THE EFFECTS OF DIETARY ANIMAL AND VEGETABLE PROTEIN ON THE SYNTHESIS AND METABOLISM OF SERUM CHOLESTEROL LIPOPROTEINS IN FISHER 344 RATS OF VARIOUS AGES

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We hereby recommend that the dissertation prepared under our supervision by Myung-Sook C. Park entitled THE EFFECTS OF DIETARY ANIMAL AND VEGETABLE PROTEIN ON THE SYNTHESIS AND METABOLISM OF SERUM CHOLESTEROL LIPOPROTEINS IN FISHER 344 RATS OF VARIOUS AGES

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CHAPTER I

INTRODUCTION

Hypercholesterolemia is a major risk facotr of coronary heart disease (CHD), the leading cause of death in the United States (8). It is known that an increased concentration of high density lipoprotein (HDL) cholesterol seems to have a protective effect in the etiology of cardiovascular disease (1,2), whereas low density lipoprotein (LDL) cholesterol is associated with the development of atherosclerotic lesions (3,4). Serum cholesterol concentrations have been shown to increase with age in man and in a number of experimental animals (5, 6, 7, 49, 91).

The effects of diet on the course of human atherosclerosis and on other chronic disease states remain to be determined. Dietary protein has been shown to be one factor which can modulate both the amounts and the composition of lipoproteins in the serum (9, 10, 11). The low serum lipid levels of vegetarians have been cited by some as evidence that the source of dietary protein may effect blood lipid levels (12, 13).

Recently, the effects of dietary protein on hypercholesterolemia and atherosclerosis have received much attention. Vegetable protein appears to be less cholesterol-

emic than animal protein in both man and experimental animals (9-13), although the mechanism of action by which plant protein exerts its hypocholesterolemic effect is not clear.

Age-related hyperlipidemia has been reported for both the human man and rats (5,7). Because the incidence of CHD increases with age and because changes in serum lipids and lipoproteins are associated with many diseases that afflict the elderly, it is important to establish the factors and conditions that influence the changes in the levels of LDL and HDL-cholesterol throughout life. It is expected that the changes in dietary protein (animal vs. vegetable) are reflected in changes in serum lipoprotein cholesterol metabolism. Dietary proteins should be examined to determine their effects on age-related changes in the metabolism of serum cholesterol and other serum lipids.

It is also important to realize that proteins from plant sources are useful in reducing the amount of expensive animal protein that is consumed by man. Relative costs of utilizable vegetable proteins are lower than those of meat proteins. With an increased use of dietary vegetable proteins, it is reasonable to expect less costly foods in the years ahead.

Objectives

The general objective of this study was to determine how dietary proteins alter both total serum cholesterol and serum lipoprotein cholesterol metabolism. Specifically, the objectives of the experiments described in this dissertation were:

1. To compare the distribution of serum lipoprotein-cholesterol between rats fed animal or vegetable proteins.
2. To examine the effects of various dietary proteins on fecal sterols and lecithin:cholesterol acyltransferase (LCAT) activity.
3. To determine if aging has any effect on dietary protein induced modification in cholesterol and lipoprotein metabolism.

CHAPTER II

REVIEW OF LITERATURE

Cholesterol metabolism

The sterols and their derivatives are essential components of the body. Unlike the triglycerides, which act simply as energy stores, cholesterol and it fatty-acyl esters are important structural components of cell membranes and give rise to derivatives (bile salt, steroid hormones, vitamin D, etc.) that play diverse and important functional roles in the body (14). Much of the biosynthetic and catabolic regulation that governs the turnover of the body pool of cholesterol takes place in the liver and involves the biliary system (enterohepatic circulation as shown in figure 1).

During lipid absorption, small amounts of cholesterol may enter bile salt micelles in the intestine and thence become absorbed by mucosal cells and eventually enter the circulation as chylomicra. In the human gut, the amount of cholesterol that may be absorbed in this manner is severely limited (the maximum is about 0.5 g/day), and excess dietary cholesterol is excreted in the feces (14). The cholesterol in chylomicra is largely taken up by the liver, where it may either be esterified with fatty acyl CoA to form the

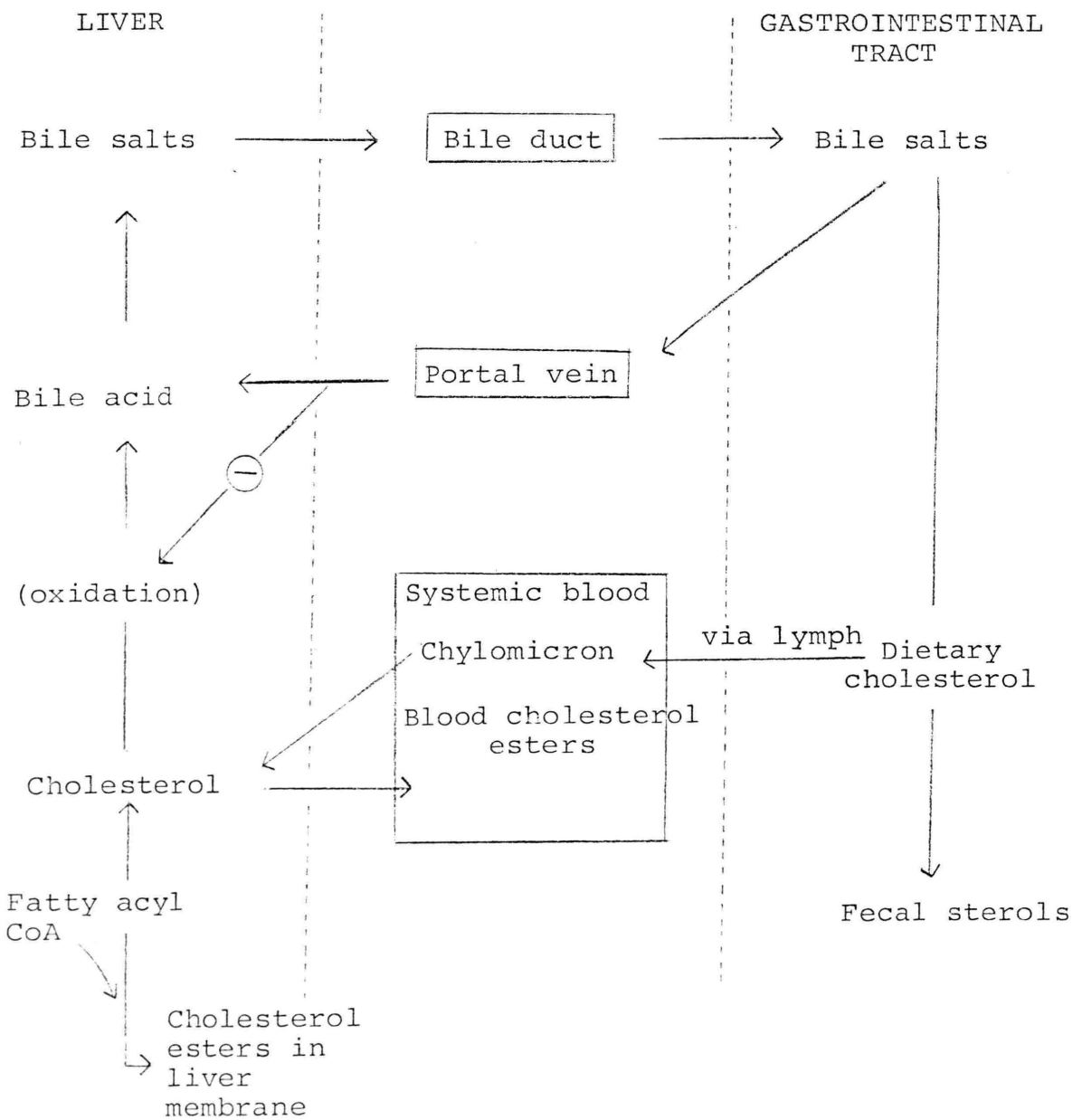


Figure 1. Role of liver and gastrointestinal tract in cholesterol and bile salt metabolism.(-; negative feedback control)

cholesterol esters of the liver cell membranes, or be converted to bile salts. The latter pathway is probably the major catabolic route in terms of the amount of sterol turned over by the body. Moreover, the production of bile salts from cholesterol by the liver is under strict negative feedback control (14). The bile salts returned via the enterohepatic circulation act as inhibitors of the enzymes in the liver that are involved in the oxidation of cholesterol to bile salts as shown in figure 1. It follows that an increase in the excretion of bile salts in the feces will, by preventing normal feedback inhibition, result in an increase in the catabolism of cholesterol by oxidation and hence a decrease in the body's cholesterol pool.

The liver and cells of the intestinal mucosa both have the capacity for de novo biosynthesis of cholesterol from acetyl CoA. The pathway is complex, consequently, the details will not be elaborated here. The important reactions in sterol biogenesis are the first steps; these distinguish the cholesterogenesis system from other lipid synthesis sequences and play the chief role in synthesis regulation. As in lipogenesis, the first step involves the combination of two acetyl groups. In the next stage, a third molecule of acetyl CoA is condensed to form the important six-carbon intermediate, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). In

the liver, the latter molecule is the common precursor of either free acetoacetic acid or cholesterol. The key reaction in sterol synthesis appears to be the next one, in which HMG-CoA is reduced by two molecules of NADPH to mevalonic acid (3,5-dihydroxy-3-methyl valeric acid). This reaction, catalyzed by the specific enzyme HMG-CoA reductase, is irreversible and is the first committed step in the long series of reactions that leads to the building of the cholesterol ring structure. A number of energy-requiring steps lead to the condensation of six isoprenoid 5 carbon intermediates, which become cyclized to a sterol ring (14). In some animals (e.g., rats), the activity of hepatic HMG-CoA reductase is subject to negative feedback control by high-cholesterol diets, the biosynthesis of cholesterol is therefore inhibited due to supression of this early reaction in the pathway (14). This mechanism appears not to be important in man, primarily because the cholesterol uptake from gut is limited and the human hepatic enzyme seems unresponsive to changes in blood cholesterol concentration. A considerable fraction of the total body cholesterol in the human is synthesized, however, by the intestinal mucosal cells. The biosynthesis of chilesterol in man is regulated via the feedback inhibition of the regulatory enzyme (HMG-CoA reductase) by bile acids rather than by cholesterol (14). In

this way, sterol synthesis, bile-acid production and bile-salt recirculation seem to form a system of self-regulation. Cholesterol produced in the liver may be exported in two ways: 1) a portion can pass into the blood system, where it is esterified at the expense of phosphatidycholine, and 2) a portion may be excreted together with bile-salt micelles in the bile fluid. An important potential consequence of this latter excretory pathway is pathologic deposition of excess cholesterol accumulations in the form of gallstones that may lead to the obstruction of the bile duct (15).

Because of its low water solubility, cholesterol does not circulate freely in the blood plasma. Rather it is a component of complex macromolecules (lipoproteins) whose physical and chemical characteristics are defined in table 1. All of these macromolecules are important in transporting lipids from the intestine and liver to adipose tissue and other target organs. Extrahepatic tissues largely derive their cholesterol from plasma LDL (16). Lesser amounts of cholesterol are provided by endogenous synthesis (17) and perhaps by direct uptake from other lipoproteins (18). Excess cholesterol located within peripheral tissues has to be transported to the liver where catabolism and removal from body occurs exclusively by conversion to bile acids.

TABLE 1

Physical Properties and Composition of Human Serum Lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Density (gm/ml)	0.95	0.95-1.006	1.006-1.063	1.063-1.21
Mobility*	origin	prebeta	beta	alpha
Molecular weight	10^3-10^4x10^6	5-10x10^6	2.1-2.6x10^6	2-4x10^5
Size (A)	750-10,000	300-800	215-220	75-100
% Protein	0.5-1.0	5-15	25	45-55
% Triglycerides	85	50-70	5-10	2
% Cholesterol	2-5	10-20	40-45	18
% Phospholipid	3-6	10-20	20-25	30
Major apoproteins	CI, CII CIII, AI	B, CI, CII CIII	B	AI, AII
Minor apoproteins	B	E		D, E, CI, CII, CIII

*Paper electrophoresis

Each of the lipoprotein classes plays a distinct role in the transport of lipids in the blood. Chylomicra are produced in the gastrointestinal mucosa and carry the fats that are absorbed from the diet. Although most of this lipid is carried from the gut to the liver and adipose tissue, any tissue may utilize the fatty acids from chylomicron fats if it possesses an active lipoprotein-lipase enzyme system (14).

The function of the very low density lipoprotein (VLDL) or the pre-beta lipoprotein fraction is also to carry triglycerides to the tissues, but in this instance, it transports exclusively those fats that are synthesized endogenously by the liver. The fatty acids from these triglycerides will therefore arise jointly from the dietary fats processed by the liver and from hepatic fatty-acid synthesis, chain elongation, and desaturation. The major recipient of these fats will be the adipose tissue. Since the VLDL fat is also susceptable to hydrolysis by lipoprotein lipase, however, the VLDL fatty acids are also used by other tissues that possess this enzyme or under circumstances where the lipoprotein lipase activity in the bloodstream is elevated (14).

The low density lipoprotein (LDL) or beta lipoprotein

fraction carries the bulk of the plasma cholesterol, most of which is present as cholesterol which is esterified (19). The process by which VLDL is converted to LDL is believed to play a significant role in removing excess unesterified cholesterol from chylomicron and VLDL (20).

Excess cholesterol in tissues that results from increased LDL uptake and internal biosynthesis must be released into extracellular fluid for return to liver. This cholesterol may be incorporated into high density lipoprotein (HDL) or directly returned to the liver where it may be converted to bile acid (20). Another possible HDL function is that it may prevent cholesterol accumulation in cells by directly blocking the uptake of LDL cholesterol by receptors (16).

The very high density lipoprotein (VHDL) fraction contains albumin-bound fatty acids. The latter represents the transport form of lipids mobilized from the fat depots by action of the hormone-sensitive lipase of adipose tissue. Although the total quantity of fatty acid in this fraction is small (10-20 mg) and represents only about 5% of the total fatty acids present in the plasma, the rapid utilization and liberation of the albumin-bound fatty acids results in an extremely fast turnover of this metabolically active pool. In the fasting state, as much as 70% of the total

energy requirement of the body may be derived from this fatty-acid fraction, with the total circulatory pool turning over completely every few minutes (14).

Transport mechanism of lipoprotein cholesterol

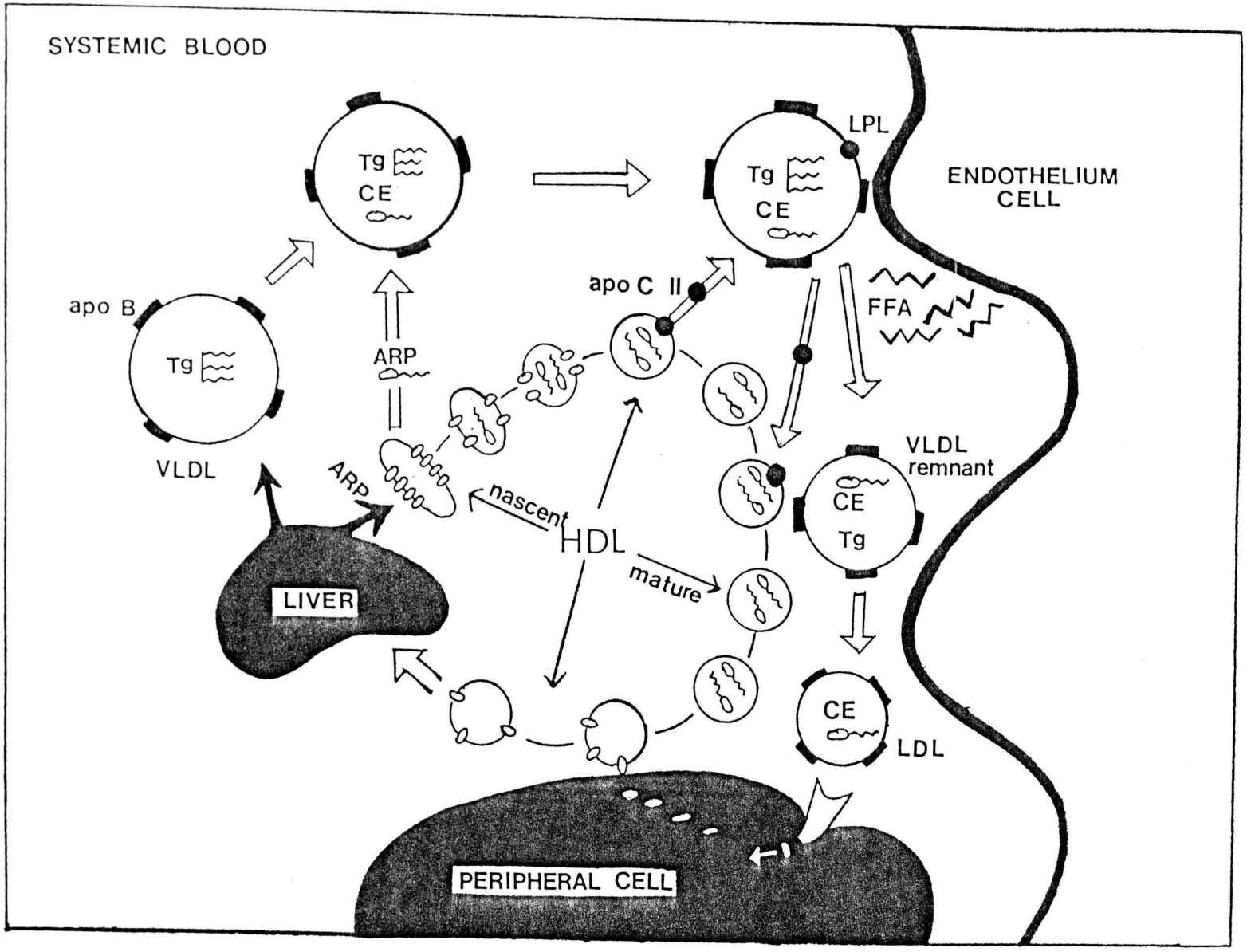
One of the most important concepts to visualize in lipid transport is that the transport of lipoproteins through the plasma is not a passive transport, but that dynamic metabolic changes are occurring. VLDL is broken down into an intermediate density lipoprotein (IDL) form in a matter of hours and, just as rapidly, the IDL is catabolized to LDL. LDL is merely the remnant of VLDL metabolism (the core apo B protein with some of its constituent lipid, cholesterol, and phospholipid) after much of the triglyceride and some of the other constituents have been removed (figure 2).

Brown and Goldstein (16) showed that the LDL (which has a half-life in the plasma of three to four days, in contrast to the VLDL and IDL, whose half lives are measured in hours) is removed by specific receptors on peripheral fibroblasts and other cells. These receptors have specific primary-affinity sites, primary receptors that bind with the LDL and are responsible for their being transported into the cell, where the lipoproteins are broken down to the constituent apoprotein and the lipid enters the lipid pool within

Figure 2. Possible role of LDL and HDL in cholesterol transport (21)

LDL carries cholesterol from its sites of origin to peripheral tissues while HDL carries cholesterol from peripheral tissues via the blood vessels to the liver.

apo B; apolipoprotein B, Tg; triglycerides ( ), CE; cholesteryl esters ( ), ARP; arginine rich protein, LPL; lipoprotein lipase, FFA; free fatty acids ( ), apo C-II; apolipoprotein C-II which is an activator of LPL., LCAT; lecithin:cholesterol acyltransferase which esterifies cholesterol in HDL., free cholesterol; ( ).


SYSTEMIC BLOOD
Tg
CE
LPL
Tg
CE
ENDOTHELIUM
CELL
apo B
Tg
VLDL
ARP
ARP
apo C II
FFA
nascent
HDL
mature
VLDL
remnant
CE
Tg
LIVER
CE
LDL
PERIPHERAL CELL

the cell (figure 3). Further, it has been demonstrated that in humans the concentrations of LDL cholesterol in our bloodstream totally saturate in vivo the primary LDL receptor sites, and thus other nonspecific receptors become involved in the clearance of lipoproteins (16). It is interesting that in most mammals such as the pig, guinea pig, sheep, cow, camel, rabbit and rat the concentration of LDL cholesterol is easily handled by the primary LDL receptors, suggesting that there is an equilibrium and that essentially all of the lipid is being cleared through this route. It had been postulated that the reason why humans are almost uniquely susceptible to atherosclerosis is related to the fact that our LDL concentrations are so much higher than they need to be, and are above the threshold for primary clearance (16).

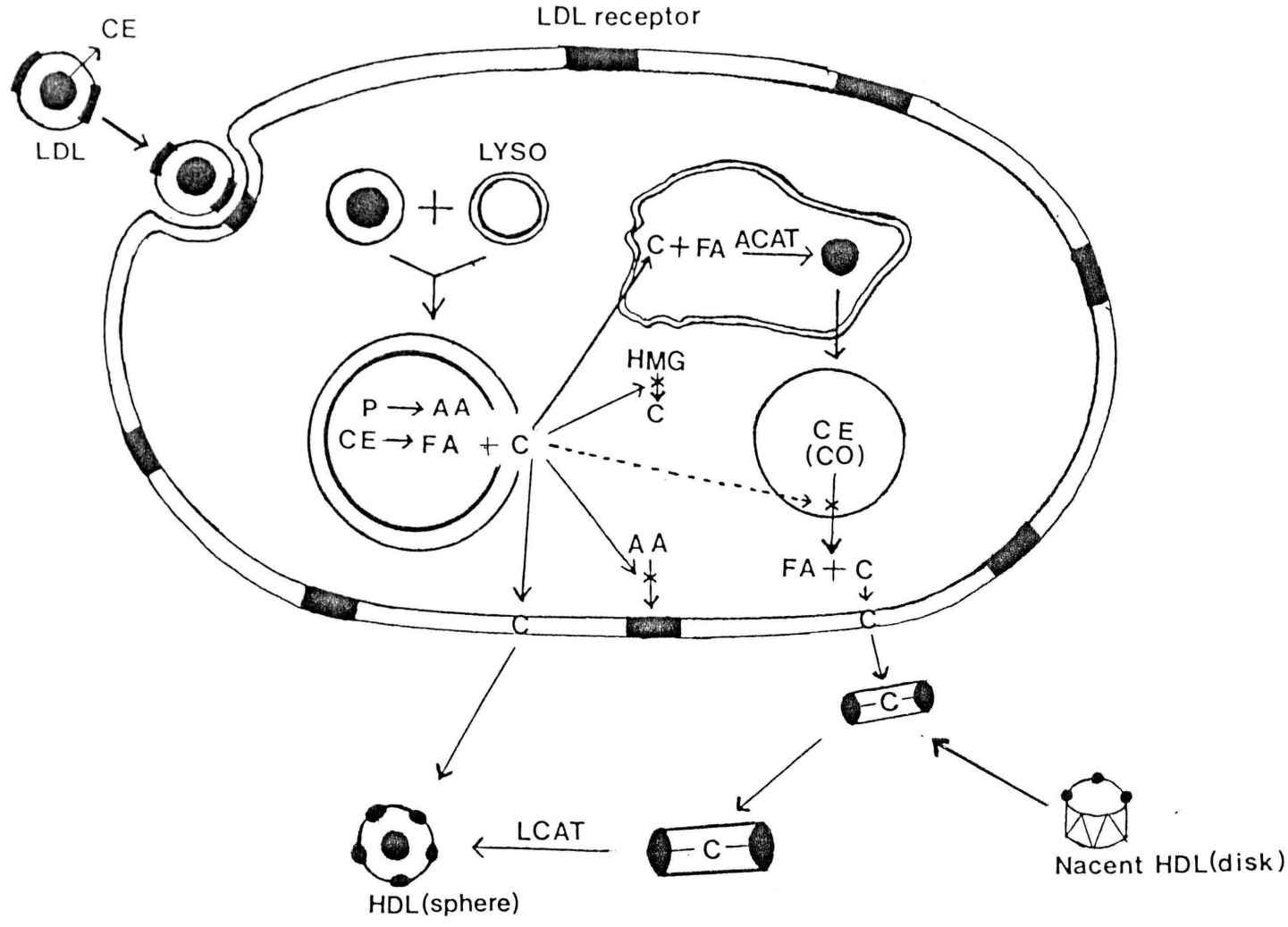
Metabolic reactions in which lecithin:cholesterol acyltransferase is involved

Lecithin:cholesterol acyltransferase (LCAT) cannot act on pure lipids and requires polypeptide cofactors to be present during the reaction (23). In vivo, LCAT acts on plasma lipoproteins which donate the lipid substrates for transesterification. HDL and VLDL were reported to be preferred substrates for LCAT (23).

Cholesteryl esters, which are one of products of the

Figure 3. Pathway for LDL breakdown in nonhepatic tissue (cellular uptake, metabolism and removal of cholesterol) (22).

Low density lipoprotein (LDL) is composed of a core of cholesterol ester (CE) and triglyceride and a shell of phospholipid (PL), free cholesterol (C) and protein (P). Once LDL binds to a receptor (R) on the cell surface, the receptor-LDL complex migrates to become an endocytotic vesicle and fuses with a lysosome (LYSO). In the acid interior of the lysosome, P is hydrolyzed to amino acids (AA) and CE to fatty acids (FA) and C. The C must then translocate to other parts of the cell. As excess C emerges from the lysosome, it first inhibits synthesis of new C from hydroxy methylglutarate (HMG) and then stimulates formation of CE, consisting mainly of cholesteryl oleate (CO), by the acyl coenzyme A:cholesterol acyltransferase (ACAT) system. In addition the excess C inhibits synthesis of new receptor and perhaps blocks cytoplasmic hydrolysis of newly formed CE. The broken line indicates that this pathway has not been fully established. The acceptors for C, disk-shaped nascent high density lipoprotein (HDL) and intact spherical plasma HDL, pick C and possibly PL from the outer leaf of the plasma membranes. As C enters nascent or intact HDL, lecithin:cholesterol acyltransferase (LCAT) converts surface C to CE, which leaves the surface to form the core of HDL. The removal of CE from the surface permits the particle to pick up more C. (22)



RECEPTOR BINDING → ENDOCYTOSIS → LYSOSOMAL CATABOLISM (hydrolysis) → HMG CoA reductase ↓
CE ↑
LDL receptor ↓

LCAT reaction, are thought to migrate towards the hydrophobic interior of the substrate HDL particles or transfer to VLDL with the aid of transfer proteins(s) (24). Serum albumin has been shown to activate LCAT by sequestering lysolecithin (24). From model studies it has been shown that LCAT reacts readily with liposomes that contain a 3:1 molar ratio of lecithin to cholesterol (25).

Relationship between HDL and LCAT

In epidemiological studies, plasma levels of HDL-cholesterol are correlated inversely with the incidence of atherosclerotic cardiovascular disease (1). There is probably an inverse correlation between concentration of plasma HDL-cholesterol and tissue cholesterol pools. It has been postulated that low plasma concentration of HDL may result in decreased cholesterol clearance from peripheral tissues, including the arterial wall, with subsequent enhancement of the atheromatous process (22). Evidences that plasma HDL promotes a net movement of cholesterol from erythrocyte membranes was provided by the demonstration that the cholesterol content of HDL could be increased substantially by incubation with erythrocyte ghost (26). Bates and Rothblat (27) similarly found that HDL promoted a greater release of desmosterol from cultured mouse fibroblast (28,29).

Another explanation for the protective role of HDL has been proposed by Carew et al. (30). They found a marked decrease in binding, internalization, and degradation of LDL into cells incubated in the presence of HDL. Therefore if uptake of LDL by arterial wall smooth muscle cells is involved in the initiation or the development of atheromatous lesion, the HDL might directly interfere with this process. An inverse relationship between HDL concentration and the size of the body cholesterol pool further supports the notion that HDL is instrumental in promoting removal of tissue cholesterol (31).

HDL, as secreted by the perfused rat liver, differs in several respects from the HDL present in the peripheral blood of intact animals (32). The nascent form of HDL consists chiefly of bilaminar discoid particles. The cholesterol of discoidal HDL isolated from rat liver perfusates or from the plasm of the inherited LCAT-deficiency patient has been shown to be esterified by LCAT in vitro at a greater rate than the cholesterol of normal plasma HDL (8,33). The esterification of nascent HDL-cholesterol by LCAT is accompanied by a change in morphology of the particles, which become transformed from discs to speres with diameters similar to normal HDL. On the basis of this observation, it seems probably that the transforma-

tion of nascent HDL to plasma HDL in vivo involves at least the following processes (34): hydrolysis of surface lecithin and the esterification of surface cholesterol by LCAT; the movement of the resultant lysolecithin to albumin; the movement of a proportion of the resultant nonpolar cholesteryl ester into the inner hydrophobic region of the phospholipid particle; and the transfer of other cholesteryl ester molecules to triglyceride-rich lipoproteins. VLDL and LDL showed little or no reactivity with LCAT in the absence of HDL (26). This finding presumably reflected the transfer of newly synthesized cholesteryl esters from HDL.

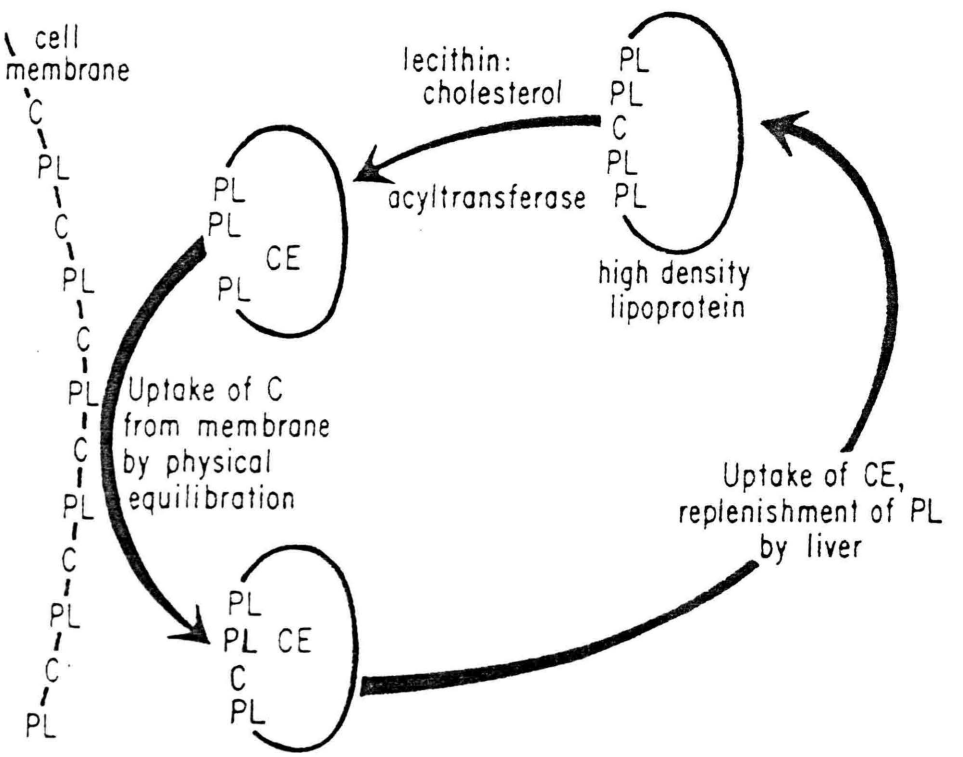
Role of LCAT in reverse cholesterol transport

The return of excess peripheral cholesterol to the liver has been designated as the "reverse cholesterol transport" pathway (figure 4). This mechanism, involving HDL and LCAT, is apparently instrumental in protecting the cardiovascular system against atherosclerosis in humans (35). LCAT may exert a controlling influence on the turnover of cholesterol in the rat plasma compartment as well, since Petersburg and Ellefson (36) showed that esterified lipoprotein cholesterol is cleared from rat plasma much faster than free cholesterol.

A finding of Glomset (24) that HDL provides the

Figure 4. Postulated mechanism for the transport of cholesterol from membranes of peripheral cells to the liver (24).

Lecithin:cholesterol acyltransferase reacts with circulating lipoproteins to form cholesteryl esters (CE) from unesterified cholesterol (C) and lecithin. The lipoproteins subsequently pick up C from cell membranes, and circulate through the liver to release CE and to pick up more phospholipids (PL).


cell membrane
C
PL
C
PL
C
PL
C
PL
C
PL
C
PL
C
PL
C
PL
lecithin: cholesterol
acyltransferase
PL
PL
C
PL
PL
high density lipoprotein
PL
PL
CE
PL
Uptake of C from membrane by physical equilibration
PL
PL CE
C
PL
Uptake of CE, replenishment of PL by liver

preferred substrates for LCAT, due probably to activation of the enzyme by apoprotein A-I, prompted the suggestion that HDL and LCAT may play concerted roles in transporting cholesterol from peripheral tissues. Glomset (24) proposed the mechanism shown in figure 4 to explain the role of LCAT in the removal of cholesterol from the peripheral tissues. According to his explanation, the esterification of cholesterol in HDL has the following consequences.

1. The esterified cholesterol is displaced toward the hydrophobic interior of the spherical HDL particle.
2. The lysolecithin generated by LCAT is eventually sequestered by serum albumin and transported to the liver.
3. Following reaction with LCAT, HDL particles interact with plasma membranes to acquire surface lipid components (phospholipid and cholesterol) or will be sequestered and catabolized by the liver and extrahepatic tissues.

The release of cholesterol from cultured cells occurs predominantly in the unesterified form. It has been confirmed that the majority of unesterified cholesterol removed from rat erythrocyte by the rat HDL in the presence of LCAT is recovered in the cholesteryl ester fraction.

Cholesterol metabolism and aging

Age related hyperlipidemia in humans may be due to environmental factors or perhaps to complications from a number of age-related metabolic diseases. In Caucasian American men there is a five fold increase in the incidence of atherosclerosis from age 30 to age 65 with more than 80% of the cases of atherosclerotic cardio vascular disease being found in individuals over age 65 (37). In general serum and tissue cholesterol levels increase with age in humans (37) and rats (38). Bloch et al. (39) reported that cholesterol output into the bile of rats decreased by 50% between 6 and 44 weeks of life. The old rats exhibited increased HDL and LDL-cholesterol concentrations (7,40,41). Not only does cholesterol synthesis decrease with age (42) but both excretion (43) and turnover of cholesterol (44) decrease as well. In relation to the previously mentioned age-related changes in cholesterol metabolism, many other changes have been reported, such as; a decrease in the biliary and fecal excretion of cholesterol and bile acids, a decrease in hepatic cholesterol 7-α-hydroxylase activity, a decrease in cholesterol absorption, and a decrease in the maximum transport capacity of bile acid through the liver (45).

Because of man's increasing susceptibility to athero-

sclerosis with age, studies using aortic tissue have received special attention (46). Lipids concentration in human aortic tissue increase in quantity with age. Between the age of 6 and 56, aortic phospholipids, triglycerides, and free cholesterol rise by 145,150 and 99%, respectively; esterified cholesterol rises by 5800% (45). Research emphasis has also been placed on studying the effect of age on serum choleserol levels. Keys et al. (47) found a definite rise of total serum cholesterol with age in a study involving 1492 man. The average cholesterol levels (mg/dl) in different age groups were 17-25(177), 25-30(192), 30-45(210), 45-60(248) and 60-72(227). The concentrations of serum lipoprotein cholesterol have also been reported to change with age. In both man and primate serum LDL-cholesterol increases with age while HDL-cholesterol decreases with age (8,48). Lifelong elevation of HDL-cholesterol concentrations have been hypothesized to be correlated with longevity.

As it has become more apparent that atherosclerosis is an age-related disease, key lipid metabolism-related enzymes have been studied to determine changes in their specific roles with age (48,49,50). LCAT is one of the enzymes since it has been postulated that LCAT facilitates the transport of peripheral cholesterol to the liver (23).

The LCAT reaction also causes many other changes in serum lipids. Goldstein and Brown (51) postulated that acyltransferase activity may be important in the regulation on lipid metabolism in vivo with an eventual effect of mortality risk. Liu et al. (52) reported that plasma LCAT activities were significantly lower than normal in hyperlipidemic (type II_a) patients with coronary heart disease. It appears that age-related changes in some plasma enzymes (substantial decrease of LCAT activity) and lipids (doubling of total serum cholesterol concentration) in rats (2 to 24 months of age) are similar to those associated with genetic and metabolic diseases (48). A consistent trend observed in experimental animals has been an age-related decrease in the fractional rate of plasma cholesterol esterification (48-50). This decrease reflects the dynamic relationship between the enzyme and its substrate (free cholesterol) in the LCAT reaction. LCAT and HDL have also been proposed to act jointly in the clearance of plasma lipids from the circulation (33). It is of interest to investigate how diet alters serum LCAT activity, since few studies have been done regarding this interaction.

Effects of dietary protein on the metabolism of cholesterol

A. Effect of dietary protein on serum cholesterol

Many experiments have indicated that type of fat,

carbohydrate or protein in the diet can influence serum cholesterol concentrations (11-13, 53-67). It has been known for some time that hypercholesterolemia and atherosclerosis can be produced in rabbits by feeding a cholesterol-free semisynthetic casein diet (68), whereas this does not occur in rabbits fed commercial stock diets (69). The first attempts to explain these effects of semisynthetic diets focused primarily on the dietary lipids (70, 71), since it was clear that atherosclerosis and increased serum cholesterol concentration could be largely prevented by including polyunsaturated fats in the diet. It appears, however, that this provides only a partial answer to the question. Kritchevsky and Tepper (69) showed that the protective effect of commercial feed was not due to the small amount of unsaturated fat in the diet. Fat extracted stock (Purina chow) diet did not produce hypercholesterolemia and atherosclerosis in rabbits, and these effects were not altered by adding the extracted fat to a semisynthetic diet.

A dietary component which recently has received significant attention regarding possible effects on cholesterol metabolism is protein. At this time the effect of dietary proteins on the serum lipid concentrations in man is not well understood. A number of reports in the literature indicate that extensive changes in the protein content of

the diet do not produce significant changes in the serum lipid levels. Keys and Anderson (71) found no significant change in the serum lipid levels of metabolically normal middle-aged men eating diets containing either 83 or 130g protein/man/day (8.6 and 17.7%, respectively, of the total caloric intake). Beveridge et al. (72) found no change in serum cholesterol concentrations of University students who went from a diet with a protein content equivalent to 15% of the total caloric intake to a diet with protein levels corresponding to 10, 20 and 25% of the total calories. The fat intake was kept constant at 20% of the total calories. They suggest that the sterol content of corn oil is responsible for its depressant effect on serum cholesterol levels.

Since the question was raised as to whether the amount and origin of dietary protein or low levels of dietary fat produced the lower concentration of serum cholesterol, there have been indications that the cholesterol levels might be influenced by the kind of protein in the diet (62). Recently epidemiological evidence derived from human populations showed that the positive correlation between animal protein diets and mortality from coronary disease was at least as strong as that between dietary fat an coronary disease (74). Studies using experimental animals have

shown that plasma cholesterol concentrations can be reduced by substituting plant protein for animal protein in the diet (62). Sirtori et al. (60) found the hypocholesterolemic effect of soya-bean protein is independent of the lipid composition of the diet. Walker et al. (75) studied how two protein diets, one deriving its protein content from a vegetable source and the other from an animal source, effected serum lipids. The mixed fat content of the two diets was the same in amount and origin. There was a significant decrease in the serum cholesterol concentrations of both groups, especially the vegetable protein group. Howard et al. (76) also found that hypercholesterolemia and atherosclerosis in rabbits fed a purified diet could be reduced by using whole soya flour or hexane-extracted soybean meal as a source of protein rather than casein. On the other hand Neves et al. (64) recently found that pure and crude plant proteins did not have a hypocholesterolemic effect in rats when compared with either pure or crude animal proteins.

B. Effect of dietary amino acids on serum cholesterol

Although the overall effect of dietary protein on cholesterol metabolism has been known for some time, little progress has been made in elucidating the mechanisms involved. Huff et al. (77), using a low-fat cholesterol-free

diet, provided evidence that rabbits fed partially-hydrolyzed soya-bean protein exhibited a greater cholesterol-lowering tendency than rabbits given the corresponding casein diet, but with an amino acid mixture equivalent to that of casein or soya-bean protein the difference was markedly neutralized. Other evidence exists that amino acid composition of dietary proteins can influence serum cholesterol levels. Olson and his associates (78) fed mixtures of L-amino acids to human subjects as part of formula diet and reported that glutamic acid exerts a hypocholesterolemic effect under experimental condition. Yadav and Liener (57) observed that, in rats, an amino acid mixture formulated to simulate the amino acid pattern of soybean isolate likewise produced levels of serum cholesterol which were lower than those obtained with an amino acid mixture corresponding to that of casein.

More recently it has been suggested that the different metabolic effects of animal and vegetable protein may be due to their amino acid ratios (11.79). Examination of the amino acid spectra of casein and soy protein reveals that the ratio of lysine to arginine in the former is twice that of the latter. Kritchevsky et al. (11) performed two experiments in which enough arginine was added to a casein diet to approximate the lysine/arginine ratio of soy protein

and enough lysine was added to a soy protein diet to give a lysine/arginine ratio seen in casein. They found that atherogenicity of a semipurified diet can be affected by addition of specific amino acids in rabbits. Addition of lysine to soy protein diet increased the diets atherogenicity by 100% and 50% in two experiments. Addition of arginine to casein had a hypocholesterolemic effect with a 41% reduction in one experiment and a 13% decrease in the other. Liepa and Park (80) also reported that an arginine-supplemented casein diet has a hypocholesterolemic effect in rats. In view of these findings it seems apparent that the addition of specific amino acid(s) to diets seems to influence serum cholesterol concentration.

C. Effect of dietary protein on fecal excretion of neutral sterols

The effect of dietary protein on serum cholesterol concentration can be explained to some extent by changes in the excretion patterns of fecal steroids (59,65,81). Nagata et al. (65) found that rats fed soya-bean protein excreted significantly greater amounts of neutral sterols and showed lower serum cholesterol concentrations than animals fed casein. In a recent study, Pathirana et al. (82) found that rats fed soya-bean protein excreted a significantly greater amount of both total biliary acid and total neutral sterols

than those given milk protein. However, several studies in rats (59,65,83) and rabbits (84) have been shown that the cholesterol-lowering properties of dietary plant proteins compared to casein are mediated by an increased drainage of neutral sterols. Data regarding fecal removal of lipids is complicated by the reabsorption of bile acids and cholesterol via the enterohepatic circulation. Sklan et al. (85) reported that proteins that are not completely digested in the small intestine interfere with the absorption of bile acids and cholesterol. It has been shown that the major pathway for the disposal of sterols from the body is the excretion of bile acids and neutral sterols in the feces (86).

D. Effect of dietary protein on LCAT activity

Dietary protein can also affect plasma LCAT activity (63,87). Yashiro and Kimura (87) have shown an increase in LCAT activity with an increase in dietary protein and in exercise levels in rats. Forsythe et al. (63) reported that pigs fed animal protein showed decreased plasma LCAT activity and increased free cholesterol concentrations when compared to animals fed plant protein. But Neves et al. (64) failed to demonstrate that plasma LCAT activity of rats fed crude and pure animal proteins is different from that of animals fed crude and pure plant proteins. Liepa and Park (80)

have shown that dietary protein may play a role in the regulation of cholesterol metabolism via effect on LCAT as described in appendices of this dissertation. According to their findings, the feeding of casein protein increased serum LCAT activity and the level of serum cholesterol in rats when compared to cottonseed protein. LCAT activity seemed to be influenced by the concentration of free cholesterol which was higher in animals fed the cottonseed protein.

CHAPTER III

MATERIALS AND METHODS

Thirty immature (three-month old) and thirty-nine mature (nine-month old) male Fisher 344 rats were obtained from Charles Rivers Breeding Laboratories Inc., North Wilmington, MA. Animals were individually housed in metabolic cages in an air-conditioned room with controlled temperature (20-23^{o} C) and lighting (alternating 12 hours period of light and dark). All the animals were fed a pellitized commercial diet (#5001 Purina Lab. Chow diet) for three days after arrival. The rats in each age group were randomly divided into three groups, and assigned to either an animal protein diet (casein) or one of two plant protein diets (soy bean or cottonseed protein). Concentrated plant proteins were used on the diets of three month old rats whereas isolated proteins were used for nine month old rats. All diets were isoenergetic and isonitrogenous (tables 2 and 3). The animals were given food and distilled water ad libitum during the experimental period (28 days).

This study was originally developed from a preliminary study (80) which was done prior to this study. Animal model and experimental techniques used in this study were tested in the preliminary study. Throughout this experiment feces

TABLE 2

Composition of diets fed to three-month old rats[1]

Ingredient	Casein	Soy protein	CSP[2]
	%	%	%
Casein[3]	20.0	0.0	0.0
Soy flour[4]	0.0	30.6	0.0
Cottonseed flour[5]	0.0	0.0	28.4
D,L-Methionine	0.3	0.0	0.0
Corn Starch	15.0	12.9	13.2
Sucrose	50.0	42.8	43.9
Celufil	5.0	4.1	4.0
Corn oil	5.0	4.9	4.9
Mineral mix[6]	3.5	3.5	3.5
Vitamin mix[7]	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2

[1]Supplied by Nutritional Biochemicals, Cleveland, Ohio.

[2]CSP: Cottonseed protein diet.

[3]Vitamin free casein, supplied by Nutritional Biochemicals, Cleveland, Ohio, contained the following: moisture, 9.5%; protein (N x 6.25), 85.5%; ash, 2.1%; fat, 1.2%; carbohydrate, 1.7%.

[4]Supplied by Nutritional Biochemicals, Cleveland, Ohio, contained the following: moisture, 8.0%; protein (N x 6.25), 65.3%; ash, 4.66%; fat, 0.3%; carbohydrate, 19.8%; fiber, 2.92%.

[5]Supplied by Oilseed Protein Research Center at Texas A&M University, College Station, Texas, contained the following: moisture, 5.1%; protein (N x 6.25), 70.52%; ash, 6.60%; fat, 0.10%; carbohydrate, 17.28%; fiber, 0.4%.

[6]AIN mineral mixture 76 contained (g/kg mixture): Calcium phosphate, dibasic, 500; Sodium Chloride 47; Potassium Citrate Monohydrate, 220; Potassium Sulfate, 52; Magnesium Carbonate, 3.5; Ferric Citrate, 6.0; Zinc Carbonate, 1.4; Cupric Carbonate, 0.3; Potassium Iodate, 0.01; Sodium Selenite, 0.01; Chromium Potassium Sulfate, 0.55; Sucrose, finely powdered, 118.

[7]AIN vitamin mixture 76 contained (in g/kg mixture): Thiamin HCL, 0.6; Riboflavin, 0.6; Pyridoxine HCL, 0.7; Nicotinic Acid, 0.003; D-Calcium Pantothenate, 0.0016; Folic Acid, 0.2; D-Biotin, 0.02; Cyanocobalamin (Vit. B-12), Retinyl Palmitate (Vit. A), DL-α-Tocopheryl Acetate (Vit. E), pre-mix, 20; Cholecalciferol (Vit. D_3), 0.0025; Menaquinone (Vit. K), 0.005; Sucrose, finely powdered, 972.9.

TABLE 3

Composition of diets fed to nine-month old rats[1]

Ingredient	Casein	Soy protein	CSP[2]
	%	%	%
Casein[3]	20.0	0.0	0.0
Soy isolate[4]	0.0	20.0	0.0
CSP isolate[5]	0.0	0.0	20.0
D,L-methionine	0.3	0.0	0.0
Corn starch	15.0	15.0	15.0
Sucrose	50.0	50.0	50.0
Celufil	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0
Mineral mix[6]	3.5	3.5	3.5
Vitamin mix[7]	1.0	1.0	1.0
Choline Bitartrate	0.2	0.2	0.2

[1]Supplied by U.S. Biochemicals, Cleveland, Ohio.

[2]CSP: Cottonseed protein diet.

[3]Vitamin free casein, supplied by U.S. Biochemicals, Cleveland, Ohio, contained the following: moisture, 9.8%, protein (N x 6.25), 85.5%; ash, 1.8%; fat, 1.0%; carbohydrate, 1.8%; fiber, 0.1%.

[4]Supplied by U.S. Biochemicals, Cleveland, Ohio, contained the following: moisture, 5.5%; protein (N x 6.25), 86.0%; ash, 3.8%; fat, 0.8%; carbohydrate, 3.8%; fiber, 0.1%.

[5]Supplied by Oilseed Protein Research Center at Texas A&M University, College Station, Texas, contained the following: moisture, 3.7%; Protein (N x 6.25), 87.8%; ash, 6.5%; fat, 0.6%; carbohydrate, 3.8%; fiber, 0.6%.

[6]AIN mineral mixture 76.

[7]AIN vitamin mixture 76-A contained (in g/kg mixture): Thiamine HCl, 0.6; Riboflavin, 0.6; Pyridoxine HCl, 0.7; Nicotinic Acid, 0.003; D-Calcium Pantothenate, 0.0016; Folic Acid, 0.2; D-Biotin, 0.02; Cyanocobalamin (Vit. B-12), 0.001; Retinyl Palmitate (Vit. A) pre-mix, 0.8; DL-α-Tocopheryl Acetate (Vit. E), pre-mix, 20; Cholecalciferol (Vit. D_3), 0.0025; Menaquinone (Vit. K), 0.05; Antioxidant, 0.01; Sucrose, finely powdered, 072.8.

were collected for the determination of fecal neutral sterols. Food consumption and weight gain were measured every three days. At the end of the experimental period, animals were anesthetized with ether following a 12 hour fast. Blood samples were taken from the inferior vena cava for determination of total serum cholesterol and HDL-cholesterol concentrations and LCAT activities. Both feces and serum samples were stored at -20°C until analyses could be done. HDL-fractions were separated using a heparin-manganese precipitation procedure (88) and measured quantitatively using an enzymatic method (BMC reagent set cholesterol, Biodynamic/BMC, Indianapolis, IN). Fecal neutral sterols were quantitatively analyzed by gas-liquid chromatography (89) using a 3% SP on 100/120 mesh Supelcoport column (Supelco Inc., Bellefonate, PA). The internal standard used was 5-α-cholestane (Supelco Inc.). A Bendix 2500 gas chromatograph equipped with a flame ionization detector was used for this assay. LCAT activity was measured by determining the initial rate of cholesterol esterification using the method described by Stokke and Norum (90) and modified by Lacko (91). The number of nmoles cholesterol esterified per milliliter per hour, as used in determining LCAT activity, are calculated by multipling the fractional rates by the endogenous free cholesterol concentration present in serum.

Detailed procedures for each analysis are described in the attached appendices.

Analysis of variance, correlations and the student t-test were done using the statistical package for the social sciences (92). The means were compared using least significant differences as calculated from analysis of variance. Comparisons were made as 3 month old animal vs. 9 month old animal and animal protein vs. plant proteins.

CHAPTER IV

RESULTS

All animals of like ages showed similar food consumption patterns. Body weights of 3-month old rats increased more rapidly than those of 9-month old rats (figure 5). Total serum cholesterol concentrations were higher in animals fed the casein diet than in animals fed the plant protein diets (soy protein and cottonseed protein) (table 4). Soybean and cottonseed protein produced comparable reductions in total serum concentration (table 4). Total serum cholesterol concentrations were significantly higher in mature rats than in immature rats (table 4). There was no significant difference between the concentrated and isolated plant proteins in reduction of serum cholesterol. When HDL-cholesterol concentrations were compared between plant protein dietary levels they were significantly higher in the serum of animals fed cottonseed protein than in those fed soy protein. Unlike the profile of total serum cholesterol, HDL-cholesterol concentrations of mature rats were not significantly different from those of immature rats. Percentages of serum HDL-cholesterol concentrations were similar between groups of immature rats fed different diets. Among groups of mature animals they were significantly

Figure 5. Growth performance of 3 month and 9 month old Fisher 344 rats maintained on isocaloric diets containing various dietary proteins (casein, o ; soy protein, Δ ; cottonseed protein, ●)

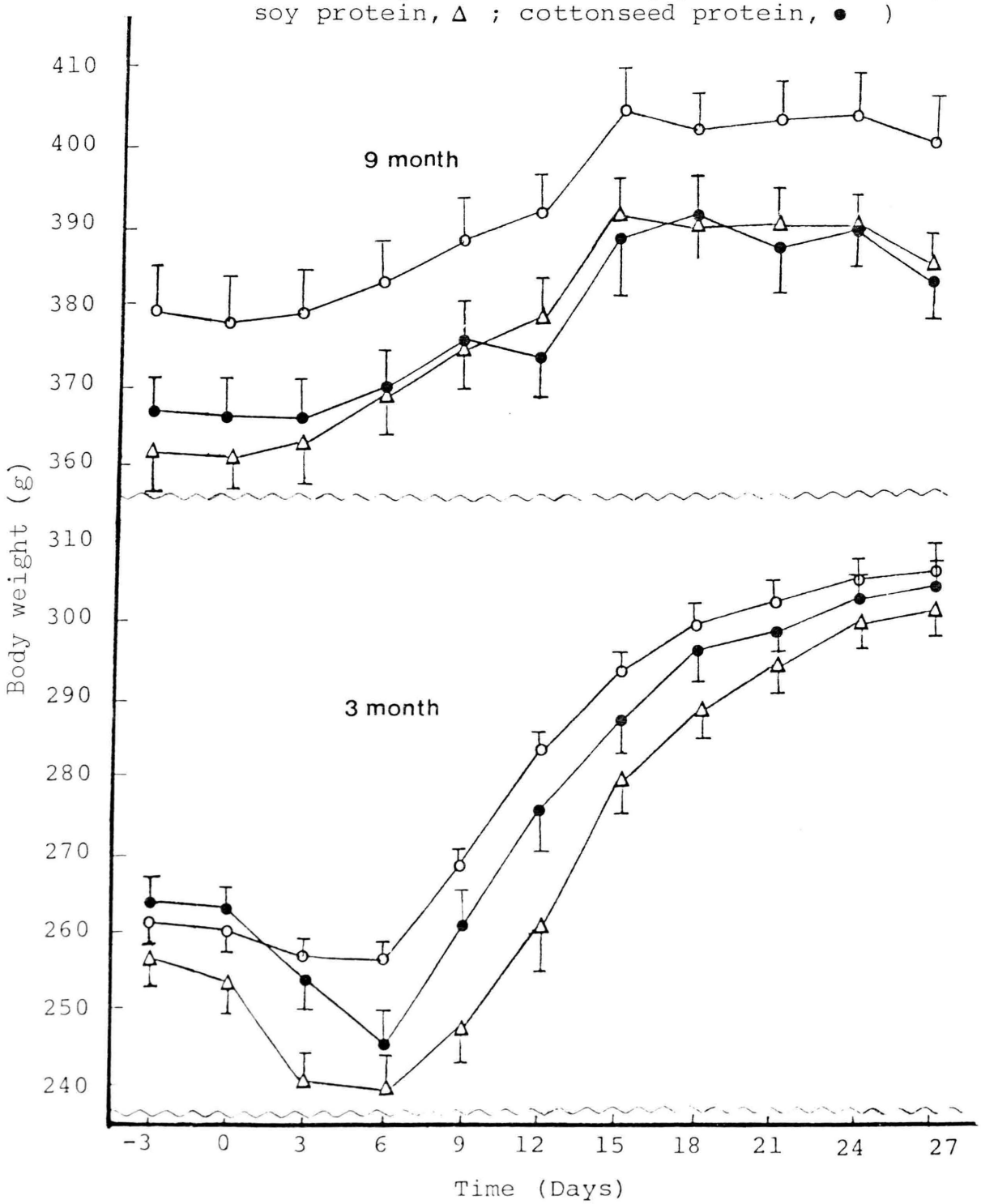


TABLE 4

Effect of Dietary Protein and Age Difference on Serum and High Density Lipoprotein (HDL) Cholesterol (Mean ± SEM)

Age (months)	Diet	No. of Animals	Cholesterol (mg/dl) Serum	Cholesterol (mg/dl) HDL	HDL-cholesterol Serum Cholesterol (%)
3	Casein (N=10)	10	89.1 ± 6.1	56.3 ± 6.2	62.3 ± 3.9
	Soy protein (N=10)	10	45.0^{a} ± 2.5**	26.7^{a} ± 1.9***	60.7 ± 4.8
	Cottonseed protein (N=10)	10	67.4^{a} ± 3.8*	$43.6^{(a)b}$ ±3.0$^{(*)***3}$	64.7 ± 2.8
9	Casein (N=13)	13	104.1^{2} ± 3.5	62.1 ± 2.8	59.8 ± 2.5
	Soy protein (N=13)	13	$58.4^{1,a}$ ± 3.2***	30.3^{a} ± 1.5***	52.6^{a} ± 2.0*
	Cottonseed protein (N=13)	13	$51.5^{1,a}$ ± 2.0***	$36.5^{a(b)}$ ±2.5***(*)	$68.7^{(a)b}$ ±3.9$^{(*)***}$

[a,b]Letters indicate diets used for comparison; (a, casein diet; b, soy protein diet) to indicate significant difference.

***Significant difference ($p < 0.0002$)

**Significant difference ($p < 0.005$)

*Significant difference ($p < 0.05$)

[1,2]Numbers indicate age used for comparison with 3 month of same dietary group to indicate significant difference (1, $p < 0.005$; 2, $p < 0.05$).

[3]Comparison of significance in parenthesis was done at significance level of corresponding parenthesis.

higher only in rats fed cottonseed protein diet when compared to those fed casein diet.

Both mature and immature animals fed plant protein diets excreted significantly higher coprostanol than those fed casein diet (table 5). Rates of fecal coprostanol excretion were significantly higher in immature rats relative to mature rats (table 5).

In this study concentration of free cholesterol and the franctional esterification rate of cholesterol were measured to determine the activity level of LCAT. The concentration of free cholesterol was higher in animals fed casein diet than those fed plant protein diets (table 6). The fractional esterification rates, which are the percentage of cholesterol esterified per hour, were significantly higher in the animals fed plant proteins than in those fed animal protein. The percentages of cholesterol esterification per hour were higher in immature animals than in mature animals (table 6). Fractional LCAT activity was negatively correlated with both free cholesterol ($r= -0.64$) and cholesteryl ester ($r= -0.57$) in serum (figure 6). This observation is in agreement with previously reported results (63) and may explain part of the decrease in fractional esterification rate that occurred when animal protein was fed. Both immature and mature animals had higher free cholesterol levels when fed animal protein: and thus, on a

TABLE 5

Effect of Dietary Protein and Age Difference on Fecal Neutral Sterols in Fisher 344 Rats (Mean ± SEM)

Age (month)	Diet	Dry fecal weight (g/day)	Fecal neutral sterols		
			Coprostanol (mg/day)	Cholesterol (mg/day)	Total (mg/day)
3	Casein (N=10)	1.2 ± 0.0	1.8 ± 0.1	0.9 ± 0.1	2.7 ± 0.1
	Soy protein (N=10)	$1.7^{a} \pm 0.0^{***}$	$2.5^{a} \pm 0.1^{***}$	$1.3^{a} \pm 0.1^{**}$	$3.8^{a} \pm 0.2^{***}$
	Cottonseed protein (N=10)	$1.6^{a} \pm 0.0^{***}$	$2.3^{a} \pm 0.1^{**}$	$0.9^{b} \pm 0.1^{*}$	$3.2^{a} \pm 0.2^{**}$
9	Casein (N=13)	$1.3^{2} \pm 0.0$	$1.2^{1} \pm 0.1$	0.7 ± 0.0	$2.0^{1} \pm 0.1$
	Soy protein (N=13)	$1.4^{1} \pm 0.0$	$1.5^{1,a} \pm 0.1^{**}$	$0.9^{1,a} \pm 0.0^{**}$	$2.4^{1,a} \pm 0.1^{*}$
	Cottonseed protein (N=13)	$1.6^{ab} \pm 0.0^{***}$	$1.5^{1,a} \pm 0.1^{**}$	$0.9^{a} \pm 0.0^{**}$	$2.5^{1,a} \pm 0.1^{***}$

^{a,b}Letters indicate diets used for comparison; (a, casein diet; b, soy protein diet) to indicate significant difference.

^{1,2}Numbers indicate age used for comparison with 3 month of same dietary group to indicate significant difference (1, $p < 0.005$; 2, $p < 0.05$).

TABLE 6

Effect of Dietary Protein and Age Difference on Serum Cholesterol Esterification and Lecithin: Cholesterol Acyltransferase Activity in Fisher 344 rats (Mean ± SEM)

Age	Diet	Serum Cholesterol		HDL[4]-cholesterol		LCAT activity	
		Free	Esterified	Free	Esterified	%/hr	nMole/ml/hr[5]
		(mg/dl)		(mg/dl)			
3	Casein (N=10)	25.2 + 1.4	64.0 ± 5.1	16.0 ± 1.6	40.1 ± 4.9	14.1 ± 1.0	90.0 ± 4.9
	Soy protein (N=10)	15.7^{a} ± 1.0***	29.4^{a} ± 2.0***	9.7^{a} ± 0.6**	17.0^{a} ±1.8***	21.4^{a} ±0.8***	92.2 ± 7.6
	Cottonseed protein (N=10)	$21.1^{(a)b}$±1.1(*)**[3]	46.3^{ab}± 2.8*	14.8^{b} ±0.9**	28.8^{ab}± 2.7*	20.7^{a} ±0.9**	114.5 ± 13.8
9	Casein (N=13)	32.1^{1} ± 1.1	72.4 ± 2.7	16.3 ± 0.6	45.6 ± 3.2	9.5^{1} ± 0.8	79.2 ± 6.6
	Soy protein (N=13)	$21.0^{1,a}$±1.0**	$37.2^{2,a}$±2.5***	9.7^{a}± 0.7**	20.5^{a}±2.5***	17.9^{a}±1.7***	90.7 ± 5.7
	Cottonseed protein (N=13)	18.1^{a}±1.2***	$33.0^{1,a}$±1.7***	$11.4^{2,a}$±0.9***	35.9^{ab}±2.6**	18.8^{a}±1.4***	85.0^{2}± 3.8

[a,b] Letters indicate diets used for comparison; (a, casein diet; b, soy protein diet) to indicate significant difference.

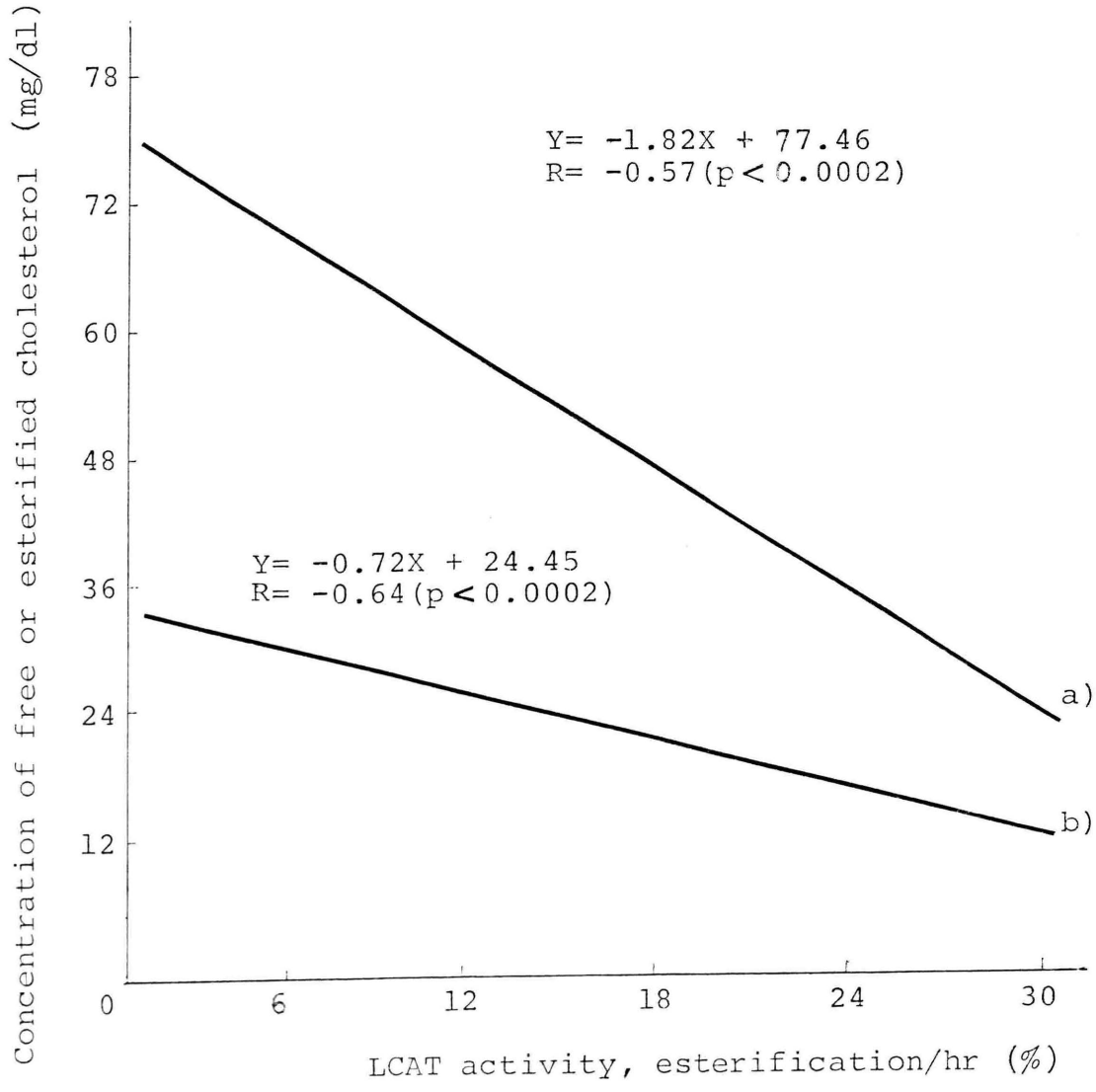
[1,2] Number indicate age used for comparison with 3 month of same dietary group to indicate significant difference (1, $p < 0.005$; 2, $p < 0.05$).

[3] Comparison of significance in parenthesis was done at significance level of corresponding parenthesis.

[4] High density lipoprotein.

[5] The molar rate of esterification is the product of the respective free cholesterol levels and fractional rates.

Figure 6. Correlations between a) serum cholesterol ester and LCAT activity, b) serum free cholesterol and LCAT activity in combined dietary group.



percentage basis, esterified less cholesterol than animals with lower cholesterol levels. The molar rate of esterification is the product of the respective free cholesterol levels and fractional rates, thus the changes in the fractional rate of esterification, when paralleled by an increase of free cholesterol values, did not result in a significant decrease in the molar rate of esterification of the animals fed plant protein diets (table 6).

DISCUSSION

This research project was designed to determine how various types of dietary protein altered cholesterol metabolism in immature and mature rats. Surprisingly, there was little variation in growth response to the various dietary protein used (figure 5).

In various animal species, it has been found that the incorporation of casein in the diet resulted in elevated levels of serum cholesterol. On the other hand, low levels of serum cholesterol were maintained, when dietary casein was replaced by soy protein (11,57,61,87). From the data shown in table 4, it is evident that soybean protein and cottonseed protein cause a significant reduction in the total serum cholesterol and HDL-cholesterol concentrations of rats. The reduction in serum cholesterol produced was similar for both types of dietary plant protein. In mature rats, rats fed casein or soy protein diet had greater concentrations of total serum cholesterol than those of immature rats fed the same diets, but this observation was somewhat opposite in animals fed cottonseed protein diet (table 4). This may be due to the fact that the weight gain of mature rats fed cottonseed protein diet was relatively poorer than that of immature rats fed the same diet and than that of mature rats fed the soy protein diet.

Animals fed plant protein diets excreted more feces than animals fed animal protein diet. Findings of our study are in agreement with Nagata's report (59) which demonstrated that soybean protein caused an increase in fecal excretion of neutral sterols. In the present study, soybean protein and cottonseed protein increased the excretion of fecal neutral sterols, especially coprostanol. Age also had a dramatic effect on the excretion of fecal coprostanol. Immature rats excreted more fecal coprostanol than mature rats.

LCAT activity in the rats decreased slightly with age. This result is in agreement with Lacko's findings (48,50). Animals fed plant proteins showed significantly higher LCAT activity than animals fed animal protein in each age group. The overall increment is probably in response to decreased substrate (free cholesterol) which is a function of dietary protein source. A more detailed investigation to explain this decrease and the interdependence of LCAT activity and HDL-cholesterol on dietary protein is needed. LCAT is more active with lower concentrations of free cholesterol in rats. This trend is also in agreement with previous findings from Park and Liepa (80).

Overall there was no obvious difference between the concentrated and the isolated plant proteins in their

ability to influence the cholesterol concentration of the blood. Non-protein contaminants in plant protein sources probably were not responsible for the cholesterol-lowering potential. Age-related trends are evident based on the low p-values of serum cholesterol, fecal excretion of the coprostanol and fecal excretion of the coprostanol plus cholesterol (tables 4 & 5). However, significant age-related trends were not shown in the concentrations of serum free and esterified cholesterol.

The present results indicate that the increase in fecal excretion of coprostanol in mature rats and in rats fed plant proteins appears to be counterbalanced partly by the decrease in the pool of hepatic cholesterol. It is also thought that an impairment of liver function due to the increase of lipids in liver may have an effect on LCAT activity or serum lipoproteins (87). Another possible factor associated with hypocholesterolemic effect of plant proteins may be an increased turnover of cholesterol in the rapidly-exchangeable pool. Carroll et al. (93) observed that feeding soya-bean protein to rabbits resulted in accelerated oxidation and turnover of labelled cholesterol than observed with casein. It has been known that feeding soya-bean protein to rats, in comparison with casein, results in the elevation of the activity of 3-hydroxy-3-

methylglutaryl coenzyme-A reductase, the rate limiting enzyme in cholesterogenesis (94).

CHAPTER 5

SUMMARY

This experiment was conducted to examine various dietary factors that influence changes in cholesterol metabolism. Animal protein (casein) and two plant proteins (soy protein and cottonseed protein) were fed to Fisher 344 rats of two different ages. Proteins used from plant sources were different with regard to content of proteins. Concentrated proteins were used in the diets of the 3-month old rats and isolated proteins were used for 9-month old rats. The body weights of 3-month old rats increased more rapidly than those of 9-month old animals. The analyses included measurement of total serum cholesterol, high density lipoprotein (HDL) cholesterol, activity of lecithin: cholesterol acyltransferase (LCAT) and excretion of fecal neutral sterold. Concentrations of total serum cholesterol were significantly higher in mature rats. Rats fed diets containing plant proteins showed significantly lower total serum and HDL-cholesterol concentrations than animals fed animal protein diet. Rates of fecal coprostanol excretion were significantly higher in immature rats. Excretion of coprostanol was significantly higher in animals fed plant proteins. A comparison of fractional LCAT activity with

serum free and esterified cholesterol concentrations revealed significant negative correlations; $r = -0.64$ ($p < 0.0002$) and $r = -0.57$ ($p < 0.0002$), respectively. There was no detectable difference between the concentrated and the isolated plant proteins in terms of their cholesterol-lowering effect.

APPENDICES

A. Method for determining HDL-cholesterol

B. Method for determining fecal neutral sterols

C. Method for measuring LCAT activity

APPENDIX A

Method for Determining HDL-cholesterol

Principle

Serum HDL-cholesterol concentration was determined following heparin-manganese precipitation of apo B-containing lipoproteins (LDL + VLDL). Serum was treated with heparin sulfate and $Mncl_2$ and allowed to stand in an ice bath for one hour to precipitate the apo B-containing lipoproteins. The precipitate was removed by centrifugation and an aliquot of the clear, HDL-containing fraction, was removed for cholesterol analysis.

Reagents

1. $Mncl_2$(1M) : dissolve 197.91g of $Mncl_2.4H_20$ in 1 liter volumetric flask with distilled water.
2. Heparin(5000 units/ml) : dissolve 1 ml of heparin (40,000 units/ml) in 7 ml of saline (0.15M)

Procedure

1. Use 3 ml glass centrifuge tubes or 6 x 50 mm glass culture tubes.
2. Add 200 μl serum, 8 μl heparin solution, and 10 μl $Mncl_2$ solution into tubes.
3. Vortex carefully to mix.

4. Cover all samples and incubate on ice or in refrigerator for at least 1 hour at $4^{o}C$.
5. Place tubes into rubber liner before centrifugation.
6. Centrifuge samples in refrigerated Sorvall for 10 minutes at 10,000 rpm.
7. Remove supernatant with micropipette and add into BMC reagent to perform enzymetic assay using BMC reagent set cholesterol (Biodynamics BMC, Indianapolis, IN).
8. Calculate HDL-cholesterol concentration.

   Corrected HDL-cholesterol concentration

   = HDL-cholesterol conc. x 1.09 (dilution factor).

APPENDIX B

Method for Determining Fecal Neutral Steroids (89)

Principle

Fecal sterol esters are first saponified by heating with alkali. The resulting free sterols are extracted using petroleum ether. The organic soluble fecal neutral sterols and a known amount of 5-α-cholestane (internal standard) are quantitatively measured using gas-liquid chromatography.

Reagents

1. 5-α-cholestane (Supelco product): dissolve in chloroform.
2. Cholesterol (Supelco product): dissolve in chloroform.
3. Coprostanol (Supelco product): dissolve in chloroform.
4. Chloroform: J. T. Baker solvent.
5. Petroleum ether: J. T. Baker solvent.
6. 1 N NaOH in 90% ethanol.
7. Column: U shaped glass column, 3 ft. x 2 mm interior diameter (I.D) (Supelco Inc.)
8. Packing: 3% SP-2250 on 100/120 Supelco Support (Supelco Inc.).

Procedure

I. Homogenation and saponification

1. Add water into feces mexture (equal dilution).
2. Homogenization for 3 min.
3. Take aliquots (0.5 - 1.0g) of the homogenates and place into 125 ml glass bottles with plastic screw tops.
4. Add a few boiling chips and 20 ml of 1 N NaOH in 90% ethanol.
5. Reflux for 1 hour.
6. Add 10 ml H_2O to sample mixture.
7. Centrifuge sample mixture for 5 minutes at 1,000 rpm.

II. Extraction of neutral sterols.

1. Wash precipitates with 50 ml of petroleum ether.
2. Centrifuge for 5 min. at 1,000 rpm and retain supernatant.
3. Repeat step 1 and 2 three times.
4. Evaporate the combined supernatant (petroleum ether) to dryness in a round bottom flask under nitrogen stream.
5. Transfer solutes quantitatively to conical centri-

fuge tube with 5 - 10 ml of petroleum ether.

6. Evaporate under a stream of nitrogen to dryness.

III. Sample injection to GLC

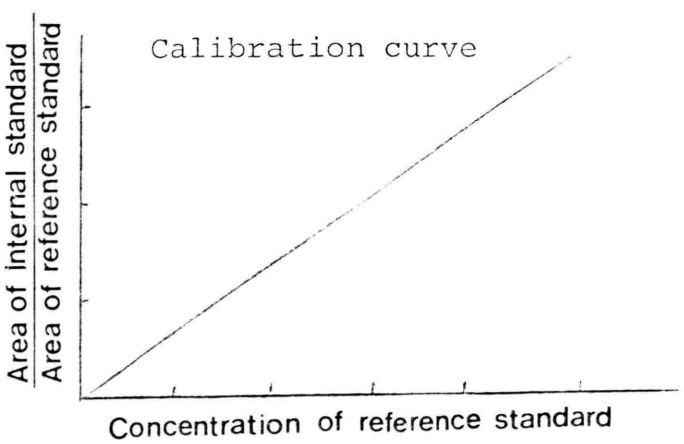
1. Column conditions: Oven temperature - 250°C
   Inlet or injection temperature - 260°C
   Detector (Flame ionization detector) temperature - 350°C
   Transfer temperature - 340°C

2. At the time of injection, add 200 µl of chloroform to dried tubes and vortex. Inject 3 µl of sample.

IV. Calculations

1. All quantitative work should be performed by comparison of the peak heights or area responses of neutral sterols to those of internal standard (5-α-cholestane).

2. Calibrate the concentration of sample using standard calibration.



Internal standard; 5-α-cholestane

Reference standards; coprostanol and cholesterol

APPENDIX C

Method for Determining LCAT Activity (90,91)

Principle

The transfer of ^{3}H-cholesterol from albumin to plasma lipoprotein is facilitated using deactivated lecithin: cholesterol acyltransferase. When the activation of LCAT occurs by adding beta mercaptoethanol, aliquots of samples are removed at 5 minute intervals over a 20 minute period and lipids are then removed. Activity of ^{3}H-cholesterol in each aliquot is counted for calculation. The fractional rates of esterification (% cholesterol esterification/hour) are calculated from the slopes of the line by least square analysis.

Reagents

1. 4.2 mM DTNB (5.5' Dithiobis-2-nitrobenzoic acid)
2. 5% Bovine serum albumin (BSA)
3. ^{3}H-cholesterol labeled at 1 & 2 positions (New England Nuclear Inc.)
4. 0.1M Beta mercaptoethanol
5. Chloroform:methanol (2:1)
6. Heptane
7. Petroleum ether
8. Ether
9. Acetic acid

10. Iodine
11. Toluene
12. Triton X-100 (Eastman Kodak Inc.)
13. Eastman concentration (Eastman Kodak Inc.)
14. Scintillation fluid (Eastman conc. I:120 ml + Toluene: 2880 ml + Triton X-100:1000 ml)
15. ^{3}H-cholesterol-BSA: make 5 μci/ml in 5% BSA

Procedures

1. Incubate 350 μl serum with 70 μl DTNB for 30 min. at 37^{o}C.
2. Add 105 μl BSA-^{3}H-cholesterol and allow samples to incubate for 4 hours at 37^{o}C.
3. Remove a 100 μl aliquot and place in chloroform:methanol (2:1) solution at zero time (before the activation of LCAT occurs)
4. Add 70 μl beta mercaptoethanol.
5. Remove subsequent aliquots (123 μl) and place into chloroform:methanol (2:1) solution at 5, 10, 15 and 20 min. intervals.
6. Heat extract for 15 min. at 60^{o}C following overnight incubation at 4^{o}C.
7. Filter using a sintered glass funnel.
8. Wash residue twice with 2 ml chloroform:methanol (2:1) solution and combine extracts.

9. Evaporate under a stream of nitrogen and rinse tube walls with 1 ml chloroform.
10. Evaporate chloroform again.
11. Dissolve the dried residue in a minimum amount of heptane and spot on Silical gel TLC plates.
12. Develop the TLC plates in petroleum ether:ether:acetic acid (90:10:1) solution.
13. Cut discs containing cholesterol and cholesteryl ester, place each disc in scintillation vials.
14. Count activities using scintillation counter.

Calculations

1. The fractional rate of esterification (% cholesterol esterified/hour) is calculated from the slope of the line by least square analysis.

$$\% \text{ cholesterol esterified} = \frac{\text{CPM of cholesteryl esters (CE)}}{\text{CPM of (free cholesterol + CE)}}$$

CPM: counts per minute

2. The number of nMoles cholesterol esterified per ml per hour is calculated by multiplying the fractional rate by endogenous free cholesterol concentration present in the serum. The nMole cholesterol esterified/ml/hour is obtained using following formula:

$$\frac{\text{Concentration of free cholesterol}}{\text{Molecular weight of cholesterol}} \times 10^9 \times \text{fractional rate}$$

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