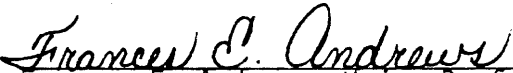


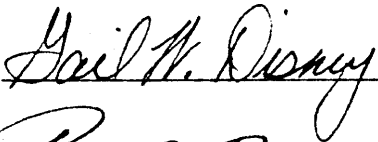
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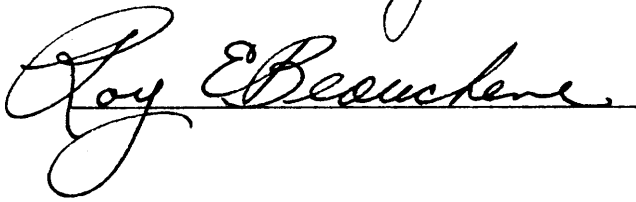
I am submitting herewith a thesis written by Gary Wayne Young entitled "The Influence of Age, Dietary Protein, and Calorie Restriction on Serum Cholesterol and Triglycerides in the Male Rat." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.



Frances E. Andrews, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

THE INFLUENCE OF AGE, DIETARY PROTEIN, AND CALORIE
RESTRICTION ON SERUM CHOLESTEROL AND
TRIGLYCERIDES IN THE MALE RAT

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Gary Wayne Young

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ABSTRACT

The effects of age, dietary protein, and caloric restriction on serum cholesterol and triglycerides were studied in 1 and 2 year old male Wistar rats fed ad libitum or restricted. Ad libitum animals were fed diets low (12%), medium (20%), or high (28%) in casein throughout the life cycle. Restricted animals were fed diets either low (18%), medium (30%), or high (42%) in casein. The latter groups were fed the same absolute amounts of protein but two-thirds of the calories as determined from the feed intakes of the ad libitum-fed rats. Two additional ad libitum-fed groups were fed diets increased or decreased in protein. After the second month of life animals fed variable amounts of protein were fed a 20% casein diet which was either increased or decreased at the rate of 0.8% per month until a level of 28 or 12% casein was obtained.

Age had an effect on both serum cholesterol and triglycerides. The young animals had a significantly ($p < 0.05$) lower mean serum cholesterol level than the old animals whereas the old animals had a lower ($p < 0.01$) mean serum triglyceride level than their younger counterparts.

The various levels of dietary protein (low, medium, and high) resulted in no significant differences in either serum cholesterol or triglycerides. The overall effect of protein on serum cholesterol was quadratic ($p < 0.01$) but not linear. The overall effect of protein on triglycerides was neither linear nor quadratic, but the interaction of protein and calories on triglycerides was linear by group ($p < 0.05$) and quadratic by group ($p < 0.05$).

Combined dietary treatments, regardless of age, significantly ($p < 0.01$) affected both serum cholesterol and triglycerides. When all ad libitum-fed versus restricted-fed animals were contrasted, both parameters were significantly ($p < 0.01$) lower for restricted animals. Variation in the ad libitum level (20→12% casein and 20→28% casein) had no effect on the serum cholesterol or the triglyceride level. In general, the significantly lower serum lipids recorded can be attributed to calorie restriction.

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CHAPTER I

INTRODUCTION

Increasing the length and quality of life is a basic goal shared by nutritionists and other health professionals. The main approach is the prevention or eradication of the diseases responsible for premature death. Diseases of the heart and arteries are the leading cause of death in the United States. Epidemiological data show a strong positive association between coronary vascular disease and diet. Hyperlipidemia, or more specifically hypercholesterolemia and hypertriglyceridemia are the principal indicators of a susceptibility for atherosclerotic coronary heart disease (1-5). As a result, various attempts have been implemented to control blood lipid levels through dietary modifications.

Most attempts to regulate blood lipids have concentrated on the fat content of the diet. Recent advances have shown that other dietary constituents such as protein can also contribute to blood lipid levels. It is well documented that Americans consume levels of protein far above their requirements (4). Furthermore, the trend toward increasing mortality from coronary heart disease in the United States during this century coincides with a doubling in the ratio of animal to vegetable protein in the diet (6).

The restriction of calories has been shown to be an effective means of lowering blood lipid levels. Calorie restriction in rats has delayed the onset of the age-associated rise in serum cholesterol and triglycerides (7). Although the data from animal studies cannot

be freely extrapolated to humans, the implications that caloric restriction presents should not be overlooked.

The main emphasis of this study was to investigate the effects of protein and calorie restriction and age on serum cholesterol and triglycerides in rats. Paired groups of animals were fed the same absolute amounts of protein. One group was fed ad libitum a lower percentage of protein, whereas its respective paired group was fed a higher percentage of protein but caloric intake was restricted to two-thirds of that consumed by the ad libitum-fed group. The effect of either increasing or decreasing the amount of protein fed during the period of growth and development was also studied to determine if this could alter the serum lipids.

CHAPTER II

REVIEW OF LITERATURE

I. FACTORS AFFECTING SERUM TRIGLYCERIDES

A. Effects of Aging

It is important to be aware that serum lipid levels of rats have been shown to be a function of the strain used. The large number of breeds, stocks, substocks, and strains within a species such as the rat complicate the establishment of normal lipid values. Physiological variations among different stocks of rats make it necessary to stress the importance of accurately identifying and properly defining the rat stocks used (8). In a comparison study using BN, DA, Lewis, and Wistar rats, Kritchevsky and Tepper (9) reported significantly different mean serum cholesterol levels for each group among the different strains. It has been shown that not all rat strains display an increase in the blood lipids as the animal ages. Using Fisher 344 rats, Story and co-workers (10) reported that this strain did not display the age-associated increase in blood lipid levels.

During the aging of the rat pronounced biochemical changes occur resulting in the alteration of serum lipid levels. In the last decade much information has been reported about the synthesis, transport, and catabolism of blood lipids. Unfortunately the primary regulatory mechanism of lipid metabolism is still unknown. An investigation of the circulating level of triglycerides should include the dietary as well as endogenous sources, and lipoprotein formation and catabolism.

The concentration of each lipoprotein series in the circulation is the result of a balance between the rates of synthesis and degradation. Since each lipoprotein is a complex macromolecule consisting of several lipid classes and one or more apolipoproteins, the mechanisms involved in metabolism are complex and incompletely understood (11).

Triglyceride formation and catabolism in rats has been extensively reviewed. Most literature suggests that aging is characterized by a reduced ability to clear lipoproteins from the plasma resulting in a rise in plasma triglyceride levels (12, 13). In a study by Jamdar et al. (14), it was demonstrated that hepatic triglyceride formation was most active 24 hours after birth. By the 10th postnatal day, values decreased to adult levels and remained constant thereafter. Jamdar et al. concluded from this study that hepatic triglyceride formation increased dramatically after birth due to increased synthesis of esterifying enzymes. The activity of these enzymes was lower in adult rats. Carlson and co-workers (15) demonstrated with rats that plasma triglyceride levels rose slightly in the first 4 months after birth and then increased sharply until the 9th month when levels stabilized. One factor of great importance in hepatic formation of plasma triglycerides is the rate of mobilization of free fatty acids (FFA) from adipose tissue. In the Carlson et al. study (15), the plasma level of FFA and free glycerol from adipose tissue did not increase with age. It is likely that the reason for the increase in plasma triglycerides with age is not an increase in lipoprotein formation, but rather a decrease in the efficiency of the mechanisms responsible for removing triglyceride rich lipoproteins from the

circulatory system. Lipoprotein lipase is the enzyme system believed to facilitate removal of triglycerides from the blood. It is conceivable that a low activity of this enzyme could cause an elevation in the level of plasma triglycerides. A study by Benjamin et al. (16) substantiated the claim of Carlson et al. (15) that elevated plasma triglycerides are probably the result of inadequate clearing from the blood rather than over synthesis by the liver. Benjamin et al. (16) noted that with aging there is a depression of both de novo fat synthesis and the synthesis of fatty acid glycerides from preformed fatty acids. These authors reported that adipose tissue of 2 year old rats was characterized by a marked decrease in lipid synthesis from acetate and a lower rate of palmitic acid incorporation into mixed lipids as compared to 1 month old animals.

B. Effects of Dietary Protein

The effect of various levels of dietary protein on serum triglycerides has been studied in both humans and experimental animals. The first clear demonstration of the role of diet in atherosclerosis was recorded in 1909 by Ignatowski (17). He postulated that atherosclerosis was due to the injurious effects of animal protein on the arterial wall. Although his conclusion was later regarded as incorrect (18) it stimulated interest in the role of protein in lipid metabolism.

In this survey of literature on the effect of protein on serum triglycerides, several common factors were found: the amount of dietary protein consumed; the source of the dietary protein; and the carbohydrate to protein ratio. Using Sprague-Dawley rats Leelamma and co-workers (19) showed an inverse relationship between the level

of dietary protein and the level of serum triglycerides. In this study intakes of casein at 40% of diet by weight as compared to 5% casein, generally produced a lower level of triglycerides in rats fed normal and atherogenic diets. In the same study a dietary level of 5% casein compared to the 40% level produced a higher level of serum triglycerides in animals fed both the normal and atherogenic diets. Hevia and colleagues (20) investigated the effects on serum and liver lipids when rats were fed casein or soybean protein with sucrose or dextrin. Male rats were fed 15, 30, or 45% casein or soy protein, respectively, for 14 days. Serum triglycerides were not different for the two protein sources, but were depressed 18% when either protein was raised to 45% of the diet with sucrose as the carbohydrate source. Increasing protein to 45% did not change liver triglycerides. This study revealed that dietary carbohydrate had a role in modifying serum lipids. Based on this finding the results of Leelamma et al. (19) should be reevaluated.

Rather than generally looking at protein as a contributing factor to serum triglyceride levels, a few researchers have examined other possible variables such as the effect of individual amino acids. Torre and co-workers (21) postulated that hyperlipidemia resulted from the feeding of imbalanced protein. Adult male rats were fed a powdered laboratory diet of 23.4% protein with gelatin included at various concentrations (5, 15, and 25% by weight) for 30 days. Significant increases in serum triglyceride levels occurred at all levels of gelatin except when 5% gelatin was added. Lipid levels of rats fed L-tryptophan-supplemented diets containing the same levels of gelatin

(5, 15, and 25%) did not differ from the control group of animals that were fed a standard laboratory diet. These data indicate that hyperlipidemia can be controlled by supplementing with the limiting amino acid.

Leelamma et al. (19) measured the activity of lipoprotein lipase in aortic, liver, and heart tissue when rats were fed diets containing casein at 5, 16, and 40% by weight. As the level of protein in the diet was increased the activity of the lipoprotein lipase enzyme also increased in each of the tissues studied. This increase in lipoprotein lipase activity could account for the low level of serum triglycerides associated with the higher levels of dietary protein.

C. Effects of Total Dietary Restriction

Little research using rats has been conducted to determine the effect of calorie restriction on serum triglyceride levels. However, several studies using humans as the experimental model have shown that acute caloric restriction caused a reduction in plasma triglycerides. In a study by Bhalchandra and co-workers (22) a 41% reduction in plasma triglycerides was attributed to caloric restriction of hyperlipidemic subjects. These authors suggested there was an increased oxidation of circulating free fatty acid (FFA) and a decrease in their synthesis in subjects on calorie restriction. As a result of decreased availability of FFA there could have been a decrease in hepatic synthesis and/or secretion of very low density lipoproteins (VLDL) into the plasma. Since this metabolic pathway is common to both humans and rats, this study could be used to anticipate the effects of calorie restriction on the serum lipid levels of the rat. Masoro et al. (7) studied feed

restriction in rats as it relates to adipocyte function. Results showed that feed restriction delays the loss of lipolytic responsiveness of adipocytes to both glucagon and epinephrine. Both of these hormones are important in regulating the rate of lipolysis (23). Plasma triglyceride response to caloric intake was studied in humans by Nestel and co-workers (24). They reported that the total intake of calories was highly relevant to the level of plasma triglycerides. Eucaloric carbohydrate rich diets produced only modest triglyceride response, doubling the caloric intake led to marked hypertriglyceridemia. These researchers suggested that a strong correlation existed between carbohydrate level of the diet and the level of plasma triglycerides. This relationship was also supported by Fallon and Kemp (25). In the Tecumseh study, Nichols and co-workers (26) did not find any significant differences in serum lipids with regard to the quantity, quality, or proportions of fat, carbohydrate, or protein consumed in the 24-hr recall period. The majority of research indicates that calorie level is a determinant in the level of serum triglycerides.

II. FACTORS AFFECTING SERUM CHOLESTEROL

A. Effects of Aging

Age-related changes in serum cholesterol levels have been extensively studied using the rat as the experimental model. The number of possible contributing factors involved in cholesterol metabolism have made it very difficult to identify the primary regulatory mechanism. Some of the phenomena reported by Uchida and co-workers (27) include a decrease in cholesterol synthesis; in cholesterol

turnover; in biliary and fecal excretion of cholesterol; in activity of controlling enzymes; in cholesterol absorption; and in sensitivity and/or secretion of hormones such as thyroxine and androgens. The end result tends to be a progressive increase in the serum cholesterol level with advancing age. Kritchevsky (11) suggests that the metabolism of plasma lipoproteins has a more direct effect on plasma levels of cholesterol than does the absorption and synthesis of cholesterol. It must be stated at this time that no consensus exists among researchers as to the role or activity of the possible contributing factors on plasma cholesterol.

Cholesterol is synthesized in most tissues of the body but the liver is almost totally responsible for the cholesterol homeostasis of the blood. When serum cholesterol biosynthesis is discussed, it must be done so in reference to the liver. It has been repeatedly demonstrated in both rats and humans that the hepatic biosynthesis of cholesterol decreases with age. Rosenman and Shibata (28) measured the daily bile output and determined its cholesterol concentration in an in vitro study. Their results indicated that rats aged 2 to 3 months had consistently higher concentrations of cholesterol and/or increased rate of synthesis than rats aged 5 to 11 months. Trout and co-workers (29), in an in vitro study, determined the rate of incorporation of 1-¹⁴C-acetate into cholesterol in young rats aged 2 days to 10 weeks and old rats 15 months to 3 years. The livers of young rats aged 8 weeks to 10 weeks did not show any greater ability to synthesize cholesterol than the old rats. In 1971, Yamamoto and Yamamura (30) studied the synthesis and excretion of cholesterol in rats aged 2, 5,

and 18 months. The results showed that at 5 and 18 months, there was a 50 to 70% reduction in both synthesis and excretion of cholesterol. There seems to be a definite downward trend in the rate of hepatic synthesis of cholesterol, but a decrease in cholesterol synthesis cannot explain hypercholesterolemia in aged rats.

Not only does hepatic cholesterol synthesis decrease in aging rats, but excretion and turnover are believed to decrease as well. According to experiments by Hruza and co-workers (31, 32) and Hruza (33), higher blood levels of cholesterol in older animals can be caused by decrease in cholesterol excretion and/or by a decrease of tissue uptake of cholesterol. Hruza and Wachtlová (31) stated that cholesterol turnover is apparently slowed down in old animals and this may explain why old animals are more susceptible to atherosclerosis. Hruza (33) dramatically demonstrated that cholesterol turnover in the serum and tissue decreases during aging in parabiotic studies of old with young rats; cholesterol turnover was increased in the old partner. The exact mechanism for this phenomena is uncertain but it may be involved with increased hormonal activity of the young rat. Hormones, such as thyroxine and insulin, can influence cholesterol excretion. Hruza (34) has suggested that the reduced turnover of cholesterol is due to the reduced sensitivity to insulin and thyroxine as well as reduced secretion of the latter with age. An experiment conducted by Malhotra and Kritchevsky (35) measured the cholesterol exchange between the red blood cells and plasma of young and old rats. The results expressed a paradox in that the red blood cells and plasma of old rats exhibited a greater turnover rate of cholesterol than the red blood cells and

plasma of the younger animals. This was attributed to a higher plasma level of free cholesterol in the older rats. This turnover pattern does not follow Hruza and Wachtlová's (31) slower turnover pattern.

Bile acid production, secretion, and resorption has a regulatory effect on hepatic cholesterol biosynthesis. A study in 1971 by Yamamoto and Yamamura (30) showed that biliary and fecal excretions of cholesterol decreased as did the gastrointestinal absorption of cholesterol in aged rats. Similarly in 1978, Uchida and co-workers (27) studied the age-related changes in cholesterol and bile acid metabolism in rats. They reported that bile flow and excretion of cholesterol and bile acids decreased in aged rats when expressed per kg of body weight. However, when these same data were expressed per rat no such age-associated decrease was found. Thus, it was concluded that bile flow, and biliary secretion of cholesterol and bile acids were almost constant regardless of age. It should be mentioned that Yamamoto and Yamamura's (30) data were not presented in terms of per rat but based on body weight.

It becomes more difficult to explain the elevated serum cholesterol levels characteristic of aged rats when biliary function remains constant. To help resolve this ambiguity, Uchida et al. (27) studied an age-related change in the composition of bile acids. They reported that chenodeoxycholic acid decreased with age while cholic acid increased. The significance of this alteration of composition is that absorption of cholesterol is enhanced by cholic acid while chenodeoxycholic acid has little effect. It can therefore be stated that the composition, as well as the amount of bile acids passing through

the liver, may influence the synthesis of cholesterol. If this premise is true then cholesterol absorption should be more efficient in the aged rat. This statement however, is inconsistent with Yamamoto and Yamamura's (30) findings that cholesterol absorption decreases with aging.

A discussion of lipid metabolism would be incomplete without considering the influence of enzymatic activity and hormonal control. Hruza (34) stated that cholesterol metabolism was regulated by the thyroid gland, hypophysis, and pancreas, much more so in the young animal than in an aged animal. He made the assumption that cholesterol metabolism of old rats was far less sensitive to thyroxine and insulin, the hormones that normally accelerate it. Yamamoto and Yamamura (30) reported that androgens may be partly responsible for changes in lipid metabolism in aging rats. Androgens accelerate the biosynthesis, degradation, and excretion of cholesterol. It is not known what role hormonal control plays in cholesterol metabolism of the older animal since there is generally a reduction in the basal metabolic rate and sensitivity to hormones as aging progresses.

The review of literature thus far implies a reduced ability to metabolize lipid as an organism ages. Recently, a wide survey of the activity of enzymes involved in carbohydrate, protein, and fat metabolism in aging rat livers was presented. Wilson (36) concluded from this survey that the activities of 45% of enzymes decreased, 22% remained unchanged, and the rest were increased. She did not specifically state what changes occurred in the liver lipid metabolizing enzymes. Kritchevsky and co-workers (37) examined the aortic

cholesterol esterase activity in rats. Their results indicated that both the synthesis and hydrolysis of cholesterol esters increased with age; synthetic activity increased (from the 2 month level) 41% at 12 months and 79% at 24 months; hydrolysis increased by 345% and 1160% at 12 and 24 months, respectively. In 18 month old rats, Story et al. (10) found that cholesterol 7 α hydrolase, the rate limiting enzyme of bile acid synthesis, was 68% of that observed in 2 month old rats.

B. Effects of Dietary Protein

The mechanism(s) by which dietary protein influences serum lipid levels remains unresolved at this time. However, several interesting and logical theories have been suggested for further investigation. Several studies have shown that the effect of protein on lipid level is not whether it is plant or animal, but rather other factors, e.g., a protein's amino acid composition, digestibility, the nature and concentration of its carbohydrate moieties, and the possibility of it being absorbed intact. The majority of studies, however, reported that the lowest concentration of serum cholesterol is generally associated with the intake of plant proteins (19, 20, 38, 39). Sirtori and Agradi (38) conducted a study in which the intake of a soybean-textured protein induced a 21% decrease in plasma cholesterol levels after 3 weeks in a group of 20 patients with hyperlipoproteinemia. In another study using rats, Hevia et al. (20) worked with casein in addition to soybean protein. They demonstrated that rats fed soybean protein had 12% less serum cholesterol than rats fed casein containing diets of the same protein level. Using the same strain of Sprague-Dawley rats, Leelamma and co-workers (19) have shown that intakes of

casein at 40% of diet by weight, as compared to 5 and 16% casein, generally caused an increase in serum cholesterol.

The quantity or percentage of total calories derived from proteins appears to be the controlling factor in the effect of protein on lipid metabolism. According to Bagchi (40) if the amount of protein consumed daily is more than the specific need, the excess may be metabolized to raw material for cholesterol synthesis. Elson and co-workers (41) investigated the influence of the level of dietary protein intake on serum lipids in humans. The subjects, when fed a low protein diet, experienced a reduction in serum cholesterol on the average of 42 mg/dl. When isocaloric diets containing either 48 or 141 g protein were fed, the mean serum cholesterol level for six subjects was 229 and 271 mg/dl, respectively. Another study involving human subjects demonstrated similar effects of low protein diets on serum cholesterol levels. Olson et al. (42) placed nine subjects on a control diet containing 100 g of protein, chiefly animal; 80 g of fat; and 300 to 350 g of carbohydrate for 1 week. At the end of the week the subjects were placed on an isocaloric, isofat diet containing 25 g of vegetable protein. All subjects showed a decrease in serum cholesterol during the low protein diet period. It should be noted that the protein source used in the low protein diet regimen was vegetable not animal as had been the chief source of protein in the high protein diet regimen.

Rather than generally looking at protein as a contributing factor to serum lipid level, a few researchers have narrowed the effects to individual amino acids (21, 40, 42, 44, 45). Of the 9

essential amino acids for humans, methionine has received the most attention in relation to its effects on lipid metabolism. Two aspects of the methionine molecule, the methyl group and the sulphur group, have offered different possible mechanisms in lipid level regulation. In vegetable protein, methionine is generally the limiting amino acid (41). Conversely, with animal protein, methionine is in much greater levels. This may partly explain why plant proteins tend to suppress blood lipids.

The biosynthesis of choline plays a significant role in fat metabolism. As stated by Harper and colleagues (43), the amino acid serine is decarboxylated to ethanolamine in a pyridoxal-dependent reaction. Ethanolamine can be progressively methylated to choline. Experiments with the rat liver have shown that the methyl group from methionine was apparently the sole source of the choline methyl groups. The decreased availability of labile methyl groups and subsequent decreased choline synthesis in a low protein vegetable diet was suggested by Ahrens (44) as being responsible for decreased serum cholesterol levels. According to Olson et al. (42) a choline deficiency in the adult rat causes a marked alteration in fat transport with an increase in liver fat and a decrease in the level of serum cholesterol and triglycerides. Elson et al. (41) stated that of all the amino acids, methionine alone could induce an increased output of adrenocortical hormones. The adrenocortical hormones promote the catabolism of amino acids and synthesis of cholesterol. Therefore, a diet low in methionine will depress the level of serum cholesterol and triglycerides by suppressing choline synthesis and adrenocortical hormone induction.

The presence of sulphur in methionine also has important implications on the level of serum cholesterol. Although several tissues can synthesize cholesterol, only the liver can influence the serum cholesterol level. In order for the liver to do this its integrity must be protected; methionine helps maintain the sulphydryl content of the liver and thus protects the liver. In a study by Bagchi et al. (40) methionine sulfoximine, a methionine analogue, was administered to rats on a 18% protein diet. The result was a marked fall in the sulphydryl content of the liver and a simultaneous reduction of serum cholesterol by about 30%. In the same study when methionine supplements were given, the serum cholesterol level increased. Three functions of methionine in lipid metabolism have been discussed in the literature: a source of labile methyl groups; a source of sulfur; and, a source of the functional unit of coenzyme A which is necessary for cholesterol synthesis (44).

In another study by Olson et al. (45) the effects of amino acids other than methionine on plasma lipids were evaluated. They suggested that methionine itself was not the regulator of plasma lipids since in several studies the low protein diets had ample levels of methionine to meet physiological needs of the animal. They fed 8 healthy male subjects a formula diet containing 8 essential amino acids in adequate amounts plus glutamate as the source of nonessential amino nitrogen. This diet produced a marked fall in serum cholesterol and had an indifferent effect on triglycerides. Formula diets identical in all respects except for replacement of glutamate by glycine and ammonium acetate did not display any lowering affect. A lower level

of serum cholesterol was accomplished by Torre and co-workers (21) by balancing the amino acid intake in the rat. Adult male rats fed rations with added gelatin for 30 days showed significant increases in serum cholesterol. However when these same diets were supplemented with the limiting amino acid tryptophan, the serum cholesterol level returned to the same level as the control animals. They reasoned that the addition of an incomplete protein would result in a larger quantity of systemic amino acids being converted to lipids by intermediary metabolic processes. Most studies implicate dietary protein as having an effect on serum cholesterol levels; the explanation may lie with the amino acid composition of the protein source.

C. Effects of Total Dietary Restriction

The concept of calorie restriction having an influence on serum cholesterol is a result of fairly recent investigations. In 1972, Hruza and Zbozkova (33) restricted the total calorie intake of young rats to equal the average intake of older rats. Excretion and tissue turnover of ^3H -cholesterol was then studied in these animals. These data showed that calorie restriction in the young animals had no effect on excretion of cholesterol. Old animals excreted much less cholesterol than young animals with calorie restriction. Bhalchandra and co-workers (22) studied the effect of acute calorie restriction on cholesterol metabolism in man. They reported that calorie restriction in humans decreased plasma cholesterol by 11% and total fecal bile acids 36%. The declines in serum lipids occurred before weight was lost. Bennion and Grundy (46) noted decreases in biliary secretion as well as decreases in pool sizes of bile acids during calorie

restriction in man. Masoro et al. (7) explored age-related changes in rats fed on either an ad libitum or restricted calorie basis. Results from this study showed that feed restriction delayed the age-related increase in serum cholesterol and also markedly reduced the magnitude of increase. A study by Nolen (47) compared the effects of various restricted regimens, 80% of ad libitum intake, or 60% of ad libitum intake, on several physiological parameters in the rat. Although the ad libitum and restricted rats experienced a small increase in serum cholesterol with advancing age; there was no distinct effect of dietary restriction on the level of serum cholesterol. Jones et al. (48) are in agreement with Nolen's findings. Investigations were made into the specific mechanism(s) of the influence of caloric restriction on serum cholesterol level. Many studies have shown that calorie restriction effects the activity of various hepatic enzymes of male rats. Barrows and Roeder (49-51) indicated that dietary restriction did not retard the normal biochemical processes associated with growth. It was also stated in these studies that little information is available on the response of adult or senescent rats to reduced dietary intake.

With all the numerous interplay of factors, much confusion and contrast in study results have been reported. The general trend agreed upon is that aging animals exhibit a progressive inability to handle lipids, while they may synthesize less they also catabolize less, the result being an age-related increase in the blood and tissue lipids. Much more research is needed to study the effects of dietary restriction on serum cholesterol and triglyceride level in aging animals.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

This study is an outgrowth of a comprehensive study on the effect of calorie and protein restriction on aged rats conducted by R. E. Beauchene, C. Bales, and T. Davis.¹ The overall experiment was near completion before the present author became actively involved. The present author did, however, assist in collecting all the blood samples and performed the analyses of the same for cholesterol and triglycerides.

Male Wistar weanling rats, (National Research Laboratories, Creve Coeur, Missouri) aged approximately 32 days were assigned to 8 dietary groups based on their weights so that the mean weight of each group was similar. Each dietary group consisted of 36 animals which were designated as old rats or those animals to be sacrificed at 2 years of age. When the old rats reached 13 months of age, 12 additional weanling rats were added to each dietary group and were referred to as young rats or those animals to be sacrificed at 1 year of age.

Three of the dietary groups were ad libitum-fed (A) diets containing low (12%), medium (20%), or high (28%) casein by weight, respectively (Table 1). These were considered the control groups. Each control group had a corresponding group fed the same absolute

¹T. A. Davis (1980) The effect of dietary protein and calorie restriction on growth, kidney function, and survival of male rats (unpublished dissertation, The University of Tennessee, Knoxville).

TABLE 1
DIETARY TREATMENTS¹

Dietary Protein Designation	Casein in Diet	
	Ad Libitum-Fed	Restricted-Fed ²
	%	%
Low	12	18
Medium	20	30
High	28	42
Medium/low ³	20→12	
Medium/high ³	20→28	

¹Taken from: Davis, T. A. (1980). (See footnote on page 19.)

²Restricted animals were fed two-thirds of the mean feed intake of their ad libitum-fed controls.

³Percent dietary casein was changed 0.8% per month for 10 months and maintained at constant levels thereafter.

amount of protein, but two-thirds of the calories as determined from the dietary intake of the ad libitum-fed rats. These groups were fed diets containing low (18%), medium (30%), or high (42%) casein by weight, respectively and were the restricted (R) animals (Table 1). All the diets were semisynthetic containing the same level and type of fat and were cholesterol free (Table 2). The other two dietary groups of animals were fed a 20% casein diet which was either increased or decreased in the level of protein at a rate of 0.8% per month for 10 months until a level of 28% or 12% casein was obtained, respectively. The two dietary groups were then maintained at a constant casein level for the remainder of the study.

All the animals were individually housed in 7" X 10" X 7" stainless steel cages with ad libitum access to water. Cages were positioned on a movable rack which had five levels for cages. Cages were rotated within the rack and the position of the rack itself was moved within the room weekly. The feed intake was monitored weekly on one-third of the ad libitum-fed animals on each diet until the sixth month of the study. This practice was used to determine the level of calories to feed the restricted rats. After 6 months, this was done biweekly until the twelfth month, and thereafter once every 4 weeks. The animals were weighed on the same schedule.

II. BLOOD COLLECTION

One and 2 year old animals were randomly selected from the 8 dietary treatments for sacrificing. Euthanasia was administered by a sharp blow at the base of the skull followed by immediate decapitation

TABLE 2
COMPOSITION OF 20% CASEIN DIET¹

Dietary Component	Percent of Diet
Casein ^{2,3}	20.0
Sucrose	29.0
Cornstarch	29.0
Crisco	6.0
Wesson oil	2.0
Vitamin mix ^{2,4}	2.0
Salt mix ^{2,5}	3.0
Alphacel ²	9.0

¹Taken from: Davis, T. A. (1980). (See footnote on page 19.)

²Nutritional Biochemical Corporation, Cleveland Ohio 44128.

³Determined by the Kjeldahl method to contain 91.5% protein.

⁴Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin mix): vitamin A, 4.5; vitamin D, 0.25; thiamin hydrochloride, 1.0; riboflavin, 1.0; niacin, 4.5; p-aminobenzoic acid, 5.0; calcium pantothenate, 3.0; pyridoxine hydrochloride, 1.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; biotin, 0.02; folic acid, 0.09; vitamin B₁₂, 0.00135; alpha-tocopherol, 5.0; and sufficient dextrose to make 1 kg.

⁵Formulated to supply the following amounts of minerals (g/kg salt mixture): CaCO₃, 543.0; MgCO₃, 25.0; MgSO₄, 16.0; NaCl, 69.0; KCl, 112.0; KH₂PO₄, 212.0; FePO₄·4H₂O, 20.5; KI, 0.08; MnSO₄, 0.35; NaF, 1.00; Al₂(SO₄)₂K₂SO₄, 0.17; and CuSO₄, 0.09.

using a guillotine. Blood was collected in 15 ml centrifuge tubes and allowed to clot for 30 minutes. The blood was then centrifuged (Model V, International Equipment Co., Boston, MA.) at 600 x g for 30 minutes. Serum was removed and the remaining blood recentrifuged at 600 x g for 10 minutes and any additional serum removed. The serum was stored in labeled 5 ml test tubes and placed under -50⁰ refrigeration until time of analysis.

III. SERUM TRIGLYCERIDE DETERMINATION

Serum triglyceride concentration was determined using the Oxford Tri-Chol Reagent Set (Oxford Laboratories, Foster City, CA. 94404). This method is based on the extraction of triglycerides from the serum using alumina powder to adsorb phospholipids (52), glycerol, and glucose; saponification with potassium hydroxide; oxidation of the glycerol to formaldehyde with sodium metaperiodate; and reaction of formaldehyde with ammonia and acetylacetone to produce diacetyldihydrolutidine (53). The amount of this compound formed was determined spectrophotometrically at 409 nm.

A. Reagents

1. Isopropyl alcohol (IPA).
2. Alcoholic acid solution: 628 g of IPA diluted to 1 liter with 0.07 M acetic acid.
3. Alcoholic ammonia solution: ammonium acetate, 115.5 g/l; IPA, 20 g/l.
4. Sodium metaperiodate: 6.4 g of sodium metaperiodate diluted to 1 liter with 1.0 M acetic acid.

5. Acetylacetone.
6. Potassium hydroxide: 6.25 M.
7. Alumina powder.
8. Lipid standards:
 - No. 1: Triolein, 100 mg/dl in IPA.
 - No. 2: Triolein, 200 mg/dl in IPA.
 - No. 3: Triolein, 300 mg/dl in IPA.

B. Working Solution Preparation

Sodium metaperiodate dilution was prepared fresh daily. The working solution was prepared by making a 1:10 dilution of sodium metaperiodate stock with the alcoholic acid solution. Acetylacetone dilution was prepared as needed by adding 0.3 ml of stock acetylacetone to 40 ml of alcoholic ammonia. This reagent mixture was stable for 1 month if stored in an amber colored bottle under refrigeration. The triglyceride and cholesterol dilutions were prepared simultaneously by adding 0.3 ml of each of the stock standards to 5.0 ml of IPA. Each test tube was then sealed and the contents mixed for 30 seconds.

C. Procedure

Two extraction tubes containing 2.5 g of premeasured alumina powder were appropriately labeled for each unknown serum specimen. Five ml of IPA were delivered to each extraction tube. Serum specimens were allowed to come to room temperature and then thoroughly mixed before a 0.3 ml sample was removed and delivered to the appropriate extraction tubes. The extraction tubes were then recapped and mixed for 30 seconds. All extraction tubes were then placed on a circular

rotator for 15 minutes. After this agitation the extraction tubes were centrifuged for 10 minutes at 900 x g. Two-1 ml aliquots of lipid extract were removed from each extraction tube and delivered to sealable glass test tubes designated for triglycerides and cholesterol. The first aliquot removed from each extraction tube was used for triglyceride determination and the second lipid extract aliquot was used for cholesterol determination.

Sealable glass test tubes were labeled appropriately for blanks, triplicate standards, and duplicate unknowns. One ml of IPA was delivered to the test tubes labeled blanks. One ml aliquots of diluted standards were delivered to the test tubes designated for each of the standards. One drop of potassium hydroxide was added to every tube, the tube plugged and mixed by vortexing. All tubes were then incubated at 68⁰ for 5 minutes. One ml of working sodium metaperiodate solution was then added to each tube and the contents mixed well. The last reagent, 0.5 ml of working acetylacetone solution was added followed by thorough mixing and incubation for 10 minutes at 68⁰. After the incubation period the tubes were placed in a room temperature water bath for 5 minutes. This cooling period stopped further reaction from taking place and made the standards and unknowns less susceptible to drift in absorbance values. The absorbance values were determined using a spectrophotometer (Model 24, Beckman Instruments, Palo Alto, CA.) at 409 nm. The absorbance values were averaged and plotted for each unknown giving the concentration in mg/dl.

IV. SERUM CHOLESTEROL DETERMINATION

The determination of serum cholesterol was accomplished using the Oxford colorimetric method based on the Kiliani-Zak reaction (54). A portion of the lipid extract obtained in the triglyceride procedure was added with the cholesterol reagent. This reaction produced a purple chromophore proportional to the concentration of cholesterol present after incubation.

A. Reagents

1. Cholesterol reagent: ferric chloride, 20 g/l; acetic acid, 13.8 M; sulphuric acid, 5.9 M.

2. Lipid standards:

- No. 1: cholesterol, 150 mg/dl.

- No. 2: cholesterol, 300 mg/dl.

- No. 3: cholesterol, 450 mg/dl.

B. Procedure

A 1 ml portion of lipid extraction mixture, obtained in the first step of the triglyceride determination was used for the analysis of cholesterol for each rat. One ml of IPA was delivered to the tubes labeled blanks. Duplicate 1 ml aliquots for each standard were added to the labeled test tubes. Using a repipet, 3.0 ml of cholesterol reagent were added against the side of each tube and the tube plugged. The cholesterol reagent was very viscous and required thorough mixing on a vortex to avoid layering. All tubes were then incubated for 5 minutes at 68°. After incubation the tubes were immediately placed in a room temperature water bath for 5 minutes to stabilize and prevent

further reaction. The absorbance was then measured spectrophotometrically at 450 nm.

V. STATISTICAL METHODS

The main emphasis of this study was to investigate the effects of (1) dietary protein (linear and/or quadratic for protein), (2) calories (A vs R), (3) the interaction of protein and calories (linear and/or quadratic), and (4) age on serum cholesterol and triglyceride levels of rats. The animal data were analyzed by procedures available in general linear models (GLM) of SAS 1979 (55). Differences among group means were tested with contrasts developed to test specific research questions. Probability levels (α -risk) of less than 0.05 were considered statistically significant. The least squares means option available in GLM was used to estimate group means and standard errors.

CHAPTER IV

RESULTS

Data were analyzed for the effect of diet independent of age; age independent of diet; and the interaction of diet and age on serum cholesterol and triglyceride levels. Table 3 is a summary of these initial analyses.

Dietary treatment, regardless of age, significantly ($p < .01$) affected serum cholesterol levels (Table 3). The mean $\pm$ SEM serum cholesterol level of all ad libitum, including variable protein, animals was 134 ± 6 mg/dl (Table 4). Restricted animals had a mean cholesterol level of 90 ± 11 mg/dl, significantly lower ($p < .01$) than ad libitum animals. Variation in the ad libitum protein level (20 $\rightarrow$ 12% casein and 20 $\rightarrow$ 28% casein) had no effect on serum cholesterol levels. The overall effect of protein on cholesterol was quadratic ($p < .01$) but not linear (Figure 1). The interaction of protein and calories on cholesterol was linear ($p < .01$) but not quadratic.

Diet of the animals significantly ($p < .01$) effected serum triglyceride levels (Table 3). The mean serum triglyceride level of all ad libitum animals was 180 ± 13 mg/dl (Table 4). The mean triglyceride level for all restricted animals was 119 ± 10 mg/dl which was significantly ($p < .01$) less than the level for all ad libitum animals. There was no significant effect on triglyceride levels when constant ad libitum protein groups were compared with variable (20 $\rightarrow$ 12% casein and 20 $\rightarrow$ 28% casein) protein groups. The overall effect of protein on

TABLE 3
SUMMARY OF STATISTICAL ANALYSES FOR SERUM
CHOLESTEROL AND TRIGLYCERIDES

Source of Variation	Significance ¹	
	Cholesterol	Triglycerides
Diet	***	***
Contrast statement-independent of age		
All ad libitum (A) vs restricted (R)	***	***
Constant A vs changing A	NS	NS
Protein intake		
Linear	NS	NS
Quadratic	***	NS
Protein intake X caloric level		
Linear	***	**
Quadratic	NS	**
Age	**	***
Diet-age interaction	NS	NS
Contrast statements age-related		
Young		
Constant protein A (12, 20, 28% casein) vs variable protein A (20→12%, 20→28% casein)	NS	NS
Change to low (20→12% casein) vs change to high (20→28% casein)	NS	***
A vs R (12, 20, 28% casein) vs (18, 30, 42% casein)	***	***
All A vs R (12, 20, 28, 20→12, 20→28% casein) vs (18, 30, 42% casein)	***	***

TABLE 3 (Continued)

Source of Variation	Significance ¹	
	Cholesterol	Triglycerides
Protein intake		
Linear	NS	NS
Quadratic	**	NS
Protein intake X caloric level		
Linear	***	NS
Quadratic	NS	NS
Old		
Constant protein A (12, 20, 28% casein) vs variable protein A (20→12%, 20→28% casein)	NS	NS
Change to low (20→12% casein) vs change to high (20→28% casein)	NS	NS
A vs R (12, 20, 28% casein) vs (18, 30, 42% casein)	***	*
All A vs R, (12, 20, 28, 20→12, 20→28% casein) vs (18, 30, 42% casein)	***	***
Protein intake		
Linear	NS	NS
Quadratic	NS	NS
Protein intake X caloric level		
Linear	NS	NS
Quadratic	*	NS

¹Statistical significance is indicated by * (p < 0.10), ** (p < 0.05), and *** (p < 0.01). Statements not statistically significant are indicated by NS (p > 0.10).

TABLE 4
MEANS FOR SERUM CHOLESTEROL AND TRIGLYCERIDES
FOR DIET INDEPENDENT OF AGE

Dietary Group		Serum Cholesterol	Serum Triglycerides
		mg/dl	mg/dl
Ad libitum			
12 ¹	(14) ²	126±10 ³	170±18
20	(14)	132±10	197±18
28	(13)	160±9	140±18
20→12	(12)	130±11	166±19
20→28	(16)	120±9	228±17
Mean ± SEM		134±6	180±13
Restricted			
18	(17)	114±10	107±18
30	(14)	69±9	105±18
42	(13)	88±8	144±16
Mean ± SEM		90±11	119±10

¹Percent dietary casein.

²Number of animals in each dietary group regardless of age.

³Mean ± SEM.

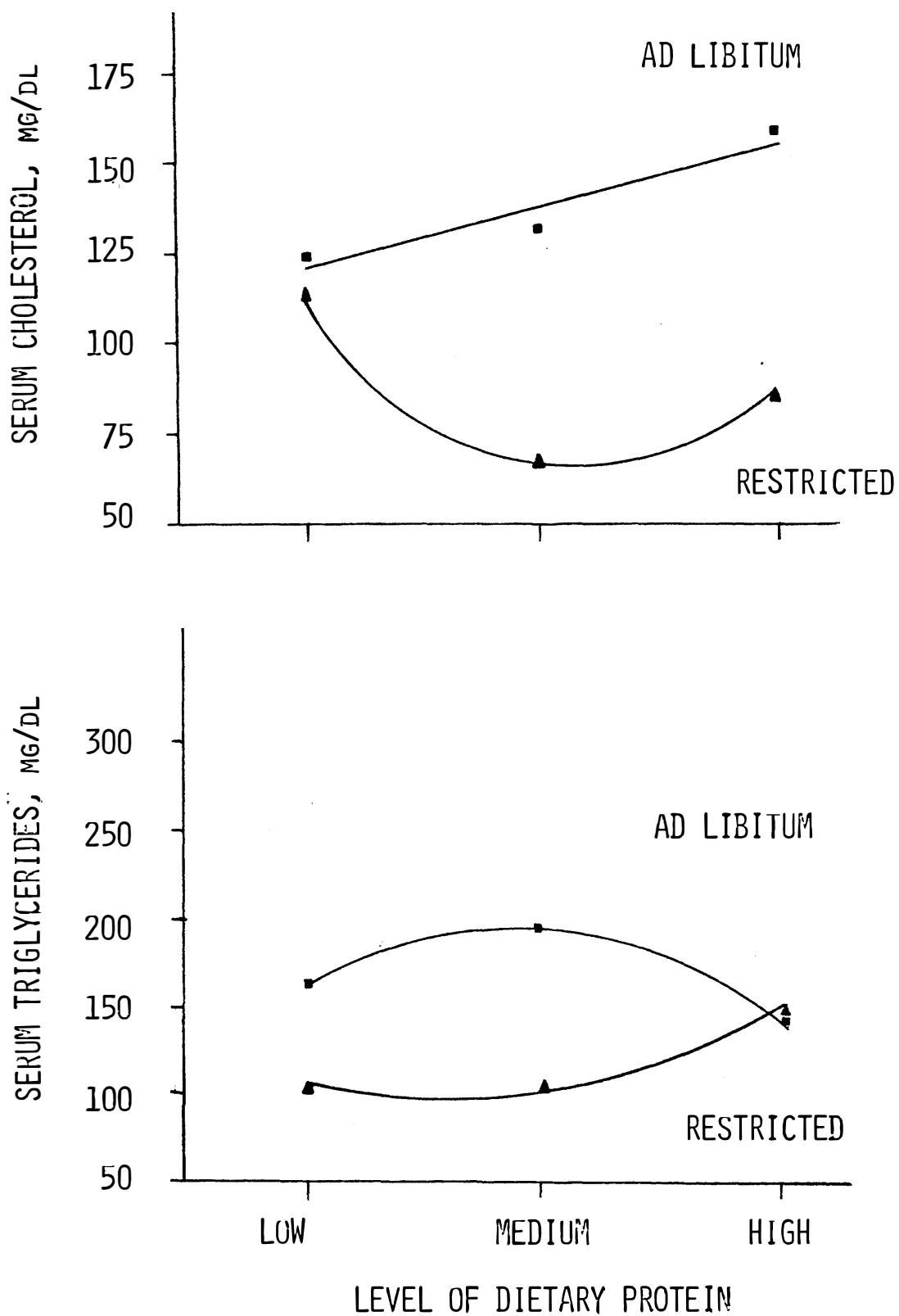


Figure 1. Effect of dietary protein and calories on serum cholesterol and triglyceride levels, independent of age.

triglycerides was neither linear nor quadratic (Figure 1) but interaction of protein and calories on triglycerides was linear by group ($p < .05$) and quadratic by group ($p < .05$).

The age effect, independent of diet, significantly effected both parameters: ($p < .05$) for cholesterol and ($p < .01$) for triglycerides (Table 3). The mean serum cholesterol level of all (ad libitum and restricted) young animals was 110 ± 4 mg/dl which was lower ($p < 0.05$) than 125 ± 5 mg/dl recorded for all old animals (Table 5). The opposite was true for serum triglyceride values. Young animals had a mean serum triglyceride level of 173 ± 8 mg/dl which was significantly greater ($p < .01$) than the value of 140 ± 10 mg/dl recorded for old animals.

A significant diet-age interaction was not observed for cholesterol values when combined means were considered. Triglyceride values were not significantly effected by a diet-age interaction either (Table 3).

Specific contrast statements were developed to test for differences between age groups and among dietary treatments. In young ad libitum animals, the contrast between constant protein and variable protein was not significant for either cholesterol or triglycerides (Table 5). This was also true for old ad libitum animals (Table 5). Changing from medium to low (20→12%) or from medium to high (20→28%) casein did not significantly effect serum cholesterol of young or old animals or serum triglycerides of old animals. This dietary manipulation significantly ($p < .01$) effected triglyceride levels of the young animals. The mean serum triglyceride level of the 20→28% casein young animals was 258 ± 20 mg/dl, significantly ($p < .01$) higher than the mean value of 178 ± 25 mg/dl recorded for the 20→12% casein animals.

TABLE 5
MEANS FOR SERUM CHOLESTEROL AND
TRIGLYCERIDES BY DIET AND AGE

Dietary Group		Serum Cholesterol	Serum Triglycerides
		mg/dl	mg/dl
Young			
Ad libitum (A)			
12 ¹	(8) ²	113±13 ³	181±23
20	(9)	113±11	207±22
28	(8)	168±12	174±23
Mean (all A constant protein)		131±12	187±23
20→12	(7)	119±13	178±25
20→28	(11)	118±10	258±20
Mean (all A variable protein)		118±11	218±22
Mean (all A constant and variable protein)		126±9	199±14
Restricted (R)			
18	(8)	99±12	117±23
30	(8)	73±12	121±23
42	(10)	78±11	149±21
Mean (all R constant protein)		83±12	129±22
Mean (all A and R)		110±4	173±8
Old			
Ad libitum (A)			
12	(5)	140±15	158±29
20	(5)	150±17	187±29

TABLE 5 (Continued)

Dietary Group	Serum Cholesterol	Serum Triglycerides
	mg/dl	mg/dl
28 (6)	152±13	106±26
Mean (all A constant protein)	147±15	150±28
20→12 (5)	141±17	154±29
20→28 (5)	123±15	196±29
Mean (all A variable protein)	132±16	175±29
Mean (all A constant and variable protein)	141±4.6	160±14
Restricted (R)		
18 (5)	129±15	97±29
30 (6)	65±12	87±26
42 (7)	99±13	139±25
Mean (all R constant protein)	98±13	107±27
Mean (all A and R)	125±5	140±10

¹Percent dietary casein.

²Number of animals in each dietary group.

³Mean ± SEM.

The mean cholesterol level of all young ad libitum-fed animals was 131 ± 12 mg/dl which was significantly ($p < .01$) greater than 83 ± 12 mg/dl recorded for all restricted-fed young animals (Table 3, page 29). The same trend was observed for the old ad libitum versus old restricted animals. When the variable protein level diets were included in the calculations, the mean cholesterol level of young ad libitum-fed animals was slightly less than that obtained when the variable protein levels were not considered but still significantly ($p < 0.01$) greater than the level of restricted-fed animals. This same effect was observed for old animals (Table 4).

The overall effect of dietary protein level on cholesterol for young and old animals was not linear. There was however a quadratic effect ($p < 0.05$) of protein on cholesterol for young animals. There was no overall quadratic effect of protein on cholesterol for old animals. The interaction of protein and calories on cholesterol was linear ($p < 0.01$) for young but quadratic ($p < 0.10$) for old animals (Figure 2).

When the contrast of 20→12% casein versus 20→28% casein was considered for triglycerides for young and old animals, there was a significant ($p < 0.01$) effect only for young animals (Table 3). In both ages of animals, the 20→12% casein group had a lower mean triglyceride level than the 20→28% group (Table 5).

For all young ad libitum-fed animals, on constant levels of dietary protein, the mean serum triglyceride level was 187 ± 23 mg/dl which was significantly ($p < 0.01$) higher than that (129 ± 22 mg/dl) recorded for young restricted-fed animals (Table 5). In the old

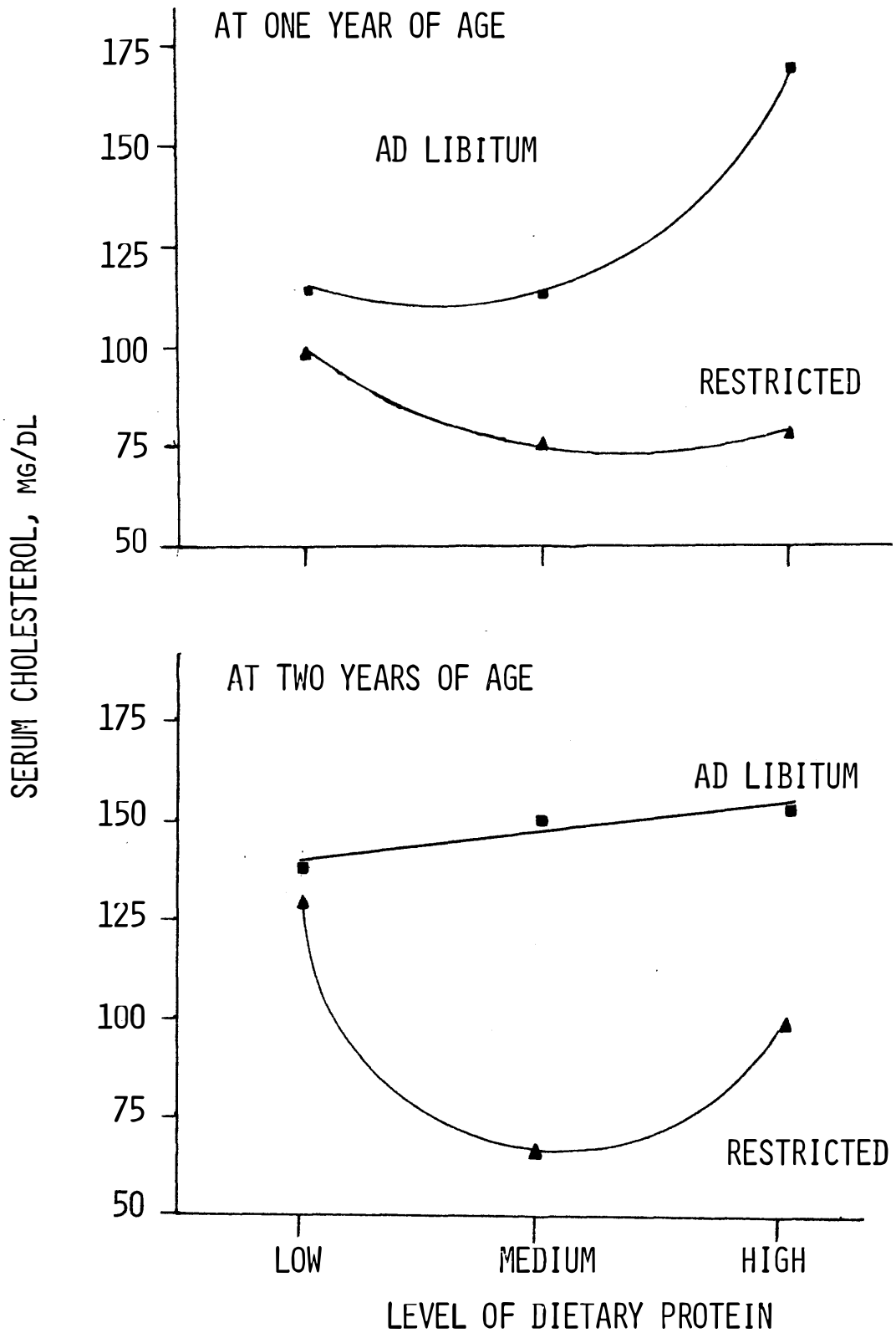


Figure 2. Influence of dietary protein and calories on serum cholesterol at 1 and 2 years of age.

animals, the mean triglyceride level of the restricted-fed animals was 107 ± 27 mg/dl, significantly ($p < 0.01$) less than 150 ± 28 mg/dl recorded for old ad libitum animals fed constant protein diets.

There was a significant ($p < 0.01$) effect of calories on triglycerides in young and old animals. The mean serum triglyceride level of all ad libitum-fed young animals was 199 ± 14 mg/dl which was significantly higher ($p < 0.01$) than 129 ± 22 mg/dl recorded for young restricted-fed animals. For old ad libitum-fed animals, the mean triglyceride level of 160 ± 14 mg/dl was also significantly ($p < 0.01$) greater than 107 ± 27 mg/dl of restricted old animals.

There was neither a linear nor quadratic effect of protein on serum triglycerides of young or old animals. The interaction of protein and calories on triglycerides in young and old animals was neither linear nor quadratic.

CHAPTER V

DISCUSSION

When the effects of diet were analyzed, regardless of age, a highly significant ($p < 0.01$) effect on both serum cholesterol and triglycerides was found. The low levels (12% or 18% casein) of dietary protein were associated with similar mean levels of serum cholesterol. This was not the trend for the other groups of animals, however. Animals receiving the 28% casein diet had almost twice the mean serum cholesterol level of the comparable fed R group (42% casein). This same trend was true for animals fed 20% (A) and 30% (R) casein, respectively. In the A fed animals, the highest level of dietary protein was associated with the greatest level of serum cholesterol. Bagchi et al. (40) suggested that if the amount of protein is more than the specific need, the excess may be metabolized as raw material for cholesterol synthesis. Leelamma and co-workers (19) have shown that high levels of dietary protein generally caused an increase in the level of serum cholesterol in ad libitum-fed rats. This pattern was not seen in the restricted-fed rats; e.g., the lowest level of dietary protein was associated with the highest level of serum cholesterol.

A high level of significance was noted when ad libitum versus restricted feeding were contrasted (Table 3, page 29). The mean cholesterol level of the R animals was 90 ± 11 mg/dl which was significantly ($p < 0.01$) lower than 134 ± 6 mg/dl for the A fed animals. This is consistent with other studies (7, 21). Feeding variable levels of

protein during periods of growth and development did not produce a significant difference when compared to animals fed constant levels of protein. Overall, protein had a quadratic effect on the serum cholesterol level ($p < 0.01$). There was a significant ($p < 0.01$) degree of linear interaction between calorie and protein levels on serum cholesterol.

Serum triglyceride values were also highly effected by dietary treatment. Animals receiving the 20% casein diet had a mean serum triglyceride level of 197 ± 18 mg/dl, the highest value for any other constant A group but less than the mean level of 228 ± 18 mg/dl recorded for the animals fed the 20-28% casein diet. Mean triglyceride levels of restricted animals fed either the 18% or 20% casein diets were not significantly different. The highest level of dietary protein (28%) fed on a constant basis ad libitum was associated with the lowest level of triglycerides. According to Leelamma and co-workers (19) and Hevia et al. (20) high levels of protein fed ad libitum generally produced a low level of triglycerides whereas a low level of protein tended to elevate serum triglycerides. Leelamma noted a direct relationship between lipoprotein lipase activity and the level of dietary protein. The R fed animals had a mean level of triglycerides, 119 ± 10 mg/dl, significantly lower than that of the A fed animals, 180 ± 13 mg/dl. Nestel and co-workers (24) and Masoro et al. (7) reported similar findings. There were no significant differences in serum triglycerides when constant protein versus variable protein were contrasted. Protein had neither a linear nor quadratic effect on triglycerides. There was a significant ($p < 0.05$) degree of linear

and quadratic interaction between calorie and protein levels on triglyceride levels.

There is a general consensus in the literature that the aging process is characterized by a progressive increase in serum cholesterol and triglycerides in the rat. This relationship appears to be stronger for cholesterol than for triglycerides. In the present study the mean serum cholesterol level of all of the older animals (125 ± 5 mg/dl) was significantly ($p < 0.05$) greater than that of all the younger animals (110 ± 4 mg/dl). This is consistent with other literature findings (11, 27, 28, 30-33). Due to the number of controlling mechanisms of cholesterol metabolism it would be difficult at present to identify the regulatory mechanism responsible for this age-related rise in serum cholesterol. The changes with age for serum cholesterol and triglycerides did not parallel each other.

In this study the age effect, regardless of diet, significantly effected both parameters: ($p < 0.05$) for cholesterol and ($p < 0.01$) for triglycerides. Without exception, the serum triglyceride level of 1 year old rats fed A or R diets, was consistently greater than that of the 2 year old animals. This result is contrary to other literature findings (12, 13). It must be noted that many of the studies on aging and serum lipids are based on conclusions where an animal was considered as adult (old) when they were 2 to 12 months of age. Animals considered old in this study were 2 years of age.

Most literature suggests that aging is characterized by a reduced ability to clear lipoproteins from the plasma. The results indicate that such was not the case in the present study. Either the efficiency

of clearing lipoproteins remained constant or increased; there was less endogenous synthesis of triglycerides by the liver; or there was a decrease in the mobilization of free fatty acids from the adipose tissue. Carlson et al. (15) reported that the plasma level of free fatty acids and glycerol from adipose tissue did not increase with age. Benjamin et al. (16) noted that with aging there was a depression of both de novo fat synthesis and the synthesis of fatty acid glycerides. A constant or increased efficiency by which the lipoprotein lipase enzyme system cleared the plasma of very low density lipoproteins may explain the lower level of triglycerides in the old animals of this study.

In evaluating the data another possible contributing factor to consider is the effect of infection on plasma lipids. At the time of sacrificing approximately 60% of the animals had pneumonia or some type of neoplasm. According to Fiser (56), during acute infections the liver takes up fatty acids from the plasma at an accelerated rate and accelerates the synthesis of both triglycerides and cholesterol. In the present study, some ill animals had markedly higher levels of triglycerides and cholesterol than healthy members of the same dietary group, but some ill animals had very low levels of serum triglycerides and cholesterol. Reasons for this ambiguity are unknown but it does imply that illness may have some effect on the blood lipid levels.

Specific age-related contrast statements were used to test for significance of differences between age groups and among dietary treatments. In almost every instance, regardless of the animal's age, feeding constant levels of protein versus variable protein did not

significantly effect either serum cholesterol or triglycerides. The change to low (20→12% casein) versus the change to high (20→28% casein) had a highly significant effect on triglycerides ($p < 0.01$) but only at 1 year of age. This dietary manipulation produced the highest level of triglycerides of all the A fed animals, 258 ± 20 mg/dl. A possible explanation for this increase is that by the time the level of 28% casein was obtained the animal had nearly completed its growth and development so additional protein was diverted to lipid. The various levels of dietary protein resulted in little difference in serum cholesterol levels. As the level of protein increased, with the exception of the 1 year 28% casein group little difference in serum cholesterol was noted. The 28% casein group had the highest level of serum cholesterol. In reviewing the raw data for this group little variance was found between the animals' serum cholesterol values so as to exclude the possibility that the large group mean was due to an extremely large value from a deviant animal. The hypothesis given earlier by Bagchi et al. (40) offers an explanation for this high cholesterol mean. Many of the studies which have shown protein to have an effect on the serum cholesterol level used dietary protein sources which were limiting or lacking in an amino acid. In addition, several researchers (42-44) have reported that a low methionine level causes a decreased availability of labile methyl groups and subsequently a decrease in choline synthesis. A decrease in choline in the adult rat can cause a marked alteration in fat transport with an increase in liver fat and decrease in the level of serum cholesterol and triglycerides. With the exception of the 12% casein diet this

should not have been the case in this study (methionine is limiting in a 12% casein diet fed ad libitum) (57).

The diets for this present study were designed to meet the physiological needs of healthy animals. The majority of animals were determined to be ill, to various degrees, at sacrifice. Nitrogen requirements increase during periods of illness and immobilization (58). In considering the state of health of most of the animals it is conceivable that the values could have been more variable since an unknown amount of protein was needed to compensate for illness.

Although the statistical analysis determined that dietary protein level had neither a linear nor quadratic effect on serum triglycerides the data show several noteworthy trends. In the ad libitum-fed animals of both age groups, serum triglycerides were the greatest when the medium protein level was fed while the highest level of protein produced the lowest level of serum triglycerides (Table 5, page 34). This inverse relationship is consistent with the results of Leelamma and co-workers (19) and Hevia et al. (20). In the restricted-fed animals this inverse relationship did not exist. In the latter animals, at both ages, as the level of protein increased the serum triglyceride level also generally increased. Although the mean serum triglyceride values between restricted groups were not significantly different, the highest serum triglyceride values were associated with the highest percentage of dietary protein.

One year restricted-fed animals had a mean serum triglyceride level of 58 mg/dl lower than similar ad libitum-fed animals ($p < 0.01$). Mean serum triglyceride level of old restricted-fed animals was

43 mg/dl lower than comparable aged animals fed ad libitum. When the triglyceride data of variable protein groups were added to that of the constant protein ad libitum-fed groups, a higher level of significance ($p < 0.01$) was found between old A and R groups. Nestel and co-workers (24) reported that the total intake of calories was highly relevant to the level of plasma triglycerides. The lower level of serum triglycerides associated with restricted feeding is probably due to an increased oxidation of free fatty acids and a decrease in their synthesis. As a result of decreased availability of free fatty acids there could have been a decrease in hepatic synthesis and/or secretion of very low density lipoproteins into the plasma (22). Even as the restricted-fed animals aged, serum triglyceride levels remained significantly lower than the animals fed ad libitum. According to Masoro et al. (7) feed restriction delayed the loss of lipolytic responsiveness of adipocytes to both glucagon and epinephrine.

Calorie restriction had a similar effect on the serum cholesterol level. One and 2 year restricted-fed animals had a mean serum cholesterol, 48 and 49 mg/dl, respectively, lower than that of their ad libitum-fed counterparts fed constant protein levels ($p < 0.01$). Similar effects of calorie restriction on serum cholesterol were demonstrated by Bhalchandra et al. (22) and Masoro et al. (7). They reported that feed restriction delayed the age-related increase in serum cholesterol and markedly reduced the magnitude of the increase.

The data indicate that caloric restriction significantly reduced the levels of serum cholesterol and triglycerides in rats at 1 and 2 years of age. This could be interpreted as being consistent with

delaying the degenerative atherosclerotic process associated with aging. Dietary protein had no significant linear or quadratic effect at either age group on triglycerides. Protein at 1 year of age had a significant quadratic effect on cholesterol ($p < 0.05$) and a strong linear interaction with calories ($p < 0.01$) on cholesterol but at age 2 this relationship was lost.

CHAPTER VI

SUMMARY

The effects of age, dietary protein and calorie restriction on serum cholesterol and triglycerides were studied in male rats. Dietary treatments included various protein levels fed with and without concomitant calorie restriction; calorie restriction with and without protein restriction; and protein level either increased or decreased during growth and development without calorie restriction.

When all dietary treatments were combined, for statistical purposes, independent of age, both serum cholesterol and triglycerides were significantly effected ($p < 0.01$). Feeding an ad libitum versus restricted regimen resulted in the restricted-fed animals possessing a significantly lower level ($p < 0.01$) of serum cholesterol and triglycerides. The various levels of dietary protein, fed either ad libitum or restricted, produced an overall quadratic effect on serum cholesterol and no effect on triglycerides. There was a significant linear protein and calorie interaction for both cholesterol ($p < 0.01$) and triglycerides ($p < 0.05$) and quadratic interaction for triglycerides ($p < 0.05$).

Both serum cholesterol and triglycerides were significantly effected by the age of the animal. Serum cholesterol levels of animals 2 years of age were significantly greater than those of the 1 year old animals. However, the reverse was found for triglycerides; i.e., values

of 1 year old animals were consistently greater than those of 2 year old animals.

More specific relationships were made apparent when the dietary treatments were analyzed dependent of age. Feeding constant levels of protein as compared to variable levels (20→12% casein and 20→28% casein) had no significant effect on either cholesterol or triglyceride values at 1 or 2 years of age. Changing from low (20→12% casein) to high (20→28% casein) had a significant effect on triglycerides ($p < 0.01$) but only at 1 year of age; these dietary changes had no effect on cholesterol. When all ad libitum-fed animals were compared to all restricted-fed animals the restricted-fed animals had significantly lower cholesterol and triglyceride values at both ages. Protein intake had a quadratic effect on cholesterol ($p < 0.05$) but only at age 1 year. No linear relationship between either serum cholesterol or triglycerides and protein at either age group was found. The protein and calorie interaction was found to be linear at 1 year of age but only for cholesterol ($p < 0.01$). At 2 years of age the protein and calorie interaction had a tendency to be quadratic ($p < 0.1$) but only for cholesterol.

The data from this study imply that calorie restriction can be a viable method of lowering both serum cholesterol and serum triglycerides in rats. Hypercholesterolemia and hypertriglyceridemia, the principal indicators of a susceptibility for atherosclerotic coronary heart disease in humans, may be controlled through dietary modifications such as calorie restriction.

LITERATURE CITED

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VITA

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