STUDIES OF THE MECHANISM OF PLASMA
CHOLESTEROL ESTERIFICATION IN AGED RATS

DISSERTATION

Presented to the Graduate Council of the
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For the Degree of

DOCTOR OF PHILOSOPHY

By

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The study was performed to determine factors influencing the esterification of plasma cholesterol in young and aged rats. The distribution of LCAT activity was determined following gel filtration chromatography and ultracentrifugation of whole plasma respectively. When rat plasma was fractionated on a Bio-Gel A-5 M column, LCAT activity was found to be associated with the HDL fraction. A similar result was observed upon 24 hr density gradient ultracentrifugation of the plasma. However, following prolonged 40 hr preparative ultracentrifugation, the majority of the LCAT activity was displaced into the lipoprotein-free infranatant fraction (d>1.225 g/ml). The dissociation of LCAT from the HDL fraction occurred to a smaller extent in aged rat plasma than in young rat plasma. Plasma incubation (37°C) experiments followed by the isolation of lipoproteins and the subsequent analysis of their cholesterol content revealed that in vitro net esterification of free cholesterol (FC) by LCAT as well as the fractional utilization of HDL-FC as substrate were lower in the plasma of the aged animal as compared to that of the young animal despite the fact that the total pool of FC was higher in the former. The net
transfer of FC from lower density lipoproteins (d<1.07 g/ml) to HDL provided the FC (in addition to HDL-FC) for esterification in the plasma of both young and aged rats, and this process was not substantially affected by aging. Substrate specificity studies indicated that HDL from young rats was a better substrate for LCAT than the HDL from aged rats. The HDL isolated from the plasma of aged rats was enriched with apo E and had a considerably higher molecular weight than the HDL from young rat plasma. The ratio of phosphatidylcholine/sphingomyelin was lower in the HDL of aged rats. These data suggest that the decreased plasma cholesterol esterification in aged rats is due to changes in the composition and size of the lipoprotein substrate (HDL).
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CHAPTER I

INTRODUCTION

Role of Plasma Lipoproteins in Cholesterol Transport

The major classes of plasma lipids—cholesterol, triglycerides, cholesteryl esters, and phospholipids—are very poorly soluble in water; consequently, these lipids are complexed with one or more specific proteins (apolipoproteins) to form transportable water-soluble macromolecules (pseudomicellar particles) referred to as plasma lipoproteins. Lipoproteins fulfill essential physiological roles by supplying peripheral tissues with lipids for the purpose of energy generation and biogenesis of hormones and membranes. In addition, lipoproteins are instrumental in maintaining the appropriate steady state cholesterol levels of nearly all tissues by removing excess quantities to the liver for elimination by way of bile acid metabolism and fecal excretion. Lipoproteins are spherical particles which contain free (unesterified) cholesterol, protein (apolipoproteins), phospholipids as surface components, and triglycerides and cholesteryl esters as core constituents. Lipoproteins can be separated into distinct classes based on their respective buoyant densities (Fredrickson, 1966). They may be further classified on the basis of
<table>
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<tr>
<th>Electrophoretic definition</th>
<th>particle size (nm)</th>
<th>molecular weight ($\times 10^3$)</th>
<th>density (g/ml)</th>
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<tr>
<td>Chylomicron</td>
<td>Remains at origin $^b$</td>
<td>75-1200</td>
<td>~400,000</td>
</tr>
<tr>
<td>VLDL</td>
<td>Pre-beta</td>
<td>30-80</td>
<td>10-80,000</td>
</tr>
<tr>
<td>IDL</td>
<td>Slow pre-beta $^c$</td>
<td>25-35</td>
<td>5-10,000</td>
</tr>
<tr>
<td>LDL</td>
<td>Beta</td>
<td>18-25</td>
<td>2.300</td>
</tr>
<tr>
<td>HDL$_2$</td>
<td>Alpha</td>
<td>9-12</td>
<td>360</td>
</tr>
<tr>
<td>HDL$_3$</td>
<td>Alpha</td>
<td>5-9</td>
<td>175</td>
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$^b$On paper.

$^c$On geon pevikon or agarose.
particle size, electrophoretic mobility or affinity chromatography as shown in Table I. Human and rat plasma lipoproteins are generally divided into four major classes: high density lipoproteins (HDL); low density lipoproteins (LDL); very low density lipoproteins (VLDL); and chylomicrons. The major classes of lipoproteins in humans and rats and their basic properties are listed in Table II. Each specific lipoprotein class fulfills unique metabolic functions in the body.

Chylomicrons, the largest lipoproteins (>100 nm in diameter) are synthesized by the intestine to transport dietary lipids (triglyceride and cholesterol) from the site of absorption (intestinal epithelium) to the site of utilization (mainly adipose tissue and muscle cells). The triglycerides in the core of these particles are hydrolyzed by the action of lipoprotein lipase (LPL), which is attached to endothelial surfaces of the capillary vessels. The hydrolysis of chylomicron triglycerides by LPL produces lipoprotein particles relatively deficient in triglycerides, referred to as chylomicron remnants. The remnants are enriched in cholesterol and are rapidly and efficiently cleared from the plasma by specific receptors in the liver (Havel, 1982).

Very low density lipoproteins (VLDL, d<1.006 g/ml) are also large particles (30-90 nm in diameter) that are secreted by the liver to transport triglycerides and cholesterol to peripheral tissues. Within the plasma compartment, the triglycerides of VLDL are hydrolyzed to free fatty acids by lipoprotein lipase (and hepatic triglyceride lipase)
<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Density Range (g/ml)</th>
<th>TG (%)</th>
<th>CHOL (%)</th>
<th>PL (%)</th>
<th>Protein (%)</th>
<th>Apolipoproteins</th>
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<tr>
<td></td>
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<td>major</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>minor</td>
</tr>
<tr>
<td>Chylomicron</td>
<td>0.94</td>
<td>83</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>C-I,C-II,C-III</td>
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<tr>
<td>VLDL</td>
<td>0.93-1.006</td>
<td>50</td>
<td>22</td>
<td>18</td>
<td>9</td>
<td>B, C-I,C-II</td>
</tr>
<tr>
<td>LDL</td>
<td>1.006-1.063</td>
<td>11</td>
<td>46</td>
<td>22</td>
<td>21</td>
<td>B</td>
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<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>8</td>
<td>20</td>
<td>22</td>
<td>50</td>
<td>A-I, A-II</td>
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**RAT**

<table>
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<tr>
<th>Lipoprotein Class</th>
<th>Density Range (g/ml)</th>
<th>TG (%)</th>
<th>CHOL (%)</th>
<th>PL (%)</th>
<th>Protein (%)</th>
<th>Apolipoproteins</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>major</td>
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<td></td>
<td></td>
<td></td>
<td>minor</td>
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<tr>
<td>VLDL</td>
<td>0.93-1.006</td>
<td>66</td>
<td>11</td>
<td>13</td>
<td>10</td>
<td>B,C-I,C-II</td>
</tr>
<tr>
<td>LDL</td>
<td>1.006-1.063</td>
<td>15</td>
<td>40</td>
<td>23</td>
<td>22</td>
<td>B</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>3</td>
<td>32</td>
<td>25</td>
<td>40</td>
<td>A-I,C-I,C-II</td>
</tr>
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generating a series of smaller, cholesterol-enriched lipoproteins called intermediate density lipoproteins (IDL, d=1.006-1.019 g/ml) which are later converted to low density lipoproteins (LDL, d=1.019-1.063 g/ml).

The LDL represent the end product of VLDL catabolism (~20 nm particles) and are the major lipoproteins transporting cholesterol to peripheral tissues and the liver. The uptake of LDL by cells is accomplished via the LDL receptor pathway (Brown and Goldstein, 1976). In a large number of epidemiological studies, a strong positive correlation has been noted between levels of LDL cholesterol and coronary heart disease.

High density lipoproteins (HDL, d=1.063-1.21 g/ml) are a heterogeneous class of lipoproteins containing several subclasses. Their components arise from a number of tissues (mainly the liver and the intestine). The mature, circulating HDL are produced within the plasma compartment as a consequence of the lipolytic processing of chylomicrons and VLDL, yielding lamellar phospholipid/protein rich structures (Tall and Small, 1978). Much interest has been focused recently on HDL as there is a strong negative correlation between HDL levels and accelerated vascular disease in man, i.e., the higher the HDL levels, the lower the incidence of coronary artery disease (Miller and Miller, 1975; Steinberg, 1978; Heiss et al., 1980). It has been postulated that HDL are involved in a process of reverse cholesterol
transport. According to this hypothesis, HDL acquire cholesterol from extrahepatic tissues and facilitate their transport to the liver for excretion from the body (Glomset, 1968). Whether the protective effect of HDL against cardiovascular disease is accomplished via reverse cholesterol transport is not yet known.

**Apolipoproteins and Their Respective Roles in Lipoprotein Metabolism**

The polypeptide components of plasma lipoproteins (apolipoproteins) can be isolated as the residue when the lipids are extracted from lipoproteins with organic solvents or detergents. The ABC nomenclature system based on immunoreactivity, proposed by Alaupovic (1971), is now widely employed. In addition to their lipid-binding properties, apolipoproteins play specific roles in lipid transport and lipoprotein metabolism by controlling the activity of a number of enzymes and by mediating the uptake of the lipoproteins by specific lipoprotein (apolipoprotein) receptors. Apolipoproteins A through E are well understood and considered here including their distribution, molecular weights, polymorphism, origins, concentrations and function as summarized in Table III.

**Apolipoprotein A-I:** Apo A-I is the major protein component of HDL. Apo A-I functions as an activator (cofactor) for lecithin: cholesterol acyltransferase (LCAT), a plasma enzyme catalyzing the
### TABLE III

**HUMAN PLASMA APOLIPOPROTEINS**

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<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight (mg/dl)</th>
<th>Plasma Concentration (mg/dl)</th>
<th>Origin</th>
<th>Function</th>
</tr>
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<tr>
<td>A-I</td>
<td>28,300</td>
<td>100-150</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>A-IV</td>
<td>46,000</td>
<td>15</td>
<td>Intestine, liver</td>
<td>LCAT activator</td>
</tr>
<tr>
<td>B-100</td>
<td>~549,000</td>
<td>80-100</td>
<td>Liver</td>
<td>LDL receptor recognition</td>
</tr>
<tr>
<td>B-48</td>
<td>~264,000</td>
<td>&lt; 5</td>
<td>Intestine, liver</td>
<td>Lipoprotein biosynthesis/secretion</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>
</tr>
<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>3-7</td>
<td>Liver</td>
<td>Chylomicron remnant receptor recognition</td>
</tr>
</tbody>
</table>
conversion of cholesterol and phosphatidyl choline to cholesteryl esters and lysophosphatidylcholine (Glomset et al., 1962; Fielding et al., 1972). In vitro, there appears to be an absolute requirement for apo A-I for the expression of LCAT activity. However, it is not likely that this is also the case in vivo (Pritchard et al., 1988). The mechanism by which apo A-I activates LCAT is not yet understood, although it is likely that amphiphilic helical structures are involved. Synthetic peptides of apo A-I that encompass the amphiphilic repeats can serve as LCAT activators (Sparrow and Gotto, 1982), as can model amphiphilic peptides that have no sequence homology to apo A-I (Sparrow and Gotto, 1982; Kaiser and Kezdy, 1984). Furthermore, certain of the human apo A-I variants are deficient in their ability to activate LCAT in vitro (Rall et al., 1984), and in these cases the primary structural abnormality can be predicted to perturb the structure of the amphiphilic region (Utermann et al., 1984; Rall et al., 1984). Proapolipoprotein A-I is as efficient as apo A-I in activating LCAT in vitro (Rall et al., 1984), and it also binds well to lipid. Apo A-I is synthesized in the liver and in the intestine (Windmueller et al., 1973). The intestinally derived apo A-I enters the circulation associated with chylomicrons and it is rapidly transferred to HDL during the lipase hydrolysis of chylomicrons. Hepatic apo A-I enters the circulation probably associated with nascent HDL particles having little or no core of cholesteryl ester. 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 their total protein and 15 per-cent of HDL mass. However, several animal species do not have a significant amount of apo A-II associated with their HDL (Chapman, 1980). In humans, apo A-II 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. The physical and chemical properties of apo A-II are well characterized (Morrisett et al., 1975). Apo A-II is known to be able to completely displace apo A-I from HDL (Lagocki and Scanu, 1980), but it is not known whether this effect has any physiological significance.

**Apolipoprotein A-IV**: Human apo A-IV is a prominent component of newly secreted chylomicrons but is not found in significant amounts associated with chylomicon remnants, VLDL, or LDL. Although it is only a minor component of human HDL, it is a major apolipoprotein constituent of rat HDL (Swaney et al., 1977). The significance of this difference is not yet clear. Unlike most other apolipoproteins, the majority of apo A-IV is found in plasma in the lipoprotein-free rather than lipoprotein-bound fraction and redistributes readily between these two fractions (Fidge, 1980). Apo A-IV is synthesized mostly by
the liver and the intestine. Under certain conditions (transfer of saturated fatty acyl groups) apo A-IV has been shown to be a potent activator of LCAT in vitro (Steinmetz and Uterman, 1983). Furthermore, when LCAT is active, rat apo A-IV preferentially associates with lipoproteins. Inactivity of LCAT results in apo A-IV redistributing to the lipoprotein-free fraction (DeLamatre et al., 1983).

**Apolipoprotein B:** Apo B is a primary apolipoprotein 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 incomplete. However, evidence for heterogeneity of human apo B has been provided. Apo B exists primarily in two forms; apo B-100 and apo B-48. Apolipoprotein B-100 containing 4,563 amino acids is synthesized by the liver and is an obligatory constituent of VLDL and LDL. In the blood stream, VLDL is acted on by lipoprotein lipase, which removes the triglycerides. This reaction converts the VLDL to LDL, which is cleared from the circulation when the carboxy-terminal domain of apo B-100 binds to LDL receptors. Therefore, apo B-100 is the protein determinant of LDL that recognizes the apo B,E receptors. This recognition of the receptor is the first step in the receptor-mediated catabolism of LDL (Brown and Goldstein, 1976; Goldstein and Brown, 1977). In man, apo B-48 is synthesized by the intestine and is
found in chylomicrons and chylomicron remnants (Kane, 1983). Metabolic, biosynthetic, and genetic evidence indicate that apo B-48 is a distinct protein that is closely related structurally to apo B-100 (Kane, 1983; Elovson et al., 1981). Apo B-48 contains only the amino terminal 2,152 amino acids of apo B-100 and is 48 per-cent as large as apo B-100 (Powell et al., 1987; Chen et al., 1987; Kane, 1983). Apo B-48, which lacks the carboxy-terminal domain, does not bind to LDL receptors. Accordingly, the catabolism of chylomicrons becomes dependent on another protein, apo E, and this allows these dietary particles to be diverted to a receptor that is different from the receptor that clears LDL.

The C apolipoproteins: The C apolipoproteins are represented by three low molecular weight apolipoproteins, designated as apo C-I, C-II, and C-III, that are surface components of chylomicrons, VLDL, and HDL. Although they appear to be diverse in their metabolic functions, they share the common property of redistributing among lipoprotein classes (Nestel and Fidge, 1982). In the fasting state, the C apolipoproteins are mainly associated with HDL. During absorption of dietary fat by the intestine with the production of chylomicrons or during the active synthesis of VLDL by the liver, the C apolipoproteins preferentially redistribute to the surface of the triglyceride-rich chylomicrons and VLDL. As the triglyceride cores of the VLDL and chylomicrons are hydrolyzed and depleted by the action of
lipoprotein lipase, excess surface components (phospholipid, unesterified cholesterol, and apolipoproteins) are generated, and the C apolipoproteins along with other excess surface components are transferred back to HDL. Thus, the C apolipoproteins are associated with the dynamic metabolic "remodeling" of plasma lipoproteins. It appears that the liver is the major site of synthesis of the apo C proteins, with the intestine contributing a minor portion (Wu and Windmueller, 1979; Krause et al., 1981).

1. Apolipoprotein C-I: Apo C-I comprises approximately 10 per-cent of the protein of VLDL and 2 per-cent of HDL. Apo C-I has been shown to activate LCAT in vitro (Soutar et al., 1975). The relative importance of apo A-I and apo C-I for the physiological activation of LCAT remains to be resolved.

2. 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. Its prime metabolic function appears to be associated with its ability to act as a cofactor in activating lipoprotein lipase, the enzyme catalyzing the hydrolysis of triglyceride in chylomicrons and VLDL (Havel et al., 1970; LaRosa et al., 1970). Patients with a familial apo C-II deficiency have severe hypertriglyceridemia and impaired plasma clearance of VLDL and chylomicrons, in spite of the presence of a functional lipase (Breckenridge et al., 1978). Apo C-II has also been reported to activate LCAT (Jonas et al., 1984).
3. **Apolipoprotein C-III**: Apo C-III is the most abundant of 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 occurs in plasma in three forms depending on the level of sialylation: C-III₀, C-III₁, and C-III₂. The subscript indicates the number of sialic acid residues that are present. The precise metabolic role of apo C-III and the significance of the sialic acid heterogeneity are unclear. It has been suggested that the presence of apo C-III may modulate the uptake of triglyceride-rich remnants by hepatic receptors (Shelburne *et al.*, 1980; Windler *et al.*, 1980). Apo C-III has also been suggested to inhibit apo C-II activation of lipoprotein lipase (Breckenridge *et al.*, 1978).

**Apolipoprotein D**: The apolipoprotein designated as apo D (McConathy and Alaupovic, 1973) or apo A-III (Kostner, 1974) comprises less than 5 per-cent of the HDL apolipoproteins. Apo D (apo A-III) was originally thought to stimulate LCAT (Kostner, 1974), and Olofsson and Gustafson (1974) 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. Not much is known about this apolipoprotein.

**Apolipoprotein E**: Apo E is a constituent of chylomicrons, chylomicron remnants, VLDL, and HDL with apo E (HDL₁, HDL₂). This apolipoprotein displays a complex isoform pattern that is due to the
presence of multiple, genetically determined alleles at a single locus and to the presence of post-translational sialylation (Zannis et al., 1981, 1982; Utermann et al., 1982). Six common phenotypes of apo E are revealed by isoelectric focusing: three homozygous (E4/4, E3/3, and E2/2) and three heterozygous (E4/3, E4/2, and E3/2) phenotypes. The most common phenotype in the human population is E3/3, which is present in approximately 60% of the subjects studied (Utermann et al., 1982; Havel, 1982; Zannis and Breslow, 1981; Menzel et al., 1983; Wardell et al., 1982; Ghiselli et al., 1982). Apo E appears to have numerous functions in cholesterol metabolism (Mahley et al., 1984). The major function of apo E is as a protein determinant for the receptor-mediated uptake of specific lipoproteins. It was observed that not only apo B-containing lipoproteins, e.g., LDL, but also apo E-containing lipoproteins, e.g., HDLc interacted with apo B, E (LDL) receptors (Mahley and Innerarity, 1983). In addition, a unique receptor that interacts with apo E-containing lipoproteins (but not normal LDL) has been described in the liver (Hui et al., 1981; Mahley et al., 1981). These apo E receptors may represent the chylomicron remnant receptors of the liver.

The E apolipoproteins are increased in concentration in the plasma of animals that have been fed high fat and high cholesterol diets. Under these conditions, apo E becomes a major protein constituent of the two cholesterol-rich lipoproteins, β-VLDL
(intestinal and hepatic remnants) and HDLc (cholesterol-rich HDL with apo E) (Mahley and Innerarity, 1983; Mahley, 1982; Mahley, 1979). Available data now suggest that the β-VLDL may be atherogenic lipoproteins by virtue of their ability to cause massive cholesteryl ester accumulation in macrophages (Mahley, 1981; Mahley, 1982; Mahley, 1983). On the other hand, HDLc may be formed in cholesterol-fed animals in response to the deposition of cholesterol in peripheral tissues and the need to transport the cholesterol from these tissues to the liver for excretion of the cholesterol from the body (Mahley and Innerarity, 1983; Mahley, 1981; Mahley, 1982; Mahley, 1983). The HDLc thus may be considered protective or anti-atherogenic lipoproteins that participate in redistribution of cholesterol among various cells and in the delivery, either directly or indirectly, of cholesterol to the liver. Apolipoprotein E is the determinant responsible for the cellular uptake of both the HDLc and β-VLDL (Mahley and Innerarity, 1983).

Apolipoprotein E-containing lipoproteins, specifically the HDL with apo E (HDL1, HDLc), play a major role in cholesterol transport in subjects with abetalipoproteinemia (Blum et al., 1982; Innerarity et al., 1984). These subjects, who lack apo B-containing lipoproteins (Malloy and Kane, 1982), possess significant quantities of the HDL with apo E, which are capable of delivering cholesterol to cells via the apo B, E (LDL) receptors (Innerarity et al., 1984). In addition, it is of
interest that HDL-containing apo E are a major class of lipoproteins in the plasma of human neonates (Innerarity et al., 1984). Both neonates and abetalipoproteinemic subjects may depend, to a different extent, upon lipoproteins containing apo E to deliver cholesterol to specific tissues.

Apolipoprotein E-containing lipoproteins bind to heparin (Mahley et al., 1979), as is also true for the apo B-containing lipoproteins. The same positively charged region of apo E that mediates interaction with the lipoprotein receptors has been shown to mediate binding to heparin (Mahley et al., 1984). The binding of these lipoproteins to heparin (or to other glycosaminoglycans) has been considered as an important physiological mechanism for lipoprotein binding to endothelial surfaces (in association with lipolytic processing) or to the ground substance of the arterial wall (in association with atherogenesis).

The requirement of apo E for the formation of large, cholesteryl ester-rich particles was suggested by Koo et al. (1985). In the presence of active LCAT, the small HDL (without apo E; ~10 nm) are increased in size by a parallel increase in the cholesteryl ester and apo E content. As the particle size increases, three distinct sizes of HDL with apo E are formed: small HDL₁ (~15 nm); large HDL₁ (~20 nm); and HDLₑ (~25 nm) (Gordon et al., 1983). Apo E is involved in the formation of these large, cholesteryl ester-rich particles, and it
apparently functions to stabilize the surface components or to allow for an increase in core size. It appears that large, cholesterol-rich HDL particles cannot be formed in the absence of apo E. The presence of apo E also targets these cholesterol-rich lipoproteins to cells with apo B, E (LDL) or apo E receptors (Mahley et al., 1984).

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 different types of lipoprotein molecules in circulation.

The Role of Lecithin: Cholesterol Acyltransferase in Reverse Cholesterol Transport

Nearly all tissues synthesize cholesterol. However, the liver is the only organ capable of catabolizing and excreting cholesterol in significant quantities. Hence, there must be a mechanism for removing excess cholesterol from peripheral tissues to the liver via the plasma. This process is generally referred to as reverse cholesterol transport, as originally proposed by Glomset (1968).

The flux of cholesterol through cells in culture has been studied extensively in the 1960's. Rothblat (1969) suggested the following scheme for cholesterol metabolism in tissue culture cell. Cholesterol enters cell from lipoproteins. However, the net cholesterol content of
these cells remains unchanged because of excretion of surplus free cholesterol from the cell. When an appropriate acceptor is present in the medium. It was suggested by Glomset (1968) and subsequently verified by Bates and Rothblat (1974) that HDL play a major role as an acceptor of cholesterol in the excretion process. The nature of the acceptor for free cholesterol from tissues in vivo is of considerable interest and several possible acceptors have been postulated. HDL have also been proposed to fulfill this role (Bates and Rothblat, 1974, 1975) and subsequent studies in cell culture by Stein et al. (1975, 1977) have so far supported this hypothesis. Recently, insights into the mechanism of cholesterol efflux have been reported using phospholipid vesicles as acceptors (Backer and Dawidowicz, 1981; Phillips et al., 1980; McLean and Phillips, 1981). In these studies, efflux was shown to depend on desorption of free cholesterol molecules into the aqueous phase and, through the unstirred water layers in cells (Phillips et al., 1980). Binding of the acceptor molecule to the cell membrane facilitates the latter process and enhances efflux. Binding of HDL to specific sites on cell membranes such as described by Oram et al. (1983) and others (Wu and Bailey, 1980) could provide an adequate explanation for the central role that HDL plays in cholesterol efflux and reverse cholesterol transport.

A key role for LCAT in reverse cholesterol transport was also suggested by Glomset (1968). LCAT, a mammalian plasma enzyme that
is synthesized in the liver and secreted into the plasma, generates most of the plasma cholesteryl esters by transferring fatty acid residues from phosphatidyl choline to cholesterol (Glomset et al., 1962) (Figure 1). Akanuma and Glomset (1968) reported that HDL provide the preferred substrate for LCAT. This finding prompted Glomset (1968) to suggest that HDL and LCAT function jointly in transporting cholesterol from the peripheral tissues to the liver. According to his postulate, LCAT plays a role in shifting the equilibrium of cholesterol by converting unesterified cholesterol to
cholesteryl esters (Figure 2). This mechanism has been modified to accommodate subsequent findings (Glomset, 1979) including: 1) in plasma, unesterified cholesterol in HDL is converted to esterified form by the action of LCAT; 2) newly formed cholesteryl esters are subsequently displaced toward the hydrophobic core of HDL particles; 3) a continuing esterification of free cholesterol by LCAT on the surface of the HDL molecule 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); and 5) in humans, cholesteryl esters generated by LCAT are
The coupling of efflux, esterification and transfer in maintaining the free-cholesterol potential gradient between cell membranes and plasma in the presence of lecithin : cholesterol acyltransferase (LCAT) activity.

FIGURE 3. The coupling of efflux, esterification and transfer in maintaining the free-cholesterol potential gradient between cell membranes and plasma in the presence of lecithin : cholesterol acyltransferase (LCAT) activity.


transferred from HDL to lower density lipoproteins (LDL and VLDL) by the action of cholesteryl ester transfer protein (CETP) (Figure 3). This non-enzymatic transfer (or exchange) process of cholesteryl esters (Nichols and Smith, 1965) allows the regeneration of the substrate lipoprotein (HDL) to accept more cholesterol from the surface of the cell membrane for the LCAT reaction.

The above considerations are based on substrate specificity studies
conducted in vitro (Fielding and Fielding, 1971; Marcel and Vezina, 1973; David et al., 1976) and studies of abnormal lipoproteins from patients with congenital (Forte et al., 1971; Norum et al., 1971; Glomset and Norum, 1973) or acquired (Sabesin et al., 1977) LCAT deficiency.

Since a considerable amount of LDL-cholesterol is probably taken up by peripheral cells each day (Brown and Goldstein, 1976), this amount of cholesterol plus that synthesized in extrahepatic tissues must find its way back to liver. One of the main functions of the proposed reverse cholesterol transport system is to prevent the accumulation of excess cholesterol in peripheral tissues. Because cholesterol exchanges freely between lipoproteins and cell membranes, its efficient removal from cells requires the establishment of a cholesterol gradient between peripheral tissues and the liver. Accordingly, the cholesterol in HDL has to be 'trapped' in the interior of the lipoprotein particle to be rendered unavailable for exchange (Figure 2). This entrapment of cholesterol is facilitated by the LCAT catalyzed esterification reaction. Therefore, the activity of LCAT in the blood controls the extent to which cholesterol is trapped in HDL, and also the rate at which cholesterol is subsequently removed from the plasma.

Fielding and Fielding (1981) have suggested that the enzyme LCAT, specific components of HDL and cholesteryl ester transfer protein
(CETP) function as a cholesteryl ester transfer complex that facilitates reverse cholesterol transport. The proposed role of this transfer complex in reverse cholesterol transport is shown in Figure 3.

**Relationship between Lecithin: Cholesterol Acyltransferase and High Density Lipoproteins**

While a great deal of information is available concerning the mechanism of the action of LCAT, the nature and composition of the substrates for LCAT *in vivo* have not yet been adequately described. Similarly, the respective roles of lipoproteins regarding the origin of lipid substrates and the fate of the product (CE) of the LCAT reaction are still not known.

Most of the available evidence suggests that HDL are the best substrates for LCAT *in vivo* (Akanuma and Glomset, 1968). HDL are formed in the liver and in the intestine, consisting of several subfractions isolated 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.*, 1972, 1974). In the studies of Gofman *et al.* (1954), HDL were separated into three subpopulations: HDL₁, HDL₂, HDL₃. HDL₂ and HDL₃ are the major HDL populations present in the plasma of most, if not all, animal species. Several studies strongly suggest that constituents originating from the surface coat of partially
degraded triglyceride-rich lipoproteins constitute the major, if not only, source of HDL precursors (Eisenberg et al., 1978 a; Tall and Small, 1978; Eisenberg et al., 1978 b). In these studies, HDL precursors are defined as apolipoprotein/phospholipid/free cholesterol complexes that are formed in vivo and can be transferred to spherical particles by the LCAT reaction. The hydrolytic action of lipoprotein lipase (LPL) on the triglyceride core of chylomicrons and VLDL leads to the accumulation and subsequent release of free cholesterol, phospholipid and apolipoprotein C from their polar surfaces. These components are then transferred to higher density fractions (Glangeaud et al., 1977; Eisenberg and Olivecrona, 1979; Chajek and Eisenberg, 1978).

Plasma HDL, especially HDL₃, may act as an acceptor for free cholesterol and phospholipid released from the surfaces of triglyceride-rich particles during their hydrolysis by lipoprotein lipase (LPL) (Patsch et al., 1978). The addition of lipid to HDL₃ facilitates their conversion to HDL₂ (Patsch et al., 1978; Schmitz et al., 1981).

Although most of the cholesterol that enters the plasma in nascent lipoproteins (Hamilton, 1972; Hamilton et al., 1976) is unesterified, more than two thirds of the circulating cholesterol is found to be esterified with long chain fatty acids (Marsh, 1976). As soon as nascent HDL molecules (discoidal HDL directly secreted from hepatic and intestinal cells) enter the circulation, they are rapidly acted upon
by LCAT, resulting in the formation of esterified cholesterol. Some of the cholesteryl esters move toward the space between the two layers of the discoidal particle, converting it into a spherical (HDL\textsubscript{3}) particle. The continued action of LCAT may further convert HDL\textsubscript{3} into HDL\textsubscript{2}. 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 (CETP) (Zilversmit \textit{et al.}, 1975; Chajek and Fielding, 1978). Therefore, HDL, LCAT and CETP comprise a system to esterify and transport the free cholesterol originally secreted in triglyceride-rich lipoproteins.

The biological significance of this process is revealed when the system fails. The classic example of the consequences of the absence of LCAT activity (disc to sphere transformation) is familial LCAT deficiency (Glomset and Norum, 1973). These patients' plasma contain abundant amounts of discoidal and vesicular structures (Forte \textit{et al.}, 1971; Glomset and Norum, 1973; Forte \textit{et al.}, 1974) and 'surface remnants', released during the lipolysis of VLDL and chylomicrons, especially after a fatty meal (Glomset \textit{et al.}, 1975). 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 has been recognized in patients with familial HDL deficiency, called Tangier disease. Cholesterol accumulation in many tissues, including the blood vessel walls, has been found in these patients (Fredrickson, 1966).

The action of LCAT on plasma lipoproteins is based on the assumption that the enzyme associates with a small number of specific (HDL-type) macromolecules (Glomset, 1968; Fielding and Fielding, 1985). These lipoproteins apparently represent the key elements in reverse cholesterol transport (Glomset, 1968; Fielding and Fielding, 1985), and have been referred to as the cholesteryl ester transfer complex (Fielding and Fielding, 1980; Fielding and Fielding, 1985) (Figure 3). Park et al. (1987) have shown that upon molecular sieve gel chromatography of normal human plasma, most of the LCAT activity was consistently associated with a population of HDL molecules recovered at the elution volume of HDL. In the plasma of LCAT deficient patients, the LCAT activity, derived from the in vitro supplementation of the plasma, was located at or near the elution volume of the LCAT activity of the control samples. These data indicate that the LCAT molecules in normal (endogenous) as well as in LCAT deficient patients (exogenous) assemble into lipoprotein complexes that resemble HDL in size. These observations appear to be similar to those seen in Tangier disease, where the endogenous LCAT
activity was also found associated with HDL size particles, even though normal HDL was virtually absent from these plasma samples (Pritchard et al., 1988). These data strongly support the notion that the enzyme/substrate complex for the LCAT reaction consists of a specific subfraction of HDL and LCAT. These observations, taken together with other findings obtained with liposome substrates (Jonas et al., 1984; Nishida et al., 1984) indicate that the HDL/LCAT complex is held together by strong forces so that dissociation of this complex is unlikely under physiological conditions. This complex may thus function as a unit until it is removed from the circulation.

Studies of the substrate specificity of LCAT using both artificial and lipoprotein substrates (Fielding et al., 1972 b; Glomset and Norum, 1973; Jonas, 1986) have indicated that HDL were preferred over other lipoproteins as a substrate (Glomset and Norum, 1973) and that LCAT required at least one polypeptide cofactor in addition to lipid substrate (Fielding et al., 1972). Experiments carried out with partially purified LCAT showed that HDL₃ (not HDL₂) were the preferred substrate for the enzyme (Fielding and Fielding, 1971; Pinon et al., 1980). Studies utilizing synthetic lipid mixtures and purified enzyme have shown that the major apolipoprotein of HDL (apo A-I) was an activator for the LCAT reaction (Fielding et al., 1972 a). Other investigations utilized partially purified enzyme and showed that with sonicated dispersions of phosphatidyl choline and cholesterol as
substrates, the rate of esterification was dependent on the molar ratio of phosphatidyl choline to cholesterol and on the amount of apo-HDL added to the reaction mixture (Nakagawa and Nishida, 1973).

Indeed, numerous studies have been conducted with artificial substrates in order to elucidate the factors that might control the rate of the LCAT reaction in vivo. These investigations have established some of the factors that control the specificity of the enzyme in vitro, although the relevance of these studies to cholesterol esterification in vivo is yet to be established. There appeared to be some discrepancy in the correlation between LCAT activity and phosphatidyl choline/cholesterol, cholesteryl ester/unesterified cholesterol, and apo A-I/A-II ratios. Fielding et al. (1972 a) found that a phosphatidyl choline to cholesterol molar ratio of approximately 3:1 was most favorable for the LCAT reaction and that excess amounts of triglycerides and cholesteryl esters were inhibitory in a phosphatidyl choline-cholesterol-apo A-I liposome system. Using single bilayer vesicles as substrates, Chung et al. (1979 a) showed that apo A-II, due to its greater affinity for the lipid surface, inhibited the LCAT reaction in the presence of phospholipids, cholesterol, and apo A-I. Furthermore, they reasoned that the apo A-I/A-II ratio at the vesicle surface could thus control the rate of the LCAT reaction in vitro. In other experiments, Chung et al. (1979 b) prepared hybrid particles, by incubating dog HDL with human apo A-II. These particles had apo A-
II/A-I ratios varying from 0.05 to 2.0. The LCAT activity, when tested against these lipoprotein hybrids, was inversely proportional to the apo A-II/A-I ratio. Fielding and Fielding (1971) on the other hand showed that HDL₃ were a considerably better substrate than HDL₂, even though the apo A-I/A-II ratio is higher in HDL₂ than in HDL₃ (Kostner et al, 1974). Further, Pinon et al. (1980) found the relatively apo A-I-rich HDL₂ particles to be inhibitors of LCAT. Jahani and Lacko (1981) have studied the interaction of HDL subpopulations (obtained by ion-exchange chromatography) with highly purified LCAT and the properties of the subfractions as substrates for the enzyme. Their data showed no correlation between LCAT activity and the phosphatidyl choline/cholesterol or the apo A-I/A-II ratios. However, the cholesteryl ester/unesterified cholesterol ratio (positively) and the molecular weight of the HDL fractions (negatively) showed some correlation with LCAT activity. The discrepancy of data could be explained by differences that exist between the substrates used for those studies. Therefore, it must be considered that physiochemical properties and overall morphology of the artificial lipid/protein complexes are likely to be quite different from plasma HDL. More information is required to understand the precise nature of the interaction of LCAT with HDL and its possible significance in vivo.
Role of Tissue Lipoprotein Receptors in Cholesterol Metabolism

In the past decade, the importance of various lipoprotein receptors in regulating cholesterol and triglyceride catabolism has become apparent. The lipoprotein receptors bind plasma lipoproteins on the cell surface to be subsequently internalized and metabolized by the cell. Receptors not only provide an efficient mechanism for removing lipoproteins from the plasma, but they also provide an effective delivery system for supplying specific cells and tissues with cholesterol and other lipids. By far the most extensively characterized system is the 'LDL (apo B, E receptor) pathway', the details of which were initially worked out by Brown and Goldstein in studies with cultured fibroblasts (Brown and Goldstein, 1974; Goldstein et al., 1974; Brown et al., 1974; Goldstein and Brown, 1976; Goldstein and Brown, 1977). The sequential steps in the LDL receptor pathway are summarized in Figure 4. The LDL bind to the cell surface receptors (LDL receptors) and are localized in areas of 'coated pits' (Anderson et al., 1982). These coated pits invaginate, forming coated vesicles, and the LDL proceed through a pathway of endocytotic vesicles to their final destination, the lysosomes. There, the LDL are hydrolyzed, liberating amino acids from apo B and free cholesterol from the lipoproteins' cholesteryl esters. It appears that the free cholesterol migrates into the cytoplasm, where it elicits three intracellular regulatory responses that maintain cholesterol homeostasis: (1) the

suppression of hydroxymethyl glutaryl-CoA (HMG CoA) reductase, the rate limiting step in cholesterol biosynthesis; (2) the activation of the enzyme that esterifies free cholesterol (Acyl CoA cholesterol acyltransferase, ACAT), enabling excess cholesterol to be stored as cholesteryl ester lipid droplets; and (3) the down-regulation of the expression of the LDL receptors. The extent of binding of LDL is controlled by the number of receptors which in turn is regulated by the cell's need for cholesterol. This provides both the means for acquiring the cholesterol for various cellular functions and a mechanism to protect against excessive accumulation of cholesterol or cholesteryl esters (Goldstein and Brown, 1976; Goldstein and Brown, 1977). This mechanism is also the basis for the lowering of plasma
cholesterol by a number of pharmacological agents (Kovanen et al., 1979; Windler et al., 1980).

Initial studies demonstrated that LDL, in which apo B is the predominant (if not exclusive) apolipoprotein, bind to the LDL (apo B, E) receptor. Some laboratories reported that circulating HDL molecules were incapable of binding to the LDL (apo B, E) receptor (Goldstein and Brown, 1977; Mahley and Innerarity, 1977; Weisgraber and Mahley, 1980), while other groups suggested that HDL could interact with this receptor (Carew et al., 1976; Miller et al., 1977). The discrepancies in these findings were resolved by experiments with swine HDL<sub>C</sub>, which contain significant quantities of apo E. The swine HDL<sub>C</sub> were capable of binding to the LDL (apo B, E) receptor and initiate the same intracellular events as LDL (Bersot et al., 1976). Competitive binding studies with fibroblasts clearly demonstrated that LDL and HDL<sub>C</sub> bound to the same receptor, i.e. the LDL (apo B, E) receptor. Thus those HDL capable of binding to the LDL (apo B, E) receptor contained apo E, and the extent of binding was proportional to the content of apo E (Mahley and Innerarity, 1977). Normal human and animal HDL that bind to the LDL (apo B, E) receptors of fibroblasts contain the E apolipoproteins (Mahley, 1978; Mahley and Innerarity, 1977; Weisgraber and Mahley, 1980; Innerarity et al., 1978). By contrast, the HDL that lack apo E are incapable of interacting with the LDL (apo B, E) receptors.
The interaction of the lipoproteins with cells is dependent on a mechanism involving either apo B or apo E. The importance of these apolipoproteins in the interaction between the lipoproteins and the LDL (apo B, E) receptors was established by the use of selective chemical modification of specific amino acid residues (Mahley, 1978; Mahley et al., 1977; Weisgraber et al., 1978; Basu et al., 1976; Mahley et al., 1980a). According to these studies, arginine residues and lysine residues of the apolipoproteins were important and significant in the recognition and uptake processes.

The presence of apo B and/or apo E in a lipoprotein does not always guarantee that a particular lipoprotein will a priori interact with the LDL (apo B, E) receptor. It is likely that the lipid content or the size of the lipoprotein particles may modify their ability to interact with the receptors. For example, normal VLDL and chylomicrons, though they contain apo B and apo E, are not well recognized by the LDL (apo B, E) receptors (Mahley and Innerarity, 1983).

Several organs or cell types possess other lipoprotein receptors in addition to the LDL (apo B, E) receptor. The liver has an apo E, or chylomicron remnant receptor. It has been established that chylomicron remnants are rapidly cleared from the plasma by receptor-mediated endocytosis and catabolized by the liver. This has been demonstrated in various systems, including the perfused liver, isolated liver membranes, and hepatocytes (Windler et al., 1980;
Sherrill and Dietschy, 1978; Carrella and Cooper, 1979; Nestel et al., 1963; Cooper, 1977). Several studies have indicated that the uptake of chylomicron remnants is mediated by the presence of the apo E molecule in the lipoprotein (Winder et al., 1980; Sherrill et al., 1980; Shelburne et al., 1980).

The liver plays a key role in cholesterol and lipoprotein metabolism. It is through the liver that cholesterol can be eliminated from the body via the bile either as free cholesterol or bile acids. Therefore, considerable attention has been focused on the hepatic high affinity, receptor-mediated lipoprotein uptake processes. It appears that the liver possesses at least two different receptors (the LDL [apo B, E] receptors and the remnant [apo E] receptors) capable of interacting with specific lipoproteins. In addition to the characteristic receptor-mediated uptake processes, nonreceptor-mediated hepatic catabolism of certain plasma lipoproteins has also been described (Attie et al., 1982). However, more information is needed to establish the physiological significance of the specific hepatic lipoprotein receptors and the nonreceptor-mediated lipoprotein catabolism.

It has been shown that some of the hepatic receptors bind apo E-HDL₃, chylomicron remnants, and HDL with apo E, but not normal LDL (Mahley et al., 1981; Hui et al., 1981). Such receptors, referred to as remnant or apo E receptors, have been described in the livers of
the dog, swine, and man using a membrane binding assay (Mahley et al., 1981). The precise role of the apo E receptor and the possible relationship of this receptor with chylomicron remnant catabolism will require further clarification.

In addition to apo E receptors, the presence of typical LDL (apo B, E) receptors has been demonstrated in the livers of animals and man (Mahley et al., 1981; Hui et al., 1981; Windler et al., 1980; Kovanen et al., 1981). Furthermore, rat livers treated with ethinyl estradiol have shown increases in LDL (apo B, E) receptor activity. These receptors have been documented in studies involving both perfused rat liver and isolated hepatic membrane fractions (Windler et al., 1980; Kovanen et al., 1981). Hui et al. (1981) reported that as dogs mature, their LDL (apo B, E) receptors decrease in number to barely detectable levels in adult animals. The hepatic LDL (apo B, E) receptors can be markedly, and in some cases very rapidly, down-regulated. Their expression can be suppressed to very low levels in young dogs by (1) cholesterol feeding, (2) the intravenous infusion of lymph lipoproteins, and (3) the infusion of bile salts. Under these conditions, apo E receptor activity remains essentially unchanged (Hui et al., 1981; Kovanen et al., 1981; Angelin et al., 1983).

In vivo receptor-mediated catabolism has been studied by several investigators by injection of chemically modified lipoproteins into animals. The difference between the clearance rates of native and
chemically modified lipoproteins was calculated as the removal rate due to receptor-mediated clearance. The data obtained with rats and monkeys indicated that approximately 50 per-cent of the clearance of LDL was receptor-mediated (Mahley et al., 1980 a; Mahley et al., 1979; Mahley et al., 1980 b). Bilheimer et al. (1982) demonstrated that normal rabbits clear about 70 per-cent of their LDL via the LDL (apo B, E) receptor pathway. Shepherd and Packard (1979) calculated that the normal human subjects cleared about one-third of the LDL via the receptor pathway. Brown and Goldstein estimated that about two-thirds of the plasma LDL are cleared via the LDL (apo B, E) receptor pathway (Goldstein and Brown, 1977).

It is now apparent that macrophages have receptors, or binding sites, for modified lipoproteins (e.g., negatively charged LDL, which can be generated by a chemical modification of the protein) (Goldstein et al., 1979; Fogelman et al., 1980). The macrophage is a cell of particular importance, in that it may be one of the progenitors of foam cells that occur in atherosclerotic lesions. Cultured macrophages can become massively loaded with cholesteryl esters when incubated with these lipoproteins (Mahley, 1981; Mahley, 1982).

Several important characteristics of the major lipoprotein receptors are summarized in Table IV.
### TABLE IV

**SUMMARY OF LIPOPROTEIN RECEPTORS**

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Cells or tissues</th>
<th>Lipoproteins bound</th>
<th>Ligands involved</th>
<th>Receptor regulation</th>
<th>Functional roles</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>Fibroblasts</td>
<td>LDL</td>
<td>Apo B</td>
<td>Regulated</td>
<td>Regulation of LDL levels.</td>
</tr>
<tr>
<td>(apo B,E)</td>
<td>Smooth muscle cells</td>
<td>HDL&lt;sub&gt;C&lt;/sub&gt;</td>
<td>Apo E</td>
<td>by delivery of lipoprotein</td>
<td>Redistribution of cholesterol by apo B and apo E lipoproteins to various tissues.</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>VLDL</td>
<td></td>
<td></td>
<td>Cholesterol utilized for membrane or hormone production.</td>
</tr>
<tr>
<td></td>
<td>Adrenal Cortex</td>
<td>Chylomicron remnants</td>
<td></td>
<td></td>
<td>Uptake of chylomicron remnants and cholesterol loaded HDL-with apo E.</td>
</tr>
<tr>
<td></td>
<td>Ovaries</td>
<td>Lp(a)</td>
<td></td>
<td></td>
<td>Delivery of cholesterol to the liver for excretion.</td>
</tr>
<tr>
<td></td>
<td>Testes</td>
<td>β-VLDL</td>
<td></td>
<td></td>
<td>Potential role in foam cell production in atherogenesis.</td>
</tr>
<tr>
<td></td>
<td>Adipocytes</td>
<td></td>
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<td>Uptake of diet induced lipoproteins.</td>
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<tr>
<td></td>
<td>Lymphocytes</td>
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<td></td>
<td>Potential role in foam cell production in atherogenesis.</td>
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<td>Macrophage-</td>
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<tr>
<td></td>
<td>monocytes</td>
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</tr>
<tr>
<td>Apo E</td>
<td>Liver</td>
<td>Chylomicron remnants</td>
<td>Apo E</td>
<td>Not subject to maked down-regulation</td>
<td></td>
</tr>
<tr>
<td>(Chylomicron remnants)</td>
<td>HDL&lt;sub&gt;C&lt;/sub&gt;</td>
<td></td>
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</tr>
<tr>
<td>β-VLDL</td>
<td>Macrophage</td>
<td>β-VLDL</td>
<td></td>
<td>Poorly regulated</td>
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<tr>
<td></td>
<td>Macrophage</td>
<td>Modified LDL</td>
<td>Charge modified apo B</td>
<td>Not regulated</td>
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<td></td>
<td>Chemically modified</td>
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<td>lipoprotein</td>
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Comparison of Human and Rat Plasma Lipoproteins

The rat has been frequently used as an animal model for lipoprotein research and especially for studying age-related changes of lipid metabolism (Lacko et al., 1984). It has the advantage of small size, modest upkeep, short life span and initial cost of obtaining the animals. Data obtained in rats are frequently extrapolated to the lipoprotein systems of other mammals including humans. However, lipoprotein metabolism in rats differs from that in humans in several respects. There are three major differences. The first of these differences is the remnant removal pathways. The remnants of triglyceride-rich lipoproteins (VLDL and chylomicrons), following the lipolytic action of lipoprotein lipase, are rapidly cleared by the liver in rats (Eisenberg and Rachmilewitz, 1973; Faergeman et al., 1975; Sherrill and Dietschy, 1978; Windler et al., 1980), whereas in humans, some components of chylomicrons are transferred to other lipoproteins (Havel et al., 1973; Patsch et al., 1978) and a large accumulation of circulating LDL is generated as the result of VLDL catabolism (Eisenberg et al., 1973; Sigurdsson et al., 1975). The second major difference is observed in the cholesterol esterification system. In humans, all the cholesteryl esters found in plasma lipoproteins are generated within the plasma compartment by the action of the enzyme LCAT (Glomset, 1968; Kudchodkar and Sodhi, 1976; Lacko et al., 1974; Nestel and Monger, 1967) whereas, in rats,
the liver also contributes to the synthesis of cholesteryl esters found in plasma lipoproteins (Gidez et al., 1965; Goodman and Shiratori, 1964). The third major difference lies in the absence of cholesteryl ester transfer protein activity (CETP) in the rat (Barter and Lally, 1978; Oschry and Eisenberg, 1982). In humans, as much as 80 percent of the LCAT-derived cholesteryl esters are transferred to lower density lipoproteins (VLDL or LDL) by the action of this protein (Nestel et al., 1979; Hopkins and Barter, 1980). The lack of cholesteryl ester transfer protein (CETP) causes the accumulation of cholesteryl esters in HDL and a relative paucity of cholesterol in other lipoprotein classes. In spite of these differences, much of the knowledge concerning lipoproteins has emerged from investigations using the rat as the experimental model.

Although there are some differences between the human and rat systems, many similarities remain, particularly in the area of the reverse cholesterol transport pathway (Davis et al., 1982). As postulated for humans, the LCAT reaction participates in the reverse cholesterol transport system in the rat (Davis et al., 1982). In addition, Sabesin et al. (1978) found that inhibition of LCAT secretion in the liver of rats elicited lipoprotein abnormalities that were remarkably similar to those observed in patients with congenital LCAT deficiency (Glomset and Norum, 1973). Moreover, the absence of cholesteryl ester transfer protein activity in the rat makes this animal
specifically suitable for studying the lipoprotein/LCAT system. In other species the cholesteryl ester transfer protein activity significantly complicates the investigation of this system by remodeling lipoproteins (especially HDL) rapidly. The recent findings of Groener et al. (1989) support this postulate. When they injected cholesteryl ester transfer protein intravenously into rats, profound effects were observed on the chemical composition of HDL subspecies, the size of HDL and on the plasma turnover and hepatic uptake of cholesteryl esters originally present in apo A-I-rich HDL.

The animal species selected for this study is the Fischer-344 male rat, a strain that has proven to be a reliable model system for the studies of age-related changes, including those of cholesterol metabolism. The age of the experimental animals has been specifically selected to achieve maximal changes in plasma lipids and to avoid potential pathological complications arising from testicular tumors and renal diseases. These two symptoms become prominent in Fischer-344 rats past 20 months of age. However, the changes in plasma lipids and plasma lipid metabolism appear to plateau at about 18 months of age (Carlile et al., 1986; Carlile and Lacko, 1985; Liepa et al., 1980). Therefore, 18-22 month-old rats were designated as the "aged rats" in this study. The control group (young rats) was composed of 6 month-old animals that are already past their growth
phase and comparable to young human adults in terms of their physiological development.

**Purpose of This Study**

Age-related increases of serum lipids (including cholesterol and triglycerides) have been observed in both humans and rats but the mechanism(s) of these changes is not known.

A number of observations in humans suggest that the fractional rate of endogenous plasma cholesterol esterification (FRE) (% cholesterol esterified/time) is decreased in conditions where there is an increase in the plasma cholesterol pool (Soloff et al., 1978; Lacko et al., 1978). Hypercholesterolemia and the decreased fractional rate of cholesterol esterification have also been observed in aged rats (Carlile et al., 1986; Story et al., 1976; Liepa et al., 1980; Carlile and Lacko, 1981). However, the biochemical and physiological mechanisms involved in these age-related changes and/or the interrelationship between these observations are not yet to be elucidated. Therefore, this study was designed to examine the potential relationship between the observed age-related hypercholesterolemia and the decreased fractional rate of endogenous plasma cholesterol esterification in rats.

Fractional rate of plasma cholesterol esterification was found to be an important parameter for the age-related changes in lipid and lipoprotein metabolism since it is related to the rate of removal of
cholesterol from the blood (Kudchodkar, 1983). LCAT, in concert with HDL, have been proposed to facilitate the removal of cholesterol from the peripheral tissues to the liver via the plasma otherwise known as reverse cholesterol transport.

While a significant amount of information is now available concerning the structure and mechanism of LCAT, relatively little is known about the properties of the LCAT substrate in vivo. Equally little or no information is available concerning the mechanism whereby substrate lipids are transferred from lower density lipoproteins (VLDL and/or LDL) to HDL. More knowledge is required regarding the mechanisms involved in the delivery of substrate to the enzyme surface, the nature of the enzyme/substrate complex and the mode of product removal in this reaction.

The rat was chosen as an animal model for this study because rats are highly resistant to atherosclerosis. Therefore, changes of the altered lipoprotein metabolism in aged animals can be studied without the physiological deterioration related to vascular disease. Moreover, the rat is an attractive model for the investigation of enzyme (LCAT)/lipoprotein substrate (HDL) interactions in isolation in vivo because of the absence of cholesteryl ester transfer protein activity. In this system, the LCAT/lipoprotein (enzyme/substrate) interrelationships can be studied without the continuous changes in the product lipoprotein pool due to remodeling by cholesteryl ester transfer.
The overall goal of this study was to investigate the specific role of LCAT/ lipoprotein interactions in the plasma of young and aged rats in order to understand the mechanism(s) underlying the decreased fractional rate of plasma cholesterol esterification and hypercholesterolemia in aged rats.
CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Male, specific pathogen-free (SPF), Fischer-344 rats were obtained at the appropriate age from Charles River Breeding Laboratories (Wilmington, MA). The animals [six and 18-22 month old] were designated as young and aged rats, respectively. Upon arrival, they were housed in special quarters to minimize exposure to pathogens. This limited barrier facility is equipped with a lamellar flow hood where the animal cages are kept until the completion of each experiment. The animal room was maintained at 25°C on a 12 hour light-dark cycle. The animals were given Purina Rodent chow (22 % protein, 4 % fat) and water ad libitum. The rats were maintained and fed for a maximum of 30 days. A period of 10 days was sufficient for the animals to adjust to their new environment and to reestablish their feeding patterns. The rats were weighed and examined before they were sacrificed to assess their physical condition and health.

The animals were fasted for 18 hrs with access to water only and from five to six animals of the same age were sacrificed on the same
day. The animals were anesthetized with ether and blood was collected from the inferior vena cava in chilled tubes containing EDTA (ethylene diamino tetra acetic acid) at a final concentration of 4.5 mM. Plasma was obtained by low speed centrifugation (1000 x g) at 4⁰C. Lipoprotein isolation and gel chromatography were performed as soon as possible after obtaining the plasma.

**Chemicals**

Source of chemicals and reagent supplies were as follows: [1,2-³H] cholesterol (40.7 Ci/mmol) was purchased from Amersham, Arlington Heights, IL. Ecolite scintillation fluid was obtained from West Chem, San Diego, CA. Bio-Gel A-5 M and all the chemicals for gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA. Crystalline bovine serum albumin (essentially fatty acid free), human serum albumin, cholesterol, triglycerides and phospholipids standards, and silica gel plates for thin layer chromatography, 17 - β- ethinyl estradiol were purchased from Sigma Chemicals Co., St. Louis, MO. Aquacide IA was from Calbiochem, La Jolla, CA. Enzymatic Kits for cholesterol and calibration protein for chromatography were obtained from Boehringer Mannheim, Indianapolis, IN. Enzymatic kits for triglyceride were purchased from Amresco, South Euclid, OH. Heparin-sepharose CL-6B and electrophoresis calibration kit were obtained from Pharmacia Inc., Piscataway, NJ. All other chemicals
were obtained from Fischer Scientific Company, Dallas, TX and were of reagent grade or better.

Methods

**Distribution of LCAT Activity in Rat Plasma**

(1) *Gel Filtration Chromatography*: Two ml of rat plasma (pooled from six animals in each group) was fractionated on a Bio-Gel A-5 M column (1.5 x 90 cm). The fresh plasma labeled with $[^3]$H-cholesterol (0.01 mCi/ml plasma) for 4 hours at 4°C was applied to the Bio-Gel A-5 M column. The column was previously equilibrated with a 0.01 M Tris-HCl buffer (pH 7.4) containing 0.01 % EDTA and 0.15 M NaCl. The plasma was chromatographed with a flow rate of 20 ml/hr; two ml fractions were collected. The absorbance (280 nm) and radioactivity of each fraction were monitored to establish protein and lipoprotein profiles, respectively. LCAT activity was assayed in alternate fractions according to Chen and Albers (1982).

(2) *Density Gradient Ultracentrifugation*: Rat plasma was also fractionated by density gradient ultracentrifugation essentially as described by Terpstra *et al*. (1981). Two ml of pooled plasma (from six animals in each group) were adjusted to a density of 1.225 g/ml with solid KBr (0.3517 g of KBr per 1 ml of plasma) and transferred to
ultracentrifuge tubes with 1 ml markings. The plasma was then sequentially layered under NaCl-KBr salt solutions of densities 1.225 g/ml (2 ml) and 1.10 g/ml (4 ml) and the tube was filled with distilled water. The tubes were placed in Sorvall SW-40 Ti rotor and centrifuged at 39,000 rpm (200,000 x g) for 24 hours in a Sorvall OTD-B ultracentrifuge at 15°C. The procedure was calibrated in our laboratory by repeatedly determining the density of successive 1 ml fractions that were removed from the top of the tubes by a narrow-bore Pasteur pipette. LCAT activity was determined in each fraction according to Chen and Albers (1982).

(3) Preparative Ultracentrifugation and Bio-Gel A-5 M Chromatography: Lipoproteins were isolated from pooled rat plasma by preparative ultracentrifugation and Bio-Gel A-5 M chromatography according to the procedure of Rudel et al. (1974). Plasma samples from 6 rats sacrificed on the same day were combined to give a 6 ml sample of plasma. The plasma sample was labeled with [3H]-cholesterol (0.01 mCi/ml) and adjusted to a density of 1.225 g/ml by adding solid KBr (0.3517 g/ml of plasma). A 5 ml volume of the solution containing 0.01 % EDTA and 0.15 M NaCl-KBr (d=1.225 g/ml) was layered over the plasma. Ultracentrifugation was carried out at 39,000 rpm (200,000 x g) in a Sorvall OTD-B ultracentrifuge using an SW-40 Ti rotor for 40 hours at 15°C. The contents of the ultracentrifuge tube were fractionated by using capillary tubing and a
peristaltic pump; 200 µl fractions were collected. The absorbance
(280 nm) and radioactivity of each fraction were monitored to
establish protein and lipoprotein profiles respectively; LCAT activity
was assayed in alternate fractions according to Chen and Albers
(1982).

The other set of plasma samples (6 ml, pooled from 6 rats) which
were not labeled with [3H]-cholesterol was adjusted to a density of
1.225 g/ml with KBr (0.3517 g/ml) and subjected to preparative
ultracentrifugation as described above. After ultracentrifugation, the
lipoproteins (d < 1.225 g/ml) were removed by collecting the top 1.5
ml of the ultracentrifuged sample and subsequently fractionated on a
Bio-Gel A-5 M column (1.5 x 90 cm, 0.01 M Tris-HCl buffer, pH 7.4
containing 0.15 M NaCl, 0.01 % EDTA) as described earlier.
Absorbance at 280 nm was monitored to establish elution pattern of
lipoproteins (VLDL, LDL and HDL), and LCAT activity was assayed in
alternate fractions according to Chen and Albers (1982).

**Measurement of LCAT Activity**

LCAT activity was assayed by the method of Chen and Albers (1982)
using an exogenous substrate. Proteoliposome vesicles were prepared
containing apo A-I, phosphatidyl choline, and cholesterol (including
[3H]-cholesterol) in a molar ratio of 0.8/250/12.5 using the cholate
dialysis method (Chen and Albers, 1982). These proteoliposomes were subsequently used as substrates for the LCAT reaction. The assay was carried out in screw-capped culture tubes (16 x 125 mm) to which 0.23 ml Tris-HCl buffer (pH 7.4), 0.125 ml 2 % human serum albumin (HSA), and 0.1 ml proteoliposomes were added. The substrate mixture was preincubated at 37°C for 20 min, followed by the addition of the enzyme source (aliquots from ultracentrifuged fractions or chromatographic fractions). The assay mixture was then mixed immediately on a vortex mixer and incubated in a 37°C waterbath for 30 min. The enzyme reaction was stopped by the addition of 4 ml chloroform:methanol (2:1, v/v). The extracts were filtered on a sintered glass funnel and the precipitate washed twice with 2 ml aliquots of chloroform:methanol (2:1, v/v).

All filtrates were combined and the solvent evaporated under a stream of nitrogen. The dried lipids were dissolved in heptane and spotted on silica gel thin layer chromatography plates and developed in petroleum ether:ether:acetic acid (90:19:1, v/v/v). The lipids were visualized by exposing the plate to iodine vapors and the spots containing the cholesterol and cholesteryl esters were removed for liquid scintillation counting.

The fractional rate of esterification (% cholesterol esterification/time) was calculated. For this calculation the radioactive counts of the cholesteryl esters are divided by the sum of the radioactive counts of
the free cholesterol and cholesteryl ester and multiplied by 100.

**Plasma Incubation study**

Fresh plasma (2 ml) from each animal of both age groups was incubated for 0 and 2 hours at 37°C to determine the lipoprotein cholesterol changes during the incubation period. Following incubation, the plasma was adjusted to a density of 1.225 g/ml with solid KBr (0.3517 g/ml plasma) and was subjected to density gradient ultracentrifugation as described earlier (Terpstra et al., 1981). After collecting successive 1 ml fractions from the top of the tube, the following four fractions of different volumes and densities were pooled.

- **Fraction 1**: \(d < 1.006 \text{ g/ml (1ml)}\); VLDL
- **Fraction 2**: \(1.006-1.025 \text{ g/ml (2 ml)}\); IDL
- **Fraction 3**: \(1.025-1.07 \text{ g/ml (2 ml)}\); HDL\(_1\) + LDL
- **Fraction 4**: \(1.07-1.21 \text{ g/ml (6 ml)}\); HDL\(_2\) + HDL\(_3\)

The total cholesterol (Parekh and Jung, 1970) and free cholesterol (Allain et al., 1974) concentrations were determined in each fraction. The concentration of cholesteryl esters (CE) was calculated by subtracting the free cholesterol (FC) values from the total cholesterol (TC) values.
Lipoprotein Substrate Specificity of LCAT

Preparation of lipoprotein substrates: Lipoproteins were isolated using preparative ultracentrifugation (40 hrs) and Bio-Gel A-5 M chromatography as described earlier (Rudel et al., 1974) from pooled rat plasma in which the endogenous LCAT activity was previously inhibited by diisopropyl fluorophosphate (DFP, 1 mM). The lipoprotein fractions (VLDL and HDL) were pooled and concentrated to 3 ml using Aquacide. After dialysis against 0.01 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.01 % EDTA, these preparations were used as substrates for LCAT.

Plasma HDL was further subfractionated into apo A-I and apo E enriched fractions by heparin-Sepharose chromatography as described by Quarfordt et al. (1978). Same amount of HDL protein (230 μg) from young and aged rat plasma were applied to a heparin-Sepharose column (2.5 x 12 cm) and eluted with a stepwise salt gradient (0.05 M and 1.5 M NaCl in 0.005 M Tris-HCl, pH 7.4). The flow rate was 30 ml/hr and 2 ml fractions were collected. The HDL subfractions were pooled, concentrated, and dialyzed as described above and subsequently were used as substrates for LCAT.

Partial purification of rat LCAT: The infranatant (d >1.225 g/ml) obtained from preparative ultracentrifugation of rat plasma (without DFP treatment) was concentrated to 5 ml using Aquacide and dialyzed
exhaustively against 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.01 % EDTA. This preparation was used as a source of LCAT.

**Measurement of LCAT activity using isolated lipoproteins as substrates:** The rate of cholesterol esterification was determined for the individual lipoprotein preparations as described by Jahani and Lacko (1982). The free cholesterol concentrations in each lipoprotein preparation were determined by an enzymatic method (Allain et al., 1974) and aliquots of each lipoprotein preparation containing 12.3-98.4 nmol/ml free cholesterol were preincubated with 20 μl of a BSA [³H]-cholesterol emulsion for 4 hours at 4°C. The final assay volume was adjusted to 210 μl and the enzyme reaction was initiated by the addition of 20 μl of partially purified LCAT. The incubation was carried out at 37°C and enzyme activity was calculated as nmol of cholesterol esterified per ml per hour. The incubation time and the amount of enzyme in the reaction mixture were selected to obtain a linear rate of cholesterol esterification. The rate of cholesterol esterification was calculated by multiplying the fractional rate (% cholesterol esterification/time) by the free cholesterol concentration of each sample.

**Characterization of Lipoprotein Substrate for LCAT**

**Molecular weight determination of HDL:** Molecular weights of HDL
from both age groups were determined by molecular sieve gel filtration chromatography (Bio-Gel A-5M, 1.5 x 90 cm). HDL from both young and aged rat plasma were isolated according to Rudel et al. (1974) as described earlier. The elution of all samples was performed with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 0.01 % EDTA. Ferritin (mol.wt.=450,000), catalase (mol.wt.=240,000), egg albumin (mol.wt.=45,000), chymotrypsinogen (mol.wt.=25,000) were used as standards. Apparent molecular weight of HDL was estimated from the relative elution volume.

**Analysis of the composition of lipoprotein substrates:** Free cholesterol (FC) was determined by enzymatic method (Allain et al., 1974) and the total cholesterol (TC) was measured by the method of Parekh and Jung (1970). Esterified cholesterol levels were calculated by subtracting the FC values from the TC values. Triglycerides were determined by enzymatic reagent kits supplied by Amresco and phospholipids were measured by the method of Raheja et al. (1973) following extraction of the lipoprotein fraction with isopropanol.

Total phospholipids of plasma HDL were subfractionated by thin layer chromatography using the solvent system described by Gilfillan et al. (1983). Plates were developed using chloroform-methanol-petroleum ether (bp 35-60°C)-acetic acid-boric acid 40:20:30:10:1.8 (v/v/v/v/v/w). The phospholipid bands were visualized by iodine vapor and identified by comparison to standards run alongside the samples.
The bands corresponding to phosphatidyl choline, phosphatidyl inositol and phosphatidyl serine, phosphatidyl ethanolamine and sphingomyelin were scraped into tubes and phospholipids were extracted by chloroform:methanol (2:1, v/v). The extracts were evaporated to dryness and the lipids were redissolved in a known volume of chloroform. Aliquots were then taken for the measurement of phospholipids by the method of Raheja et al. (1973).

Protein determinations were carried out according to Markwell et al. (1981) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 0.1 % (w/v) SDS, according to the method of Laemmli (1970) to establish the apolipoprotein profile of the isolated lipoprotein fractions. A slab gel of 10 % acrylamide was used and the gels were stained for 4 hours with 0.02 % Coomassie Blue. Apolipoproteins were identified by their apparent molecular weights as determined by comparisons with molecular weight standards.

**Preliminary Study of Ethinyl Estradiol Treatment**

**in Young and Aged Rats**

For the study of ethinyl estradiol treatment, male Fischer-344 rats were obtained at the appropriate age from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The animals of both age groups were divided into
two groups (3 rats in each group) 10 days after their arrival. Body weight of the animal and the amount of food consumed were measured daily. The groups receiving ethinyl estradiol were allowed free access to food. Control group was given the same quantity of food consumed by the group which received ethinyl estradiol (pair-fed) to compensate for the reduced food intake induced by ethinyl estradiol treatment. All animals were allowed water ad libitum.

Ethinyl estradiol (40 mg) was dissolved in absolute ethanol (1.6 ml) and diluted with dimethyl sulfoxide (DMSO, 6.4 ml). The final concentration was 5 mg ethinyl estradiol/ml. The animals were administered 5 mg ethinyl estradiol/kg body weight per day by intraperitoneal injection. The control groups received diluent alone. At the end of the 7 day treatment period, the animals were sacrificed under light ether anesthesia after 18 hour fast. Blood was drawn from the inferior vena cava and plasma was obtained by low speed centrifugation at 4°C. Aliquots of plasma were taken for the determination of lecithin:cholesterol acyltransferase (LCAT) activity and for the isolation of lipoproteins. After the removal of blood, the body was perfused with cold saline. The liver was removed, rinsed with cold saline, blotted, and weighed. Plasma lipoproteins were isolated according to Rudel et al. (1974) as described earlier. Each lipoprotein fraction (VLDL, LDL, and HDL) was pooled, concentrated using Aquacide, and dialyzed against 0.01 M Tris-HCl (pH 7.4) buffer
containing 0.15 M NaCl, 0.01 % EDTA. The concentrations of lipids (free cholesterol, esterified cholesterol, triglycerides, and phospholipids) and protein in each lipoproteins fraction (VLDL, LDL, and HDL) were measured as described for the analysis of the composition of lipoprotein substrates. The size of the plasma lipid pool was calculated as the product of the cholesterol concentration (mg/ml) and the plasma volume (ml). Plasma volume was assumed to represent 4 per cent of the body weight (Edelman and Liebman, 1959).

The initial rate of endogenous cholesterol esterification was measured by the method of Stokke and Norum (1971). The plasma samples taken for the measurement of endogenous LCAT activity were kept in the presence of dithiobis-2-nitrobenzoic acid (DTNB) at a final concentration of 4.2 mM. A 5 per cent solution of bovine serum albumin (BSA) (w/v) in 0.01 M Tris-HCl buffer, pH 7.4 was prepared and [3H]-cholesterol in acetone was added gradually to achieve a specific activity of 0.1 μCi per mg of BSA. The solution was stirred under nitrogen until all of the acetone was evaporated and then stored at 4°C. To a 0.60 ml sample of plasma, 150 μl of [3H]-cholesterol-BSA emulsion was added. This mixture was incubated at 37°C for 4 hours after which a 150 μl aliquot was removed as a zero time blank and the LCAT reaction was started by adding 100 μl of β-mercaptoethanol (BME). Subsequently, 175 μl samples were removed at 5, 10, 15, and
20 minutes and the reaction was stopped by the addition of 2 ml of chloroform:methanol (2:1, v/v). The lipids were extracted as described for the proteoliposome assay (Chen and Albers, 1982).

The fractional rate of esterification (% cholesterol esterified/time) was calculated at each time point (0, 5, 10, 15, and 20 minutes). The fractional rate at the zero time was subtracted from all time points. These corrected fractional rates were plotted as the function of time and the fractional rates for 60 minutes were extrapolated from the graph or calculated from the slope of the line by least square analysis.

To estimate the amount of LCAT present, the plasma samples were also assayed by the method of Chen and Albers (1982) using an exogenous substrate (proteoliposome substrate) as described above. Mass of LCAT by immunoassay is proportional to LCAT activity measured by this exogenous substrate method (Albers et al., 1981).

Statistical Analysis

The student's t-test was performed to examine the differences between the young and aged rats. A probability of 0.05 or less was accepted as statistically significant. Values reported in the text and in the tables represent mean ± standard error of the mean (SEM).
CHAPTER III

RESULTS

Distribution of LCAT Activity in Rat Plasma

In order to determine the distribution of LCAT activity among lipoproteins in rat plasma, plasma samples from young and aged rats were labeled with $[^3]$H-cholesterol and then chromatographed on a Bio-Gel A 5-M column. Figure 5 shows the representative radioactivity and LCAT activity pattern of rat plasma. The two distinct radioactivity peaks appeared corresponding to the elution volume of lipoprotein standards (VLDL and HDL) isolated from normal human plasma by sequential ultracentrifugation. The LCAT activity was measured in alternate fractions by using exogenous proteoliposomes as substrate (Chen and Albers, 1982). Most of the LCAT activity was associated with the HDL containing fraction and there was no difference in this pattern between samples of plasma from young and aged rats.

In order to confirm the results obtained by gel filtration chromatography, the distribution of LCAT activity in plasma was determined after fractionation of plasma lipoproteins by 24 hour density gradient ultracentrifugation as described in methods (Terpstra et al., 1981). LCAT activity was determined in each fraction after
FIGURE 5. DISTRIBUTION OF LCAT ACTIVITY IN RAT PLASMA

Pooled rat plasma (2 ml) was labeled with $[^3]$H]-cholesterol (0.01 mCi/ml of plasma) and subjected to gel-chromatography (Bio-Gel A-5 M; 1.5 X 90 cm; 0.01 M Tris-HCl, pH 7.4 containing 0.01 % EDTA, 0.15 M NaCl). The flow rate was 20 ml/hr and fractions (2 ml) were collected. $A_{280}$ ( ————), radioactivity (—— ———) were monitored and LCAT activity (— — — — ) was measured using 20 μl aliquot from chromatographic fractions.
Two ml of pooled plasma were adjusted to a density of 1.225 g/ml with KBr and then sequentially layered under NaCl-KBr salt solutions of densities 1.225 g/ml (2 ml) and 1.10 g/ml (4 ml) and the tube was filled with distilled water. The tubes were placed in Sorvall SW-40 Ti rotor and centrifuged at 39,000 rpm (200,000 g) for 24 hrs in a Sorvall OTD-B ultracentrifuge at 15°C. 1 ml fractions were removed from the top of the tubes. LCAT activity was determined in each fraction.

FIGURE 6. DISTRIBUTION OF LCAT ACTIVITY AFTER DENSITY GRADIENT ULTRACENTRIFUGATION
density gradient ultracentrifugation. Figure 6 shows the distribution of LCAT activity in the plasma lipoprotein fractions. The results show that most of the LCAT activity was associated with HDL containing fraction (d = 1.125-1.21 g/ml).

Distribution of LCAT activity in rat plasma was also determined following preparative ultracentrifugation to compare with the results obtained above. Plasma samples labeled with [³H]-cholesterol were subjected to 40 hours of preparative ultracentrifugation and the contents of the ultracentrifuge were fractionated as described under Methods. Absorbance at 280 nm, radioactivity and LCAT activity (Chen and Albers, 1982) were determined in the isolated fractions. The results are shown in Figure 7. The radioactivity ([³H]-cholesterol) was associated with only the top 1.5 ml (d < 1.225 g/ml) representing the lipoprotein containing fractions. The data presented here (Figure 7) indicate that most of the LCAT activity was associated with the lipoprotein free infranatant (d > 1.225 g/ml) accounting for 90% of the total enzyme activity. This result suggests that upon 40 hour ultracentrifugation, most of the LCAT activity which was previously associated with the HDL containing fraction is displaced into the plasma protein containing infranatant (d > 1.225 g/ml) fraction.

In order to examine the distribution of LCAT activity that is left in the lipoprotein containing fraction after preparative ultracentrifugation, the top 1.5 ml of ultracentrifuged sample (d < 1.225
Pooled rat plasma (6ml) was labeled with [3H]-cholesterol and brought to a density of 1.225 g/ml by the addition of KBr. The sample was then overlayed with a KBr solution (d=1.225 g/ml) containing 0.15 M NaCl and 0.01 % EDTA. Ultracentrifugation was carried out at 39,000 rpm in a Sorvall-OTD ultracentrifuge using an SW-40 rotor for 40 hrs at 15°C. The contents were fractionated and 200 μl fractions were collected. Radioactivity (—▲—), A 280 (—■—) and LCAT activity (—○—) were monitored.
g/ml; lipoprotein containing fraction) was further fractionated on a Bio-Gel A-5 M column. Absorbance at 280 nm was monitored and LCAT activity was determined in alternate fractions (Chen and Albers, 1982). Figure 8 shows the absorbance (A₂₈₀) and LCAT activity profile of the lipoproteins isolated from young (Panel A) and aged rat (Panel B) plasma. The two distinct absorbance peaks corresponded to the elution volume of VLDL and HDL standard isolated from normal human plasma by sequential ultracentrifugation. But the LDL region was not clearly discernible. The data indicate that the LCAT activity which remained in the lipoprotein containing fraction after preparative ultracentrifugation was found associated with the HDL fraction only in both young and aged rats. Furthermore, the HDL associated LCAT activity was higher in the plasma samples obtained from aged rats (Panel B) as compared to those from young rats (Panel A), even though the original enzyme levels assayed in whole plasma (Chen and Albers, 1982) were nearly the same in both plasma samples.

The Distribution of Cholesterol Content in Lipoproteins

In order to determine the distribution of cholesterol content in the respective lipoprotein fractions, plasma samples obtained from each animal of both age groups (four animals in each group) were subjected to density gradient ultracentrifugation as described under Methods.
Lipoproteins (d<1.225 g/ml) were isolated from pooled plasma of young and aged rats by 40 hr preparative ultracentrifugation and subsequently fractionated by gel filtration chromatography (Bio-Gel A-5 M ; 0.01 M Tris-HCl, pH 7.4 containing 0.15 M NaCl and 0.01 % EDTA). The flow rate was 20 ml/hr and 2 ml fractions were collected. Panel A and B show the distribution of lipoprotein fraction (A 280 ; —O—) and LCAT activity (——#——) for young and aged rats respectively.
The following four fractions of different volumes and densities were collected: fraction 1, \( d < 1.006 \) g/ml (1ml); fraction 2, \( d=1.006-1.025 \) g/ml (2 ml); fraction 3, \( d=1.025-1.07 \) g/ml (2 ml); fraction 4, \( d=1.07-1.21 \) g/ml (6 ml). The total cholesterol (TC) and free cholesterol (FC) concentrations of each fraction were determined and the concentrations of cholesteryl ester (CE) were calculated as the difference between total and free cholesterol values. Table V shows cholesterol levels in the respective density fractions of plasma lipoproteins. Total cholesterol levels of the plasma were significantly higher (\( P<0.05 \)) in aged rats (70.4±1.33 mg/dl) as compared to those in young rats (54.5±2.5 mg/dl). The most significant increases in the levels of plasma cholesterol as well as its distribution among the lipoprotein fractions were seen in the fractions of \( d=1.025-1.07 \) g/ml in aged rats. The relative amount of cholesterol (% of the total pool) was significantly lower (\( P<0.05 \)) in the fraction of \( d=1.07-1.21 \) g/ml from aged rats. There was no significant difference in the percentage of cholesteryl esters among the isolated lipoproteins fractions. However, the cholesteryl ester (CE) content of \( d <1.006 \) g/ml fraction of aged rats tended to be somewhat higher than the same fraction from young rats (\( P<0.1 \)).

**Origin of Lipid Substrates and the Fate of the Product**

**Cholesteryl Esters of the LCAT Reaction**
### TABLE V

**CHOLESTEROL LEVELS IN DIFFERENT DENSITY FRACTIONS OF PLASMA LIPOPROTEINS IN YOUNG AND AGED RATS**

<table>
<thead>
<tr>
<th>Density Fraction</th>
<th>Parameters</th>
<th>Young rats</th>
<th>Aged rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g/ml</td>
<td></td>
</tr>
<tr>
<td>&lt;1.006</td>
<td>Total cholesterol (mg/dl)</td>
<td>10.1±1.50</td>
<td>14.0±2.53</td>
</tr>
<tr>
<td></td>
<td>(18.5±2.12)</td>
<td></td>
<td>(19.6±3.46)</td>
</tr>
<tr>
<td></td>
<td>Esterified cholesterol (%)</td>
<td>59.4±3.75</td>
<td>63.8±3.32</td>
</tr>
<tr>
<td>1.006-1.025</td>
<td>Total cholesterol (mg/dl)</td>
<td>2.4±0.22</td>
<td>3.7±0.44</td>
</tr>
<tr>
<td></td>
<td>(4.5±0.55)</td>
<td></td>
<td>(5.2±0.64)</td>
</tr>
<tr>
<td></td>
<td>Esterified cholesterol (%)</td>
<td>65.0±3.88</td>
<td>58.5±3.46</td>
</tr>
<tr>
<td>1.025-1.07</td>
<td>Total cholesterol (mg/dl)</td>
<td>9.7±0.71</td>
<td>18.8±0.82*</td>
</tr>
<tr>
<td></td>
<td>(17.8±1.83)</td>
<td></td>
<td>(26.3±1.34)*</td>
</tr>
<tr>
<td></td>
<td>Esterified cholesterol (%)</td>
<td>74.2±3.27</td>
<td>76.2±2.30</td>
</tr>
<tr>
<td>1.07-1.21</td>
<td>Total cholesterol (mg/dl)</td>
<td>32.3±2.23</td>
<td>33.9±0.51</td>
</tr>
<tr>
<td></td>
<td>(59.1±2.92)</td>
<td></td>
<td>(47.5±0.85)*</td>
</tr>
<tr>
<td></td>
<td>Esterified cholesterol (%)</td>
<td>80.5±1.98</td>
<td>78.0±1.50</td>
</tr>
</tbody>
</table>

Data represent Mean± SEM values for four animals in each group. Numbers in parentheses represent the mean ± SEM value for the percentage distribution of lipid among the different density fractions.

*Denotes significant difference (P<0.05) between young and aged groups.
Free cholesterol (FC) from lower density lipoproteins (VLDL and LDL) can be transferred to HDL and become the substrate for LCAT (Fielding and Fielding, 1980; Park et al., 1987). The following experiments were performed to determine whether age-related changes in plasma lipoproteins influenced this process. Aliquots of plasma from both age groups were incubated at 37°C for 0 and 2 hours, respectively, to study the effect of the LCAT reaction on the changes in the free cholesterol and cholesteryl ester content of the isolated lipoprotein fractions. After incubation, the lipoproteins were fractionated by density gradient ultracentrifugation and the different density fractions were collected as described under Methods. The total cholesterol and free cholesterol concentrations were determined and the concentrations of cholesteryl ester were calculated. The data in Table VI show that during the incubation period, the free cholesterol content decreases while the cholesteryl ester content increases. The total amount of cholesteryl ester formed during 2 hour incubation was 5.5 mg/dl in young rat plasma and 3.8 mg/dl in aged rat plasma. These data show that the in vitro net esterification of plasma cholesterol is lower in the plasma of the aged animal as compared to that of the young animal, even though the initial free cholesterol pool is higher in the former (the total amount of FC present at the beginning of incubation is 14.0 mg/dl and 18.6 mg/dl in the young and aged rat plasma, respectively). Fractional utilization of
### TABLE VI

CHANGES IN THE CHOLESTEROL CONTENT OF LIPOPROTEIN DURING THE INCUBATION OF RAT PLASMA FROM YOUNG AND AGED RATS

<table>
<thead>
<tr>
<th>Density Fraction (g/ml)</th>
<th>Young rats</th>
<th></th>
<th></th>
<th></th>
<th>Aged rats</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0hr FC CE</td>
<td>2hr FC CE</td>
<td>2hr-0hr FC CE</td>
<td>0hr FC CE</td>
<td>2hr FC CE</td>
<td>2hr-0hr FC CE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.006</td>
<td>4.1 6.0 2.7 6.4 -1.4 0.4</td>
<td>5.1 8.9 4.0 9.1 -1.1 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.006-1.025</td>
<td>0.8 1.6 0.3 0.9 -0.5 -0.7</td>
<td>1.5 2.2 1.1 1.7 -0.4 -0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.025-1.07</td>
<td>2.5 7.2 1.2 6.8 -1.3 -0.4</td>
<td>4.5* 14.3* 3.2 13.9 -1.3 -0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.07-1.21</td>
<td>6.6 26 4.3 32.2 -2.3 6.2</td>
<td>7.5 26.5 6.4 31 -1.1* 4.5*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14.0 40.8 8.5 46.3 -5.5 5.5</strong></td>
<td><strong>18.6 51.9 14.7 55.7 -3.9 3.8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent the mean value for four animals in each group. For visual clarity SEM value have not been given.

*Denotes significant difference (P<0.05) between young and aged groups.
the free cholesterol of the d=1.07-1.21 g/ml fraction showed considerable differences between the two age groups. Thirty-five % of the total FC available in d=1.07-1.21 g/ml fraction was esterified during 2 hour incubation period in young rat plasma; however, only 14.6 % was converted to cholesteryl ester in the aged rat plasma. The amount of cholesteryl ester formed in d=1.07-1.21 g/ml fraction during incubation exceeded the amount of free cholesterol decrease in the same fraction. At the same time, the free cholesterol content of the lower density fractions (d <1.07 g/ml) decreased during incubation whereas the cholesteryl ester content of these fractions did not show marked changes. These findings show that a net transfer of free cholesterol from the lower density fractions (d <1.07 g/ml) to higher density fraction (d=1.07-1.21 g/ml) has taken place during the incubation period. The net transfer of free cholesterol from the lower density fraction (d <1.07 g/ml) to higher density fraction (d=1.07-1.21 g/ml) was about the same in both age groups. However, the fraction of the total pool transferred was significantly decreased (P<0.05) in aged rats (41 % vs 25 %).

**Substrate Specificity studies**

In order to determine age-related changes in LCAT substrate potential of individual lipoprotein fractions, the following experiment was performed. In this experiment, the endogenous LCAT activity of
the plasma was first inhibited by DFP. Plasma lipoproteins were isolated by preparative ultracentrifugation and subsequently, HDL and VLDL were separated by Bio-Gel A-5 M column (Rudel et al., 1974). The isolated lipoprotein fractions were concentrated, dialyzed, and subsequently tested as LCAT substrates. The lipoprotein-free infranatant from aged rat plasma (without DFP treatment) served as the LCAT enzyme source.

Figure 9 shows the reactivity of individual lipoproteins from young and aged rats with partially purified LCAT. Enzyme activity was determined for a range of free cholesterol concentrations (12.3-98.4 nmol/ml) of the isolated lipoprotein substrates. When HDL from young rats was used as the substrate, the rate of cholesterol esterification was substantially higher than with the HDL from the aged rats (especially at higher FC concentrations). VLDL, whether from young or aged rats, showed only marginal reactivity with LCAT. The activity using LCAT from lipoprotein-free infranatant prepared from the plasma of young rats gave identical results. These observations suggest that HDL from young rat plasma provides a better substrate for LCAT than the HDL from aged rat plasma.

**Characterization of Lipoprotein Substrates for LCAT**

As a first step to investigate the different properties of lipoprotein substrates with age, the molecular weights of HDL from young and
FIGURE 9. REACTIVITY OF LIPOPROTEINS ISOLATED FROM THE PLASMA OF YOUNG AND AGED RATS WITH PARTIALLY PURIFIED LCAT

Aliquots containing (12.3-98.4 nmol/ml) HDL (—), VLDL (—o—) from young rat plasma and HDL (—), VLDL (—o—) from aged rat plasma were preincubated with 20 µl of serum albumin/[^3H]-cholesterol emulsion for 4 hr at 4°C. The final assay volume was adjusted to 210 µl and the LCAT reaction was initiated by adding 20 µl of partially purified enzyme.
FIGURE 10. DETERMINATION OF MOLECULAR WEIGHT OF HDL

HDL from young and aged rat plasma were isolated by preparative ultracentrifugation and Bio-Gel A-5 M column chromatography as described in methods. Molecular weights of HDL from both age groups were determined by Bio-Gel A-5 M column chromatography (1.5 x 90 cm; 0.01 M Tris-HCl, pH 7.4, 0.01 % EDTA, 0.15 M NaCl). The standard proteins used were A: Ferritin (450,000), B: Katalase (240,000), C: Egg albumin (45,000), D: Chymotrypsinogen (25,000).
aged rats were determined by gel chromatography on a calibrated Bio-Gel A-5 M column. The results presented in Figure 10 show that the apparent molecular weights of the HDL from young and aged rats are 240,000 and 600,000 daltons, respectively. Molecular weight of HDL from aged rats was 2.5 times greater than that of HDL from young rats. The substantial difference in molecular weight of HDL prompted additional studies including compositional analysis of the lipoproteins of young and aged rats.

The chemical composition of the plasma VLDL and HDL isolated from the pooled plasma of young and aged rats by Bio-Gel A-5 M chromatography is shown in Table VII. Free cholesterol, cholesteryl esters, triglycerides, phospholipids and protein concentrations were determined and the relative proportion of each component (weight %) were calculated. There was no marked difference in the relative proportion of each lipid component between the HDL from young and aged rats. The parameters previously related to LCAT activity such as the ratio of cholesteryl ester to free cholesterol (CE/FC) and the ratio of phospholipids to free cholesterol (PL/FC) in HDL (Fielding and Fielding, 1972) showed no appreciable differences between the respective HDL samples of young and aged rats.

In order to investigate the differences in phospholipid fractionation, the total phospholipids of HDL samples were taken for the separation of different phospholipid type by thin layer
TABLE VII
COMPOSITION OF LIPOPROTEINS ISOLATED FROM YOUNG AND AGED RATS

<table>
<thead>
<tr>
<th>Component</th>
<th>HDL Young rat</th>
<th>HDL Aged rat</th>
<th>VLDL Young rat</th>
<th>VLDL Aged rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>29</td>
<td>27</td>
<td>11</td>
<td>6.0</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>37</td>
<td>36</td>
<td>8.4</td>
<td>9.9</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>24</td>
<td>27</td>
<td>7.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Free Cholesterol</td>
<td>8.3</td>
<td>9.8</td>
<td>5.4</td>
<td>5.6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.9</td>
<td>0.3</td>
<td>68</td>
<td>72</td>
</tr>
</tbody>
</table>

Lipoproteins (d<1.225 g/ml) were isolated from pooled plasma by ultracentrifugation and subsequently separated on a Bio-Gel A-5 M column. VLDL and HDL peaks were pooled according to the elution volumes of standard human lipoprotein fractions (isolated by sequential ultracentrifugation), concentrated and dialyzed. The concentration of each component (mg/dl plasma) was determined and the relative proportion of each component (wt %) was calculated.
chromatography. Plates were developed in a special solvent system as described under Method (Gilfillan et al., 1983). The phospholipid bands corresponding to sphingomyelin (SM), phosphatidyl choline (PC), phosphatidyl inositol (PI) and phosphatidyl serine (PS), phosphatidyl ethanolamine (PE) were visualized and measured. Table VIII shows weight per cent (wt %) of each phospholipid type present in HDL from young and aged rat plasma. The amount of sphingomyelin increased in HDL from aged rat plasma, while the amount of phosphatidyl choline decreased in the same HDL sample compared to those of HDL from young rat plasma. Accordingly, the ratio of phosphatidyl choline to sphingomyelin (PC/SM) in the HDL of aged rats was markedly lower compared to that in the HDL of young rats (2.9 and 5.4 in the HDL of aged and young rats respectively).

In addition to phospholipid composition, apolipoprotein composition of HDL samples was compared in young and aged rats. HDL isolated from young and aged rat plasma was subjected to 10 % polyacrylamide gel electrophoresis in the presence of 0.1 % SDS (SDS-PAGE). Figure 11 shows the SDS-PAGE pattern of the respective HDL. Apo A-I, apo A-IV, apo E and apo C's were shown as major apolipoprotein components of HDL from both age groups. Apo A-I bands showed about the same intensity in HDL from both young and aged rats. Compared to that, there was a substantial difference in apo E bands between the two HDL samples. The HDL from aged rats was
TABLE VIII
PHOSPHOLIPID FRACTIONATION OF THE HDL FROM
YOUNG AND AGED RATS

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Young rat HDL wt (%)</th>
<th>Aged rat HDL wt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>14.3</td>
<td>24.1</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>77.2</td>
<td>69.4</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>4.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>3.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Total phospholipids of HDL samples were extracted. An aliquots was taken for the separation of phospholipids by thin-layer chromatography. Plates were developed using chloroform-methanol-petroleum ether (bp 35-60°C)-acetic acid-boric acid 40:20:30:10:1.8 (v/v/v/v/w). The phospholipids bands were visualized by iodine vapor and identified by comparison to standards run alongside the samples. The bands corresponding to PC, PI, PE and SM were scraped into tubes and phospholipids were extracted and measured.
FIGURE 11. APOLIPOPROTEIN PROFILE OF HDL BY SDS-PAGE

HDL from young and aged rat plasma were isolated by preparative ultracentrifugation and gel chromatography (Bio-Gel A-5 M). Same amount of HDL protein (50μg) was used for SDS-PAGE. A slab gel of 10% acrylamide was used in the presence of 0.1% (w/v) SDS.
found to be enriched with apo E.

Subsequently the HDL from both age groups was further subfractionated by heparin-Sepharose chromatography as described under Methods (Quarfordt et al., 1978). The heparin-Sepharose column is known to retain arginine-rich, apo E containing HDL by charge interaction (Mahley et al., 1979). Equal amounts of HDL protein (230 ug) were loaded on the column and the HDL subfractions were eluted with a stepwise salt gradient (0.05 M NaCl and 1.5 M NaCl). Figure 12 shows the A280 pattern of HDL subfractions by heparin-Sepharose column chromatography. The major subfraction, representing the HDL without apo E eluted in the void volume. The apo E containing HDL were eluted from the column upon increasing the NaCl concentration of the elution buffer. The data presented in Figure 12 indicate that the HDL from aged rat plasma contains a higher proportion of apo E enriched HDL compared to the HDL from young rat plasma.

Next, the reaction of the individual subfractions of HDL (HDL-A and HDL-E) with partially purified LCAT was investigated. In this experiment, each subfraction was first labeled with [3H]-cholesterol (Stokk and Norum, 1971) and then incubated with partially purified LCAT preparations. The rate of cholesterol esterification was determined as described under Methods (Jahani and Lacko, 1982). Figure 13 shows the substrate potential of individual HDL subfractions
Equal amount of HDL protein (230 μg) from young and aged rat plasma were applied to a heparin-sepharose column (2.5 X 12cm) and eluted with a stepwise salt gradient (0.05 M and 1.5 M NaCl in 0.005 M Tris-HCl, pH 7.4). The flow rate was 30 ml/hr and 2 ml fractions were collected.
FIGURE 13. REACTIVITY OF HDL-A AND HDL-E WITH LCAT

The eluted fractions from heparin-sepharose column chromatography were pooled, concentrated and dialyzed against 0.01 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.01 % EDTA. The rate of cholesterol esterification was determined for the individual preparation in the same way as described for "Reactivity of lipoproteins isolated from the plasma of young and aged rats with partially purified LCAT".
(HDL-A and HDL-E). The results presented here indicate that apo E containing subfracton of HDL has lower substrate capability than its apo A-I enriched counterpart.

**Study of Ethinyl Estradiol Treatment**

**In Young and Aged Rats**

The expression of the hepatic apo B, E receptors is known to be reduced in mature animals (Mahley and Innerarity, 1983). It is also known that its expression can be enhanced by pharmacological doses of ethinyl estradiol. In order to examine if such changes can be induced in aged animals, young and aged rats were treated with ethinyl estradiol (5 mg/kg body weight/day) for 7 days. Control animals were pair fed to compensate for the reduced food intake induced by ethinyl estradiol treatment.

**Effects of ethinyl estradiol on body weight and liver weight:** Table IX shows the effects of ethinyl estradiol treatment on body weight and liver weight in young and aged rats. Because of reduced food consumption during the treatment period, body weights of both control and ethinyl estradiol treated groups decreased. The liver weight, however, increased significantly (P<0.05) with ethinyl estradiol treatment in both young and aged rats (9.73±0.25 to 12.6±0.19 and 11.4±0.03 to 15.4±0.60, respectively). Since the increase in liver weights is related to the change of body weights, the
TABLE IX

EFFECTS OF ETHINYL ESTRADIOL TREATMENT (E.E. Rx.)
ON BODY WEIGHT AND LIVER WEIGHT
IN YOUNG AND AGED RATS

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body wt. (g)</th>
<th>Liver wt. (g)</th>
<th>Liver wt. per 100 g B.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>YOUNG RATS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>371±4.9\textsuperscript{a}</td>
<td>296±22</td>
<td>9.73±0.25\textsuperscript{ab}</td>
</tr>
<tr>
<td>E.E. Rx.</td>
<td>380±8.9</td>
<td>316±8.5</td>
<td>12.6±0.19\textsuperscript{ad}</td>
</tr>
</tbody>
</table>

AGED RATS

| Control         | 423±16\textsuperscript{a} | 376±11        | 11.4±0.03\textsuperscript{bc} | 3.03±0.08\textsuperscript{a} |
| E.E. Rx.        | 428±12        | 338±19        | 15.4±0.60\textsuperscript{cd} | 4.57±0.20\textsuperscript{a} |

Ethinyl estradiol (5 mg/kg body weight) was administered to young (6 month) and aged (22 month) rats for 7 days. The control rats were pair fed based on the amount of food consumed by the treatment group (Rx). Data are means ± SEM. Numbers in each column with identical letter superscripts are significantly different from one another, with P<0.05.
liver weight was normalized in terms of liver weight per 100 g body weight. The results of Table IX suggest that upon ethinyl estradiol treatment, the liver weights increase to a similar extent in both young and aged rats.

Effects of ethinyl estradiol on the concentrations of lipids and protein components of lipoprotein fractions: In order to determine the extent of the hypolipidemic effects caused by hepatic apo B, E receptor expression upon ethinyl estradiol treatment, the concentrations of lipids and protein in each lipoprotein fraction of young and aged rat plasma were measured and compared. Lipoproteins were isolated from pooled plasma by preparative ultracentrifugation and separated on a Bio-Gel A-5 M column. VLDL, LDL, and HDL peaks corresponding to the elution volume of standard human lipoprotein fractions isolated by sequential ultracentrifugation were pooled, concentrated, and dialyzed prior to analysis. Total cholesterol, free cholesterol, cholesteryl esters, triglycerides, phospholipids, and proteins were determined as shown in Table X. Upon ethinyl estradiol treatment, the extent of plasma total cholesterol reduction was smaller in aged rats (47 %) than that in young rats (57 %). However, the reduction of HDL-cholesterol was nearly the same in both age groups (64 % in young and 63 % in aged rats, respectively). LDL-cholesterol levels were very low in the young rat plasma and were not affected by ethinyl estradiol treatment (1.5 vs
TABLE X
EFFECTS OF ETHINYL ESTRADIOL (E.E.Rx.) ON THE
CONCENTRATIONS OF LIPIDS AND PROTEIN IN EACH
LIPOPROTEIN FRACTION OF YOUNG AND AGED RAT PLASMA

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
</tr>
<tr>
<td>YOUNG CONTROL</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>6.62</td>
</tr>
<tr>
<td>LDL</td>
<td>1.52</td>
</tr>
<tr>
<td>HDL</td>
<td>43.1</td>
</tr>
<tr>
<td>YOUNG E.E. Rx.</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>4.91</td>
</tr>
<tr>
<td>LDL</td>
<td>1.56</td>
</tr>
<tr>
<td>HDL</td>
<td>15.4</td>
</tr>
<tr>
<td>AGED CONTROL</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>7.93</td>
</tr>
<tr>
<td>LDL</td>
<td>18.0</td>
</tr>
<tr>
<td>HDL</td>
<td>99.1</td>
</tr>
<tr>
<td>AGED E.E. Rx.</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>19.0</td>
</tr>
<tr>
<td>LDL</td>
<td>10.7</td>
</tr>
<tr>
<td>HDL</td>
<td>36.8</td>
</tr>
</tbody>
</table>

Lipoproteins (d<1.225 g/ml) were isolated from pooled plasma by ultracentrifugation and separated on a Bio-Gel A-5 M column. VLDL, LDL, and HDL peaks, corresponding to the elution volumes of standard human lipoprotein fractions isolated by sequential ultracentrifugation were pooled for analysis.
1.5 mg/dl). The LDL-cholesterol levels were high in aged rat plasma and were markedly reduced by ethinyl estradiol treatment (18.0 vs 10.0 mg/dl). On the other hand, VLDL-cholesterol, triglycerides, and phospholipids were markedly increased in aged rats upon ethinyl estradiol treatment, but were decreased in young rats. The HDL-protein levels were markedly decreased upon ethinyl estradiol treatment in both young and aged rats.

Based on the results obtained in Table X, the changes in the cholesterol content of plasma lipoprotein pools upon ethinyl estradiol treatment were estimated. The plasma lipid pool was calculated as the product of the cholesterol concentration (mg/ml) and the plasma volume which is assumed to be 40 ml/kg body weight; \[\text{plasma lipid pool} = \text{cholesterol concentration (mg/ml)} \times \text{plasma volume (40 ml/kg body weight)}\]. Table XI shows the amount of cholesterol cleared from the plasma upon ethinyl estradiol treatment. This was calculated as the difference of plasma lipid pool between the control group and treatment group; \[\text{changes} = \text{control group} - \text{treatment group}\]. Upon ethinyl estradiol treatment, the total amount of cholesterol cleared from the plasma in aged rats was about three times that from young rats (9.8 mg/7 day in aged rats and 3.29 mg/7 day in young rats, respectively). The effect of ethinyl estradiol treatment on the cholesterol pool cleared from the plasma was most profound in HDL-cholesteryl esters in both age groups.
TABLE XI

CHANGES IN THE CHOLESTEROL CONTENT OF PLASMA LIPOPROTEIN POOLS UPON ETHINYL ESTRADIOL TREATMENT

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>TC</th>
<th>FC</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/7 days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YOUNG RATS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>-0.14</td>
<td>+0.14</td>
<td>-0.28</td>
</tr>
<tr>
<td>LDL</td>
<td>0</td>
<td>+0.14</td>
<td>-0.14</td>
</tr>
<tr>
<td>HDL</td>
<td>-3.15</td>
<td>-0.35</td>
<td>-0.28</td>
</tr>
<tr>
<td>AGED RATS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>+1.33</td>
<td>+0.35</td>
<td>+0.98</td>
</tr>
<tr>
<td>LDL</td>
<td>-1.26</td>
<td>+0.70</td>
<td>-1.96</td>
</tr>
<tr>
<td>HDL</td>
<td>-9.87</td>
<td>-1.40</td>
<td>-8.47</td>
</tr>
</tbody>
</table>

Changes in the cholesterol content of plasma lipoprotein pools upon ethinyl estradiol treatment were calculated based on the results obtained in TABLE X. Plasma lipid pool was calculated as the product of the cholesterol concentration (mg/ml) and the plasma volume (ml). Plasma volume was assumed to be 40 ml/kg body weight. Changes in the cholesterol content of plasma lipoprotein pools = Control group - Ethinyl estradiol treatment group.
The effects of ethinyl estradiol treatment on the activity of LCAT: Plasma LCAT activity was determined by two different methods using exogenous and endogenous substrates as described in Methods (Chen and Albers, 1982 and Stokke and Norum, 1971, respectively). The results are shown in Table XII. LCAT activity determined using exogenous (proteoliposome) substrate was decreased in the ethinyl estradiol treated group in both young and aged rats. As reported by Carlile et al. (1986), the data here also show that LCAT activity measured by endogenous substrate method (the fractional rate of endogenous cholesterol esterification) is lower in aged rats than that of young rats (10.0 %/hr vs 6.8 %/hr). However, upon ethinyl estradiol treatment, fractional rate of LCAT activity was found to be similar in both age groups (4.8 %/hr vs 4.3 %/hr).
TABLE XII

THE EFFECTS OF ETHINYL ESTRADIOL TREATMENT ON THE ACTIVITY OF LECITHIN : CHOLESTEROL ACYLTRANSFERASE

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>LCAT activity (^a) CE(%)/4 hrs</th>
<th>LCAT activity (^b) CE(%)/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOUNG RATS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.3±0.3</td>
<td>10</td>
</tr>
<tr>
<td>E.E.Rx.</td>
<td>4.0±0.3</td>
<td>4.8</td>
</tr>
<tr>
<td>AGED RATS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.2±0.3</td>
<td>6.8</td>
</tr>
<tr>
<td>E.E.Rx.</td>
<td>3.2±0.9</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Data are based on determination of triplicate samples and expressed as mean ± SEM.

\(^a\) Measured by exogenous (proteoliposome) substrate

\(^b\) Measured by endogenous substrate
CHAPTER IV

DISCUSSION

It is now generally accepted that the LCAT reaction takes place mainly on the surface of HDL molecules (Glomset et al., 1966; Lacko et al., 1974; Chen and Albers, 1982; Chung et al., 1982). Further evidence available in the literature supports the notion that the enzyme substrate complex for the LCAT reaction consists of a specific subfraction of HDL composed of LCAT and a number of discrete lipid and polypeptide components. Fielding and Fielding (1981b) have suggested that LCAT, specific components of HDL and cholesteryl ester transfer protein (CETP) function as a cholesteryl ester transfer complex that facilitates reverse cholesterol transport. One of the main functions of the proposed reverse cholesterol transport system is to prevent the accumulation of excess cholesterol in peripheral tissues. Because unesterified cholesterol (UC) is in a dynamic equilibrium between lipoproteins and cell membranes, the efficient removal of cellular cholesterol requires the establishment of a UC gradient between peripheral tissues and plasma lipoproteins. Accordingly, UC in lipoprotein has to be 'trapped' in the interior of the lipoproteins and thus made unavailable for equilibration with the peripheral cells.
In the plasma, the trapping of UC is accomplished by its esterification catalyzed by the enzyme LCAT. Thus the activity of LCAT in the blood controls the extent to which UC is trapped in HDL and the rate at which UC is subsequently transported from the peripheral cells to the liver or from lipoproteins to the cells (Glomset, 1968; Fielding, 1984; Rothblat and Phillips, 1982).

Earlier studies showed that in rats, the fractional rate of endogenous cholesterol esterification decreased with age (Carlile and Lacko, 1981; Carlile et al., 1986). Although this reaction is catalyzed by the enzyme LCAT, the activity of this enzyme in the plasma does not appear to change with age when an exogenous lipoprotein substrate was used to measure plasma LCAT activity. Since plasma LCAT activity is directly proportional to LCAT mass measured by radioimmuno assay (Albers et al., 1981), these observations suggest that circulating LCAT concentration does not change with age.

The studies presented here were mainly designed to provide information on the mechanism(s) of the age-related decrease of the fractional rate of cholesterol esterification (FRE) in rats, specifically:

1. the site of esterification of cholesterol by LCAT in the plasma;
2. transfer of lipid substrate(s) from donor lipoprotein(s) to the substrate lipoprotein(s);
3. the reactivity of the enzyme with major and minor subfractions of plasma lipoproteins; and
4. the properties of lipoprotein substrate(s) for LCAT.

In addition, preliminary studies were carried out to gain additional insights on the mechanism(s) of hypercholesterolemia in aged rats.

Previous studies have shown that most of the plasma LCAT activity is associated with the HDL fraction when human or hog plasma was subjected to molecular sieve chromatography (Park et al., 1984; Park and Lacko, 1986; Cheung et al., 1986). These observations taken together with other findings obtained with liposome substrates (Jonas et al., 1984; Nishida et al., 1984) indicate that, the HDL/LCAT complex may be held together such that the dissociation of this complex may be unlikely under physiological conditions. Accordingly, this complex may function as a unit from the time it is formed and until it is removed from the circulation. The chromatography pattern shown in Figure 5 represents similar data for rat plasma; LCAT activity mainly associates with HDL containing lipoprotein fraction. Similar results were observed upon 24 hour density gradient ultracentrifugation of the plasma. The data presented in Figure 6 show that most of the LCAT activity is found associated with the lipoprotein containing fraction, which is also characteristic of HDL (d=1.125-1.21 g/ml density fraction).

However, when the plasma was subjected to more prolonged (40 hour) ultracentrifugation, more than 90 % of the total LCAT activity
was recovered from the lipoprotein free infranatant (Figure 7). These observations suggest that prolonged ultracentrifugation results in the dissociation of the enzyme from its complex with HDL as was found before in human plasma (Fielding and Fielding, 1980a; Chen and Albers, 1982; Chung et al., 1982). The minor portion of the LCAT activity remaining with the lipoprotein containing fraction following 40 hour preparative ultracentrifugation was still found associated with the HDL fraction upon gel chromatography (Figure 8). Interestingly, the amount of lipoprotein-associated LCAT activity that remained with the HDL fraction was considerably higher in the samples from aged rats as compared to those of young rats (Figure 8). These data suggest that LCAT may be more tightly bound to HDL in the plasma of aged rats than in young rats. Whether this "tighter" binding of LCAT to HDL in aged rats, is related to the decreased fractional rate of esterification of free cholesterol with age remains to be determined.

Although plasma HDL is known to be the physiological substrate for the LCAT reaction (Glomset, 1968), VLDL and LDL are also known to contribute lipid substrates for the reaction by first transferring them to HDL (Fielding and Fielding, 1980a; Park et al., 1987). In accord with earlier findings (DeLamatre et al., 1983; Swaney et al., 1987), the data presented in Table VI indicate that in vitro net transfer of FC from the lower density fractions (d <1.07 g/ml) to the higher density fraction (d= 1.07-1.21 g/ml) provides most of the free cholesterol
substrate for esterification in rat plasma during prolonged incubation. Furthermore, these data suggest that the net transfer of free cholesterol from the lower density fractions (d <1.07 g/ml) to higher density fraction (d=1.07-1.21 g/ml) is not substantially affected in aging. The exact mechanism of the net movement of FC from either VLDL or LDL to the HDL-LCAT complex is presently unknown. Free cholesterol exchanges rapidly among lipoproteins. However, this pathway does not lead to net changes in the amount of free cholesterol content of lipoproteins. The mechanism of exchange is believed to involve desorption of FC molecules from the donor lipid/water interface and subsequent diffusion through the aqueous phase and finally collision with an acceptor particle (Lund-Katz et al., 1982). A similar process, controlled by the free cholesterol/phospholipid (FC/PL) ratio, is involved in the net movement of FC from cell membrane surfaces to lipoproteins and vice versa (Rothblat and Phillips, 1982). According to Tall (1986) the spontaneous exchange of lipoprotein lipids is insufficient to account for the net changes observed during lipoprotein metabolism. Thus, a process promoted by plasma (apolipoprotein) factors is the favored mechanism for the transfer of plasma lipids among lipoproteins. At present, the details of this process are not known. Whatever the mechanism of transfer of FC from the lower density fractions to higher density fraction is, it does not appear to be affected in aging rats.
Upon incubation of the whole plasma, the free cholesterol decrease in d=1.07-1.21 g/ml fraction from aged rats was lower than the decrease seen in the same density fraction from young rats (1.1 mg/dl in aged vs. 2.3 mg/dl in young). These changes occurred despite the fact that the total pool of FC in this density fraction was higher in aged rats compared to the FC pool of young rats. These data suggest that HDL from aged rats may be a relatively poor substrate for the LCAT reaction.

The decrease in the fractional rate of plasma cholesterol esterification often coincides with a substantial expansion of the plasma free cholesterol pool (Soloff et al., 1978; Lacko et al., 1978). Therefore, the LCAT substrate potentials of individual lipoprotein fractions obtained from both young and aged rats were compared to elucidate the nature of the decline of FRE with age. The data presented in Figure 9 suggest that HDL is a much better substrate than VLDL for LCAT and the HDL from young rats is a better substrate than HDL from aged rats especially at higher FC concentrations. The results were the same regardless of whether the LCAT was prepared from the plasma of young or aged rats. These data suggest that the decreased FRE observed in aged rats (Carlile et al., 1986; Carlile and Lacko, 1981) is not a direct consequence of FC pool size or some defect in the LCAT enzyme itself but it is likely to be due at least in part to changes in the nature of the substrate lipoproteins.
HDL represent a family of particles differing in composition, size and density due to extensive remodeling during its maturation (Eisenberg, 1984). The decreased reactivity of LCAT towards the HDL substrate from aged rats could be due to (1) changes in substrate composition, (2) change in particle size, (3) and accumulation of product lipoproteins or to a combination of all these factors. The compositional analysis of lipoprotein substrates (Table VII) suggests that the relative proportion of total protein and lipid components is nearly the same in the HDL substrate isolated from young and aged rats. The parameters previously suggested to modulate LCAT activity such as the ratio of cholesteryl ester to free cholesterol (CE/FC) and the ratio of phospholipid to free cholesterol (PL/FC) in HDL (Jahani and Lacko, 1981) were also similar. However, there were several significant differences between properties of the lipoproteins of young and aged rats. Firstly, the molecular weight of aged rat HDL was much higher (x 2.5) than that of young rat HDL (Figure 10). This may indicate that aged rat HDL particles become larger than young rat HDL particles without marked differences in composition of their respective major lipid components. Earlier studies from our own and from other laboratories suggested that smaller particles of HDL were better substrates for LCAT (Barter et al., 1985; Jahani and Lacko, 1981; Glomset, 1972; Fielding and Fielding, 1971). Secondly, there was a marked difference in the phospholipid component of HDL
substrates between young and aged rats. Earlier, Malhotra and Kritchevsky (1978) had shown that all lipoprotein classes from plasma of young rats contained more phosphatidyl choline and less sphingomyelin than that of plasma from aged rats. Our data also showed increased amounts of sphingomyelin in HDL fraction of aged rat plasma. Accordingly, the ratio of phosphatidyl choline/sphingomyelin (PC/SM) was lower in aged rat plasma (Table VIII). The increased sphingomyelin levels in aged rat HDL may reflect a general change in substrate specificity for LCAT by affecting some changes in the fluidity of substrate lipoprotein molecules. Thirdly, one of the most pronounced age-related changes observed was the enrichment of apo E in the HDL of aged rats (Figures 11 and 12). These findings are in good agreement with the large increase in the lipoproteins of d=1.025-1.07 g/ml fraction in aged rat plasma (Table V) as most of the apo E containing HDL$_1$ has been isolated in this fraction (Eisenberg, 1984). "Nascent" apo E-rich, cholesteryl ester poor HDL, isolated from rat liver perfusate is known to be a superior substrate for LCAT (Hamilton et al., 1976). However, these discoidal (nascent) lipoprotein particles circulate only for a very brief period (Turner et al., 1979) as they are rapidly converted to spherical (mature) HDL via the action of LCAT. Consequently, the nascent HDL species have only been found in significant amounts in the plasma of patients with liver disease (Sabesin et al., 1979) or with inherited
LCAT deficiency (Glomset and Norum, 1973; Forte et al., 1971).

However, HDL\textsubscript{1} accumulating in plasma from aged rats are cholesteryl ester-rich particles. Our data show that the apo E containing HDL isolated from the rat plasma has a lower substrate potential for LCAT as compared to the apo A-I-rich HDL isolated from the same plasma (Figure 13). These findings are in agreement with earlier reports with human lipoproteins (Marcel et al., 1980). Consequently, the increased levels of the apo E-rich HDL subclass in aged rats may also contribute to the decreased LCAT substrate potential of the total HDL pool. This postulate is supported by the findings that the fractional rate of plasma cholesterol esterification in rats is decreased upon feeding of a semisynthetic diet with casein, in copper deficiency, hypothyroidism, and cholesterol feeding. These conditions are also known to be associated with increased levels of apo E-rich HDL (Kudchodkar et al., 1988; Lefevre et al., 1986; Lau and Klevay, 1981; Delamatre and Roheim, 1981). Further support for this suggestion comes from the findings obtained in preliminary studies using ethinyl estradiol treatment. The fractional rate of endogenous cholesterol esterification became similar in young and aged rats upon ethinyl estradiol treatment (Table XII). It has been shown that apo E containing HDL was markedly decreased in both young and aged rats upon ethinyl estradiol treatment (Lee et al., 1987).

Apo E enriched lipoproteins are generally removed more rapidly
from the circulation by hepatic receptor-mediated mechanism (Mahley et al., 1984). It has been found that hepatic receptor activity is decreased in hypothyroidism and upon feeding a semisynthetic diet containing casein. Under these two conditions, apo E-rich HDL accumulated in the plasma. Therefore, the accumulation of apo E-rich HDL in the plasma of aged rats suggests that hepatic receptor-mediated uptake of these lipoproteins could be decreased during aging. Experiments performed with dogs suggests that the expression of hepatic apo B, E receptors is reduced in mature animals (Mahley and Innerarity, 1983). Possible causes for the age-related decline of hepatic apo B, E receptor activity include: (1) the feedback inhibition of apo B, E receptor synthesis due to an increase in the cholesterol content of the cell, [for example, when excess cholesterol accumulates in the liver, the liver responds by decreasing the production of apo B, E receptors (Goldstein and Brown, 1977; Brown and Goldstein, 1986)]; (2) the defective or inadequate synthesis of receptors by the aging liver as a result of the generalized loss of the biosynthetic capability of the aging liver cell; or (3) both. Experiments conducted with mature animals indicate that the expression of apo B, E receptor can be induced by reducing the cholesterol content of the cell by dietary means or by injecting pharmacological doses of ethinyl estradiol. Administration of pharmacological amounts of ethinyl estradiol (5 mg/kg body weight daily for 5 days) increases the number
of LDL (apo B, E) receptors in rat hepatocytes ten to twentyfold as a result of proliferation of the liver cell (Windler et al., 1980; Kovanen et al., 1979; Kovanen et al., 1981; Mahley and Innerarity, 1983). Treated animals develop a profound hypolipidemia, which encompasses all of the major classes of lipoproteins (Chao et al., 1979; Hay et al., 1971). Preliminary studies were performed to test if the defective or inadequate synthesis of receptors by the aging liver was responsible for the age-related decrease of cholesterol removal from the plasma. The data presented in Table IX suggest that the liver weight increases to a similar extent upon ethinyl estradiol treatment in both age groups indicating that the proliferation of liver cells by ethinyl estradiol treatment is not affected by aging. The data presented in Tables X and XI suggest that both plasma HDL and LDL cholesterol levels decrease to a similar extent in young and aged rats upon ethinyl estradiol treatment. This may be due to the induction of specific high affinity lipoprotein receptors in the liver. Thus, the study described here, using ethinyl estradiol treatment, provides indirect evidence that the pupative age-related changes in the receptor activity are not due to the defective receptor synthesis. It could, however, be due to the increased cholesterol ester content of the liver cell as has been shown to increase in aged rats (Story et al., 1981). The mechanism by which the cholesterol ester content of the liver increases in aged rats is presently unknown.
Upon ethinyl estradiol treatment, both VLDL-cholesterol and VLDL-triglycerides decreased in young rats but increased in aged rats (Table X). Administration of ethinyl estradiol to rats has been shown to increase the hepatic synthesis and secretion of VLDL (Weinstein et al., 1986; Harvel, 1986). However, the concentrations of VLDL in plasma are lowered by high doses of ethinyl estradiol due to their increased clearance from the plasma (Weinstein et al., 1986). Tissue lipoprotein lipase activity plays a major role in the catabolism and clearance of VLDL from the plasma. Therefore, the accumulation of VLDL in the plasma of aged rats upon ethinyl estradiol treatment could be due to the decreased tissue lipoprotein lipase activity seen earlier in aged rats (Carlile and Lacko, 1985).

It has been shown that dietary factors may be the major determinants in the age-related changes of cholesterol metabolism. Liepa et al. (1980) have found no age-related changes in plasma lipids upon food restriction. Similarly, diets containing plant proteins prevented the age-related increase in the plasma cholesterol levels as well as the decrease of fractional rate of cholesterol esterification (Park et al., 1987b). Recently, Spady and Dietschy (1989) have shown that when hamsters were maintained on a low-cholesterol, low saturated triglyceride diet, the rates of LDL transport into the various tissues of the body and plasma total as well as LDL-cholesterol concentrations remained constant over the entire life span (1-24
months). These studies support the view that changes seen in the composition and the levels of plasma lipoproteins with age are not necessarily the direct consequences of the aging process per se but may be caused by long term diet induced metabolic effects. These findings make the study of the "age-related" alterations in plasma lipids potentially even more important because they imply that these age related changes in plasma lipids and the subsequent cardiovascular pathology seen during aging may be preventable by dietary intervention.

The specific roles of the HDL/LCAT complexes in reverse cholesterol transport in vivo have been proposed to include the conversion of FC to CE in HDL, rendering the HDL particle capable to accept additional FC molecules from peripheral tissues including the arteries, and thus prevent accumulation of cholesterol in those tissues (Glomset and Norum, 1973; Fielding and Fielding, 1980b). The lower rate of esterification of HDL-FC in aging as the result of changes in composition and size, could impede this "reverse cholesterol transport" process and thus may have a significant impact on cholesterol homeostasis.
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