ISOLATION, PHYSICAL AND CHEMICAL CHARACTERIZATION
OF LECITHIN:CHOLESTEROL ACYLTRANSFERASE
FROM HUMAN PLASMA

DISSERTATION

Presented to the Graduate Council of the
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For the Degree of

DOCTOR OF PHILOSOPHY

By

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Denton, Texas
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Lecithin:cholesterol acyltransferase (LCAT) was isolated from a human plasma fraction, in a homogeneous state, by an efficient and highly reproducible procedure. The purification scheme involved: (1) hydrophobic affinity chromatography, (2) DEAE-agarose chromatography, (3) delipidation, (4) a second DEAE-agarose chromatography, and (5) a combination of hydroxylapatite and antibody adsorption chromatography. The final product was purified 20,000-fold over the starting material and was found to be free of polypeptide contaminants as indicated by gel electrophoresis, sedimentation equilibrium ultracentrifugation, immunodiffusion and immunoelectrophoresis.

The physical and chemical properties of LCAT were investigated. The partial specific volume ($\bar{\nu}$) of LCAT was determined by sedimentation equilibrium ultracentrifugation experiments ($\bar{\nu} = 0.702$ ml/gm) and by compositional analysis ($\bar{\nu} = 0.708$ ml/gm). An average molecular weight of 67,000 daltons was obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis as compared to the value of 60,000
daltons determined by sedimentation equilibrium experiments. The discrepancy between the two sets of data is presumably due to the glycoprotein nature of the enzyme. Studies of the ultraviolet spectrum revealed twelve moles of tyrosine and eleven moles of tryptophan per mole of LCAT. Ten moles of tryptophan per mole of the enzyme were calculated from fluorometric measurements under denaturing conditions. Human LCAT was found to have a relatively high extinction coefficient \( E_{1	ext{cm}(280	ext{nm})} = 21 \) at neutral pH. From the circular dichroism study, 24% \( \alpha \)-helix, 27% \( \beta \)-sheet, and 49% random structure were calculated. Upon isoelectric focusing, LCAT exhibited four protein bands with apparent PI values ranging from 4.2 to 4.5. LCAT was also found to contain about 25% (w/w) carbohydrate: hexoses, 13% (w/w); hexosamines, 6.2% (w/w); and sialic acids, 5.4% (w/w). Based on four half-cystines, the total number of 408 amino acid residues per mole of LCAT was calculated. Two residues of cysteine/mole of LCAT were found when titrated with 5,5'-dithiobis-2-nitrobenzoic acid.
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CHAPTER I

INTRODUCTION

Cholesterol Metabolism And
Cholesterol Transport

Cholesterol is an essential structural component of all cell membranes. The metabolism of cholesterol is reasonably well understood with regard to its biochemical reaction mechanisms, while much less is known about the transport processes that insure the adequate cellular supply and orderly catabolism of cholesterol in mammals. There are at least two major reasons for the existence of mammalian cholesterol transport systems.

1. Nearly all tissues are capable of synthesizing cholesterol from acetyl-CoA but the major producers are the liver and the small intestine where biosynthetic rates generally exceed the rate of local utilization. The cholesterol supply of extra-hepatic tissues appears to be derived from receptor-mediated uptake of plasma low-density lipoproteins (LDL)\(^1\) (Goldstein and Brown, 1974), to a lesser extent from endogenous synthesis (Dietschy and Wilson, 1970), and

\(^1\)Abbreviations used: LDL, low-density lipoprotein; VLDL, very low-density lipoproteins; HDL, high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; apo, apoproteins; DTNB, 5-5'‐Dithiobis-2-nitrobenzoic acid.
perhaps from direct transfer from other lipoproteins (Shattil et al., 1976).

2. Excess cholesterol from peripheral tissues has to be transported to the liver where catabolism and disposition of cholesterol from the body occurs exclusively by conversion to bile acids. Because of the low water solubility of cholesterol, its transport presents an intriguing biochemical problem. Cholesterol in the lymphatic and blood circulation is thus transported as a component of complex macromolecules referred to as plasma lipoproteins. Some of the basic properties of these lipoproteins are shown in Table I.

**Plasma Cholesterol Metabolism and Lecithin: Cholesterol Acyltransferase (LCAT)**

Human plasma LCAT (EC 2.3.1.43) is an enzyme that is synthesized in the liver (Simon and Boyer, 1970) and acts on high-density lipoproteins (Glomset, 1968). About 70% of the cholesterol in human plasma is in the esterified form and the source of plasma cholesteryl esters has been shown (Glomset, 1979) to be the result of the LCAT reaction. By catalyzing fatty acid transfer from the C:2 position of phosphatidylcholine to unesterified cholesterol, it promotes the formation of lysophosphatidylcholine and of cholesteryl esters. This reaction is considerably more complicated than other acyltransfer mechanisms (such as acetylcholine esterase) since both substrates and products have limited
**TABLE I**

CLASSIFICATION AND COMPOSITION OF HUMAN SERUM LIPOPROTEINS

<table>
<thead>
<tr>
<th></th>
<th>Density Range</th>
<th>% Protein</th>
<th>% Triglycerides</th>
<th>% Cholesterol*</th>
<th>% Phospholipid</th>
<th>Major Apo-Proteins</th>
<th>Minor Apo-Proteins</th>
</tr>
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<tr>
<td>Chylomicrons</td>
<td>0.94</td>
<td>2</td>
<td>83</td>
<td>8</td>
<td>7</td>
<td>CI, CII, CIII, AI</td>
<td>B</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.94 -1.00</td>
<td>9</td>
<td>50</td>
<td>22</td>
<td>18</td>
<td>B, CI, CII, CIII</td>
<td>E</td>
</tr>
<tr>
<td>LDL</td>
<td>1.00 -1.063</td>
<td>21</td>
<td>11</td>
<td>46</td>
<td>22</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.20</td>
<td>50</td>
<td>8</td>
<td>20</td>
<td>22</td>
<td>AI, AII</td>
<td>D, E, CI, CII, CIII</td>
</tr>
</tbody>
</table>

*Includes unesterified cholesterol and cholesterol esters.
solubility in water, and yet the physiological site of the reaction is in a hydrophilic medium (human plasma). LCAT, indeed, cannot act on pure lipids and requires polypeptide cofactors (Fielding et al., 1972) to be present during the reaction. In vivo, LCAT acts on plasma lipoproteins which donate the lipid substrates for transesterification.

High-density lipoproteins (HDL) and very high-density lipoproteins (VHDL) were reported to be preferred substrates for LCAT (Akanuma and Glomset, 1968a; Fielding and Fielding, 1971). Of the HDL apoproteins, apo-AI (a major polypeptide component of HDL) is the main activator of LCAT (Fielding et al., 1972). Soutar et al. (1975) and Sigler et al. (1976) have reported that apo-CI (a minor polypeptide component of HDL) may also activate LCAT. In contrast to all the evidence concerning preferential action of LCAT on HDL, patients of Tangier disease (almost complete absence of HDL) showed plasma cholesterol esterification in the normal range (Assman, 1976).

The two products of the LCAT reaction are cholesteryl ester and lysolecithin. The cholesteryl esters are thought to migrate towards the hydrophobic interior of the substrate HDL particles (Glomset, 1968) or transfer to VLDL with the aid of transfer protein(s) (Chajek and Fielding, 1978). Serum albumin has been shown to activate LCAT by sequestering lysolecithin (Glomset, 1968). Switzer and Eder (1965) and Klopfenstein (1969) found that in mammalian plasma, most of
the lysolecithin was associated with the serum albumin frac-
tion. Fielding et al. (1972) reported that serum albumin
was an essential component of a sonicated lecithin/cholesterol
substrate mixture for LCAT. From model studies it has been
shown that LCAT reacts readily with liposomes that contain a
3:1 molar ratio of lecithin to cholesterol (Nichols and
Gong, 1971). Aron et al. (1978) reported that in the pre-
sence of pure lecithin liposomes, serum albumin, and apoAI,
LCAT exhibited phospholipase activity at a rate comparable
to that of cholesteryl ester synthesis in the presence of
liposomes containing lecithin and cholesterol. They showed
that both phospholipase and transferase activities co-migrated
during anionic gel electrophoresis.

Relationship Between HDL and LCAT

In epidemiological studies, plasma levels of HDL are
correlated inversely with the incidence of atherosclerotic
cardiovascular disease. There is probably an inverse cor-
relation between levels of HDL cholesterol and tissue cho-
lesterol pools. It has been postulated that low plasma
concentration of high-density lipoproteins may result in
decreased cholesterol clearance from peripheral tissues,
including the arterial wall, with subsequent enhancement of
the atheromatous process (Miller and Miller, 1975). Con-
firmation 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 ghosts (Glomset, 1970). Stein and Stein (1973) subsequently reported that HDL had a greater capacity for removing $^{3}$H-cholesterol from Landschutz ascites cells in vitro than did LDL at a similar lipoprotein concentration. Bates and Rothblat (1974) similarly found that HDL promoted a greater release of desmosterol from cultured mouse fibroblasts than did LDL. The effectiveness of HDL in enhancing cholesterol efflux from peripheral cells has since been demonstrated in studies with cultured rat aortic smooth muscle cells and human skin fibroblasts (Stein et al., 1976a,b). Another explanation for the protective role of HDL has been proposed by Carew et al. (1976). They have found a marked decrease in binding, internalization, and degradation of LDL into the cell 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 lesions, then HDL might directly interfere with this process. An inverse relationship between HDL concentration and the size of body cholesterol pool further supports the notion that HDL is instrumental in promoting removal of tissue cholesterol (Miller and Miller, 1975).

HDL as secreted by the perfused rat liver differs in several respects from the HDL present in the peripheral
blood of intact animals (Hamilton, 1972; Marsh, 1974, 1976; Hamilton et al., 1976). The nascent form of HDL consists chiefly of bilaminar discoid particles. The cholesterol of discoidal HDL, isolated from rat liver perfusates containing an LCAT inhibitor (Hamilton et al., 1976) or from the plasma of patients with inherited (Glomset, 1978) or acquired (Ragland et al., 1978) LCAT deficiency, was esterified by LCAT in vitro at a greater rate than the cholesterol of normal plasma HDL. This difference in LCAT activity can be attributed to the lower cholesteryl ester content of nascent HDL, since this has been shown in artificial systems to inhibit enzyme activity (Fielding et al., 1972). The esterification of nascent HDL cholesterol by LCAT was accompanied by a change in morphology of the particles, which became transformed from discs to spheres with diameters similar to normal plasma HDL. On the basis of these observations, it seems probable that the transformation of nascent HDL to plasma HDL in vivo involves at least the following processes (Nicoll et al., 1980): 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 "bilayer," transforming it into a spherical pseudomicellar particle; and the transfer of other cholesteryl ester molecules to triglyceride-rich lipoproteins.
When VLDL and/or LDL were included in the incubation mixture, most of the cholesteryl ester formed was recovered in these lipoproteins (Norum et al., 1975). VLDL and LDL showed little or no reactivity with LCAT in the absence of HDL (Glomset, 1970). This finding presumably reflected the transfer of newly synthesized cholesteryl esters from HDL. The cholesteryl ester transfer from HDL to VLDL was also observed early by Nichols and Smith (1965). Chajek and Fielding (1978) have reported a specific protein which catalyzes the net transport of cholesteryl esters from HDL to VLDL and LDL, and found the transport protein to be apo D which is one of the apoprotein components usually found in HDL. This finding has recently come under intense criticism (Cheung et al., 1981).

Role of LCAT in Reverse Cholesterol Transport

A finding of Akanuma and Glomset (1968a) that HDL provides the preferred substrate for LCAT, due probably to activation of the enzyme by apoprotein AI (Fielding et al., 1972), prompted the suggestion that HDL and LCAT may play concerted roles in transporting cholesterol from peripheral tissues. Glomset (1968) postulated the mechanism to explain the role of LCAT in the removal of cholesterol from the peripheral tissues. (Fig. 1). While this scheme is twelve years old and it has had to be modified to accommodate recent findings (Glomset, 1979), it still represents a fairly accurate description of our current state of understanding of reverse
Figure 1. Postulated mechanism for the transport of cholesterol from membranes of peripheral cells to the liver. LCAT reacts with circulating lipoproteins to form cholesteryl esters from unesterified cholesterol and lecithin. The lipoproteins subsequently pick up unesterified cholesterol from cell membranes, circulate through the liver, and release esterified cholesterol. C, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid. (From J. A. Glomset (1968), J. Lipid Res. 9, 155.)
Uptake of C from membrane by physical equilibration

Uptake of CE, replenishment of PL by liver
cholesterol transport. Accordingly, 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, the cholesterol component of cholesteryl esters being excreted after prior hydrolysis (Stein et al., 1976b; Rothblat and Kritchevsky, 1967). Few data exist concerning the mechanism by which unesterified cholesterol is transferred from cells to HDL. The suggestion that the esterification of cholesterol in HDL by LCAT might facilitate the uptake of further cholesterol molecules by vacating surface binding sites was examined by Glomset (1970). In the absence of LCAT the unesterified cholesterol content of human plasma HDL increased by 122% during incubation with human erythrocyte ghosts, without any change in HDL esterified cholesterol. Addition of purified LCAT to the system led to an increase in HDL unesterified cholesterol of 152% and a 107% increase
in HDL esterified cholesterol, representing a 200% greater total uptake of cholesterol compared to that without LCAT. d'Hollander and Chevallier (1972) subsequently confirmed that the majority of unesterified cholesterol removed from rat erythrocytes by rat HDL in the presence of LCAT was recovered in the cholesteryl ester fraction.

Glomset et al. (1970) have also examined the relative capacities of the discoidal and small globular HDL from LCAT-deficient patients to remove cholesterol from erythrocyte ghosts. In the absence of LCAT, cholesterol was transferred from both lipoproteins to the ghosts. Addition of LCAT reversed this process suggesting that LCAT converts surface cholesterol of HDL into core cholesteryl ester, thereby creating a gradient for transfer of membrane unesterified cholesterol into HDL. The most marked effect was observed with discoidal particles, in which a 3-fold increase in total cholesterol content, and an 18-fold increase in cholesteryl ester occurred.

In addition to the role of LCAT in reverse cholesterol transport, Schumaker and Adams (1969) proposed that LCAT may participate in the maintenance of the spherical configuration of triglyceride-rich lipoproteins. They reasoned that LCAT would facilitate the removal of excess surface lipids that accumulate upon the hydrolysis of core triglyceride-rich lipoproteins by lipoprotein lipase.
Clinical data from patients with congenital LCAT deficiency substantiated the predictions of Schumaker and Adams (1969), since the majority of these patients exhibited hypertriglyceridemia in addition to abnormally low levels of plasma cholesteryl esters and other lipid abnormalities.

The physiological role of LCAT in lipoprotein metabolism is not yet adequately defined. LCAT might affect the chemical properties of lipoproteins and consequently the metabolism of circulating lipoproteins by changing their polar and non-polar lipid components. However, since most of the cholesterol of freshly isolated lipoproteins is already esterified, it is difficult to investigate the LCAT reaction using lipoproteins by in vitro studies. With the discovery of familial LCAT deficiency (Norum and Gjone, 1967) most of the current information concerning the metabolic role of LCAT have been derived from studies of the abnormal lipoproteins obtained from patients with congenital LCAT deficiency.

Isolation and Physicochemical Properties of LCAT

LCAT has been one of the most difficult enzymes to isolate in the homogeneous state. Several investigators have reported on the isolation of the enzyme (Albers et al., 1979; Utermann et al., 1980; Aron et al., 1978; Chung et al., 1979;
Kitabatake et al., 1979) yielding 0.065 mg to 0.31 mg of LCAT per preparation. These preparations have been purified 15,000 to 34,000-fold from human plasma. The molecular weight of LCAT by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was determined to be from 66,000 - 69,000, while Chung et al. (1979) reported that molecular weight of LCAT was 59,000 determined by the analytical sedimentation equilibrium ultracentrifugation. The discrepancy between the above sets of data is probably due to the glycoprotein nature of the enzyme, since Chung et al. (1979) reported that LCAT contained 25% (w/w) carbohydrate. Amino acid composition analysis has been reported by several investigators (Chung et al., 1979; Albers et al., 1979; Aron et al., 1978), although the data from respective laboratories do not agree very well with one another.

Purpose of This Investigation

The physiological role of LCAT has been the subject of a number of recent articles (Glomset, 1979; Nilsson-Ehle et al., 1980). According to most current theories, the enzyme functions in combination with high-density lipoproteins in the reverse cholesterol transport pathway which presumably returns peripheral cholesterol to the liver where cholesterol catabolism takes place. Despite the exciting potential for studies on the catalytic function and the nature of the enzyme-substrate complex, the mechanism
of action of LCAT remains largely unexplored. The relatively slow progress in the elucidation of the LCAT reaction mechanism is likely to be due to the difficulties in the isolation of the enzyme in sufficient quantities. Consequently, considerably less is known about the physical and chemical properties of the enzyme.

Therefore, the first objective of this investigation was to isolate and purify sufficient amount of enzyme for subsequent characterization studies.

The second objective of this investigation was to characterize the physical properties of the enzyme by techniques including analytical ultracentrifugation, ultraviolet spectroscopy, and circular dichroism and fluorescence spectroscopy.

The third objective of this investigation was to characterize the chemical properties of the enzyme which deals with the amino acid and carbohydrate composition and with some basic structural features that are related to the chemical composition of LCAT.

In the present studies I have attempted to answer these questions and open the way for future studies on the mechanism of action of LCAT.
CHAPTER II

MATERIALS AND METHODS

Materials

Plasma

A human plasma product (the supernatant from cryoprecipitate preparations) was generously provided by the Wadley Blood Bank of Dallas and used as the starting material for the LCAT purification procedure. This material appeared to have essentially the same LCAT activity as whole human plasma.

Chemicals

Bis-acrylamide, acrylamide, riboflavin, ammonium persulfate, N,N,N',N'-tetramethylethylene diamine, β-mercaptoethanol, Coomassie Blue G-250 and R-250 were obtained from Eastman-Kodak, Rochester, N. Y. Polyethylene glycol (PEG) 6000 (M.W. approx. 6,000), was purchased from Matheson, Coleman & Bell, East Rutherford, N. J. DEAE-agarose, hydroxylapatite, and protein assay kits were purchased from Bio-Rad Labs. Cyanogen bromide-activated Sepharose 4B, bovine serum albumin, deuterium oxide (99.8%), calf serum fetuin, ovalbumin aldolase, chymotrypsinogen, pronase, anthrone, 2-thiobarbituric acid, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and N-acetylneuraminic acid were purchased from Sigma Chemical
Co., St. Louis, Missouri. $[^1,2-^3H]$ cholesterol was obtained from Amersham/Searle. Ampholine gels were obtained from LKB. Amino acid standards and tryptophan were purchased from Pierce Chemical Co. D(+)-mannose and D(+)-galactose were obtained from Pfanstiehl Lab., Waukegan, Illinois. D(+)-glucosamine-HCl was from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals were products of Fisher Scientific Co., and were of reagent grade.

Methods

**Protein Determination**

The determination of protein concentration was carried out by amino acid analysis as well as protein-dye binding method (Bradford, 1976) using bovine serum albumin as a standard.

**Cholesterol Determination**

Enzymatic kits from Boehringer-Mannheim (colorimetric determination) were used to determine the unesterified cholesterol content of the high density lipoprotein (HDL) substrate. A cholesterol stock solution (Sigma Chemical Co.) was used to standardize the method.

**Polyacrylamide Gel Electrophoresis**

Analytical gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS) was performed according to the method described by Maizel (1971). Gels were stained with 0.05% (w/v) Coomassie Blue R-250 in isopropanol/acetic
acid/water(25:10:65, v/v/v). Destaining was carried out in a diffusion destainer using 10% (v/v) acetic acid. Analytical polyacrylamide gel electrophoresis was also performed in the presence of 7 M urea. Gels containing 7.5% (w/v) acrylamide were prepared according to Kane (1973). LCAT samples of 20–40 μg in 100 μl were mixed with an equal volume of 10 M urea solution containing 1.4% (v/v) β-mercaptoethanol, 0.05% (w/v) Bromphenol Blue and 20% (w/v) sucrose. The samples were then boiled for 5–10 minutes and cooled to room temperature. A constant current of 1.25 mA per gel was applied until the tracking dye entered the lower gel. Current was then increased to 2.5 mA per gel. Electrophoresis was conducted for 1–2 hr. at 23°C. The gels were stained for 8 hours in a 0.01% (w/v) Coomassie Blue G-250 solution as described by Blakesley and Boezi (1977). The gels were destained by several consecutive changes of distilled water.

**Preparation of HDL Substrate**

HDL was prepared as described previously (Jahani et al., 1980). An HDL solution, containing 50 mg of apoprotein, was mixed with 10 ml of an emulsion of [1,2-3H] cholesterol (5 μCi/ml) in 10% (w/v) fatty acid free BSA (Lacko et al., 1973). To this emulsion, a solution of BSA in buffer was added to achieve a final volume of 100 ml, a final BSA concentration of 2% (w/v), and a final buffer concentration of 0.01 M Tris, 0.005 M EDTA, 0.15 M NaCl and 0.005 M β-mercaptoethanol, and a final pH of 7.2.
Enzyme Assay

LCAT assays were conducted using a modified procedure of Glomset and Wright (1963). Samples of 5-10 μl of the enzyme containing 0.02-0.5 μg of LCAT were incubated with 200 μl of labeled HDL substrate at 37°C. The reaction time was adjusted so that initial rates (not exceeding 5% esterification) were obtained. Recovery studies were performed in duplicate yielding an average error of less than 5%. The assay mixtures contained approximately 740,000 dpm/ml amounting to a cholesterol concentration of 0.35 nmoles/ml. The tracer cholesterol contributed less than 1.0% to the total unesterified cholesterol pool and thus was not expected to influence the rate of esterification by LCAT. At the end of the incubation period the reaction was stopped by the addition of 20 volumes of chloroform:methanol (2:1, v/v). The lipid extracts were analyzed for unesterified cholesterol (UC) and cholesteryl ester (CE) as described by Lacko et al., (1972). The lipid extract of the assay mix was heated for fifteen minutes at 60°C and filtered on a 30-ml coarse sintered glass funnel. The residue was washed twice with aliquots of 2 ml chloroform:methanol (2:1, v/v) and then evaporated under a stream of nitrogen. The dry residue was dissolved in a small amount of chloroform and the solvent was evaporated again. The lipids were finally dissolved in 30 μl of n-heptane and applied to silica gel sheets (20 x 20 cm) with a plastic backing for thin layer chromatography (TLC). TLC was carried out in a
solvent mixture of petroleum ether:diethyl-ether:acetic acid (90:10:1, v/v/v). Staining was accomplished by exposing the air-dried plate to iodine vapors. After evaporation of the excess iodine, the zones containing cholesterol and cholesteryl esters were cut out of the plates and placed in scintillation vials for analysis. The radioactive zones containing cholesteryl esters were analyzed and the percent esterification established for each assay. The rate of esterification was computed as the product of the percent esterification/hr and the unesterified cholesterol concentration present in the sample.

**Preparation of the Antibody-Agarose Column**

The last stage of the purification procedure involved an antibody adsorption step using antibodies against the most persistent contaminants (presumably HDL apoproteins) in the highly purified LCAT preparations. Antisera against lipoprotein A and lipoprotein D (Curry et al., 1977) were generously donated by Dr. Walter J. McConathy of Oklahoma Medical Research Foundation. Aliquots of 5 ml of these antisera were made 33% (w/v) with regard to ammonium sulfate in deionized water, pH 7.8, and the crude immunoglobulin precipitates were dissolved in and dialyzed against 0.2 M sodium phosphate, pH 7.8. The antibodies were immobilized by coupling to CNBr-activated Agarose beads (Pharmacia) and used for obtaining additional antigens. When highly purified
LCAT preparations were applied to this column, the LCAT activity appeared in the void volume while most of the contaminants appeared to be retained by the immobilized antibodies. The column was regenerated with a solution of 2 M NaSCN and the eluted material was concentrated and dialysed. These concentrated eluates (1.5 mg in 1 ml of 0.1 M sodium phosphate buffer, pH 7.2) were emulsified with 1 ml of Freund's complete adjuvant and injected intradermally into the back of rabbits at multiple sites. The injection was scheduled for the 1st, 3rd, and 4th weeks, then at the end of each subsequent month. Blood was collected and tested every two weeks until satisfactory antibody titer was obtained, and then the animals were bled and the serum was collected by centrifugation.

Partial purification was performed by ammonium sulfate precipitation. The immunoglobulin fraction was extensively dialyzed against 0.175 M sodium phosphate buffer, pH 7.4.

Cyanogen bromide (CNBr) - activated Agarose (2.5 gm) was suspended in 1 mM HCl, pH 2.8. The slurry was stirred for 5 minutes then poured onto a sintered glass funnel, washed with 200 ml of 1 mM HCl, pH 2.8 followed by water. After the wash, the moist, compact slurry was transferred to a plastic centrifuge tube (29 mm x 115 mm) and immunoglobulin fraction in 0.175 M sodium phosphate, pH 7.4 was added. Coupling was conducted at 4°C for 24 hours. The slurry was packed into a column (1.5 x 10 cm), the column was equilibrated with 1 mM sodium phosphate buffer, pH 7.2. This general procedure
Figure 2. A flow chart of preparation of Antibody-Agarose column. Step 1: highly purified LCAT preparations were applied to the antibody column (1.0 cm x 10 cm) which had been equilibrated with 1 mM sodium phosphate buffer, pH 7.2. Step 2: LCAT activity passed through the column while most of the contaminants were retained in the column. Steps 3, 4, and 5 were continued until satisfactory antibody titer was obtained. Steps 6 and 7 were used to increase the overall capacity of antibody column.
Highly purified LCAT Preparations

1

↓

Antibody column (antilipoprotein A and D)

Eluted with 1 mM sodium phosphate pH 7.2

Eluted with 2 M NaSCN

LCAT

Contaminants

4

↓

Emulsified with Freund's complete adjuvant

↓

Injected into rabbit

5

↓

Checked antibody titer

↓

Antisera (15 - 20 ml)

6

↓

Ammonium sulfate precipitation

↓

Immunoglobulin fraction

7

↓

CNBr-activated Agarose

↓

Antibody-Agarose column
was used to increase the overall capacity of the purification procedure (Figure 2).

**Chromatography Resins and Packing of Columns**

The dodecylamine-agarose (DDA-agarose) column which had been saturated with lipoproteins, was operated in a continuous cycle. Up to fifteen preparations could be processed on this column without the need for repacking. However, once the flow rate of the enzyme elution fell below 100 ml/hr, the resin was resuspended and sedimented repeatedly under gravity in deionized water until the supernatant appeared clear. The DEAE-agarose columns were also operated in a continuous cycle; the regeneration was accomplished by washing with 100 ml of 4 M NaCl. These columns did not need to be repacked.

The hydroxylapatite columns were repacked as follows: the hydroxylapatite was removed from the column and suspended in 1 liter of degassed, deionized water. The hydroxylapatite was gently shaken for 10 minutes and then allowed to sediment and the suspended fine particles were removed. This procedure was repeated four times and the hydroxylapatite was subsequently packed into the column under gravity. All procedures were carried out at 4°C.

**Determination of Partial Specific Volume**

The partial specific volume ($\bar{\nu}$) of the enzyme was determined according to Edelstein and Schachmann (1973) employing
analytical sedimentation equilibrium ultracentrifugation in the presence of H$_2$O and D$_2$O. The calculation of $\bar{v}$ is accomplished with the aid of the equation:

$$
\bar{v} = \frac{k\left[\frac{(dlnc/dr^2)D_2O}{(dlnc/dr^2)H_2O}\right]}{\rho D_2O - \rho H_2O \left[\frac{(dlnc/dr^2)D_2O}{(dlnc/dr^2)H_2O}\right]}
$$

where $k$ is the ratio of the molecular weight of the macromolecules in the deuterated solvent to that in the non-deuterated solvent, $\rho$ is the density of the solution, and $dlnc/dr^2$ is the change in protein concentration with respect to the square of the distance from the center of the rotation. While the $k$ value has been determined for some proteins (Martin et al., 1959), no such data appeared to be available for glycoproteins. Chung et al. (1979) have reported that LCAT may contain up to 25% (w/w) covalently linked carbohydrate, thus necessitating the calculation of the $k$ value for glycoprotein preparations. Calf serum fetuin was thus employed as a standard glycoprotein for the calculation of the $k$ value. Fetuin is available commercially in a highly purified form and has a molecular weight (48,000) and carbohydrate content (25%, w/w) similar to LCAT (Spiro, 1960). The purity of calf serum fetuin was assessed to be 90-95% by SDS-polyacrylamide gel electrophoresis. One ml (0.75 mg/ml) of calf serum fetuin was dialyzed extensively against deionized H$_2$O. The dialyzed sample was divided into two portions of equal volume, which were dispensed into pyrex
test tubes (13 x 100 mm) and lyophilized. One sample was redissolved in 0.5 ml of deionized water and the other redissolved in 0.5 ml of deuterium oxide (99.8%). They were extensively dialyzed against deionized water and deuterium oxide, respectively.

Meniscus depletion sedimentation equilibrium experiments using 12-mm double sector cells were carried out essentially as described by Yphantis (1964) in a Beckman Model E analytical ultracentrifuge. Samples were run at 28,000 rpm at 18°C. The equilibrium was considered established when the meniscus was depleted (approximately 18-24 hours) and no further changes of fringe displacement were observed in the interference patterns. Photographs were taken at selected exposure times using Spectroscopic II-G plates (Kodak). A suitable equilibrium photograph was selected and aligned in the microcomparator (Nikon), and the deflection of a fringe in the Y direction was measured as a function of the r positions from the center of rotation. The slopes (both in H₂O and D₂O) were determined from the plot of dlnc vs. dr² by a linear regression computer program (Texas Instrument Inc., "Calculator Decision-Making Source Book"). The slopes of (dlnc/dr²) H₂O and (dlnc/dr²) D₂O were used to calculate the value of k.

The purified human LCAT (0.64 mg/ml) was subjected to the meniscus depletion sedimentation equilibrium experiments in H₂O and D₂O solutions as described in the method portion
for the determination of k value (page 25) using calf serum fetuin. Both of the slopes \( \frac{dlnc}{dr^2} \) from sedimentation equilibrium experiments in H\(_2\)O and D\(_2\)O obtained for LCAT and K values of fetuin (obtained from the fetuin experiment) were used to calculate the \( \bar{v} \) of human plasma LCAT employing the formula as described on page 25.

**Molecular Weight Determinations**

A sample of purified LCAT (0.8 mg/ml) was lyophilized and redissolved in 1 mM sodium phosphate buffer, pH 7.2 and in 6 M guanidine-HCl, 1 mM sodium phosphate buffer containing 0.1M \( \beta \)-mercaptoethanol, respectively. The enzyme samples were dialyzed to equilibrium against the respective buffers. Solvent densities were determined by weighing 10 ml aliquots of the respective buffer solutions in a 10-ml volumetric flask on a semi-micro analytical balance and the weights were established to 4 significant figures. The respective slopes \( \frac{dlnc}{dr^2} \) established by sedimentation equilibrium in the presence of 1 mM sodium phosphate, pH 7.2 and in the presence of 6M guanidine-HCl in 1 mM sodium phosphate buffer containing 0.1 M \( \beta \)-mercaptoethanol were determined and used to calculate the molecular weight.
**Molecular Weight Determination by SDS-Polyacrylamide Gel Electrophoresis**

The molecular weight of human plasma LCAT was also determined by polyacrylamide gel (7.5%, w/v) electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (Maizel, 1971) as described by Weber and Osborn (1972). Phosphorylase B, ovalbumin, human serum albumin, aldolase, and chymotrypsinogen were used as standards. The distance of migration for each protein was established by the Quick-Scan gel scanning apparatus (Helena Laboratories).

**Ultraviolet Spectroscopy**

One ml (0.2 mg/ml) of the purified human LCAT was dialyzed extensively against 1 mM sodium phosphate buffer, pH 7.2 and 0.1 N NaOH, pH 12.4, respectively. The enzyme solutions were analyzed in a Varian 210 double beam spectrophotometer equipped with 10-mm cells. The scanning was performed between 320 nm and 240 nm. The contents of tyrosine and tryptophan were determined by the method of Bencze and Schmid (1957) from the ultraviolet absorption spectrum of the enzyme in 0.1 N NaOH, pH 12.4.

**Circular Dichroism (CD) Spectroscopy**

LCAT was subjected to CD studies using the Jasco J-40 CD Spectropolarimeter. A solution of LCAT (0.13 mg/ml) in a 1-mm cuvette was used for detection of far ultraviolet spectra (250-190 nm). Spectral data were analyzed according to
the method of Chen et al. (1974) as modified by Thompson et al. (1976) using the non-linear regression analysis package HELIX 1.2. The three component model describes the data as percentage of \( \alpha \)-helix, \( \beta \) -pleated sheet, and random structures.

**Analytical Isoelectric Focusing**

Analytical isoelectric focusing on LKB Ampholine polyacrylamide gel plates, pH range 3.5 - 9.5 (LKB producter, Bromma, Sweden), was carried out by applying 20 \( \mu l \) of LCAT (1.0 mg/ml), and 20 \( \mu l \) of bovine serum albumin (1.0 mg/ml) as a pH marker according to the manufacturer's instructions using LKB 2117 multiphor electrophoresis equipment. The anode and cathode electrode solutions were 1 M \( \text{H}_3\text{PO}_4 \) and 1 M \( \text{NaOH} \), respectively. Electrofocusing was conducted at 1500 volts for 2 hours. The plate was removed from the apparatus, and stained in 0.12% (w/v) Coomassie Blue R-250 in 25% (v/v) ethanol/8% (v/v) acetic acid for 30 minutes at 65°C, and was destained in 25% (v/v) ethanol/8% (v/v) acetic acid. The PI of LCAT was determined from the calibration plot of pH vs. the distance traveled by bovine serum albumin as a standard pH marker (Righetti and Caravaggio, 1976).

**Amino Acid Analyses**

Samples of purified LCAT (60-90 \( \mu g \)) were dialyzed against deionized water for 24 hours, and then lyophilized. Hydrolysis was carried out in 6 N HCl containing 0.02% (v/v)
β-mercaptoacetic acid for 24, 48, and 72 hours at 110 °C. The amino acid analyses of the hydrolysates were performed in a Durrum amino acid/peptide analyzer equipped with o-phthaldialdehyde detection system. Norleucine was added to each sample prior to hydrolysis and the values of the respective amino acid residues were corrected based on norleucine recovery. Half-cystine was determined in a separate experiment as cysteic acid following performic acid oxidation as described by Hirs (1967). Proline was determined independently using a ninhydrin detection system (Spackman et al., 1958)

Carbohydrate Analyses

Hexose was determined by the anthrone reagent as described by Spiro (1966) using an equimolar mixture of D(+)mannose and D(+)galactose as standards. Calf serum fetuin was employed as a glycoprotein standard and was subjected to the same experimental conditions as LCAT in order to correct for the recovery of carbohydrate residues. Prior to colorimetric determination, the hexosamine of LCAT and fetuin was liberated by acid hydrolysis (4 N HCl, 6 hrs, 100 °C). The hydrolysates were then lyophilized to remove HCl and redissolved in deionized water. The neutralized samples were applied to a Dowex 50-X8 (H⁺ form, column size, 0.5 cm x 5 cm). The column was then washed with 30 ml of deionized water, and the hexosamines were eluted with 1.5 M
NH₄OH. The eluates were lyophilized to remove NH₄OH and then were redissolved in deionized water. The hexosamines liberated from both LCAT and fetuin were subjected to colorimetric determinations as described by Johnson (1971) using glucosamine-HCl as a standard.

Sialic acid was liberated from both LCAT and fetuin by acid hydrolysis (0.05 N H₂SO₄, 1 hr, 80°C). The hydrolysate was neutralized by adding excess Ba₂CO₃. After removal of Ba₂SO₄ by filtration it was passed through Dowex 1-X8 (formate, column size, 0.5 cm x 5 cm) which had been prepared as described by Spiro (1960). The column was washed with deionized water and the liberated sialic acid was eluted with 0.3 N formic acid. The eluate was lyophilized to remove formic acid and was redissolved in deionized water. The samples were analyzed by the thiobarbituric acid assay procedure as described by Warren (1959) using N-acetylneuraminic acid as a standard.

**Fluorescence Spectroscopy**

Fluorescence measurements were made with the Aminco Spectrophotofluorometer equipped with an Aminco ratio photometer (American Instrument Co., Silver Springs, Missouri). LCAT was dissolved in 20 mM triethanolamine-20 mM ethanolamine buffer, pH 8.0 and 10 mM sodium phosphate, pH 7.4 respectively. For quantitation of the tryptophan content of LCAT, the denaturation of enzyme was carried out
in 12-ml conical centrifuge tubes in a boiling water bath for 5 minutes. The tubes were cooled to room temperature and 5 μl of a freshly prepared solution containing 0.02% (w/v) each of chymotrypsin and pronase were added as described by Sasaki et al. (1975). Three ml of the digested and undigested enzyme solution were transferred to a quartz cell respectively for the fluorescence measurements. The tryptophan content of LCAT was evaluated by adding increasing amounts of a standard tryptophan solution as described by Sasaki et al. (1975).

**Partial Specific Volume by Composition**

Partial specific volume (\(\bar{v}\)) of LCAT was also estimated from an analysis of the amino acid and carbohydrate compositions. \(\bar{v}\) of the protein portion was obtained from Edelstein and Schachmann (1973), and \(\bar{v}\) for the carbohydrate portion from the following values: hexoses = 0.60 ml/g, hexosamines = 0.647 ml/g, and sialic acids = 0.681 ml/g\(^1\).

**Extinction Coefficient**

The absorbance of LCAT in 1 mM sodium phosphate buffer (pH 7.2) at 280 nm was established in a Varian 210 Spectrophotometer. An aliquot of the LCAT solution was subjected to protein determination as described by Bradford (1976).

\(^1\)I am grateful to Dr. David C. Teller, University of Washington, Seattle, Washington for supplying us the individual \(\bar{v}\) values of carbohydrates.
and amino acid analysis using norleucine as internal standard to establish the protein concentration of the solution.

**DTNB Titration**

The reaction of LCAT with DTNB was carried out with respect to time as essentially described by Kemp and Forest (1968) at 23°C. Titrations were performed in the presence or absence of sodium dodecyl sulfate in a buffer containing 0.1 M sodium phosphate, pH 8.0. The reported extinction coefficient of 13.6 (Ellman, 1959) at 412 nm for the thionitrobenzoic acid ion of DTNB was employed to compute the number of reactive sulfhydryl groups per mole of enzyme.
CHAPTER III

RESULTS

Dodecylamine (DDA)-Agarose Chromatography

This was the initial step in the purification scheme and was carried out according to a previously published procedure (Lacko and Chen, 1977). Two liters of human plasma were treated with polyethylene glycol 6000 (final concentration: 6%, w/v) to precipitate the triglyceride and cholesterol-rich plasma lipoproteins. The mixture was stirred for 3-4 hours at 4°C, and then centrifuged at 9,000 rpm for 30 minutes at 4°C. The supernatant solution was collected, and the precipitate was discarded. The conductivity of polyethylene glycol (PEG) supernatant was adjusted to 21-28 x 10^{-3} micro MHOS with 5 M NaCl and the pH was adjusted to 7.4 with 2 M citric acid.

Dodecylamine-agarose (DDA-agarose) was prepared according to Deutch et al. (1973) using the coupling procedure of March et al. (1974) as described in the Methods section. A DDA-agarose column (5 x 70 cm) was equilibrated with high conductivity buffer (10 mM sodium phosphate, 5 mM EDTA, 0.3 M NaCl, 0.025% NaN₃, pH 7.4), and the PEG supernatant solution was applied. Next the column was washed with the high conductivity buffer to remove excess plasma proteins until the
absorbance (at 280 nm) of the eluate became negligible. The enzyme was eluted with deionized water at a flow rate of 125 ml/hr and the fractions of the eluate were analyzed for absorbance (at 280 nm) and LCAT activity (Figure 3). The LCAT containing fractions were pooled for the next step and directly applied to a DEAE-agarose column.

DEAE-Agarose Chromatography

The first two steps in this purification scheme were very efficient, since due to its low ionic strength the pooled material from the DDA-agarose step could be processed directly without concentration and/or dialysis. DEAE-agarose chromatography was carried out on a column (2.5 x 25 cm) using a salt gradient. First the DDA pool was allowed to enter the column which had previously been equilibrated with a buffer containing 5 mM sodium phosphate, 0.025% NaN₃, pH 7.2. Next, 700 ml of 5 mM sodium phosphate, 0.05 M NaCl, 0.025% NaN₃, pH 7.2 was allowed to flow through the column resulting in the elution of a large protein peak that lacked LCAT activity (Figure 4). The enzyme activity was eluted at a flow rate of 100 ml/hr using a linear gradient consisting of 1 liter of 5 mM sodium phosphate, 0.025% NaN₃ and 0.05 M NaCl, pH 7.2 and 500 ml of 5 mM sodium phosphate, 0.025% NaN₃ and 0.2 M NaCl, pH 7.2. The fractions containing LCAT were pooled and concentrated for delipidation using an XM-50 Diaflo Ultrafiltration membrane at 50 p.s.i. (Amicon Corp.)
Figure 3. Chromatography of LCAT on DDA-Agarose column (5 x 70 cm). ●-●-● (A_{280}), ○-○-○ (LCAT activity), △-△-△ (conductivity). 15 ml fractions were collected at a flow rate of 125 ml/hr.
Figure 4. Chromatography of LCAT on a DEAE-agarose column (2.5 x 25 cm). •••• (A280), 0-0-0 (LCAT activity), ΔΔΔ (Conductivity). 9 ml fractions were collected at a flow rate of 100 ml/hr.
The pooled material from the first DEAE column was reduced to about 20 ml and divided into four 5 ml portions. The 5 ml aliquots were frozen and 15 ml of butanol:diisopropyl ether (40:60, v/v) were added to each tube to extract the lipids (Cham and Knowles, 1976). The mixtures were gently rotated for at least 4 hours at 4°C to achieve a thorough delipidation. At the end of the extraction, the samples were frozen, thawed and centrifuged at 2,000 xg at room temperature. The organic layer was carefully removed by aspiration, the aqueous layer was placed in an ice bath, and dialyzed against 0.01 M sodium phosphate, 0.025% NaN₃, pH 7.2.

Second DEAE-Agarose Chromatography

The delipidated and dialyzed LCAT preparation from the first DEAE column (Figure 4) was applied to a smaller (1.5 x 20 cm) DEAE-agarose column which had previously been equilibrated with 0.01 M sodium phosphate, 0.025% NaN₃, pH 7.2. The chromatography on this second DEAE-agarose column was accomplished by a stepwise gradient at a flow rate of 50 ml/hr as shown on Figure 5. The application of 900 ml of the first buffer (0.01 M sodium phosphate, 0.015 M NaCl, 0.025% NaN₃, pH 7.2) generally resulted in the elution of some protein but no LCAT. Elution of the enzyme was accomplished with 900 ml of the next buffer (0.01 M sodium phosphate, 0.05 M NaCl, 0.025% NaN₃, pH 7.2). Subsequently, a large protein peak
Figure 5. Chromatography of LCAT on the second DEAE-agarose column (1.5 x 20 cm). Concentrated and delipidated fractions containing LCAT from the first DEAE-agarose step were used in this experiment. •-•-• (A280), O-O-O (LCAT activity), △-△-△ (conductivity). 9 ml fractions were collected at a flow rate of 50 ml/hr.
containing little or no LCAT activity, was eluted when the last buffer was applied to the column (0.01 M sodium phosphate, 0.025% NaN₃, 0.2 M NaCl, pH 7.2). The LCAT containing fractions from the second DEAE column were pooled, concentrated (by vacuum dialysis) to about 15 ml and were finally dialyzed for 12 hours against 4 liters of 1 mM sodium phosphate, 0.025% NaN₃, pH 7.2.

Hydroxylapatite/Antibody-Agarose Chromatography

In this purification step a hydroxylapatite column (2.5 x 15 cm) and an antibody-agarose column were connected together so that the effluent from the former was directly introduced to the latter. This column system was equilibrated with 1 mM sodium phosphate, pH 7.2 and the enzyme pool from the second DEAE column was applied. A single peak of LCAT activity emerged from the columns (flow rate: 20 ml/hr) coinciding with a peak of absorbance at 280 nm (Figure 6). Following the elution of the LCAT containing fractions, the columns were disconnected and regenerated. The hydroxylapatite column was washed with 0.2 M sodium phosphate, pH 7.2 which resulted in the elution of HDL apoproteins and serum albumin; the antibody-agarose column was washed with 2 M NaSCN which eluted a number of polypeptides that were subsequently used as antigens for additional production as described in the "Materials and Methods" section. The hydroxylapatite was discarded once the flow rate fell below
Figure 6. Hydroxylapatite (2.5 x 15 cm)/Antibody-Agarose (1.5 x 20 cm) Chromatography. •-•-• ($A_{280}$), O-O-O (LCAT activity), Δ-Δ-Δ (Conductivity). 3 ml fractions were collected up to a total volume of 105 ml and then the fraction size was adjusted to 6 ml/fraction. Flow rate of the column was 20 ml/hr.
10 ml/hr. The enzyme purification was not affected by using different batches of hydroxylapatite.

Evaluation of the Purification Procedure

The enzyme preparation obtained after the hydroxylapatite/antibody-agarose step was found to be consistently homogeneous by several criteria. Figure 7 shows the results of polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate and 7M urea. Application of up to 30 μg of enzyme to these gels failed to reveal any contamination. In addition, data from sedimentation equilibrium analytical ultracentrifugation experiments (Chong et al., 1981b) showed a high degree of homogeneity of LCAT preparations. In addition, the purified enzyme was found to be free of serum albumin and apolipoproteins, AI, AII, B, CI, CII, D, and E as indicated by highly sensitive immunodiffusion and immunoelectrophoresis studies.1

Table II shows the yield and the degree of purification obtained with the procedure used. Generally up to 1.0 mg of enzyme could be prepared from about 2 liters of cryoprecipitate supernatant solution with an approximately 20,000-fold increase in specific activity. Every step of this procedure was found to be highly reproducible over a six

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1I thank Dr. Walter J. McConathy for performing these experiments at Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma.
Figure 7. Polyacrylamide gel electrophoresis of purified human plasma lecithin:cholesterol acyltransferase. The gel was loaded with 30 μg of protein and electrophoresis was carried out in the presence of 7M urea (A) and 0.1% (w/v) sodium dodecyl sulfate (B). The experimental conditions for electrophoresis in the presence of 7M urea were described in Methods. The electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate was conducted for 6 hours with the current of 4 mA/gel. The staining and destaining procedures were described in Methods.
### TABLE II

**Purification of Human Plasma Lecithin:Cholesterol Acyltransferase**

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Enzyme Nmoles h⁻¹</th>
<th>Specific Activity Nmoles h⁻¹ mg⁻¹</th>
<th>Recovery %</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>2,000</td>
<td>166,537</td>
<td>30,616</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>Polyethylene-glycol Supt.</td>
<td>2,210</td>
<td>152,243</td>
<td>29,614</td>
<td>96.7</td>
<td>1.1</td>
</tr>
<tr>
<td>DDA-agarose</td>
<td>300</td>
<td>7,245</td>
<td>26,130</td>
<td>85.3</td>
<td>20</td>
</tr>
<tr>
<td>DEAE-agarose</td>
<td>490</td>
<td>905</td>
<td>18,385</td>
<td>60.1</td>
<td>113</td>
</tr>
<tr>
<td>2nd DEAE-agarose</td>
<td>277</td>
<td>100</td>
<td>7,115</td>
<td>23.2</td>
<td>396</td>
</tr>
<tr>
<td>Hydroxylapatite/antibody column</td>
<td>75</td>
<td>1.07</td>
<td>3,875</td>
<td>12.7</td>
<td>20,117</td>
</tr>
</tbody>
</table>

Enzyme activity is expressed in terms of nanomoles of free cholesterol esterified h⁻¹ at 37°C using the standard assay described under "Materials and Methods."
month period and the whole purification cycle could be completed in less than ten days.

Following the final purification step, the enzyme was concentrated on a small (1 x 5 cm) DEAE-agarose column reducing the volume from about 75 ml to 10 ml. The purified enzyme was applied directly to the DEAE-agarose column, and a buffer containing 1 mM sodium phosphate, 0.2 M NaCl, pH 7.2 was used to elute the LCAT preparation in the concentrated form. Since the highly purified enzyme appears to be unstable at high ionic strength (Furukawa and Nishida, 1979), the LCAT concentrates were dialyzed against 1 mM sodium phosphate, pH 7.2 immediately following their elution from the DEAE column and frozen at -20°C. No loss of LCAT activity was observed during this concentration step.

Partial Specific Volumes ($\bar{\nu}$)

When macromolecules are dissolved in D$_2$O, their density is increased as a result of deuterium exchange and their partial specific volume is decreased by the same relative amount. The $k$ value (see equation on page 25) is the ratio of the molecular weight of the macromolecules in the deuterated solvent to that in the non-deuterated solvent. The $k$ value calculated for calf serum fetuin (a control glycoprotein) was found to be 1.016, which is similar to other $k$ values reported for proteins containing no carbohydrate (Martin et al., 1959). The apparent partial specific volume of human plasma
LCAT was calculated to be 0.702 ml/g using a k value of 1.016. This $\bar{v}$ value of LCAT was used in the calculation of molecular weight by sedimentation equilibrium analysis.

**Molecular Weights of LCAT**

The molecular weight of human plasma LCAT determined by sedimentation equilibrium experiments in 1 mM sodium phosphate, pH 7.2 using a $\bar{v}$ of 0.702 ml/g was 60,000 ± 1,400 (3 determinations). Figure 8 shows the plot of $d\ln c$ (LCAT concentrations) vs $dr^2$. The linearity of the plot is an indication of the homogeneity of the enzyme preparation which had already been established by other methods (Chong et al., 1981a). Molecular weight values obtained in the presence of 6 M guanidine-HCl were 61,000 and 60,300 (two determinations). Such data indicate that the enzyme possesses a monomeric structure. Molecular weight determinations were also carried out by polyacrylamide gel electrophoresis of 20 µg of LCAT in the presence of 0.1% (w/v) SDS which yielded a value of 67,000 ± 2,000 (Figure 9). The discrepancy between the values from SDS-PAGE analysis and those from sedimentation equilibrium experiments is likely to be due to the relatively high carbohydrate content of the enzyme (Mickelson and Petra, 1978 and Kisiel and Davie, 1975).
Figure 8. Sedimentation equilibrium ultracentrifugation of purified human plasma lecithin:cholesterol acyltransferase (0.8 mg/ml) in 1 mM sodium phosphate buffer, pH 7.2. Ultracentrifugation was carried out at 18°C at 22,000 rpm in the An-D rotor in a 12-mm double sector cell with sapphire windows. The molecular weight of LCAT was determined using $\bar{v}$ of 0.702 g/ml.
Figure 9. Determination of molecular weight by poly-acrylamide gel (7.5%, w/v) electrophoresis in the presence of 0.1% (w/v) SDS. The standards were: 1) phosphorylase (94,000); 2) human serum albumin (68,000); 3) ovalbumin (43,000); 4) aldolase (40,000); 5) chymotrypsinogen (24,000).
Ultraviolet Absorption Spectrum

Human plasma LCAT in 1 mM sodium phosphate, pH 7.2 exhibited an ultraviolet spectrum with a maximum at 280 nm and a maximum at 291 nm in 0.1 N NaOH, pH 12.4. Figure 10 shows the absorption spectrum of LCAT at pH 7.2 and pH 12.4. Employing the method of Bencze and Schmid (1947), the tyrosine content of LCAT was found to be 4.8% (w/w) and the tryptophan content 5.0% (w/w) corresponding to approximately 12 tyrosine and 11 tryptophan residues per mole of LCAT. At pH 7.2, \( \frac{A_{295}}{A_{274}} \) nm ratio was calculated to be 0.42, while at pH 12.4, \( \frac{A_{295}}{A_{274}} \) nm ratio was calculated to be 0.87. These data suggest that the increase in the \( \frac{A_{295}}{A_{274}} \) nm ratio with increasing pH probably represents the ionization of some or all of the tyrosine residues which has been shown to result in an increase of the wavelength of maximum absorption and the extinction coefficient for tyrosine (Tanford et al., 1955).

Circular Dichroism Spectrum

Circular dichroism data obtained on the purified LCAT sample are shown in Figure 11. Human plasma LCAT (0.13 mg/ml) was calculated to contain 24% \( \alpha \)-helix, 27% \( \beta \)-sheet and 49% random structure in 1 mM sodium phosphate buffer, pH 7.2 (Chong et al., 1981b). The C.D. spectrum of LCAT was also examined in a buffer of 0.2 M NaCl containing 1 mM sodium phosphate, pH 7.2. Under these conditions, the enzyme
Figure 10. Ultraviolet absorption spectrum of purified human LCAT. Effect of solvent on the spectrum of tryptophan and tyrosine. ——— 0.1 N NaOH, pH 12.4; ———— 1 mM sodium phosphate, pH 7.2.
Figure 11. Circular dichroism spectrum of purified human LCAT in 1 mM sodium phosphate, pH 7.2. The content of α-helix, β-sheet, and random structure was calculated by computer program.
appeared to have 23% α-helix, 34% β-sheet and 45% random structure. While the change to the high ionic strength medium was accompanied by a slight increase in β-sheet structure, these data indicate no significant change in the overall conformation of the enzyme.

Isoelectric Focusing

Figure 12 shows the electrofocusing of purified LCAT, indicating the presence of at least four bands having pI values from 4.2 - 4.5. The microheterogeneity of LCAT is probably due to the presence of protein isozyme species or a variation in carbohydrate content.

Table III summarizes the physical properties of LCAT found in this study.

Amino Acid Composition

Table IV shows the amino acid composition of human LCAT. The polypeptide molecular weight of LCAT (45,000) was obtained by subtracting the weight of carbohydrate moiety (25%,w/w; see Table V) from 60,000 daltons determined by sedimentation equilibrium analytical ultracentrifugation (Chong et al., 1981b). The assumed molecular weight (45,000) appears to be approximately correct since the sum of the gram residue weight (based on four half-cystines/LCAT) adds up to a molecular weight of 45,156 (Table IV). The enzyme showed a relatively high content in glutamic, aspartic acid, glycine and leucine.
Figure 12. Analytical isoelectric focusing in thin layer (pH range 3.5 - 9.5) of polyacrylamide gels of purified human LCAT. 20 μg of LCAT was applied. The experimental conditions were described in Methods.
TABLE III

PHYSICOCHEMICAL PROPERTIES OF HUMAN PLASMA LCAT

<table>
<thead>
<tr>
<th>Methods and Conditions</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td></td>
</tr>
<tr>
<td>Sedimentation Equilibrium in 1 mM sodium phosphate, pH 7.2</td>
<td>60,000</td>
</tr>
<tr>
<td>Sedimentation Equilibrium in 6 M guan HCl</td>
<td>60,700</td>
</tr>
<tr>
<td>SDS gel electrophoresis</td>
<td>67,000</td>
</tr>
<tr>
<td>Partial Specific Volume Composition analysis</td>
<td>0.708 ml/gm</td>
</tr>
<tr>
<td>Parallel Sedimentation Equilibrium Measurement in H2O and D2O solutions</td>
<td>0.702 ml/gm</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.2-4.5 (4 bands)</td>
</tr>
</tbody>
</table>
| Circular Dichroism in 1 mM sodium phosphate, pH 7.2 | \( \alpha \)-helix 24%  
\( \beta \)-sheet 27%  
random structure 49%  |
| in 0.2 M NaCl/1 mM sodium phosphate, pH 7.2 | \( \alpha \)-helix 23%  
\( \beta \)-sheet 32%  
random structure 45%  |
| U.V. Spectrum |             |
| \( A_{295}/A_{274} \) nm in 1 mM sodium phosphate, pH 7.2 | 0.42 |
| \( A_{295}/A_{274} \) nm in 0.1 N NaOH, pH 12.4 | 0.87 |
TABLE IV
AMINO ACID COMPOSITION OF HUMAN PLASMA LCAT

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues/10⁵g of Protein</th>
<th>Mol. Wt./10⁵g of Protein</th>
<th>Residues/45,000g of Protein</th>
<th>Nearest Int./45,000g</th>
<th>Integral No. Mol. Wt. of Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>31.1 (30.6-31.6)</td>
<td>3,981</td>
<td>13.7 (13.5-13.9)</td>
<td>14 (14-14)</td>
<td>1,792</td>
</tr>
<tr>
<td>Histidine</td>
<td>28.2 (27.5-28.8)</td>
<td>3,863</td>
<td>12.4 (12.1-12.7)</td>
<td>12 (12-13)</td>
<td>1,644</td>
</tr>
<tr>
<td>Arginine</td>
<td>41.5 (37.5-45.6)</td>
<td>6,474</td>
<td>18.3 (16.5-20.0)</td>
<td>18 (17-20)</td>
<td>2,808</td>
</tr>
<tr>
<td>Aspartic acid*</td>
<td>93.4 (85.6-101.1)</td>
<td>10,741</td>
<td>41.1 (37.7-44.5)</td>
<td>41 (38-45)</td>
<td>4,715</td>
</tr>
<tr>
<td>Threonine</td>
<td>53.9 (50.4-57.4)</td>
<td>5,444</td>
<td>23.7 (22.2-25.3)</td>
<td>24 (23-25)</td>
<td>2,424</td>
</tr>
<tr>
<td>Serine</td>
<td>55.1 (53.7-56.4)</td>
<td>4,794</td>
<td>24.2 (23.6-24.8)</td>
<td>24 (24-25)</td>
<td>2,088</td>
</tr>
<tr>
<td>Glutamic Acid**</td>
<td>87.1 (85.9-88.2)</td>
<td>11,236</td>
<td>37.3 (37.8-38.8)</td>
<td>38 (38-39)</td>
<td>4,902</td>
</tr>
<tr>
<td>Prolinea</td>
<td>71.1</td>
<td>6,897</td>
<td>31.3</td>
<td>31</td>
<td>3,007</td>
</tr>
<tr>
<td>Glycine</td>
<td>85.0 (84.2-85.8)</td>
<td>4,845</td>
<td>37.4 (37.0-37.8)</td>
<td>37 (37-38)</td>
<td>2,109</td>
</tr>
<tr>
<td>Alanine</td>
<td>57.0 (56.1-57.9)</td>
<td>4,047</td>
<td>25.1 (24.7-25.5)</td>
<td>25 (25-26)</td>
<td>1,775</td>
</tr>
<tr>
<td>Valine</td>
<td>63.2 (60.8-65.6)</td>
<td>6,257</td>
<td>27.8 (26.8-28.9)</td>
<td>28 (27-29)</td>
<td>2,772</td>
</tr>
<tr>
<td>Methionine</td>
<td>15.4 (15.1-15.6)</td>
<td>2,017</td>
<td>6.8 (6.6-6.9)</td>
<td>7 (7-7)</td>
<td>917</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Residues/10⁵g of Protein</td>
<td>Mol. Wt./10⁵g of Protein</td>
<td>Residues/45,000g of Protein</td>
<td>Nearest Int./45,000g</td>
<td>Integral No. Mol. Wt. of Residue</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>-----------------------------</td>
<td>---------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>32.9 (31.3-34.4)</td>
<td>3,718</td>
<td>14.5 (13.7-15.1)</td>
<td>15 (14-15)</td>
<td>1,695</td>
</tr>
<tr>
<td>Leucine</td>
<td>114.0 (110.3-117.6)</td>
<td>12,882</td>
<td>50.2 (48.5-51.7)</td>
<td>50 (49-52)</td>
<td>5,650</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>25.6 (25.4-25.8)</td>
<td>4,173</td>
<td>11.3 (11.2-11.4)</td>
<td>11 (11-11)</td>
<td>1,793</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>42.4 (41.4-43.6)</td>
<td>6,233</td>
<td>18.7 (18.2-19.2)</td>
<td>19 (18-19)</td>
<td>2,793</td>
</tr>
<tr>
<td>Half-cystine b</td>
<td>7.9 (7.6-8.2)</td>
<td>814</td>
<td>3.5 (3.3-3.6)</td>
<td>4 (3-4)</td>
<td>412</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>23.0 (22.0-24.0)</td>
<td>4,278</td>
<td>10.1 (9.7-10-6)</td>
<td>10 (10-11)</td>
<td>1,860</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>102,694</strong></td>
<td></td>
<td><strong>45,156</strong></td>
</tr>
</tbody>
</table>

The values are average of three independent determinations.

a. Single determination (Beckman amino acid analyzer was used; ninhydrin detection.

b. Average of two determinations. Determined by performic acid oxidation (Hirs, 1967)

c. Average of two determinations. Determined by method of fluorescence spectroscopy (Sasaki et al., 1975).

*Aspartic acid is sum of aspartic acid and asparagine.

**Glutamic acid is sum of glutamic acid and glutamine.
The total number of 408 residues per mole of LCAT polypeptide (45,000) was found. The mean residue weight was calculated to be 110.3.

Carbohydrate Composition

From Table V it may be seen that LCAT has a large carbohydrate content with the following composition (w/w): hexoses, 13%; hexosamines, 6.2%; and sialic acids, 5.4%. The colorimetric methods employed for carbohydrate determinations were consistently reproducible and the recovery was over 90% based on the recovery of a standard fetuin sample.

Fluorescence Emission Spectrum

Figure 13A shows the fluorescence emission spectrum of native LCAT in a buffer containing 10 mM sodium phosphate, pH 7.4. The wavelength of maximum excitation was 288 nm while the maximum emission was at 335 nm. Figure 13B shows the evaluation of tryptophan content of LCAT with increasing addition of the standard tryptophan. The quantity of tryptophan was found to be 4-5 nmoles (duplicates) which correspond to 6-7 moles of tryptophan per mole of LCAT. Figure 14A shows the emission spectrum of LCAT pretreated with chymotrypsin and pronase. The excitation maximum was 288 nm while the emission maximum was 355 nm. From Figure 14B tryptophan contents were calculated to be 7.5-8 nmoles (duplicates) which correspond to approximately 10-11 moles of tryptophan/
### TABLE V

**CARBOHYDRATE COMPOSITION OF HUMAN PLASMA LCAT**

<table>
<thead>
<tr>
<th>Composition</th>
<th>Contents % (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptide</td>
<td>75.4</td>
</tr>
<tr>
<td>Carbohydrate*</td>
<td>24.6 (24.2 - 24.8)</td>
</tr>
<tr>
<td>Hexoses</td>
<td>13.0 (12.8 - 13.2)</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>6.2 (6.0 - 6.4)</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>5.4 (5.3 - 5.4)</td>
</tr>
</tbody>
</table>

*The values are average of three independent determinations.
Figure 13. A: The fluorescence emission spectrum of human plasma LCAT. The protein sample was in a buffer containing 10 mM sodium phosphate, pH 7.4. 33 µg of LCAT was used. The buffer yielded no fluorescence.

B: Evaluation of the tryptophan content of LCAT. The fluorescence emission from Figure 13A was measured at 335 nm, with excitation at 288 nm. To each sample, 3 nmole quantities of tryptophan (5 µl) were added. The quantity of tryptophan present was measured by extrapolating the fluorometer readings to the X-axis, as indicated by the arrows.
Figure 14. A: The fluorescence emission spectrum of human plasma LCAT. The protein samples were digested as described in Methods. The buffer contains 6 M urea/20 mM triethanolamine-20 mM ethanolamine, pH 8.0. Samples containing the chymotrypsin-pronase mixture alone yielded negligible fluorescence at 355 nm.

B: Evaluation of the tryptophan content of enzymatically digested LCAT in 6 M urea/20 mM triethanolamine-20 mM ethanolamine, pH 8.0. The fluorescence emission was measured at 355 nm, with excitation at 288 nm. See explanation of Figure 13B for quantitative evaluation of tryptophan.
mole of LCAT. From the fluorescence spectroscopic studies it was calculated that approximately 60% of the total tryptophan residues may be readily accessible to the solvent under non-denaturing conditions at neutral pH.

Partial Specific Volume by Composition

A partial specific volume of 0.708 ml/gm was calculated from the polypeptide and carbohydrate composition analysis. The $\bar{v} = 0.702$ ml/gm determined by parallel $D_2O-H_2O$ sedimentation equilibrium analytical ultracentrifugation (Chong et al., 1981b), agrees well with the chemical data.

Extinction Coefficient ($E_{1cm}^{1%}$)

A relatively high extinction coefficient ($E_{1cm}^{1%}(280nm)=21$) of human LCAT was found based on the protein concentration determined by amino acid composition analysis. The relatively high content of tryptophan (5%, w/w) may be responsible for the high extinction coefficient.

Sulfhydryl Groups of LCAT

The native and denatured LCAT were titrated with DTNB. A time course of the titration of LCAT at pH 8.0 in the presence and absence of sodium dodecyl sulfate is shown in Figure 15. In the presence of SDS the protein was denatured, and the average value from two independent determinations for the number of titrable SH groups was approximately 2 residues per mole of LCAT. The amino acid analysis data of
Figure 15. Reaction of LCAT with DTNB in the presence and absence of sodium dodecyl sulfate. Reactions were carried out at 22 °C with a protein concentration of 0.25 mg/ml and a DTNB concentration of 1 mM in a buffer containing 0.1 M sodium phosphate, pH 8.0. Solid line: no sodium dodecyl sulfate; dotted line: 2% (w/v) sodium dodecyl sulfate.
Chung et al. (1979), Albers et al. (1979), and this study indicated 4 half-cystine residues/mole of LCAT (Table VII). Hence, the presence of a disulfide bond in LCAT seems likely. In the native enzyme, approximately 2 cysteine residues were also rapidly titrated, indicating that free sulfhydryl groups are exposed and readily accessible to DTNB in the reaction solution under non-denaturing conditions.
CHAPTER IV

DISCUSSION

Purification of the Enzyme

A highly efficient purification procedure was described for the enzyme LCAT that yields milligram quantities of homogeneous enzyme. The current procedure represents several advancements over many published procedures, since it (a) avoids the requirement for an ultracentrifuge; (b) uses a starting material (cryoprecipitate supernatant) which is more readily available in large quantities and less expensive than whole human plasma; (c) is designed so that the chromatography sequence involves a minimum number of dialysis and concentration steps; and (d) offers the potential for scaling up the procedure.

LCAT has been one of the most difficult enzymes to isolate in the homogeneous state. Attempts to purify the enzyme have been made since 1964, and several procedures are published to date (Lacko et al., 1973; Fielding et al., 1971; Lacko et al., 1974; Kostner, 1974; Albers et al., 1976; Varma et al., 1976; Marcel et al., 1980; Kitabatake et al., 1979; Chung et al., 1979). Most of the previously reported procedures, however, appear difficult. Therefore, a large number of laboratories chose to develop their own purification
schemes rather than relying on the information in the literature. Some of the steps described in earlier publications were found difficult to reproduce since the success of the individual chromatography steps were highly dependent on the efficiency of the preceding steps in a particular scheme. For example, it was found that the HDL-agarose affinity columns (Akanuma and Glomset, 1968b) will release varying amounts of contaminating lipoprotein material along with the elution of purified LCAT. Such contamination is very difficult to control from batch to batch, since the composition of HDL will vary with the individual donors. Furthermore, the HDL-agarose columns are very easily overloaded unless the bulk of the HDL is removed; however, under these conditions the stability of the enzyme is adversely affected (Furukawa and Nishida, 1979). Some of the published procedures involved the use of Cibacron-Blue-agarose chromatography. The successful operation of these columns for LCAT purification also mandates the prior removal of most of the HDL from the preparation, since, as reported by Wille (1976), Cibacron-Blue-agarose will retain serum albumin as well as plasma lipoproteins. It was found that the LCAT fraction will generally accompany the bulk of the HDL resulting in little or no purification.

In our procedure, the first step removes nearly all plasma protein contaminants and results in the isolation of the HDL-LCAT complex (Lacko and Chen, 1977). This first
step involves a highly efficient chromatography procedure that requires minimum labor and could be further scaled up with ease. In the subsequent steps, the components of the HDL complex are gradually removed to produce homogeneous enzyme. The first DEAE-step eliminates the bulk of the HDL, and another apparently lower molecular weight HDL-LCAT complex (Jahani and Lacko, 1981) is isolated. The affinity of the enzyme for this HDL subfraction is changed upon delipidation, since during the second DEAE-agarose chromatography (Figure 5) the enzyme elutes at a lower ionic strength than earlier (Figure 4). Finally, elimination of the remaining contaminants is accomplished by the hydroxylapatite/antibody-agarose chromatography. The current method describes a highly efficient and reproducible procedure for the purification of LCAT resulting in the isolation of milligram quantities of homogeneous enzyme. The isolation of these enzyme preparations has made the physical and chemical characterization of LCAT in detail possible (Chong et al., 1981b, 1981c).

Physical Properties of the Enzyme

In this study, several physical properties of human plasma LCAT are reported. Table VI compares the physical parameters that resulted from this investigation and data published from other laboratories. The partial specific volume ($\bar{v}$) of LCAT is lower than that of proteins containing
### TABLE VI

**COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF HUMAN PLASMA LCAT**

<table>
<thead>
<tr>
<th>Methods and Conditions</th>
<th>Observations</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular Weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedimentation Equilibrium in 1mM sodium phosphate, pH 7.2</td>
<td>59,000</td>
<td>Chung et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>60,000</td>
<td>This study</td>
</tr>
<tr>
<td>Sedimentation Equilibrium in 6M guan HCl/0.1M β-mercaptoethanol</td>
<td>60,700</td>
<td>This study</td>
</tr>
<tr>
<td><strong>SDS gel electrophoresis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>66,000</td>
<td>Albers et al. (1979)</td>
</tr>
<tr>
<td>&quot;</td>
<td>69,000</td>
<td>Chung et al. (1979)</td>
</tr>
<tr>
<td>&quot;</td>
<td>66,000</td>
<td>Aron et al. (1978)</td>
</tr>
<tr>
<td>&quot;</td>
<td>67,000</td>
<td>Uterman et al. (1980)</td>
</tr>
<tr>
<td>&quot;</td>
<td>65,000</td>
<td>Kitabatake et al. (1979)</td>
</tr>
<tr>
<td>&quot;</td>
<td>67,000</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Partial Specific Volume composition analysis</strong></td>
<td>0.71 ml/g</td>
<td>Chung et al. (1979)</td>
</tr>
<tr>
<td>parallel D$_2$O-H$_2$O</td>
<td>0.708 ml/g</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>0.702 ml/g</td>
<td>This study</td>
</tr>
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</table>
TABLE VI--Continued

<table>
<thead>
<tr>
<th>Isoelectric point</th>
<th>4.28-4.37</th>
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<tbody>
<tr>
<td></td>
<td>4.1-5.5</td>
<td>Albers et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>4.2-4.5</td>
<td>This study</td>
</tr>
</tbody>
</table>

Circular Dichroism
in 1 mM sodium phosphate, pH 7.2

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24%</td>
<td>27%</td>
<td>49%</td>
</tr>
</tbody>
</table>

in 0.2 M NaCl/1 mM sodium phosphate, pH 7.2

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23%</td>
<td>32%</td>
<td>45%</td>
</tr>
</tbody>
</table>

U.V. Spectrum

A<sub>295</sub>/A<sub>274</sub> nm in 1 mM sodium phosphate, pH 7.2

<table>
<thead>
<tr>
<th></th>
<th>A&lt;sub&gt;295&lt;/sub&gt;/A&lt;sub&gt;274&lt;/sub&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.42</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

A<sub>295</sub>/A<sub>274</sub> nm in 0.1 N NaOH, pH 12.4

<table>
<thead>
<tr>
<th></th>
<th>A&lt;sub&gt;295&lt;/sub&gt;/A&lt;sub&gt;274&lt;/sub&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.87</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
only amino acid residues (0.74 ml/g) due to the presence of carbohydrate in the enzyme. The $\bar{v}$ calculated from amino acid and carbohydrate composition agreed very closely with the $\bar{v}$ value obtained from D$_2$O/H$_2$O experiments (Edelstein and Schachmann, 1973) and also was in good agreement with the findings of Chung et al. (1979), (Table VI).

The discrepancy between the molecular weight determined by sedimentation equilibrium analytical ultracentrifugation (60,000 daltons) and by SDS-polyacrylamide gel electrophoresis (67,000 daltons) is probably due to the carbohydrate content of LCAT. Similar discrepancies in molecular weights obtained by SDS-PAGE and sedimentation analysis were reported by Chung et al. (1979) for LCAT and by other investigators for two other serum glycoproteins (Mickelson and Petra, 1978; Kiesel and Davie, 1975). Earlier reports from our laboratory suggested that LCAT may have a dimeric structure (Lacko et al., 1974). These earlier studies, however, were conducted with partially purified enzyme and radioactively labeled diisopropyl fluorophosphate was employed to establish the elution of the enzyme from agarose columns. Due to the very small amount of enzyme protein used in these experiments, in addition to the other factors listed above, the generation of artifacts was a likely possibility. Therefore, data presented in this study (Table VI) as well as from other laboratories (Chung et al., 1979; Albers et al., 1976) strongly indicate
that LCAT is a glycoprotein consisting of a single polypeptide chain with a molecular weight of 60-61,000.

Circular dichroism studies revealed that LCAT has a relatively high content of random structure. Only a small increase in β-sheet structure (from 27% to 32%) was observed when the C.D. spectrum was obtained with an LCAT sample in a buffer containing 0.2 M NaCl. Recently, Doi and Nishida (1981) have reported similar findings. Furukawa and Nishida (1979) reported that LCAT activity was progressively lost when the enzyme was dissolved in a buffer containing ionic strength higher than 4 mM sodium phosphate. Since in the presence of 0.2 M NaCl only small changes in overall structure were indicated by the C.D. analysis, the inactivation of LCAT at high ionic strength is more likely to be due to alterations in the active site region rather than to large conformational changes of the enzyme molecule.

The near ultraviolet absorption spectra obtained at pH 7.2 and at pH 12.4 showed apparent peak shifts. While these changes could have resulted from a pH-induced change in LCAT conformation, it is also likely that the increase in the 295/274 nm absorption ratio resulted from the ionization of solvent accessible tyrosine residues. The pH titration of free aqueous tyrosine has been shown to result in an increase in both wavelength of maximum absorption (from 274 nm at pH 6 to 295 nm at pH 13) and an increase in molar extinction coefficient (by approximately two-fold). The
absorption spectrum due to tryptophan will dominate the near-UV wavelength range in LCAT since the molar absorptivity of tryptophan is greater than tyrosine and there are eleven tryptophan residues per LCAT monomer compared with twelve tyrosine residues. Thus the pH-dependent tyrosine spectrum will be superimposed on the pH-independent tryptophan spectrum. While a change in the polarity of the environments of tyrosine and tryptophan can induce near-UV spectral shifts, the observed increase in $\lambda_{\text{max}}$ and $\varepsilon$ would have resulted from a decrease in environment polarity. This is unlikely since the tyrosine content calculated from the absorption spectrum at pH 12.4 is in close agreement with the tyrosine content determined from amino acid analysis. The method of tyrosine determination is based on the absorption spectrum of free tyrosine (Bencze and Schmid, 1957) and therefore should agree with amino acid analysis results only if all of the tyrosine residues are exposed to solvent. Thus the observed increase in $\lambda_{\text{max}}$ and $\varepsilon$ with increasing pH probably result in an increase rather than a decrease in polarity in the neighborhood of the tyrosine residues.

The analytical isoelectric focusing pattern of LCAT shown in Figure 12 is similar to that reported by Uterman et al. (1980), but is slightly different from the data of Albers et al. (1979). The latter investigators reported a higher pI and five instead of four components of the enzyme. Uterman et al. (1980) suggested that the pre-mixing of the
enzyme protein with the ampholine solution might cause alterations in the electrofocusing pattern. Recently, Doi and Nishida (1981) reported that the treatment of LCAT with neuraminidase converged the multiple bands during isoelectric focusing (representing the putative isozyme species) into a single band, indicating that the microheterogeneity is due to a variation in sialic acid content.

Chemical Properties of the Enzyme

Table VII shows the comparison of amino acids and carbohydrate reported by different investigators. The amino acid composition analysis shown here (Table VII) differs appreciably from that reported by Albers et al. (1979) and Aron et al. (1978), but it showed a good similarity to that reported by Chung et al. (1979). Four residues of half-Cys per mole of LCAT have been observed by a number of investigators (Chung et al., 1979; Albers et al., 1979).

The enzyme contains a relatively high amount of carbohydrate as shown in Table VII. Recently, Doi and Nishida (1981) have shown that the treatment of LCAT with neuraminidase enhances the enzyme activity. The presence of carbohydrate moiety in the enzyme may be responsible for some of the observed, unique physicochemical properties as compared to proteins that contain only amino acids. The molecular weight and partial specific volume of LCAT are the examples of these parameters reported by the current study.
<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Chung et al. (1979)</th>
<th>Albers et al. (1979)</th>
<th>Aron et al. (1978)</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>14 (12-15)</td>
<td>15 ± 1</td>
<td>14 ± 1</td>
<td>14 (14-14)</td>
</tr>
<tr>
<td>His</td>
<td>12 (11-13)</td>
<td>13 ± 2</td>
<td>12 ± 1</td>
<td>12 (12-13)</td>
</tr>
<tr>
<td>Arg</td>
<td>19 (16-20)</td>
<td>19 ± 2</td>
<td>17 ± 2</td>
<td>18 (17-20)</td>
</tr>
<tr>
<td>Asx</td>
<td>37 (34-40)</td>
<td>43 ± 2</td>
<td>33 ± 1</td>
<td>41 (38-45)</td>
</tr>
<tr>
<td>Thr</td>
<td>25 (23-27)</td>
<td>27 ± 0</td>
<td>20 ± 1</td>
<td>24 (23-25)</td>
</tr>
<tr>
<td>Ser</td>
<td>25 (22-28)</td>
<td>28 ± 2</td>
<td>23 ± 2</td>
<td>24 (24-25)</td>
</tr>
<tr>
<td>Glx</td>
<td>42 (38-45)</td>
<td>46 ± 5</td>
<td>41 ± 2</td>
<td>38 (38-39)</td>
</tr>
<tr>
<td>Pro</td>
<td>35 (30-39)</td>
<td>36 ± 2</td>
<td>34 ± 1</td>
<td>31</td>
</tr>
<tr>
<td>Gly</td>
<td>38 (35-42)</td>
<td>44 ± 6</td>
<td>39 ± 2</td>
<td>37 (37-38)</td>
</tr>
<tr>
<td>Ala</td>
<td>27 (26-28)</td>
<td>28 ± 2</td>
<td>23 ± 1</td>
<td>25 (25-26)</td>
</tr>
<tr>
<td>Val</td>
<td>27 (25-29)</td>
<td>29 ± 1</td>
<td>21 ± 1</td>
<td>28 (27-29)</td>
</tr>
<tr>
<td>Met</td>
<td>7 ( 7-8 )</td>
<td>9 ± 1</td>
<td>7 ± 1</td>
<td>7 ( 7-7 )</td>
</tr>
<tr>
<td>Ile</td>
<td>18 (17-19)</td>
<td>19 ± 1</td>
<td>13 ± 1</td>
<td>15 (14-15)</td>
</tr>
<tr>
<td>Leu</td>
<td>46 (44-49)</td>
<td>51 ± 5</td>
<td>41 ± 1</td>
<td>50 (49-52)</td>
</tr>
<tr>
<td>Tyrosine (Tyr)</td>
<td>13 (12-15)</td>
<td>18 ± 2</td>
<td>17 ± 1</td>
<td>11 (11-11)</td>
</tr>
<tr>
<td>Phenylalanine (Phe)</td>
<td>14 (13-16)</td>
<td>20 ± 1</td>
<td>17 ± 0</td>
<td>19 (18-19)</td>
</tr>
<tr>
<td>Half-Cystine (1/2 Cys)</td>
<td>4</td>
<td>4 ± 1</td>
<td>...</td>
<td>4 (3-4)</td>
</tr>
<tr>
<td>Tryptophan (Trp)</td>
<td>7</td>
<td>...</td>
<td>...</td>
<td>10 (10-11)</td>
</tr>
</tbody>
</table>

| Carbohydrate | % (w/w) | 24% | 24.6% (w/w) |
| Hexoses | 51 (mol/59,000g) | | 13.0% |
| Hexosamines | 17 (mol/59,000g) | 31 ± 4 (mol/10⁵g Protein) | 6.2% |
| Sialic Acids | 13 (mol/59,000g) | | 5.4% |
The partial specific volume determined by two independent methods (D$_2$O/H$_2$O sedimentation equilibrium and compositional analysis) gave values that were in close agreement with one another.

The tyrosine residues of 12 moles/mole of LCAT determined by absorption spectroscopy gave values that were in close agreement with the data from amino acid analysis (Table VII). LCAT was found to contain a relatively high amount of tryptophan. The absorption and fluorescence spectral methods used for determining tryptophan gave values that agree well with one another. Chung et al. (1979) have found 7 residues of tryptophan per mole of LCAT by methanesulfonic acid hydrolysis of the protein. The difference between the data of Chung et al. (1979) and this study thus might be due to the different methods employed. The tryptophan content in 10 mM sodium phosphate, pH 7.2 determined by fluorescence spectroscopy was found to be approximately 60% of total tryptophan content in LCAT. These findings may explain the relatively high value for the extinction coefficient ($E_{1%}^{\infty}$) since a considerable proportion (60% of total tryptophan) of tryptophan residues appeared to be easily accessible to the solvent at neutral pH.

Doi and Nishida (1981) have shown that the sequence of amino acid residues in the NH$_2$-terminal region of LCAT conveys an unusually strong hydrophobic character. They proposed that strongly hydrophobic sequence of the N-terminal region
may participate in the binding of the hydrophobic substrates although currently no evidence is available to support this hypothesis. In addition to the highly hydrophobic NH$_2$-terminal region, the exposed tryptophan residues may also play a role in the substrate binding mechanism.

Studies of the sulfhydryl groups of a number of proteins have indicated that not all cysteiny1 residues are equally available for reaction with a variety of reagents. However, LCAT readily reacts with DTNB, indicating that the SH groups are located at the outer surface of the molecule. LCAT has been known to lose its activity upon reaction with DTNB. Thus it is probable that at least one SH group is located at or near the active site of the enzyme. Recently, Verdery (1981) reported that LCAT activity was enhanced by β-mercaptoethanol but inhibited by free cysteine and by reduced glutathione. He interpreted his results by suggesting that LCAT contained both sulfhydryl and disulfide linkage(s) although he did not provide direct evidence to support this hypothesis. Indeed it is likely that LCAT contains one disulfide linkage and two SH groups based on the data that 4 half-cystines/mole of LCAT were observed by amino acid analysis (Table VII), while 2 half-cystines/mole of LCAT reacted during DTNB titration (Figure 15).

Future studies such as the role of tryptophan, cysteine, and carbohydrate moiety in the function of LCAT, the composition and sequence of the active site region of the enzyme
will be necessary to further extend the knowledge of the mechanism of action of LCAT.


