increased during the first five weeks of overeating and remained at essentially the same elevated level for the remaining fifteen weeks, even though the weight gain continued at the same rate throughout the twenty weeks. The concentration of S_f 12-20 lipoprotein tended to increase from the tenth to the twentieth week, a time when serum cholesterol remained constant. The calorie increases were due largely to added carbohydrates, so that the per cent of calories furnished by fat was actually decreased during the weeks of overeating. In discussing their results, the authors stated that under controlled conditions, serum cholesterol concentration is determined by the fat transport load imposed on the blood. At caloric equilibrium, the fat transport load is determined by the proportion of calories furnished by fat. In positive caloric balance the transport load is increased by fat synthesized in the liver. However, if the weight gain is steady, there is no further

increase in fat transport and serum cholesterol should remain constant at the elevated level. This line of reasoning seems compatible with the suggestion made by Danowski (40) that subtle hypothyroidism occurring in aging contributes to elevated serum lipids and atherosclerosis.

Similar conclusions are reached when serum lipids are considered relative to long term habits of caloric intake as indicated by degree of obesity. Albrink (9, 11, 12, 41) reported positive correlation between obesity and elevated serum triglycerides in large numbers of middle aged male factory employees. Furthermore, when the heavy individuals were divided into two groups, those who had a life-long history of obesity and those who had gained ten pounds or more since age twenty-five, significantly higher triglyceride measurements are found in the latter group. The serum cholesterol levels showed some tendency toward elevation in the weight gainers, but the results were not as clear cut as with the triglyceride measurements. Albrink (9) states that the cholesterol-rich lipoproteins are most responsive to type and quantity of dietary fat, while the triglyceride-rich, very low density lipoproteins are more influenced by total carbohydrate and total calories. This statement adds weight to the theory mentioned above that the relationship among the various circulating lipids is more complex than a simple reduction in particle size via removal of triglycerides.

Perhaps of less practical importance but of great theoretical interest, are various studies done on serum lipids during starvation. Ende (42, 43) reported that healthy young men and women responded to starvation of two to five days duration with sharp increases in serum cholesterol. Males over fifty years of age show less marked and less

consistent elevations, while males with atherosclerosis and/or hypercholesteremia showed no significant increases in serum cholesterol.

The current attitude toward the relationship of calories and serum lipids seems to be that while calorie intake undoubtedly exerts some influence, particularly on triglycerides, this influence is not great enough to account for observed differences in serum lipid levels. There is consistent recognition that the quality and quantity of dietary fat is of great significance. Jolliffe and others (36) stated that the Prudent Reducing Diet is successful in maintaining lowered serum cholesterol levels, not because of reduced calorie intake, but because of a high proportion of polyunsaturated fatty acids.

Keys and others (29), having studied obesity and serum cholesterol in many population groups around the world, stated that any effect of calorie intake may be secondary to fat metabolism differences commonly associated with differences in calorie intake. These workers have taken the stand that serum cholesterol is more dependent on the intake of saturated fatty acids than of any other dietary component.

This is in contrast to the earlier theories that put emphasis on quantity rather than quality of fat. Kempner's rice-fruit diet (44) which was extremely low in fat and cholesterol was widely reported (29, 45) to produce prompt and substantial decreases in serum cholesterol. It was shown that the absence of dietary cholesterol was not responsible (45). The additions of small amounts of vegetable oil to the rice regimen resulted in increases in serum cholesterol (46).

Keys and others (29) observed the serum lipid responses to

reduced fat intake in thirty-five to forty-eight healthy men. Each man was stabilized for at least a month on a diet furnishing 39 per cent of calories from fat and then transferred for three to four weeks to a diet furnishing either 9, 18, or 24 per cent of calories from fat. The lowered fat intakes resulted in decreases of total cholesterol and of cholesterol in both the alpha and beta lipoproteins in serum. The changes were not consistently proportional to the degree of reduction of dietary fat. Serum cholesterol levels remained lowered during a thirty-two week period of consuming a low fat diet.

In another study by the University of Minnesota group (31) the fat content of the diet eaten by eighteen Japanese coal miners was increased from 10.3 to 24.9 per cent of calories by the substitution of butter or margarine for part of their customary rice. The result was an 11.6 per cent increase in serum cholesterol in eleven days. There was no significant difference between the effects of the two fats.

In spite of the numerous reports of a direct relationship between quantity of dietary fat and levels of serum lipids, there was indication that this was an over-simplification of the situation (47). Jolliffe et al. (36) pointed out that when the quantity of fat in the typical American diet is reduced, there is almost always a simultaneous change in its composition due to the relative ease of eliminating the visible fats. Goldsmith (48) reviewed a number of studies which showed that the effects of a reduction in dietary fat depends upon the quality as well as the quantity of fat. If the fat was composed largely of saturated fatty acids, a reduction in quantity produced a decrease

in serum cholesterol, phospholipid, and beta lipoprotein S_f 0-12. A reduction from 40 to 10 per cent of calories furnished by a largely polyunsaturated fat resulted in a slight increase in cholesterol and phospholipid in serum and no change in beta lipoprotein and triglyceride. Goldsmith suggests that these increases are due to the increase in dietary carbohydrate used to replace fat. In view of the recent indications of an influence of dietary carbohydrate on serum lipids, it may be necessary to re-evaluate many of the older studies, taking into consideration the type and quantity of carbohydrate used to replace dietary fat.

Many investigators have attempted to characterize the qualities of fat affecting serum lipids in man. Although animal fats were frequently found to elevate serum cholesterol and vegetable fats to lower it, several workers (49-51) have shown that the significant differences in these fats were not simply their origin. Forty adults in an investigation by Ahrens *et al*. (49) consumed for four to six months formula diets in which the type of fat was varied. In each diet fat furnished 40 per cent of total calories. In contrast to the hypolipidemic effect of corn oil, coconut oil increased serum lipids in these subjects. In a similar study (50) by the same group menhaden oil was shown to be as effective as corn oil. The obvious difference between these fats is their content of saturated and unsaturated fatty acids, corn oil, and menhaden oil being highly unsaturated. Such findings led to characterizing the fat content of diets in terms of iodine number (49), square root of the iodine number (51), or ratio of polyunsaturated to saturated

fatty acids, hereafter referred to as P/S (36, 52, 53). While all three measurements are valid under certain conditions, they all have definite weaknesses (54, 55). None of these measurements takes into consideration the amount of fat in the diet, implying that a low fat diet and high fat diet would produce the same effect providing the degree of unsaturation was equal. Both iodine number and its square root give equal weight to all double bonds. It has been shown that the monoenes, oleic and erucic acids, have little or no effect on serum lipids and that the highly unsaturated fatty acids are no more effective than linoleic acid (55). The P/S ratio has some advantage over iodine values in that it does not include the monoenes. To be applicable to ordinary diets, the P/S ratio must also exclude iso-acids, conjugates, or trans isomers from the polyunsaturated category (54).

Major emphasis has been placed on the polyunsaturated fatty acids, particularly linoleic acid, as the causal factors in fats and oils. There is undeniable evidence that the polyunsaturated fatty acids do have a depressing effect on serum cholesterol (45-50, 53-58). The results are not so consistent as far as the other serum lipids are concerned. For example, Campbell and others (58) fed diets in which the fatty acid content of the dietary fat was either 12 or 40 per cent linoleic acid to older men of fifty-three to seventy years of age. In both diets the total fat content was 40 per cent of the calories. After fifty days of eating the diet containing 40 per cent linoleic acid, the men had significantly lower serum cholesterol levels than they had when ingesting the diet containing less linoleic acid. However, there were

no significant differences in total serum lipids, sterol esters, glycerides, phospholipids, or unesterified fatty acids. The fatty acid content of these fractions did reflect the amounts of dietary linoleic acid.

Although the polyunsaturated fatty acids do tend to lower serum cholesterol, they do not outweigh the effects of the saturated fatty acids. Keys, Anderson, and Grande (55), after extensive study of the response of men to various fat mixtures and examination of similar studies in the literature, have proposed that certain saturated fatty acids are twice as effective in raising serum cholesterol as the polyunsaturated fatty acids are in lowering it. This group states that the serum cholesterol response to a dietary fat change can be predicted from the percentages of total calories provided by saturated (S) and polyunsaturated (P) fatty acid glycerides, using the formula, Δ cholesterol mg./100 ml. = 1.35 (2S-P). In calculation of S, these workers neglected the percentage of calories furnished by stearic acid and by saturated fatty acids having less than twelve carbon atoms (59). The decision to ignore these acids was based on controlled studies with cocoa butter which had previously mystified investigators (49) because of its failure to elevate serum cholesterol in spite of its high content of saturated fatty acids. Stearic acid amounts to 35 to 36 per cent of the fatty acids of cocoa butter. When the stearic acid content of cocoa butter was not included in the calculations, the observed serum cholesterol levels agreed closely with those predicted

by the above formula. The same type of criteria suggested the inertness of the relatively short chain fatty acids.

Pinter and Karle (60) recently observed responses in the same individuals to ingestion of saturated and unsaturated fatty acids of equal chain length. After consumption of emulsions containing saturated fatty acids, serum triglyceride concentrations were higher than those obtained during fasting or at comparable time intervals after ingestion of partially or completely unsaturated fats. Emulsions rich in stearic acid were consistent in their failure to produce an increase in serum triglycerides. The authors point out that stearic acid is a solid at body temperature and does not enter readily into micellar formation in the intestinal lumen unless low melting point fatty acids are available.

In most diets stearic acid and the fatty acids of less than twelve carbon atoms are rarely consumed in any significant amounts, except in combination with other fatty acids. Stearic acid seldom accounts for more than 3 per cent of the calories of the usual American diet. Of the more common saturated fatty acids, lauric, myristic, and palmitic, palmitic is much more abundant in most diets and is probably primarily responsible for the effect of elevating serum lipids (50). Likewise, by virtue of its relative abundance, linoleic is the most important of the cholesterol-lowering polyunsaturated fatty acids.

The influence exerted on serum lipids by the various fatty acids is not consistent throughout the animal kingdom. In rats and calves, increases in intake of unsaturated fat will result in increased plasma

cholesterol levels (61, 62). Mice and dogs react similarly to man (61). This difference is one of many special variations in lipid metabolism. Therefore, care must be taken when drawing inferences from work with laboratory animals.

Much recent research (57, 60, 63-67) has been focused on elucidation of the mechanism or mechanisms by which dietary fatty acids influence the serum lipids. For each lipid fraction there are at least four possible mechanisms for the lowering of serum level of that fraction: (a) a decreased synthesis, (b) increased net excretion or catabolism, (c) shift from the serum to some other tissue, and (d) some combination of these factors (63).

In the case of cholesterol, the rates of removal via excretion of bile acids and of reabsorption of these acids have been suggested as serum level controlling mechanisms. Apparently, the fatty acid composition of the cholesterol-bearing lipoproteins influences the rate of bile acid and sterol excretion (62, 65, 67), polyunsaturated fatty acids favoring excretion.

In contrast, Spritz and others (63) found that when serum cholesterol was altered in human subjects by feeding various fats, there was no significant reciprocal change in fecal steroid excretion. They could account for the decrease in serum cholesterol only by increased removal to other tissues.

The question of the mechanisms by which the various fatty acids exert their effects is far from resolved. The multiplicity of results suggests that there is probably more than one site of influence.

Early in this century it was shown that rabbits fed cholesterol developed hypercholesteremia and eventually lesions similar to those of atherosclerosis in man. From these results began a still uncompleted attempt to demonstrate such a relationship in man. Cholesterol is found only in animal products and is unevenly distributed among them. Therefore, ordinary diets may vary widely in their cholesterol content. This leads to an attractive hypothesis that the different serum cholesterol levels observed in humans are due to their different cholesterol intakes. Unfortunately, the situation is complicated by the fact that differences in cholesterol content of self-selected diets are almost always accompanied by differences in protein and fat content. In 1955 Keys and his group (29) after extensive population studies stated that their work confirmed the lack of response in man to the cholesterol level of the diet. Numerous studies of feeding cholesterol in controlled experiments upheld this statement. For example, Ahrens and others (49) reported that adding 600 mg. cholesterol daily to lard-containing diets produced no higher levels of serum lipids than lard alone. In a 1961 review (54) Jolliffe stated that it has been repeatedly shown that the cholesterol content of any practical dietary had no significant effect on the serum cholesterol levels in man.

However, in the late fifties and early sixties there appeared more and more reports of a direct relationship between dietary and serum cholesterol (68-73). These studies differed from some of the earlier ones that had negative results in that the cholesterol was always fed in combination with fat and that the comparisons were made

between cholesterol-free and cholesterol-containing diets. Apparently the presence of dietary fat is necessary for absorption of cholesterol. Some of the earlier investigators added additional cholesterol to diets already high in it. There seems to be a definite plateau effect on serum cholesterol when dietary cholesterol is increased beyond the amount, approximately 600 mg. per day, found in ordinary non-vegetarian diets. One study (70) compared serum cholesterol levels of subjects fed cholesterol-free diets with those of subjects consuming daily diets containing 475 to 1,425 mg. cholesterol furnished by egg yolks. All subjects consuming cholesterol had similar elevations in their serum cholesterol levels. Beveridge and others (69) found similar flattening of the response curve when dietary cholesterol was increased beyond 634 mg. per day.

Keys et al. (74) have modified their earlier stand on the significance of dietary cholesterol. After studying the responses of twenty-two schizophrenic men to various doses of cholesterol administered in the fat portion of the diet, they derived the following relationship:

Δ cholesterol, mg./100 ml. serum = 1.5 Δ Z, where Z is the square root of daily dietary cholesterol in mg. Obviously, according to this equation, small differences in cholesterol intake would produce extremely small changes in serum cholesterol. These authors point out that intraindividual average standard deviation in serum cholesterol level is 20 mg./100 ml. and interindividual average standard deviation is 45 mg./100 ml. In view of this high degree of variability, it is no wonder that the relatively small effects of dietary cholesterol often

go undetected. Other dietary differences in uncontrolled diets could easily mask this small effect.

Keys and his group (74) further stated that a given intake of dietary cholesterol produces the same effect on serum regardless of the fat composition of the diet. Therefore, they have combined the two formulas given above to produce the following equation: Δ serum cholesterol mg./100 ml. = $1.5\ \Delta\ Z + 2.7\ \Delta\ S - 1.3\ \Delta\ P$. On the surface, this relationship may appear contradictory to an earlier conclusion of Beveridge and others (68) that the serum response to cholesterol depends upon the nature of the dietary fat with which the sterol is associated. However, Beveridge's group reached this conclusion after feeding eighty-three young men and women various distillation fractions of butter and corn oil. Sterol and fatty acid content differed simultaneously and since there were no trials with cholesterol-free fractions, there was no opportunity to distinguish between the effects of the fatty acids and of the cholesterol.

Animals vary greatly in their response to dietary cholesterol (29). Rabbits and chicks at one extreme of the sensitivity scale can respond to added cholesterol by increases in serum cholesterol of as much as 3000 per cent (29). Man is at the other extreme of the sensitivity scale. It has been suggested (75) that lower animals compensate for ingested cholesterol by rapid suppression of hepatic cholesterol synthesis. This compensatory action would obviously be limited to the capacity of liver to synthesize cholesterol. Estimations of hepatic cholesterol synthesis in man are of the order of 1.5 to 2.0 g. per day

(75, 76). If the liver is responsive to dietary cholesterol in man as in lower animals, there is a potentially powerful compensatory mechanism.

Taylor and others (75), in order to test this possibility, fed seven healthy adults diets containing 1 to 4 g. cholesterol daily for eight weeks. The cholesterol was labelled with ^{14}C at the fourth carbon. Radioactivity of serum cholesterol permitted direct calculation of the exogenous portion which was found to be 24 to 31 per cent of total serum cholesterol. The authors interpret this to mean not that the liver had failed to respond by decreased synthesis, but that synthesis by extrahepatic tissues was supplying about three-fourths of the serum cholesterol. Presumably, extrahepatic synthesis is unaffected by cholesterol ingestion.

Assuming that the liver does reduce cholesterol synthesis when the diet provides significant amounts of that sterol, then loss or reduction of this compensatory mechanism would produce increases in the serum level. Beveridge and others (69) point out that in normal young individuals there is an effective control system that prevents hypercholesteremia even when relatively large amounts of cholesterol are consumed. They further suggest that this mechanism may break down in hypercholesteremic individuals.

Extensive studies of lacto-ovo and pure vegetarians (77, 78) showed that both vegetarian groups consumed less animal fat and cholesterol than similar non-vegetarian groups. In the mature individuals these differences resulted in higher serum cholesterol levels among

non-vegetarians. In adolescents and pregnant women dietary differences seemed to have no effect on serum cholesterol. It was suggested that as the compensatory mechanism fails, dietary cholesterol may play an increasingly significant role in elevating serum lipids.

Most of the studies of response to dietary cholesterol have been concerned with serum cholesterol levels only. However, the current awareness of serum lipid interrelationships has led to examination of simultaneous effects on phospholipids and triglycerides, as well as on cholesterol. In general, the phospholipid responses parallel those of serum cholesterol, but the absolute value of the changes may not be as large (70-73). The serum triglyceride response if often variable and is usually considered unrelated to cholesterol intake (70-73).

In contrast to the responses to the animal sterol, cholesterol, are the serum lipid reactions to plant sterols, particularly beta sitosterol. Corn oil has frequently been shown to be particularly effective in lowering serum cholesterol. In one study (79) the serum influences of corn oil, sunflower seed oil, sardine oil, and butter were observed in healthy men. Corn oil had the greatest cholesterol depressing effect. This effect could not be explained on the basis of iodine value or linoleic acid content.

Beveridge and others (80) fed diets in which butter provided 60 per cent of calories to fifty-two male students for eight days. Then for eight subsequent days the rations were supplemented with alpha tocopherol or beta sitosterol. The tocopherol had no effect on plasma cholesterol, but the sitosterol caused highly significant decreases in

plasma cholesterol. In a later study (81) the same group estimated that approximately 300 mg. sitosterol/1000 Calories would significantly lower serum cholesterol. A diet in which corn oil provided 15 per cent of calories would produce this effect. Keys and others (55) estimated an average decrease in serum cholesterol of 2 to 3 mg./100 ml. for each 10 per cent of calories from corn oil, the effect being independent of the influence of the fatty acids in corn oil.

Sitosterol is poorly absorbed (77). That which is absorbed is not converted to cholesterol, nor does it affect biosynthesis of cholesterol (57). It seems that the influence of sitosterol occurs in the intestine, probably by interfering with absorption of both endogenous and exogenous cholesterol. It has been reported (57) that feeding sitosterol with equal amounts of cholesterol prevents absorption of 66 per cent of the cholesterol.

On the other hand, some workers (27, 54) feel that the amount of sitosterol required to produce any significant effect is so great, 10 g. or more per day, as to render it impractical in ordinary diets. Jolliffe (54) reports that the sitosterol content of 1 to 3 oz. of corn oil is without effect on serum cholesterol.

As already mentioned, studies of population groups often show that the poorer groups have lower serum lipid levels and less atherosclerosis. Comparisons of the diet of these peoples with those living in the more developed countries usually show differences in intakes of quantity and quality of fat, carbohydrate, and protein. It has been shown that fat content of the diet can be influential to serum lipids.

Various studies have attempted to show whether or not dietary protein is also an important factor.

Dietary protein supplies from 13 to 14 per cent of the total calories in the current United States diet (82). This protein is largely of animal origin, animal protein furnishing 8 to 9 per cent of total calories. According to a ranking presented by Keys and Anderson (82), only primitive Eskimos exceed the people of the continental United States in proportion of calories furnished by protein and by fat. The list of thirty-one population groups cites ten groups which have been shown to have low serum cholesterol levels. Of these ten, all have lower fat intakes than Americans. This ranking does not take into consideration the animal protein content of the various diets. Keys and his group point out that most populations receiving low-fat diets eat relatively small amounts of protein from animal sources. Therefore, it is likely that the ten low serum cholesterol groups are characterized not only by low dietary fat, but also by low animal protein intake.

Annand has made an intensive search for a dietary factor in the increase of atherosclerosis in the United Kingdom (83). Pointing out that atherosclerosis is not a new disease, but one of greatly increased incidence, he has set up two general criteria of a dietary factor that could be considered as correlated to the disease: first, the food must be common to all countries having a high incidence of the disease and second, the intake of the food must have increased parallel to the atherosclerosis incidence. By consideration of death records against

food technology advances, economic influences, and other indicators of food intake, he has concluded that heated animal protein is implicated. Special emphasis is placed on the high cyanocobalamine content of this food group, but no further explanation is made for the hypothesized deleterious effect of heating on animal protein. The inferences drawn are thought-provoking, but without further evidence must be regarded only as speculation.

Stronger indication of a protein involvement in serum lipid regulation is found in a report (84) by Luyken of dietary composition and biochemical criteria, i.e. serum cholesterol, serum albumin, and urea nitrogen, of eight groups living in tropical areas. The tuber diets of some of these people were quite low in protein in general, and in methionine and cystine, in particular. The lowest serum cholesterol levels were found in those people suffering from protein malnutrition as witnessed by low albumin and urea values. Although stressing the correlation of serum cholesterol with dietary protein in this study, Luyken pointed out the possible implication also of fat and calorie content.

The rigidly controlled life and diet of monasteries have provided an opportunity to observe the results of long term ingestion of diets low in fat and animal protein by men following their normal routine. One study (85) compared the diet and serum cholesterol of Benedictine and Trappist monks who consumed respectively 13 and 14 per cent of total calories from protein. The Trappist monks are vegetarians who consume very little animal protein or fat. The Benedictines eat a more liberal

diet, including some meat. Serum cholesterol was significantly higher among the Benedictines.

However, another study (86) of Trappist monks conducted by Calatayud and others showed serum cholesterol levels no lower than those of ordinary men matched in age. Measurement was also made of serum protein and of cholesterol in the alpha and beta lipoprotein fractions. None of these indices showed significant differences. Elevated serum cholesterol levels among the monks or the controls could be correlated only with excessive weight, age, or family history of coronary artery disease.

In a somewhat earlier study (87) of six middle aged men who had lived for about twenty years on a meatless diet, Mirone found the same apparent lack of effect on serum cholesterol. These men ate only 10 to 23 g. animal protein and 22 to 24 g. total fat daily. However, the intakes of total protein were about 60 to 75 g. and no evidence of protein deficiency was found. Two of the six men were overweight. Average serum cholesterol was 208 mg./100 ml., a level which can not be called unusually low. However, cholesterol levels of the middle aged Trappist monks and controls reported by Calatayud *et al.* (86) were 260 and 243 mg./100 ml., respectively. Once again a definition of normal serum lipid values is crucial in interpretation of data.

The studies by Hardinge and coworkers (77, 78) of vegetarians and non-vegetarians are in contrast to those vegetarian groups discussed above. These workers reported significantly lower serum cholesterol among the vegetarian adults, the pure vegetarians having lower values

than the lacto-ovo-vegetarians. Calculation of the amino acids eaten by both vegetarian groups and by non-vegetarians showed that all groups exceeded twice their minimum requirement (88). Therefore, the results can hardly be attributed to amino acid deficiency. As in all the epidemiological studies reviewed here, dietary fat varied as well as dietary protein.

The Framingham Study (89) represents another epidemiological approach, the prospective study. The occurrence of hypertension and coronary heart disease has been followed since 1949, via medical histories, physical examinations, laboratory determinations, and dietary histories of approximately 5000 adults. The results show incontrovertible evidence linking serum cholesterol levels and coronary heart disease. On the other hand, no significant correlation was found between serum cholesterol levels and daily intakes of calories, total fat, animal fat, plant fat, total protein, animal protein, plant protein, or cholesterol.

Studies with rats repeatedly have shown an involvement of dietary protein in the regulation of serum lipid levels. Such studies often involve the rat made hypercholesteremic by use of a diet containing from 1 to 5 per cent cholesterol, about 1 per cent cholic acid, and relatively high levels of fat, usually highly saturated. Choline may be added to heighten the cholesteremia. This regimine may elevate the serum cholesterol to as high as 1000 mg./100 ml. (84). This is in contrast to a typical rat serum cholesterol level of 90 mg./100 ml. (33). Moyer and others (90) fed such a diet plus 10 to 60 per cent casein or

soybean protein to 180 to 200 g. rats for four weeks. With either protein, increasing the protein content of the diet caused a progressive drop in serum cholesterol. Diets to which choline was added produced higher serum cholesterol levels than choline-free diets of equal protein composition.

Nath and others (91) compared casein, wheat gluten, and butanol-extracted wheat gluten as to their effect on serum lipids of weanling rats. The basal hypercholesteremic diet containing 25 per cent casein produced the expected high serum cholesterol levels after three weeks. Substitution of 25 per cent wheat gluten produced serum cholesterol levels of approximately half those of the group consuming casein. The hypocholesteremic effect of the wheat gluten appeared to be due to its lipid content because butanol-extracted gluten showed no such effect. When 10 to 67.5 per cent of the diet was extracted wheat gluten, the serum cholesterol levels were inversely related to the protein intake. The authors stated that this progressive reduction was due to the higher intake of sulfur-containing amino acids when the gluten content increased.

Luyken (84) also reported a hypocholesteremic effect of an unextracted wheat gluten supplement to rats on an otherwise hypercholesteremic diet. The effective component of wheat gluten appeared to be the protein, rather than the lipid content because a mixture of amino acids corresponding to those in gluten was as effective as the intact protein. In other trials, supplementation with groups of three of the essential amino acids was effective only when methionine was included. Luyken concluded that the sulfur-containing amino acids are of particular significance in maintaining low blood cholesterol levels.

Fillios and Mann (92) produced hypercholesteremia in both mice and rats by feeding diets containing 20 per cent soybean protein, 5 per cent corn oil, and 5 per cent cholesterol. Increases in S_f 3-20 lipoprotein accompanied the hypercholesteremia. Soybean protein has been shown to be deficient in sulfur-containing amino acids. Addition of 0.6 per cent DL-methionine partially prevented the cholesterol elevations. Later Portman and Mann (93) showed that taurine could be substituted for methionine or cystine in preventing hypercholestermia in rats fed soybean protein.

This same group has done extensive study of sulfur amino acid deficiency in various species of monkeys (94-96). With these animals even a cholesterol-free soybean protein diet produced elevation of serum cholesterol. Substitution of casein for the soy protein lowered the cholesterol levels. Furthermore, the casein-fed monkeys were better able to compensate for dietary cholesterol without elevation of serum cholesterol. When compared on the basis of sulfur content, cystine, methionine, cysteine, and glutathione supplements were equally effective in preventing hypercholesteremia and promoting growth (95). Taurine was somewhat less effective.

In spite of the consistency of these studies in implicating a deficiency of sulfur-containing amino acids in elevation of serum lipids, other studies indicate a more complex situation. Jones and Hoffman (97) fed three-month-old rats diets containing in per cent: cholesterol 1.2, lard 15.0, choline 0.6, casein 7.5 to 25.0, and dextrin up to 100.0. After twenty-five weeks the serum cholesterol of the rats receiving 12.5

per cent casein was significantly lower than that of rats eating 7.5, 9.0, or 25.0 per cent protein diets. One group of older rats fed a 40 per cent casein diet showed a three-fold elevation in serum cholesterol over that of chow-fed controls. Rats receiving a diet containing 25 per cent casein and 15 per cent lard had higher cholesterol levels than animals on 18.75 per cent casein and 30 per cent lard. In this situation apparently elevation of dietary protein above 12.5 per cent was more significant than an increase in intake of highly saturated fat.

Bagchi and others (98) have demonstrated a cholesterol-elevating effect of dietary protein. Weanling rats were given diets containing either 4, 10, or 18 per cent casein, resulting in serum cholesterol levels of 71, 88, and 121 mg./100 ml. Simultaneous determination of liver sulfhydryl content showed parallel increases. Addition of 50 mg. methionine to the 4 and 10 per cent casein diets caused 18 to 33 per cent increases in serum cholesterol, while addition of sulfoximine to the 18 per cent casein diet resulted in a 30 per cent decrease. The conditions of this study are in sharp contrast to those reviewed above. In most of the other investigations adult rats were used; this group studied weanling rats. These rats were not fed the usual hypercholesteremic diet; their cholesterol levels even after the demonstrated increases were often approximately 10 per cent of the levels of the animals in the other studies. Bagchi's group suggested that the effect of protein on the livers of the animals in their study was due to promotion of cholesterol synthesis either by maintaining the functional

integrity of the liver or by providing the sulfydryl groups of enzymes necessary for this synthesis.

Olson and others (33) have produced further evidence of development of marked hypocholesteremia, hypolipidemia, and hypobetalipoproteinemia in young adult rats fed soy protein low in methionine and choline. Addition of choline removed these effects; substitution of casein for soy protein partially did so. Since the total organic sulfur content of the diets was kept constant by varying the amount of cystine, the effect of the higher quality protein was attributed to its content of labile methyl group donors, particularly methionine.

The effects of dietary protein and choline on cholesterol metabolism are not entirely interchangeable (99). Depending on other conditions such as dietary composition, health, and age, the significant effect of adding high quality protein to rat diets may be either to remove a relative deficiency of organic sulfur, or to furnish labile methyl groups. Several of the studies (90, 91, 97) in which a sulfur-containing amino acid deficiency was implicated were based on diets already high in choline. Okey and Lyman (99) suggested that in young animals methionine may be used preferentially to support growth and only secondarily to prevent fatty livers.

Rats are, by no means, the only laboratory animals used in studies of dietary protein and serum lipid levels. As mentioned earlier, Mann and his group have worked extensively with monkeys (94-96). Barnes and others have shown an age difference in response of serum cholesterol to dietary protein in swine (100, 101). Adult swine showed no response

to low protein diets, but young animals responded with severe protein deficiency symptoms and elevated serum cholesterol.

Studies with chicks or mature birds have been numerous, particularly at the University of Illinois. Day old chicks were fed for three weeks diets of 10 to 40 per cent soy protein. They responded to increased protein with serum cholesterol decreases (102). Arginine supplementation of a 10 per cent casein diet lowered serum cholesterol, apparently by promoting growth. Methionine lowered serum cholesterol somewhat, but had no effect on growth. Combined supplements of arginine and methionine were most effective both in promoting growth and lowering serum cholesterol levels. This work by Johnson, Leveille, and Fisher supports the theory of the diphasic influence of protein. In agreement with responses found in monkeys by Mann (95), this study showed also that increased protein intake produced resistance to elevation of serum cholesterol by dietary cholesterol in chicks. Birds fed a 7 per cent soy protein diet responded to 2 per cent cholesterol with levels almost triple those of chicks on the same diet without cholesterol. At 31 per cent protein, the same cholesterol intake caused no significant difference in serum levels.

In a later study (103), Kokatnur and Kummerow observed growth and serum cholesterol levels in week-old chicks consuming various mixtures of essential and nonessential amino acids. Results confirmed the earlier findings of Johnson's group (102). An increase in nitrogen intake produced by manipulating the amount of a balanced amino acid mixture lowered serum cholesterol. However, an imbalance caused by a

small excess of one amino acid resulted in poor growth and high cholesterol levels. Leveille and Sauberlich (104) reported that when serum cholesterol was lowered by increasing dietary protein, increases in serum proteins paralleled the cholesterol decreases in young chicks.

Marion and others (105) found no influence on serum cholesterol of the protein content of diets very low in fat. Only when corn oil was present did the quantity of dietary protein exert an inverse effect on blood cholesterol levels of the chicks.

Kokatnur et al. (106) demonstrated a somewhat different interrelationship between dietary fat and protein. Male birds, 12 to 18 months old were fed for twenty-one days diets containing 7.5 to 30.0 per cent soybean meal and either 0.1 or 15.0 per cent corn oil. At either fat intake, increased protein intake lowered serum cholesterol. The high fat diet showed an elevating effect on blood cholesterol only when the protein intake was inadequate. This finding is reminiscent of observations in monkeys (95) and day-old chicks (102) of a protective effect of protein against dietary cholesterol and fat. The authors interpreted their results as indicating that reduction in serum cholesterol may be produced more efficiently by increasing dietary protein intake than by decreasing fat intake.

The influence of dietary protein in chicks has been further pinpointed by a study of the metabolism of intraperitoneally administered acetate-1-^{14}C (107). Five-month-old chicks were fed diets of 15 to 30 per cent protein, with or without 1 per cent cholesterol. After three

weeks on these diets, the birds were injected with the labeled acetate and sacrificed at intervals up to two hours. Livers and blood were analyzed for the relative content of labeled carbon in cholesterol and in fatty acids. A low dietary protein level tended to increase at least initial incorporation of acetate into liver cholesterol. The low protein also increased the rate of fatty acid biosynthesis. The effect on fatty acid synthesis was more pronounced than on cholesterol formation. The serum changes paralleled the effects on liver lipid synthesis.

Thus, numerous studies have shown that both chicks and older birds respond to relatively high protein levels with lowered serum cholesterol. Part of this influence might be attributed to an improved ability to cope with dietary fat and cholesterol without elevating blood cholesterol levels. In addition, there seems to be an inverse relationship between dietary protein and liver lipid biosynthesis (107).

Results of work with rats, monkeys, chicks, and swine, as well as other species, indicate an involvement of dietary protein in regulation of serum lipid levels. Provocative though these indications may be, they cannot be extrapolated directly to man. Differences in lipid metabolism in man and in lower animals have been mentioned. Barnes and others (100) observed the responses of swine, rats, and humans to similar dietary changes and concluded that rats and swine are more sensitive to variations than are humans. Also it must be borne in mind that, with the exception of the work implicating lipotropic substances found with protein, most of the animal work suggests that a high

protein diet produces lowered serum cholesterol. The self-chosen diets of men exhibiting high serum lipid levels generally furnish seemingly adequate protein intakes (45, 82-84).

Keys and Anderson (82) observed the responses of middle aged men to dietary fat when an already moderate protein intake, 70 g. per day, was doubled. An increase in dietary fat from 55 to 125 g. daily caused in serum equal and highly significant increases in total cholesterol and in cholesterol in the beta lipoprotein fraction, regardless of the protein content of the diet. In a second study, men were maintained for sixteen weeks on a dietary regimen so arranged that each man served as his own control before and after four week periods of doubled protein intake and of increased cholesterol intake. Once again, the elevation of protein was ineffective, as was an increase in dietary cholesterol, providing little substantiation of the theory of a protein protective effect against dietary fat and cholesterol.

In a later study Wilcox and others (108) also employed high intakes of protein, 90, 122, or 154 g. daily. The study was so constructed that eight university athletes consumed each of nine diets which varied in protein content and in the amount of milk consumed daily, 0, 1, or 2 qt. Each diet was served for seventeen days. Serum cholesterol, total lipid, or lipid phosphorus values were not affected by either the quantity of milk or the amount of protein consumed. Nor was there any effect of the vigorous exercise performed by the subjects during a portion of each dietary period.

Both the Keys and Anderson study (82) and the one by Wilcox's group (108) employed high protein diets. As pointed out by the Framingham Study group (89), the lowest protein intakes studied may have already passed a critical threshold level, increases beyond which cause no further changes. Numerous groups (109-115) have conducted studies using a lower range of protein intake. One such study (109) by Leveille and others involved seven healthy young men ingesting rigidly controlled diets containing either 30 or 100 g. protein per day. The diets were composed of ordinary foods with the exception of dried, desugared egg white which was substituted isocalorically for sucrose in the high protein diet. The amount and type of fat remained constant throughout the study at approximately 100 g. per day from mixed sources. The subjects received low, high, and low protein diets successively during three four-week periods.

The low protein diets produced negative nitrogen balances, but the subjects were essentially in nitrogen equilibrium by the end of each four week low protein trial. Serum protein levels dropped progressively throughout the first low protein trial, rose during the high protein period, and remained at the same high level during the repeat period on low protein. This same pattern was seen in the plasma glyceride response. The glycerides were significantly elevated when the subjects were changed from the low to the high dietary protein level, but remained elevated when the protein intake was returned to the low level. Free and total cholesterol and phospholipid levels were unchanged. Likewise, no significant differences were noted in bile acid or sterol

excretion. Weight loss occurred throughout the study, but apparently this loss had no effect on the serum lipids.

In an experiment at the University of Maryland (110), young college women consumed diets supplying 26 or 100 g. protein daily. The diets were composed of ordinary foods, except for 73.7 g. vitamin free casein per day in the high protein diet. The fat content of the two diets was comparable. The subjects were divided into two groups and assigned to one of the two diets. Because each subject consumed only one diet and because no pre-experimental blood analyses were made, there were no control values. Values for serum cholesterol, phospholipid, and cholesterol/phospholipid ratio showed no consistent effect of the two diets during the eight week periods. Serum cholesterol levels of the group on the high protein diet were considerably above those of the other group after one week on the diet but after two weeks this difference had disappeared. Leveille's group (109) observed a similar initial serum cholesterol increase in subjects fed a high protein diet. In the Maryland study, the lack of control values makes it impossible to judge whether the cholesterol differences observed in the first week were real or merely represented differences in the usual cholesterol levels of the subjects.

A different approach to the protein-serum lipid problem was taken in one of the Institute of Nutrition for Central American and Panama studies (111). It has been observed that poor Central American children have unusually low serum cholesterol levels. These children ordinarily consume a diet consisting largely of whole corn tortillas.

The content of animal protein and of fat is very low. While continuing on their usual diets one hundred children received daily for twenty-eight weeks supplements of reconstituted dried skim milk, furnishing 20 g. protein. Other children were given 30 g. fat per day in the form of lard, hydrogenated cottonseed oil, or plain cottonseed oil for periods of eighteen to forty-five weeks. None of the supplements produced significant differences in serum cholesterol levels. The authors suggest that the hypercholesteremic effect of the supplements was counteracted by the tortilla diet. Such a diet furnished relatively large quantities of sitosterol, cornstarch, crude fiber, and calcium, all which have been shown to favor low serum cholesterol.

In quite different a situation, Albanese and others (114) observed the effects of a milk protein supplement to the diets of convalescent men and women of sixty years or older. This additional protein was more frequently associated with serum cholesterol increases than with decreases, but the results were not clear-cut or consistent. Feeling that perhaps the ineffectiveness of milk concentrate was due to a relative deficiency of methionine and cystine, the researchers then administered orally a commercial lipotropic preparation containing betaine, pyridoxine, nicotinamide, and vitamin B 12. This treatment also had no consistent effect on serum cholesterol. The authors concluded that lipotropic substances may be effective in growing animals and children but not in adults.

In the research reviewed thus far, controlled dietary studies of humans give little or no indication of an influence of dietary protein,

its content of sulfur amino acids, or its associated lipotropic agents on serum lipid levels. However, these studies represent only one segment of the related research. An equally impressive array of studies presents contrary results. For example, one of the early low protein diets, Kempner's rice diet (44), has been observed (116) to produce decreases in serum cholesterol that are greater than can be explained on the basis of the low fat content alone.

Another example involved metabolic studies similar to those in some of the work (108-110) affording negative results. Prather (113) reported the responses of six university women to daily protein intakes of 50 or 91 g. Both diets contained only ordinary foods, the protein variation being made primarily by manipulation of the quantities of lean beef and skim milk. About 36 per cent of the total calories was furnished by fat in both diets, and the calculated linoleic acid content of each was the same. The cholesterol content of the moderate protein diet and of the high protein diet was 310 and 416 mg., respectively. Fasting blood samples were drawn weekly and the plasma analyzed for total lipids, cholesterol, and phospholipids. The study was designed so that each subject consumed one diet for four weeks and then the other diet for an equal time, thereby serving as his own control. The order in which the diets were eaten was shown to be unimportant.

After one week either diet produced serum lipid levels lower than the initial values. On the moderate protein intake, serum cholesterol levels continued to fall until after four weeks the mean value was 132 mg./100 ml. Four weeks of the high protein diet produced a

mean cholesterol level of 183 mg./100 ml., a value significantly different from that of the other group. Phospholipids were not significantly different after four weeks of the two diets, although four of the six subjects had higher levels on the higher protein intake. Likewise, although the higher protein diet increased total plasma lipids in five subjects, the mean increase of 12 per cent was not statistically significant. In addition to suggesting that large amounts of high quality protein increase serum cholesterol in young women, this study points out the importance of individuality of response to dietary change, emphasizing the advantages of a study in which each subject can serve as his own control.

The hypolipotropic quality of a low protein diet has been of interest to Olson and others from the University of Pittsburgh. This group studied (115) the serum cholesterol response to such a diet in nine subjects, five of whom were hypercholesteremic. For two weeks the subjects ate a control diet supplying daily 100 g. protein, largely from animal sources. Fat, also mainly animal, provided 30 per cent of total calories. After a control period on this diet, the subjects were transferred for one week to an isocaloric isofatty diet containing 25 g. vegetable protein per day. The low protein diet furnished all essential amino acids except methionine in amounts which met Rose's tentative minimum requirement. The choline content of the high and low protein diets was 1.2 and 0.2 g. per day, respectively. At the end of the low protein period the subjects returned to the control diet. Serum cholesterol levels decreased by an average of 44 mg./100 ml. during the

period of low protein intake. These values resumed their original levels when the subjects returned to the control diet.

As was observed in the studies by Leveille and others (109) and by Watkins (110), serum cholesterol may respond to a dietary variation with an initial change which is not sustained for more than a week or two. Therefore, Olson's group (115) continued one of their subjects on the low protein intake for ten weeks while observing changes in his body weight, serum lipids, and serum beta lipoproteins, as well as nitrogen balance. Serum lipid levels were lowest after four weeks on the low protein diets. By ten weeks they were slightly increased, but were still definitely lower than the control values. The degree of hypocholesteremia did not parallel the degree of negative nitrogen balance or weight loss. Extensive laboratory tests of liver function gave no indication of liver dysfunction. The researchers concluded that it is unlikely that the effect on serum lipids is due to a net catabolism of body protein. More likely the changes represent an amino acid imbalance in which methionine and other lipotropic agents are factors.

Olson et al. (115) also described the responses of a middle aged alcoholic female to various dietary treatments. The subject responded to the high and low protein diets in the same manner as the other subjects. Kempner's rice-fruit regimen produced cholesterol levels similar to those of the low protein period. Adding butterfat to the rice diet produced no serum lipid response. While still consuming the rice plus butterfat diet, the subject was given a lipotropic supplement of choline, methionine, inositol, liver concentrate, and vitamin B 12.

This supplement restored serum cholesterol to the subject's original mildly hypercholesteremic level. Return to the high protein diet produced no further hypercholesteremia.

Others have studied the effect of lipotropic agents on serum lipids. Albanese's failure to find any significant effect (114) has been noted above. Labecki (116) administered a combination of choline, methionine, and inositol to patients with demonstrated myocardial infarction. This treatment resulted in significant depression of chylomicron levels and a slight depression in total serum cholesterol. Feeding the lipotropic supplement daily with 4 g. safflower oil raised the low ratio of alpha to beta lipoprotein which is characteristic of coronary patients. The subjects continued to eat their usual self-selected diets while receiving the supplements. Labecki described their diets as typically southern, and he suspected them to have a low lipotropic content due to the large amounts of vegetables and relatively little meat and dairy products. Studies of self-selected dietaries of Wisconsin women (117) and of women in the central states (118) showed that methionine appeared to be the first limiting essential amino acid. Neither study measured other sulfur amino acids. Labecki (116) stated that despite its high calorie content, the usual American diet is frequently inadequate, both relatively and absolutely, in specific vitamins, essential fatty acids, minerals, and amino acids. There are others (18) who feel that a relative deficiency of lipotropic agents in the diet of Americans is highly unlikely.

Mann and others (119) state that there is no sound evidence to

justify the use of lipotropic substances as treatment for atherosclerosis. They point out that such agents lower the cholesterol level in the liver and increase it in the serum of laboratory animals. Therefore, they feel that is illogical to expect that lipotropic treatments will be beneficial in treatment of human vascular disease. Their skepticism is based upon their observation of lack of serum lipid response in twenty-four males to daily methionine supplements. Like the Labecki subjects (116) these men continued on their usual diets. The supplements used by Mann's group contained three times as much methionine as the Labecki therapy, but furnished no choline or inositol.

Bagchi and others (98) have demonstrated a hypercholesteremic response to increased protein in malnourished hypocholesteremic children in India. The children received daily for two years supplements of 21 g. protein of either animal or vegetable origin. Both proteins increased serum cholesterol and serum albumen levels. Liver function, as measured by thymol turbidity, was greatly improved by the additional protein, that from animal sources being slightly more effective. The workers concluded that the children were initially suffering from hepatic dysfunction, the relief of which allowed more normal rates of cholesterol biosynthesis leading to serum cholesterol levels like those of healthy, well-nourished children.

The influence of dietary protein origin upon serum lipids has been studied also by Walker and others (120). In this study two diets of ordinary foods were planned so that they were nearly identical in all nutrients except protein and those nutrients closely associated with

protein. Thus, the daily diet furnishing 45 to 50 g. protein from animal sources contained not only high quality protein, but also more cholesterol, about 4.3 g. more animal fat, and 0.7 g. more methionine than the diet with the same quantity of protein from vegetable sources. Choline was neither calculated not analyzed, but it could be expected that the animal protein diet would contain more of this lipotropic agent. The vegetable protein furnished more fiber, more starch, and more sitosterol than the animal protein regimen. In general, the two dietaries were representative of freely chosen vegetarian and non-vegetarian intakes with the exception that the protein sources were selected for minimal fat content. Fat in both diets furnished 36 per cent of calories and came largely from margarine, salad oil, and hydrogenated shortening.

Twelve young women each consumed one of the two diets for six weeks during which blood samples were taken weekly. Serum cholesterol levels were consistently lower in the vegetable-protein group than in the animal-protein group, but the differences were significant only at the second and the fifth weeks. In both groups the final serum cholesterol levels were lower than those of the young women on their pre-experimental diets, a finding which may have been due either to the relatively low protein intake, to the high percentage of polyunsaturated fat, or to other factors. A control period during which the subjects consumed diets identical to the experimental ones except in protein content would have defined this point. Serum lipid phosphorus was significantly lower in the vegetable-protein group at the end of two weeks,

but by the end of the experiments there was no significant difference in the levels of the two groups.

This experiment, then, suggests a difference in lipid metabolism in young women consuming animal protein sources and in those young women on a vegetable-protein diet. However, the inherent differences in the diets made it impossible to identify the factor or factors responsible. In an attempt to elucidate the situation, a member of Walker's group, E. H. Morse, has continued the work with certain modifications (121). In the study by Morse and others, the basic daily diet used contained 60 g. protein all from plant sources except that furnished by 10 g. dried skim milk. Fat, mainly margarine, provided 40 percent of total calories. This vegetable diet was high in bulk and fiber. Daily vitamin and mineral supplements furnished, in addition to other nutrients, 5 mg. pyridoxine, but no choline. Fifteen healthy young men consumed this diet for a two-week standardization period. Then they were randomly divided into three groups. For the subsequent three weeks one group received daily 4 g. methionine; another 3.24 g. cystine; the third group got placebos. Weekly fasting blood samples were analyzed for total serum lipids, cholesterol, and lipid phosphorus.

Average serum cholesterol values had dropped in all three groups by the end of the two-week standardization period, but decreased no further when the supplementation period began. There was no consistent significant difference in any of the serum lipid levels among the three groups. The results were reminiscent of the mixed response to lipotropic substances reported by Albanese and others (114).

It may be worthy of note that of several similar studies (82, 108, 109, 113, 116, 120, 121) in which carefully controlled diets were fed to groups under metabolic study conditions, only those in which the subjects were females of child-bearing age (113, 120) showed a sustained hypocholesteremic effect of a low protein diet. The possibility of a sex-related difference is particularly important in consideration of the studies by Walker and others (120) and by Morse and others (121) which were especially designed to eliminate differences in conditions and procedures. The most obvious difference between these two investigations is the sex of the subjects. Although Morse's subjects did receive 60 g. protein per day as compared to a daily intake of 45 to 50 g. in the other study, this difference would probably be very small when related to the body weight of the subjects.

In an effort to eliminate some of the inherent differences when ordinary foods are used to construct diets varying in protein content, formula diets have recently been used in studying protein-lipid interrelationships. By using a purified protein source, the researcher can avoid the complicating differences in sterol, fiber, or fat content. Beveridge and others (122) used formula diets of butter, Dextri-maltose, sucrose, skim milk powder, and calcium caseinate, which was substituted for carbohydrate to provide diets in which 5, 10, 15, 20, and 25 per cent of calories came from protein. Fat provided 30 per cent of calories. Half of the subjects on each diet also received 500 mg. cholesterol/950 Calories. The diet providing 15 per cent of calories from protein was used as a standardization diet for an eight day pre-period. During the

subsequent eight days the subjects each consumed one of the experimental diets. Fasting blood samples were taken every four days.

When seventy-nine male students were studied, there was no significant serum cholesterol response except in the group receiving the lowest amount of protein, 5 per cent of calories. This group showed a significant increase in cholesterol levels. The presence of dietary cholesterol raised the average serum cholesterol levels, but had no effect on the degree of response to low protein intakes. When thirty-two female students were subjects, the level of protein intake had no effect on serum cholesterol, suggesting a sex-related difference in response, although of a different nature than that pointed out earlier. The results need to be substantiated by use of longer experimental periods.

In a study with older men, Campbell and others (58) used semi-purified diets in which 4.8 g. of the 6.5 g. daily nitrogen intake was provided either by wheat gluten or a casein-lactalbumin mixture. Two fat assortments were used; either provided 40 per cent of calories. Assortment I was designed to approximate the fat composition of the usual American diet and contained 12 per cent linoleic acid. The other fat composite had, in addition, safflower oil bringing the linoleic acid content up to 40 per cent. Ordinary foods of low protein and fat content made up the rest of the diet. Thus, the quality of fat or protein could be varied without affecting the intake of other nutrients. The study design consisted of a five-day pre-period and four 25 day experimental periods, each of the two proteins being fed with each of the

two fat mixtures. Six men were subjects for each dietary treatment.

The variation in dietary protein source had no significant effect on serum content of total lipids, sterol esters, glycerides, phospholipids, or unesterified fatty acids. The higher level of linoleate was reflected in the fatty acid composition of the serum lipids and produced lower serum cholesterol. The protein quality had no effect on the response to the increased linoleate intakes.

The results of these two studies, although not conclusive, would suggest that effects observed on low protein diets of usual foodstuffs are due not to the protein itself, but to factors associated with the protein. There is a need for more long term studies on semi-purified diets or diets in which the complicating factors of ordinary foods are minimized.

In most of the studies reviewed thus far, variation in quantity of fat and protein have been made at the expense of dietary carbohydrate. Such studies not only vary protein or fat, but also the third major dietary component, carbohydrate. Until rather recently this variable was considered unimportant; dietary carbohydrates were assumed to be bland constituents all treated alike after intestinal breakdown. In many metabolism studies individual caloric needs beyond that furnished by the basal diet are supplied by sucrose or lactose supplements. Recent research from many laboratories is producing evidence that such variations in carbohydrate are not as insignificant as was thought earlier.

Albrink (9) states that postabsorptive serum triglyceride levels are greatly influenced by dietary carbohydrates. Drastic reduction of total fat intake with an isocaloric increase in carbohydrate will result in hypertriglyceridemia, even though serum cholesterol may be lowered. Apparently the very low density lipoproteins are dependent upon carbohydrate metabolism while the cholesterol-rich S_f 0-20 lipoproteins are more closely related to cholesterol metabolism. However, there is some indication of an adaptation to a high carbohydrate diet. Antonis and Bersohn (123) reported that the triglyceride elevation produced by a high carbohydrate diet receded in normal persons after several months. Triglyceride levels have been shown to be slightly but significantly correlated with blood sugar levels of men on their usual diets (41).

Recent evidence suggest that both the quantity and type of carbohydrate intake is significant. MacDonald (124) fed seven adult males low fat diets of ordinary foods with the exception of a daily intake of 500 g. carbohydrate furnished by cornstarch or sucrose. Each carbohydrate was consumed for twenty-five days during which time serum lipid and depot fat analyses were made. On the starch diet all serum lipids were decreased except glycerides which remained unchanged. On the other hand, the sucrose diet increased total serum lipids and glycerides while amounts of phospholipids and total cholesterol were unchanged. The proportion of linoleic acid in serum lipids was decreased and that of palmitic acid increased by both diets. McGandy and others (125) found that substitution of sucrose for starch slightly raised serum

cholesterol levels among men. There was no interaction between type of carbohydrate and type of dietary fat. Others report (126) no effect of isocaloric variations in dietary carbohydrate on serum cholesterol levels of young men.

Grande and others (127) substituted sucrose and gluten isocalorically for bread and potatoes in the diets of middle-aged mental patients. In a second experiment sucrose and soybean protein were substituted for leguminous seeds. The sucrose diet and the bread and potato diet produced identical serum cholesterol levels, whereas consumption of the bean diet resulted in a 9 per cent decrease in cholesterol levels as compared to those produced by the comparable sucrose diet. The addition of cellulose actually raised the serum cholesterol somewhat, but pectin was without effect. No triglyceride data were reported. Prather (128) showed that the daily addition of 13 g. cellulose to the diets of young women had no effect on serum cholesterol, total serum lipids, or lipid phosphorus.

In contrast to the earlier study (124) by MacDonald, a later study (129) from the same laboratory reports that young women respond to high sucrose diets with decreases in total serum lipids, mainly as triglyceride and cholesterol. The male subjects in the earlier study responded in just the opposite manner. This difference led MacDonald to observe the reaction of young men, young women, and postmenopausal women to the same dietary variations (130). The fat-free diet consisted daily of 50 g. calcium caseinate, a vitamin-mineral supplement, and 7.5 g./kg. body weight of a carbohydrate mixture. The carbohydrate

mixtures were (a) 40 per cent fructose, 60 per cent corn starch, (b) 40 per cent glucose, 60 per cent corn starch, and (c) 40 per cent fructose, 60 per cent glucose. Each mixture was eaten for five days with nine-day intervals on self-selected diets between periods.

The men and postmenopausal women responded to dietary fructose with an increase in serum glycerides; the young women showed no such increase. Dietary glucose when compared with dietary fructose or starch seemed to be associated with an increase in fasting serum phospholipids in men and a decrease in this fraction in both groups of women. Serum cholesterol did not seem to be influenced at all by these dietary carbohydrate changes. MacDonald concluded that both the male and female sex hormones have an active role in dietary carbohydrate-lipid interrelationships, estrogens or progesterones preventing the fructose-induced rise in serum glycerides, and androgens having a positive effect on the phospholipid response to dietary carbohydrate.

Yudkin (131) points out that increased sugar consumption parallels increased fat intake and increased incidence of coronary heart disease of population groups. He suggests that, of all dietary components, sugar intake is most significantly correlated with the production of atherosclerosis. Lopez and others (132) have drawn similar conclusions from data from Interdepartmental Committee of Nutrition for National Defense surveys of sixteen countries. They found a highly significant correlation between consumption of simple carbohydrates and average concentration of blood cholesterol. Consumption of complex carbohydrates showed a significant negative correlation with cholesterol levels.

In the United States, consumption of sugars has increased while intake of complex carbohydrates has decreased in the last few decades (133). Calculations based on available food supplies suggest that Americans may derive as much as 48 per cent of total calories from carbohydrate, complex carbohydrates and sugars furnishing approximately equal portions. Observation (134) of dietary records of ninety-nine men and women showed that 40 per cent of total calories came from carbohydrate. Approximately one-fourth of the total carbohydrate intake was purchased as sucrose. This study reported wide variation among individuals as to the proportion of dietary carbohydrate furnished by simple or complex forms. Men usually ate a higher proportion of complex carbohydrate than did women.

MacDonald states that both the extent of carbohydrate influence on serum lipids and the metabolic pathways involved are still unknown (124). Various modes of action have been proposed. Lopez and others (132) list the following postulates: (a) changes in intestinal flora, (b) differences in rate of conversion of cholesterol into bile acids and excretion of them in the feces, (c) variations in the amount of pectins and other nonabsorbable carbohydrates in the diet, (d) differences in the amount of available riboflavin, (e) differences in the intake of plant sterols including sitosterol, and (f) differences in the rate of absorption of carbohydrate. Obviously, some of these factors would be operative only in diets of natural foodstuffs; they would not be involved in studies using highly purified carbohydrate sources.

It has been shown that the quality and quantity of all three dietary components, fat, carbohydrate, and protein, are involved to some extent in the regulation of serum lipid levels, suggesting a complex system of interrelationships. The obvious question arises--are not some or all of the micro-nutrients also involved? It has already been mentioned above that dietary contribution of cholesterol, sitosterol (and possibly other plant sterols), riboflavin, choline, or inositol may have an effect on serum lipids. Mendez and others (111) suggested that a high calcium intake might have a depressing effect on serum cholesterol.

Nicotinic acid has been referred to as the most effective cholesterol-lowering agent known today (135). In amounts of 3 to 6 g. daily, this vitamin maintained lowered serum cholesterol in somewhat less than 75 per cent of the cases in a study (135) where administration was continued for periods up to two years. The treatment does have some undesirable side effects including the flushing sensation, gastro-intestinal irritation, and possibly liver dysfunction, decreased carbo-hydrate tolerance, and increased serum uric acid after prolonged therapy. The quantity required for cholesterol lowering is far greater than the amount found in any ordinary diet. Therefore, this use of nicotinic acid must be considered therapeutic, rather than nutritional (57).

Very large doses of ascorbic acid have also been reported to have a cholesterol-lowering effect, possibly by a synergistic action when administered with fats high in linoleic acid (27). Use of large

doses of pyridoxine has given very little evidence of a beneficial effect on serum cholesterol levels in man (27).

Recent research has suggested that consideration must be given not only to the dietary content, but also the meal pattern. Gwinup and others (136) fed to five adult men and women a constant diet which was consumed as one meal, three meals, or ten meals per day. Gorging raised serum lipid levels while nibbling decreased them. Males and females were equally affected.

Not only dietary components influence serum lipid levels; non-dietary factors such as hormonal activity, exercise, stress, genetic makeup, climate, and others are important also. Of these factors, the endocrine influences are possibly the most significant and may be the agent of the other factors. It is not possible in this review to discuss in even a superficial manner the relative role of the various hormones in lipid metabolism. However, the subject can not be completely ignored.

Apparently, the sex hormones play a highly significant role in regulating serum lipid levels. It has already been pointed out that the human female during the child-bearing years is less susceptible to atherosclerosis, has a different serum lipid pattern, and responds differently to dietary carbohydrates than the male of the same age. A possible sex-related difference in response to dietary protein has been suggested. It may be that other evidence of sex-related differences requires only additional experimentation subjecting numbers of men and women to the same dietary conditions.

Administration of sex hormones to male survivors of myocardial infarction resulted in lowering serum lipids to approximately those levels found in healthy young males (16). There was an increase of cholesterol in alpha lipoproteins and a decrease of that in the beta fraction. When medication was discontinued serum lipids returned to previous levels. Female hormones seem able to change the balance of lipoproteins in blood toward a higher proportion of the smaller relatively stable molecules. Rosenfeld (16) suggests that this function may be associated with the biological need for efficient transport of large amounts of fat in women during pregnancy and lactation. Male sex hormones promptly reverse the effects of estrogen even when both are given together.

Thyroxin also plays a role in lipid metabolism and consequently affects serum lipid levels. Plasma cholesterol rises in hypothyroidism and falls in hyperthyroidism without any close or immediate relation to basal metabolic rate (137). There is no evidence of changes in intestinal absorption of cholesterol in either condition. A low cholesterol diet will not lower serum cholesterol in the hypothyroid individual. The rate of synthesis of cholesterol is increased in hyperthyroidism and decreased in hypothyroidism. Studies with rats (137) suggest that cholesterol excretion is so increased in the hyperthyroid state as to more than offset the increased synthesis rate.

Administration of desiccated thyroid to healthy male prisoners lowered serum cholesterol and phospholipids but did not affect triglycerides in an experiment by Danowski (40). The author points out that

in aging the size of the thyroid gland decreases, and there is a trend to lower basal metabolic rate and to lower serum protein bound iodine and ^{131}I uptake values. He suggests that prophylactic administration of desiccated thyroid to youthful adults might defer onset of hyperlipemia and related cardiovascular aspects of aging. On the other hand, Boyd (27) stated that thyroid administration disproportionately increased the oxygen consumption rate of the myocardium, thereby limiting the usefulness of such therapy. In recent years a number of thyroxin derivatives and isomers have been discovered to have varying degrees of success in reducing serum cholesterol with little or no increase in basal metabolic rate (57).

Pancreatic and adrenal hormones exert an effect on circulating lipids, but their influence is not as well established as are the effects of the gonads and the thyroid (138).

It has been shown in this review that many dietary components influence the levels of the serum lipids. There is an ever increasing awareness of the complexity of the regulatory system and of the possibility of an intricate system of interrelationships both within dietary components and between the diet and other factors, especially endocrine influences. The current state of knowledge concerning a possible role of dietary protein in serum lipid control is one of contradiction and inconsistency. Epidemiological studies suggest such a role, but also implicate high animal fat and sugar intakes in elevated serum lipid levels. Work with laboratory animals suggests a diphasic influence of protein; one, a cholesterol-lowering influence due to an adequate

supply of essential amino acids, and two, a cholesterol-raising influence due to lipotropic action. Controlled human matabolic studies varying protein quality and quantity have produced contradictory results.

Therefore, a series of carefully controlled metabolic studies has been conducted in the Nutrition Department of The University of Tennessee to further test the hypothesis that alterations in dietary protein will cause variation in lipid levels. Diets were composed largely of ordinary foods and alterations in quantity and origin of protein were made with variations in fat, cholesterol, mineral, and vitamin content kept at a minimum. Healthy premenopausal women served as subjects, thus providing data on the responses of the group known to be most resistant to elevated serum lipids. The use of these women also offered an opportunity to compare their reactions to those of male subjects used in similar studies (109, 121).

CHAPTER III

PROCEDURE

The effect of quality and quantity of dietary protein on serum lipids in premenopausal women has been investigated in three controlled studies involving five different diets. These studies, thirty-three to thirty-six days in length, were conducted in January-February, 1962 (Study I), October-November, 1962 (Study II), and October-November, 1964 (Study III). Each study was divided into six-day periods. They were part of a series of metabolic experiments begun in 1961 to investigate the influence of level and source of dietary protein on the metabolism of various other nutrients, including energy, magnesium, iron, copper, and zinc (139-142). All diets were composed of ordinary foods with the exception of a small supplement of "vitamin free" casein. Menus used have been published elsewhere (139-142). The typical American meal pattern was adhered to as closely as possible. A total of twenty-one subjects participated, five in Study I and eight in both Study II and Study III.

The subjects were all women of ages twenty to forty-one years, associated with the College of Home Economics at The University of Tennessee. Two were undergraduates; one was a faculty member; the remainder were graduate students. One subject served on all three studies and three subjects served twice. Prior to each study, the subjects were given a physical examination and judged to be in good

health. The subjects weighed on the same scale in the Department of Nutrition during the week immediately preceding each study and at three day intervals throughout the studies. Age, height, and weight data on the subjects are presented in Table XVII in the Appendix.

All subjects were engaged in academic pursuits and led relatively sedentary lives. They continued their usual routines while participating in the experiments. All experienced the mental and emotional stresses and anxieties associated with college studies.

Prior to each study, all subjects kept a two-week record of their food intakes. Before Study III, the participants were requested to reduce their protein intakes in order to adjust partially to a diet lowered in nitrogen content. In addition, four subjects weighed their food and recorded the exact weight eaten for three 3-day periods between Studies I and II. Energy, protein, and fat content of these self-selected diets were calculated (143, 144).

Diets were planned to meet the National Research Council's recommended levels (145) for all nutrients for which recommendations have been made, with the exception of protein and iron. Nutrients falling below recommended levels were supplied by the addition of primary calcium phosphate to recipes of mashed potatoes, cream topping, cookies, and soups, and by the administration of specially prepared vitamin and mineral supplements by capsule. Vitamin D was supplied by Drisdol[1] in a solution added to breakfast cream. Each of the five

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[1]Drisdol, calciferol in propylene glycol; 6.25 μg./drop, prepared by Winthrop, New York, N. Y.

diets provided daily approximately 10 mg. of iron. Dietary fat, approximately 99 g. per day, was derived mainly from margarine, vegetable shortening, corn oil, and cream. The percentages of fat from animal, vegetable and hydrogenated vegetable sources were held constant in all the diets. From 40 to 44 per cent of the total calories were furnished by fat. The cholesterol content of the diets was kept at a constant low level by the exclusion of foods rich in cholesterol.

Protein quantity and quality were varied by manipulation of low-fat protein sources. Calories were adjusted to individual needs by the addition of essentially fat-free foods, fondant, sugar cubes, or a cola beverage. Those who did not need additional calories were allowed an artificially sweetened cola beverage daily. Because of its magnesium content, coffee was restricted. Demineralized water was allowed ad libitum, but the amount consumed was recorded. A summary of level and source of calories, protein, and fat furnished by the five diets is presented in Table I.

During the Study I three-day pre-period and the first nine experimental days (one and one-half periods), the daily diet furnished 20 g. protein, largely of vegetable origin. On the tenth day, 5 g. purified "vitamin-free" casein per subject per day were added. Some subjects approached nitrogen equilibrium only when 50 g. chopped cooked egg white were included on the first day of the third 6-day period and continued for the remainder of the study. This addition brought the calculated daily protein intake up to 31 g., 50 per cent of which came from animal sources.

TABLE I

SUMMARY OF MAJOR COMPONENTS OF EXPERIMENTAL DIETS

Study Diets	Avg. total energy intake (Cal./24 hr.)	Total protein (g./24 hrs.)	Protein sources		Total fat (g./24 hr.)	Fat sources		
			Animal (%)	Plant (%)		Animal (%)	Veg. (%)	Hydrogenated (%)
I	2180	20 (initial)	25	75	104	39	15	46
		31 (final)	50	50				
II A	2264	35	50	50	97	37	16	47
B	2219	48	65	35	99	37	16	47
III A	2521	36	33	67	99	37	16	47
B	2422	47	37	63	97	37	16	47

In Study II two diets were used with four subjects consuming each diet. The subjects had restricted their protein intakes to approximately 50 g. per day during the two weeks prior to the study. A six-day preliminary period during which all subjects ate the same diet containing 42 g. protein per day was employed. The eight young women were then randomly assigned to the two experimental diets, A and B. Diet A was the basal diet, furnishing daily approximately 35 g. protein, equally from animal and vegetable sources. It differed from the diet used in the final periods of Study I, in that breast of turkey, lean fish, and small increases in ground round of beef, together with small amounts of skim milk and dry cottage cheese were used to provide additional animal protein and to replace the casein and egg white. Additional plant protein came from small increases in the servings of vegetables. Diet B was the basal diet plus the daily addition of 7.5 g. casein and 75 g. cooked egg white, furnishing a daily total of 48 g. protein, 65 per cent of animal origin. The calorie intake of two subjects, who gained weight, was lowered by the removal of cola beverages, 10 g. margarine, 10 g. brown sugar, and 40 g. grape juice per day.

Study III was designed to maintain the same quantity of protein as Study II, but to alter the sources so that approximately 65 per cent would be of plant origin. This alteration was made by replacing part of the animal protein foods with plant protein sources, including bread, dried beans, and spaghetti. All subjects ate 43 g. protein daily during the six-day preliminary period. Thereafter, the four subjects on

diet A received 36 g. protein, while those on diet B received 47 g. protein daily.

All meals were prepared and served in the metabolic laboratory kitchen in the Department of Nutrition. Foods were weighed, served, and consumed in a manner as quantitative as was practical. During each period composites were prepared of half portions of all foods and beverages, except margarine, salad oil (Study I only), coffee, and the extra calorie sources. These foods were sampled individually from each lot, producing pooled composites. Period food composites were prepared, homogenized, and frozen in small lots for analysis. Details of procedures used in compositing food, urine, and feces are reported elsewhere (139, 142).

Carmine capsules, taken before breakfast at the beginning of each period during Studies I and II, were used to mark the feces. During Study III a mixture of brilliant blue[2] and methyl cellulose (146) was found to give much better separation. Fecal samples containing markers were separated before freezing. After a period collection was completed, the feces were composited and portions were frozen for later analysis.

Capillary blood samples were taken by finger prick every three days during preliminary periods, experimental periods, and periods of recording self-selected diets. The samples were usually obtained between 5:00 and 6:00 p.m., approximately five hours after the noon meal.

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[2] FD&C Blue #1, Bates Chemical Company, Lansdowne, Pa.

The blood was collected in capillary tubes which were stoppered with Critocaps,[3] allowed to clot 10-15 minutes and centrifuged 5 minutes at 2000 r.p.m. in an International Centrifuge Model SBV. Using a file or diamond point pencil, the tubes were scratched at the interface of clot and serum, and broken. The serum was blown out into 3 ml. test tubes which were capped and placed in freezer storage. At the time of analysis, the serum samples were thawed and usually an individual's samples from days 1 and 4 were combined to give a composite period sample.

During Study III venous blood samples of approximately 10 ml. were collected immediately before the study, mid-study, and on the last experimental day. Several weeks after the study a fourth sample was collected. Thus, two samples represented the effects of the subject's non-experimental diets and ways of life, and two represented the experimental procedure. The venipunctures were made by laboratory technicians at the University Clinic at times approximately four to five hours after eating. The venous blood was allowed to clot and was centrifuged. The serum was poured off and frozen in capped tubes. A few capillary collections were made within four hours of venipunctures on the same subject. Comparison of the serum cholesterol levels of the serum obtained by the two methods showed only small inconsistent differences. Thereafter, capillary collections were eliminated on those experimental days when venous blood was drawn.

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[3]Critocaps, disposable hematocrit tube closures, distributed by Aloe Scientific Co., St. Louis 3, Missouri.

Samples of food composites of each of the five diets were analyzed for total fats by two different methods. One method, a modification of the AOAC technique of acid hydrolysis (147), involved digestion with yeast to remove sugars. Following fermentation, the slurries were hydrolyzed with concentrated hydrochloric acid for thirty minutes. Extraction involved shaking each slurry vigorously with equal parts of diethyl and redistilled petroleum ether in a stoppered cylinder. The ether layer was removed with suction and was filtered through fat-free cotton into weighed beakers and the remaining residue was extracted twice again with the two ethers. The entire filtrate was evaporated to dryness, and the fat weighed in the beakers. The other method (148) employed extraction with methanol and chloroform with no previous digestion. Weighed portions of the food composites were blended in a Waring blendor with equal portions of methanol and chloroform, and half that volume of distilled water. Zinc acetate was used to precipitate the protein. After filtering through Whatman No. 1 filter paper in a Buchner funnel under suction, the volume of the chloroform layer was measured in a graduated cylinder and a portion pipetted into a weighed beaker. The subsample was dried and weighed as in the acid hydrolysis method. Some samples were analyzed by both methods in order to compare results. Margarine and salad oil composites were analyzed by the same procedures. Total fat intake was determined by adding the fat content of the food composites, the margarine, and the salad oil. The cola beverages, coffee, and fondant, which contributed negligible amounts of fat, were not subjected to any lipid analysis.

Fecal composites from Study II were analyzed for total fat by the acid hydrolysis technique with the omission of the digestion step.

Serum and food samples were analyzed for total cholesterol by modification of a micro-method (149) based on the Sperry and Webb procedure (150), a modification of the Liebermann-Burchard reaction in which a blue color is formed after the addition of acetic anhydride and sulfuric acid to a cholesterol solution. Acetone-absolute ethanol (1:2 by volume) was added to 0.04 ml. serum in 1 ml. volumetric flasks. Immediately the flasks were buzzed on a Vortex Junior Mixer until the precipitate was finely dispersed. The mixture was brought to a boil in a water bath. After cooling, the mixtures were made up to volume and centrifuged at 2500 r.p.m. for 20 minutes in an International Centrifuge Model SBV. The supernatant fluid was decanted, and a 0.2 ml. portion pipetted into each of three 1 ml. centrifuge tubes. The precipitate was re-extracted in the same manner and again 0.2 ml. of extract were added to the same three tubes. To these tubes were added 0.01 ml. of 33 per cent potassium hydroxide. The tubes were buzzed, fitted with rubber caps, and incubated in a 37-40° C. sand bath for 30 minutes. Blanks containing 0.4 ml. acetone-absolute ethanol were given the same treatment. After cooling to room temperature, each tube received 0.25 ml. acetone-absolute ethanol, 0.01 ml. phenolphthalein solution, and 0.06 ml. 10 per cent acetic acid. Next 0.2 ml. digitonin solution (5 g. digitonin in a mixture of 475 ml. water and 525 ml. 95 per cent ethanol) were added. After being capped and buzzed, the tubes were allowed to stand overnight at room temperature.

The next day the tubes were centrifuged in the same centrifuge for 30 minutes at 2800 r.p.m. The supernatant fluid was carefully removed by means of a fine tipped transfer pipette and suction and then discarded. The precipitate was washed once with acetone-ether and once with ether only, followed each time by centrifugation and removal of the supernatant fluid. The tubes containing the dry cholesterol digitonide were heated in a sand bath at 110-115° C. for 30 minutes. While still in the hot sand, the tubes received 0.1 ml. glacial acetic acid. In the meantime, a series of standards containing cholesterol in glacial acetic acid was prepared by diluting a stock solution so as to have three tubes containing 5, 10, and 15 µg. cholesterol respectively. Color was developed by the addition of 0.4 ml. color reagent (10 ml. acetic anhydride plus 0.5 ml. concentrated sulfuric acid). The reagent was made up within 10 minutes of use and kept on ice. The color reaction was time, temperature, and light sensitive. Therefore, the tubes were kept covered in a temperature controlled room until exactly 30 minutes after the addition of the color reagent to each tube.

Optical density was read at a 625 mµ setting on the Beckman DU spectrophotometer, equipped with micro adapter and micro cuvettes, and without a filter. Glacial acetic acid was used to zero the instrument. Readings were corrected with a reagent blank.

The optical density values for the series of standards were used to construct a standard curve from which the concentrations of the samples were read. Although there was little variation in the standard curve, standards were included in each series of determinations. The

serum cholesterol concentrations were expressed as mg. per 100 ml. serum. Reproducibility of analyses and recovery of standards added to serum are reported in Table II.

The analysis of food composites for total cholesterol was very similar to determinations on the serum except that larger quantities were used. A 2-3 g. sample of each composite was weighed into a 50 ml. centrifuge tube. The samples were extracted with acetone-absolute ethanol, brought to a boil, and cooled. The extract was filtered into 25 ml. volumetric flasks. The tubes, funnels, and filter papers were rinsed and the extracts made up to volume with acetone-absolute ethanol. Triplicate 1 ml. portions of the filtrate were placed in 3 ml. test tubes. Thereafter, the procedure was identical to that used for serum, except that the results were expressed in mg./g. food and mg./day. Interference by plant sterols prevented use of this technique for the analysis of the margarine and salad oil composites. They being plant products, were assumed to make no appreciable contribution to the cholesterol content of the diets (143).

Esterified fatty acids were determined in venous serum samples and in all capillary serum samples of sufficient volume by the method of Stern and Shapiro (151). These workers have developed a method substituting an ethanol-water solution in the strictly anhydrous method developed by Bauer and Hirsch (152). Both procedures involve the reaction of extracted carboxylic acid esters with hydroxylamine in alkaline solution to form hydroxamic acids. The hydroxamic acids produce a red-violet color with ferric chloride. The optical density was

TABLE II

REPRODUCIBILITY AND RECOVERABILITY IN SERUM CHOLESTEROL ANALYSIS

Sample	Corrected OD readings	Avg. OD readings	Cholesterol (µg/tube)	Recovery (%)	Reproducibility (%)
Serum RS (1)	.130 .125 .138	.131	16.0		
Serum RS	.132 .132 .128	.131	16.0		100
Cholesterol standard (1)	.082 .082 .080	.082	9.0		
Cholesterol standard (2)	.090 .080 .085	.085	9.3		97
Serum RS & standard (1)	.228 .185 .208	.207	25.3	101	
Serum RS & standard (2)	.208 .212 .212	.210	27.0	107	94

measured on the Beckman DU spectrophotometer set at 525 mμ without a filter. Ninety-five per cent ethanol was used to zero the instrument and a reagent blank was used to correct the readings. Triacetin in 95 per cent ethanol was used to prepare a series of six standards, varying in concentration from 0.0002 to 0.0016 mEq. triacetin per tube. The optical densities of the standards were used to construct a standard curve. The results were read off this standard curve and are expressed as ester mEq./liter serum. The size of the serum samples used varied from 1 ml. to 0.05 ml. without any apparent loss of accuracy. Modifications in the volumes of reagents were made for use with the very small samples. Reproducibility of analyses and recovery of standards added to serum are reported in Table III.

Venous serum samples and food composites were analyzed for fatty acid composition by gas-liquid chromatography. Serum samples of 1-2 ml. were extracted with three parts 95 per cent ethanol and one part redistilled diethyl ether as was done in determination of the esterified fatty acids. The total extract was evaporated on a flash evaporator, redissolved in petroleum ether, and stored under nitrogen at -20° C. The food, margarine, and salad oil samples were extracted with equal volumes of methanol and chloroform and evaporated. No attempt was made to fractionate the lipids before analysis.

The fatty acids in all samples were converted to their methyl esters by interesterification procedure which involved refluxing the fats with 5 per cent sulfuric acid in methanol (v:v) for one hour in a 70° C. water bath (153). Approximately 0.05 to 0.10 g. of food lipid

TABLE III

REPRODUCIBILITY AND RECOVERABILITY IN ANALYSIS OF FATTY ACID ESTERS IN SERUM

Sample	Corrected OD readings	Avg. OD readings	Triacetin equivalents (mEq. x 10^4/tube)	Recovery (%)	Reproducibility (%)
Serum BT	.228 .230 .235	.231	20.8		
Triacetin standard	.048 .045 .043	.045	4.0		
Serum BT & triacetin standard	.285 .275 .265	.275	24.8	100	
Serum RS	.163 .175 .170	.169	14.7		
Triacetin standard	.040 .053 .044	.046	4.0		
Serum RS & triacetin standard	.200 .225 .213	.212	18.8	100	
Serum ESP(1)	.120 .123 .123	.122	10.6		
Serum ESP(2)	.120 .125 .123	.123	10.7		99

was used for each methylation. In the case of the serum samples, the entire extract was used, the amount being minimal. Fat from both food and serum were methylated in the same manner. The methyl esters in petroleum ether were flushed with nitrogen, stoppered, and frozen until immediately prior to analysis at which time they were evaporated and redissolved in hexane.[4]

Gas-liquid chromatography of the methyl esters was done on a Barber-Colman model 61 C Gas-Liquid Chromatograph, equipped with an argon ionization detector with radium source. The seven foot coiled packed column had a polar liquid phase.[5] The column temperature was 175° C. for both serum and food samples. The food samples were also run at a column temperature of 140° C. in order to improve resolution of the short chain fatty acids. The other operating conditions were as follows: cell temperature 210° C., flash heater temperature 220° C., cell voltage setting 900, gas pressure 20 p.s.i., and sensitivity setting 10. Attenuation was varied with the concentration of the samples. Duplicate chromatograms were obtained for each sample. Fatty acids were identified by comparison of retention times with those of a standard fatty acid mixture, obtained from National Institutes of Health.

The concentrations of the various fatty acids were calculated

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[4] Matheson, Coleman, and Bell, Chicago, Ill., chromatoquality.

[5] Applied Science Lab, Inc., State College, Pa.: 13 % diethylene glycol succinate polyester on 80-100 mesh Gas Chrom P.

from the "area" under each peak on the chromatogram. The "areas" were obtained by multiplying the height by the width at half height of each peak, correcting for changes in attenuation if necessary. The following formula was used to calculate the percentage of each fatty acid:

$$\% \text{ fatty acid in sample} = 100 \times \frac{\text{"area" of peak}}{\text{total "area" of all peaks}} .$$

In the food samples, the range of fatty acids present prevented measurement at one column temperature. Therefore, chromatograms of each sample were made at 175° C. to measure fatty acids of at least 14 carbon chain length and at 140° C. to measure the fatty acids of less than 14 carbons. At least one peak, usually C 14 was always measurable under both sets of conditions. Therefore, the "areas" of all other fatty acid peaks were expressed in ratio to the "area" of this fatty acid in order to produce a composite chromatogram including all the fatty acids present in the sample. This calculation was made with the following equation:

$$\text{"Area" of peak y at 175° C.} =$$

$$\text{"Area" of C 14 peak on 175° C. chromatogram} \times \frac{\text{"Area" of y on 140° C. chromatogram}}{\text{"Area of C 14 on 140° C. chromatogram}} .$$

The percentages of the various fatty acids were then calculated as above.

CHAPTER IV

RESULTS

Calculation of the nutritive content of self-selected diets recorded by the subjects at periods before and after the experimental periods produced unexpectedly low figures (Table IV). For example, average total calorie consumption was often 500 Calories below the energy intake required to prevent weight loss on the experimental diets. Prior to Study II the subjects had been instructed to reduce their daily protein intake to approximately 50 g. Their dietary records suggested that they made this alteration by merely decreasing their total intake of food without any great qualitative changes. Therefore, in order to make more realistic comparisons, the average nutrient intakes of each individual are shown as per cent of total calories in Table IV. Each value is the average daily consumption during seven to fifteen day periods. Subject ESE joined the Study I squad at a late date and did not keep a dietary record.

Blood samples were collected concurrently with periods of recording dietary intakes. The number of samples from each individual varied from one to eight, the larger numbers coming from those subjects who served on more than one study. In only five cases was there only one non-experimental sample. Non-experimental serum cholesterol values, representing a total of sixty-one determinations, are presented in Table V. The average values of the members of each group were used

TABLE IV

DESCRIPTION OF NON-EXPERIMENTAL DIETARY INTAKES[a]

Group / Subject	Avg. daily Cal. intake	% Calories furnished by: Total protein	Animal protein	Total fat[b]	Animal fat	Veg. fat	Sugars[c]
I							
GMB	1956	15	11	39	20	4	36
SH	1590	21	14	32	18	8	32
ESP	1809	21	18	37	25	9	14
MN	1435	13	10	34	23	3	11
avg.	1697	18	13	36	22	6	23
II A							
SH	1689	10	6	35	12	12	25
FG	1410	13	8	43	28	4	21
PM	1398	13	8	24	9	9	13
MR	1800	10	6	31	6	5	37
avg.	1574	11	7	33	14	8	24
II B							
ESP	1327	15	10	44	23	10	21
MN	1460	12	7	31	14	9	22
CE	1447	12	7	36	16	9	20
PT	1626	11	8	30	22	4	27
avg.	1465	12	8	35	19	8	23
III A							
MN	1682	20	14	44	23	8	14
CP	2364	12	8	34	19	7	17
RS	1713	13	9	37	19	7	16
RL	2451	15	10	41	31	7	17
avg.	2053	15	10	38	23	7	16
III B							
PM	1915	18	14	38	25	5	5
ESP	1832	16	11	59	40	6	11
BT	1442	18	14	33	21	6	23
HR	1968	15	9	28	12	14	24
avg.	1789	17	12	40	25	7	16

[a]Calculated (143, 144) from dietary records of seven to fourteen days intake.

[b]Total fat included three categories; animal fat, vegetable fat, and hydrogenated vegetable fat. The latter is not shown separately.

[c]Mono- and disaccharides.

TABLE V

NON-EXPERIMENTAL SERUM CHOLESTEROL VALUES

Subject	Study I	Study II A	Study II B	Study III A	Study III B	No. determinations	Mean value (mg./100 ml.)	SE
GMB	X					4	179	2
ESE	X					1	138	
SH	X	X				5	163	10
ESP	X		X			4	196	25
MN	X		X			5	164	11
CE			X			1	228	
PT			X			1	134	
FG		X				1	234	
MR		X				1	216	
MM				X		3	141	2
CP				X		3	182	9
RS				X		3	185	6
RL				X		3	201	17
PM					X	3	245	10
ESP					X	3	227	23
BT					X	3	150	8
HR					X	3	187	8
All study I subjects						19	168	10
All study II A subjects						7	204	21
All study II B subjects						11	180	20
All study III A subjects						12	177	13
All study III B subjects						12	202	21
All subjects						61	186	9

to calculate a group average. Although group means varied from 168 (group I) to 204 mg./100 ml. (group IIA), single random factor analysis (154) showed no significant difference between groups. The grand mean was 186 mg. cholesterol/100 ml. serum, a value well within normal ranges. Intra-individual variability was frequently as large as inter-individual variability.

Non-experimental serum fatty acid ester values, representing fifty-six determinations, are shown in Table VI. In this case variation between individuals was usually greater than variation among determinations on a single individual. However, in both cases serum esterified fatty acid level variations were rather high. Group means ranged from 7.4 (III B) to 9.7 mEq./liter serum (II A and II B); the grand mean was 7.9 mEq./liter serum. Analysis of variance (154) showed no significant difference among groups. There appeared to be no consistent relationship between serum cholesterol and esterified fatty acid levels.

Fatty acid analysis of two serum samples collected from each Study III subject while consuming her self-chosen diet showed the largest percentage to be linoleic acid, followed by palmitic and oleic acids. Approximately equal percentages of total saturated, monounsaturated, and polyunsaturated fatty acids were found. There was no evidence of significant differences between Group A and Group B subjects; neither did any single individual deviate radically from the over all pattern. The actual values are shown in comparison with experimental values in a later table.

TABLE VI

NON-EXPERIMENTAL SERUM FATTY ACID ESTER VALUES

Subject	Study I	Study II A	Study II B	Study III A	Study III B	No. determinations	Mean value (mEq./liter[a])	SE
GMB	X					7	8.7	0.6
ESE	X					1	4.4	
SH	X	X				7	8.4	0.5
ESP	X		X			8	9.4	0.6
MN	X		X			7	9.3	0.5
CE			X			1	9.6	
PT			X			2	10.6	0.1
FG		X				1	10.0	
PM		X				1	11.6	
MR		X				2	8.5	0.1
MM				X		2	6.5	1.4
CP				X		3	8.0	1.0
RS				X		3	6.9	0.5
RL				X		3	10.2	0.6
PM					X	2	8.7	0.1
ESP					X	2	9.2	2.4
BT					X	2	5.2	0.6
HR					X	2	6.7	0.6
All study I subjects						30	8.0	1.1
All study II A subjects						19	9.7	0.9
All study II B subjects						11	9.7	0.3
All study III A subjects						11	7.9	0.7
All study III B subjects						8	7.4	0.8
All subjects						56	7.9	0.6

[a]Expressed in terms of triacetin standard.

Analyzed total fat and cholesterol content of the experimental diets is shown in Table VII. The average total fat content of the various diets was 99 g. per day. About one-fourth of this total fat was furnished by margarine. In Study I salad oil was composited and analyzed separately. In the other studies the salad oil was included in the food composites. Cholesterol analysis showed period variations of a similar magnitude in diets prepared simultaneously, i.e., II A and B, and III A and B.

Analyzed fatty acid content of the composited foods is presented in Table VIII. These figures are based on duplicate analyses of one period composite from each diet. Limitations of time and facilities did not permit analysis of several composites of each diet. The food composites contained numerous short chain fatty acids which could not be positively identified. They were grouped together as all fatty acids of less than fourteen carbon chain length. This group made up from 9 to 21 per cent of total fatty acids in the food composites. In all the diets palmitic acid and oleic acid were the most predominant fatty acids, each accounting for approximately one-fourth of the total. Linoleic acid content ranged from 22 per cent of fatty acids in food composite II B to 11 per cent in composite I. No measurable amounts of linolenic or arachidonic acids were found.

The fatty acid contents of the margarines used in each study and of the salad oil used in Study I are shown in Table IX. The diets of Studies II and III contained different lots of the same margarine, but in the earlier study a different brand, which was later discontinued

TABLE VII

TOTAL FAT AND CHOLESTEROL FURNISHED BY EXPERIMENTAL DIETS

Diet	Total cholesterol[a] (mg./day ± SE)	Fat			
		Composited foods[b] (g./day ± SE)	Margarine (g./day)	Salad oil[c] (g./day)	Total fat (g./day ± SE)
I	146 ± 5	74 ± 1	24	6	104
II A	210 ± 18	73 ± 2	24		97
II B	217 ± 10	75 ± 1	24		99
III A	182 ± 18	75 ± 2	24		99
III B	181 ± 15	73 ± 2	24		97
Avg. all diets	173 ± 14	74 ± 1	24		99 ± 1

[a]Based on analysis of composited foods only.

[b]Average of analyses of three period composites of each diet.

[c]Dressings carrying salad oil were included in the food composite except in Study I.

TABLE VIII

ANALYZED FATTY ACID CONTENT OF EXPERIMENTAL DIETS[a]

Fatty acid	Diets					Avg.
	I	II A	II B	III A	III B	
	(% of total fatty acids)					
< C14	9	9	10	18	21	14
C14	6	6	6	9	12	8
C14:1[b]	4	1	2	4	2	3
C16	30	22	22	24	26	25
C16:1	4	8	8	6	4	6
C18	6	5	3	3	3	4
C18:1	30	31	26	24	20	26
C18:2	11	18	22	13	12	15
Total saturated	51	42	42	54	62	51
Total mono-unsaturated	38	39	36	34	26	33
Total poly-unsaturated	11	18	22	13	12	15

[a]Based on analysis of one composite for each diet.

[b]This fatty acid could not be positively identified.

TABLE IX

ANALYZED FATTY ACID CONTENT OF MARGARINES AND SALAD OIL

Fatty acid	Margarine used in study			Salad oil
	I	II	III	
	(% of total fatty acids)			
C16	7	9	7	9
C16:1	4	4	4	3
C18	1	6	3	
C18:1	57	67	62	16
C18:2	30	15	24	72
Total saturated	8	15	10	9
Total mono-unsaturated	62	70	66	19
Total poly-unsaturated	30	15	24	72

on the market, was used. The analyzed linoleic acid content of the margarines used in Studies I, II, and III was respectively 30, 15, and 24 per cent of total fatty acids. Because the fatty acid contents are measured as per cent of total fatty acids rather than as per cent of fat, it is not feasible to calculate the actual total intake of any one fatty acid. However, rough calculations of the intake of linoleic were made by weighting the percentage of that acid in each source for its portion of total fat intake and adding the weighted percentages. This procedure showed that linoleic acid was roughly 19, 17, 21, 16, and 15 per cent of the total fatty acid intake of the subjects on diet I, II A, II B, III A, and III B, respectively. The same method of calculation indicated that saturated fatty acids made up roughly 39, 35, 35, 43, and 49 per cent of the total fatty acid intake of each group.

The amino acid content of the individual foods composing each experimental diet was calculated (144, 155) and totaled. Approximately 70 to 80 per cent of the total protein content of each diet was accounted for in the calculations. The remaining protein was furnished largely by fruits and other low protein foods for which no amino acid figures were available. The amino acid contents are compared with minimum amino acid requirements for young women (156) in Table XVIII in the Appendix. The initial diet used in Study I was deficient in sulfur-containing amino acids and borderline in tryptophan and valine. The Study III A diet also contained a minimal amount of tryptophan. The amino acid content of all the other diets exceeded the minimum requirements for all the essential amino acids. The pattern of

amino acids in diets II B and III B differed only slightly, in spite of the fact that the proportion of protein from animal sources in the two diets was 65 and 35 per cent, respectively.

During all the experimental periods the subjects maintained good health and were, with the exception of one subject, able to complete the experiments. The single exception, MR, suffered an intestinal upset during period five of Study II A and was forced to discontinue participation. Hemoglobin measurements before and after each study showed no consistent effect of the diets. Likewise, serum protein determinations before and after Study III gave no indication of a dietary influence.

During all the studies there was a tendency for weight loss. However, in most cases the changes were small and did not exceed the subjects' usual weight fluctuations. Weight changes were kept to a minimum by the addition to or removal from the diet of fondant, sugar cubes, or a cola beverage. This calorie manipulation resulted in variable intakes of sugars. Table X shows average total calorie intake and weight changes as well as the calculated intake of sugars. Analysis, carried out by another student, of food, urine, and fecal energy content during Study III showed no influence of the differences in protein intake upon excretion of energy containing substances (141).

Table XI compares the average non-experimental nutritive intakes of each group with the experimental diet consumed by that group. The experimental diets all furnished fewer calories from protein than did the subjects' usual diets. In the first two studies, the experimental

TABLE X

CALCULATED CALORIE AND SUGAR INTAKES AND WEIGHT CHANGES OF SUBJECTS

Group	Subject	Weight change[a] (kg./30 days)	Avg. Calorie intake (Cal./day)	Avg. intake sugars[c] (g./day)
I				
	GMB	0.0	2097	89
	ESE	-1.6	2293	131
	SH	0.0	2097	89
	ESP	-1.3	2170	115
	MN	-1.7	2244	104
	avg.	-0.9	2180	106
II A				
	SH	-0.1	2241	113
	FG	-0.4	1981	42
	PM	-0.7	2466	170
	MR	0.2	2368	146
	avg.	-0.2	2264	118
II B	ESP	-0.2	2377	130
	MN	-2.0	2173	69
	CE	-0.4	2022	12
	PT	-0.4	2305	112
	avg.	-0.7	2219	81
III A				
	MM	-1.1	2456	104
	CP	0.0	2561	117
	RS	-1.3	2528	108
	RL	-1.3	2537	114
	avg.	-0.9	2521	111
III B				
	PM	0.0	2497	105
	ESP	-0.9	2430	93
	BT	-0.8	2455	92
	HR	-0.8	2304	65
	avg.	-0.6	2422	88

[a]Subject's weight on final experimental day minus her weight on first experimental day.

[b]Calculated (143, 144).

[c]Calculated consumption of all mono- and disaccharides.

TABLE XI

COMPARISON OF NON-EXPERIMENTAL AND EXPERIMENTAL DIETARY INTAKES

Group	Avg. Cal. intake (Cal./day)	% Calories furnished by					
		Total protein	Animal protein	Total fat[a]	Animal fat	Veg. fat	Sugars
I non-exp	1697	18	13	36	22	6	23
I exp P 1-2	2101	4-5	1-2	44	17	7	20
I exp P 3-5	2190	5	3	42	16	6	19
II A non-exp	1574	11	7	33	14	8	24
II A exp[b]	2358	6	3	39	14	6	24
FG exp	1981	7	4	42	17	7	9
II B non-exp	1465	12	8	35	19	8	23
II B exp[c]	2285	8	5	40	15	6	18
CE exp	2022	9	6	40	17	7	2
III A non-exp	2053	15	10	38	23	7	16
III A exp	2521	6	2	36	13	5	18
III B non-exp	1789	17	12	40	25	7	16
III B exp	2422	7	3	38	14	6	15

[a]Including animal, vegetable, and hydrogenated vegetable fats.

[b]Excluding subject FG.

[c]Excluding subject CE.

diets contained a somewhat larger proportion of fat calories than the non-experimental intakes. There is little difference in the proportion of fat in Study III diets. Study II subjects CE and FG consumed less fat and fewer calories than other members of their respective groups. However, since the ratio of fat to total calories in their diets was very similar to that of the other group members, their serum lipid data were not excluded.

Because of the discrepancy between the subjects' estimation of their usual caloric intakes and the calories required to prevent weight loss during the experimental periods, fecal fat was measured during Study II to determine the efficiency of fat absorption. These data appear in Table XII. Average fecal fat among Group A subjects was 4.4 g./24 hr., while that of Group B subjects was 3.8 g./24 hr. Although these two means are statistically different at the 5 per cent level according to the Student "t" test (154), they both represent greater than 95 per cent absorption of dietary fat.

Other workers also determined nitrogen balance of all subjects in all three studies (139, 140). Their findings are summarized in Table XIX in the Appendix. During the first two periods of Study I, the subjects were consuming approximately 20 g. protein daily and were unable to achieve nitrogen balance. When the protein was increased during periods three, four, and five, the subjects were essentially in nitrogen equilibrium. With the exception of group III A, all other groups had nitrogen balance means within the range usually considered to represent equilibrium. Group III A, consuming daily 36 g. protein

TABLE XII

FECAL FAT OF STUDY II SUBJECTS

Subject	Periods I	II	III	IV	V	Subject avg.	SE
	(g./24 hr.)						
Group A							
FG	4.2	3.3	4.4	4.1	3.8	4.0	
SH	5.9	3.9	3.7	6.0	3.2	4.6	
PM	4.0	4.6	3.7	3.8	3.0	3.8	
MR	4.7	4.6	5.8	5.9	4.3[a]	5.1	
avg.	4.7	4.1	4.4	4.9	3.6	4.4[b]	0.21
Group B							
CE	3.1	2.6	3.6	3.1	3.5	3.2	
MN	3.7	3.4	3.7	4.1	3.7	3.7	
ES	4.4	4.2	3.7	4.6	5.3	4.4	
PT	3.7	4.3	4.4	3.0	4.5	4.0	
avg.	3.7	3.6	3.8	3.7	4.2	3.8[b]	0.13

[a]Calculated value.

[b]Statistically different at the 0.05 level.

of predominantly vegetable origin, had an average nitrogen balance in negative range.

Capillary blood from finger pricks was used for serum cholesterol and esterified fatty acid analysis during Studies I and II. The extremely small volume of the samples made it impossible to analyze all samples for both groups of serum lipids. Since it seemed desirable to do paired comparisons between an individual's non-experimental and experimental values, it was necessary to determine first whether an individual's period values could be averaged together without obscuring or exaggerating any response due to the length of time on the diet.

For this purpose, data from Studies II and III were arranged in blocks according to diet and to number of days, less than seven, eight to eighteen, and more than nineteen, of consuming that diet. Two factor analysis of variance (154) was applied to these data. This analysis for the serum cholesterol values, presented in Table XIII, showed no effect of length of time, four to thirty days, on the various diets. However, the variance ratio due to dietary treatment was significant at the 5 per cent level. There was no evidence of interaction. Similar analysis of the esterified fatty acid values is shown in the same table. In this case, neither dietary treatment, time, nor interactions produced significant variations. Data from Study I were not included in these analyses because of the difference in number of subjects and because of the shorter period of time on the final experimental diet. However, a separate analysis of the values from periods three, four, and five of Study I showed no effect of time on either

TABLE XIII

ANALYSIS OF VARIANCE OF EFFECTS ON SERUM LIPIDS OF TYPE AND OF LENGTH OF DIETARY TREATMENT

Source	Degrees of freedom	Mean squares	F	$F_{0.05}$
Serum cholesterol				
Main effect of diets	3	6264.3	4.03	3.59
Main effect of time	2	1527.1	0.98	4.18
Interaction	6	1065.0	0.68	2.87
Deviations	34[a]	1552.8		
Total	45	10409.2		
Serum esterified fatty acids				
Main effect of diets	2	0.024	0.40	4.18
Main effect of time	2	0.020	0.33	4.18
Interaction	4	0.063	1.05	3.59
Deviations	27	0.060		
Total	35	0.167		

[a]Adjusted for loss of two degrees of freedom due to use of two calculated values.

serum cholesterol or serum esterified fatty acids. Therefore, each subject's experimental values for all periods were averaged and paired comparisons were made between each individual's non-experimental and experimental values.

In Table XIV, a comparison of non-experimental and experimental serum cholesterol values of all groups is presented. The values for period 2 and periods 3, 4, and 5, Study I, were not averaged together since the dietary protein content was changed from 21 to 31 g. at the beginning of period 3. The data obtained from subject PM was omitted from Study II A calculations because only one experimental value was available on her. The average differences were tested for significance by the Student "t" test (154). The group mean responses to the various diets were all positive, although some individuals showed a decrease from their non-experimental levels. The group mean differences were significant at the 0.05 level in group II A and group III A. Both of these groups consumed experimental diets containing 34 g. protein per day; the II A protein was of 50 per cent animal origin while the III A protein was of 33 per cent animal origin. The group II B response was of as great magnitude as that of group II A, but was not statistically significant at the 5 per cent level because of the high variance.

Serum esterified fatty acid levels were submitted to the same statistical analysis as the serum cholesterol data. Paired comparison between the average non-experimental and experimental values of each subject is shown in Table XV. In this case, figures were available only for periods 3, 4, and 5 of Study I. The change from the subjects'

TABLE XIV

COMPARISON OF PAIRED NON-EXPERIMENTAL AND EXPERIMENTAL SERUM CHOLESTEROL VALUES

Group Subject	Serum cholesterol Avg. non-exp. (mg./100 ml. serum ± SE)	Serum cholesterol Avg. exp. (mg./100 ml. serum ± SE)	Avg. difference (mg./100 ml. serum ± SE)	t
I Period 2				
GMB	179	175		
ESE	138	153		
SH	163	196		
MN	164	156		
ESP	196	203		
avg.	168 ± 10	177 ± 13	9 ± 7	1.216
I Period 3-5				
GMB	179	203		
ESE	138	156		
SH	163	170		
MN	164	146		
ESP	195	186		
avg.	168 ± 10	172 ± 13	4 ± 8	0.540
II A				
SH	163	211		
FG	234	274		
MR	216	255		
avg.	204 ± 21	245 ± 18	41 ± 3[a]	15.483
II B				
CE	228	234		
ESP	196	262		
PT	134	213		
MN	164	206		
avg.	181 ± 20	230 ± 25	49 ± 16	3.030
III A				
RL	201	209		
MM	141	151		
CP	182	194		
RS	185	187		
avg.	177 ± 13	185 ± 12	8 ± 2[a]	3.386
III B				
PM	245	213		
ESP	227	201		
BT	150	163		
HR	187	234		
avg.	202 ± 21	203 ± 15	1 ± 18	0.031

[a]Significantly different at the 0.05 level.

TABLE XV

PAIRED COMPARISON OF NON-EXPERIMENTAL AND EXPERIMENTAL SERUM TOTAL FATTY ACID ESTER VALUES

Group / Subject	Esterified fatty acids: Avg. non-exp. (mEq./liter serum ± SE)	Avg. exp. (mEq./liter serum ± SE)	Avg. difference (mEq./liter serum ± SE)	t
I				
GMB	8.7	6.5		
ESE	4.4	5.5		
SH	8.4	5.2		
MN	9.3	7.9		
ESP	9.4	7.7		
avg.	8.0 ±1.1	6.6 ± 0.4	-1.4 ± 0.7	-1.972
II A				
SH	8.4	8.4		
FG	10.0	11.7		
MR	8.5	13.5		
PM	11.6	8.3		
avg.	9.7 ± 0.3	10.5 ± 1.3	0.8 ± 0.3	0.497
II B				
CE	9.6	12.6		
ESP	9.4	11.5		
PT	10.6	8.0		
MN	9.3	8.4		
avg.	9.7 ± 0.9	10.1 ± 1.1	0.4 ± 1.3	0.305
III A				
RL	10.2	14.1		
MM	6.5	8.8		
CP	8.0	9.7		
RS	6.9	9.7		
avg.	7.9 ± 0.7	10.6 ± 0.6	2.7 ± 0.5[a]	5.933
III B				
PM	8.7	10.0		
ESP	9.2	10.1		
BT	5.2	8.9		
HR	6.7	9.8		
avg.	7.4 ± 0.8	9.7 ± 0.3	2.3 ± 0.7[a]	3.333

[a]Significant at 0.05 level.

usual diets produced a statistically significant difference at the 0.05 level only in groups III A and III B which both experienced an increase in serum esterified fatty acids.

Serum fatty acid data from Study III were submitted to paired comparison Student "t" tests (154) to determine any differences due to the change from subjects' usual diets to the experimental diets. The group means of each fatty acid are shown in Table XVI. The experimental values represented the means of samples collected in the middle of period 3 (twelve to eighteen days on the diets) and at the end of period 5 (thirty days on the diets). The only significant differences ($P < 0.05$) between non-experimental and experimental values were a decrease in percentage of palmitoleic acid among group A subjects and an increase in oleic acid among group B subjects.

TABLE XVI

FATTY ACID CONTENT OF SERUM OF STUDY III SUBJECTS

Fatty acid	Group A means		Group B means	
	Non-exp.	Exp.	Non-exp.	Exp.
	(% of total fatty acids ± SE)			
C14	2.5 ± 0.3	6.1 ± 1.6	3.2 ± 1.1	4.1 ± 0.7
C16	26.4 ± 0.9	26.0 ± 1.0	27.6 ± 1.5	25.6 ± 2.2
C16:1	13.6 ± 1.1	10.8 ± 1.7[a]	14.9 ± 1.3	12.9 ± 1.3
C18	6.1 ± 1.3	4.6 ± 0.7	8.8 ± 2.0	8.6 ± 2.4
C18:1	19.1 ± 2.3	17.8 ± 1.2	17.4 ± 1.7	21.0 ± 1.4[a]
C18:2	32.8 ± 3.6	34.7 ± 2.5	30.1 ± 4.7	30.1 ± 0.6
Total saturated	34.8	36.7	37.2	38.3
Total mono-unsaturated	32.7	28.6	32.2	33.9
Total poly-unsaturated	32.8	34.7	30.1	30.1

[a]Significantly different from comparable non-experimental value at the 0.05 level.

CHAPTER V

DISCUSSION

The purpose of the research reported in this paper was to observe the response of serum lipids in young women when their protein intake was reduced in quantity and/or quality. Both animal and human experiments have produced evidence of a lipid-protein interrelationship. Therefore, it was reasonable to expect a change in dietary protein to be reflected in serum lipid levels. The direction such a change might take was not clearly indicated by the literature. Various researchers have reported both elevations and depressions in serum lipids when dietary protein was decreased. Perhaps the predominant feeling among the workers in the field was that low protein intakes tend to produce relatively low serum lipid levels. The present study produced little evidence to support such a theory.

The subjects in this study were healthy premenopausal women. Such women as a group are reported to have lower serum lipid concentrations than males of the same age (15, 16). There is no evidence of qualitative differences in the usual dietary habits of the two sexes. Young men and women have been reported to differ in their serum lipid responses to experimental variation in dietary protein (120-122) and in dietary carbohydrate (130). Such findings make young women an interesting group for study, particularly since several similar studies have involved male subjects only (108, 109, 119, 121).

Interestingly enough, the women in this study showed serum lipid responses more similar to those of the young men in the experiments by Leveille et al. (109), and by Morse and others (121) than the young women studied by Walker et al. (120).

Young women, although they may be protected from development of extremely high serum cholesterol levels, do show considerable variation in their "normal" cholesterol levels. The subjects, while consuming their self-selected diets had serum cholesterol levels ranging from 131 to 294 mg./100 ml. with a mean of 186 mg./100 ml. This mean value is certainly well within the range considered normal for this age group. The young women studied by Walker and others (120) had similar initial cholesterol levels. Keys and others (157) reported serum cholesterol levels of 564 physically normal young women to average 176.5 mg./100 ml., while Adlersberg and others (24) reported a mean of 201.9 mg./100 ml. for a similar group of forty young women.

Although the mean initial serum cholesterol level was considered normal for young women, the range of these non-experimental values implied considerable variation among the subjects, even though the group was reasonably homogeneous in most other regards. Presumably the wide range of serum cholesterol levels represents differences in genetic make-up, hormonal influence, amount of exercise, emotional stresses, and other factors. Such factors can not be controlled in studies of normal human beings in their usual environments. Their influence can be minimized by the use of quite large numbers of subjects or by studying the same subjects under several experimental conditions.

Because the present study employed only small groups of subjects, its major contribution probably lies in the paired comparisons between each individual's serum lipid levels on his usual diet and on the experimental regimen. The usual finding was either no change or an increase in serum lipids when the subjects consumed the relatively low protein experimental diets. Since the results did not show consistently significant increases in serum lipid levels, one can not justify a conclusion of a hypercholesteremic effect of protein intakes of 48 g. or less daily.

The most usual serum cholesterol response during any of the experimental periods in the present study was an increase over the level produced by the individuals' self-selected diets. Although these changes were significant only in groups II A and III A, the consistency with which increases occurred is worthy of note. Similar studies of young adults consuming low protein diets showed either decreases in blood cholesterol (113, 115, 120) or no changes (109, 110, 121, 122). Beveridge et al. (122) reported cholesterol elevation in males on low protein diets, but this effect was not shown with young women. These differing responses can not be explained solely by differences in protein intake.

The results of the present study gave no indication of a serum lipid response pattern within the range of protein intake studied. However, the study was not designed to quantitate serum lipid responses to specific protein intakes. In view of the numerous factors affecting serum lipid levels, such an effort would necessitate the use of much

larger experimental groups and undoubtedly more rigid control of the diets than could be obtained with ordinary foods.

Further elucidation of the relationship between dietary protein and serum lipid levels may require more thorough examination of the subjects' protein nutriture, both during the experimental periods and during the weeks preceding the experiment. Few of the studies reported in the literature give any consideration of the subjects' usual dietary intakes. If one wishes to observe the effects of varying the quality or quantity of a component found in ordinary diets, one must consider not only the absolute level of that component, but also the change that is involved. In the present study, comparison of the experimental intakes with the subjects' usual diets showed that the difference between the usual protein intakes and the various experimental levels was greater than the differences among the experimental diets.

In 1961, Mann (95) stated that critical human trials of the relationship between sulfur-containing amino acids and cholesterol metabolism should be conducted with less complex dietary conditions than were currently in use. He recommended that a diet rich in fat and choline, limited in cystine precursors, with marginal levels of methionine, and with graded levels of cholesterol be fed for long periods to human subjects, preferably young females. The diets used in the present studies met some, but not all of these conditions. Their fat content was probably more normal than that used in similar studies in other laboratories. However, there was no control over choline content and sulfur-containing amino acids were controlled only by the

manipulation of natural protein sources. While these diets were hardly "less complex," they are realistic and do represent the usual American combination of nutrients.

It seems most likely that the observed differences in serum cholesterol can be attributed, not simply to one factor, such as the amount of protein or methionine, but to a combination of factors. The subjects were consuming relatively large amounts of fat and sucrose; they were subjected to the usual stresses of college work plus the inhibitions of a balance study. Undoubtedly all these factors and others contributed to the responses observed. Perhaps, under certain conditions of protein nutrition, the young female body is unaffected by such stresses, while under other conditions, it responds with elevated serum cholesterol. The numerous seeming contradictions in the literature, when serum lipids are measured in subjects on diets varying in protein content, and in the results of this work, would suggest that the protein-serum lipid relationship is a very intricate, delicate one which will require quite refined methods to elucidate it.

For example, it seems that it will be necessary to study either quite large groups or one group under many different dietary conditions. Otherwise, differences in initial levels and individual response patterns are so great as to obscure variations caused by the dietary treatment. This difficulty is particularly evident in the case of serum fatty acid esters. In the present study the problem was partially alleviated by the use of paired comparisons between each individual's non-experimental and experimental serum lipid values. Since only a few

subjects participated in more than one study, it was not possible to use paired comparisons to analyze the results of the different experimental diets. Only rarely were the data linear, so analysis of covariance was not applicable. Even the use of paired comparisons occasionally indicated statistical significance in cases where the differences were of dubious biological significance. For example, group III A showed a serum cholesterol increase of 8 mg./100 ml. This difference was statistically significant at the 0.05 level, while a 49 mg./100 ml. change observed in group II B was not. The use of larger groups of subjects would probably prevent this type of situation.

The fat content of the experimental diets used in this study furnished from 36 to 44 per cent of total calories. The low protein diets reported in the literature to have lowered serum cholesterol contained daily from 80 to 95 g. of fat which accounted for 30 to 36 per cent of total energy intake. In similar studies where serum cholesterol was unchanged, fat furnished 24 to 40 per cent of calories and ingestion ranged from 68 to 111 g. per day. Therefore, no discrimination between these studies can be made on the basis of fat quantity. However, the diets used in the current experiment did contain more fat than several of the reported diets and possibly somewhat more than that to which some of the subjects were accustomed. It is possible that while consuming diets restricted in protein, the subjects responded differently to a typical fat intake.

With the exception of the studies from this laboratory and the

work by Morse et al. (121), most dietary regimens reported in the literature contained lower proportions of fat calories than is considered usual for Americans. The fat assortment most often used was similar to that employed in the present research, a mixture of fats common to the American table. An exception was the diet used by Beveridge et al. (122) which furnished 60 g. of butter per day as the sole fat source. Such a fat intake is certainly not usual and may account for the differences in the serum lipid response by that group.

The lipid content of the various experimental diets was controlled as nearly as possible. However, the use of ordinary foods did result in some small variations. For example, the Study II B diet contained daily 71 mg. more cholesterol than Study I diet. According to the formula developed by Keys et al. (74) this difference would result in a change in serum cholesterol of roughly 9 mg./100 ml., a value insignificant in comparison to the magnitude of day to day variations in cholesterol levels. Likewise, the range in total fat intake from 97 g. per day in diet III B to 103 g. per day in diet I is not of sufficient magnitude to cause concern.

Of greater potential significance is the variation in fatty acid content of the diets. The individual fatty acids were measured in terms of percentage of total fatty acids of the composite or of the food. Such figures are not easily converted to absolute amounts for purposes of comparison. The relatively large amounts of the short chain fatty acids in the food composites were expected because of the large amount of dietary fat furnished by cream. The great variability may reflect

the degree of oxidative breakdown of the longer chain unsaturated fatty acids. Also measurement of the short chain fatty acids required the combining of chromatograms made under different temperature conditions. Any such manipulation obviously increases the opportunity for experimental error.

According to Keys and others (59) the saturated fatty acids, palmitic, lauric, and myristic, exert a cholesterol elevating influence twice as strong as the cholesterol lowering effect of the polyunsaturated fatty acids. Of the saturated fatty acids, they considered palmitic to have the greatest practical significance, due to its predominance in most diets. The palmitic acid content of the diets used in the present research was relatively constant.

All studies such as this are plagued by the possibility that in altering the protein content of the diet, there is also a significant alteration in fatty acid consumption. It was highly desirable that the experimental diets have a fatty acid composition similar to that of the subjects' previous intakes. In the present research there is little evidence of a change in fatty acid composition, certainly not toward increased consumption of saturated fatty acids which could account for the observed increase in serum cholesterol. Comparisons of non-experimental and experimental serum fatty acids during Study III showed essentially no change. Although the fatty acid content of cholesterol esters and phospholipids is relatively constant, the fatty acid profile in the triglyceride fraction is reported to reflect dietary intake (23, 47). Therefore, determination of total serum fatty

acid pattern could be expected to show any significant differences in fatty acid intake.

No attempt was made to measure the dietary content of lipotropic agents. However, calculation of the amino acid content of the diets indicated that, with the exception of the initial Study I regimen, all the diets exceeded minimum amino acid requirements (156) including those for total sulfur-containing amino acids and for threonine. Cereals and cereal products which are reported (158) to contain moderate amounts of choline were daily constituents of all the diets. Dried beans, considered to be quite good sources of choline (158), were consumed every other day by the subjects of Study III. Therefore, there is little reason to suspect any abnormalities of supply of lipotropic agents.

Except for the planned variation in protein intake, those differences between the subjects' previous diets and the experimental diets were kept to the minimum possible with ordinary foods. It is not possible to plan isocaloric diets in which the quantity of one macronutrient is varied without altering the amounts of one or both of the other energy sources. The recent implication of dietary carbohydrate, as well as fat and possibly protein, in regulation of serum lipid levels, has given such alterations new importance. It becomes necessary to make a decision as to which dietary variation will have the least complicating effect on serum lipids. Some workers feel that it is the relative amount of a nutrient that is critical. Therefore, those workers make their dietary variations in terms of percentage of total calories.

The current research was planned not only for observation of serum lipid responses, but also for study of the interrelationship of dietary nitrogen to magnesium, iron, copper, and zinc metabolism. Therefore, it seemed best to vary the absolute amounts of dietary protein and make up the differences in caloric requirements with supplements of sucrose.

Researchers at Harvard School of Public Health (125) reported that, although low intakes of sugar produced lower serum cholesterol levels in schizophrenic men than did high sugar consumption, the differences were quite small. They concluded that dietary carbohydrate was far less important than dietary fat in regulating blood cholesterol. Serum neutral fat is reported to be especially sensitive to increases in carbohydrate intake (9). In this study, no such influence could be shown; there appeared to be no relationship between the level of esterified fatty acids and intake of sucrose supplements. The individuals' non-experimental dietary records indicated that they were accustomed to a high sucrose intake.

In all the periods of collecting and determining serum cholesterol, there was always a day-to-day variability. Although an individual on his self-chosen diet seems to have a characteristic range into which his serum cholesterol values almost always fall, the magnitude of this range may often be large enough to prevent accurate estimation by analysis of one or two serum samples. Keys et al. (74) reported intra-individual average standard deviation in serum cholesterol levels of healthy American males to be 20 mg./100 ml., while the inter-individual

deviation was 45 mg./100 ml. In the present study intra-individual standard deviation in the non-experimental cholesterol values of those subjects from whom three or more samples were available ranged from 3 to 49 with an average of 21 mg./100 ml. The inter-individual standard deviation of the entire group of subjects was 35 mg./100 ml. This somewhat lower inter-individual variability probably reflects the absence of any extremely elevated values among this group of healthy young women.

Part of the variation observed in these young women may have been produced by the hormonal changes during the menstrual cycle. A rise in serum cholesterol immediately before and after ovulation in young women has been reported (159). Since the subjects in the present study entered the experiments at any point in their menstrual cycles and since the length of the studies was only thirty to thirty-six days, it was not possible to distinguish any hormonal influences from that of the dietary treatment.

In this laboratory serum esterified fatty acids, rather than triglycerides, were measured. Although the two groups are not strictly comparable, they both reflect changes in the neutral fat content of the blood. One of the chief proponents of triglyceride determinations, Albrink, measures this fraction by difference (9, 11, 12), using the formulas developed by Peters and Man (160) for calculating the amount of fatty acid esterified with cholesterol and phospholipid respectively. While this approach is more specific than measurement of total

esterified fatty acids, it does compound any errors in the determination of cholesterol and phospholipid.

The fatty ester determinations in this laboratory were not done on serum collected from fasted subjects. The capillary serum samples were routinely collected before the evening meal both during the non-experimental and experimental periods. Venous samples were drawn before the noon meal. There is evidence (20, 21) that postprandial triglyceride levels are affected by the previous intake of fat. However, in order to demonstrate this effect, fatty test meals had to contain at least 30 g. of fat. Since the subjects followed the typical American pattern of eating their heaviest meal at night, it is doubtful the earlier meals, consumed three to five hours before the serum collections, contained enough fat to show any effect.

The measurements of serum esterified fatty acids of the subjects, while consuming their self-selected dietary intakes, showed the same high degree of variability as is reported for triglycerides. The inter-individual standard deviation of the whole group was approximately one-third of the mean. Walker and others, studying similar groups of young women, measured fatty esters and reported a comparable degree of variability (120, 161). Such variability makes it difficult to demonstrate a statistically significant change in serum lipid levels.

Each experimental dietary period was preceded by a three to six day preliminary period. In Study I the preliminary period diet was the same low protein intake as employed in the first experimental periods. In Studies II and III, the diet consumed during the

adjustment period differed from the experimental diets in that the protein content was approximately 42 g. daily, rather than 35 or 50 g. These periods allowed a time for adjustment to the dietary changes. Some of the serum samples collected during preliminary periods were lost due to instrumental difficulties during subsequent analysis.

The responses to the change from the subjects' usual diets were prompt, apparently occurring by the end of the first six days on the experiments. Although there was considerable fluctuation in the bi-weekly serum samples, the serum lipid levels achieved in period I were essentially maintained during the remainder of each study. This relative stability may reflect response to the non-protein dietary factors during the three to six day preliminary periods. There was no apparent serum lipid readjustment to the dietary conditions during the thirty day experiments, in spite of the fact that nitrogen balance data (139, 140) indicated an adaptation to the low protein intakes. This lack of consistent change in serum lipid levels suggested also that there was no effect of the small weight changes during the course of the studies. Most subjects showed the greatest weight loss at the beginning of the studies and had stabilized by the end. These findings do not eliminate the possibility that after several months of consuming low protein diets, the subjects might return to their usual lower serum cholesterol levels. Other workers (109, 110, 119) have found evidence of a temporary serum lipid response which is not maintained over several weeks.

On the other hand, Prather (128) showed that young women

consuming diets containing 55 g. protein per day showed serum cholesterol depressions which increased in magnitude with increased time on the four week long experiment. When the same subjects were transferred to daily intakes of 91 g. protein, their serum cholesterol levels progressively increased.

The results of the various studies in the literature indicate that although the serum lipid response to an alteration in dietary protein may be quite prompt, observations after only one or two weeks on the experimental diets are not adequate bases for drawing conclusions. A number of studies have used experimental periods of only seven or eight days. Short term changes in blood lipids are of little biological importance.

The time required to show a response to dietary change and the stability of that response may be influenced by the type of dietary alteration. Keys and others (74), in reviewing a study (69) by Beveridge et al. in which eight day periods were used to observe response to various levels of dietary cholesterol, stated that after such a brief period of time only 70 per cent of the ultimate effect would have been achieved. Klein (61) in a 1960 review stated that the major portion of the change brought about by unsaturated fat in the diet occurs within three to eight days, although a slow decrease continues for some time. Mann and others (38) estimated a response lag of seven to fourteen days when medical students consuming a high calorie diet discontinued an intensive exercise program. During the three weeks of observation one student failed to show any change in serum lipid

levels, even though he was rapidly gaining weight. The authors suggested that his response lag may have been greater than three weeks.

The mechanisms of dietary effects on cholesterol and other serum lipids have not yet been elucidated. They may be due to changes in rates of absorption, synthesis, removal to other tissues, or degradation. Only in a few human studies have dietary alterations been combined with measures of these actions. Although the techniques involved in such measurements are complicated, the results would contribute greatly to the knowledge of the interrelationships between dietary constituents and lipid metabolism.

In the study of so intricate a relationship as that between the various dietary components and the serum lipid levels, it is necessary not only to give careful attention to control of dietary intake and accuracy of analytical techniques, but also to consider the response pattern expected and how to best treat that response statistically. The most usual procedure is to assume that the reaction to a dietary change will be a linear response. The literature shows little support for this theory, except in those cases where the subjects all have consumed a standardized diet for several weeks before being transferred to the experimental regimen. In spite of the rarity of a truly linear response, many research groups persist in comparison of serum cholesterol means, a statistically significant difference being their only criterion of a real change in cholesterol levels. Such treatment assumes that all the subjects have comparable initial values and that they all respond in the same manner and degree. In the case of serum cholesterol this

assumption can not be justified. For example, the mean non-experimental and experimental values of the group III A subjects (Table XIV, p. 101) are the same. However, the individual subjects showed definite responses to the dietary treatment, the tendency being toward a leveling out of inter-individual differences. In a larger group this tendency might have resulted in a significant decrease in the group variance. Comparison of the means themselves would lead to the conclusion of no significant effect of the dietary treatment. On the other hand, the magnitude of the individual responses would suggest a biological significance. Jolliffe and others (36) stated that they consider a persistent 10 per cent change in serum cholesterol levels of middle-aged men as evidence of a biologically significant change. Only rarely will such small changes be statistically significant.

One might hypothesize that the mixed response of the group III A subjects represented the individual differences in their usual or previous dietary intake. It is upon such a theory that the formula developed by Keys and others (55, 59, 74) for predicting the effects of dietary fatty acids and cholesterol on serum cholesterol levels is based. This formula uses, not the absolute dietary content of these nutrients, but the change in intakes of them. Thereby, the degree of change in serum cholesterol produced by a change in the intakes of certain fatty acids and of cholesterol can be predicted with reasonable accuracy. In order to make predictions from the absolute intakes of these lipids, the formula must include an unknown factor, the sum of all non-fat influences on serum cholesterol.

In addition, the ability to respond to a hypocholesteremic diet with a lowering of serum cholesterol levels does not necessarily depend upon the initial level. Prather (113) observed six young women whose initial plasma cholesterol levels ranged from 145 to 250 mg./100 ml. The greatest decreases occurred in those young women who originally had the highest and the lowest blood cholesterol levels.

Such a lack of pattern in responses was observed in some of the work of this laboratory. For example, in group II B (Table XIV, p. 101) both ESP and PT showed increases in serum cholesterol of approximately 70 mg./100 ml. while MN whose non-experimental average value was half way between the other two showed an increase of only 40 mg./100 ml. It might be hypothesized that those individuals who showed the greatest variation in non-experimental levels were particularly susceptible to fluctuations in serum cholesterol levels and the magnitude of their responses to dietary alterations merely reflected this susceptibility. The diet, however, would be considered responsible for the direction of the change. Obviously, such a theory does not explain every case of unusually high or low, but still "normal" serum lipid levels. Neither does it offer any explanation as to cause of the supposed susceptibility.

Although the current research does not warrant conclusion as to the nature of the interrelationship of dietary protein and serum lipid levels, it does, by no means, rule out such an interrelationship. It does suggest that the influence of protein may depend not solely on the quantity or quality of intake, but also on the needs of the body.

It also suggests that the role of dietary protein may be indirect, perhaps asserting itself through the handling of dietary fat and cholesterol. It appears that serum cholesterol was more sensitive to the dietary alterations than were serum fatty acid esters.

The results do strongly indicate that decreasing the intake of protein is not an effective method of lowering serum lipid levels. At least among these young women, reduction of daily protein intake to 48 g. or less with minimal alteration of the other dietary constituents did not lower serum lipids. Likewise, a decrease in the proportion of animal protein in the diet did not produce hypocholesteremia as reported by other workers. On the contrary, these dietary changes tended to elevate blood lipid levels.

CHAPTER VI

SUMMARY

The effect of quality and quantity of dietary protein on serum lipids in healthy premenopausal women was investigated in the Department of Nutrition at The University of Tennessee by means of three controlled dietary studies. A total of twenty-one subjects participated, five in Study I and eight each in Study II and Study III. Each study lasted thirty days and was preceded by a three to six day adjustment period.

The experimental diets were based on the usual American meal pattern and were varied in quantity and quality of protein by manipulation of ordinary foods, with the exception of a small supplement of casein. Daily fat intake from a variety of sources was held constant at approximately 99 g. and furnished 36 to 44 per cent of total calories. Daily cholesterol intake was 217 mg. or less. Analysis of dietary fatty acids showed no great differences among the various experimental diets.

The subjects kept a record of their food consumption during one to two week periods before each study. Serum samples were collected during these periods and at intervals of several weeks after the experiments.

Serum cholesterol and esterified fatty acid levels of the subjects while consuming their self-selected diets showed considerable

variation both among individuals and between various samples collected from a single subject. However, the means of both lipid fractions were well within the ranges reported as normal for that age group.

In Study I the daily protein intake was initially 20 g., largely of vegetable origin. On this intake the subjects were in negative nitrogen balance. Additions of casein and egg white increased the protein content to 31 g. per day, so that the total protein intake then came equally from animal and vegetable sources. During eighteen days of the higher protein consumption, the subjects approached nitrogen equilibrium. There was no significant difference between the mean serum cholesterol levels of the Study I subjects while consuming their self-selected diets, the diet containing 21 g. protein per day, or the 31 g. protein daily regimen. The dietary alterations also had no effect of serum fatty esters.

In Study II two diets with four subjects in each dietary group were used. Diet A, the basal diet, contained equal amounts of animal and vegetable protein totaling 35 g. per day. Diet B was the basal diet plus sufficient casein and cooked egg white to bring the total daily protein content up to 48 g., 65 per cent coming from animal sources. Both dietary groups maintained nitrogen equilibrium. Every subject in Study II responded to the dietary treatment with an elevation over her usual serum cholesterol level. These elevations, averaging about 40 mg./100 ml., were significant ($P < 0.05$) for Group II A but not Group II B. Serum fatty ester levels were essentially unchanged

by either dietary alteration. Fecal fat determinations indicated normal fat excretion levels in both II A and II B subjects.

In Study III the protein quantity was 36 and 47 g. daily, but vegetable sources replaced some animal products so that 67 per cent of the protein intake was of plant origin. Group III B was in nitrogen equilibrium, but the over-all average nitrogen balance of group III A was in the negative range. Group III A subjects showed small, but significant ($P < 0.05$), increases in serum cholesterol levels but the level of that sterol was unchanged in group III B subjects. Esterified fatty acids showed significant increases ($P < 0.05$) over the non-experimental levels of both groups. Determination of the fatty acid content of serum collected before, during, and after Study III showed no significant difference due to either diet.

The three diets, I, II A, and III A, all furnishing from 31 to 34 g. of protein per day, resulted in group average serum cholesterol levels which varied from 172 to 244 mg./100 ml. These differences can not be explained on the basis of differences in protein quantity, quality, or origin. Serum esterified fatty acids appeared less sensitive to dietary protein.

These results suggest an intricate relationship between the body's state of protein nutrition, the dietary content of protein, perhaps the dietary lipid load, and serum lipid levels. However, the current data are not sufficient to warrant any real conclusion as to this relationship. They do strongly suggest that serum lipid levels

can not be lowered in women by merely decreasing their protein intake while other dietary factors remain relatively unchanged.

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APPENDIX

TABLE XVII

AGE, HEIGHT, AND WEIGHT OF SUBJECTS

Subject	Age (yr.)	Height (cm.)	Initial Weight (kg.)	Final Weight (kg.)
Study I				
GMB	25	159	60	56
ESE	35	170	65	64
SH	41	166	58	58
MN	22	166	81	79
ESP	24	162	74	73
Study II Diet A				
FG	25	159	48	47
SH	41	166	59	59
PM	33	158	63	62
MR	27	156	65	65
Study II Diet B				
CE	22	158	52	51
MN	22	165	79	77
ESP	24	162	77	77
PT	28	159	56	55
Study III Diet A				
RL	24	164	70	69
MM	20	158	54	54
CP	21	171	61	60
RS	25	163	45	45
Study III Diet B				
PM	35	160	63	63
ESP	26	165	83	83
HR	29	168	63	63
BT	21	168	62	61

TABLE XVIII

CALCULATED[a] AMINO ACID CONTENT OF EXPERIMENTAL DIETS

Diet	% Intake nitrogen accounted for	Amino acids							
		Tryp-tophan	Phenyl-alanine	Leucine	Iso-leucine	Lysine	Valine	Total S containing	Threo-nine
		g./day							
I (initial)	74	0.2	0.6	0.9	0.6	0.7	0.6	0.3	0.5
I (P 3-5)	79	0.3	1.2	1.8	1.2	1.4	1.3	0.8	0.9
II A	79	0.5	1.1	2.0	1.5	2.0	1.3	0.8	1.0
II B	86	0.6	1.7	2.9	2.1	2.6	1.9	1.5	1.7
III A	79	0.2	1.2	2.0	1.3	1.6	1.4	0.8	1.0
III B	85	0.4	1.8	3.1	1.9	2.3	2.0	1.2	1.6
Minimum requirements[b]		0.16	0.22	0.62	0.45	0.50	0.65	0.55	0.31

[a]Bowes and Church (144) and Orr and Watt (155).

[b]Minimum amino acid requirements for young women, as established by Leverton and by others (156).

TABLE XIX

NITROGEN INTAKE AND BALANCE DATA
STUDIES I, II, AND III

Group	No. subjects	Days	Avg. nitrogen intake (g./24 hr. ± SE)	Avg. nitrogen balance (g./24 hr. ± SE)
I[a]				
P 1-2	5	12	3.45 ± .09	-0.84 ± .43
I[a]				
P 3-5	5	18	5.13 ± .04	-0.11 ± .32
II A[a]	4	30	5.56 ± .04	0.15 ± .13
II B[a]	4	30	7.81 ± .08	0.71 ± .32
III A[b]	4	30	5.85 ± .08	-0.42 ± .38
III B[b]	4	30	7.77 ± .07	0.40 ± .25

[a]Hunt, S. (139).

[b]Gomez, V. (140).