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Title: Effect of Dietary Fat and Cholesterol on Plasma Lecithin:Cholesterol Acyltransferase Activity in Man

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Effect of diets varying in amount of cholesterol and polyunsaturated to saturated fatty acid ratio (P/S) on plasma lipid levels and lecithin:cholesterol acyltransferase (LCAT) activity was determined in fourteen young adult male subjects during a 13-week experimental period in a crossover design.

Control diet was fed for two weeks prior to 11-week experimental period. Two experimental diets were sequentially fed to each group during the 11-week experimental period switching the diet at the midpoint. The blood was drawn five times and LCAT activity and plasma lipid levels were measured. The effect of fat modifications in the diets on fractional LCAT activity showed a systematic change in LCAT activity independent of diet. Plasma total cholesterol levels were significantly ($p < 0.05$) elevated by diets containing high saturated fat with either high (22%) or low (7%) cholesterol while diets high in polyunsaturated fat with either high or low cholesterol levels lowered

the total cholesterol in Groups I and II but increased it in Groups III and IV. Trend of changes in plasma triglyceride was similar to that of plasma LCAT in Groups I and IV. However, there was inconsistency in the relationship between the LCAT activity and plasma lipid levels. LCAT enzyme was purified by 436-fold from plasma with 14% yield.

Effect of Dietary Fat and Cholesterol on Plasma Lecithin:Cholesterol Acyltransferase Activity in Man

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS USED IN TEXT

APO	Apolipoprotein
CHD	Coronary heart disease
FC	Free cholesterol
HDL	High density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
P/H	High P/S ratio and high cholesterol diet
P/L	High P/S ratio and low cholesterol diet
P/S	Polyunsaturated/saturated fat ratio
PUFA	Polyunsaturated fatty acid
S/H	Low P/S ratio and high cholesterol diet
S/L	Low P/S ratio and low cholesterol diet
TC	Total cholesterol
TG	Triglyceride
VLDL	Very low density lipoproteins

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INTRODUCTION

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43), an enzyme which catalyzes the transfer of fatty acids from lecithin to cholesterol on the surface of plasma lipoproteins (Glomset, 1962), performs several unique functions in the vascular lipoprotein metabolism. The balance between free and esterified cholesterol is maintained by LCAT (Glomset and Norum, 1973; Glomset et al., 1980). LCAT also creates the gradient necessary for the transfer of free cholesterol from tissues to the plasma (Tall and Small, 1980) and, at the same time, the transfer of cholesterol ester to the tissue. In tissue, cholesterol esters undergo hydrolysis and the resulting free cholesterol can be utilized, catabolized, or returned to the recirculation process. Little is known of the factors that regulate plasma LCAT activity.

A positive correlation has been found between diet, serum cholesterol level, and death from coronary heart disease (CHD) (Shekelle et al., 1981). Dietary fats have been shown to influence the effect of dietary cholesterol on lipoprotein cholesterol (Tan et al., 1980; Jackson et al., 1980; Oh and Monaco, 1984). Schaefer et al. (1981) investigated the effect of four different diets differing in amount of cholesterol and P/S ratio on plasma cholesterol levels, and concluded that lowering dietary cholesterol alone may cause modest decrease in plasma cholesterol levels but greater

reduction can be achieved by feeding diets low in cholesterol and fat and with a high P/S ratio. Dietary fat (P/S ratio) has been shown to affect LCAT activity. Gjone et al. (1972), Miller et al. (1975), Rosseneu et al. (1979), and Lichtenstein et al. (1980) have observed a decrease in LCAT activity when the P/S ratio was switched from low to higher ratio. Dietary cholesterol was reported to increase the molar esterification rate but the fractional rate of cholesterol esterification remained unchanged (Kostner, 1978).

The objective of the present study was to investigate the effect of dietary modifications in amount of cholesterol and degree of fat unsaturation on plasma LCAT activity and plasma lipid levels. For determining the effect of dietary fat modifications on changes in high density lipoproteins, the natural substrate for the enzyme, a method suitable for processing large quantities of human plasma that yields milligram quantity of purified LCAT for in vitro experiments was developed.

REVIEW OF LITERATURE

Overview of the Enzyme Lecithin:Cholesterol Acyltransferase (LCAT)

The plasma cholesterol esterification reaction was first described by Sperry (1935). In his experiment, he incubated blood serum samples at 37°-40° C for 3 days and determined the concentration of free and total cholesterol before and after the incubation and observed a marked decrease in amount of free cholesterol. This effect was abolished by heating the serum to 55°-60° C. He therefore concluded that it was due to an enzyme which appeared to exert its maximum activity at about pH 8.0. He also observed simultaneous hydrolysis of a fatty acid-containing compound to furnish the fatty acid required for the esterification of cholesterol. He concluded that: (1) an active cholesterol esterase is present in blood serum, (2) combined and free cholesterol are not in equilibrium in isolated serum, and (3) the hypothetical point of equilibrium lies in the direction of an increased proportion of combined to free cholesterol. However, later Sperry and Stoyanoff (1937; 1938) postulated the existence of a special cholesterol-esterifying enzyme to explain their observations of cholesterol formation in dog, human, and monkey sera.

Sperry's observation of the increase in esterified cholesterol during the incubation of serum or plasma was soon confirmed by Klein (1938), who found that plasma phospholipids were indeed the source of fatty acids in the esterification reaction.

Klein's finding was confirmed by Le Breton and Pantaleon (1947). They observed that acetone-precipitable lipids were decreased during incubation of serum, that this decrease and the decrease in unesterified cholesterol could be prevented by adding phlorizin or monobromoacetate, and that addition of ovolecithin increased the amount of cholesterol esterified. Because incubation of serum for 72 hours did not hemolyze red cells and because no detectable amount of acetone-precipitable lipids was in some samples of incubated serum, they concluded that the decrease in "phosphoaminolipid" was caused by a lecithinase B. Finally, they proposed a mechanism for the plasma cholesterol esterification reaction that involved the coupled action of the lecithinase B and a plasma cholesterol esterase: lecithin lecithinase B, 2 fatty acids + glycerophosphoryl choline, and then: cholesterol + fatty acid cholesterol esterase, cholesterol ester. Later their observation concerning the effect of added lecithin was confirmed by Etienne and Polonovski (1959; 1960), who demonstrated a decrease in lecithin and an increase in glycerophosphoryl choline in extracts of incubated serum.

Glomset et al. (1962) and Glomset (1962) subsequently obtained results which led them to propose that the initial breakdown of lecithin was not caused by a hydrolase but by an acyltransferase. They also noted that, when human plasma was incubated, the molar change in lecithin was similar to that in unesterified cholesterol. On the basis of these findings they postulated the following: lecithin + unesterified cholesterol acyltransferase, lysolecithin + cholesterol ester.

Most recently, Yokoyama et al. (1980) suggested that the LCAT reaction proceeds in two separate steps. The first is a phospholipase reaction leading to lysolecithin and an acylated enzyme which transfers the acyl group to cholesterol. Enzyme kinetics suggest that the initial step was activated by apolipoproteins or synthetic peptides. Glomset et al. (1962) and Glomset (1962) suggested that the enzyme mainly transfers fatty acids from the 2-position of lecithin. They observed that 10% or more of the fatty acids transferred to cholesterol were saturated. Therefore they suggested that saturated fatty acids (mostly palmitic acid) might largely be derived from a source other than the 2-position. Shah et al. (1964) suggested that plasma triglyceride might be one such source since cholesteryl palmitate-^{14}C was formed when plasma was incubated with tripalmitin-^{14}C.

Portman and Sugano (1964) reported that the acyltransferase of human plasma was able to distinguish between different fatty acids in the 2-position of lecithin. To verify this, they incubated mixtures of fresh plasma with heat-inactivated rat plasma labeled with cholesterol-^{14}C, and those of fresh rat plasma with heat-inactivated human plasma labeled with cholesterol-^{14}C, and studied the composition of the resulting cholesterol-^{14}C esters. In both experiments, they found that the newly formed esters more nearly resembled the preexisting cholesterol esters of the fresh plasma than in those of the unincubated mixture. They concluded that the pattern of cholesterol esters formed during the incubation was related to the specificity of the enzyme (in the fresh plasma) rather than the

composition of the fatty acid source (the lipoproteins of the mixed heated and fresh plasmas).

Fielding et al. (1972) and Fielding (1974) demonstrated that the acyl group was transferred from phospholipids which should preferentially have a basic nitrogen group. The rate of the acyl group transfer depended on the type of fatty acid in the 2-position of lecithin and on the fluidity of the acyl chains of phospholipids (Soutar et al., 1975). They found that in human plasma the rate of acyl group transfer by LCAT decreased as follows: linoleic acid > oleic acid > arachidonic acid > palmitic acid. The corresponding sequence with rat plasma LCAT was arachidonic acid > linoleic acid > oleic acid (Sgoutas, 1972). Several investigators have demonstrated that a maximal LCAT activity requires a greater molar ratio than 3:1 between lecithin and cholesterol (Albers et al., 1979; Fielding et al., 1972; Nichols and Gong, 1971; Sgoutas, 1972). This requirement may be related to the effect of cholesterol on the fluidity of phospholipid acyl chains (Soutar et al., 1975). This ratio may also be important for esterification in vivo because HDL is the only lipoprotein with a ratio greater than 3:1 between between lecithin and cholesterol (Skipski et al., 1967) and because it was also the best substrate for LCAT (Glomset, 1968).

Plasma Lipoproteins

Lipoproteins are lipid-protein complexes which serve to solubilize and transport cholesterol, triglycerides, and phospholipids

through plasma from sites of lipid absorption and synthesis to sites of storage and utilization (Oh, 1982). Lipoproteins consist of different proportions of various lipids in addition to a number of specific polypeptides or apolipoproteins, which consequently affect physico-chemical properties of intact lipoproteins. As a result of differing proportions of various lipids and apoproteins in each class of lipoprotein, the flotation properties of lipoproteins occur along a density gradient, and they are divided into the following: chylomicrons (CYM) with $d < 1.00$ g/ml, very low density lipoproteins (VLDL) with $d < 1.006$ g/ml, intermediate-density lipoproteins (IDL) with $d = 1.006\text{-}1.019$ g/ml, low-density lipoproteins (LDL) with $d = 1.019\text{-}1.063$ g/ml, high density lipoproteins (HDL) with $d = 1.063\text{-}1.21$ g/ml, and very high density lipoproteins (VHDL) with $d = 1.21\text{-}1.25$ g/ml. Almost all of these density classes have been subfractionated by stepwise ultracentrifugation at increasing densities or in a density gradient giving rise to density subclasses (De Lalla and Gofman, 1954; Laggner et al., 1977).

Another method used to separate lipoproteins is electrophoresis which separates the lipoproteins according to the charge they have. The most common electrophoretic procedures using paper, cellulose acetate, or agarose gels separate the lipoproteins into nonmigrating (Chylomicron), beta (LDL), pre beta (VLDL), and alpha lipoprotein (HDL) (Kunkel and Slater, 1952). These two systems of nomenclature can usually be considered essentially interchangeable.

On negative staining electron microscopy, isolated lipoproteins appear as spheres ranging from about 90 Å for HDL to about 5000 Å for

chylomicrons. Lipoprotein complexes are spheres in which lipid and protein are held together by largely hydrophobic interaction, with the polar head of protein, phospholipids and free cholesterol on the surface and apolar lipids such as triglycerides and esterified cholesterol in the core of the sphere (Levy, 1981).

Triglyceride in the diet is usually the major source of the daily flux of fatty acid in the circulation. Fatty acids and monoglyceride are released from dietary triglycerides by enzymic hydrolysis in the small intestine. They are absorbed by the intestinal epithelium, esterified to triglycerides and combined with cholesterol, cholesteryl esters, phospholipids and specific apolipoproteins into triglyceride-rich lipoproteins, chylomicrons and VLDL. Through a complex series of processes, these particles are released into the circulation for transport of triglycerides to individual tissues (Eisenberg et al., 1973). Chylomicrons and VLDL are the main carriers of triglyceride and LDL is the main carrier of cholesterol in plasma, although HDL also carries substantial cholesterol but in an opposing direction against LDL. HDLs are the heaviest and the smallest (90 Å) of the lipoproteins and contain the highest proportion of protein.

Chylomicrons are synthesized in the endoplasmic reticulum of the intestinal mucosal, carry the dietary fat absorbed through intestinal mucosal cells, and enter the circulation via the lymph. They are the largest (5000 Å diameter) lipoproteins. They are composed of 80% to 95% triglyceride and less than 2% protein (Levy et al., 1976) and, therefore, are the lightest of all lipoproteins. About 90% of this

protein is made up of apoproteins B and C, and 12% account for apo A-I and A-II.

VLDL are synthesized and secreted both by the liver and intestinal mucosal cells (Hamilton, 1972; Mahley et al., 1969) and transport endogenous lipids. Twenty to 50% of the total plasma VLDL is attributable to the intestinal origin (Ockner et al., 1969). The VLDL share many structural features with chylomicrons though their metabolic functions differ in fundamentals. Both are primarily triglyceride carriers from liver and intestine to internal tissues. The lipoprotein fraction is composed of about 50% to 60% triglycerides, 18% to 20% phospholipids, 10% to 12% unesterified cholesterol, 4% to 6% esterified cholesterol, and 8% to 12% protein (Margolis, 1969). The VLDL apoprotein represents a mixture of apo A, apo B, apo C, ad apo E, with relative contents of trace amount, 40% to 80%, and 13%, respectively (Schaefer et al., 1978).

The origin of LDL has been attributed to VLDL. Studies using apoprotein labeling techniques have shown that VLDL were at least partially metabolized to LDL. Studies with iodinated VLDL and iodinated apo B in VLDL revealed that VLDL were initially catabolized to the intermediate density lipoproteins and later to LDL (Eisenberg et al., 1973; Reardon, et al., 1978). In human plasma, LDL is the major cholesterol carrier, as 50% of its mass consists of cholesterol of which greater than 70% is in the esterified form. Protein and phospholipids comprise about 20 to 25% each of the total mass and the remaining few percent are made up of triglycerides. The mean

diameter of LDL is 220 Å. The major apoprotein present in LDL is apo B along with small quantities of C-apoproteins (Lee and Alaupovic, 1974).

The site of HDL synthesis in man appears to be in parenchymal cells of liver (Jackson et al., 1977), the intestine (Green and Glickman, 1981), and hydrolysis vemnants of triglyceride rich lipoproteins by LPL (Havel, 1978). These HDLs eventually end up in plasma. HDLs are composed of about 50% protein, 25% to 30% phospholipids, 20% cholesterol, and 5% triglyceride. The protein moiety of HD consists of apolipoprotein A-I, A-II, B, C-I, C-II, C-III, D, E, and F (Schaefer et al., 1978; 1979). Among them, apo A-I and apo A-II comprise about 90% of HDL protein with an apo A-I/apo A-II weight ratio of 3:1. HDLs are divided into two major subclasses: HDL_2 and HDL_3. HDL_2 is composed of 45% protein while HDL_3 is 55% protein (Margolis, 1969; Skipski, 1972). The weight ratio of apo A-I/apo A-II has been reported to be greater in the HDL_2 subfraction than in HDL_3 (Cheung and Albers, 1979).

Interest in the function of HDL has been greatly increased largely due to the finding that HDL cholesterol is an independent negative risk factor for coronary artery disease. The exact mechanism by which HDL plays a protective role in coronary heart disease (CHD) is not yet known. Two theories have been proposed. The first theory, called reverse cholesterol transport, is one in which HDLs play major roles in removal of cholesterol from peripheral tissue to liver and eventual catabolism and excretion by liver (Miller and Miller, 1975). The second theory was presented by Carew et al. (1976), who suggested

the modification of LDL metabolism by HDL for cellular binding and cholesterol uptake.

Familial LCAT Deficiency and Plasma Lipid Metabolism

The discovery of subjects with an inborn deficiency of plasma LCAT has demonstrated the essentiality of this enzyme in intravascular transport of cholesterol and other lipids. Familial LCAT deficiency was first described by Gjone and Norum (1967) in Norway. The characteristics of the plasma lipids in this disease include: (1) high phosphatidyl choline and low lysophosphatidyl choline, (2) less than 10% of cholesterol as cholesterol ester, (3) cholesterol ester composition similar to that of chylomicrons in thoracic duct lymph, (4) minimal *in vivo* esterification of labeled cholesterol following the infusion of mevalonic acid-^{14}C, and (5) elevated triglycerides. These lipid abnormalities were considered to be secondary to a deficiency of LCAT. Two concepts prevail as to the role of LCAT in the metabolism of lipoproteins and in the transport of serum lipids: LCAT as part of a removal mechanism for excess cholesterol and as a participant in the removal of triglycerides in conjunction with lipoprotein lipase.

The patients with LCAT deficiency were either heterozygotes or homozygotes. Utermann et al. (1980) demonstrated that the homozygotes have a deficiency of the immunodetectable enzyme protein. Albers et al. (1981a) from further study of families with familial LCAT deficiency described that the members of the family who represent the

heterozygotes gene have approximately one-half the normal level of plasma LCAT.

With the discovery of two rare inborn errors of metabolism, the function of lipoproteins in plasma became a little more clarified. In abetalipoproteinemia, no chylomicrons, VLDL, or LDL are present. HDL is present as are the A, E, and C apoproteins. These individuals cannot produce B apoprotein or at least cannot release it into the bloodstream causing problems in fat metabolism. The other inborn error of lipid metabolism is "Tangier disease" (Fredrickson and Levy, 1972) in the plasma. Very small amounts of HDL and of the A-I and A-II apoproteins are present, but all the other lipoproteins and B, C, E apoproteins are present in plasma. In these patients the cholesterol concentration of blood is low and cholesterol esters are deposited in reticuloendothelial cells of the body while plasma triglyceride level, especially after a fatty meal, is usually increased. These observations suggest that the A apoproteins or HDL are involved in clearance of lipids from the plasma.

Interaction of Lipoproteins and LCAT

Lower density lipoproteins, VLDL and LDL, carry the bulk of unesterified cholesterol in plasma, and support plasma LCAT reaction by being the major donors of cholesterol for LCAT reaction. LCAT circulates in the plasma in close association with HDL molecules (Glomset, 1980; Rose, 1981). Recent findings by Fielding and Fielding (1980) suggested the existence of a complex consisting of one molecule

of apo A-I., two molecules of apo D, and one molecule of LCAT. Albers et al. (1981b) have shown that an immunoadsorber specific for apo D binds approximately 64% of plasma LCAT and 11% of apo A-I. In that study only about two-thirds of plasma LCAT was found in a complex with apo D. Utermann et al. (1980), who used a different approach, have also come to the same conclusions: antibodies against LCAT added to plasma totally inhibited plasma LCAT activity, whereas only 50% inhibition was obtained with antibodies against apo D. Because anti-apo D precipitated only one-half of plasma LCAT, at least one-half of the enzyme molecules were associated with apo D.

HDL complex with LCAT and apoproteins binds to its substrate and esterifies the cholesterol. The cholesterylesters (CE) produced do not remain in the HDL but seem to be distributed to other plasma lipoproteins (Lally and Barter, 1979; Nestel et al., 1979; Nichols and Smith, 1965) via a recently discovered CE transferring protein (Marcel et. al., 1980).

HDL molecules have been further subspecified into Lp A, Lp C, Lp D, Lp E. LpC_{HDL} and may represent the true substrate or interface for cholesterol esterification within HDL as concluded from the study by Kostner (1978). HDL subfractions at the lower end of its density range may be responsible for a protective effect against coronary heart disease (Barboriak et al., 1979). When isolated from perfused rat liver, the nascent HDL which initially contain only free cholesterol are discoidal particles and were shown to be much better substrates for LCAT than the circulating spherical macromolecules referred to as "Plasma HDL" (Hamilton et al., 1976). While plasma HDL

appears to be an inferior substrate to nascent HDL *in vitro*, it is much more available to LCAT than nascent HDL, due to its much longer half life *in vivo*. Nascent HDL may be related to the fecal steroid excretion.

Factors Effecting Activation or Inhibition of LCAT

Many studies have been conducted to elucidate factors affecting LCAT reaction rate. Some have been conducted with artificial substrates and purified LCAT *in vivo*. These investigations (Albers et al., 1976; Albers et al., 1979; Chen and Albers, 1981) have established some of the factors that control the specificity of the enzyme *in vitro*, although the relevance of these studies to cholesterol esterification *in vivo* is yet to be established. Fielding et al. (1972) found that a molar ratio of 3:1 between lecithin and cholesterol was most favorable for the LCAT reaction and that excess amounts of triglyceride and cholesterol esters were inhibitory for the enzyme activity in a lecithin, cholesterol, and apo A-I liposome system.

Early studies showed that in normal plasma the most significant correlation observed was between LCAT activity and plasma triglyceride levels, suggesting a role of chylomicrons and/or VLDL in the reaction (Fabien et al., 1973).

Marcel and Vezina (1973) and Wallentin and Vikrot (1975) observed that an increase of the mean LCAT activity coincided with an increase of plasma phospholipids and HDL phospholipids but not with the increase in plasma triglyceride concentration.

Albers et al. (1981b) have shown that LCAT mass was highly correlated with LCAT activity measured with either a common heated plasma substrate or radio assay with endogenous plasma as substrate and by measurement of the decrease in plasma unesterified cholesterol. The authors also reported that LCAT activity was correlated with plasma total cholesterol, unesterified cholesterol, and log triglycerides but was not highly correlated with HDL cholesterol, apo A-1, apo A-II, or apo D. Therefore, they concluded that plasma unesterified cholesterol transported by HDL may not be a good substrate for LCAT.

Since the successful partial purification of LCAT (Fielding et al., 1972), factors affecting the activity have been tested. The lack of reaction of partially purified enzyme with synthetic substrate demonstrated the requirement of the enzyme for apo A-I. For optimum LCAT reaction using single bilayer vesicles of lecithin and cholesterol as substrate of LCAT with molar ratio of 4:1, approximately 8 molecules of apo A-I per vesicle were required (Chung, Abano, Fless, and Scanu, 1979). When single bilayer vesicles of lecithin and cholesterol were incubated with LCAT, the addition of albumin to the incubation mixture nearly doubled the rate of LCAT with apo A-I as activator (Albers et al., 1979). As a result of LCAT reaction with cholesterol and lecithin, lysolecithin was produced as the metabolic end product which exerted inhibition on activity of the enzyme. Albumin probably binds lysolecithin and relieves the end product inhibition according to Fielding et al. (1972). Apo C-I was also shown to activate purified LCAT (Albers et al., 1979) but the

optimum concentration of apo C-I was only 42% of that obtained at the optimum concentration of apo A-I. Addition of albumin decreased the rate of LCAT reaction in presence of apo C-I.

Apo A-II inhibits the activity of LCAT through a mechanism of competition with the activating peptides for the surface of the lipid bilayer. Addition of apo A-II to HDL resulted in displacement of apo A-I from the HDL surface and its replacement with a precise stoichiometry of uptake-displacement of two molecules of apo A-II for one molecule of apo A-I (Scanu et al., 1980). Chung, Abano, Fless, and Scanu (1979) showed that apo A-II, due to its greater affinity for the lipid surface, inhibited the LCAT reaction in the presence of phospholipids, cholesterol, and apo A-I. Furthermore, they reasoned that the apo A-I/A-II ratio of the vesicle surface could thus control the rate of the LCAT reaction in vitro. In more recent experiments, Chung, Lagocki, Albano, and Scanu (1979) prepared hybrid particles by incubating dog HDL with human apo A-II. These particles had apo A-II/A-I ratios varying from 0.05-2.0. The LCAT activities, when tested against these lipoprotein hybrids, were inversely proportional to the apo A-II/apo A-I ratio.

Apo C can also inhibit the activation of LCAT reaction by A-I when saturation of reaction was reached with respect to apo A-I concentration. Inhibition of LCAT activity was exerted by apo C-II and apo C-III. The mechanism of inhibition by individual apo C peptides was related to a competition of the apoproteins with apo A-I for the surface of the liposome (Albers et al., 1979; Soutar et al., 1975). Similar results have also been obtained with apo D, which

inhibits LCAT reaction in the presence of saturating concentrations of apo A-I.

Human plasma HDLs have two main subfractions as prepared by ultracentrifugation and were designated as HDL_2 (d = 1.067-1.125 g/ml) and HDL_3 (d = 1.125-1.21 g/ml). Fielding and Fielding (1971) showed that HDL_3 was a considerably better substrate than HDL_2, even though the apo A-I/A-II ratio was higher in HDL_2 than in HDL_3. Pinon et al. (1980) found the relatively apo A-I rich HDL_2 particles to be inhibitors of LCAT. Therefore, plasma LCAT activity was inversely related to the ratio of HDL_2 cholesterol to HDL_3 cholesterol.

A major portion of phospholipids and apoproteins carried by chylomicrons was transferred to HDL (Wallentin and Vikrot, 1975). These transfers of lecithin to HDL increased the ratio of lecithin to unesterified cholesterol and might result in increased substrate reactivity as noted by Wallentin and Vikrot (1975), who found a positive correlation between LCAT activity and HDL phospholipids. Despite the preferential reaction of LCAT with lipoproteins contained within the HDL particle spectrum, the activity of the enzyme was clearly not correlated with plasma levels of HDL cholesterol (Rose and Juliano, 1976; Sutherland et al., 1979; Wallentin and Vikrot, 1975). However, LCAT activity was correlated with plasma levels of unesterified cholesterol of all lipoproteins (Pinon et al., 1980).

Effect of Diet on LCAT Activity

Atherosclerosis is a multifactorial disease. There are many theories surrounding its pathogenesis, none of which is more popular

than the "lipid" hypothesis (National Diet-Heart Study Research Group, 1968). There is an extensive body of evidence from epidemiological (Kannel and Gordon, 1969; Keys, 1970), experimental and clinical studies (Ahrens et al., 1957; Connor et al., 1964; Schaefer et al., 1981; Shekelle et al., 1981) linking diet to plasma cholesterol on the one hand and plasma cholesterol to heart disease on the other (Shekelle et al., 1981). Epidemiological findings (Gordon and Kannel, 1971) consistent with clinical and animal experiment data have identified at least four major risk factors which are related to a high degree of frequency in individuals who have coronary heart disease (CHD). They are high plasma cholesterol level, particularly elevated LDL cholestrol; hypertension; cigarette smoking; and diabetes. Secondary risk factors (Strasser, 1972) are high blood triglycerides, low plasma HDL, obesity, stress, lack of exercise, and personality type. It has been shown that HDL cholesterol is inversely related to the incidence of CHD (Miller and Miller, 1975). This finding suggests that total serum cholestrol can no longer be regarded as adequate in evaluating risk factors of CHD and that distribution of cholesterol among lipoproteins is a more important factor. The saturated fats and cholesterol content in diet have been closely correlated with serum cholesterol and lipoprotein levels (Keys, 1970). In several other clinical dietary studies (Schaefer et al., 1981), the cholesterol incresing effect of dietary cholesterol and saturated fats and cholesterol lowering effect of polyunsaturated fats have been shown. Early study by Ahrens et al., (1957) demonstrated the relationship between the degree of fatty acid saturation and plasma

cholesterol levels. They found that the more saturated the dietary fat, the higher the plasma cholesterol levels obtained. The diet high in unsaturated fats also lowered the mean plasma cholesterol levels (Connor et al., 1964).

Previous studies of the effect of dietary fats on plasma LCAT activity showed conflicting results. Gjone et al. (1972) observed a significant fall in the plasma concentrations of cholesterol and lecithin after 21-day feeding of soybean oil diet to two groups of five healthy subjects 23 to 42 years old. Concomitantly, a significant reduction in plasma LCAT activity also took place. They also tested the effect of medium chain triglycerides on plasma concentration and activity of LCAT, and found no significant changes. Miller et al. (1975) also observed a reduction in the ability of plasma to form cholesterol esters by a given LCAT source when dietary unsaturated fats were increased. When heat-inactivated labelled plasma as substrate and standard LCAT source were incubated together, there was a genuine decrease in plasma LCAT activity on high P/S diets, though it was not possible to determine whether enzyme or substrate was rate limiting in the given situation. Reduced esterification on high P/S diets has also been observed by Chait et al. (1974). High P/S diets were associated with reduced secretion of endogenous triglyceride into plasma. This has been confirmed more directly in rats (Morris et al., 1975). Other work by Miller (1975) suggested that variables influenced by cholestyramine resin therapy, such as hepatic cholesterol synthesis or plasma concentration or LDL, do not influence esterificaton. In contrary to above observations,

Rosseneu et al. (1979) tested the effect of polyunsaturated lecithin and saturated lecithin on lipoprotein composition and fatty acid profile in four male chimpanzees. The chimpanzees were given three isocaloric diets containing the same amount of fat with varying fat saturation from a P/S ratio of 1.0:1 in the control diet to P/S ratio of 0.2:1 in the diet enriched with saturated lecithin. The treatment with polyunsaturated lecithin increased the cholesterol esters and lysolecithin content in HDL, presumably due to activation of LCAT. These modified HDL particles had a more fluid surface and a denser core and were able to act as better cholesterol carriers. Another effect of this treatment was a decrease in the plasma triglyceride level and VLDL concentration, and an increase in the unsaturation ratio of the triglycerides. The saturated lecithin treatment also increased the plasma LDL concentration and most noticeably the saturation degree in the cholesterol esters. These effects would likely enhance the progression of atherosclerosis. The changes in lipoprotein composition should affect the organization of the lipids within lipoprotein molecules and the lipid-apoprotein interactions. Consequently, such alterations may be reflected in the fluidity or microviscosity of the isolated lipoprotein fractions. Blaton et al. (1970) studied lipid changes in the plasma lipoproteins of baboons on an atherogenic diet and looked at the changes in the total plasma lipids and in lipoprotein fractions. This study concluded that the degree of fat saturation of the diets given either to patients or to experimental animals had a significant effect on lipid and protein composition with altered fatty acid profiles of plasma lipoprotein.

Lichtenstein et al. (1980) assessed plasma LCAT activity in six squirrel monkeys (Saimiri sciureus) and six cebus monkeys (Cebus albifruns) from birth to 3 to 4 years old were given diets containing saturated fat (coconut oil) or unsaturated fat (maize oil) with or without 0.1% cholesterol. They found that LCAT activity was primarily affected by species and dietary fats, and to a lesser extent by dietary cholesterol in squirrel monkeys. Plasma from both species of monkeys fed corn oil diets had comparable overall rates of LCAT activity. In all monkeys fed the coconut oil diets, percent esterification was lower than in those monkeys fed the corn oil diets. Coconut oil feeding resulted in lower LCAT activity in all monkeys whereas cholesterol feeding had no significant effect on LCAT activity. Coconut oil feeding resulted in a lower P/S ratio in HDL phospholipids in both species. Plasma total unesterified cholesterol was also increased when coconut oil was the dietary fat. Since PUFA are thought to be the preferred substrate for LCAT esterification of cholesterol, the marked reduction in PUFA content of lecithin in monkeys fed coconut oil may have contributed to the decreased substrate fractional rates associated with that diet. The plasma lipid data obtained from these monkeys confirmed previous observations by Nicolosi et al. (1977).

Sharma et al. (1983) studied the effect of different carbohydrate diets on the activity of serum LCAT on cynomologus monkeys. They used sucrose or starch diets with or without addition of cholesterol. They observed markedly higher LCAT activity in monkeys fed sucrose than starch diets and the addition of cholesterol to starch and sucrose

diets decreased the activity of enzyme or percent esterification. They explained these results by saying that an increased plasma triglycerides turnover was responsible, and triglyceride (VLDL) flux was relatively greater with sucrose than with starch diet (Nestel and Barter, 1973), thus an increase in total LCAT activity in monkeys when fed sucrose rather than starch diets could primarily be related to triglyceride metabolism.

Purification of the Enzyme

Lecithin:cholesterol acyltransferase (Ec 2.3-1.43) is produced by the liver and secreted into the circulation (Glomset, 1968; 1972). In view of the vital importance of LCAT in the metabolism and structure of plasma lipoproteins, many attempts have been made toward purification. The labile nature and low concentration of LCAT in human plasma (around 6ug/ml) (Dobiasova, 1983) has made its purification a most difficult task. The difficulty has been compounded by the fact that enzyme is present in plasma as a lipoprotein complex found in the density range of HDL and VHDL or bound to these lipoproteins (Akanuma and Glomset, 1968; Albers et al., 1976).

Early work on LCAT purification delineated methods such as hydroxylapatite chromatography (Glomset and Wright, 1965) and HDL affinity chromatography (Akanuma and Glomset, 1968) which were later refined and shown to be the most efficient steps in the purification of the enzyme. Fielding and Fielding (1971) and Fielding et al., (1972) have made other attempts to partially purify this enzyme.

Well-documented purification of LCAT was reported by Albers et al. (1976), who presented criteria of the homogeneity of their preparation. The enzyme thus purified by ultracentrifugation, HDL affinity chromatography, hydroxylapatite chromatography, and anti-apo D affinity chromatography has a molecular weight of 68,000 and an amino acid composition different from that reported for various apoproteins. Subsequently, several groups have reported methods for the purification of LCAT (Aron et al., 1978; Chung, Abano, Fless, and Scanu, 1979; Chung, Lagocki, Albano, and Scanu, 1979; Doi and Nishida, 1981; Kitabatake et al., 1979; Suzue et al., 1980; Utermann et al., 1980) that yield purifications ranging from 15,000- to 34,700-fold with recoveries varying between 8% and 20%. The proteins that have the highest affinity for LCAT and thus are the major contaminants to be removed from the preparation have been shown to be apo A-I and apo D. Several groups have observed that apo D removal requires passage upon an immunoadsorber column for apo D or, alternatively, preparative gel electrophoresis (Suzue et al., 1980). There is good general agreement among authors on the amino acid composition for LCAT, which presents no special feature other than a high content of glutamic acid, glycine, and leucine (Aron et al., 1978; Chung, Abano, Fless, and Scanu, 1979; Chung, Lagocki, Albano, and Scanu; Albers et al., 1979).

LCAT is a glycoprotein with high carbohydrate content (24% w/w) with the following composition per mole (59,000 g of protein): mannose (31%), galactose (20%), glucosamine (17%), and sialic acid (13%) (Chung, Abano, Fless, and Scanu, 1979). Albers et al. (1979)

also reported the absence of galactosamine and the presence of glucosamine (31 moles/10 moles of protein). These observations were corroborated by the affinity of LCAT for wheat germ agglutinin (Suzue et al., 1980).

The apparent molecular weight for LCAT determined by electrophoresis on SDS-polyacrylamide gel ranges from 65,000 to 69,000 (Albers et al., 1979; Chung, Abano, Fless, and Scanu, 1979; Utermann et al., 1980). These authors have concluded that the higher molecular weight obtained by SDS-polyacrylamide gel electrophoresis is likely due to the high carbohydrate content of the enzyme, which affects its electrophoretic mobility.

There is no consensus about the optimum conditions to maintain activity of the purified enzyme upon storage. Although Albers et al. (1979) reported a 26-day half-life for LCAT at low concentration (30 mg/ml) in Tris saline, pH 7.4, at 4° C, Utermann et al. (1980) reported a half-life of 1 to 2 days in the same buffer (albeit with taurocholate and dithiothreitol at 4° C) but increasing stability with decreasing storage temperatures. Kitabatake et al. (1979) found the enzyme to be stable for 4 weeks when stored under nitrogen at 4° C in 0.4 mM phosphate buffer, pH 6.8 with 4 mM mercaptoethanol, whereas addition of physiological saline decreased the stability of pure LCAT. Furukawa and Nishida (1979) studied the stability of purified LCAT in more detail and came to the conclusion that it depended primarily on low ionic strength of the medium. At an ionic strength of 0.01 at pH 7.4 in 0.4 mM phosphate buffer, the enzyme activity remained unchanged for 6 hours when incubated at 37° C. The stability of the enzyme was

progressively decreased with increasing ionic strength up to 0.1 in 39 mM phosphate buffer. As early as 30 minutes after the start of incubation, about 90% of the enzyme activity was lost. The study further showed that inactivation of the enzyme mostly took place at the air-water interface.

MATERIALS AND METHODS

Subjects

Fourteen young adult male university students were selected from a pool of 40 applicants on the basis of medical history and physical examination and blood lipid analysis. None of the subjects were taking medications which might influence lipid metabolism. They were not allowed to smoke or drink alcohol during the study period. Prior to the start of the control diet, they were taught by a dietitian how to keep an accurate dietary record. Personal data on the subjects at admission and ranges of body weights through the study are given in Table 1.

Diet

Control Diet

Three-day diet records (two weekdays and Saturday or Sunday) were obtained from the 14 subjects to estimate the energy requirement for weight maintenance. All subjects were fed the control diet for two weeks. The composition of the control diet was the same as the experimental diet shown in Table 2 but it provided 500 mg of cholesterol per day and had a P/S ratio of 0.4:1.

Experimental Diet

The basic experimental diet was formulated to be patterned after the typical American diet containing moderate amounts of protein and high fat content from a typical variety of fat sources. In order to achieve the desired P/S ratios, beef tallow or safflower seed oil was

Table 1. The composition of the diets.

Diet	Fat % Calories	P/S Ratio	Protein % Calorie	Carbohydrate % Calories	Cholesterol mg/day
P/L	42	1.5-2.0	15	43	250-300
S/L	42	0.2-0.25	15	43	250-300
P/H	42	1.5-2.0	15	43	950-1050
S/H	42	0.2-0.25	15	43	950-1050

P/H = high P/S ratio and high cholesterol content

S/H = low P/S ratio and high cholesterol content

P/L = high P/S ratio and low cholesterol content

S/L = low P/S ratio and low cholesterol content

Table 2. A typical basic diet menu and major nutrient composition per standard servings. (P/L Diet; high P/S ratio and low cholesterol).

Mean	Food	Amount (g)	Kcal	Protein (g)	Fat (g)	CHO (g)	PUFA (g)	SFA (g)	Chol. (mg)
Breakfast	Orange juice	249	122	1.7	0.2	28.9	-	-	-
	Sugar	4	15	-	-	4.0	-	-	-
	Cornflakes	28	106	2.2	0.1	24.0	-	-	-
	Whole wheat bread	56	134	5.2	1.4	27.6	0.4	0.2	-
	Whole milk	244	157	8.0	8.9	11.0	0.3	5.6	35.0
	Bananas	150	128	1.7	0.3	33.0	-	-	-
	Subtotal		662	18.8	10.9	128.5	0.7	5.8	35.0
Lunch	Turkey breast	85	134	25.4	2.7	-	0.7	0.9	58.7
	Saffola margarine	21	150	-	16.5	-	12.0	1.5	15.0
	Lettuce	25	3	0.1	tr	0.8	-	-	-
	Hamburger bun	40	119	3.3	2.2	21.2	0.5	0.5	-
	Celery sticks	56	10	0.6	tr	2.2	-	-	-
	Pnut butter-beef fat	20	133	4.0	12.3	3.0	2.5	3.6	4.7
	Whole milk	244	157	8.0	8.9	11.4	0.3	5.6	36.0

Table [2] (continued)

	Carrot cupcakes-2		336	4.9	13.9	49.1	10.2	1.2	-
	Walnuts	8	52	1.2	5.1	1.3	3.2	0.4	-
	Subtotal		1094	47.5	61.6	89.0	29.4	13.7	113.3
Supper	Roast beef	114	214	35.5	6.9	-	0.2	3.3	103.3
	M. Potatoes-½c.		118	2.0	5.3	14.4	0.2	2.8	9.0
	Gravy, Saffola-½c.		177	0.5	19.0	3.6	14.8	1.3	-
	Green beans	135	32	1.9	0.3	7.0	-	-	-
	Roll	50	156	5.0	1.6	29.8	0.4	0.4	-
	Salad		18	0.9	0.1	4.0	-	-	-
	Celery seed dressing	17	97	tr	9.1	4.4	6.7	0.8	-
	Rye krisp-2 triple		50	1.0	-	10.0	-	-	-
	Peach half w/syrup		85	0.4	0.1	21.9	-	-	-
	Popcorn	18	69	2.4	0.9	13.8	0.6	tr	-
	Saffola margarine	23	168	0.1	18.4	0.9	9.6	3.0	-
	Butter	5	33	tr	3.8	tr	0.1	2.4	10.3
	Margarine	5	36	tr	4.0	tr	0.6	0.8	-
	Subtotal		1253	49.7	69.5	109.8	33.2	14.8	122.6
	Total		3009	116.0	142.0	327.3	63.3	34.3	270.9
	% of energy			15.4	41.6	43.0		P/S:1.8	

added to the diets. By altering the levels of animal products, the concentration of cholesterol was adjusted in the diets. The composition of the four experimental diets is shown in Table 1. The basic diet was adjusted calorically to maintain the initial weight of the subjects within a 2-pound variation, while keeping the percentage of calories supplied from fat, protein and carbohydrate constant. The experimental diets were isocalorically formulated by exchanging the amount of fat with the kinds of fat desired, and manipulating with fresh eggs as an exogenous cholesterol source to achieve the cholesterol content of the four experimental diets as outlined in Table 1. A typical menu is listed in Table 2. The calculated nutrient values of all four diets exceeded the 1980 Recommended Dietary Allowance of the major nutrients for males in the age group represented in the study. Daily intakes of protein, calcium, phosphorus, iron, vitamin A, ascorbic acid, thiamin, riboflavin and niacin were greater than 100% of the RDA for respective age groups. Agricultural Handbook No. 456 and No. 8 were the major sources of nutrient information.

Experimental Design

Outline of Experiment

The 14 subjects were randomly assigned to four groups. Each group was made up of either three or four subjects as shown in Figure 1. A crossover design was used for the study over a 13-week feeding period. Each group was sequentially placed on two different diets during the 11-week experimental diet period switching

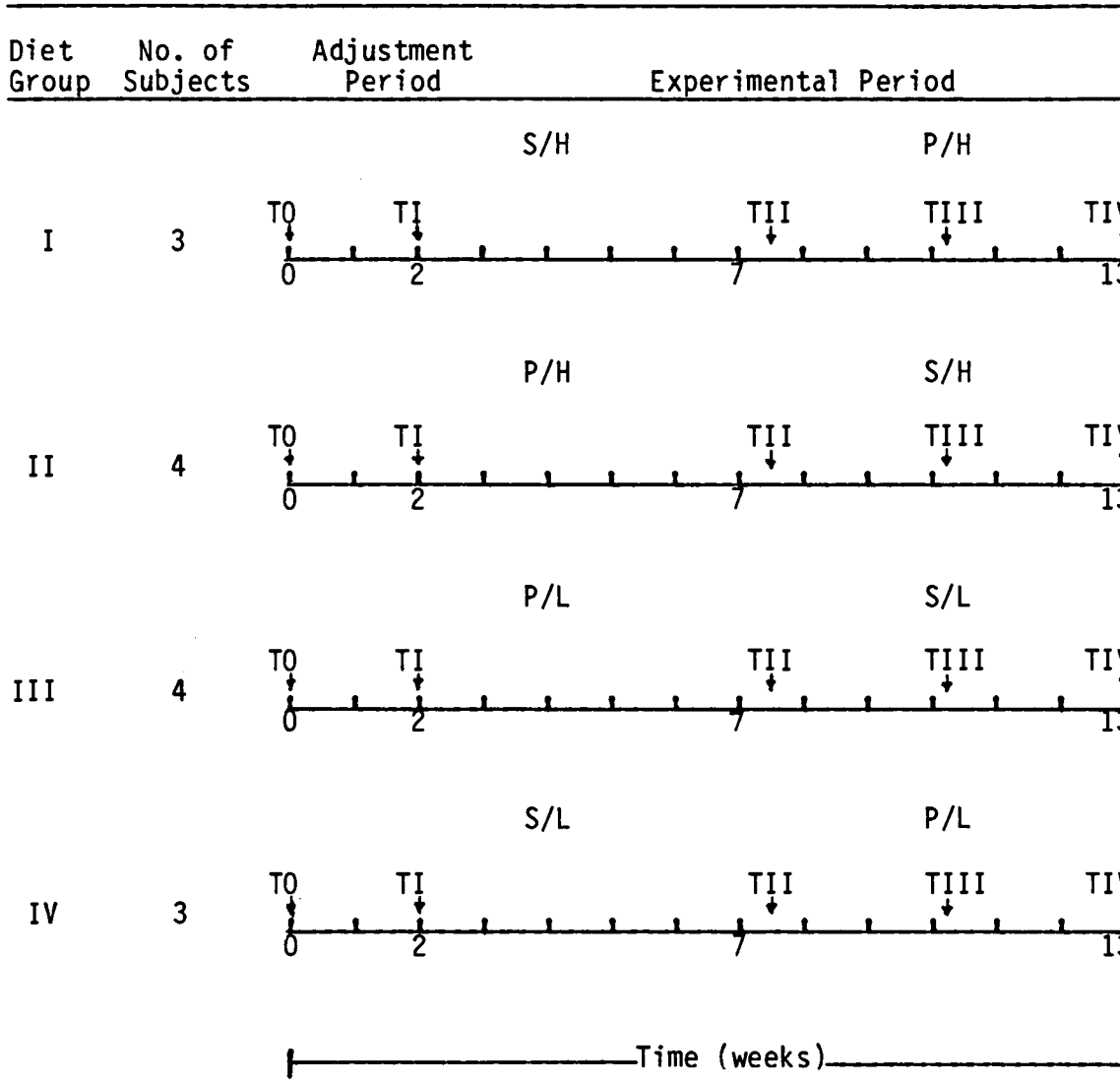


Figure 1. The experimental design for the fat modification diets.

from one diet to the other after 38 days on the first diet. This crossover experimental design resulted in a greater number of observations on each diet and enabled determination of the carryover effect due to diet change. The whole study lasted 13 weeks. The first two weeks were the adjustment period, the remaining 11 weeks were divided into two equal parts.

Sampling Period

The blood was drawn five times during the study: T=0, on the first day of the adjustment diet; T=I, on the last day of the control diet or the first day of the first experimental period; T=II, on the first day of the second diet; T=III, on the midpoint of the 5 1/2 week second-diet period and T-IV; on the last day of the second diet, which was the end of the 13th week.

Methods of Analysis

Plasma Preparation

The overnight fasting blood was drawn into 10 ml vacutainers in the presence of EDTA (0.2 mg/ml). The tubes containing the blood after gentle shaking were immediately placed in crushed ice. The tubes were immediately centrifuged at 3000 rpm, at 4° C for 35 minutes. The plasma was transferred to polyethylene tubes and was immediately frozen until assayed for LCAT activity.

Determination of Plasma LCAT Activity

The total plasma LCAT activity was determined as described by Stokke and Norum (1971). ^{14}C-cholesterol (specific activity, 54.4 Ci/mmoles) and Aquasol were purchased from New England Nuclear

(Boston, MA). Ready coat silica gel plates for TLC (250 um) were obtained from Baker (Phillipsburg, NJ). Bovine serum albumin (96-99%) and 5.5 dithiobis-2-nitrobenzoic-acid (DNTB) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals used were reagent grade.

Cholesterol Assay

Total and free cholesterol levels were determined on whole plasma as well as all lipoprotein fractions. Cholesterol was assayed colorimetrically using an enzymatic method as described by Allain et al. (1974). For total cholesterol determination, cholesterol esters were hydrolyzed to free cholesterol by cholestrol ester hydrolase (Miles Laboratories, Elkart, Indiana). The free cholesterol produced was oxidized by cholestrol oxidase (Miles Laboratories, Elkart, Indiana) to cholest-4-en-3-one with the concurrent production of hydrogen peroxide. The peroxide in the presence of peroxidase (Miles Laboratories, Elkhart, Indiana), coupled with 4-amino-antipyrine (Sigma Chemical Company, St. Louis, Missouri) and phenol to produce a chromagen with maximum absorption at 500 nm. Free cholesterol was measured by omitting the cholesterol ester hydrolase treatment.

Triglyceride Assay

Plasma triglyceride levels were determined as described by Giegel et al. (1975). In brief, triglycerides were extracted with heptane from plasma and glycerol was released from triglycerides by treatment with a transesterifying reagent. The released glycerol was oxidized to formaldehyde with periodate and formaldehyde was reacted with acetylacetone and ammonium ions to produce a lutidine (Hantzsch)

reaction. Thus, the color intensity (OD) due to the presence of lutidine was measured at 415 nm in a Beckman DU spectrophotometer.

Isolation and Purification of LCAT

For isolation and purification of LCAT, the method of Nichols and Gong (1971) was used.

Crude LCAT preparation. Crude LCAT enzyme was prepared from 355 ml of fresh human plasma. All procedures were carried out at 4° C. The first step in LCAT isolation involved ultracentrifugation. The density of plasma was raised to 1.21 g/ml by adding 115.9g of solid potassium bromide and 0.197 g of DTNB to make the final concentration of DTNB in the plasma 1.4 mM. The mixture was loaded in ultracentrifuge tubes and centrifuged at 35,000 rpm for 48 hours in 35 Ti rotor. Each ultracentrifuge tube held 65 ml and there were six tubes in the rotor. At the end of centrifugation the top 15 ml of each tube was carefully removed using pasteur pipets. The supernatant fraction (93 ml) containing all the lipoproteins was discarded. The bottom fractions were pooled (286 ml) and dialyzed against 0.01 M phosphate buffer containing 0.26 M NaCl and 0.1 mg/ml EDTA, pH 7.4 at 4° C, in cellulose dialysis bags. The dialysis solution was changed three times over 24 hours. After dialysis the volume came up to 305 ml. To this dialyzed bottom fraction, phospholipid liposome mixture (the liposome mixture was made as described below) and 1.58 g NaCl was added. Then this mixture was incubated at room temperature for 30 minutes. After incubation sucrose was added to make 16% sucrose density. This mixture was loaded in ultracentrifuge tubes and centrifuged at 35,000 rpm for 16 hours in 35 Ti rotor. After

centrifugation, the top white layer which was enzyme-liposome complexes was carefully removed with pasteur pipets so that any enzyme-liposomes on the sides would not be lost. The white liposome-enzyme complexes on the side were removed and washed with 0.01 M phophate buffer. The total volume of top liposomes enzyme complexes was 87 ml. The density of this 87 ml was raised to 1.21 g/ml by adding 28.4 g of KBr and the mixture was incubated in a cold room overnight to dissociate the enzyme from liposome complexes. The incubated mixture was loaded in centrifuge tubes and carefully overlayed with 1.15 g/ml KBr density solution. These tubes were centrifuged for 18 hours at 50,000 rpm in 50 Ti rotor.

The centrifuge tubes were cut at 4 cm from the bottom. The length of the tubes were 7.5 cm. The bottom was pooled and the tubes rinsed with 0.01 M phosphate buffer and 0.15 N NaCl. From 19 tubes 110 ml of bottom was obtained and dialyzed against 0.01 M phosphate buffer containing 0.26 M NaCl and 0.1 mg/ml EDTA, pH 7.4 in a cellulose dialysis bag. The dialysis solution was changed three times, each with 4 l of dialysis solution which was precooled at 4° C. After dialysis the sample was concentrated from 130 ml down to 13 ml and kept frozen at -20° C.

Preparation of liposomes. To make phospholipid liposomes with a final concentration of 2.5 mg/ml, 1,038 mg of lecithin were used for a total volume of 415 ml. Lecithin dissolved in chloroform was dried under nitrogen gas and 10 ml of .01 M phosphate buffer, 0.1 mg EDTA/ml, pH 7.5 were added. This mixture was sonicated for a total of 8 minutes with four intervals of each 2 minutes.

Purification of LCAT by hydroxylapetite column chromatography. Hydroxylapetite (BioGel HT) was obtained from Bio-Rad Laboratories (Richmond, California). The column material was received in 1 mM sodium phosphate buffer at pH 6.8. Approximately 100 ml of this solution after thoroughly mixing was transfered to a container and let it settle down. The upper layer was poured out. Phosphate buffer (0.01 M) containing 0.15 N NaCl was added, mixed and let to settle in a refrigerator. Then the top clear supernatant was poured out. This was repeated three times to equilibrate the column material with the phosphate buffer. The column was washed and dried. Hydrophobic surface coating solution, desicote (Altex) was poured through it, drained, rinsed with distilled water and dried.

The column with a diameter of 1.5 cm was packed with the flow rate of 5-6 ml/hours to the height of 18 cm. The column was extensively eluted with 0.01 M phosphate buffer in 0.15 M NaCl before the crude enzyme preparation was applied.

A gradient mixer was used to make linear gradient of the elution buffer solution. Two buffers with concentrations of 0.01 and 0.1 M sodium phosphate containing 0.15 M NaCl were made. The low buffer was connected to the tube connecting directly to the column and the high buffer added to the low buffer gradually through a channel.

The enzyme preparation was thawed and gently mixed. Two milliliters of enzyme preparation was mixed with 10 ml of sodium cholate solution (.01 g/ml solution). This mixture was applied on top of the column bed without disturbing the column bed.

A total of 200 fractions were collected. For the first 22 tubes 110 drops, or 7.2 ml, were collected and for the rest of the tubes 50 drops, or 2.5 ml, were collected. The total time of collection was approximately 6 days. The content of each tube was read at the wavelength of 280 nm in a dual beam Beckman spectrophotometer. The column fractions (Table 3) were pooled into nine fractions after plotting the results obtained from spectrophotometer readings (Figure 2). The nine fractions were concentrated through a Diaflo concentrator with PM 30 filter membrane.

The fractions were dialyzed in dialysis bags (cellulose acetate) against 0.01 M Na_2HPO_4/.15 M NaCl containing 0.1 mg/ml EDTA, pH 7.35. The dialysis solution was cooled in refrigerator prior to use. The dialysis solution was changed every 6 hours. The content of the bags after dialysis were then transferred to storage tubes and refrigerated.

Statistical Analysis

The collected data were analyzed in the following ways. The data were first arranged as a factorial set of treatments in a completely randomized block design to examine the interactions of four different diets (P/H, S/H, P/L, S/L) on plasma enzyme activity, plasma total cholesterol (TC), plasma-free cholesterol (FC), and plasma triglyceride (TG). Since the number of observations was not matched for F test analysis, orthogonal sums of squares (Steel and Torrie, 1980) were used to get a general idea about the beforementioned parameters and their interactions. A crossover design (Cochran

Table 3. Fractions collected from hydroxylapatite column.

Fraction	Tube No.	Approximate Volume After Concentration (ml)	Enzyme Activity % CE/hr
I	23- 32	3	--
II	33- 40	3	0.35
III	41- 53	3	0.21
IV	54- 95	5	24.8
V	96-105	3	26.0
VI	106-112	3	8.5
VII	113-140	10	0.66
VIII	141-159	4	0.64
IX	160-200	10	1.50

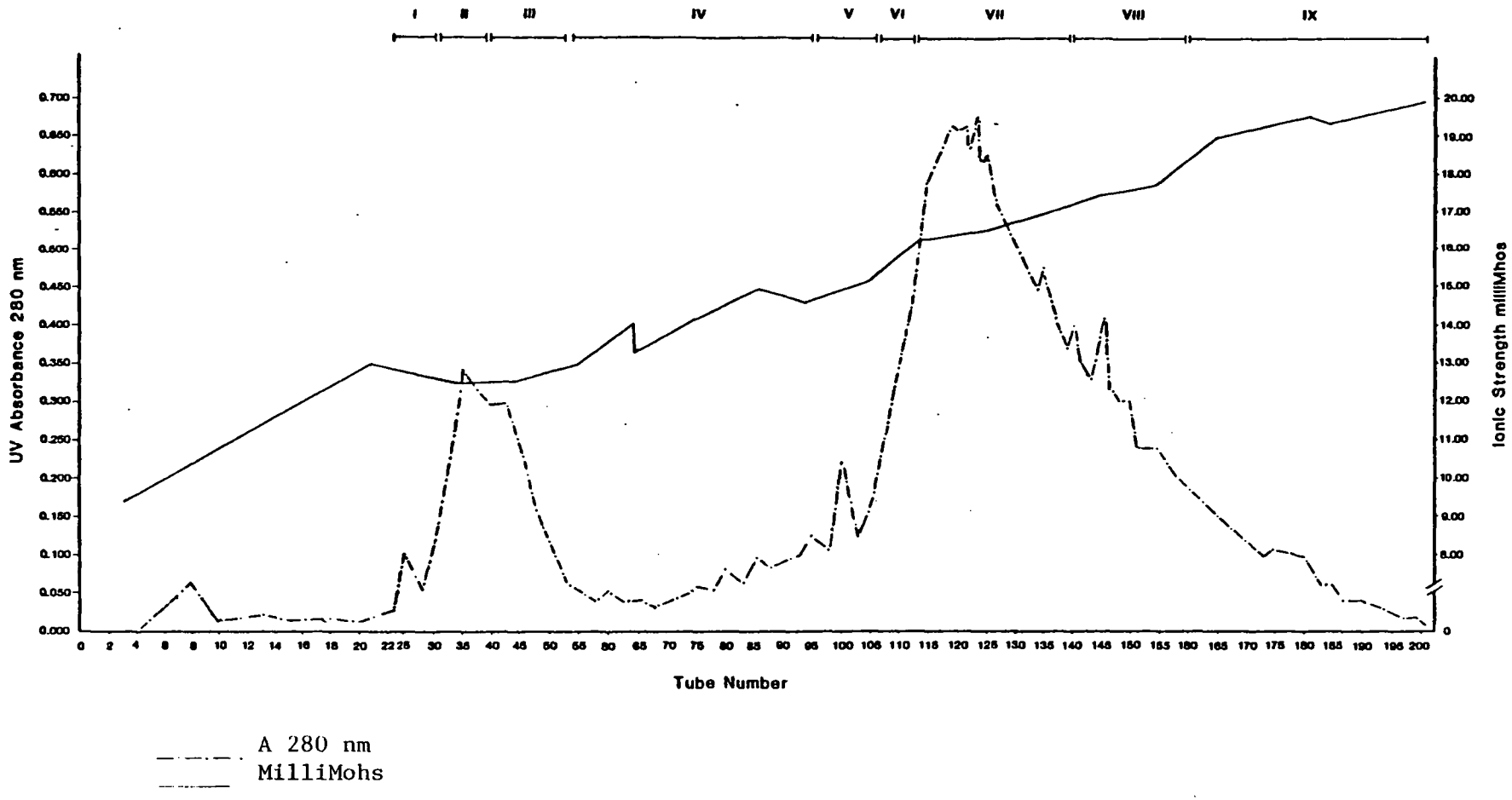


Figure 2. Chromatography of LCAT on hydroxylapatite. (Roman numerals represent the column fraction numbers. LCAT activity is found in fractions IV and V.)

and Cox, 1950; Neter and Wasserman, 1974) was used to examine the effect of dietary modification and diet order on enzyme activity, TC, FC, and TG. Since in this statistical design, the number of individuals in each group should be equal, subjects numbered 5 and 13 were omitted from groups II and III, respectively. To adjust for the initial variation observed in individuals, Time II minus Time I and Time IV minus T were used for each individual observation. This is used for indexing and removal of some of the individual variability which existed at the baseline. Secondly, for comparison Times II and IV were compared with Time I.

The paired t test (Steel and Torrie, 1980) was used to compare differences in the biochemical parameters between each period.

RESULTS AND DISCUSSION

The rate of esterification of free cholesterol in the pooled plasma of the subjects in this study was 54.0 nmole/liter/hr and the fractional esterification rate, the fractional turnover of the intravascular pool of the free cholesterol, was 3.5% per hour. These values are substantially lower than the reported normal values of 90 nmole/hr/liter of plasma for molar esterification rate (MER) and 6-7% for the fractional esterification rate, respectively (Dobiasova, 1983). The given normal values of Dobiasova (1983) were obtained by using different methods. The present values were obtained by measuring LCAT activity in self substrate, that is, by incorporating ^{14}C labelled cholesterol in the plasma pool of cholesterol during 4-hour preincubation of ^{14}C-cholesterol and the plasma while the enzyme activity was inhibited by DTNB. Using the self-substrate, Kudchodkar and Sodhi (1976) showed that the molar esterification rate of the enzyme was 82 nmole/hr/liter of plasma, which was still considerably higher than the present data.

The effect of dietary fat modification on plasma LCAT activity and plasma TC, FC, and TG during two 5 1/2 week experimental periods is shown in Table 4. The result of fat modified diet on fractional LCAT activity indicates a systematic change in LCAT activity independent of diet as shown in Figure 3. The mean percent esterification of cholesterol during the first 5 1/2 weeks of the study was increased in all four groups regardless of the kind of diet. The increases in LCAT activity in groups I, II, III, and IV were 71, 69, 42, and 69 percent, respectively. In groups I, II, and III

Table 4. Effect of dietary fat modification on plasma activity of lecithin:cholesterol acyltransferase (LCAT), total cholesterol (TC), free cholesterol (FC), and triglyceride (TG).

Group	Diet	Time	LCAT % CE/hr		TC		FC mg/100 ml		TG	
	Baseline	I	2.69	0.59	133.7	12.1	40.7	4.2	59.7	8.7
I n=3	S/H	II	4.65	1.32*	176	21.7*	47.7	3.5	78.7	25.5
	P/H	IV	2.54	0.41	126.3	11.6*	39.0	7.0	45.3	9.0
	Baseline	I	2.83	0.47	130.5	9.2	44.3	6.3	67.5	34.5
II n=4	P/H	II	4.83	1.15*	137.3	28.8	40.5	5.8	57.5	15.6
	S/H	IV	2.63	0.51*	153.8	23.2	47.8	8.2	62.8	45.5
	Baseline	I	2.43	0.10	156	24.1	61	18.2	61.0	40.5
III n=4	P/L	II	3.46	0.45*	160.8	42.9	46.5	11.9	61.0	48.7
	S/L	IV	2.35	0.27*	155	24.4	45	7.7	64.5	22.7
	Baseline	I	2.68	0.52	125.3	18.2	42	6.1	47.0	16.1
IV n=3	S/L	II	4.52	1.71	138.3	24.8	40.3	7.1	57.3	16.5
	P/L	IV	2.50	0.64	111.3	11.0	32.3	3.5	28.3	42.5

* The reported mean and standard deviation values are significant ($p < .05$).

t_2 0.05 = 4.303

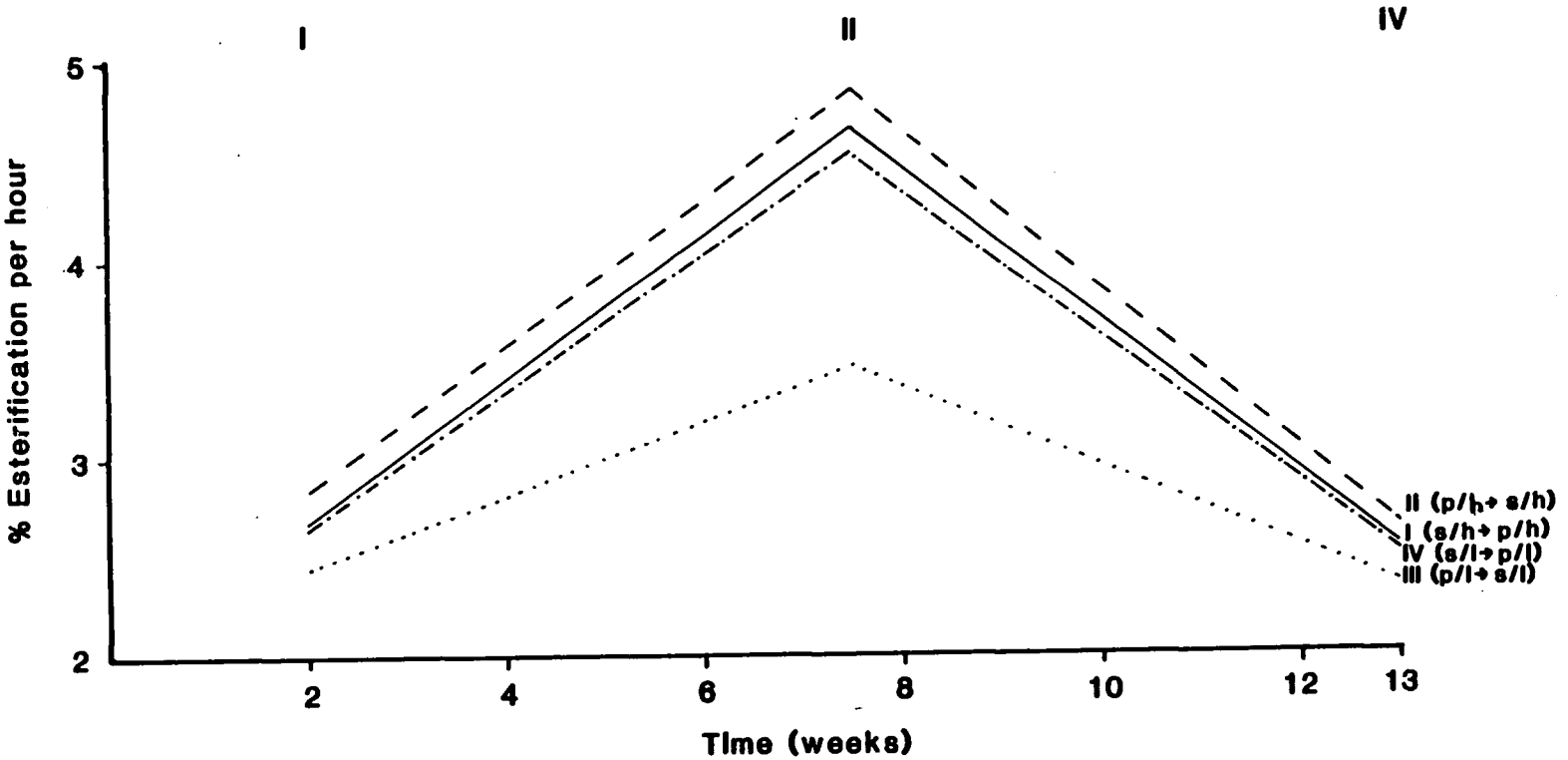
t_3 0.05 = 3.182

n = number of subjects in each group

on S/H, P/H, and P/L diets, the rises were significant from the baseline ($p < 0.05$). During the second 5 1/2 weeks of experimental period a fall in percent esterification of cholesterol was observed in all four groups, groups I, II, III, and IV on diets P/H, S/H, S/L, and P/L, respectively. The decreases in percent esterification of cholesterol were by 46, 46, 52, and 45 percent, respectively. In groups II and III on S/H and S/L diets, this fall was significant ($p < 0.05$).

Plasma total cholesterol (Figure 4) levels were significantly elevated ($p < 0.05$) by the diets containing high saturated fat with either high cholesterol (S/H) or low cholesterol (S/L) while the diets high in polyunsaturated fat with high (P/H) or low cholesterol (P/L) substantially decreased the sterol levels in plasma. Plasma LCAT activity in the groups I, III, and IV followed a similar trend of the changes in plasma total cholesterol. That is, the LCAT activity was decreased when the total plasma cholesterol level was decreased and increased when the cholesterol level was increased. However, in the group II the LCAT activity was in opposing direction of the changes in plasma total cholesterol on both P/H and S/H diets. By many laboratories (Akanuma et al., 1973; Albers et al., 1981; Fabien et al., 1973; Sutherland et al., 1979) a positive relationship between LCAT activity and the concentration of plasma cholesterol has been established.

The changes in plasma free cholesterol levels (Figure 5) were in a similar trend as those of the total cholesterol in two groups (I and II) but different in groups III and IV. There was a lack of specific



I - Baseline second week of experiment.
II - End of first experimental diet and beginning of the second experimental diet.
IV - End of second experimental diet.

_____ Group I S/H → P/H diet
_ _ _ Group II P/H → S/H diet
..... Group III P/L → S/L diet
_._._ Group IV S/L → P/L diet

Figure 3. Effect of dietary fat and cholesterol modification on activity of plasma lecithin:cholesterol acyltransferase activity in four experimental groups.

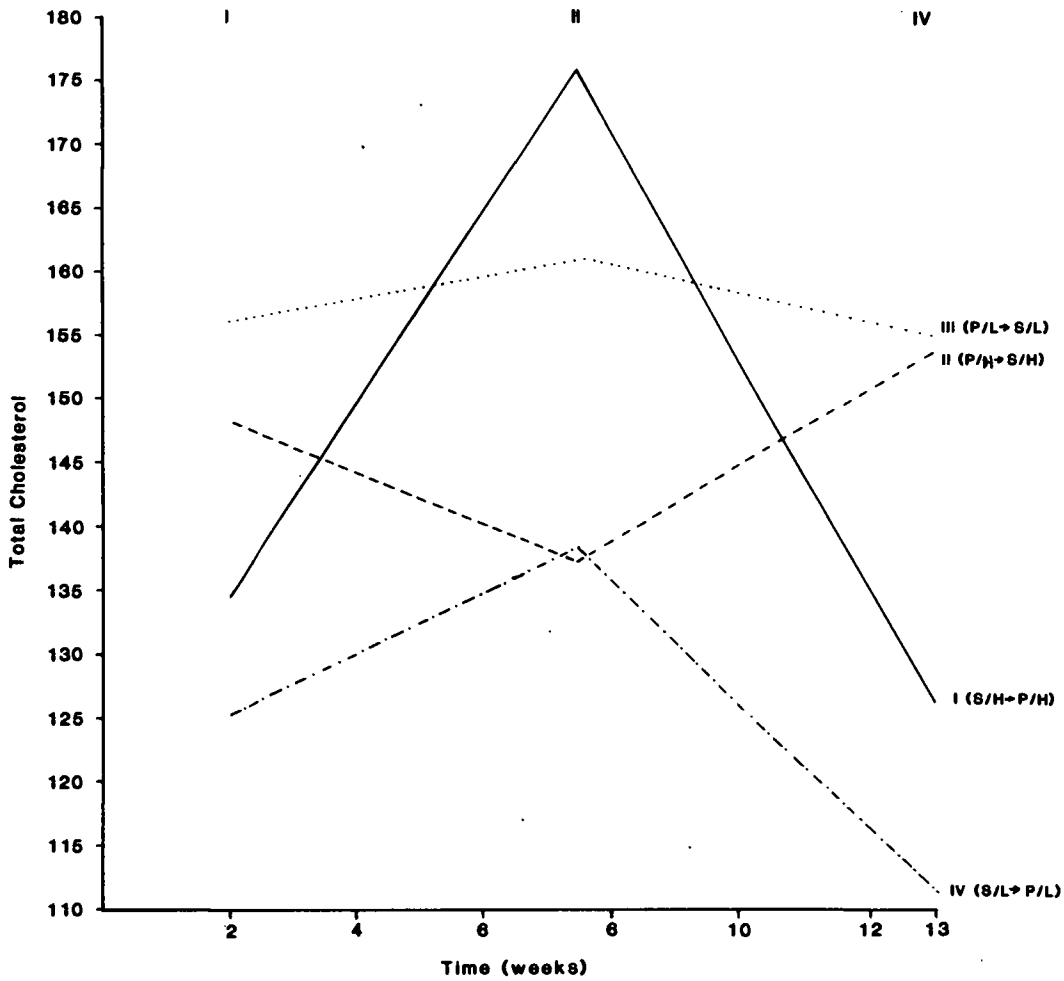


Figure 4. Effect of four experimental diets varying in amount of cholesterol and degree of fat saturation on the plasma total cholesterol measured as mg of total cholesterol per milliliter of plasma.

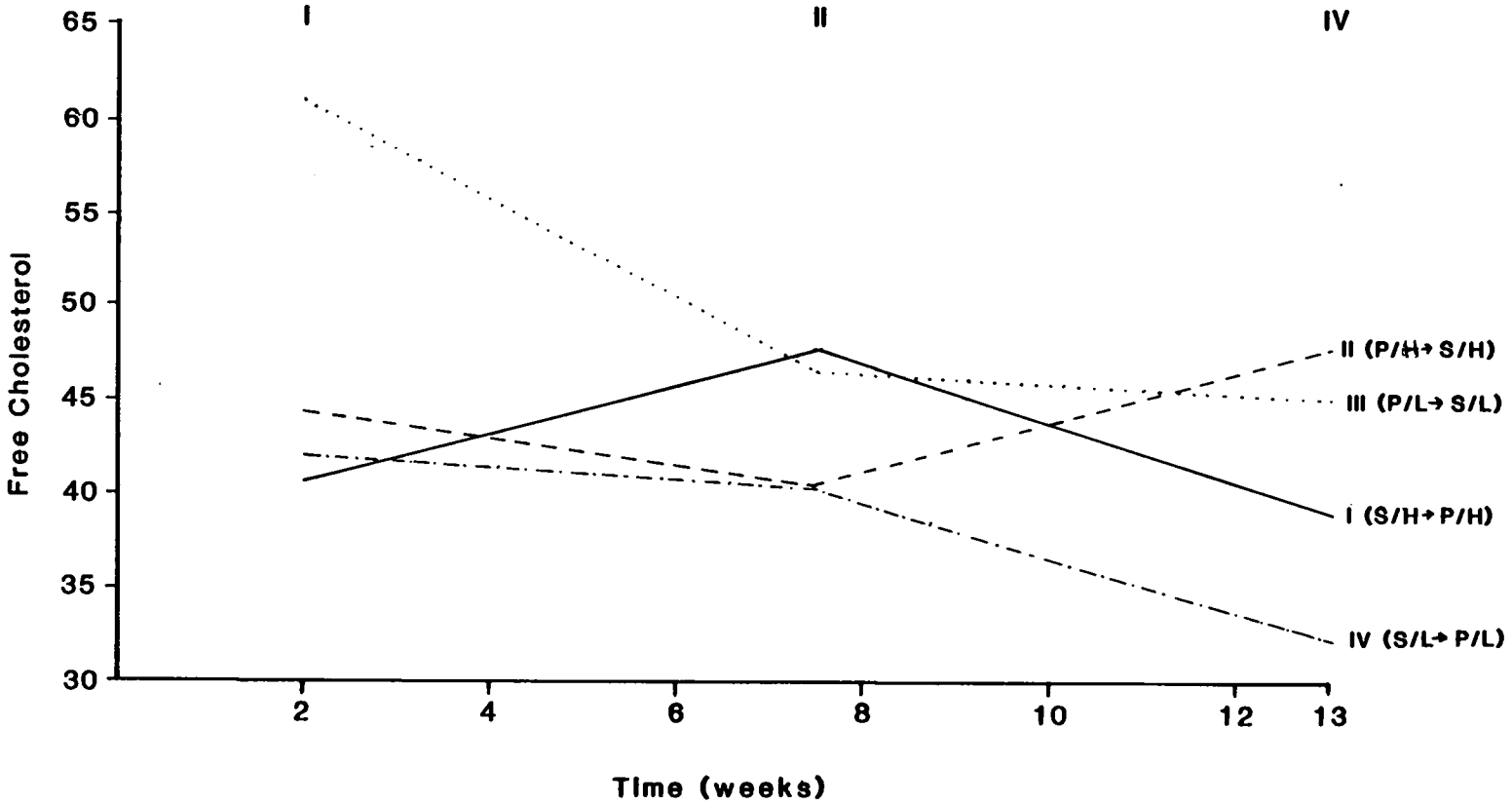


Figure 5. Effect of four experimental diets varying in amount of cholesterol and degree of fat saturation on plasma free cholesterol measured as mg of free cholesterol per milliliter of plasma.

correlation between the LCAT activity and free cholesterol levels in plasma.

Although the plasma triglyceride (Figure 6) levels were not significantly changed ($p < 0.05$) as a result of dietary fat modifications, the trend of changes in LCAT activity was similar to that of plasma triglyceride levels in groups I and IV. Again, there was inconsistency in the relationship between the LCAT activity and triglyceride levels in the groups II and III.

The analysis of variance for the major biochemical parameter (Table 5) showed that in the diets high in cholesterol (P/H and S/H) there was no significant effect of diet order on LCAT activity, plasma level of total cholesterol, free cholesterol, and triglycerides. However, the P/S ratio of the diets high in cholesterol had a significant ($p < 0.05$) effect on LCAT activity, plasma free cholesterol, and triglyceride level but not on total cholesterol. The analysis revealed that in the diets containing low cholesterol (S/L and P/L) there was statistically significant effect ($p < 0.01$) of diet order on plasma LCAT activity. The P/S ratio in the diets also had significant effect on plasma LCAT activity ($p < 0.01$) and triglyceride level ($p < 0.05$).

The response of plasma LCAT activity to dietary fat and cholesterol has been investigated in various laboratories. Gjone et al. (1972) demonstrated a reduction in cholesterol esterification rate *in vitro* when saturated fat was replaced by unsaturated fat in the diet. This finding was later confirmed by Miller et al. (1975) who conducted an experiment in which the effect of a switch from saturated

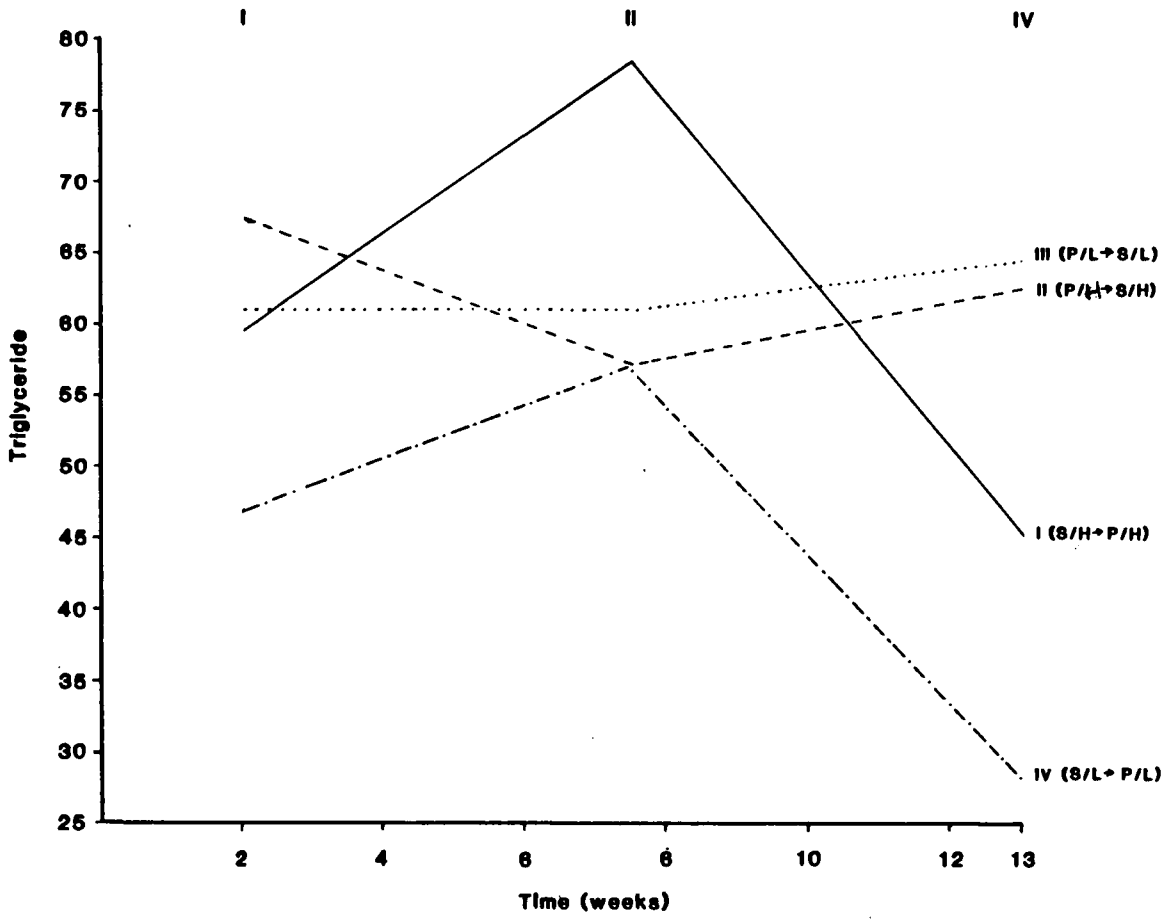


Figure 6. Plasma triglyceride (mg per milliliter of plasma) in subjects on four different diets varying in amounts of cholesterol and polyunsaturated to saturated fat (P/S) ratio.

Table 5. Crossover design values on lecithin:cholesterol acyltransferase (LCAT), total cholesterol (TC), free cholesterol (FC), and triglyceride (TG).

		LCAT		TC		FC		TG	
Diet	DF	MS	F	MS	F	MS	F	MS	F
High Cholesterol:									
Diet order	1	0.05	0.22	133.33	0.25	24.17	1.51	147	1.33
P/S	1	11.96	5257*	1541.33	2.84	140.17	8.74*	1496.33	11.63*
Error	4	0.23		541.84		16.04		128.67	
Low Cholesterol:									
Diet order	1	2.65	18.29*	96.33	0.45	16.33	0.9	602.08	3.32
P/S	1	6.84	48.86*	1200	5.65	85.33	7.72	1518.75	8.37*
Error	4	0.14		212.42		18.09		181.49	

* $p < 0.05$

$df_1 = 7.71$

fat (P/S of 0.2) to polyunsaturated fat (P/S of 2.4) for a period of 7 to 9 days decreased the plasma LCAT activity and esterification rate *in vitro* in the same individuals. The unsaturated fat diets resulted in significant decreases in the concentrations of cholesterol and triglycerides in parallel with a reduction in LCAT activity and net fraction esterification rate of cholesterol. However, Dobiasova (1983) converted this net rate of cholesterol esterification obtained by Miller to fractional esterification rate and showed that changing diet did not affect the esterification rate and the concentration of plasma lipids. Previous works revealed conflicting findings of the effect of dietary cholesterol on plasma LCAT activity. When high cholesterol diets were fed to rabbits, plasma LCAT levels were reported to decrease by Rose (1972) and Shapiro et al. (1968), but Wells and Rongone (1969) found that dietary cholesterol increased LCAT activity in rabbits. This conflict might be caused by the difference in substrate preparation between the laboratories. The effect of dietary P/S ratio on LCAT activity may be exerted as the result of changes in plasma cholesterol level by the P/S ratio in diet or the degree of fatty acid unsaturation in phospholipids by dietary P/S ratio or the reduced level of triglycerides.

Reduced esterification on high P/S diets has also been observed by Chait et al. (1974). In a study by Rosseneu et al. (1979) the dietary polyunsaturated lecithin increased the cholesterol esters and lysolecithin content in HDL, presumably due to activation of LCAT. Lichtenstein et al. (1980) reported effect on LCAT activity primarily by dietary fat and to a lesser extent by dietary cholesterol in

squirrel monkeys. Corn oil diet affected the rate of LCAT activity in both species of monkeys similarly, and coconut oil diets brought about a lower percent esterification than the corn oil diets. Coconut oil feeding also resulted in lower substrate activity in all monkeys, whereas cholesterol feeding had no significant effect on substrate activity.

Previous works have demonstrated that the mechanism of cholesterol esterification was closely related to the intravascular transformation of VLDL and chylomicrons (Glomset and Norum, 1973; Norum et al., 1982), and that hypertriglyceridemia in humans was often associated with the increased LCAT activity (Akanuma et al., 1973; Blomhoff et al., 1974; Kuczynska and Sznajderman, 1980; Kudchodkar and Sodhi, 1976; Wallentin, 1977). Rose and Juliano (1977) found a significant positive correlation between percent increase in plasma triglycerides and esterification rate in eight human subjects. The average increment in triglyceride concentration was not parallel in time. Esterification attained a rather broad optimum after 5-7.5 hours. On the other hand, the triglyceride concentration was substantially increased as early as 2.5 hours after feeding, reached a maximum in the fifth hour, and fell sharply after 7.5 hours; a normal postabsorptive level was attained after 10 hours.

Isolation and Purification of LCAT

The stepwise purification of LCAT enzyme is presented in Table 6. The specific activity of plasma LCAT activity was 1.09 unit per mg of plasma protein. One unit of enzyme activity is equivalent to

Table 6. Successive steps in LCAT purification.

Step	Source of Enzyme	Specific Activity (units*/mg)	Yield (%)	Purification (n-fold)
Plasma	23,430	1.09	100	1
Crude enzyme preparation	1,055	11.4	47	10
Hydroxyapatite (fractions no. IV & V)	8.2	425.9	14	390

* One unit of enzyme catalyzed the esterification of 1 nmole of cholesterol per hour at 37°C.

1 nmole of cholesterol esterified during 1-hour incubation at 37° C. When the plasma LCAT was partially purified by ultracentrifugation of the enzyme liposome complexes, the specific activity was increased by tenfold and the yield was 47%. The crude enzyme prep was further purified by hydroxyapatite column chromatography as described earlier (Method of Analysis section). The specific activity of the enzyme was greatly increased to 426 units per mg protein. This is 390-fold purification when compared with the enzyme specific activity in plasma and the yield was 14%. The present purification was quite a bit lower than previous reports (Aron et al. 1978; Chen and Albers, 1981; Chung et al., 1979; Kitabatake et al., 1979). Various researchers have reported methods for the purification of LCAT that yield purification ranging from 16,000- to 34,700-fold with recoveries varying between 8% and 13%, with the use of several column chromatographies, including immunoabsorption chromatography (Table 7). Aron et al. (1978) used multiple column chromatographies which purified the enzyme with the final specific activity of 10,200 unit/mg of protein before the immunoabsorption step. The protein content of this final enzyme preparation was only 0.22 mg purified from 13,939 mg of plasma protein. Compared to the above findings, the enzyme protein obtained from the present procedure had a yield 40 times greater but with about 20-fold less purification. The discrepancy might be caused by the use of specific affinity column chromachromatography.

Table 7. Successive steps in LCAT purification schemes elaborated by different authors.

Step	Protein (mg)	Specific Activity (units*/mg)	Yield (%)	Purification (n-fold)	Reference
Plasma	13,939	1.26	100		Aron et al. (1978)
First CsCl step	420	27	66	21	
Second CsCl step	52	215	63	171	
Sephadex G-100	11	610	39	477	
Hydroxyapatite	0.22	10,200	13	8,075	
Anti-apo D column	0.065	20,370	8	16,166	
Serum	58,400	0.05	100	1	Chung, Lagocki, Albano, and Scanu (1979)
d = 1.21 g/ml middle fraction	458	5.96	93	118	
Affigel Blue	81.1	19.2	53	381	
DEAE-cellulose	9.9	132.8	45	2,635	
DEAE-Sephadex A-50	2.9	192.4	19	3,817	
Hydroxyapatite	0.307	850.2	8.9	16,889	
Plasma	91,740	0.12	100	1	Chen and Albers (1981)
d = 1.21-12.5 g/ml middle fraction	2,068	3.56	66.9	30	
Phenyl-Sepharose	68.3	100	62.0	833	
DEAE-Sepharose	28.3	176	45.2	1,467	
Hydroxyapatite	0.75	1,980	13.4	16,500	
Plasma	105,000	6	100	1	Kitabatake et al. (1979)
DEAE-Sephadex	10,000	43	68	7	
Butanol-(NH4)2SO4	1,481	149	35	25	
Preceipitat, DEAE-Sephadex	464	366	27	61	
Dextran sulfate supernatant	44	2,840	20	473	
Hydroxyapatite	0.3	208,000	10	34,700	

* One unit of enzyme catalyzed the esterification of 1 nmol of cholesterol per hour at 37° C.

SUMMARY

A study of the effect of fat and cholesterol modification on the activity of plasma lecithin:cholesterol acyltransferase and other plasma lipids, total cholesterol, free cholesterol, and triglycerides was carried out. Fourteen young adult healthy male subjects were divided into four experimental groups. Each group was subjected to two experimental diets with a crisscross design. The four experimental diets varied in amount of cholesterol (250-950 mg), and polyunsaturated to saturated fat ratio (0.2-2.0).

Analysis of plasma LCAT activity indicated no significant effect of diet on this parameter. However, plasma total cholesterol levels were significantly elevated ($p < 0.05$) by the diets containing high saturated fat with either high cholesterol (S/H) or low cholesterol (S/L), while the diets high in P/S ratio and containing high (P/H) or low (P/L) cholesterol showed greatly decreased plasma cholesterol concentrations. Plasma triglyceride levels were significantly decreased by the diets high in P/S ratio but not consistently by the diets.

Since the changes in LCAT activities measured in this study were not consistent with the changes in diets differing in amount of cholesterol and P/S ratios or with those of plasma lipid levels, it is difficult to draw a conclusive summary of the relationship between dietary fats and LCAT activity.

A simple method for mass purification of LCAT resulted in a good yield (14%) with 390-fold purification, substantially below that which has been achieved by others.

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APPENDIX

Table 8. Values from plasma analysis of lecithin:cholesterol acyltransferase (LCAT), total cholesterol (TC), free cholesterol (FC), and triglyceride (TG).

Subject	%CE Formation/hr LCAT					TC				
	0	I	II	III	IV	0	I	II	III	IV
1	2.03	2.10	3.22	2.54	2.11	123	138	190	142	133
2	5.59	2.72	4.92	2.34	2.58	155	143	187	156	133
3	5.38	3.22	5.81	2.88	2.93	130	120	151	132	113
4	5.86	2.36	3.98	2.21	2.18	171	142	173	208	174
5	4.90	3.42	6.50	3.53	3.36	136	132	112	160	165
6	4.28	3.04	4.15	2.44	2.54	143	128	148	162	155
7	3.10	2.56	5.68	3.08	3.24	132	118	124	106	105
8	3.72	2.23	2.56	2.34	2.11	163	146	167	162	124
9	4.42	3.24	5.33	2.44	2.16	132	112	124	123	105
10	3.12	2.56	3.74	1.65	2.20	164	136	139	147	149
11	2.40	2.44	3.92	1.59	2.18	206	176	225	236	186
12	3.38	2.42	3.20	2.07	2.76	140	147	137	154	127
13	3.01	2.31	3.97	2.42	2.27	172	175	142	167	158
14	2.88	2.58	4.68	2.70	2.44	129	120	116	115	121
	FC					TG				
1	35	36	48	37	47	68	67	77	61	46
2	45	44	51	42	36	56	50	54	43	36
3	41	42	44	42	34	59	62	105	87	54
4	51	50	48	46	59	67	65	66	70	51
5	44	49	34	45	48	109	117	75	145	130
6	44	41	39	43	40	70	43	42	45	36
7	38	38	34	33	29	28	32	39	27	25
8	50	49	48	44	36	--	64	62	61	33
9	61	39	39	35	32	100	45	71	45	27
10	58	49	40	43	43	51	32	46	35	39
11	61	63	62	68	51	96	116	132	146	80
12	43	86	35	40	35	--	29	21	21	30
13	--	46	49	54	51	96	67	45	79	37
14	--	37	41	41	44	40	45	47	48	34