STUDIES ON HUMAN PLASMA LECITHIN:CHOLESTEROL
ACYLTRANSFERASE: PHYSICAL AND CHEMICAL
CHARACTERIZATION, AND COUPLED
SPECTROPHOTOMETRIC ENZYME
ASSAY

DISSERTATION

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Denton, Texas
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The physico-chemical properties of lecithin:cholesterol acyltransferase were investigated. The amino acid composition analysis showed a relatively high content of glutamic acid, aspartic acid, glycine and leucine. The spectrophotometric titration of phenolic groups in the enzyme showed a large increase in absorbance at 295 nm with an apparent pK of about 12.0. The largest change in molar ellipticity at 222 nm was also observed above pH 11. Circular dichroism studies revealed that human lecithin:cholesterol acyltransferase has a relatively high content of β-pleated sheet structure (48%) with 20% α-helix, and 32% remaining structure. Human lecithin:cholesterol acyltransferase has a high extinction coefficient ($E_{1cm}^{1%=280nm}=17$) at neutral pH. Microsequencing of the amino terminal residues of the enzyme revealed a hydrophobic character. Inactivation of lecithin:cholesterol acyltransferase activity was observed using diisopropylfluorophosphate with a stoichiometry of 1 mole of diisopropylphosphate incorporated per mole of enzyme. This suggests the involvement of a serine residue in the active site of the
enzyme, possibly for the formation of an acyl-intermediate.

A new quicker assay method for lecithin:cholesterol acyltransferase was developed. This assay involved coupling reaction with acyl CoA synthetase, PP$_i$-dependent phosphofructokinase, aldolase, triosephosphate isomerase and $\alpha$-glycerol-3-phosphate dehydrogenase monitoring a change in the absorbance or fluorescence intensity due to the oxidation of NADH. The activity of each coupling enzyme was accurately determined to establish the optimum assay condition for lecithin:cholesterol acyltransferase. The coupled enzyme assay for lecithin:cholesterol acyltransferase by spectrofluorometry showed a significant change in relative fluorescence intensity whereas a UV absorption spectroscopy method showed no significant absorbance change for the initial rate of lecithin:cholesterol acyltransferase reaction.
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  Protein Determination
  Extinction Coefficient
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  A. pH-Dependence of Far UV Circular Dichroism
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Liposome Substrate Preparation
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pH-Dependence of Far UV Circular Dichroism
Effect of Salt on LCAT Secondary Structure
Manual Microsequencing Using DABITC/PITC Double Coupling
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<tr>
<td>ACS</td>
<td>acyl coenzymeA synthetase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CE</td>
<td>cholesteryl ester</td>
</tr>
<tr>
<td>CoA(SH)</td>
<td>coenzymeA</td>
</tr>
<tr>
<td>DABITC</td>
<td>4-N,N-dimethylaminoazobenzene isothiocyanate</td>
</tr>
<tr>
<td>DFP</td>
<td>diisopropylfluorophosphate</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DIP-emi</td>
<td>diisopropylphosphoryl-emission</td>
</tr>
<tr>
<td>exi</td>
<td>excitation</td>
</tr>
<tr>
<td>FDP</td>
<td>D-fructose-1,6-diphosphate</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>F6P (F-6P)</td>
<td>D-fructose-6-phosphate</td>
</tr>
<tr>
<td>GAP</td>
<td>D-glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>GCAT</td>
<td>glycerolphospholipid:cholesterol acyltransferase</td>
</tr>
<tr>
<td>α-GDH</td>
<td>α-glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>α-glycerol-3P (α-G-3P)</td>
<td>α-glycerol-3-phosphate</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HMG-</td>
<td>3-hydroxy-3-methylglutaryl-</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin:cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MgPP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>magnesium pyrophosphate</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Pipes</td>
<td>piperazine-N,N'-bis[2-ethane sulfonyl acid]</td>
</tr>
<tr>
<td>PP&lt;sub&gt;i&lt;/sub&gt;-PFK</td>
<td>pyrophosphate-dependent fructose-6-phosphate kinase</td>
</tr>
<tr>
<td>PITC</td>
<td>phenylisothiocyanate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TPI</td>
<td>triosephosphate isomerase</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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</table>
CHAPTER I

INTRODUCTION

Role of Plasma Lipoproteins in Cholesterol Transport

The lipids which are found in plasma—cholesterol, triglycerides, and phospholipids—are very poorly soluble in water; consequently, these lipids are complexed with specific proteins (apolipoproteins) to form large macromolecules referred to as plasma lipoproteins. It is generally accepted that those lipoproteins are spherical particles (Fig. 1) which contain phospholipids, 'free' cholesterol, and protein (apolipoproteins) as surface components, and triglycerides and cholesteryl esters as core constituents. Each component of the lipoprotein complex has a characteristic density: triglyceride 0.9, cholesterol 1.0, phospholipid 1.0, and protein 1.3 g/ml. Internal variations in the relative amounts of the components of lipoproteins give rise to particles which exhibit a range of densities and sizes (Fig. 2). Plasma lipoproteins thus have densities ranging from 0.92 to 1.2 g/ml. These variations in densities give a spectrum of lipoproteins as depicted in Fig. 2 and Table I. There are four basic classes, namely: 'high density lipoproteins' (HDL), 'low density lipoproteins' (LDL), 'very low density lipoproteins' (VLDL), and chylomicrons. Some of the isolation
Figure 1. Simplified model of plasma lipoprotein particle.
Triglycerides
Esterified cholesterol

Lipoprotein membrane
Apolipoprotein
Phospholipids
Free cholesterol
Figure 2. Classes of plasma lipoproteins in terms of densities, Svedberg flotation unit, electrophoretic mobilities, sizes, and the distribution of four apolipoproteins. Chol, cholesterol; TG, triglyceride
<table>
<thead>
<tr>
<th>Density</th>
<th>Chylomicron</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>Albumin</th>
<th>Globulin</th>
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</thead>
<tbody>
<tr>
<td>0.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.96</td>
<td></td>
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<td>1.006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1.063</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.21</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>1.35</td>
<td></td>
<td></td>
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**Electrophoretic Mobility**

<table>
<thead>
<tr>
<th>Cholesterol</th>
</tr>
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<tbody>
<tr>
<td>apo-A</td>
</tr>
<tr>
<td>apo-B</td>
</tr>
<tr>
<td>apo-C</td>
</tr>
<tr>
<td>apo-D</td>
</tr>
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</table>
## TABLE I

COMPOSITION AND PROPERTIES OF HUMAN PLASMA LIPOPROTEINS

<table>
<thead>
<tr>
<th>Properties Classes</th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
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<tr>
<td>Density (g/ml)</td>
<td>&lt;0.95</td>
<td>0.95-1.005</td>
<td>1.006-1.063</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>Electrophoretic Mobility</td>
<td>origin</td>
<td>Prebeta</td>
<td>Beta</td>
<td>Alpha</td>
</tr>
<tr>
<td>Major apolipoproteins(^a)</td>
<td>A-I</td>
<td>B</td>
<td></td>
<td>A-I</td>
</tr>
<tr>
<td></td>
<td>A-II</td>
<td>C-I</td>
<td></td>
<td>A-II</td>
</tr>
<tr>
<td></td>
<td>A-IV</td>
<td>C-II</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>C-III</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-I</td>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor apolipoproteins</td>
<td>E</td>
<td>A-I</td>
<td>E</td>
<td>A-IV</td>
</tr>
<tr>
<td></td>
<td>A-II</td>
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<td></td>
<td>B</td>
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<td>C-I</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>C-III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D(or A-III)</td>
</tr>
<tr>
<td>Components</td>
<td></td>
<td></td>
<td></td>
<td>E</td>
</tr>
<tr>
<td>% Cholesterol(^b)</td>
<td>8</td>
<td>22</td>
<td>46</td>
<td>20</td>
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<tr>
<td>% Phospholipid</td>
<td>7</td>
<td>18</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>% Triglyceride</td>
<td>83</td>
<td>50</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>% Protein</td>
<td>2</td>
<td>9</td>
<td>21</td>
<td>50</td>
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</table>

\(^a\)Major refers to proteins comprising 5% or more of the total protein in mesenteric lymph chylomicrons and plasma VLDL, LDL, and HDL.

\(^b\)Includes unesterified and esterified cholesterol.
procedures take advantage of these differences in densities when separating lipoproteins via ultracentrifugation.

Among the lipid components of plasma lipoproteins, cholesterol is one of the most important, since it is a precursor for steroid hormones and an important constituent of the cell membrane of different tissues. Cholesterol is mainly synthesized in the liver from acetyl CoA via mevalonate and squalene. The rate of synthesis of cholesterol appears to be regulated by the enzyme, HMG-CoA reductase (Gould, 1951; Tompkins et al., 1953; Gould et al., 1953; Siperstein and Guest, 1960; Siperstein and Fagan, 1964, 1966). This enzyme catalyzes the conversion of acetyl CoA to produce mevalonate as a rate-determining step. Another major source of cholesterol is from the diet.

Cholesterol enters the plasma mainly as a component of chylomicrons and VLDL secreted by the intestine (Ashworth and Johnston, 1963; Cardell et al., 1967), in VLDL secreted by the liver (Hamilton et al., 1967; Jones et al., 1967) and in HDL secreted by the liver (Marsh, 1974; Noel and Rubinstein, 1974) and intestine (Windmueller and Spaeth, 1972). Cholesterol in plasma exists in two forms: free (unesterified) (30 per cent) and as cholesteryl esters (70 per cent). Cholesterol is removed from the plasma predominantly by the tissue uptake and catabolism of LDL (Sniderman et al., 1974, 1975; Goldstein and Brown, 1973, 1974; Brown et al., 1975, 1976), chylomicron (Stein et al., 1969; Redgrave, 1970), and
HDL (Stein and Stein, 1973; Stein et al., 1975). In each case the site and extent of uptake seems to be determined largely by the tissue distribution of receptors with specificity for a given type or apolipoprotein and by the accessibility of different tissues to the circulating lipoproteins of a given particle size.

Apolipoproteins and Their Respective Roles in Lipoprotein Metabolism

Apolipoproteins are components of the plasma lipoproteins obtained by extracting the lipid from lipoproteins with organic solvents, detergents, or chaotropic agents. The ABC nomenclature system was proposed by Alaupovic (1971) and is now widely employed. In addition to the lipid-binding properties, all apolipoproteins appear to play more specific roles in lipid transport and lipoprotein metabolism.

Apolipoproteins A through E are well understood and discussed here. Current knowledge of their distribution, molecular weights, polymorphism, origins, concentrations and functions is summarized in Table II.

Apolipoprotein A-I: Apo A-I is the major protein component of HDL. Apo A-I functions as an activator of lecithin:cholesterol acyltransferase (LCAT), a plasma enzyme catalyzing the conversion of cholesterol and phosphatidylcholine to cholesteryl esters and lysophosphatidylcholine (Glomset, 1962, 1968; Fielding et al., 1972). Apo A-I is synthesized in liver and intestine (Windmueller et al., 1973)
**TABLE II**

**HUMAN PLASMA APOLIPOPROTEINS**

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight</th>
<th>Plasma Concentration, mg/dl</th>
<th>Origin</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>A-I</td>
<td>28,300</td>
<td>90-130</td>
<td>Intestine, liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>A-II</td>
<td>17,000</td>
<td>30-50</td>
<td>Intestine, liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>B-100</td>
<td>~549,000</td>
<td>80-100</td>
<td>Liver</td>
<td>Neutral lipid transport</td>
</tr>
<tr>
<td>B-48</td>
<td>~264,000</td>
<td>&lt;5</td>
<td>Intestine, liver</td>
<td></td>
</tr>
<tr>
<td>C-I</td>
<td>6,500</td>
<td>4-7</td>
<td>Liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>C-II</td>
<td>8,800</td>
<td>3-8</td>
<td>Liver</td>
<td>LPL activator</td>
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<tr>
<td>C-III</td>
<td>8,750</td>
<td>8-15</td>
<td>Liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>E</td>
<td>35-39,000</td>
<td>3-6</td>
<td>Liver</td>
<td>?Receptor-mediated lipoprotein remnant catabolism</td>
</tr>
</tbody>
</table>
but their relative contributions to the total plasma content and the factors regulating apo A-I production are not well clarified. More than 90 per cent of the circulating plasma apo A-I is associated with HDL, less than 1 per cent with VLDL and LDL, and no more than 10 per cent with the lipoprotein-free fraction of plasma (Cheung and Albers, 1977).

**Apolipoprotein A-II:** Apo A-II is also a major component of human HDL, making up about a third of the total protein and 15 per cent of HDL mass. It is a dimer of two identical polypeptide chains, and both the monomeric and dimeric forms of apo A-II are capable of reassembling with phospholipids. Although the physical and chemical properties of apo A-II are well characterized (Morrisett et al., 1975), the specific role of apo A-II in lipid transport is not clear.

**Minor HDL Apolipoproteins:** The designation apo A-III (Kostner, 1974a) or apo D (McConathy and Alaupovic, 1973) comprises less than 5 per cent of the HDL apolipoproteins. Apo D (apo A-III) was originally thought to stimulate LCAT (Kostner, 1974b), and Olofsson and Gustafson (1974) have suggested that it may be a specific carrier of lysolecithin formed after the action of LCAT on HDL. However, Soutar et al. (1975) were unable to confirm that apo A-III activates LCAT.

Another protein, so-called apolipoprotein A-IV, first recognized as a major component of rat HDL (Swaney et al., 1974), has been identified in human plasma and lymph (Weisgraber et al., 1978; Beisiegel
and Utermann, 1979), synthesized by both intestine and liver (Wu and Windmueller, 1979), and may be cyclically reincorporated into chylomicrons (Fidge, 1980). An acidic apolipoprotein termed apolipoprotein F, has also been isolated from human plasma HDL (Olofsson et al., 1978). Functions of these minor apolipoproteins have not been identified.

**Apolipoprotein B**: Apo B is an obligatory constituent of chylomicrons, VLDL, and LDL. It comprises more than 90 per cent of the protein of VLDL and chylomicrons. Because of the difficulty in solubilizing and dissociating apo B, knowledge about its structure is still inadequate. However, evidence for heterogeneity of human apo B has been provided. One type of proteins found in plasma LDL is designated as B-100 (Kane et al., 1980). A distinct second type of apo B that is a major constituent of lipoproteins synthesized by intestines and livers represents B-48. Apo B is thought essential for the synthesis of chylomicrons (Glickman et al., 1976; Schonfeld et al., 1978) and VLDL (Swift et al., 1980). Apo B also appears critical to receptor-mediated uptake of LDL (Brown and Goldstein, 1976; Goldstein and Brown, 1977; Shepherd et al., 1979, 1980).

**Apolipoprotein C-I**: Apo C-I comprises approximately 10 per cent of the protein of VLDL and 2 per cent of HDL. It binds phospholipid and can also activate LCAT (Soutar et al., 1975). The effect of apo C-I to stimulate LCAT in vitro is less than that of apo A-I (Soutar et al., 1975; Albers, 1979).
The relative importance of apo A-I and apo C-I remains to be re-
solved for the physiological activation of LCAT. Ganesan et
al. (1975, 1971) have also shown that apo C-I activates lipo-
protein lipase (LPL) from human postheparin plasma, but this
observation remains to be confirmed.

**Apolipoprotein C-II:** Apo C-II makes up about 10 per cent
of the protein of VLDL and 1 to 2 per cent of HDL. Apo C-II
is essential for lipoprotein lipase (LPL), the enzyme cataly-
zing the hydrolysis of triglyceride in chylomicrons and VLDL
(Bier and Havel, 1970; LaRosa et al., 1970).

**Apolipoprotein C-III:** Apo C-III is the most abundant
among the C apolipoproteins, comprising about 50 per cent of
the protein in VLDL and about 2 per cent of that in HDL.
Apo C-III may serve as a regulator for the activity of the
lipoprotein lipase system (Brown and Bagnisky, 1972) and as
an inhibitor of the interaction of triglyceride-rich lipo-
proteins with hepatic receptors (Windler et al., 1980a,b).
However, the physiological functions of apo C-III are yet
uncertain.

**Apolipoprotein E:** Apo E accounts for 10 to 20 per cent
of VLDL proteins. Extensive heterogeneity of apo E has been
identified by isoelectric focussing (Zannis and Breslow,
1980; Utermann, 1975; Utermann et al., 1978). The three major
isoforms (termed E-2, E-3, and E-4, the latter being most basic)
have been reported as different allelic products at a single
locus (Zannis and Breslow, 1981). Hepatic uptake and
catabolism of apo E appear preferentially to involve the more basic isoforms (E-3 and E-4), and their recognition by hepatocyte receptor may provide a link in the normal conversion of VLDL remnants to LDL via IDL (Chao et al., 1979; Havel et al., 1980).

Each lipoprotein particle is characterized by a specific apolipoprotein profile (Eisenberg and Levy, 1975; Osborne and Brewer, 1977). This profile determines specific metabolic events that occur during the life-span of lipoprotein molecules in circulation. However, a precaution against the artifact by isolation method has to be considered for some of the apolipoproteins since the conventional isolation of lipoproteins mainly relies on an ultracentrifugation method.

Reverse Cholesterol Transport

Extrahepatic tissues also synthesize cholesterol, however, the liver is the only organ capable of metabolizing and excreting cholesterol in significant quantities. Hence, if extrahepatic tissues are to remain in balance with respect to cholesterol there must be a mechanism for removing cholesterol from the tissues to the liver via the plasma, a process referred to as reverse cholesterol transport. Removal of cholesterol from the arterial walls during regression of atheromatous lesions has also been suggested to involve the transport of cholesterol from the tissues into the plasma and eventually to the liver.
Little is known about the mechanisms responsible for reverse cholesterol transport in vivo. However, studies of cholesterol efflux from tissues in vitro (Dayton and Hashimoto, 1966) and from cultured cells (Bates and Rothblat, 1974) have established that cells that secrete lipoproteins excrete cholesterol almost exclusively in the unesterified form and only if there is a suitable acceptor for free cholesterol in the external medium. The nature of the physiological acceptor for free cholesterol from tissues is of considerable interest and several possible acceptors have been postulated. Glomset (1968) has proposed that HDL may be the agent of transport (Fig. 3). This hypothesis is supported by studies in cell culture by Stein et al. (1973, 1975). Other possible carriers of cholesterol include LDL (Sniderman et al., 1975) or another plasma protein not previously identified to transport lipids (Carew et al., 1976). Since a considerable amount of LDL-cholesterol (Grundy, 1978) is probably taken up by peripheral cells each day, this amount of cholesterol plus that synthesized in extrahepatic tissues must find its way back to liver. Future studies to further clarify the lipoprotein metabolism will give some more understandings of the nature and mechanism of reverse cholesterol transport.

**Important Features of Lecithin:Cholesterol Acyltransferase**

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43), an enzyme which catalyzes the transfer of fatty acids between
Figure 3. 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 unesterified 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

Lecithin: cholesterol acyltransferase

High density lipoprotein

Cell membrane
lecithin and cholesterol (Fig. 4) on the surface of plasma lipoproteins (Glomset, 1962), has several unique functions in the system of lipid and lipoprotein metabolisms:

1. By maintaining the balance between free and esterified cholesterol, LCAT stabilizes the normal shape and size of lipoproteins among them (Glomset and Norum, 1973; Glomset et al., 1980).

2. LCAT produces the flux necessary for the transfer of free cholesterol from tissues to the plasma (Tall and Small, 1980) and, at the same time, the transfer of cholesteryl esters to the tissues. In tissues, cholesteryl esters subsequently undergo hydrolysis and the resulting free cholesterol can be utilized for the buildup or reconstruction of cellular membranes.

3. LCAT is also a plasma enzyme which produces lysolecithin from lecithin. However, the full extent of the versatile activity of lysolecithin has not yet been fully understood.

4. The cholesterol esterification rate reflects the kinetics of interchanges, that is, the influx and efflux of cholesterol. Based on methods assessing LCAT activity (Nestel et al., 1969; Barter, 1974; Kudchodkar and Sodhi, 1976), the determination of molar and fractional esterification rates offers a unique possibility for the detection and quantitation of the deviations accompanying various disorders of cholesterol metabolism.
Figure 4. Lipid reactants in plasma lecithin:cholesterol acyltransferase reaction.
LECITHIN
$\text{CH}_2\text{O}-\text{saturated fatty acid}$
$\text{CHO}-\text{unsaturated fatty acid}$
$\text{CH}_2\text{O}-\text{P-choline}$
$\text{OH}$

+ CHOLESTEROL

LEcITHIN: CHOLESTEROL ACYLTRANSFERASE

LYSOLECITHIN
$\text{CH}_2\text{O}-\text{saturated fatty acid}$
$\text{CHOH}$
$\text{CH}_2\text{O}-\text{P-choline}$
$\text{OH}$

+ CHOLESTERYL ESTER

unsaturated fatty acid
Since Glomset (1962) first defined the function of LCAT, several important advances have been made to the general understanding of the reaction mechanism and the physiological role of this enzyme. The discovery of hereditary LCAT deficiency (Norum and Gjone, 1967) suggested the importance of cholesteryl esters in the structure of lipoproteins, and disclosed new viewpoints applicable to studying this problem. The isolation of highly purified enzyme created the necessary prerequisite to the elucidation of the three-step reaction mechanism of LCAT and also of its participation in reversible reactions (Yokoyama et al., 1980; Subbaiah et al., 1980). In essence, the LCAT reaction proceeds in the following distinct steps:

1. activation of the phospholipid bilayer by protein or peptide;
2. hydrolysis of fatty acid ester by means of phospholipase A2-like activity (formation of an intermediate acyl enzyme);
3. transfer of the fatty acyl group to the acyl acceptor molecules; and,
4. reverse LCAT reaction.

Role of Lecithin:Cholesterol Acyltransferase in Reverse Cholesterol Transport

Akanuma and Glomset (1968) reported that HDL provides the preferred substrate for LCAT. This finding prompted Glomset (1968) to suggest that HDL and LCAT may play concerted
roles in transporting cholesterol. Accordingly, Glomset (1968) postulated the mechanism to remove cholesterol from the peripheral tissue to the liver (Fig. 3). According to his postulate, LCAT plays a role in shifting a physical equilibrium of cholesterol by converting unesterified cholesterol to cholesteryl esters. Although Glomset postulated this scheme sixteen years ago, it still accounts for our current notion of understanding of reverse cholesterol transport. Moreover, the above author's original postulate has been modified to accommodate recent findings (Glomset, 1979) (Fig. 5) with the following events:

1. In plasma, unesterified cholesterol in HDL is converted to esterified form catalyzed by LCAT.

2. Newly formed cholesteryl esters are subsequently displaced toward the hydrophobic core of HDL particles.

3. A continuing esterification of the free cholesterol on the surface of HDL creates a concentration gradient for a new movement of free cholesterol from tissues to plasma.

4. HDL particles will also interact with triglyceride-rich lipoproteins to acquire their surface components (phospholipids and cholesterol).

5. In humans, cholesteryl esters are transferred from HDL to both LDL and VLDL mediated by cholesteryl ester transfer protein.

6. Finally, HDL particles will be sequestered and catabolized by the liver and extrahepatic tissues.
Figure 5. Modified model for the transport of cholesterol from peripheral tissues to liver. FC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase.
Above considerations are based on substrates specificity studies conducted in vitro (Fielding and Fielding, 1971; Marcel and Vezina, 1973; David et al., 1976) and studies of abnormal lipoprotein patients with congenital (Forte et al., 1974b; Norum et al., 1971; Glomset et al., 1973) or acquired (Sabestin et al., 1977) LCAT deficiency.

While a great deal of information has been accumulated concerning the mechanism of the action of LCAT, the physiological role of LCAT in lipoprotein metabolism including reverse cholesterol transport is not yet adequately understood. Nevertheless, the information that is currently available confirms the role of LCAT in the maintenance of physical equilibrium of cholesterol between tissues and plasma.

Relationship Between Lecithin:Cholesterol Acyltransferase and High Density Lipoproteins with Respect to Plasma Cholesterol

HDL are formed in the liver and in the intestine, consisting of several subfractions as determined by differential (Camejo et al., 1971) and rate zonal ultracentrifugation (Patsch et al., 1974) and by analytical and preparative gel electrofocusing (Mackenzie et al., 1973; Robinson, 1970; Sundaram et al., 1974, 1974). The subfractions classify HDL\textsubscript{3} and HDL\textsubscript{2} particles. Among them, HDL\textsubscript{3} particles are smaller, contain less cholesteryl ester, and have a lower apo A-I/apo A-II ratio than HDL\textsubscript{2} particles (Schaefer and Levy, 1979). The major apolipoprotein of HDL, apolipoprotein A might originate from the intestinal mucosa. The hydrolytic action of lipoprotein lipase (LPL) on the non-polar core of chylomicrons
and VLDL leads to an abundance and subsequent release of free cholesterol, phospholipid and apolipoprotein C from their polar surfaces (Chajek and Eisenberg, 1978; Eisenberg and Olivecrona, 1979; Patsch et al., 1978). It has not yet been established whether these three components are released together as particles with the density of HDL, which then act as substrates for LCAT, or whether the cholesterol and phospholipid become incorporated into pre-existing HDL particles.

Plasma HDL, especially HDL\(_3\), may act as an acceptor for free cholesterol and phospholipid released from the surfaces of triglyceride-rich particles during their hydrolysis by LPL (Patsch et al., 1978). The addition of lipid to HDL\(_3\) converts it into HDL\(_2\) (Patsch et al., 1978; Schmitz et al., 1981). Possibly, HDL\(_3\) crosses the capillary wall, picks up free cholesterol from cell surfaces and returns to the plasma as HDL\(_2\) via the lymphatic system (Yoffey and Courtice, 1970).

Although a considerable proportion of the cholesterol that enters the plasma in nascent lipoproteins (Hamilton, 1972; Hamilton et al., 1976) is unesterified, more than two-thirds of circulating lipoprotein is esterified with long-chain fatty acids (Marsh, 1976). In other words, it appears that as soon as nascent HDL molecules enter the circulation, they are acted upon by LCAT, with the formation of esterified cholesterol. Some of the cholesteryl esters move toward the space between the two layers of the discoid, converting it into a spherical (HDL\(_3\)) particle. The continued action of LCAT may further convert HDL\(_3\) into HDL\(_2\), the additional
substrate arising from the polar surfaces of triglyceride-rich lipoproteins. As cholesteryl esters are produced in the cholesterol/phospholipid monolayer of HDL, they either move into the core region of HDL or are transported to VLDL and LDL by a specific cholesteryl ester transfer protein (Zilver-smith et al., 1975; Chajek and Fielding, 1978). Thus, HDL and LCAT appear to comprise a system to esterify the free cholesterol originally transported into triglyceride-rich lipoproteins.

The biological significance of this process is revealed when the system fails. In familial LCAT deficiency (Norum and Gjone, 1967), the free cholesterol released during the lipolysis of VLDL and chylomicrons is unable to be converted into a less-polar form that can be incorporated into the core lipid of lipoprotein particles (Forte et al., 1974a; Glomset et al., 1973, 1980). Instead, together with phospholipid, albumin and other proteins, it forms abnormal lipoproteins that cause pathological changes in a variety of tissues (Glomset and Norum, 1973). These observations also support the hypothesis that LCAT may participate in the maintenance of the spherical configuration of triglyceride-rich lipoproteins proposed by Schumaker and Adams (1969). In addition, the importance of apolipoprotein synthesis in the normal production or integrity of HDL is illustrated in patients with familial HDL deficiency, called Tangier disease. Cholesterol accumulation in many tissues, including the blood vessel walls, has been found in those patients with Tangier disease (Fredrickson, 1966).
An Overview Summary of Lipoprotein Metabolism
--A Dynamic Equilibrium System

Until about one decade ago it had been assumed that the four major plasma lipoproteins, i.e., chylomicrons, VLDL, LDL, and HDL, were independent lipoprotein particles, each having discernible pathways of synthesis, intravascular metabolism and degradation. This concept, however, has been substantially modified during the last ten years (Eisenberg and Levy, 1975; Eisenberg and Schurr, 1976). Available data now suggest that a dynamic equilibrium exists in the plasma lipoprotein system. Chylomicrons and VLDL are primary secretory products of cells and carry triglycerides in the circulation. As intravascular triglycerides are hydrolyzed via LPL, the further metabolism of non-triglyceride constituents of chylomicrons and VLDL can be followed along two interrelated pathways. Along the core pathway in VLDL metabolism cholesteryl ester becomes a major core component with resultant formation of intermediate density (IDL) and eventually LDL. Concomitant with reduction of core volume, redundant surface lipid and proteins are generated and either form HDL precursors (Chajek and Eisenberg, 1978; Eisenberg et al., 1978) or become incorporated into HDL particles. Cholesteryl esters are thought to be formed mainly via the action of LCAT on HDL (Glomset, 1968; Hamilton et al., 1976). Therefore, the action of LPL and LCAT on triglyceride-rich lipoproteins and their catabolic products is sufficient and necessary for formation of LDL and HDL (Fig. 6).
Figure 6. An overview summary of plasma lipoprotein metabolism.
Once formed, all plasma lipoproteins are further remodelled by exchange and transfer reactions. In humans, a major remodelling occurs through exchange of LDL- and HDL-cholesteryl ester by VLDL- (and chylomicron-) triglyceride. The reaction is the main source of cholesteryl esters in triglyceride-rich lipoproteins and is responsible for the enrichment of triglycerides in LDL and HDL.

Therefore, the physiological and pathophysiological significance of LCAT research with respect to the plasma lipid transport system may be fully appreciated only when the interrelations of all these metabolic reactions are fully elucidated.

Physico-Chemical Properties of Human Plasma Lecithin:Cholesterol Acyltransferase

Human plasma LCAT is the enzyme responsible for producing cholesteryl esters in plasma as described above. The enzyme has been assigned to have a key role in lipoprotein metabolism (Glomset, 1979), but little is known yet of its structure, function, and mechanisms of action. Although several investigators have reported on the amino acid composition (Chung et al., 1979; Albers et al., 1979; Aron et al., 1978; Doi and Nishida, 1981) of the enzyme, the role of specific amino acid residues in the catalytic action of the enzyme has yet to be established.

The relatively slow progress in the elucidation of the LCAT reaction mechanism has likely been due to the difficulties in the isolation of the enzyme in sufficient quantities
for detailed characterization and to the unstable nature of
the highly purified enzyme preparations (Furukawa and Nishida,
1979). Several physico-chemical characteristics are summarized in Table III and IV.

The relative molecular weight of a highly purified enzyme preparation found has been estimated by various laboratories
to be in the range of 65,000–69,000 (Table III) by polyacrylamide gel electrophoresis in the presence of SDS. A somewhat lower value is obtained by sedimentation equilibrium centrifugation. The discrepancy can probably be explained by the fact that the former method of molecular weight determination often yields artifactual results with glycoproteins since the carbohydrate content of LCAT is relatively high (Table IV). Determinations of amino acid composition by several investigators yielded similar values (Table IV) with relatively high contents of glutamic acid, aspartic acid, glycine, and leucine. The carbohydrate contents were also determined by a few investigators. Currently, very little is known about the structural arrangement of the enzyme, except that human LCAT appears to contain one disulfide linkage (Chong et al., 1983b) and some data on the secondary/tertiary structure of the enzyme (Table III). LCAT can form as many as five isoforms with the isoelectric point ranging from 5.1 to 5.5, as demonstrated by analytical isoelectric focussing (Albers et al., 1979); other measurements located the pI in a lower pH region, between 4.5 to 4.8 after neuraminidase treatment (Doi and
### TABLE III

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

<table>
<thead>
<tr>
<th>Methods and Conditions</th>
<th>Observations</th>
<th>Sources</th>
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<tr>
<td>Molecular Weight</td>
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<tr>
<td>Sedimentation Equilibrium</td>
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<tr>
<td>in 1 mM sodium phosphate, pH 7.2</td>
<td>59,000</td>
<td>Chung et al. (1979a)</td>
</tr>
<tr>
<td>in 39 mM sodium phosphate, pH 7.4</td>
<td>60,000</td>
<td>Chong et al. (1983a)</td>
</tr>
<tr>
<td></td>
<td>63,000</td>
<td>Doi and Nishida (1983)</td>
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<tr>
<td>Sedimentation Equilibrium in 6 M guanidine HCl</td>
<td>60,700</td>
<td>Chong et al. (1983a)</td>
</tr>
<tr>
<td>SDS-gel Electrophoresis</td>
<td>66,000</td>
<td>Aron et al. (1978) and Albers et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>65,000</td>
<td>Kitabatake et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>69,000</td>
<td>Chung et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>67,000</td>
<td>Utermann et al. (1980) and Chong et al. (1983a)</td>
</tr>
<tr>
<td></td>
<td>65,000</td>
<td>Doi and Nishida (1983)</td>
</tr>
<tr>
<td>Partial Specific Volume</td>
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<tr>
<td>Composition Analysis</td>
<td>0.71 ml/g</td>
<td>Chung et al. (1979)</td>
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<td></td>
<td>0.707 ml/g</td>
<td>Chong et al. (1983a)</td>
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<tr>
<td></td>
<td>0.712 ml/g</td>
<td>Doi and Nishida (1983)</td>
</tr>
<tr>
<td>Parallel D$_2$O-H$_2$O</td>
<td>0.702 ml/g</td>
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<td>Isoelectric Point</td>
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<td>4.28 - 4.37</td>
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<td>4.5 - 4.8</td>
<td>Doi and Nishida (1981)</td>
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<td>4.2 - 4.5</td>
<td>Chong et al. (1983a)</td>
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<td>3.9 - 4.2</td>
<td>Doi and Nishida (1983)</td>
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<td>Stokes' Radius</td>
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<td>Doi and Nishida (1983)</td>
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<tr>
<td>Extinction Coefficient</td>
<td>21</td>
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<tr>
<td></td>
<td>20</td>
<td>Doi and Nishida (1983)</td>
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<td>Secondary Structure</td>
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<td>α-Helix, β-Sheet, Remainder</td>
<td>24%, 27%, 49%</td>
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<td></td>
<td></td>
<td>18%, 53%, 29%</td>
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<td>------------</td>
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</tr>
<tr>
<td>Lys</td>
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<td>Phe</td>
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<td>35</td>
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<tr>
<td>h(\frac{1}{2})/Cys</td>
<td>N.D.</td>
<td>6</td>
</tr>
<tr>
<td>Trp</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

| Carbohydrate % (w/w) | 24% | 24.6% |
| Hexoses | 51 (mol/10^5 g protein) | 13.0% |
| Hexosamine | 31 (mol/10^5 g protein) | 17 (mol/10^5 g protein) | 6.2% |
| Sialic acid | 13 (mol/10^5 g protein) | 5.4% |
Nishida, 1981). However, another enzyme preparation isolated by a different method and measured by the same procedure yielded just a single zone between 3.7 and 4.0 (Gustow et al., 1978). Thus, some physico-chemical properties have been investigated relatively in detail and well characterized. However, more studies in this area are required to fully elucidate the structure, function and mechanisms of action of LCAT.

Coupled Spectrophotometric Enzyme Assay for Human Plasma Lecithin: Cholesterol Acyltransferase

All assay methods for LCAT have so far involved lipid extraction, separation of the esterified cholesterol from the unesterified cholesterol counting the radioactivity of each as described by Glomset and Wright (1963) originally, and modified by Stokke and Norum (1971) and Lacko et al. (1972). Since the present assay for LCAT is somewhat tedious and time-consuming, it would be desirable to develop a quicker and continuous assay system. A coupled enzyme assay was first described for a kinetic analysis by McClure (1969), and further developed for a general expression by Easterby (1973). Thus, the coupled spectrophotometric enzyme assay was developed for LCAT (Fig. 7).

Glomset (1962) reported that LCAT hydrolyzed fatty acyl chain from the C:2 position of phosphatidylcholine and transferred it to 3β-OH group of cholesterol (Fig. 8). The phospholipase activity of the enzyme thus could be utilized for
Figure 7. Reaction scheme for coupled spectrophotometric enzyme assay.
Lecithin $\xrightarrow{\text{LCAT}}$ Lysolecithin + Free Fatty Acid

Apo A-I $\xrightarrow{\text{PPi-PFK}}$ MgPP$_i$ + AMP $\xrightarrow{\text{MgATP}}$ CoA Synthetase

Fructose-1,6-diphosphate (FDP) $\xrightarrow{\text{Mg + P}_i}$ Fructose-6-phosphate (F-6P)

Aldolase

Glyceraldehyde-3-phosphate (GAP) $\xrightarrow{TPI}$ Dihydroxyacetone phosphate (DHAP)

$\xrightarrow{\text{NADH}}$ $\alpha$-GDH

NAD$^+$ $\xrightarrow{\text{Glycerol-3-phosphate}}$

\[ \text{Mg}^+ \text{P}^: \text{Fructose-1,6-diphosphate (FDP)} \]
Figure 8. Enzymic reaction by lecithin:cholesterol acyltransferase and glycerophospholipid:cholesterol acyltransferase, with respect to two distinct activities.
the development of a coupled enzyme assay. However, LCAT is not available in large quantities and is relatively unstable in the highly purified state, although Chong et al. (1981) recently reported the purification method with a milligram-range recovery and Jahani and Lacko (1982) also reported the stabilization method to store the enzyme for extended periods.

Bacterial glycerophospholipid:cholesterol acyltransferase (GCAT), which appears to catalyze a reaction similar to that catalyzed by LCAT as described by Buckley et al. (1982), was available to establish the optimum condition of coupled enzyme assay together with LCAT. GCAT is far more stable than LCAT and is available in larger quantities (Buckley et al., 1982).

Purpose of These Investigations

LCAT catalyzes one of the three reactions that have been described for the esterification of cholesterol (Glomset, 1968). The enzyme exhibits a number of unique features including the requirement for a macromolecular lipoprotein complex (Akanuma and Glomset, 1968) and a polypeptide cofactor (Fielding et al., 1972). 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 unresolved. The paucity of definitive investigation in this area has been mainly due to the lack of procedures that consistently yield sufficient amounts of
enzyme for detailed characterization studies and to the labile nature of the preparations. Chong et al. (1981) have recently developed a highly reproducible method that allowed the isolation of LCAT in milligram quantities. These recent developments have allowed me to characterize the enzyme in more detail.

The present investigations were thus designed with the following objectives and/or goals:

(A) To characterize the physical and chemical properties of the enzyme. These include amino acid composition analysis, especially for tyrosine and phenylalanine residues, ultraviolet and circular dichroism spectroscopy, microsequencing of amino terminal region, and covalent modification of the serine residue by DFP.

(B) To develop a simple enzyme assay method for LCAT since the conventional assay is tedious and time-consuming.

The former work should provide important information for further studies on the mechanism of action of LCAT. The latter work should provide a useful tool for kinetic studies not only on human plasma LCAT but on any enzyme that has a phospholipase activity or that releases free fatty acids.
CHAPTER II

MATERIALS AND METHODS

Materials

Enzymes

Acyl coenzyme A synthetase, \( \text{PP}_{i}\text{-PFK}, \) and TPI were obtained from Sigma Chemical Co., St. Louis, Missouri. Aldolase and \( \alpha\)-GDH were from Boehringer Mannheim. Those enzymes were used without further purification. Bacterial GCAT was a generous gift from Dr. J. T. Buckley at the University of Victoria, Victoria, British Columbia, Canada. Human plasma LCAT was isolated in our laboratory according to Chong et al. (1981) and slightly modified by Torres and Lacko (1984). Apo-A-I was isolated as a by-product from LCAT purification.

Substrates

All substrates used for coupled spectrophotometric enzyme assay were obtained from Sigma.

Chemical Modification Chemicals

Iodoacetate and non-radiolabeled DFP were obtained from Sigma. Radiolabeled \([1,3-^3\text{H}]\text{DFP}\) were obtained from New England Nuclear.
Chromatographic and Electrophoretic Supplies

Sephadex G-25 was a product of Pharmacia. Sepharose CL-4B was obtained from Sigma. The amino acid standards were from Pierce Chemical Co., and the other reagents used in amino acid analysis were from Dionex. Plastic-backed polyamide thin-layer sheets for two dimensional separation of DABTH amino acids were from Pierce Chemical Co.

Sequencing and Other Chemicals

All sequencing reagents were purchased from Pierce Chemical Co. as the "Sequential Grade." Pyridine was redistilled twice over ninhydrin and KOH. 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC), quanidine HCl and SDS were obtained from Pierce as the "Sequential Grade." Bis-acrylamide, acrylamide, riboflavin, ammonium persulfate, N,N,N',N'-tetramethylene diamine, β-mercaptoethanol, Coomassie blue G-250 were obtained from Eastman-Kodak, Rochester, N.Y. BSA, calf serum fetuin, and non-radiolabeled cholesterol were purchased from Sigma. [1,2-3H]cholesterol was obtained from Amersham/Searle. All other chemicals were products of Fisher Scientific Co., and were of reagent grade.

Methods

Amino Acid Composition Analyses

Samples of purified LCAT (275 µg) were dialyzed against 5 mM sodium phosphate buffer, pH 7.2, extensively. An
aliquot of 0.1 ml of sample (15 μg) was transferred to an acid-cleaned ampules. Hydrolysis was carried out in 6 N HCl containing 0.1% (w/v) phenol for 24, 48, and 72 hours at 110° C. The addition of phenol was to improve the recovery of tyrosine (Moore, 1972). The amino acid analyses of the hydrolysates were performed in a Durrum amino acid/peptide analyzer equipped with o-phthaldialdehyde fluorescence detection system and with an automatic chromatography data station (Perkin Elmer Sigma 10). The values of the respective amino acid residues were corrected based on a standard recovery. Half-cystine was determined in a separate experiment as S-carboxymethylcysteine following the reduction of disulfide bonds with dithiothreitol and alkylation with iodacetate as described by Hirs (1967).

**Liposome Substrate Preparation**

Single bilayer vesicles of lecithin and cholesterol were prepared as described by Batzri and Korn (1973). The suspension containing egg PC and cholesterol in ethanol was concentrated on an Amicon (Lexington, Mass.) ultrafiltration device using a 43 mm diameter YM-30 membrane with rapid stirring under a N₂ pressure of 10 psi. The concentrated solution was then subjected to molecular sieve chromatography on a Sepharose CL-4B column (1.0 cm x 16 cm) as described by Huang (1969). The final liposome preparation contained 5 mg of PC and 0.5 mg of cholesterol, and PC:Cholesterol molar ratio
was 5:1. The liposome solution was kept in the refrigerator for a subsequent circular dichroism study.

**Spectrophotometric Studies**

**Protein Determination** - The determination of protein concentration was carried out by the protein dye-binding method as described by Bearden, Jr. (1978), using bovine serum albumin and fetuin from fetal calf serum (Sigma Chemical Co., St. Louis, MO) as standards. 0.5 ml of samples containing 0-10 µg of the standard were mixed with 0.5 ml of dye reagent, Coomassie Brilliant Blue G-250 (Eastman-Kodak, Rochester, N.Y.). The sample solution was placed in Cary 210 double-beam spectrophotometer in a 1 cm plastic cuvette. At 465 nm, the reading of the sample solution vs. the blank (dye reagent plus buffer) was set to zero. The wavelength was then changed to 595 nm, and the absorbance read directly. The LCAT concentration was determined by extrapolating the absorbance on the standard curve.

**Extinction Coefficient** - The absorbance of LCAT in 5 mM sodium phosphate buffer, pH 7.2, at 280 nm was established in a Cary 210 Spectrophotometer. An aliquot of the LCAT solution was subjected to protein determination as described originally by Bradford (1976) and modified by Bearden, Jr. (1978).

**Spectrophotometric Titration** - One ml (0.39 mg/ml) of purified human LCAT was dialyzed extensively against 1 mM sodium phosphate buffer, pH 7.2. The enzyme solution was placed in a Cary 210 double-beam spectrophotometer equipped with 1 cm
cells. The scanning was performed between 320 nm and 240 nm. The pH of the LCAT solution was gradually increased by the addition of small aliquots (1-30 microliters) of KOH (1-10 M) solutions. The concentration of tyrosine residues was calculated from the absorbance at 295 nm assuming a molar extinction coefficient of 2,300 as described by Beaven et al. (1952).

Circular Dichroism Studies

A) pH-dependence of Far UV Circular Dichroism

LCAT was subjected to CD studies using the JASCO J-40 CD Spectropolarimeter. The CD spectra was recorded at a sensitivity of 2 millidegree/cm in a strain-free quartz cuvette with a pathlength of 0.1 cm. The pH dependence of the CD spectrum was carried out with far ultraviolet range (250-190 nm) at room temperature as described for the Spectrophotometric Titration above. The optical activity was expressed in terms of mean residue ellipticity, \([\Theta]_{\text{mrw}}\), using a mean residue weight of 110 for LCAT. The dimension was expressed as deg·cm\(^2\)·dmol\(^{-1}\). The following equations were used to estimate the protein secondary structure:

\[
\% \alpha\text{-helix} = \frac{-[\Theta]_{208\text{nm}} - 4,000}{33,000 - 4,000} \times 100 \quad (\text{Greenfield \\& Fasman, 1969})
\]

\[
\% \beta\text{-pleated sheet} = \frac{-[\Theta]_{218\text{nm}}}{19,300} \times 100^* \quad (\text{Greenfield \\& Fasman, 1969})
\]

*The presence of \(\alpha\)-helix structure at 218 nm was not considered in this equation.

B) Effect of Salt on LCAT Secondary Structure

Samples of purified LCAT (450 \(\mu\)g) were dialyzed extensively against 5 mM sodium phosphate buffer, pH 7.2. 0.5 ml
of liposome as prepared previously was added to LCAT solution and incubated for 30 min at 37° C. The salt effect of CD spectrum was carried out as described above in Circular Dichroism section. The salt concentration of the LCAT solution was gradually increased by the addition of small aliquots of 5 M NaCl solutions. As a background, the CD spectrum of liposome solution was recorded as a baseline.

**Manual Microsequencing Study Using DABITC/PTIC Double Coupling**

The original procedure of sequence analysis using DABITC/PTIC double coupling was adopted with minor modifications as described by Chang et al. (1978) (Fig. 9). Samples of purified LCAT (735 μg, 15 nmole) eluted from antibody column chromatography as described by Chong et al. (1981) were lyophilized. Dry sample was dissolved in 1.5 ml of deionized water and then lyophilized again. Dry sample was redissolved in 80 microliters of 50% pyridine-water (v/v) and treated with 40 microliters of DABITC (25 nmole) solution in an acid washed Pierce Reacti^R^ vials fitted with stoppers. The vial was flushed with N₂ for 10 sec. and placed in a heating block at 55° C for 50 min. After the first coupling, 10 microliters of PTIC was added and the second coupling was allowed to proceed at 55° C for 30 min. After removing by-products extracting with hetane:ethylacetate (2:1, v/v), the derivatized LCAT was dried and subjected to cleavage by anhydrous trifluoroacetic acid.
Figure 9. Schematic procedure of manual microsequencing.

(a) The abbreviations used are: PITC, phenylisothiocyanate; TFA, trifluoroacetic acid (anhydrous); and ATZ, anilinothiazolinone.

(b) The use of benzene:ethylacetate (2:1) or n-heptane:ethylacetate (2:1) can substitute for 1-chlorobutane during extraction step.

(c) The extraction of thiazolinone derivatives with 1-chlorobutane can be replaced by extracting with n-butylacetate or ethylacetate.
Thiazolinone released from the cleavage was extracted with butylacetate. After collection of the butylacetate extract, the protein in aqueous phase was dried and subjected to the next degradation cycle. The butylacetate extract was evaporated and redissolved in water (20 microliters) and acetic acid saturated with HCl gas (40 microliters). Conversion of the thiazolinones of amino acids into thiohydrantoin (DABTHs) was carried out in the 52°C heating block for 50 min. The sample was dried and redissolved in a suitable volume of ethanol (5-30 microliters) and 1/40-1/5 of the ethanol extract was used for TLC identification.

The identification of DABTH-amino acids by two-dimensional thin-layer chromatography was carried out on polyamide sheets (5 cm x 5 cm). The blue marker DAB-diethylamine was co-chromatographed with each unknown to facilitate identification. The first dimension was developed in acetic acid:water (2:1, v/v), while toluene:n-hexane:acetic acid (2:1:1, v/v) was used as the solvent for the second dimensional separation. After separation, the plates were dried and exposed to HCl vapor for visualization of the red dimethylamino azobenzene thiohydrantoin. The sensitivity of this method is 10 to 20 picomoles.

Chemical Modification Study
Preparation of \[^{3}H\]Diisopropylfluorophosphate Stock Solution - 0.9 ml of DFP (Sigma, 1g/ml) was diluted to 5-fold with anhydrous isopropanol. Then an aliquot of \(1-{^{3}H}\)DFP (new England
Nuclear, 5 mCi) was added to the diluted non-radiolabelled DFP solution. The final concentration of \( ^3\text{H}\)DFP stock solution was calculated as 0.89 M. The radioactivity of \( ^3\text{H}\)DFP stock solution was measured with an aliquot amount (5-10 microliters) by Hewlett Packard Tri-Carb 300C. The \( ^3\text{H}\)DFP stock solution was found to contain 925,000 dpm/µmole.

### Isolation of \( ^3\text{H}\)DIP-LCAT

The LCAT suspended in \((\text{NH}_4)_2\text{SO}_4\) was dialyzed extensively against 5 mM sodium phosphate, pH 7.3. The total A\(_{280}\) recovery was found to be 1.32. The purity of sample of LCAT was analyzed by 10 M urea polyacrylamide gel electrophoresis (7.5% gel) and by HPLC using TSK-250 gel filtration column (7.5 mm x 300 mm) with 5 mM sodium phosphate buffer, pH 7.3. The LCAT was identified as homogeneity except with a very trace contaminant of apo A-I. The LCAT activity was assayed with a minor modification as described by Glomset and Wright (1963), and by Lacko et al. (1973).

The sample of purified LCAT (600 µg) was treated with an aliquot of \( ^3\text{H}\)DFP solution (4 mM) for 20 min. Then \( ^3\text{H}\)DIP-LCAT was separated from unreacted \( ^3\text{H}\)DFP by Sephadex G-25 column chromatography with 0.1 M sodium phosphate-0.1 M NaCl buffer, pH 7.2. After collection of samples of \( ^3\text{H}\)DIP-LCAT, the sample was analyzed by reading the absorbance at 280 nm and by counting the radioactivity. The sample of \( ^3\text{H}\)DIP-LCAT was further dialyzed extensively against deionized water, and only the radioactivity was counted again since the sample of \( ^3\text{H}\)DIP-LCAT was assumed in a denatured condition. Then the
sample was lyophilized and subjected to the alkylation of free sulfhydryl groups. The carboxymethylated \[^3\text{H}\]DIP-LCAT was saved for amino acid analyses and for tryptic digestion. The radioactivity of carboxymethylated \[^3\text{H}\]DIP-LCAT was measured before the subsequent experiments.

**Coupled Spectrophotometric Enzyme Assay**

**Optimization and Cost Analysis**

The coupled enzyme assay was optimized for a 5 min. lag and for cost per assay according to the method of Cleland (1979a). Figure 10 shows the equations to calculate the optimum amount of coupled enzymes and the cost.

The theoretical result was shown on Table V based on the data available from 1984 Sigma Chemical Company catalog and 'Enzyme Handbook' by Barman (1969).

**Enzyme Assays**

In the direction of formation of lysolecithin, the decrease in absorbance at 340 nm due to the oxidation of NADH was monitored using a Gilford 2600 spectrophotometer with a 1 cm light path. Aminco SPF-500 RATIO spectrofluorometer was also used for the LCAT assay to increase its sensitivity. Fluorescence spectra were obtained with excitation at 340 nm and emission at 460 nm. Mixed micelles of apo A-I with egg PC were also prepared by sodium cholate dispersion according to the method of Matz and Jonas (1982). The incubation mixture contained egg PC:apo A-I with molar ratio, 300 to 1. The sodium cholate was removed by an extensive dialysis and by gel
Figure 10. Equations for the optimum amounts of coupled enzymes.

\( V \), amount of enzyme (unit/ml); \( X \), cost of enzyme (¢/unit);
\( L \), lag time (min.).
### TABLE V

**OPTIMIZATION OF COUPLED ENZYME AND COST
ANALYSIS OF COUPLED ASSAY**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cost (SIGMA '84 Price List)</th>
<th>(K_m) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl CoA Synthetase</td>
<td>$115.95/10 U = 1159.5 ¢/U</td>
<td>0.46</td>
</tr>
<tr>
<td>PP(_i)-PFK</td>
<td>$ 57.60/10 U = 576 ¢/U</td>
<td>0.40</td>
</tr>
<tr>
<td>Aldolase</td>
<td>$ 27.80/900 U = 3.089 ¢/U</td>
<td>0.015</td>
</tr>
<tr>
<td>TPI</td>
<td>$ 16.90/25000 U = 0.0676 ¢/U</td>
<td>0.050</td>
</tr>
<tr>
<td>a-GDH</td>
<td>$ 19.25/8000 U = 0.241 ¢/U</td>
<td>0.0050</td>
</tr>
<tr>
<td>DHAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgPP(_i)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Lag (min.)</th>
<th>Amount of Enzyme (U/ml)</th>
<th>Assay cost (£/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl CoA Synthetase</td>
<td>5</td>
<td>0.014</td>
<td>16.23</td>
</tr>
<tr>
<td>PP(_i)-PFK</td>
<td>5</td>
<td>0.006</td>
<td>3.46</td>
</tr>
<tr>
<td>Aldolase</td>
<td>5</td>
<td>0.14</td>
<td>0.43</td>
</tr>
<tr>
<td>TPI</td>
<td>5</td>
<td>4.0</td>
<td>0.27</td>
</tr>
<tr>
<td>a-GDH</td>
<td>5</td>
<td>4.1</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Enzyme cost 21.38 £/ml

Total cost per 1 ml assay = Enzyme cost + Substrates cost at saturating level.
filtration column chromatography with Bio-gel A-5 m (1.5 x 45 cm).

A typical assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 0.1 U ACS; 0.8 U PP\textsubscript{i}-PFK; 2 U aldolase; 20 U TPI; 7 U α-GDH; 10-20 μl micelle solution; 1.5 mM CoA; 1 mM MgATP; 3 mM F6P; 0.2-0.3 mM NADH; with variable amounts of LCAT. Reactions were initiated by the addition of micelle solution, followed by LCAT. This coupled assay was optimized for 1.5 min. lag and the cost was minimized according to the method of Cleland (1979a). To determine whether the assay is performing properly, the activity of each coupling enzyme was determined accurately, and, as an initial study, bacterial GCAT, which appears to catalyze a similar reaction, was used to establish the optimum condition of coupled enzyme assay. Background activity as a control was checked with everything except an enzyme tested for each assay. The following is the determination scheme of each enzyme activity.

\[ \text{α-glycerol-3-phosphate dehydrogenase} \]

\[
\begin{align*}
PDP & \xrightarrow{\text{aldolase}} \xleftarrow{TPI} GAP \\
 DHAP & \xrightarrow{\text{aldolase}} \xrightarrow{\alpha-GDH} \text{glycerol-3P} \\
 & \xrightarrow{NADH} \xleftarrow{\text{NAD}} \\
\end{align*}
\]

An aliquot of α-GDH suspended in (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solution was spun down at around 1,000 rpm for a few min., followed by removing the supernatant and dissolving the precipitates with an appropriate amount of 10 mM Pipes, pH 7.4, used in this assay.
The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 0.4 mM FDP; 0.2 mM NADH; saturating conditions of aldolase and TPI; with variable amounts of α-GDH. The reaction was initiated by an addition of α-GDH after 5-10 min. preincubation.

![Triosephosphate isomerase](image)

About one mg of lyophilized TPI was weighed and dissolved in one ml of 10 mM Pipes, pH 7.4.

The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 5 mM GAP; 0.2 mM NADH; 1 U α-GDH; with variable amounts of TPI. The reaction was initiated by an addition of TPI after a very brief preincubation. This coupled assay was optimized for a 30 sec. lag.

![Aldolase](image)

Aldolase stock solution was prepared in a similar manner as the preparation of α-GDH.
The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 0.5 mM FDP; 0.2 mM NADH; 2 U TPI; 1 U α-GDH; with variable amounts of aldolase. The reaction was initiated by an addition of aldolase after 5 min. preincubation. This coupled assay was optimized for a 1 min. lag.

**Pyrophosphate-dependent phosphofructokinase**

\[
\begin{align*}
F-6P + MgPP_i & \overset{PP_i-PPK}{\longrightarrow} FDP + Mg + P_i \\
aldoase & \downarrow \\
GAP \overset{TPI}{\longrightarrow} DHAP & \overset{\alpha-GDH}{\longrightarrow} glycerol-3P \\
& \downarrow NADH \\
& \downarrow NAD \\
\end{align*}
\]

**PP_i-PPK** stock solution was prepared by weighing an appropriate amount of lyophilized enzyme and dissolving in 100 mM Pipes, pH 7.4, containing 20% glycerol. The enzyme stock solution was kept at -20° C.

The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 20 mM F-6P; 0.2 mM PP_i; 1 mM MgCl_2; 0.3 mM NADH; 1 U aldolase; 25 U TPI; 10 U α-GDH; with variable amounts of PP_i-PPK. The reaction was initiated by an addition of PP_i-PPK after 5 min. preincubation. This coupled assay was optimized for a 5 sec. lag.
ACS stock solution was also prepared by weighing lyophilized enzyme and dissolving it in 10 mM Pipes, pH 7.4.

The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 0.1-0.2 mM palmitate; 1-1.5 mM CoA; 10 mM MgCl₂; 5 mM ATP; 3 mM F-6P; 0.3 mM NADH; 0.4-0.8 U PP₁-PFK; 1-2 U aldolase; 10 U TPI; 4.5-7 U α-GDH; with variable amounts of ACS. This coupled assay was optimized for 30-40 sec. lag. A micellar solution was formed for fatty acids by sodium cholate freshly before an assay. Fatty acid solution was not included in the preincubation but was added just before the initiation of the reaction by an addition of ACS.
Glycerophospholipid:cholesterol acyltransferase

\[
\begin{align*}
\text{Lecithin} & \xrightarrow{\text{GCAT}} \text{Lysolecithin} + \text{FFA} \\
\text{Mg}^{++} + \text{PP}_i & \xrightarrow{\text{PP}_i-\text{PFK}} \text{FDP} \\
\text{DHAP} & \xrightarrow{\text{a-GDH}} \text{Glycerol-3P} \\
\text{Aldolase} & \quad \text{F6P} \\
\text{TPI} & \\
\text{GAP} & \\
\end{align*}
\]

The assay contained in a 1 ml volume: 10 mM Pipes, pH 7.4, 25° C; 0.02-0.04 mg egg lecithin; 0.5-1.5 mM CoA; 5-10 mM Mg; 1-5 mM ATP; 3 mM F-6P; 0.3 mM NADH; 0.1 U ACS; 0.5-1 U PP_i-PFK; 5 U aldolase; 20-30 U TPI; 6-7 U a-GDH; with variable amounts of GCAT. This coupled assay was optimized for 1.4 min lag. A micellar solution was formed for egg lecithin by sodium cholate freshly before assay. Egg lecithin solution was not included in the preincubation but was added just before the initiation of the reaction by an addition of GCAT.

Determination of Kinetic Parameters for Acyl CoenzymeA Synthetase

0.5-1 milliunit of ACS were added to 1 ml of the incubation mixture as described above. After a brief preincubation at 25° C, the reaction was initiated by an addition of CoA, MgATP, palmitic acid, stearic acid, oleic acid, and
linoleic acid, respectively, and the initial rate of absorbance change at 340 nm was monitored. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. All data were fitted to the Lineweaver-Burk equation using FORTRAN programs developed by Cleland (1979b).
CHAPTER III

RESULTS

Amino Acid Composition Analyses

Table VI shows the amino acid composition of human LCAT based on a molecular weight of 45,000. Amino acid analysis of human LCAT indicated that it contained approximately 394 amino acid residues with a molecular weight of 43,000. The mean residue weight was calculated to be 109.4 without proline and tryptophan residues. The enzyme showed a relatively high content in aspartic acid, glutamic acid, glycine and leucine. The recovery of tyrosine residues was mainly focused here in terms of the subsequent spectrophotometric titration experiment for phenolic groups.

Liposome Substrate Preparation

Figure 11 shows the elution profile of PC liposomes with cholesterol from Sepharose CL 4B. The fractions between 4 and 12 were collected (indicated by an arrow) and concentrated to 5 ml. The concentrated sample was kept in a refrigerator for a subsequent experiment.

Protein Determination

Figure 12 shows the standard curves for the determination of protein concentration with BSA and fetuin. Table VII shows the LCAT concentration recovered from the respective standard
<table>
<thead>
<tr>
<th>Amino Acids&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Number of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.03 (42)</td>
</tr>
<tr>
<td>Threonine</td>
<td>20.33 (20)</td>
</tr>
<tr>
<td>Serine</td>
<td>26.47 (26)</td>
</tr>
<tr>
<td>Glutamate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>47.60 (48)</td>
</tr>
<tr>
<td>Proline</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycine</td>
<td>52.15 (52)</td>
</tr>
<tr>
<td>Alanine</td>
<td>25.83 (26)</td>
</tr>
<tr>
<td>Valine</td>
<td>29.43 (29)</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.60 (7)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16.07 (16)</td>
</tr>
<tr>
<td>Leucine</td>
<td>42.77 (43)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>18.87 (19)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>21.53 (22)</td>
</tr>
<tr>
<td>Histidine</td>
<td>12.40 (12)</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.77 (12)</td>
</tr>
<tr>
<td>Arginine</td>
<td>17.60 (18)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>N.D.</td>
</tr>
<tr>
<td>Half-cystine&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.96 (2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Total 394</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>43,094</td>
</tr>
</tbody>
</table>

<sup>a</sup>For experimental details, see the text.

<sup>b</sup>The values are average of 24, 48, and 72-hours hydrolysis without correcting labile and hydrophobic residues.

<sup>c</sup>Aspartate is the sum of aspartic acid and asparagine.

<sup>d</sup>Glutamate is the sum of glutamic acid and glutamine.

<sup>e</sup>Average of two determinations. Determined by S-alkylation.
Figure 11. Elution profile of liposomes by Sepharose CL 4B column chromatography.
Eluent: 5 mM sodium phosphate, pH 7.2
Fraction size: 2.5 ml
Flow Rate: 25 ml per hour
Column Size: 1.0 x 16 cm
Figure 12. Standard curves for the dye-binding reaction with bovine serum albumin and fetuin.
### TABLE VII
SUMMARY TABLE OF PROTEIN ASSAY

<table>
<thead>
<tr>
<th>BSA concentration (µg/ml)</th>
<th>( \Delta A_{595} - \Delta A_{465} )^a</th>
<th>BSA ( \Delta A_{595} - \Delta A_{465} )</th>
<th>Fetuin concentration (µg/ml)</th>
<th>( \Delta A_{595} - \Delta A_{465} )^a</th>
<th>Fetuin ( \Delta A_{595} - \Delta A_{465} )</th>
<th>LCAT Standard curve</th>
<th>BSA Standard curve</th>
<th>Fetuin Standard curve</th>
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</table>

^aAverage of two readings.

\(^b\)LCAT was diluted by 20-fold.

\(^c\)These points were not included for linear regression analysis.
curve. There seems to be 3-fold difference in recovery between two standard curves. This could be due to the physico-chemical nature of two different proteins. Fetuin seems to give a better approximation to determine LCAT concentration since it is a glycoprotein which appears to have a similar carbohydrate content as LCAT.

**Extinction Coefficient**

The extinction coefficient \(E_{1\text{cm}(280\text{nm})}^{18}\) was found to be 17 determined by protein determination assay. The relatively high content of tryptophan may be responsible for the high extinction coefficient.

**Spectrophotometric Titration**

Figure 13 shows the spectrophotometric titration of LCAT between pH values 7 and 13. The large increase in absorbance at 295 nm indicates that nearly all the phenolic groups are unavailable to the solvent at neutral pH while they become fully exposed at higher pH values. The absorbance data allowed the calculation of the concentration of tyrosine residues per mole (eighteen) assuming a molar extinction coefficient of 2,300. This value agrees well with the number of tyrosine residues per mole recovered from amino acid composition analyses. It appears that the exposure of the tyrosine residues to the aqueous environment requires some type of conformational transition of unfolding of the polypeptide chain with a characteristic apparent pK
Figure 13. Spectrophotometric titration of LCAT (o) and N-acetyltirosinamide (x) at 295 nm. One milliliter of LCAT (0.39 mg/ml) was titrated by the addition of small increments (1-30 µl) of KOH solutions of appropriate concentration (1-10 M).
MOLAR EXTINCTION (295 nm) x 10^{-3}

PHENOLIC GROUPS TITRATED/MOLECULE

N-acetyl-L-tyrosylamide

L-tyrosylamide

pk 9.8

pk 12

pH
value of about 12.0 that is distinct from the apparent pK of ionization of the free tyrosine residues (Fig. 13).

**pH-Dependence of Far UV Circular Dichroism**

CD data obtained on the purified LCAT sample in the far ultraviolet region are shown in Figure 14. The native enzyme exhibited CD spectra typical for helical proteins (Adler et al., 1974). Characteristic double-negative maxima at 222 nm and 209 nm, and a positive band below 200 nm region suggest that the enzyme contains relatively abundant ordered secondary structures. Human plasma LCAT was found to have 20% α-helix, 48% β-pleated sheet, and 32% remaining structure in 1 mM sodium phosphate buffer, pH 7.1, 19% α-helix, 41% β-pleated sheet, and 40% remaining structure at pH 12.0. Figure 15 shows the change in molar ellipticity at 222 nm as the function of pH. Similar to the absorbance data shown on Fig. 13 no significant change in molar ellipticity occurred between pH 9 and 11 while a relatively large increase was apparent between pH 11 and 12.

**Effect of Salt on LCAT Secondary Structure**

Figure 16 shows the change in percentage of α-helicity with respect to the change in a salt concentration. The maximum α-helicity (24%) was obtained upon addition of liposome to LCAT without any addition of salt. The α-helicity started to decrease upon increasing the salt concentration. However, the analysis of how much the decrease in α-helicity
Figure 14. Circular dichroism (C.D.) spectra of LCAT in the far ultraviolet region at pH 7.1 (○) and at pH 12.0 (x). The C.D. spectrum was obtained using selected samples of LCAT following the recording of the ultraviolet spectrum.
Figure 15. The pH dependence of the molar ellipticity of LCAT at 222 nm. Conditions for this experiment were the same as described in Figure 14.
\[ \Delta [\theta] \times 10^{-2} \text{ deg cm}^2 \text{ dmol}^{-1} \text{ at 222nm} \]

Graph showing the relationship between pH and \( \Delta [\theta] \times 10^{-2} \text{ deg cm}^2 \text{ dmol}^{-1} \text{ at 222nm} \). The graph indicates a sharp increase in the value at pH 11.
Figure 16. Change in α-helicity of lecithin:cholesterol acyltransferase with respect to the change in the concentration of NaCl. Inset figure is the difference of α-helicity with addition of liposome and NaCl from α-helicity without liposome and NaCl.
contributed to the increase in β-pleated sheet or random structure was not done in this experiment.

Manual Microsequencing using DABITC/PTIC Double Coupling

In doing protein/peptide sequence analysis, sensitive and reliable techniques have been developed in the past decade using different approaches, such as, microtechniques of the spinning cup liquid-phase or solid-phase sequencing, or a number of other manual methods. However, many of those methods are excluded from a number of laboratories due to maintenance difficulty and high cost. Recently, H. S. Lu developed an economical, rapid sequencing method capable of analyzing 1 to 10 nanomoles of protein samples (1981, dissertation). This manual method, shown in Figure 9, utilized the PITC coupling step but shortened the extraction manipulation after coupling. The resulting thiazolinone derivatives derived from each cycle were reconverted to free amino acids by hydrolyzing the derivatives with 6N HCl containing mercaptoethanol-phenol. The amino acids were subsequently analyzed by a high-sensitivity amino acid analyzer. Thus, the amino terminal sequence of human plasma LCAT was analyzed with this technique.

DABITC/PTIC double coupling microsequencing technique allowed 4 cycles of Edman degradation leading with phenylalanine as the amino terminal. The second amino terminal was identified as tryptophan. However, after the second cycle of
Edman degradation, the spot on the TLC sheet remained in the same position as the one on the second cycle. The amino acid analysis of third and fourth cycles of Edman degradation using back hydrolysis of thiazoline derivatives showed two significant peaks of glycine and leucine on both cycles. Therefore, the four amino acid residues in the amino terminal region are likely to be the following: Phe-Trp-\(^2\text{Leu}\)\(^2\text{Gly}\)\(^2\text{Leu}\). Further microsequencing was not attempted due to an unidentified spot of the fifth cycle of Edman degradation on TLC sheet.

Isolation of \[^3\text{H}]\text{Diisopropylphosphoryl-LCAT}\n
Figure 17 shows the isolation scheme and the result of the isolation steps. Figure 18 shows the elution profile of \[^3\text{H}]\text{DIP-LCAT}\) by Sephadex G-25 column chromatography. Ten nanomoles of \[^3\text{H}]\text{DIP-LCAT}\) were recovered from gel filtration column chromatography based on absorbance assuming a molar extinction coefficient of 21. On the other hand, 36 nanomoles of \[^3\text{H}]\text{DIP-LCAT}\) were recovered from the same step based on radioactivity count. The discrepancy of the recovery between absorbance and radioactivity count is likely due to a non-specific binding of \(^3\text{H}\)DFP to LCAT. However, an extensive wash and an alkylation of \[^3\text{H}]\text{DIP-LCAT}\) by iodoacetate helped to remove nonspecifically bound \[^3\text{H}]\text{DFP}\. In fact, 11 nanomoles of carboxymethylated \[^3\text{H}]\text{DIP-LCAT}\) was recovered from the alkylation step based on radioactivity count. Therefore, approximately one mole of \[^3\text{H}]\text{DFP}\) appeared to be incorporated
Figure 17. Isolation scheme of $[^3\text{H}]$DIP-LCAT.
LCAT in (NH₄)₂SO₄ suspension

Dialysis against 5 mM phosphate, pH 7.3

A₂₈₀ = 0.307, volume = 4.3 ml, E₁%₁cm = 21

LCAT = 0.6286 mg = 630 μg

*Urea gel electrophoresis (7.5%, 10 M urea) showed LCAT band and very faint apo A-I band.

*HPLC analysis (TSK-250 gel filtration column, 300 mm x 7.5 mm) showed huge LCAT peak and two very tiny peaks.

4.1 ml LCAT + 17.8 μmole (20 μl) [³H]DFP

1.65 x 10⁷ dpm

Incubation for 20 min.

Sephadex G-25 Column Chromatography

[³H]DIP-LCAT

Fraction pool 45-65
A₂₈₀ = 0.04, volume = 23 ml

LCAT = 9.7 nmole (based on absorbance)
   = 36.2 nmole (based on radioactivity)

Dialysis against DI H₂O twice

LCAT = 16.8 nmole (based on radioactivity)

Lyophilization

Dry Sample

Carboxymethylation of [³H]DIP-LCAT

Dialysis against 3% of acetic acid three times

CM-[³H]DIP-LCAT = 10.8 nmole (based on radioactivity)
Figure 18. Elution profile of \[^3\text{H}\]DIP-LCAT from Sephadex G-25 column chromatography.
Sephadex G-25 Column Chromatography

Eluent: 0.1 M sodium phosphate
-0.1 M NaCl, pH 7.2
Fraction size: 1 ml
Flow rate: 16 ml per hour
Column size: 1.8 x 81.5 cm

A<sub>280</sub> CPM

DIP-LCAT

DFP peak

Fraction Number
into one mole of LCAT. Thus, this indicates that LCAT has an essential serine residue at the active site.

Then, the carboxymethylated $[^3H]$DIP-LCAT was digested by TPCK-treated trypsin. However, HPLC analysis failed to detect a tryptic digested peptide peak. Several possible reasons might account for the failure, such as incomplete reduction of disulfide linkage followed by alkylation of sulfhydryl group or in the incompletion of tryptic digestion. However, the definite reason remains undetermined.
Physico-chemical Properties of Human Plasma  
Lecithin:Cholesterol Acyltransferase

In this study, several physico-chemical properties of human plasma LCAT are reported. Table VIII shows the summary of some physico-chemical properties of the enzyme in this investigation. Table IX shows the comparison of amino acids by different investigators. The amino acid composition analyses shown here (Table IX) in principle showed a good agreement with that presented by K. S. Chong (1981, dissertation). However, this study revealed a relatively higher content of glutamic acid and glycine, but lower content of leucine than that reported by other investigators. This discrepancy could be due to the different methods employed. The discrepancy might have been caused by the addition of 0.1% (w/v) phenol to improve the recovery of tyrosine residues and by no addition of internal standard, norleucine, to avoid a peak overlap with leucine. The amino acid composition analyses also showed that there were 19 residues of tyrosine. This value agreed well with the value obtained from the spectrophotometric titration study.

The spectrophotometric titration of phenolic groups in the enzyme showed a large increase in absorbance at 295 nm with an apparent pK of about 12.0. In a control experiment, using N-acetyltyrosinamide, the apparent pK for the increase
<table>
<thead>
<tr>
<th>Properties</th>
<th>Observation</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four Most Amino Acid Residues</td>
<td>1 Glycine, 2 Glutamate, 3 Leucine, 4 Aspartate</td>
<td>Amino Acid Composition Analysis</td>
</tr>
<tr>
<td>Number of Tyrosine Residues</td>
<td>19</td>
<td>Amino Acid Composition Analysis</td>
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<tr>
<td></td>
<td>18</td>
<td>Spectrophotometric Titration</td>
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<tr>
<td>Apparent pK for Phenolic Groups</td>
<td>~12.0</td>
<td>Spectrophotometric Titration</td>
</tr>
<tr>
<td>Secondary Structure (pH 7.1)</td>
<td>20% α-helix, 48% β-pleated, 32% Remainder</td>
<td>Far UV Circular Dichroism</td>
</tr>
<tr>
<td>Δ Molar Ellipticity at 222nm from pH 7.1 to pH 12.0</td>
<td>$1.2 \times 10^3$ deg·cm$^2$·dmol$^{-1}$</td>
<td>Far UV Circular Dichroism</td>
</tr>
<tr>
<td>Salt Effect on α-helicity of LCAT with Liposome</td>
<td>↓ α-helicity upon ↑ salt</td>
<td>Far UV Circular Dichroism between 0.1M and 1M salt</td>
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<td>$E_{1%}^{1\text{cm}}$ (280 nm)</td>
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<td>Protein Determination Assay</td>
</tr>
<tr>
<td>Molar DFP Incororation into 1 mole of LCAT</td>
<td>1:1</td>
<td>Sephadex G-25 Column Chromatography and Alkylation of DIP-LCAT by Iodoacetate</td>
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<td>-------------------------------------------</td>
<td>-----</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>N-terminal Sequence</td>
<td>Phe-Trp-(Leu&lt;sub&gt;Gly&lt;/sub&gt;)-(Leu&lt;sub&gt;Gly&lt;/sub&gt;)</td>
<td>Manual Microsequencing</td>
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Table VIII--Continued
TABLE IX

COMPARISON OF AMINO ACID COMPOSITION ANALYSES
OF HUMAN LCAT (RESIDUES/45,000 RELATIVE M.W.)

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<td>⅓ Cys</td>
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<td>4</td>
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<tr>
<td>Trp</td>
<td>7</td>
<td>--</td>
<td>--</td>
<td>10</td>
<td>N.D.</td>
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in absorbance was about 9.8. These data indicate that the ionization of phenolic side chains in the enzyme is delayed, perhaps due to a conformation change that allowed the exposure of the tyrosine residues to the solvent. Similarly, the largest change in molar ellipticity (at 222 nm) was observed above pH 11 supporting the view that the enzyme molecule begins to undergo a conformational transition at this pH. The state of tyrosine residues (which are nearly totally buried at neutral pH) may be contrasted to the somewhat unusually exposed state (60%) of tryptophan residues in LCAT reported by Chong et al. (1983b).

The analysis of the CD spectrum of human LCAT revealed that the enzyme has approximately 20% α-helix, 48% β-pleated sheet, and 32% remaining structure. Recently, Doi and Nishida (1983) have reported similar findings using the method of Provencher and Glöckner (1981). Provencher and Glöckner claimed that the correlation coefficients between the fractions of α-helix, β-pleated sheet, β-turn and remainder, and the corresponding fractions obtained from x-ray analyses were 0.96, 0.94, 0.31, and 0.49, respectively. Therefore, although the method of Chen et al. (1972) originally gave a β-pleated sheet content of 27% reported by Doi and Nishida (1981), the value of 48% in β-pleated sheet seems to provide a better approximation, indicating that human LCAT has a relatively high content of β-pleated sheet structure.
Yamazaki et al. (1983) reported the affinity of LCAT for HDL in media of varying ionic strength. The above authors studied the interaction of the enzyme with lipoproteins by gel permeation chromatography on Sephadex G-150 or Sepharose CL 4B. These authors claimed that the affinity of HDL for LCAT was substantially enhanced upon increasing the ionic strength to 0.5 and further argued that the binding of the enzyme to HDL at higher ionic strength medium must have resulted from the change in the enzyme conformation. This study showed that the α-helicity slightly increased upon addition of liposome to LCAT with no addition of salt. It remains unclear whether or not this degree of change in α-helicity is physiologically significant. This study further showed that the α-helicity started to decrease upon increasing the salt concentration. It might be interesting to determine if the decrease in α-helicity upon increasing salt concentration enhances the binding of LCAT to liposome. However, since the liposome made for this study did not contain apo A-I, the change in α-helicity may not fully explain the enhancement of the affinity of enzyme for liposome or HDL. Nonetheless, a liposome system with and without apo A-I can be a good model to investigate the binding affinity of enzyme to lipoproteins.

LCAT contains a relatively high amount of carbohydrate (24%) as reported by Chung et al. (1979) and Chong et al. (1983b). Therefore, it might be important to consider this factor for the determination of a protein concentration.
Since fetuin is a glycoprotein both LCAT and fetuin might form a similar dye complex with the dye reagent. Accordingly, fetuin appears to be a better standard for LCAT concentration assay than BSA. Chong et al. (1983b) reported that approximately 60% of total tryptophan residues may be readily accessible to the solvent in 10 mM phosphate at pH 7.4 from the fluorescence spectroscopic study. The above authors also reported a relatively high $E_{1\text{cm}}^{1%}$ value of 21 for LCAT at 280 nm in 1 mM phosphate buffer (pH 7.2). The current study also revealed a relatively high $E_{1\text{cm}}^{1%}$ value of 17 at 280 nm in 5 mM phosphate buffer (pH 7.2). The discrepancy in $E_{1\text{cm}}^{1%}$ might be due to the differences in the respective protein determinations used.

Glomset reported that LCAT activity was sensitive to diisopropylfluorophosphate (DFP) in 1968. Our laboratory showed that DFP inhibited a highly purified LCAT preparation (Chong et al., 1983). Similarly, $10^{-3}$ M DFP was reported to inhibit LCAT activity in human plasma by Nakagawa et al. (1977). These authors suggested that the inhibition of LCAT by DFP was likely to be due to phosphorylation of the enzyme or the cofactor apoprotein (apo A-I). However, Chong et al. (1983b) indicated that the inhibition of LCAT is more likely to be due to phosphorylation of the enzyme itself, rather than that of apo A-I. Further, the current study showed that one mole of DFP appeared to be incorporated into one mole of LCAT. Therefore, the inhibition of LCAT by DFP seems to involve an essential serine residue at the active site of the enzyme.
Doi and Nishida (1981) reported eight amino acid residues in the amino terminal region, i.e., Phe-Trp-Leu-Leu-X-Val-Leu-Phe-Pro. The amino terminal region of LCAT revealed an unusually strong hydrophobic character. The above authors proposed that this hydrophobic sequence of the amino terminal region may participate in the binding of the hydrophobic substrate, although currently there is no further evidence available to support this hypothesis. The current study was aimed to identify amino acid residues in the amino terminal region using sensitive manual microsequencing technique with DABITC/PITC double coupling. This study showed findings similar to that reported by Doi and Nishida (1981). However, Park and Lacko (unpublished data) observed that the amino terminal residue in LCAT may be blocked. Thus, further studies are required to clarify whether this discrepancy was due to the different methods applied or the microheterogeneity of LCAT itself. These questions may be resolved by a detailed study of the amino acid sequence or if the DNA sequence for LCAT biosynthesis is determined from a gene library.

These studies are important for at least two reasons. First, they provide significant basic structural-function information on the enzyme which may lead to a better understanding of catalysis in general, and acyl-transfer reactions in particular. Furthermore, they may allow a better understanding of lipoprotein metabolism. Finally, the approach used in these studies, the methods developed here and the
experimental design can be applied to the study of other human enzymes available in very limited quantities.

Further studies, such as the role of tryptophan, serine, and carbohydrate moiety in the function of LCAT, the interaction of LCAT with lipoproteins, and the composition and sequence of the active site region will be necessary to further extend the knowledge of the mechanism of action of the enzyme.
CHAPTER IV

RESULTS

Coupled Spectrophotometric Enzyme Assay

After a trial in which the concentration of substrates and individual coupling enzymes were examined based on the analysis of its optimization and cost, the final optimized conditions described under "Methods" section were developed. The initial rate of absorbance change was directly proportional to the amount of enzyme added (Fig. 19 - 23).

The linearity of the rate of the reaction for acyl CoA synthetase was established up to 5 µl of the enzyme. This assay system constantly recovered 60 to 80 per cent of the activity which was specified on the catalogue by a commercial company. Then, an aliquot amount of the enzyme within the accurate range of its activity was used to determine the kinetic parameters for the enzyme. Apparent K_m values for MgATP, CoA, and several fatty acids were determined as described in "Methods" section (Table X). Palmitic, oleic, and linoleic acids were activated with almost the same reaction rate. In addition, fatty acid stock solution dispersed by sodium cholate was found stable for a day so that it must be prepared freshly before an assay. The inclusion of fatty acid solution in the preincubated mixture was also found to cause a turbidity to the solution. Therefore, the fatty acid solution was added
Figure 19. Determination of $\alpha$-glycerol-3-phosphate dehydrogenase activity.
FDP 0.4 mM
NADH 0.2 mM
Aldolase sat'd
TPi sat'd
Buffer 10 mM Pipes, pH 7.4

Velocity (µmol/min)

Glycerol-3-phosphate dehydrogenase
Figure 20. Determination of triosephosphate isomerase.
GAP 5 mM
NADH 0.2 mM
α-GDH sat'd (~1 U/ml)
Figure 21. Determination of aldolase activity.
FDP 0.5 mM
NADH 0.2 mM
TPI ~2 U/ml
α-GDH ~1 U/ml

 Velocity (μmol/min) vs Aldolase
Figure 22. Determination of pyrophosphate-dependent phosphofructokinase activity.
F-6P 20 mM
Mg 1 mM
PPi 0.2 mM
NADH 0.3 mM
Aldolase ~1 U/ml
TPI 25 U/ml (5 sec lag)
α-GDH 10 U/ml

Pyrophosphate-dependent Phosphofructokinase

Velocity (μmol/min)
Figure 23. Determination of acyl coenzyme A synthetase activity.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>V (mM/min)</th>
<th>Km (mM)</th>
<th>V/Km (S⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>0.980 ± 0.066</td>
<td>0.0292 ± 0.0092</td>
<td>0.560 ± 0.145</td>
</tr>
<tr>
<td>CoA</td>
<td>0.923 ± 0.082</td>
<td>0.155 ± 0.032</td>
<td>0.0995 ± 0.013</td>
</tr>
<tr>
<td>Stearate</td>
<td>0.877 ± 0.020</td>
<td>0.00615 ± 0.00077</td>
<td>2.374 ± 0.255</td>
</tr>
<tr>
<td>Palmitate</td>
<td>1.225 ± 0.065</td>
<td>0.0134 ± 0.0208</td>
<td>1.520 ± 0.161</td>
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<tr>
<td>Oleate</td>
<td>1.243 ± 0.075</td>
<td>0.0147 ± 0.0025</td>
<td>1.410 ± 0.154</td>
</tr>
<tr>
<td>Linoleate</td>
<td>1.219 ± 0.084</td>
<td>0.0116 ± 0.0018</td>
<td>1.753 ± 0.161</td>
</tr>
</tbody>
</table>
just before the initiation of the reaction to avoid a noise response from the spectrophotometer.

The initial rate of absorbance change for GCAT was proportional to the amount of the enzyme (Fig. 24), although the rate of the reaction became limited with increasing amounts of the enzyme. This could be depletion of one or more coupling reactions or substrates. The specific activity of GCAT was calculated as 5-6 U/mg in this assay system. This value agreed well with the cholesteryl esterification rate of GCAT measured by Dr. J. T. Buckley (1984, personal communication). More Acyl CoA synthetase was found to be required than expected to complete the reaction. When the amount of acyl CoA synthetase calculated theoretically based on 5 min. lag was added for GCAT assay, the initial rate of absorbance change was found almost identical to that of the background (data not shown here). The amount of enzyme described in "Methods" section for GCAT assay not only shortened the lag time, but appeared to be a minimum threshold to couple the reaction to completion. This may be due to the release of different fatty acids into the assay medium below a critical micelle concentration (CMC) from C:2 position of egg yolk lecithin upon the activation of GCAT.

Further, the coupled enzyme assay for LCAT by UV absorption detection method was carried out in the same manner as GCAT assay except that egg PC was emulsified with apo A-I. However, because of the lack of a sensitivity by that method,
Figure 24. Determination of glycerophospholipid: cholesterol acyltransferase activity.
Egg Lecithin  0.04 mg
CoA  0.8 mM
MgCl₂  10 mM
ATP  5 mM
F-6P  3 mM
NADH  0.3 mM
ACS  0.1 U/ml
PP₇-PFK  1 U/ml
Aldolase  ~5 U/ml
TPI  30 U/ml
α-GDH  ~7 U/ml (1.4 min lag)
the initial rate of absorbance change was independent of the amount of LCAT (data not shown here). The initial rate of absorbance change was also same as that of background rate. Then the fluorescence detection method was tested for a coupled assay of LCAT. Since NADH has a maximum emission peak around 460 nm region when excited at 340 nm, the initial rate of the reaction can be obtained by looking at the change in the relative fluorescence intensity. The coupled assay for LCAT by fluorescence method gave a significant, though small, change in a relative fluorescence intensity at the initial stage of the coupled reaction (Fig. 25). There was no significant change in relative fluorescence intensity for a background rate. However, whether or not this change in a relative fluorescence intensity reflected the enzyme activity of interest was not established since the quantitative analysis showed a relatively fast rate of decrease in the relative fluorescence intensity.

The cost analysis of LCAT assay by a conventional TLC method was found to be about 40 cents per assay. The cost of coupled enzyme assay can be comparable to the cost by TLC if the amount of ACS used for the assay can be reduced. Moreover, the coupled enzyme assay has an advantage with respect to the time required for an assay and/or a continuous monitoring of a colored substance at a specific wavelength.
Figure 25. Fluorescence spectra of coupled enzyme assay for lecithin:cholesterol acyltransferase (LCAT). Assay contains 0.1 U ACS; 0.8 U PPi-PPK; 2 U aldolase; 20 U TPI; 7 U α-GDH; 20 μl micelle solution; 1.5 mM CoA; 1 mM MgATP; 0.25 mM NADH with addition of:

a. 50 μl (13 μg) of LCAT
b. 100 μl (26 μg) of LCAT
c. 200 μl (52 μg) of LCAT

Background spectrum is shown on 'd'.
DISCUSSION

Coupled Spectrophotometric Enzyme Assay

In this study, a simpler and quicker assay for human plasma LCAT is reported. In order to facilitate a continuous monitor of the enzyme reactions which do not include a colored reactant, a product of this reaction can be coupled to one or more enzyme reactions which produce or utilize a colored reactant. Human plasma LCAT catalyzes the transfer of fatty acid from the C:2 position of phosphatidylcholine to either unesterified cholesterol or water. The rate of transfer to water with pure lecithin liposome is comparable to that of cholesterol ester synthesis in the presence of liposome containing lecithin and cholesterol (Aron et al., 1978). The proposed assay takes advantage of phospholipase A₂ activity of LCAT.

The activity of individual coupling enzymes was accurately determined. This study showed that the respective velocity of α-GDH, TPI, aldolase and PP₄-PFK was directly proportional to the amount of each enzyme whose activity was to be determined. When the activity of ACS was determined, the initial rate of absorbance change was proportional up to 5 µl of the enzyme. However, this study revealed that the rate of the reaction for ACS became limiting at 10 µl of the enzyme. This limitation may be due to the depletion of one or more coupling enzymes, or the depletion of substrates. If the former case is true, the occurrence of the hyperbolic
limitation can be ascribed to the fact that the rates of the coupling enzymes are always lower than the rate of the enzyme of an interest, because neither the length of the measuring time nor the concentrations of the coupling enzymes are infinite. This factor will be more important and critical especially when more and more coupling enzymes are added in an assay system. This situation can also be applied to the assay for GCAT and/or LCAT. On the other hand, if the latter case is true, the depletion of palmitic acids is more likely to cause a hyperbolic limitation since the concentration of palmitic acids may not be quite reaching the zero-order region of a normal Michaelis-Menten curve. This might reflect slightly lower $V_{\text{max}}$ values of MgATP and CoA than that of palmitic acid.

Apparent $K_m$ values for palmitic, stearic, and oleic acids determined from the same enzyme source as utilized in this study have been reported by Shimizu et al. (1979). This study showed a similar agreement to that reported by the above authors, although there is a slight difference between the two results. The difference may be explained by the fact that these authors dispersed fatty acids by Triton X-100. On the other hand, sodium cholate was used to disperse fatty acids in this study. This study showed that stearic acid has a relatively low $K_m$ value among fatty acids, whereas palmitic, oleic and linoleic acids have similar $K_m$ values. This study also revealed that ACS has the lowest affinity to CoA among substrates, indicating that CoA is loosely bound to the
enzyme. However, the determination of $K_m$ values was not a goal of this study. Therefore, the determination of kinetic parameters for ACS may have to be done more rigorously.

Buckley et al. (1982) reported that GCAT appeared unique among bacterial phospholipases, and that it shared a number of the characteristic features of mammalian LCAT. Moreover, GCAT exhibited 2-position specificity, both as an acyltransferase and as a phospholipase. This study showed that an initial velocity of GCAT was proportional to the amount of the enzyme, although there became a slight limitation with increasing amounts of the enzyme. This study also showed that there was a background rate for GCAT assay. This can be simply due to a noise response from the spectrophotometer or to the decomposition of egg lecithin, followed by a release of fatty acids which will be activated by ACS. Nonetheless, this study established the coupled spectrophotometric enzyme assay for GCAT. Further, this assay system gave a similar value of the specific activity to the rate of cholesterol esterification measured by Dr. J. T. Buckley (personal communication).

However, this study also revealed that more ACS was required than theoretically expected and resulted in an increased expenditure. This may be due to the low concentration of egg lecithin. Holwerda et al. (1936) recognized that one of the most characteristic and intriguing features of lipolytic enzymes are their activation by interfaces. Further,
Pieterson et al. (1974) have shown that the porcine pancreatic phospholipase A\textsubscript{2} is strongly activated by substrate aggregation. The above authors observed that below the critical micelle concentration (CMC), where only monomeric lecithin molecules are present, phospholipase A\textsubscript{2} activity remains very low and seems to follow normal Michaelis-Menten kinetics. When the monomers aggregate above the CMC, there occurs a strong increase in lipolytic activity indicating that the micellar aggregates are a much better substrate for the enzyme than the molecules dispersed in water. Therefore, the above observation might suggest that the low concentration of egg lecithin - sodium cholate micelles upon the activation by GCAT may release low concentrations of fatty acids which may be dispersed in an aqueous buffer as the monomeric state. Then each fatty-acid substrate could have a variable affinity and/or susceptibility for the enzyme at the interface. Thus, in order to observe the absorbance change at 340 nm, more ACS has to be added to increase pseudo-first-order rate, and, at the same time, the lag time will be shortened for an increased V/K.

Finally, the coupled enzyme assay for LCAT was carried out using a fluorescence spectrophotometer. Fluorescence spectroscopy has a great advantage over absorption spectroscopy with respect to its sensitivity. The fluorescence assays, in which fluorescent substances are converted into non-fluorescent substances, or vice-versa, exist for numerous hydrolytic
enzymes, oxidative enzymes, transaminases, dehydrogenases, isomerases, kinases, and decarboxylases. Since NADH and NADPH have a strong fluorescence (ex. 340 nm, em. 460 nm), these pyridine nucleotides were utilized to determine the activity of a large number of dehydrogenases. When LCAT was assayed by absorption spectroscopy, the oxidation rate of NADH was the same as that of the background indicating that absorption spectroscopy may not be sensitive enough for LCAT assay by coupled enzyme assay method. On the other hand, the fluorescence method detected the change in the initial rate of LCAT reaction with different amounts of the enzyme. Therefore, this study indicates that fluorescence spectroscopy is currently the method of choice for assay of LCAT.

This study also revealed that the cost of the coupled assay may not be a consideration if an assay system was developed that reduces the amount of ACS needed. Moreover, if ACS can be purified in the laboratory, since about 0.1 unit of ACS was found to be required, this cost could be further reduced. Further, the coupled enzyme assay for LCAT can be more simplified with the following scheme if each of coupling enzymes were commercially available.
Lecithin (+ Cholesterol) $\xrightarrow{\text{LCAT}}$ FFA (CE) + Lysolecithin

Lysolecithinase

Glycerol-3-phosphate $\xleftarrow{\text{diiesterase}}$ Glycerophosphorylcholine

NAD$^+$ $\xrightarrow{\alpha$-GDH} NADH

$\xleftarrow{\text{DHAP}}$

Future studies such as kinetic parameters LCAT and competition experiment of phospholipase activity vs. transferase activity will be necessary to extend the knowledge of enzymic action of LCAT. This study also indicated that the coupled enzyme assay system can be developed not only for LCAT and GCAT but for any enzyme that has a hydrolytic activity to release free fatty acids.
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BIBLIOGRAPHY


