THE EFFECT OF TWO TYPES OF PROTEIN, SUPPLEMENTARY METHIONINE AND DIETARY CHOLESTEROL ON PLASMA AND LIVER CHOLESTEROL LEVELS

by

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ABSTRACT

High serum cholesterol levels have been identified as an important risk factor for atherosclerosis in humans. The purpose of this study was to investigate the effect of protein quality and quantity and dietary cholesterol supplementation on plasma and liver cholesterol levels, using a rat model. Male Wistar rats, weighing approximately 150 g, were fed ad libitum, with food intakes recorded daily and body weights weekly. At the end of six weeks, plasma and liver samples were collected and analyzed enzymatically for cholesterol content.

Dietary protein was fed at levels of 10%, 15% and 20%, using peanut protein, peanut protein with methionine and casein. Methionine was added to one group of peanut protein diets to increase the amount of this amino acid to the same level as is found in a casein diet of equivalent protein quantity. Cholesterol was fed to three diet groups: 20% peanut protein, peanut protein with methionine, and casein. Rats were also fed a laboratory chow diet to evaluate the effect of time on their tissue cholesterol levels.

The results reveal no significant effect of protein quality on plasma cholesterol and only at 15% and 20% protein levels does liver cholesterol increase with improvements in protein quality. Time and the amount of protein in the diet, whether casein or peanut protein, had no effect on plasma and liver cholesterol levels although a trend revealed by Pearson correlation analysis showed a negative correlation between plasma cholesterol and peanut protein, and a positive correlation

between plasma and liver cholesterol and casein. The effect of protein on tissue cholesterol appeared to be a minor one in comparison with the effect of dietary cholesterol, although accumulation of cholesterol in the livers of rats fed diets containing cholesterol could be decreased by dietary methionine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER I

INTRODUCTION

Atherosclerosis is one type of arteriosclerosis ('hardening of the arteries') characterized by an abnormal accumulation of smooth muscle cells, lipids especially cholesterol, and calcium in the intima of large- and medium-sized arteries. The clinical manifestation of the subtle degeneration of the circulatory system is a spectrum of vascular diseases involving the heart, brain, kidneys and peripheral tissues (Marx, 1976).

A number of theories have been proposed to account for the development of atherosclerosis and a composite of these suggests that an injury to the endothelium may encourage platelet adherence and smooth muscle cell proliferation. Uptake of lipid occurs at the damage site and is probably enhanced when blood cholesterol levels are high and when the blood pressure is elevated (Cantwell, 1977).

Despite the multi-directional research devoted to testing the current theories, concrete means of either preventing or curing atherosclerosis have not been found. The problem of atherosclerosis remains a very real one since coronary heart disease, a kind of cardiovascular disease, is the principal cause of death in this country (Canada, 1976) and in most industrialized Western countries (World, 1972).

High serum cholesterol levels have been identified as a major risk factor for atherosclerosis, along with high blood pressure, cigarette smoking and diabetes (Mustard Report, 1976; Kannel et al., 1979). Attempts have been

made to lower serum cholesterol in order to moderate or prevent the development of atherosclerosis because of the strong relationship that exists between the level of blood cholesterol and atherosclerosis. Both human and experimental animals have been used to this end, and the influence of inherent physiological factors and environmental factors has been evaluated. The diet is undoubtedly the most significant of the environmental factors, and the components of the diet that have been investigated include cholesterol and other fats, protein, carbohydrate, fiber, and trace minerals.

With particular reference to protein, epidemiological studies have shown that a positive correlation exists between animal protein intake and the incidence of heart disease (Yudkin, 1957; Yerushalmy and Hilleboe, 1957; Olson et al., 1958). Connor and Connor (1972) observed a similar association between animal protein and the mortality from coronary heart disease in men aged 55 - 59 years, and a strong negative correlation between plant protein and mortality from coronary heart disease in this group. Studies with vegetarians also indicate that their serum cholesterol levels are significantly lower than in the non-vegetarian group, and as the degree of non-vegetarianism increases, so also does the serum cholesterol (Hardinge and Stare, 1954; West and Hayes, 1968).

In light of the epidemiological evidence linking elevated blood cholesterol levels with heart disease, and the influence protein has been shown to have on these levels, the purpose of this study was to further investigate the qualitative and quantitative effects of plant and animal proteins in an animal model using peanut protein and casein to represent these proteins respectively. Each was fed at three levels in an otherwise adequate diet,

and at the highest level, the effect on serum cholesterol of dietary cholesterol was also studied. Blood and liver samples were collected and analyzed for cholesterol; the blood because it represents a cholesterol pool and the liver because it is the site of lipoprotein and endogenous cholesterol synthesis and cholesterol catabolism.

The rat was chosen as the experimental animal, since its cholesterol metabolism is more like a human's metabolism, than that of a rabbit or bird (Keys and Anderson, 1955). The use of humans would have been ideal, but this was neither practical nor feasible. Animal studies can be performed with greater ease since the subjects are more readily available. Variability among them is reduced by using animals from the same strain, controlled diets and comparable housing conditions. The time period for an animal experiment need not be as long as for a human experiment, and the ethics of sample-taking and experimental design are of lesser importance.

Results obtained from these experiments with rats contribute to the information available about cholesterol metabolism and the influences of diet upon it. They do, however, apply only to this species, and unwarranted inferences and implications for humans should not be drawn from them.

CHAPTER II

REVIEW OF LITERATURE

Our current understanding of the effects of dietary protein on blood and liver cholesterol levels is fragmentary and fragmented by the plethora of studies using different species of animals in countless variations of experimental design. Furthermore, the results obtained are not necessarily unanimous in determining the extent and direction of the response. The problem is also complicated by other components of the diet, such as cholesterol. This review focuses on the effects of protein on tissue cholesterol levels in the rabbit, rat, mouse, pigeon , chicken, several non-human primates and the human.

Rabbits

In 1908, Ignatowski studied the effects of high protein diets on rabbits, and observed fatty plaques on the internal surface of the aorta in some of these animals, as well as fatty accumulation in the liver. The diets contained meat, milk, and eggs and he attributed the pathological changes to a diet rich in animal proteins. In an attempt to pinpoint more precisely the cause of the arterial lesions, Stuckey (1911) fed diets consisting of milk, egg white, egg yolks and meat juice in various combinations. He found that only the diets containing egg yolks produced fatty changes in the aorta, while milk, egg white and meat juice alone had no damaging effect. These results suggested that the lesions in the arteries were more likely due to the fatty fraction of the diet rather than to a protein fraction.

More support was given to these results when Anitschow and Chalatow (1913) demonstrated in rabbits that the feeding of cholesterol dissolved in sunflower seed oil was capable of producing lesions identical with those described by Ignatowski (1908) and Stuckey (1911). Since no lesions developed when the rabbits were fed a cholesterol-free diet, they concluded that the cholesterol was the active component and that the oil acted as a solvent. Wacker and Hueck (1913) reported that pure cholesterol in solid form when added to the ordinary food of rabbits could produce this arterial damage. The consequence of these cholesterol-feeding experiments was that attention was diverted from protein and the mainstream of research concentrated on other components of the diet as the causative factors of atherosclerosis.

However, not all experimental evidence has supported the theory that cholesterol alone is responsible for the atherosclerosis. In 1920, Newburgh and Squier found atherosclerotic plaques in the aortae of rabbits fed a powdered beef diet for four weeks. If the protein source was casein, aortae were normal at ten weeks, but did show the signs of atherosclerosis at eleven months. In a later study, Newburgh and Clarkson (1923) produced fatty lesions in the arteries of rabbits by feeding them diets which contained 27% protein from powdered beef. This occurred after one year. In addition, they found that if the diet contained 36% protein, atherosclerosis could be seen at two months. The only cholesterol in the diet was in the dried beef (about 30 mg per day at the 27% protein level), and since this amount, when fed alone, did not result in arterial damage, the conclusion reached was that protein, not cholesterol, was responsible for the damage observed (Clarkson and Newburgh, 1926).

It also seemed that the rate of development of atherosclerosis was dependent on the amount of protein in the diet.

Although Wacker and Hueck (1913) had shown the influence of cholesterol in solid form, Duff (1935) felt that the conclusion reached by Newburgh and Clarkson (1923) was invalidated by the fact that the cholesterol, when fed alone, was in the form of a dry powder which probably was not as well absorbed as its equivalent in the meat diet, and that a direct experiment of feeding a diet rich in proteins and free from cholesterol was not carried out.

It was not until the early 1940s that this direct feeding of a cholesterol-free, high protein diet was tried. Meeker and Kesten (1940, 1941) fed such a diet containing 38% of calories from casein to rabbits for six months. They noted hypercholesterolemia and the development of atheromatous lesions indistinguishable from those produced by cholesterol. By contrast, rabbits fed a soybean diet, at approximately the same protein level, and for the same length of time had the higher serum cholesterol levels but the lower incidence and severity of atherosclerosis. The addition of cholesterol (60 or 250 mg per day) magnified the response of the rabbits to the two proteins, but did not change the direction of the response.

Although these studies provided evidence that animal proteins, such as casein and dried beef, could produce hypercholesterolemia and atherosclerosis in rabbits, research was again directed away from protein and into another area when Lambert et al. (1958), and Malmros and Wigand (1959) reported that hypercholesterolemia and atherosclerosis could be produced in rabbits by feeding cholesterol-free, purified diets

containing 25% casein. Since both effects could be prevented by the addition of safflower or corn oils, it was thought that the problem was a relative deficiency of essential fatty acids. Kritchevsky (1964) showed that the atherosclerosis developed by rabbits fed a purified diet but not the commercial feed could not be attributed to the fat content of the diet. Kritchevsky and Tepper (1968) extracted the lipid from Purina rabbit chow and added it to a semisynthetic diet containing 25% casein and 14% fat. They found that substitution of the fat usually present in the chow for 2% of the hydrogenated coconut oil used in the semisynthetic diet did not affect the atherogenicity of the diet. They further suggested that the differences observed may be due to non-lipid components of the diet, such as carbohydrate, fiber and protein.

Not only does the level of a protein appear to affect the incidence and severity of hypercholesterolemia and atherosclerosis, but the substitution of one type of protein for another also appear to exert an influence. Howard et al. (1965) varied the composition of the basic semisynthetic rabbit diet and found that none of the alterations had any effect on atherosclerosis, except for the substitution of casein in the original diet by either whole soya flour or hexane-extracted soybean meal. These soy products were effective in preventing the hypercholesterolemia and atherosclerosis of rabbits fed semisynthetic diets. Carroll (1971) showed that the addition of casein to commercial feed, in a ratio of 1:3, produced a definite hypercholesterolemia. These results substantiate those of Howard et al. (1965) which also demonstrated a hypercholesterolemic response of rabbits to casein even in the absence of cholesterol.

In another set of experiments, the kind of protein used in the diet determined the extent of a hypercholesterolemic response. (Carroll and Hamilton, 1975; Hamilton and Carroll, 1976; Carroll, 1978a) These diets were low in fat and contained no cholesterol. In general, the results showed that proteins derived from animals produced higher plasma cholesterol levels in rabbits, while diets containing plant proteins gave a normocholesterolemic response. Unexpected results were obtained with some of the animal proteins, pork protein and egg white, since the animals fed the diets containing these proteins had plasma cholesterol levels which were not significantly different from the plant protein-fed group. Huff et al. (1977) found that the hypercholesterolemic response of rabbits to a semisynthetic diet containing casein could be counteracted by the addition of soy protein. No elevation of plasma cholesterol occurred when the diet contained 1:1 casein:soybean protein isolate, whereas a diet containing a 3:1 mixture of these proteins gave a plasma cholesterol level which was higher than that produced by the diet containing the 1:1 mixture but lower than that produced by the casein diet.

Huff et al. (1977) also found that an enzymatic digest of casein or a mixture of L-amino acids equivalent to casein produced high plasma cholesterol levels similar to those obtained with the intact protein. By contrast, plasma cholesterol levels remained low in rabbits fed on enzymatic digest of soy protein, and were moderately, but not significantly, higher when the protein source was in the form of L-amino acids corresponding to soy protein. These results suggest that the hypercholesterolemic response to casein was probably due to the protein constituents of the diet, although the possiblity remains that something other than

protein in the isolated soy protein was contributing to the low level of plasma cholesterol.

Casein is more hypercholesterolemic than soybean protein for rabbits, but the effect of protein, per se, appears to be secondary to the effect of dietary cholesterol. Munro et al. (1965) fed adult rabbits diets containing 8% or 30% casein, with and without 1% cholesterol. The rabbits fed diets containing cholesterol had higher blood cholesterol levels than those fed diets with no cholesterol added, regardless of the level of protein. Similarly, a large amount of cholesterol accumulated in the livers of rabbits fed the cholesterol-enriched diets. When cholesterol was part of the diet, the hepatic deposition of cholesterol was greater in the 30% casein-fed group than in the group consuming the 8% casein diet, suggesting that the effect of cholesterol is enhanced by higher levels of protein in the diet. These results, however interesting, must be kept in perspective since rabbits after weaning and under non-experimental circumstances are exposed to neither casein nor cholesterol in their diets.

Rats and Mice

An early stimulus to studies concerning the relationship of dietary protein to liver cholesterol metabolism was the work of Channon et al. (1938), who noted that cholesterol accumulated in the livers of rats fed low protein diets containing either 8% albumin or 8% casein. This problem could be overcome by the addition of casein (Best et al., 1936) or methionine (Tucker and Eckstein, 1937) to the diets that induced fatty livers. An explanation for this seems to lie in the provision of labile

methyl groups from either the methionine in casein or "free" methionine. Harper (1958) suggests that these methyl groups are used for the synthesis of choline which is an integral part of some phospholipids, which, in turn, are part of lipoproteins used in fat transport.

More recently, Aoyama et al. (1969) studied the lipid level in the livers of rats fed amino acid mixtures simulating 7% egg albumin and 7% casein. The liver lipid levels were higher in the rats fed the egg albumin amino acid mixture than in the casein amino acid mixture, and they attributed the difference to the higher methionine content of the casein amino acid mixture.

Fillios and Mann (1954) studied the relationship of the quality of dietary protein to plasma cholesterol metabolism. In both rats and mice, they found that hypercholesterolemia resulted when soybean protein was substituted for casein at both 10% and 20% protein levels. The addition of methionine to the soybean diets containing the two levels of protein caused a drop in the serum cholesterol levels of rats fed these diets. In the mice, methionine added to the 10% soybean diet resulted in serum cholesterol levels between those from animals fed the 10% soybean diet without added methionine and the 10% casein diet.

Portman and Mann (1955) have suggested that the hypercholesterolemia of rats fed a soybean protein diet is due to a sulphur amino acid deficiency in the diet. This type of deficiency, in turn, limits the production of taurine, and hence of taurine-conjugated bile acids. When this conversion is inhibited by a sulphur amino acid deficiency

in the diet, cholesterol tends to accumulate in the plasma. This, however, suggests that a priority system is in operation for the use of methionine not involved in protein synthesis and that it will be channelled preferentially for the synthesis of choline, before being used for the synthesis of taurine.

This effect seems to hold also when the quantity of protein in the diet is changed. Moyer et al. (1956) found that additional dietary protein stimulated a drop in serum cholesterol values in rats, whether the additional protein was casein or soybean protein. This so-called "protective effect of protein" was also observed by Fillios et al. (1956) who evaluated diets containing 10, 20, and 60% casein. They found the highest serum cholesterol levels in those rats fed a 10% casein diet and the lowest in the 60% protein group. Nishida et al. (1956) used 15, 20, 30, and 35% soybean protein diets, and, in agreement with the two previous studies, noted an inverse relationship between serum cholesterol and the level of protein in the diet. In addition, the incidence of atherosclerosis paralleled the serum cholesterol, or in other words, it was highest at the lowest level of protein. De Groot (1959), Seidel et al. (1960), Nath et al. (1961), Leveille and Sauberlich (1964), and Renaud and Allard (1964) also found that the addition of protein to a diet was capable of lowering plasma cholesterol.

The effect of protein on tissue cholesterol levels in rats can be explained in terms of methionine availability but not all studies have lent support to this theory. Jones et al. (1957) observed that rats consuming diets containing either 14% or 19% casein had serum cholesterol

levels of 164 and 218 mg %, respectively, when measured at the end of six months. However, Jones and Huffman (1956) suggest that a reduction or elevation of dietary protein beyond a modest range (12 - 18%) will lead to an increase in serum cholesterol. They found very high levels of cholesterol, 490 mg %, in the serum of rats fed 40% casein, as well as lesions in the coronary arteries of one-third of these animals. Nath et al. (1958) noted similarly high serum cholesterol levels in rats fed a 40% casein diet. Corroborating the results of Jones and Huffman (1956), but contradicting their earlier work (Fillios et al., 1956), Fillios et al. (1958) indicated a downward trend in serum cholesterol when the casein level was increased from 5% to 20%, but they found what seemed to be a rebound effect when the protein levels were increased to 40% and 60%.

Yadav and Liener (1977) obtained opposite results using essentially the same type of diet as Fillios and Mann (1954). They found that replacement of casein by soybean flour, soybean isolate or roasted navy bean flour led to a significant reduction in serum cholesterol. An amino acid mixture simulating soybean isolate likewise produced levels of serum cholesterol which were lower than those obtained with an amino acid mixture corresponding to casein. They support the suggestion that the cholesterol-lowering effect of soybean protein is due to a difference in its amino acid profile without mentioning a possible mechanism for this effect.

Méndez (1964) investigated the effect of dietary protein level and cholesterol supplementation on the rat's serum and liver cholesterol levels,

in a study similar to one designed for rabbits by Munro et al. (1956). He found no significant difference in the serum cholesterol of rats fed 5% or 20% casein. This lack of difference in the serum cholesterol levels existed whether or not cholesterol was added to the diet. However, the rats fed diets containing cholesterol had serum cholesterol levels that were much higher than those of rats fed a cholesterol-free diet. This would suggest, as in the rabbit, that the effect of protein may be secondary to the effect of dietary cholesterol. In the liver, the cholesterol levels were much higher in the low protein groups, and the addition of cholesterol amplified this difference. This may be due to an increased availability of such lipotropic agents as methionine at the higher levels of protein, although Morris and Chaikoff (1959) reported a significant increase in liver cholesterol accumulation in rats fed a diet containing 25% casein and 2% cholesterol. A similar increase was noted by Frantz et al. (1954) with a diet of laboratory chow and 1% cholesterol.

Birds

In chickens, an indirect linear relationship seems to exist between serum cholesterol and the intake of protein. Low protein diets enhance hypercholesterolemia and atherosclerosis while high protein diets suppress these effects (Stamler et al. 1958a, b; Kokatnur et al., 1958; Nishida et al., 1956; Nishida et al., 1958; Leveille and Fisher, 1958; Leveille et al., 1960). Johnson et al. (1958) also found higher plasma cholesterol levels in chicks fed 10% protein diets as compared to those fed 25% and 40% protein diets. The proteins used were either casein or soybean. As the protein level was increased to 25% and 40%, there was a corresponding decrease in plasma cholesterol. The hypercholesterolemia of the birds

on the low protein diet could be reduced by the addition of methionine, but in the growing chick supplementary methionine was without effect at the 25% and 40% protein levels. The hypocholesterolemic effect of methionine in growing chicks was also observed by Leveille et al. (1962) and Kokatnur and Kummerow (1961). It may be that any hypocholesterolemic advantage gained by the addition of methionine to diets containing low levels of proteins is masked when the amount of protein in the diet is increased.

Chaikoff et al. (1961) examined the role of protein in the naturally-occurring aortic atherosclerosis that chickens develop even when their diet is low in cholesterol. In this study, two levels of protein, 14.5% and 7.3%, were used. The results indicate that average plasma cholesterol levels were higher in the birds fed the low protein diets, in agreement with previous studies. However, the protein level did not affect spontaneous atherosclerotic lesions in chickens.

The decrease in serum cholesterol observed in chickens as the amount of protein in the diet is increased may be the result of three processes: a decrease in cholesterol synthesis as measured by the incorporation of radioactive acetate into liver cholesterol (Nishida et al., 1960); an increased conversion of cholesterol to bile acids (Leveille and Sauberlich, 1961); and amino acid balance. With regard to the last process, Kokatnur and Kummerow (1961) observed that crystalline amino acid mixtures which were deficient or made deficient by eliminating a specific essential amino acid led to elevated serum cholesterol levels. Conversely, when the deficiency was corrected, the result was a lower serum cholesterol level.

As Carroll and his co-workers studied the effects of plant and animal proteins on the serum cholesterol levels of rabbits, Hevia and Visek (1979) looked at the same parameters in chickens. The diets contained lactalbumin, soybean protein or egg white solids fed at 25% and 50% levels. Although no significant differences were observed between the plasma cholesterol values at the two levels of protein, soybean protein depressed plasma cholesterol, as compared to lactalbumin and egg white solids.

In pigeons, the picture is somewhat different, especially when considering the studies of Lofland and his associates, using atherosclerosis-susceptible pigeons. Feeding diets to these birds containing 5, 15, or 30% casein, Lofland et al. (1961) and Clarkson et al. (1962) found that the 30% casein diet was more atherogenic and hypercholesterolemic than the 5% and 15% diets, when 0.25% cholesterol was added to these diets. A more recent study by Little and Angell (1977) using the same breed of pigeons, noted a significant increase in serum cholesterol concentrations, as the protein level was increased from 10% to 20% and 40%. They, however, failed to observe the increased atherogenicity of diets containing higher amounts of protein. It seems that pigeons respond like rabbits to protein and cholesterol in their diets, whereas chickens respond like rats.

Non-human Primates

Serum cholesterol response to dietary protein has been measured in several different species of primates. Mann and co-workers (1953) observed that Cebus monkeys fed diets containing 10% soybean protein had higher serum

cholesterol than monkeys fed casein at the same level. Later work has focussed on the level of protein in the diet, with little attention being paid to the effect of proteins with different amino acid compositions.

Middleton et al. (1967) fed squirrel monkeys 25% or 9% protein diets and found that the level of protein had little effect on serum cholesterol levels. However, the severity of atherosclerosis was influenced by dietary protein when cholesterol (0.5%) was added. Under these circumstances, the high protein diet was more than twice as atherogenic. This would again suggest that, in squirrel monkeys, the degree of atherosclerosis is more readily influenced by dietary cholesterol than by dietary protein. In another primate study, Strong and McGill (1967) fed baboons diets containing 10% or 25% casein and 0.5% or 0.01% cholesterol. Although the animals receiving the high protein diets tended to have higher serum cholesterol values, Strong and McGill (1967), in agreement with Middleton et al. (1967), concluded that the effect of protein was small in comparison with the effect of dietary cholesterol, as measured by changes in serum cholesterol levels.

Srinivasan et al. (1979) studied serum cholesterol levels in spider monkeys fed diets containing 4, 8, or 25% protein from laboratory chow and casein and 0.5% cholesterol. Their results indicate that the lowest serum cholesterol level was found in those monkeys consuming the 8% protein diet, and the highest in the 4% protein diet.

The differences observed among the primates may be a species-specific response to protein levels in the diet. Varied responses of serum lipids in several species of primates have been documented by Srinivasan et al., 1976; Nicolosi et al., 1977; and Srinivasan et al., 1978.

Humans

The evidence that dietary protein can influence human serum cholesterol levels is equivocal. Olson et al. (1957) fed 100 g protein/day (mainly of animal origin) to seven subjects, five of whom were hypercholesterolemic, for periods of several weeks. They were then fed a low protein diet, 25 g protein per day (mainly of plant origin) for one week and returned thereafter to the control diet. All showed a decrease in serum cholesterol during the low protein feeding, with values returning toward original values when the control diet was reintroduced. Keys and Anderson (1957) maintained two groups of physically healthy men on a lower protein intake, 83 g protein per day, for a four-week period, changed them to a higher protein diet, 130 g protein per day, and then back to the low protein regimen. There was no significant change in the serum cholesterol level in either group during the experimental period. In a similar study by Leveille et al. (1962), seven healthy males were fed a rigidly controlled diet varying in the amount of protein for twelve weeks. The subjects started on a lower protein diet, 32 g protein per day, switched to a higher protein diet, 106 g protein per day, and then returned to the lower protein diet. As before, dietary protein had no significant influence on plasma cholesterol.

The type of dietary protein may exert a stronger influence on plasma cholesterol levels than the actual amount of protein. Walker et al. (1960) reported differences in cholesterol levels when the quantity of protein was constant, but derived from two different sources. In this study, young women consuming a daily diet containing 50 g vegetable protein had lower serum cholesterol levels after five weeks than did a group eating 50 g animal protein per day. The difference in serum cholesterol levels was approximately 20 mg % with the final values for both diets still within normal physiological limits. Hodges et al. (1967) observed an average drop of 100 mg % in the serum cholesterol of six males fed a diet based on soybean protein, but containing almost no fat or cholesterol. Although part of this decrease can be attributed to the absence of dietary fat and cholesterol, there appeared to be a difference between the predicted and observed decrease, which may have been due to dietary protein.

Tripathy et al. (1970) studied the effect of high protein diets on the serum cholesterol levels of twelve malnourished adults. The control diets consisted of 15 - 30 g protein per day, and after four to six weeks, the dietary protein level was raised to 35 - 100 g per day. Within three weeks, there was a highly significant increase in serum cholesterol levels. This increase was likely due to increased synthesis of the protein moiety of lipoproteins in the presence of a higher protein diet. A recent study by Rickman et al. (1974) has also shown that a high protein diet can lead to high serum cholesterol levels. The diet in this case is extreme, in that it consists almost entirely of protein and animal fat, with carbohydrate contributing only about 2% of total

calories. This diet raised the serum cholesterol in all subjects studied and it seems likely that the protein component of the diet is responsible for at least part of this response.

Sirtori et al. (1977) reported a 14% decrease in plasma cholesterol levels after two weeks and a 21% decrease after three weeks when a textured soybean protein was substituted for animal protein in the diets of twenty patients with Type II hyperlipoproteinemia. Initially, their diets were also low in cholesterol, but the addition of 500 mg cholesterol per day in the form of egg powder did not modify the hypocholesterolemic response. Munoz et al. (1979) also tested the effect of textured vegetable protein on serum cholesterol levels of ten healthy men. They found that adding 26 g of textured vegetable protein per day to a mixed diet containing 16% protein had no significant effect on total plasma cholesterol. The contradictory findings of these two studies are probably due to the replacement of almost all the animal protein by vegetable protein in the first experiment, as compared to only partial replacement in the second. Sirtori and co-workers used subjects who were hypercholesterolemic and provided little dietary cholesterol, so that any dietary manipulations would likely result in more profound changes in serum cholesterol in those patients who had a genetic predisposition to high serum cholesterol levels.

Summary

Rabbits and pigeons respond to an increase in the quantity of protein in their diets by an increase in blood cholesterol levels. By contrast, rats and chickens demonstrate a hypocholesterolemic response to both the addition of protein and the amino acid methionine to their diets. In non-

human primates and man, the level of dietary protein does not seem to significantly influence blood cholesterol levels.

In rabbits, rats, chickens and humans, the substitution of plant protein for animal protein leads to a decrease in blood cholesterol levels, as compared to Cebus monkeys who respond to this change with an increase. It would seem that the human hypocholesterolemia in response to plant protein in the diet is best seen in those individuals who have aberrant cholesterol levels to begin with.

The available data (excepting humans) indicate that the role protein plays in determining blood cholesterol levels may be a relatively minor one, especially when dietary cholesterol is present.

In light of the equivocal results of experiments documented in the literature, this study with rats will determine the influence on plasma and liver cholesterol of the quality and quantity of a plant protein, as found in peanut meal, when compared to an animal protein, casein. The addition of methionine to the peanut meal to improve its protein quality will provide information as to whether the differences seen in the tissue cholesterol of rats are due to inherent differences in the two proteins, rather than some other component of the diet. The nature of a tissue cholesterol response to dietary cholesterol will determine which exerts a greater influence, dietary protein or dietary cholesterol.

CHAPTER III

MATERIALS AND METHODS

Animal Care

Male Wistar rats, weighing approximately 150 g, were obtained from Canadian Breeding Farms, Ste. Constance, Quebec, housed in wire-bottom stainless steel cages, and provided with food and water ad libitum. They were weighed weekly during the six-week experimental period, and food intake was recorded daily.

Three groups of rats were used in this diet study. The first group was fed Purina laboratory chow for a two-week period, and examination by a veterinarian from the Animal Care Centre, U.B.C. confirmed the presence of respiratory infection in these animals. Consequently, tetracycline was added to the drinking water for five days, followed by another five-day treatment with chloramphenicol. Their condition was stabilized and these animals were assigned to a diet group. As a precautionary measure, they were housed in a separate room which was maintained at about 23^{o} C. Under these conditions, lighting control was not possible.

After one to three days acclimatization with Purina laboratory chow and water, the second and third groups of rats $\sim$ 150 g were assigned to a diet group and housed in an air-conditioned room maintained at about 22^{o} C. Lighting was regulated automatically to provide alternating light (6 am to 6 pm) and dark (6 pm to 6 am) periods.

Diets

Thirteen diets were prepared. Twelve contained 10% lard and 5% corn oil, 2.2% vitamins (Vitamin Diet Fortification Mixture), and 5% minerals (Bernhardt Tomarelli Salt Mixture, modified). The protein source was either peanut meal (approximately 55% protein) or casein (approximately 90% protein). The casein diets also contained 2% alphacel (cellulose). The thirteenth diet was ground Purina laboratory chow, with a protein content of approximately 20%.

The abbreviations used to denote the diets, detailed vitamin and mineral contents of the diet, and the composition of the twelve diets are shown in Tables 3.1 to 3.6.

Tissue Collection

In the first group of animals, fed diets P2O, P2OCh, C2O, and C2OCh, six rats per diet were killed on day 42. The animals were anaesthetized with anhydrous diethyl ether and blood was collected by heart puncture using a heparinized syringe. Plasma was obtained by centrifugation, drawn off with Pasteur pipettes and stored frozen in plastic tubes, until analyzed for cholesterol content. The livers were removed immediately after the heart puncture, rinsed in distilled water, blotted dry and weighed. They were stored frozen in plastic bags until analyzed for cholesterol content.

The rats in the second group were fed the P1O, P15, C1O and C15 diets; in the third group, the P1OM, P15M, P2OM, P2OChM and LC diets. They were sacrificed according to the procedure described for the first group, with seven rats per diet.

Table 3.1

Abbreviations Used for Diets

P	:	diet made with peanut meal (protein content approximately 55%)
C	:	diet made with casein (protein content approximately 90%)
10	:	10% protein diet
15	:	15% protein diet
20	:	20% protein diet
Ch	:	cholesterol added to diet at 2% level
M	:	DL-methionine added to diet
LC	:	Purina laboratory chow

Table 3.2

Vitamins Added to Diet[1]

Vitamin[2]	Amount Added per Kg Diet
Vitamin A (200,000 U per g)	99.0 mg
Vitamin D (400,000 U per g)	5.5 mg
Alpha tocopherol	110.0 mg
Vitamin C	0.99 g
Inositol	110.0 mg
Choline Chloride	1.65 g
Menadione	49.5 mg
Para-aminobenzoic Acid	110.0 mg
Niacin	99.0 mg
Riboflavin	22.0 mg
Pyridoxine Hydrochloride	22.0 mg
Thiamine Hydrochloride	22.0 mg
Calcium Pantothenate	66.0 mg
Biotin	0.44 mg
Folic Acid	1.98 mg
Vitamin B-12	0.0297 mg

[1]Total vitamin content of diet was 2.2%.

[2]Vitamin Diet Fortification Mixture, ICN Pharmaceuticals, Cleveland, Ohio.

Table 3.3

Minerals Added to Diet[1]

Mineral[2]	Amount Added per Kg Diet
Calcium Carbonate	1.05 g
Calcium Phosphate	36.75 g
Citric Acid	113.5 mg
Cupric Citrate · $2\frac{1}{2}$ H_2O	23 mg
Ferric Citrate · 5 H_2O	279 mg
Magnesium Oxide	1.25 g
Manganese Citrate	417.5 mg
Potassium Iodide	0.50 mg
Potassium Phosphate Dibasic	4.05 g
Potassium Sulphate	3.40 g
Sodium Chloride	1.53 g
Sodium Phosphate	1.07 g
Zinc Citrate · 2 H_2O	66.5 mg

[1]Total mineral content of diet was 5.0%.

[2]Bernhardt Tomarelli Salt Mixture, modified, ICN Pharmaceuticals, Cleveland, Ohio.

Table 3.4

Composition of Peanut Meal Diets without Added Methionine

	Diet			
Composition	P10	P15	P20	P20Ch
Protein Level	10%	15	20	20
Peanut Meal	18%	27	37	37
Vitamins	2.2%	2.2	2.2	2.2
Minerals	5%	5	5	5
Lard	10%	10	10	10
Corn Oil	5%	5	5	5
Cholesterol	-	-	-	2%
Starch	59.8%	50.8	40.8	38.8

Table 3.5

Composition of Peanut Meal Diets with Added Methionine

	Diet			
Composition	P10M	P15M	P20M	P20ChM
Protein Level	10%	15	20	20
Peanut Meal	18%	27	37	37
Vitamins	2.2%	2.2	2.2	2.2
Minerals	5%	5	5	5
Lard	10%	10	10	10
Corn Oil	5%	5	5	5
L-Methionine[1]	0.22%	0.34	0.46	0.46
Cholesterol	-	-	-	2%
Starch	59.58%	50.46	40.34	38.34

[1]Added as DL-Methionine.

Table 3.6

Composition of Casein Diets

	Diet			
Composition	C10	C15	C20	C20Ch
Protein Level	10%	15	20	20
Casein	11%	17	23	23
Vitamins	2.2%	2.2	2.2	2.2
Minerals	5%	5	5	5
Lard	10%	10	10	10
Corn Oil	5%	5	5	5
Cholesterol	-	-	-	2%
Alphacel	2%	2	2	2
Starch	64.8%	58.8	52.8	50.8

Biochemical Analysis

Determination of Plasma Cholesterol:

Plasma cholesterol was determined by an enzymatic method using a single aqueous reagent, as described by Allain et al. (1974). Using this method, esters are hydrolysed to free cholesterol by cholesterol ester hydrolase. The free cholesterol is oxidised by cholesterol oxidase to cholest-4-ene-3-one with the simultaneous production of hydrogen peroxide. The latter oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromagen with a maximum absorption at 500 nm.

In the assay for total plasma cholesterol, 30 µl of plasma was incubated with 3.0 ml of enzyme reagent (described in Table 3.7) for 10 minutes at 37^{o} C and the absorbance at 500 nm was measured against a reagent blank (30 µl water plus 3.0 ml enzyme reagent). Concentrations of unknown samples were determined from a linear regression curve constructed by using cholesterol (BDH (Canada) Ltd., Toronto) standards in isopropanol, in concentrations from 50 mg% to 500 mg%. 30 µl of each standard was used.

Determination of Liver Cholesterol:

Carlson and Goldfarb (1977) adapted the enzymatic method of Allain et al. (1974) for the analysis of total liver cholesterol. They found that the addition of a detergent, Leconal Wetting Agent, (Leco Corp., St. Joseph, Mich.) was necessary for use with lipid extracts of the liver samples.

In this assay, samples of liver (0.2 g ± .01 g) were hydrolysed for one hour at 75^{o} C in capped tubes containing 1 ml of a KOH solution (KOH/H_2O, 1:2 w/v). The tubes were cooled by refrigeration, generally overnight.

Table 3.7

Enzyme Reagent for Determination of Tissue Cholesterol

sodium cholate[1]	3 mmol/liter
4-aminoantipyrine[1]	0.82
phenol[2]	14
Na_2HPO_4[1]	50
NaH_2PO_4[1]	50
carbowax-6000[1]	0.17
cholesterol ester hydrolase[3] (E.C.3.1.1.13)	33 U
cholesterol oxidase[3] (E.C.1.1.3.6)	117 U
peroxidase[1] (E.C.1.11.1.7)	67000 U

[1]Sigma Chemical Company, St. Louis, Mo.

[2]Fisher Scientific Company, Vancouver, B.C.

[3]ICN Pharmaceuticals, Cleveland, Ohio

After the addition of 1.5 ml 95% ethanol and 3 ml petroleum ether (b.p. 30 - 60^{o} C), the tubes were tightly capped, vortexed for one minute, and centrifuged at low speed for 5 minutes to separate the phases. Triplicate aliquots (50 µl) of the petroleum ether phase were removed and allowed to evaporate in the air. Isopropanol (5 µl), enzyme reagent (500 µl) and leconal wetting agent (5 µl) were added to the dried aliquots. The samples were vortexed and incubated for 15 minutes at 37^{o} C. Colour development was measured at 500 nm against a reagent blank (5 µl H_2O plus 5 µl leconal wetting agent plus 500 µl enzyme reagent). Cholesterol standards were prepared in isopropanol in concentrations from 1 mg/ml to 5 mg/ml, and 5 µl of each standard was used. Concentrations of the unknown samples were determined from a linear regression curve constructed from these standard values.

Statistical Analysis

The data collected in this study were statistically analyzed using the Dalhousie University computer facilities and the Statistical Package for the Social Sciences, version 7.0, as described by Nie et al. (1975). The procedures used were Student's t, analysis of variance, Pearson product moment correlation, and a contrast test using least-significant difference. Student's t is the statistic used to test whether or not the difference between two sample means is significant. Analysis of variance is used to detect a difference in a set of more than two means. It is able to cope with a nonorthogonal design, such as this study is, i.e. one with unequal cell frequencies. Pearson product moment correlation is a measure of linear correlation for pairs of variables and the

coefficient r measures the strength of the relationship between the variables. A contrast test is a means of comparing all possible pairs of group means. The groups are ranked in homogeneous subsets, in which the means of the first and last groups do not differ by more than a critical value for a subset of that size. The least-significant difference procedure was used for the contrast test, with a significance level of $p = 0.01$.

CHAPTER IV

RESULTS

The tables and figures used in this chapter present data collected from the animals, described in Materials and Methods, in order to determine the effect of protein quality and quantity, and dietary cholesterol on plasma and liver cholesterol levels. Unless stated otherwise, the significance level used throughout was $p = 0.01$.

Food Consumption

The rats used in these experiments were fed their respective diets for a six-week period. Average daily food intake of the diet groups ranged from 15.34 g (P10M) to 23.34 g (LC), as shown in Table 4.1. Those animals fed the 10% peanut protein diets ate the least amount of food, and while this may be explained on the basis of a poorer quality protein such as peanut protein, one might predict that a similarly low food intake would also be recorded for rats fed the C10 diet. This was not the case, as the daily food intake for the animals fed the 10% casein diet was higher, although not significantly so, than for those animals fed the C15 and C20 diets ($F = 3.2062$, $p = .0658$). Apart from the average daily food intake of the P10 and P10M diet groups, the food intake of all other diet groups compared favourably with food consumption data from rats fed 20% casein diets (Sugano et al., 1978). These animals ate 18.5 - 21.3 g/day.

Those animals fed the P20M diet ate significantly more than those fed the P20 diet ($t = 3.21$, $p = .008$). However, a significant difference did not

occur between the groups fed P10 and P10M diets ($t = .22$, $p = .830$), and P15 and P15M diets ($t = 1.74$, $p = .107$).

The effect of dietary cholesterol on food intake was significant only when the daily consumption of rats fed C20 and C20Ch diets was compared ($t = 4.19$, $p = .002$), in contrast to those animals fed the P20 and P20Ch diets ($t = 2.03$, $p = .069$), and the P20M and P20ChM diets ($t = 1.54$, $p = .150$).

Although the contrast test (Table 4.1) and analysis of variance ($F = 10.0656$, $p < .001$) reveal significant differences among the food intakes of the diet groups, differences in plasma and liver cholesterol cannot be attributed to differences in food consumption. Pearson correlation indicates no significant correlation between these variables and food intake, when the peanut protein and casein diets are considered separately or together (Table 4.2).

Body Weights and Weight Gain

The data in Table 4.3 show the initial mean weight of rats within each diet group, weight gained during the experimental period, and standardized weight gain (weight gain as a function of initial weight). The latter was used to compensate for differences in initial weights and to represent weight gain with a kind of factor system. For example, a standardized weight gain of 0.5 would indicate that the diet group gained half its initial weight whereas a value of 2 means the group gained double its initial weight.

The largest weight gain was recorded for those animals fed the P20ChM diet, and the smallest for those fed the P10 diet. Whether the protein was from

Table 4.1

Average Daily Food Intake

Diet Group	n	Average Daily Food Intake (g)
P10	7	15.58 ± 2.02 [a] [1,2]
P10M	7	15.34 ± 2.11 [a]
P15	7	18.48 ± 2.33 [b]
P15M	7	18.80 ± 2.32 [b]
P20	6	17.58 ± .41 [ab]
P20M	7	19.77 ± 1.61 [c]
P20Ch	6	18.53 ± 1.06 [b]
P20ChM	7	21.41 ± 2.31 [cd]
C10	7	19.27 ± 1.19 [bc]
C15	7	18.77 ± 1.90 [b]
C20	6	17.43 ± .13 [ab]
C20Ch	6	17.93 ± .25 [ab]
LC	7	23.34 ± 2.28 [d]

[1]Value represents mean ± standard deviation.

[2]Values not sharing a common superscript letter are significantly different ($p \leq 0.01$) by contrast testing, using least-significant difference.

Table 4.2

Pearson Correlation Coefficients and Probability Levels for Tissue Cholesterol Levels and Average Daily Food Intake

	Average Daily Food Intake		
	Peanut Protein Diets	Casein Diets	Peanut Protein and Casein Diets
Plasma Cholesterol	r = .2047 p = .069 n = 54	r = .0913 p = .329 n = 26	r = .1364 p = .114 n = 80
Liver Cholesterol	r = .2729 p = .023 n = 54	r = -.1999 p = .164 n = 26	r = .1350 p = .116 n = 80

Table 4.3

Initial Weights, Weight Gain and
Standardized Weight Gain of Diet Groups

Diet Group	n	Initial Weight (g)	Weight Gain (g)	Standardized Weight Gain
P10	7	159.14 ± 4.18 cde [1,2]	85.85 ± 14.08[1]	0.5508 ± .079[1,3]
P10M	7	166.42 ± 9.07 e	95.85 ± 36.75	0.5821 ± .244
P15	7	155.71 ± 5.93 cde	174.71 ± 44.07	1.1284 ± .317
P15M	7	167.00 ±12.85 e	188.71 ± 39.73	1.1338 ± .245
P20	6	129.16 ±10.22 a	221.83 ± 29.82	1.7235 ± .237
P20M	7	148.57 ±13.95 bcd	249.85 ± 31.00	1.6925 ± .242
P20Ch	6	142.66 ± 9.28 b	205.83 ± 24.77	1.4390 ± .091
P20ChM	7	147.42 ± 7.48 bc	302.28 ± 43.50	2.0439 ± .217
C10	7	160.71 ± 4.78 de	159.85 ± 23.24	0.9985 ± .173
C15	7	159.85 ± 7.10 de	210.42 ± 31.16	1.3157 ± .183
C20	6	139.00 ±10.25 ab	246.16 ± 14.77	1.7758 ± .123
C20Ch	6	150.00 ± 7.51 bcd	224.66 ± 15.75	1.5022 ± .149
LC	7	149.71 ± 7.88 bcd	240.00 ± 48.16	1.6168 ± .390
Initial	9	159.77 ± 7.44 cd	-	-

[1]Value represents mean ± standard deviation.

[2]Values not showing a common superscript letter are significantly different (p = .01) by contrast testing, using least-significant difference.

[3]Standardized weight gain represents weight gain as a function of initial weight. See text for further explanation.

an animal source or vegetable source, weight gain was directly proportional to the amount of protein in the diet. Pearson correlation analysis shows a strong positive correlation ($p < 0.001$) between weight gain and peanut protein, peanut protein with methionine, and casein diets with correlation coefficients of 0.8693, 0.8786, and 0.8369, respectively.

Those rats fed casein diets gained more weight than their peanut protein-fed counterparts, but this difference was only significant at the 10% protein level (Table 4.4). This is also illustrated in Figure 4.1, using level of protein on the y-axis and standardized weight gain on the x-axis.

Methionine was added to the P10, P15 and P20 diets to increase their methionine content to the same level as is found in a casein diet of equivalent protein content, i.e. to improve the quality of the peanut protein. Although methionine appeared to enhance the weight gain of those rats fed the peanut protein diets containing additional methionine, the differences in weight gain are not significant for those animals fed peanut protein diets with and without methionine, at all three protein levels (Table 4.4).

Dietary cholesterol also had no significant effect on weight gain, although the rats fed peanut protein and casein diets with cholesterol added tended to gain less weight than those fed equivalent diets without cholesterol. Dietary cholesterol and methionine did not influence weight gain when considered separately, but when added to the same diet (P20ChM), weight gain for animals fed this diet was significantly greater than for animals fed the P20Ch ($t = 4.79$, $p = .001$) and C20Ch diets ($t = 4.12$, $p = .002$).

Table 4.4

Statistical Analysis of
Standardized Weight Gain of Groups Fed Dietary Protein at Three Levels

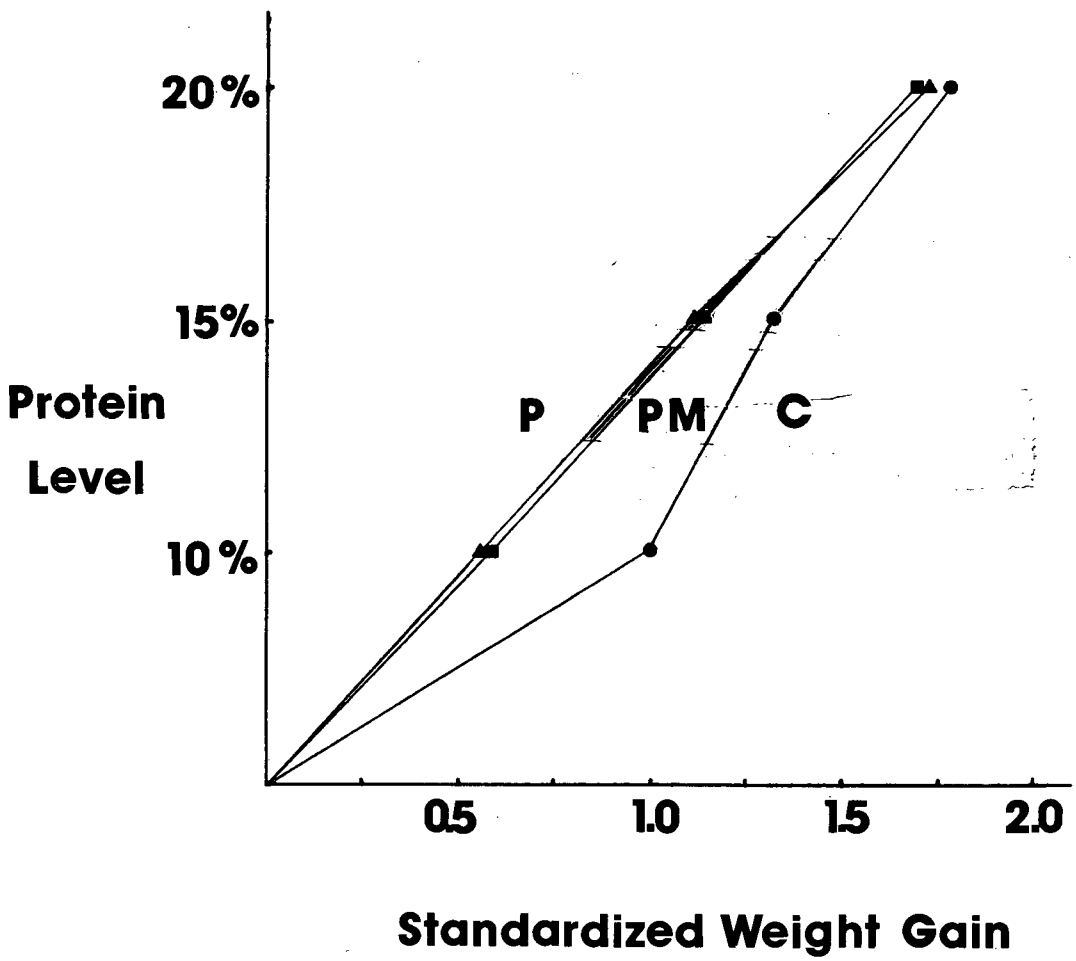
Standardized Weight Gain	Dietary Protein			
	Peanut Protein	Peanut Protein with Methionine	Casein	
at 10% level of dietary protein	.5508 ± .079 [1]	.5821 ± .244	.9985 ± .173	F ratio = 13.6907[2] p = .0002
	t = .32 [3]	p = .752		
at 15% level of dietary protein	1.1284 ± .317	1.1338 ± .245	1.3157 ± .183	F ratio = 1.2278 p = .3163
	t = .04	p = .972		
at 20% level of dietary protein	1.7235 ± .237	1.6925 ± .242	1.7758 ± .123	F ratio = .2549 p = .7781
	t = .23	p = .821		

[1] Value represents mean ± standard deviation.

[2] F ratio and probability level for analysis of variance.

[3] t value and probability level for t-test between P & PM diets.

Figure 4.1. Standardized weight gain at three protein levels


20%
15%
10%
Protein
Level
P
PM
C
0.5
1.0
1.5
2.0
Standardized Weight Gain

Quality of Dietary Protein and Methionine Supplementation

The effect of protein quality on tissue cholesterol levels is presented in Table 4.5. Only at the 15% protein level was there a significant difference among the plasma cholesterol levels of the rats fed the three proteins. This was probably due to the anomalous plasma cholesterol value from animals fed the P15M diet.

The addition of methionine to the peanut protein diets resulted in an increase in plasma cholesterol at all three levels of protein. Although this was only significant at the 15% level of protein, it points out a trend related to methionine supplementation of the diet.

Protein quality had no influence on liver cholesterol values at the 10% protein level, but, at both the 15% and 20% protein levels, the better the quality of the protein, the higher the liver cholesterol level (Table 4.6).

The group of animals fed the LC diet had plasma and liver cholesterol levels which were very similar to initial values (Tables 4.5 and 4.6). This group was included in this study to eliminate the effect of time on tissue cholesterol levels. Since no significant effect was evident, the changes in both plasma and liver cholesterol occurring in the tissues of the rats fed the other diets cannot be accounted for on the basis of an increase or decrease over the time period used in this study.

Table 4.5

Effect of Protein Quality and Methionine Supplementation on Plasma Cholesterol Levels

Diet Group	n	Plasma Cholesterol (mg%)	
P10	7	113.98 ± 10.71[1]	
P10M	7	117.13 ± 19.68	F ratio = 3.5443[2]
C10	7	84.28 ± 37.99	p = .0504
P15	7	109.49 ± 4.84	
P15M	7	134.21 ± 25.45	F ratio = 6.4808
C15	7	96.48 ± 22.79	p = .0076
P20	6	102.51 ± 8.10	
P20M	7	116.90 ± 18.67	F ratio = 1.5600
C20	6	107.69 ± 15.16	p = .2405
LC	7	80.42 ± 7.54	t = .80[3]
Initial	9	77.87 ± 5.26	p = .438

[1] Value represents mean ± standard deviation.

[2] F ratio and probability level for analysis of variance.

[3] t value and probability level for t-test.

Table 4.6

Effect of Protein Quality and Methionine Supplementation on Liver Cholesterol Levels

Diet Group	n	Liver Cholesterol (mg/g liver wt)	
P10	7	6.60 ± 2.14[1]	
P10M	7	8.67 ± 3.49	F ratio = .3216[2]
C10	7	7.49 ± 7.32	p = .7290
P15	7	3.81 ± 2.14	
P15M	7	6.59 ± 1.37	F ratio = 5.9992
C15	7	14.21 ± 9.74	p = .0101
P20	6	7.16 ± 3.83	
P20M	7	10.61 ± 2.94	F ratio = 5.5318
C20	6	14.91 ± 5.22	p = .0149
LC	7	5.12 ± 2.26	t = 2.56[3]
Initial	9	7.51 ± 1.47	p = .023

[1]Value represents mean ± standard deviation.
[2]F ratio and probability level for oneway analysis of variance.
[3]t value and probability level for t-test.

Quantity of Dietary Protein

The effect of protein quantity on plasma cholesterol is presented in Table 4.7. Although there were no significant differences in plasma cholesterol within each group, two opposing trends can be seen. As the amount of peanut protein increased, there was a corresponding decrease in plasma cholesterol. By contrast, as casein levels increased, so did the plasma cholesterol level. These trends can also be seen graphically in Figure 4.2. It is interesting to note that, in spite of these opposing trends, plasma cholesterol values for animals fed the 20% protein diets were very similar ($t = .77$, $p > .40$). Pearson correlation analysis (Table 4.8) shows a strong negative correlation between plasma cholesterol and peanut protein and a somewhat weaker positive correlation between plasma cholesterol and casein.

The amount of protein in the diet had no significant effect on liver cholesterol (Table 4.9). Although no particular trend was apparent in the liver cholesterol levels of animals fed the peanut protein diets, analysis revealed a moderately strong positive correlation between casein in the diet and liver cholesterol (Table 4.8).

Dietary Cholesterol

The addition of cholesterol to each of the protein diets, had no effect on plasma cholesterol (Table 4.10). Dietary methionine appeared to moderate the effect of dietary cholesterol since the plasma level of the animals fed the P2OChM diet tended to be lower than those of animals fed the P2OCh and C2OCh diets.

Table 4.7

Effect of Protein Quantity on Plasma Cholesterol Levels

Diet Group	n	Plasma Cholesterol (mg%)	
P10	7	113.98 ± 10.71[1]	
P15	7	109.49 ± 4.84	F ratio = .1029[2]
P20	6	102.51 ± 8.10	p = .7523
C10	7	84.28 ± 37.99	
C15	7	96.48 ± 22.79	F ratio = .0014
C20	6	107.69 ± 15.16	p = .9702
Initial	9	77.87 ± 5.26	

[1]Value represents mean ± standard deviation.
[2]F ratio and probability level for analysis of variance.

Figure 4.2. Relationship between protein and plasma cholesterol levels for peanut protein and casein diets

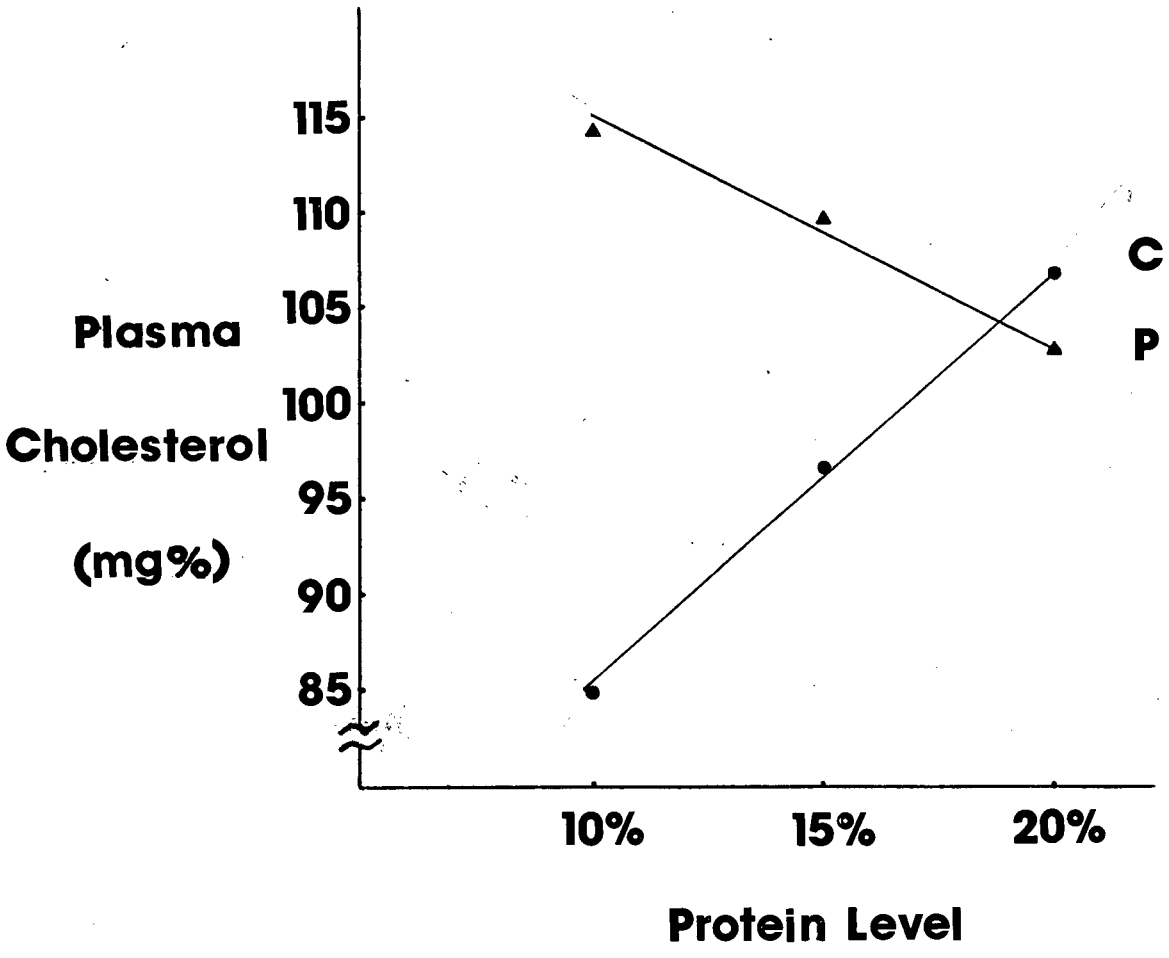

Plasma
Cholesterol
(mg%)
115
110
105
100
95
90
85
C
P
10%
15%
20%
Protein Level

Table 4.8

Pearson Correlation Coefficients and Probability Levels for Plasma Cholesterol, Liver Cholesterol and Dietary Protein

	Dietary Protein	
	P	C
Plasma Cholesterol	r = -.5153 p = .010 n = 20	r = .3476 p = .067 n = 20
Liver Cholesterol	r = .0543 p = .410 n = 20	r = .3854 p = .047 n = 20

Table 4.9

Effect of Protein Quantity on Liver Cholesterol Levels

Diet Group	n	Liver Cholesterol (mg/g liver weight)	
P10	7	6.60 ± 2.14[1]	
P15	7	3.81 ± 2.14	F ratio = 5.6269[2]
P20	6	7.16 ± 3.88	p = .0298
C10	7	7.49 ± 7.32	
C15	7	14.21 ± 9.74	F ratio = 1.8822
C20	6	14.91 ± 5.22	p = .1826
Initial	9	7.51 ± 1.47	

[1]Value represents mean ± standard deviation.
[2]F ratio and probability level for oneway analysis of variance.

Table 4.10

Effect of Dietary Cholesterol on Plasma Cholesterol Levels

Diet Group	n	Plasma Cholesterol (mg%)	
P2O	6	102.51 ± 8.10[1]	t = 1.95[2]
P2OCh	7	116.67 ±15.87	p = .080
P2OM	7	116.90 ±18.67	t = .78
P2OChM	7	109.06 ±18.84	p = .450
C2O	6	107.67 ±15.16	t = 2.22
C2OCh	7	124.14 ± 9.98	p = .051
Initial	9	77.87 ± 5.26	

[1]Value represents mean ± standard deviation.
[2]t value and probability level for t-test.

In contrast to the lack of difference between plasma cholesterol levels of rats in groups fed diets with and without dietary cholesterol, a highly significant difference could be seen between the liver cholesterol values of these three pairs of diet groups (Table 4.11). The change in liver cholesterol accumulation when cholesterol was added to the P20, P20M, and C20 diets ranged from about a four- to ten-fold increase, with the greater accumulation being found in the livers of those rats fed the casein diet. As in the plasma, dietary methionine appeared to moderate this effect since animals fed this diet had liver cholesterol values that were lower than the other diets to which cholesterol was also added.

Table 4.11

Effect of Dietary Cholesterol on Liver Cholesterol Levels

Diet Group	n	Liver Cholesterol (mg/g liver weight)	
P2O	6	7.16 ± 3.83[1]	t = 8.30[2]
P2OCh	7	76.01 ±19.94	p < 0.0001
P2OM	7	10.61 ± 2.94	t = 6.06
P2OMCh	7	45.97 ±15.16	p < 0.0001
C2O	6	14.91 ± 5.22	t = 15.20
C2OCh	7	101.11 ±12.87	p < 0.0001
Initial	9	7.51 ± 1.47	

[1]Value represents mean ± standard deviation.
[2]t value and probability level for t-test.

CHAPTER V

DISCUSSION

Protein Quality and Dietary Methionine

The first objective of this study was to determine the effect of the quality of dietary protein on plasma and liver cholesterol levels. The results obtained fail to show any particular effect of protein related to plasma cholesterol, although the literature has suggested on one hand that hypercholesterolemia is a consequence of feeding rats a plant protein and that lower plasma cholesterol levels can be achieved by using an animal protein such as casein. On the other hand, it has been found that a plant protein or an amino acid mixture equivalent to it was responsible for lower plasma cholesterol levels than casein or an amino acid mixture equivalent to it. It does not seem, on the basis of the present evidence, that differences in amino acid profiles are reflected in differences in plasma cholesterol levels.

Altering the quality of a protein by adding a limiting amino acid, in this case methionine, was carried out on the suggestion that relative amounts of methionine will influence plasma cholesterol levels. Only at the 15% level of protein did the addition of methionine to the peanut protein diet significantly change the plasma cholesterol of rats fed this diet. However, the increase observed was opposite to a predicted hypocholesterolemic response expected from improvement in protein quality by the addition of methionine. Plasma cholesterol levels of rats have been shown to be fairly resistant to change and the dietary conditions imposed with regard to protein quality may bear no relation to the

factors that will influence cholesterol levels, despite previous studies that suggest otherwise.

The effect of protein quality on liver cholesterol levels has also been related to the methionine content of the diet. The present data show that improving the methionine content either by the addition of dietary methionine as the free amino acid or substituting a protein with a lower methionine content for one with a higher content, eg. casein, led to increased liver cholesterol accumulation at the 15% and 20% protein levels.

The literature provides no explanation for this observation primarily because the influences on plasma cholesterol have received the greater attention. It may be that improving the quality of a dietary protein stimulates an overall growth or anabolic response which has been measured in the liver as increased endogenous cholesterol synthesis. It would be interesting to determine if this is indeed the case, perhaps by measuring labelled acetate incorporation into cholesterol.

There seems to be no consistent relationship between plasma and liver cholesterol levels according to the present study. Such a relationship would be anticipated if the relative rates of choline and taurine synthesis were dependent upon protein quality and the availability of methionine, as has been suggested. If methionine were in short supply, it would tend to produce an increase in tissue cholesterol accumulation since the synthesis of choline and taurine was being inhibited. Conversely, with methionine readily available, plasma and liver cholesterol

levels would not increase since lipoproteins would presumably be made to remove cholesterol from the liver to the blood and enough bile acids would be available to remove excess cholesterol from general circulation, maintaining a sufficient rate of cholesterol turnover to prevent a "back-up" of cholesterol in the blood. The present results lend no support to these suggestions and relegate the effect of protein whether from an animal or plant source and dietary methionine on tissue cholesterol levels to minor positions.

Protein Quantity

The second objective was to study the effect of the quantity of dietary protein on plasma and liver cholesterol levels. A number of studies have indicated that increasing the amount of protein in the diet decreased plasma cholesterol levels, whether the additional protein was from an animal or vegetable source. Others have found that increases in dietary protein were paralleled by increases in plasma cholesterol. In this study, the plasma cholesterol levels of rats were fairly resistant to change when the factor being varied in their diets was the amount of protein. The animals fed the peanut protein diet had plasma cholesterol levels that showed a downward trend as the amount of protein increased, while those fed casein diets had plasma cholesterol levels that demonstrated an upward trend. However, within each diet group, differences among the plasma cholesterol levels were not significant.

It is interesting to note that the direction of the trend as the amount of peanut protein increases supports the premise that protein has a

protective effect against hypercholesterolemia. By contrast, the direction of the trend as the amount of casein in the diet increases tends to support the opposite view that plasma cholesterol is directly proportional to the amount of protein in the diet. Overall, the results agree with the work of Méndez (1964) who observed no significant difference in plasma cholesterol as a result of feeding diets with different levels of protein.

Best et al. (1936) reported that the addition of casein to an 8% casein diet resulted in a decrease in liver cholesterol accumulation, indicating that an improvement in the quantity of protein would be followed by a decline in liver cholesterol. Why this occurred was again thought to be related to the amount of methionine available. The data on the effect of protein on liver cholesterol levels provided by this study do not support this suggestion, since liver cholesterol was not significantly different at each level for the two proteins examined. With increases in the amount of the better quality protein, casein, the liver cholesterol tended to be higher, not lower as suggested by Best et al. (1936). The accumulation of cholesterol in the liver may be a general growth response to casein similar to the one suggested for the effect of protein quality on liver cholesterol. However, this does not account for the relative stability of the liver cholesterol levels of the rats fed the three levels of peanut protein.

Dietary Cholesterol

The third objective of this study was to evaluate the effect of dietary cholesterol on tissue cholesterol levels. The addition of cholesterol to

the diet has been reported to lead to an increase in both plasma and liver cholesterol (Méndez, 1964). In the present study, the feeding of diets with and without cholesterol did not result in significant differences in plasma cholesterol although the increase over initial plasma values was significant.

When the protein source was either peanut protein or casein, the animals fed the diets containing cholesterol tended to have higher plasma cholesterol levels. However, the addition of both cholesterol and methionine to the peanut protein diet produced the opposite effect. This suggests that methionine was exerting a moderating influence, albeit a mild one, on plasma cholesterol levels in the presence of dietary cholesterol. This is an effect it did not exert by itself, since plasma cholesterol values of rats fed the P2OM diet were higher than the levels of those fed the P2OChM diet.

The accumulation of cholesterol in the liver was significantly greater when cholesterol was added to the diet, and the effect of cholesterol was more pronounced with the better quality protein. As in the plasma, the effect of methionine added to a diet also containing cholesterol was to moderate the deposition of cholesterol in the liver. There is about a four-fold increase in liver cholesterol accumulation for rats in the P2OChM group, in contrast to about a ten-fold increase in the P2OCh diet group and about a seven-fold increase in the C2OCh diet group.

Frantz et al. (1954), Morris and Chaikoff (1959) and Méndez (1964) have also reported increases in liver cholesterol subsequent to the

addition of dietary cholesterol. It may be that the metabolism of a rat is not equipped to handle the influx of exogenous cholesterol because of a relative shortage of lipoproteins or inadequate catabolism and excretion via the bile. It may also be a transitory situation that eventually returns toward normal with perhaps a compensating reduction in endogenous cholesterol synthesis. It would be interesting to investigate this possibility further, since others have found that dietary cholesterol inhibited the synthesis of cholesterol from acetate (Siperstein and Guest, 1960).

In evaluating the relative influences of dietary protein and cholesterol on tissue cholesterol levels, the effects of either animal or vegetable protein and dietary methionine on tissue cholesterol and dietary cholesterol on plasma cholesterol are relatively minor compared to the effect of dietary cholesterol on liver cholesterol accumulation. Although the use of the lab chow diet ruled out the probability that the increases in cholesterol observed were only due to time and the aging of the rat, it does not eliminate the possibility that an extended experimental period would reveal significant differences between the proteins with respect to quality and quantity, or reduce the significance of the liver cholesterol accumulation. It is a frequent criticism and a justifiable one in many cases that short-term dietary studies are just that, and do not allow for sample collections that might more accurately reflect adaptations to dietary manipulations.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In light of the current concern about the risk that high serum cholesterol levels present for the development of atherosclerosis in humans, this study was undertaken to determine the role dietary protein quality and quantity and cholesterol may play in modifying both plasma and liver cholesterol levels in rats.

Male Wistar rats, weighing approximately 150 g were divided into groups of 6 or 7 and fed diets containing protein at levels of 10%, 15%, and 20%. The sources of protein were peanut protein (P), peanut protein with methionine (PM), casein (C) and lab chow (LC). Methionine was added to the peanut protein to improve its quality, and the amount added brought the concentration of this amino acid to the same level as a casein diet of equivalent protein content. Cholesterol was also added at the 20% protein level to the P, PM, and C diets. Thus the thirteen diets used in this study were: P10, P10M, P15, P15M, P20, P20M, P20Ch, P20ChM, C10, C15, C20, C20Ch and LC. The last diet was fed to determine the effects of time and aging on the tissue cholesterol levels of the rat.

Food consumption was recorded daily and body weights weekly. Plasma and liver samples were collected at the end of six weeks and analyzed enzymatically for cholesterol.

Plasma cholesterol was unaffected by protein quality and only at the 15% and 20% protein levels did liver cholesterol increase with improvements

in protein quality. The amount of protein in the diet, whether casein or peanut protein, did not significantly influence plasma and liver cholesterol levels, although Pearson correlation analysis revealed a negative correlation between plasma cholesterol and peanut protein, and a positive correlation between plasma and liver cholesterol and casein. Time does not appear to influence tissue cholesterol levels and the effect of protein seems to be secondary to the effect of additional dietary cholesterol. A highly significant increase in liver cholesterol accumulation resulted from cholesterol supplementation of the diet, although this effect could be moderated by additional dietary methionine.

Since coronary heart disease is most likely caused by a number of factors working together, it is both narrow and simplistic to look at just a few components of the diet in isolation. Nonetheless, under the conditions of this experiment it does seem that protein quality and quantity are not influences of primary importance on tissue cholesterol levels, and that the influence of dietary cholesterol is limited to its effect on liver cholesterol accumulation.

Although the results of this research do not support earlier experiments that showed a distinct effect of protein on rat tissue cholesterol levels, they do point out the need for further investigation into the mechanisms by which cholesterol metabolism is regulated. It would be instructive to know the effect of protein and dietary cholesterol on endogenous cholesterol synthesis, whether changes in the amounts of these two components of the diet influence the turnover of cholesterol and bile salts, and whether the catabolism of cholesterol is enhanced in rats fed peanut

protein diets. The fibre content may be interfering with the enterohepatic circulation of bile acids since the peanut protein was in the form of a meal rather than a purified protein.

The implications for humans cannot be drawn directly from this study but in light of the results from the LC diet group, it seems that components of this non-purified diet other than protein, fat and cholesterol are responsible for the lack of difference between the plasma and liver cholesterol values of this group and the initial tissue values. Perhaps research in the future will focus on these other components as a means of discovering a cure for atherosclerosis.

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