

Lawrence Berkeley National Laboratory

Recent Work

Title

INTERACTION OF SERUM ENZYME LECITHIN: CHOLESTEROL ACYLTRANSFERASE WITH LIPOPROTEINS AND MODEL LIPID SYSTEMS

Permalink

<https://escholarship.org/uc/item/67v7z7sg>

Author

Ho, Walter Kwok Keung.

Publication Date

1972-07-01

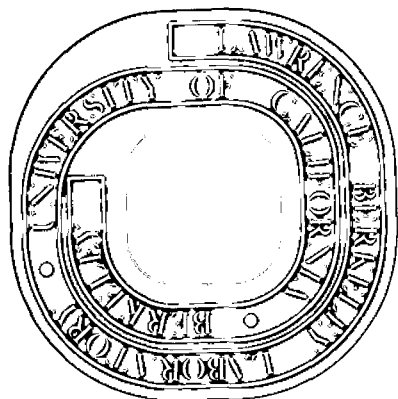
INTERACTION OF SERUM ENZYME LECITHIN:
CHOLESTEROL ACYLTRANSFERASE WITH
LIPOPROTEINS AND MODEL LIPID SYSTEMS

Walter Kwok Keung Ho
(Ph.D. Thesis)

DONNER LABORATORY

July 1972

AEC Contract No. W-7405-eng-48



For Reference

Not to be taken from this room

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

TABLE OF CONTENTS

	Page
Chapter 1. INTRODUCTION	1
I. General Considerations	1
II. Physical and Chemical Characteristics of Serum Lipoproteins	2
a. Chylomicra	3
b. Very Low Density Lipoproteins (VLDL)	8
c. Low Density Lipoproteins (LDL)	10
d. High Density Lipoproteins (HDL)	11
III. Lipoprotein Lipase	13
a. Historical Background	13
b. Physiological Function of Lipoprotein Lipase	14
c. Site of Lipoprotein Lipase Activity	15
d. Interaction of Lipoprotein Lipase with Its Substrate	15
e. Effect of Lipoprotein Lipase on Lipoprotein Distributions	16
f. Significance of Work Done on Lipoprotein Lipase	18
IV. Lecithin: Cholesterol Acyltransferase (LCAT)	18
a. Historical Background	19
b. Site of LCAT synthesis	20
c. Possible Physiological Function of LCAT	20
d. LCAT Deficiency and Its Implications to the Physiological Role of LCAT	22
e. General Objectives of Present Thesis	25
Chapter 2. INTERACTION OF LECITHIN: CHOLESTEROL ACYL-TRANSFERASE WITH SERUM HIGH DENSITY LIPO-PROTEINS AND SONICATED DISPERSIONS OF LECITHIN	26
I. Background and Objectives	26
a. HDL as Primary Substrate for LCAT in Human Serum	26
b. Changes in Physical and Chemical Properties of HDL Following Interaction with LCAT	26
c. Factors Influencing the Reactivity of HDL with LCAT	28
d. Complex Formation of HDL with LCAT	28

e. Interaction of LCAT Activity with Model Lipid Substrates	32
f. Specific Objectives of the Present Chapter	33
II. Materials and Methods	34
a. Preparation of Ultracentrifugal $d > 1.21$ Protein Fraction	34
b. Preparation of Lipoprotein Fractions	34
c. Preparation of Sonicated Dispersions of Lipids	36
d. Preparation of Sonicated Substrates for Assay of LCAT Activity	36
e. Determination of LCAT Activity in Samples	37
f. Lipid Analysis	37
g. Radioassay	40
h. Protein Determination	41
i. Molecular Sieving Chromatography	41
j. Polyacrylamide Gel Electrophoresis	41
III. Results	42
a. Dissociation and Association of Serum LCAT Activity with Lipoproteins	42
b. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin. Study Performed in Sucrose Medium of $d 1.065$ gm/ml	47
c. Effect of Prior Treatment with Mercaptoethanol on LCAT Activity in Ultracentrifugal ($d 1.065$ gm/ml-sucrose) Fractions	50
d. Evaluation of the Effect of Excess Lecithin on Assay of LCAT Activity	53
e. Effect of Lecithin Concentration on Ultracentrifugal ($d 1.065$ gm/ml-sucrose) Flotation of LCAT Activity	53
f. Factors Influencing Formation of the LCAT-Lecithin Dispersion Complex	56
g. Dissociation of LCAT-Lecithin Dispersion Complexes	61
IV. Discussion	64
a. Nature of the LCAT Activity in the $d > 1.21$ Protein Fraction	64
b. Association of LCAT Activity with HDL	65
c. Interaction of LCAT with Sonicated Dispersions of Lecithin	66

Chapter 3.	INTERACTION OF LECITHIN: CHOLESTEROL ACYL-TRANSFERASE WITH SONICATED DISPERSIONS OF LECITHIN. EVALUATION OF COMPLEX FORMATION BY ULTRACENTRIFUGAL FLOTATION IN SALT MEDIA OF d 1.065 and d 1.21 gm/ml	70
I.	Background and Objectives	70
	a. Effect of Salt on the Ultracentrifugal Properties of LCAT Activity in the Presence of Sonicated Dispersions of Lecithin	70
	b. Nature of the LCAT Activity Not Floated in a Salt Medium (KBr) of d 1.065 gm/ml	72
	c. Objectives of the Present Chapter	74
II.	Materials and Methods	75
	a. General	75
	b. Preparation of HDL Protein	75
	c. Preparation of HDL Apolipoproteins	76
	d. Identification of the Sephadex Fractions	79
III.	Results	79
	a. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin. Ultracentrifugation Performed at d 1.21 gm/ml (KBr)	79
	b. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin. Effect of Ultracentrifugation in Salt Solutions (KBr) at Different Densities	82
	c. Effect of HDL Apolipoproteins on Assay of LCAT Activity	88
	d. Effect of Duration of Ultracentrifugation (d 1.21 gm/ml-KBr) on the Association of LCAT Activity with Lecithin Dispersions	88
	e. Ultracentrifugal (d 1.21 gm/ml) Flotation of Lecithin Dispersions Containing LCAT Activity. Low vs. High Ionic Strength	90
	f. Mechanism of ApoLP-gln I Activation	92
IV.	Discussion	101
	a. Recapitulation of the Results on the Ultracentrifugal Flotation of LCAT Activity Using Sonicated Dispersions of Lecithin	101
	b. Implication of the Present Work to Considerations of the Physiological Function of LCAT	103

APPENDIX	111
I. Foreword	111
II. Some Characteristics of LCAT Activity and Its Purification	111
a. Molecular Sieving Chromatography of the $d > 1.21$ Protein Fraction	111
b. DEAE-Cellulose Chromatography of the $d > 1.21$ Protein Fraction	114
c. Ammonium Sulfate Precipitation of LCAT Activity in the $d > 1.21$ Protein Fraction	114
d. Purification of LCAT Activity	117
III. Interaction of LCAT Activity with Sonicated Substrate	124
a. Effect of Size of Sonicated Dispersions on Assay of LCAT Activity Contained in the $d > 1.21$ Protein Fraction	124
b. Effectiveness of Sonicated Dispersions of Mixtures of Cholesterol and Lecithin as Substrates for LCAT. Effects of Charge	127
c. Effect of Mercaptoethanol on Assay of LCAT Activity	128
d. Effect of Mercaptoethanol and Excess Lecithin on Assay of LCAT Activity	130
ACKNOWLEDGMENTS	146
REFERENCES	147

INTERACTION OF SERUM ENZYME LECITHIN: CHOLESTEROL ACYLTRANSFERASE
WITH LIPOPROTEINS AND MODEL LIPID SYSTEMS

ABSTRACT

Walter Kwok Keung Ho

In the work described in this dissertation, I have studied the interaction of lecithin: cholesterol acyltransferase with human serum lipoproteins and sonicated dispersions of lipids. The purpose of these studies was to provide information on 1) the interrelationship between lecithin: cholesterol acyltransferase and serum lipoprotein structure and metabolism, 2) the nature of the association between lecithin: cholesterol acyltransferase and its substrates, 3) the mechanisms by which lipids, as found in lipoproteins, are utilized as substrates for LCAT, and 4) the role of apolipoproteins in enzyme activity.

The dissociation and association of serum lecithin: cholesterol acyltransferase activity with human serum high density lipoproteins were investigated under different experimental conditions. Results of these studies indicate that the association between lecithin: cholesterol acyltransferase activity and high density lipoproteins is sensitive to high ionic strength, and that the affinity of the enzyme for high density lipoproteins is not altered when most of the substrate lipids (i. e., unesterified cholesterol and lecithin) in the high density lipoproteins have been transesterified.

During incubation with essentially lipoprotein-free serum,

sonicated dispersions of lecithin form complexes containing lecithin: cholesterol acyltransferase activity. These complexes can be isolated by ultracentrifugal flotation in a sucrose solution of density 1.065 gm/ml. Determination of lecithin: cholesterol acyltransferase activity contained in these complexes does not require addition of a specific cofactor to the assay medium.

The formation of complexes between sonicated dispersions of lecithin and active lecithin: cholesterol acyltransferase is not affected by inhibition of the enzyme's activity with hydroxymercuribenzoate. However, the ultracentrifugal flotation of such complexes, in a sucrose solution of density 1.065 gm/ml, is highly dependent on the pH and the concentration of lecithin dispersion in the ultracentrifugal medium. Dissociation of the complexes formed between sonicated dispersions of lecithin and active lecithin: cholesterol acyltransferase can be achieved by treatment with sodium taurocholate and high salt concentrations (potassium bromide).

When incubation mixtures of lipoprotein-free serum and lecithin dispersions are ultracentrifuged in potassium bromide solutions of density 1.065 gm/ml and density 1.21 gm/ml, lecithin: cholesterol acyltransferase activity, associated with lecithin dispersions, can be isolated in the density 1.065 gm/ml to 1.21 gm/ml fraction. Assay of enzyme activity in this fraction does not require addition of a specific cofactor. On the other hand, lecithin: cholesterol acyltransferase activity associated with the density greater than 1.21 gm/ml fraction can only be demonstrated after addition of a

high density lipoprotein apolipoprotein (apoLP-gln I). Based on these observations, it is proposed that apoLP-gln I is a major requirement in the transesterification reaction catalyzed by lecithin: cholesterol acyltransferase. The mechanism whereby apoLP-gln I activates lipid dispersions for transesterification probably involves the formation of a high density lipoprotein-like structure between apoLP-gln I and sonicated dispersions of lipids.

When lecithin: cholesterol acyltransferase activity is assayed in the presence of mercaptoethanol and sonicated dispersions of lecithin, an activation of enzyme activity is observed. The basis for such activation is still unknown.

Attempts to purify lecithin: cholesterol acyltransferase, using a combination of ultracentrifugal and chromatographic procedures, resulted in a maximal purification of approximately five hundred fold. Chromatography of lipoprotein-free serum on a Sephadex G-200 column indicates that the molecular weight of lecithin: cholesterol acyltransferase is probably below 100,000 daltons. Ammonium sulfate precipitation of lecithin: cholesterol acyltransferase activity, contained in a lipoprotein-free serum, shows that this enzyme precipitates in the same saturation range as the alpha and beta globulin proteins.

CHAPTER 1.

INTRODUCTION

I. General Considerations

In higher animals serum lipids are complexed with proteins forming lipoproteins which circulate throughout the bloodstream. Lipids, such as triglyceride and cholesterol, synthesized or absorbed in one organ can be transported in lipoprotein form to other organs for either metabolism or storage. The transport of lipids between organs is highly responsive to physiological demands (96). For example, after the ingestion of a fatty meal, the chylomicron level in the bloodstream is usually markedly elevated. Such elevation is believed to result from absorption of dietary fat by the intestine and its subsequent transport within lipoprotein particles to the adipose tissue or liver for storage or metabolism (96). The enzymic and physiological mechanisms by which circulating lipids are channeled into various organs for metabolism and storage are currently under intensive investigation. Both direct and indirect effects of hormones on lipid metabolism have been demonstrated (96).

Two enzyme systems found in the bloodstream or associated tissues have been shown to participate in the metabolism of serum lipoproteins; these are lipoprotein lipase and lecithin: cholesterol acyltransferase (LCAT). The specific mechanisms by which these enzymes channel the lipids of circulating lipoproteins in and out of various organs are under active investigation.

The interaction between lipoproteins and these two enzyme systems raises interesting questions with respect to lipoprotein metabolism as well as lipoprotein structure. Thus, by reacting with the lipid moiety of specific classes of serum lipoproteins, both lipoprotein lipase and LCAT can produce marked changes in the lipid compositions of lipoproteins. Since the lipid moiety of lipoproteins has been shown to play a crucial role in determining lipoprotein structure (48), changes in lipid composition mediated by lipoprotein lipase and LCAT can significantly influence lipoprotein structure.

In the present thesis, I shall describe my investigation of the interaction between LCAT and lipoproteins. It is hoped that these studies may provide a basis for better understanding of 1) the physiological role of LCAT, 2) the importance of this enzyme in the metabolism of lipoproteins, 3) the role of lipoprotein structure in determining the mode of enzyme action, and 4) the possible interrelationship between LCAT and lipoprotein lipase in the metabolism of lipids and lipoproteins.

In order to provide an overview of the present state of knowledge on serum lipoprotein metabolism, I have attempted, in the following sections, to summarize the available data on serum lipoprotein structure, lipoprotein lipase and LCAT. Although these sections may not contain all of the most recent developments on these topics, they provide a general background upon which the present research was based.

II. Physical and Chemical Characteristics of Human Serum Lipoproteins

It is well established that serum lipoproteins are the major complex proteins in the bloodstream which are responsible for the transport of lipids. Since their discovery a great deal of knowledge has been accumulated on their structure and function. In general, serum lipoproteins are designated into four major classes; they are the chylomicra, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Their physicochemical properties are summarized in Tables 1, 2 and 3.

a. Chylomicra.

Chylomicra are the largest of the serum lipoproteins. They are secreted mainly by the gastrointestinal tract during the absorption of dietary fats. The chemical composition of chylomicra consists predominantly of triglyceride with smaller amounts of phospholipid (3-6%), unesterified cholesterol (1-3%), cholesteryl ester (2-4%), and protein (0.5-2.5%). Their protein composition is uncertain; however, they are believed to contain proteins which are usually associated with HDL and VLDL (Table 3) (68). Chylomicra are generally characterized by particle size, electrophoretic mobility, S_f rates and hydrated density. They range in size from 750 Å to 10,000 Å with molecular weights ranging from $10^3 \times$ to $10^4 \times 10^6$ daltons. On paper and agarose gel electrophoresis they remain at the origin, while on starch and cellulose acetate they have an α_2 mobility and migrate with VLDL. Their S_f rate is in the range of from 400 to greater than 10^5 Svedberg flotation units, and their hydrated

TABLE 1

PHYSICAL CHARACTERISTICS OF THE FOUR MAJOR CLASSES OF NORMAL HUMAN SERUM LIPOPROTEINS¹.

Lipoprotein	Flotation Rate ²	Density ³	Mobility ⁴	Molecular Wt. ⁵	Size (Å) ⁶	Conformation ⁷
Chylomicrons	S _f >400	<0.95	Origin	10 ³ -10 ⁴ X 10 ⁶	750-10,000	-
VLDL	S _f 20-400	0.95-1.006	Prebeta	5-10 X 10 ⁶	300-800	-
LDL	S _f 0-20	1.006-1.063	Beta	2.1-2.6 X 10 ⁶	205-220	Mostly beta, some alpha helix and random coil.
HDL	F _{1.20} 0-20	1.063-1.21	Alpha	1.7-3.6 X 10 ⁵	75-100	60-65% alpha helix, 35-40% random coil.

¹ Data obtained from references 47, 68, 71, 78, 80 and 98.

² S_f, lipoprotein flotation rate in Svedberg units (10⁻¹³ cm/sec/dyne/gm) in a NaCl solution of density equal to 1.063 gm/ml at 26°C, 52,640 rpm. F_{1.20}, lipoprotein flotation rate in Svedberg units in a NaCl-NaBr solution of density equal to 1.20 gm/ml at 26°C, 52,640 rpm.

³ In gm/ml.

⁴ As determined by paper electrophoresis.

⁵ In daltons.

⁶ As determined by electron microscopy.

⁷ As determined by circular dichroism and optical rotatory dispersion analysis of native lipoproteins.

TABLE 2

COMPOSITION¹ OF MAJOR LIPOPROTEIN CLASSES OF NORMAL HUMAN SERUM².

Lipoprotein Constituents	Chylomicron	VLDL	LDL	HDL
Protein	1-2	10	25	45-55
Triglyceride	80-90	50-70	10	3
Unesterified cholesterol	1-3	10	8	15
Cholesteryl ester	2-4	5	37	22
Phospholipid	3-6	15-20	22	30
Carbohydrate	-	<1	1	<1

¹ % of dry weight.² Data obtained from reference 68.

TABLE 3

THE APOLIPOPROTEINS OF THE FOUR MAJOR LIPOPROTEIN CLASSES OF NORMAL HUMAN SERUM¹.

Apolipoprotein ²	Chylomicron	VLDL	LDL	HDL
ApoLP-ser	Unknown	Major ³	Minor	Minor ⁴
ApoLP-glu	Unknown	Major ³	Minor	Minor ⁴
ApoLP-ala	Unknown	Major ³	Minor	Minor ⁴
ApoLDL	Unknown	Major	Major	Absent
ApoLP-gln I	Unknown	Minor	Trace	Major (approx. 68% of HDL protein)
ApoLP-gln II	Unknown	Minor	Trace	Major (approx. 20% of HDL protein)

¹ Data obtained from references 6, 66, 68 and 108.

² According to nomenclature suggested by Levy et al. (68). ApoLP-ser, apoLP-glu, apoLP-ala and apoLP-gln (either I or II) designate apolipoprotein with a carboxyl-terminal amino acid serine, glutamic acid, alanine and glutamine respectively. See Table 4 for correlation of apolipoprotein nomenclatures of other authors.

³ These three apolipoproteins in combination make up approximately 50% of the VLDL protein. "Major" refers to proteins making up 10% or more of the total protein.

⁴ These three apolipoproteins in combination make up approximately 12% of the HDL protein.

TABLE 4
CORRELATION OF APOLIPOPROTEIN NOMENCLATURES OF VARIOUS AUTHORS.

Levy et al. ¹	Alaupovic ²	Scanu et al. ³	Shore ⁴
ApoLP-ser	Apo C	Fraction V	R-valine
ApoLP-glu	Apo C	Fraction V	R-glutamic acid
ApoLP-ala	Apo C	Fraction V	R-alanine
ApoLDL	Apo B	-	R-serine
ApoLP-gln I	Apo A	Fraction III	R-threonine
ApoLP-gln II	Apo A	Fraction IV	R-glutamine

¹ Data obtained from 18, 19, 20, 55 and 68. Abbreviations for carboxyl-terminal amino acids are described in Table 3.

² Data obtained from reference 66.

³ Data obtained from reference 101.

⁴ Data obtained from references 106, 107 and 108.

density is less than 0.95 gm/ml (68, 71, 116). Data obtained from electron microscopy support the idea that chylomicra are spherical particles consisting of an outer surface, composed primarily of phospholipid and protein, with an internal core of triglyceride in which some cholesteryl ester and possibly unesterified cholesterol are dissolved (116).

b. Very Low Density Lipoproteins (VLDL).

VLDL are triglyceride-rich particles derived mainly from the liver. A main function of this lipoprotein class is the transport of triglyceride to various tissues for metabolism (96). The chemical composition of VLDL consists predominantly of triglyceride (50-70%), with smaller amounts of phospholipid (15-20%), unesterified cholesterol (10%), cholesteryl ester (5%), and protein (10%). Recent studies indicate that the VLDL protein moiety mostly consists of four major apolipoproteins (18, 19, 20, 55). Characteristics of these apolipoproteins are summarized in Table 5. In general, VLDL have a hydrated density ranging between 0.95 and 1.006 gm/ml, S_f rate of 20 to 400 Svedberg flotation units, molecular weight of 5 X to 10 X 10^6 daltons, and prebeta or α_2 electrophoretic mobility on most media (68, 71, 74). The detailed substructure of VLDL is still unknown; however, VLDL have been shown to be of spherical form with diameters ranging from 300 Å to 800 Å (31, 68, 80). As in the case of chylomicra, VLDL probably consist of a spherical apolar core of triglyceride with phospholipid and protein on the surface (74).

TABLE 5

SOME CHARACTERISTICS OF APOLIPOPROTEINS¹.

Apolipoprotein	Molecular Wt. (daltons)	C-terminal Amino Acid	N-terminal Amino Acid	Missing Amino Acids	Conformation	Sialic Acid
ApoLP-ser	7,000	Serine	Threonine	Cysteine, Tyrosine, Histidine	Primarily alpha helix	0
ApoLP-glu	10,000	Glutamic acid	Threonine	Cysteine, Histidine	Primarily random coil	-
ApoLP-ala ₁	10,000	Alanine	Serine	Cysteine, Isoleucine	Primarily random coil	1 mole/ mole
ApoLP-ala ₂	10,000	Alanine	Serine	Cysteine, Isoleucine	Primarily random coil	2 mole/ mole
ApoLDL ²	27,000	Serine	Glutamic acid	-	-	-
ApoLP-gln I	15,000	Glutamine	Aspartic acid	Isoleucine, Cysteine	>90% alpha helix	-
ApoLP-gln II	15,000	Glutamine	Aspartic acid	Histidine, Arginine, Tryptophan, Cysteine	Approximately 40% alpha helix	-

¹ Data obtained from references 18, 19, 20, 55, 66, 68, 101, 106, 107 and 108.

² The chemical and physical nature of this apolipoprotein has not been well characterized. The values given in this Table are derived from results available in the current literature (48, 59, 108).

c. Low Density Lipoproteins (LDL).

Serum LDL are characterized by their high cholesterol content, particularly in the form of cholesteryl ester. Recent experimental evidence described by Levy et al. (68) indicates that this class of molecules may derive mainly from the breakdown of VLDL during which hydrolysis of VLDL-triglyceride occurs. The earlier idea that some LDL are synthesized in the liver has not been ruled out (69). The physiological function of LDL is still obscure; it has been suggested that the development of atherosclerosis is significantly related to increased blood levels of this class of lipoproteins (71, 78). The chemical composition of LDL is approximately as follows: 25% protein, 10% triglyceride, 8% unesterified cholesterol, 37% cholesteryl ester, and 22% phospholipid. The exact apolipoprotein composition of LDL-protein is still uncertain; however, it has been shown to contain some VLDL apolipoproteins and apolipoproteins whose C-terminal amino acids are not yet characterized (Table 3) (66, 68, 108). Physical features defining this class of lipoproteins include hydrated density ranging from 1.006 to 1.063 gm/ml, S_f rate from 0 to 20 Svedberg flotation units, beta mobility on paper electrophoresis, molecular weight from 2.1×10^6 to 2.6×10^6 daltons, and a mean diameter of 212 \AA (68, 71, 80). Various models have been proposed for the structure of LDL. The most recent one, proposed by Pollard et al. (88), consists of a 20 subunit dodecahedron with each subunit made up of an apolar cholesteryl ester core coated by a phospholipid-protein surface.

d. High Density Lipoproteins (HDL).

Serum HDL are the smallest of all the major lipoproteins. They are made up of approximately 50% protein and 50% lipid. Of the lipid components present, phospholipid and cholesteryl ester predominate. The protein moiety of HDL has been fractionated into its constituent apolipoproteins; apoLP-gln I (68%) and apoLP-gln II (20%) are the major ones (101, 106, 107, 108). The minor apolipoproteins of HDL-protein are the major apolipoproteins of VLDL (Table 3) (66, 68, 108). The presence of these apolipoproteins in both classes of lipoproteins has led to the speculation that HDL and VLDL might be metabolically interrelated. The physical characteristics of HDL include hydrated density ranging from 1.063 to 1.21 gm/ml, $F_{1.20}$ rate of 0 to 20 Svedberg flotation units, alpha mobility on paper electrophoresis, molecular weight ranging from 2×10^5 to 4×10^5 daltons, and particle diameters ranging from 75 Å to 100 Å (68, 71, 80). The high density lipoproteins have been divided into three subclasses; they are HDL₁¹, HDL₂ and HDL₃. Some properties of the latter two subclasses are summarized in Table 6 (71, 78, 100). The detailed structure of HDL is still unknown; however, recent electron micrographs of negatively stained HDL indicate that this lipoprotein is probably made up of several subunits (31). The role of the different apolipoproteins in determining the structure of HDL is under investigation.

¹ HDL₁ is excluded in the present discussion because it is not well characterized, and it is present in serum only at low concentration.

TABLE 6

SOME CHARACTERISTICS OF THE MAJOR HDL SUBCLASSES¹.

Subclass	Density ²	Flotation Rate ³	S _{20,w}	Ave. Mol. Wt. ⁴	Size (Å) ⁵	Protein/Lipid ⁶	Apolipoprotein ⁷
HDL ₂	1.063-1.12	F _{1.20} 3.5-9.0	4.79	3.6 X 10 ⁵	60-140	40/60	ApoLP-gln I (68%); apoLP-gln II (20%); apoLP-ala, apoLP-ser and apoLP-glu (12%).
HDL ₃	1.12-1.21	F _{1.20} 0-3.5	5.0	1.7 X 10 ⁵	45-100	55/45	ApoLP-gln I (74%); apoLP-gln II (16%); apoLP-ala, apoLP-ser, and apoLP-glu (10%)

¹ Data obtained from references 6, 31, 71, 78 and 100.² In gm/ml.³ As defined in Table 1.⁴ In daltons.⁵ As determined by electron microscopy.⁶ w/w ratio.⁷ Values in parentheses indicate the approximate apolipoprotein content. Abbreviations for carboxyl-terminal amino acids are explained in Table 3.

Although information on the physical and chemical characteristics of the serum lipoproteins is rapidly accumulating, the specific role of lipoproteins in lipid transport and their metabolism in the bloodstream and associated tissues is still not clear. Some insight into lipoprotein metabolism derives from the study of the two enzyme systems which are involved in lipid and lipoprotein metabolism in the bloodstream and adjoining tissues. These are the lipoprotein lipase and LCAT systems. Investigation of the manner in which these enzyme systems work and of their interaction with serum lipoproteins continues to yield interesting information on the dynamic aspects of lipoprotein metabolism.

III. Lipoprotein Lipase

a. Historical Background.

It is well known that after ingestion of a fatty meal the serum of many animal species becomes turbid. This turbidity is due to the presence of chylomicra which are synthesized in and secreted by the intestinal cells. In 1943, Hahn (51) observed that when lipemic dogs were infused with blood from dogs which had previously been injected with heparin, a clearing of the turbidity occurred. This observation was subsequently confirmed by Weld (115). The nature of this phenomenon was not apparent until 1950 when it was shown that the addition of heparin to lipemic serum in vitro did not induce clearing of the turbidity; however, when the non-lipemic serum of a dog which had been previously injected with heparin was

added, a clearing occurred (7). Thus, it was suggested that the injection of heparin into the bloodstream released a certain factor which was responsible for the clearing of lipemic serum.

b. Physiological Function of Lipoprotein Lipase.

It is now generally accepted that the clearing of lipemic serum is due to the hydrolysis of the triglyceride component of the chylomicra by a lipase which is released into the blood after the injection of heparin (63, 95). This lipoprotein lipase, as it is now generally called, is believed to play a very important role in energy metabolism since one of its main functions is the channeling of fatty acids into tissues for metabolism or storage (96). In light of this vital role, it is not surprising that lipoprotein lipase activity has now been located in a variety of tissues. Some of these are: spleen, lung, heart, kidney, aortic wall, diaphragm and adipose tissue (95). In addition to its wide occurrence, lipoprotein lipase activity has been shown to fluctuate in different tissues under different physiological conditions (86).

The role of lipoprotein lipase as a factor in controlling serum lipid levels is further exemplified in the case of Type I familial hyperlipoproteinemia (35). Patients with this disease show abnormally low post-heparin lipoprotein lipase activity and a markedly reduced rate of removal of chylomicra from the circulation. On normal diets, highly elevated levels of chylomicra are present in the plasma of these patients. Substitution of a diet low in fat and rich in carbohydrate leads to a dramatic reduction in plasma chylomicron and

triglyceride concentrations. In addition to the above, the Type I familial hyperlipoproteinemia patients show a variety of other symptoms (e.g., swollen liver and spleen, milky serum and unusually prominent abdomen) which may or may not be directly related to the deficiency of lipoprotein lipase. There are other diseases which are associated with a decrease of plasma post-heparin lipoprotein lipase activity. Some of these include nephrosis (97), diabetes (96), hepatic cirrhosis (10, 11, 60), and obstructive jaundice (11). The underlying causes by which these diseases affect lipoprotein lipase activity are still unknown; however, it is certain that these diseases affect the normal metabolism of lipids.

c. Site of Lipoprotein Lipase Activity.

The exact cellular site where lipoprotein lipase is located is still uncertain. Because of the ease with which lipoprotein lipase is released after intravascular injection of heparin or other polyanions, this enzyme is probably located at sites which are immediately accessible in the vascular lumen (96).

d. Interaction of Lipoprotein Lipase with Its Substrate.

Since triglycerides are not readily soluble in aqueous systems, the nature of the interaction between lipoprotein lipase and its substrate, namely lipoprotein-triglyceride, is more complex than in the case of water soluble enzyme-substrate systems. There are several types of substrates one can use to study the activity of

lipoprotein lipase; these include the chylomicra (94), lipoproteins with S_f rates greater than 10 Svedberg flotation units (62, 104), and artificial emulsions of triglyceride activated with serum lipoproteins (62). The role of lipoproteins in the lipoprotein lipase reaction was first studied by Korn (62). He observed that lipoprotein lipase from an acetone extract of rat heart muscle would not hydrolyze triglyceride emulsions unless these emulsions were activated by serum lipoproteins. Recent data from Bier and Havel (15) indicated that both HDL and VLDL could activate soybean oil emulsions as substrates for lipoprotein lipase, and the degree of activation per mg of VLDL protein was approximately 13 times greater than that of HDL protein. Furthermore, when various apolipoproteins were tested for activation, apoLP-glu was the only apolipoprotein which showed appreciable effect in promoting the hydrolysis of triglyceride by lipoprotein lipase (28, 54, 64). Based on this observation and the possible absence of apoLP-glu in LDL (68), it is not surprising that LDL cannot activate triglyceride emulsions for hydrolysis. The specific role of apoLP-glu in activating the hydrolysis of triglyceride emulsions by lipoprotein lipase is still unclear. Current work in this area will undoubtedly yield interesting information on both lipoprotein structure and function.

e. Effect of Lipoprotein Lipase Activity on Lipoprotein Distributions.

In addition to its role in lipoprotein-triglyceride metabolism, lipoprotein lipase probably participates in the interconversion

of different lipoprotein classes. In vivo studies indicated that after injection of heparin into lipemic patients the concentration of the S_f 20-100 lipoprotein class decreased while that of the S_f 10-20 class increased (49). This is in agreement with earlier as well as recent in vitro studies which all showed similar shifts in VLDL distributions (from lower densities to higher densities) when VLDL were incubated in the presence of lipoprotein lipase (1, 2, 70). Recently, LaRosa et al. (65), using more modern methods, studied the effects of post-heparin lipoprotein lipase activity on serum lipoprotein distributions. They found that injection of heparin into subjects produced a reduction in plasma triglyceride and VLDL-cholesterol, an increase in plasma free fatty acids and in LDL- and HDL-cholesterol. Relative increases of cholesterol were greater in LDL than HDL. Furthermore, the concentration of HDL, isolated after heparin injection, was consistently higher than before (at 60 minutes after injection, the increase was from 1.89 mg/ml to 2.63 mg/ml). Paper electrophoresis data indicated disappearance of the prebeta band, and an increase in mobility and sharpening of the alpha band. Moreover, it was shown that the amount of apoLP-ala in the HDL fraction was increased during the process of lipolysis in serum; however, this was only qualitatively demonstrated by the intensification of the apoLP-ala band in immunoprecipitin reactions and polyacrylamide gel electrophoresis. Carboxypeptidase digestion of the post-heparin plasma HDL fraction indicated a threefold increase of apoLP-ala. The above results support the idea that there is a

shift of at least one apolipoprotein from the VLDL to the HDL fraction during heparin-induced intravascular lipolysis. However, the possibility that other VLDL apolipoproteins, such as apoLP-glu and apoLP-ser, may also be involved was not excluded in these experiments.

f. Significance of Work Performed on Lipoprotein Lipase.

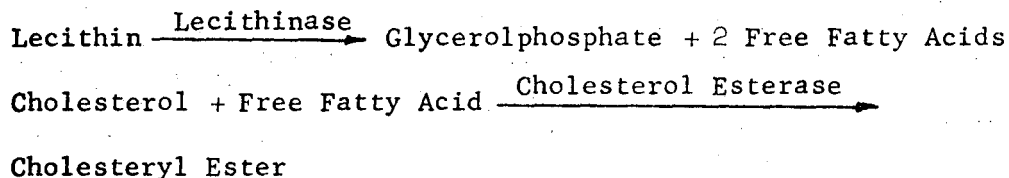
Under controlled experimental conditions lipoprotein lipase provides a unique means for studying lipid and lipoprotein metabolism. Investigation of its interaction with lipoproteins promises to provide us with a better understanding not only of lipoprotein structure and function, but also of the interaction between lipids and proteins.

Since lipoprotein lipase and LCAT are the only enzyme systems presently known which utilize substrates in serum lipoprotein form, it is possible that elucidation of the characteristics of one may provide valuable information on the other. Furthermore, the hydrolysis of VLDL-triglyceride by lipoprotein lipase may produce higher density by-product lipoproteins which are rich in the substrates (lecithin and unesterified cholesterol) of LCAT. Whether the actual removal of such by-product lipoproteins is dependent on LCAT activity is still unknown. Knowledge gained in this area will undoubtedly promote a better understanding of the mechanisms of lipoprotein metabolism as well as of removal of lipoproteins from bloodstream.

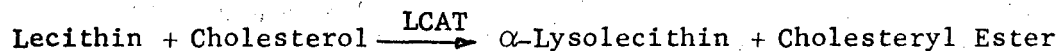
IV. Lecithin: Cholesterol Acyltransferase (LCAT)

a. Historical Background.

In 1935 Sperry (111) observed that during the incubation of either human or dog plasma a decrease in free cholesterol and an increase in cholesteryl ester occurred. This initial observation was later confirmed (67). LeBreton and Pantaleon (67) obtained evidence that the decrease in free cholesterol was accompanied by a decrease in phospholipid. Based on this result and other supplementary data they proposed the presence in plasma of a lecithinase and a cholesterol esterase which promoted the hydrolysis of lecithin and the esterification of cholesterol in the following manner:



In contrast to the view that the production of cholesteryl esters was brought about by the combined action of a lecithinase and a cholesterol esterase, Glomset (37, 38) proposed that a single acyltransferase was responsible for the overall reaction. Thus,



Supporting his argument Glomset observed 1) radioactively labeled cholesteryl esters were formed in plasma incubated with C^{14} -linoleoyl lecithin but not in plasma incubated with C^{14} -linoleic acid complexed to albumin (37), 2) during incubation of rat plasma, the fatty acids esterified to cholesterol were similar to those found in the β

position of lecithin (38), 3) when human plasma was incubated, the molar change in lecithin content was similar to the change in unesterified cholesterol content (38).

b. Site of LCAT Synthesis.

Although LCAT activity has been detected in various tissues, the activity found in plasma is always several fold higher (41). It has been proposed that LCAT may be synthesized or stored in tissues as an inactive enzyme and may be activated upon release of the enzyme into bloodstream (43). The site of synthesis of LCAT is still not clear. Data obtained from evisceration studies indicated that there was a decrease in enzyme activity in the plasma 11 hours after the operation (43). In this respect, liver perfusion studies showed conflicting results. Quarfordt and Goodman (89) were not able to detect LCAT activity in rat liver when a 50% whole plasma was used as perfusate; however, more recently, Simon and Boyer (109) were able to show a low level of LCAT activity in rat liver when a Krebs-Ringer bicarbonate buffer was used as perfusate. The latter authors suggested that the discrepancy between their result and that of the former authors was probably due to the masking of the low liver enzyme activity by plasma LCAT activity when plasma was used as the perfusing medium.

c. Possible Physiological Function of LCAT.

Compared with lipoprotein lipase our knowledge of the physiological role of LCAT is relatively meager. Since most lipoprotein

surfaces are probably made up of a significant amount of phospholipid and unesterified cholesterol (48), changes in surface composition resulting from the transesterification reaction will no doubt alter the physical and metabolic properties of the lipoproteins. Thus, it has been proposed that LCAT might participate in the metabolism of VLDL by regulating the surface content of polar lipids, and as a consequence, enabling lipoprotein lipase to interact with the triglyceride moiety below the surface (103). In support of this idea, Fielding (27) has demonstrated that the reactivity of lipoprotein lipase with triglyceride emulsions was strongly dependent on the proportion of phospholipid to triglyceride in the emulsions. Furthermore, addition of cholesterol to these emulsions inhibited lipolytic activity; substitution of cholesterol by cholesteryl oleate reduced the inhibitory effect.

In addition to a possible role in VLDL metabolism, it has been suggested that LCAT may somehow be involved in the metabolism and homeostasis of cell membranes (43). When normal whole blood was incubated in vitro, the cholesterol content of red blood cells showed a significant reduction associated with the esterification of lipoprotein cholesterol by LCAT (75). This depletion of cholesterol in red blood cells was probably due to a transfer of cholesterol from the red blood cells to the lipoproteins whose cholesterol had been transesterified to cholesteryl esters by LCAT. These observations suggested a possible process of cholesterol removal, involving LCAT, which may participate in regulating the cholesterol content

of red blood cells as well as other tissues.

It is now reasonably well established that LCAT is the main enzyme system which is responsible for the production of cholesteryl esters in the bloodstream (44). The role of cholesteryl esters in normal and abnormal physiology is still obscure. Goodman (46) has suggested that cholesteryl esters may be a precursor of a number of steroid hormones. If this is the case, it is very possible that LCAT may have a more far reaching physiological influence than has been proposed so far.

d. LCAT Deficiency and Its Implications to the Physiological Role of LCAT.

A small number of people who are deficient in LCAT has been discovered recently in Scandinavia (52, 83, 84). Studies performed on these patients have provided valuable data on the possible physiological role of this enzyme in lipid and lipoprotein metabolism. Patients deficient in LCAT usually show a marked elevation of serum cholesterol, triglyceride and lecithin, and almost complete absence of cholesteryl esters and lysolecithin. Their ultracentrifugal serum lipoprotein distributions are generally very abnormal and are characterized by extremely low concentrations of HDL, and by elevations of VLDL. Furthermore, the cholesterol and lecithin content of these patients' red blood cells are approximately twice normal. The morphology of their red blood cells appears abnormal also. In addition to the above abnormalities, a variety of other symptoms

occurs which may or may not be directly associated with the absence of LCAT. These symptoms include anemia, proteinuria, corneal opacity and the appearance of foam cells in both bone marrow and kidney tubules.

More detailed biochemical studies have been recently performed on the lipoproteins of these LCAT deficient patients (32, 85). Their HDL fraction was separated into a high molecular weight and a low molecular weight fraction on a Sephadex G-200 column. The high molecular weight fraction had a mean $F_{1.20}$ rate of approximately 7 Svedberg flotation units and elevated amounts of phospholipid and unesterified cholesterol. The low molecular weight fraction had an $F_{1.20}$ rate that ranged from 0 to 3.5 Svedberg flotation units, and normal lipid composition. When viewed in the electron microscope the high molecular weight fraction showed a discoidal structure with diameters ranging from 150 Å to 200 Å. The low molecular weight fraction showed spherical structures with diameters ranging from 45 Å to 60 Å. The LDL fraction of the patients was also separated into two different molecular weight fractions in a 2% agarose column. The high molecular weight LDL contained 4 to 10 times the normal amount of triglyceride, phospholipid and unesterified cholesterol, and had a mean S_f rate equal to 30 Svedberg flotation units. The low molecular weight fraction contained 1.5 to 3 times the normal amount of phospholipid and unesterified cholesterol, and 10 times the normal amount of triglyceride. Its S_f rate was within the normal range. Electron microscopy of the high molecular weight fraction

indicated large flattened structures with diameters in the range of 900 Å to 1,200 Å. The low molecular weight fraction appeared normal in size and shape.

Based on the results of the above studies, the presence of LCAT activity in the bloodstream apparently plays an important role in regulating the lipid composition of serum. In addition, the gross abnormalities of the ultracentrifugal lipoprotein distributions in LCAT deficiency probably reflect the importance of LCAT activity in providing the appropriate lipid components for maintaining normal lipoprotein distributions and morphology.

One interesting aspect in the study of LCAT deficiency was the apparent role of this enzyme in the exchange of lipids among various classes of lipoproteins. Glomset et al. (45) have provided evidence that the lipoproteins from LCAT deficient patients could serve as substrates for this enzyme. Furthermore, they also demonstrated that normal transfer of lipid moieties, promoted by LCAT, among different classes of lipoproteins could also take place among the lipoproteins of LCAT deficient patients under appropriate conditions. Thus, it is possible that the gross compositional abnormalities of lipoproteins from LCAT deficient patients might be due, at least partially, to a lack of LCAT and resultant inability to promote the transfer of certain lipid components among the various classes of lipoproteins. In this respect, it is interesting that the discoidal structures seen in the HDL fraction of the LCAT deficient patients could also be converted to the spherical appearance of

normal HDL after incubation with the enzyme (33).

e. General Objectives of the Present Thesis.

Although a sizable amount of knowledge is being accumulated on the interaction of LCAT with lipoproteins, the physiological role of this enzyme is still speculative. The function of cholesteryl esters in both normal and abnormal physiology is still uncertain. Since LCAT activity is mainly responsible for the production of this compound in the bloodstream, elucidation of the physiological significance of cholesteryl ester will probably reveal pertinent information on the function of LCAT. The discovery of LCAT deficient patients has provided valuable data on the interaction of LCAT with lipoproteins. Additional studies in this area will undoubtedly provide insight into various biochemical and physiological problems concerning the role of LCAT in lipoprotein metabolism.

In the present study, I propose to investigate various aspects of interactions between LCAT and lipoproteins, and between LCAT and sonicated dispersions of lecithin. It is hoped that the results of these studies may provide a better understanding of 1) the nature of LCAT's interaction with its substrate, 2) the nature of the complex formed between LCAT and its substrate, 3) the role of lipoprotein apolipoproteins in the transesterification reaction catalyzed by LCAT, and 4) the function of LCAT and its influence on lipoprotein morphology as well as lipoprotein metabolism.

CHAPTER 2.

INTERACTION OF LECITHIN: CHOLESTEROL ACYLTRANSFERASE (LCAT) WITH SERUM
HIGH DENSITY LIPOPROTEINS (HDL) AND SONICATED DISPERSIONS OF LECITHIN

I. Background and Objectives

a. HDL as Primary Substrate for LCAT in Human Serum.

During incubation of human serum, the substrates of LCAT are the unesterified cholesterol and lecithin of the serum lipoproteins (39). When each of the three major classes of serum lipoproteins is incubated separately with a partially purified LCAT preparation, the highest esterification rate has been observed with HDL followed by LDL and VLDL (3, 42). On the other hand, the largest increase in cholesteryl ester content is detected in the LDL fraction even though this lipoprotein by itself serves poorly as substrate (3, 39). Since it has been shown that lipid components do transfer between the major lipoprotein classes, the occurrence of an increased cholesteryl ester content in LDL after incubation of serum is probably due to a redistribution of product cholesteryl esters among HDL and LDL (44, 77, 92). Based on these results, it is evident that HDL are probably the preferred substrates of LCAT even though most of the product cholesteryl esters are found in the LDL fraction after incubation of serum.

b. Changes in Physical and Chemical Properties of HDL Following
Interaction with LCAT.

Since the surface structure of HDL probably includes a significant amount of phospholipid and unesterified cholesterol (48), changes in surface composition mediated by LCAT will no doubt alter the physical chemical properties of these lipoproteins. In support of this, Glomset et al. (42) have shown that serum HDL could be separated into subfractions of different cholesterol and lecithin contents. Subfractions whose lecithin content was high tended to be eluted at the lower molecular weight region from a gel filtration column than those whose lecithin content was low. Since the reaction catalyzed by LCAT promotes the breakdown of lecithin, it was suggested that LCAT might be responsible for the production of the lecithin-poor subfractions of HDL. The reason why the lecithin-poor subfractions had higher molecular weights than the lecithin-rich subfractions is still unknown. Glomset et al. (42) suggested that this was possibly due to the aggregation of HDL whose lecithin and cholesterol had undergone transesterification. When whole human serum is incubated for 24 hours at 37°C, a shift in the high density lipoprotein ultracentrifugal distribution from HDL₃ to HDL₂ has been observed by Nichols et al. (79). No definite conclusion has been made whether such shift is due to LCAT activity or not.

Further evidence demonstrating that LCAT activity can promote structural changes in abnormal HDL as well as normal HDL has been obtained during study of LCAT deficient serum. When the HDL fraction of patients with LCAT deficiency was observed under electron microscopy, disc shaped particles (aggregated into stacks) were

the predominant structure. Upon incubation with a LCAT preparation a conversion of the discoidal structures to spherical particles resembling normal HDL resulted (Figure 1) (33). This phenomenon was probably brought about by the conversion of cholesterol to cholesteryl esters, and the subsequent reorganization of HDL apolipoproteins and polar molecules around the newly formed apolar cholesteryl ester core. From these results, it is possible that the transesterification reaction catalyzed by LCAT might be crucial in determining the normal morphology of HDL.

c. Factors Influencing the Reactivity of HDL with LCAT.

The physical chemical factor or factors responsible for the apparently high reactivity of HDL as substrate for LCAT is not clear. However, in view of the high lecithin to unesterified cholesterol molar ratio in these molecules, it has been suggested that structural arrangements of lipid components on lipoprotein surface may be critical in promoting the appropriate orientation of substrates at the catalytic site (81). The recent demonstration of specific apolipoproteins within the different classes of lipoproteins suggests the possibility that the apolipoproteins of HDL may also play a role in enhancing the reactivity of these lipoproteins against LCAT. In support of this idea, Akanuma and Glomset (4) have obtained evidence demonstrating the binding of LCAT activity to both native and delipidated HDL.

d. Complex Formation of HDL with LCAT.

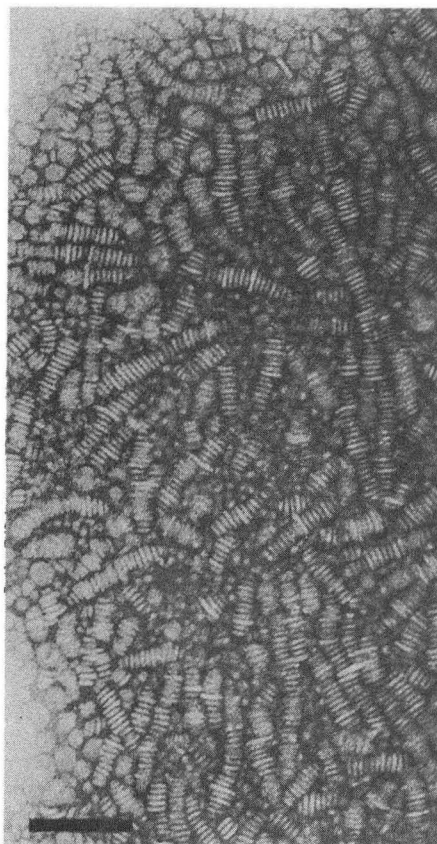
FIGURE 1

TRANSFORMATION OF d 1.063-1.21 gm/ml FRACTION FROM LCAT DEFICIENT
PATIENT AFTER INCUBATION WITH SERUM LCAT ACTIVITY.

Plasma - LCAT enzyme incubation

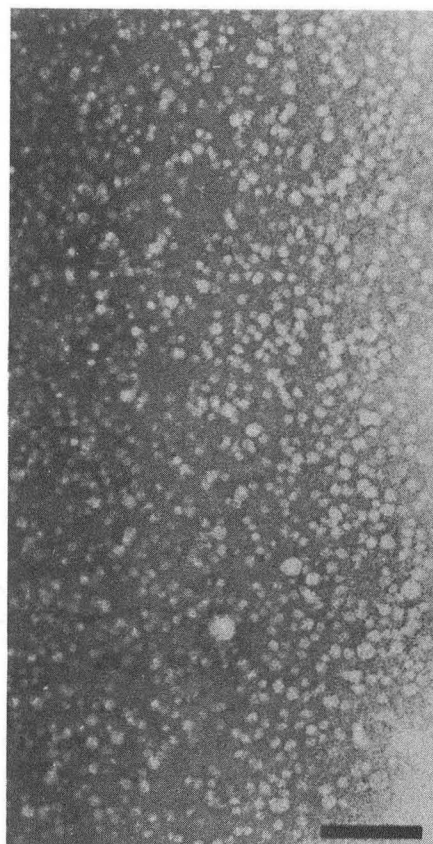
Before

(d 1.063-1.21)



After

(d 1.063-1.21)



L.G.

XBB 714-1698

Evidence demonstrating the formation of a stable complex between LCAT and HDL has been conflicting. Glomset (39) and Lossow et al. (72) have reported the presence of LCAT activity in association with HDL of both human and rat sera. However, more recently Raz et al. (90) have suggested that such enzyme activity, associated with ultracentrifugally isolated HDL, might be due to contamination resulting from incomplete ultracentrifugal flotation of HDL from LCAT activity in serum. Nevertheless, it is still possible, as suggested by Raz et al. (90), that HDL might form a complex with the enzyme in plasma, but that during the process of repeated ultracentrifugal flotation at high ionic strength such complexes are dissociated. Akanuma and Glomset (4) have demonstrated complex formation, at low ionic strength, between LCAT and HDL when the lipoproteins were attached to agarose.

Although data on the conditions promoting the association of LCAT with HDL are still accumulating there are strong evidences from studies on other enzyme-lipid substrate systems showing that complex formation does occur and is strongly influenced by various environmental factors. Thus, phospholipase A (23) and serum lipoprotein lipase (16) showed no association with their lipid substrates at low pH, and maximum association at or near neutral pH. In the case of lipoprotein lipase, the addition of 1 M NaCl prevented the formation of an enzyme-chylomicron complex (16). Lossow et al. (72) have shown that significantly higher amounts of LCAT activity could be ultracentrifugally floated within the HDL fraction when

rat serum was processed at $d = 1.21$ gm/ml in a solution of D_2O and NaCl instead of H_2O and a greater amount of salt. Judging from their results, the association between LCAT and HDL appears to be sensitive primarily to the ionic strength of the medium.

Currently there are no data on the nature of the specific forces that bring LCAT and HDL together. In the case of phospholipases, it has been shown that the binding of the enzyme onto micellar phospholipid substrates is highly dependent on the surface charge characteristics of the micelles. Phospholipase C will not hydrolyze emulsions of lecithin unless long chain cations (e.g., cetyltrimethylammonium bromide) have been incorporated into the emulsions (13). On the other hand, phospholipase B will only hydrolyze emulsions of lecithin which bear a net negative electrokinetic potential (12). Bangham (14) has suggested that the specific charge on the substrate surface might promote the binding of an oppositely charged enzyme.

e. Interaction of LCAT Activity with Model Lipid Substrates.

Recently, in this laboratory, sonicated dispersions of mixtures of lecithin with unesterified cholesterol have been shown to be effective substrates for LCAT activity associated with the ultracentrifugal $d > 1.21$ gm/ml protein fraction (81). The $d > 1.21$ gm/ml protein fraction was obtained after ultracentrifugal flotation of the serum lipoproteins. Electrophoresis of this preparation showed only trace amounts of alpha lipoproteins; however, the presence of HDL apolipoproteins (especially the major HDL apolipoprotein, apoLP-gln I) was not excluded (6).

When sonicated dispersions of mixtures of lecithin with unesterified cholesterol were used as substrates for LCAT, it was discovered that their reactivity was highly dependent on the ratio of lecithin to unesterified cholesterol (81, 91). At a molar ratio of one lecithin molecule to one cholesterol molecule, a low amount of transesterification was detected; and at a ratio of three or more lecithin molecules to one cholesterol molecule significantly higher amounts of transesterification occurred. The authors suggested that such a ratio might be critical in mimicking an HDL-like surface structure (81). In support of this idea, initial reaction rates obtained when sonicated dispersions of mixtures of lecithin with unesterified cholesterol were used as substrates compared favorably with those obtained when HDL were used as substrates (81). The influence of the HDL apolipoproteins, probably present in small amounts in the $d > 1.21$ gm/ml protein fraction, on the reactivity of the sonicated dispersions of lecithin with cholesterol was not established (81).

f. Specific Objectives of the Present Chapter.

In view of the many remaining unanswered questions concerning the interaction between HDL and LCAT, and between sonicated dispersions of specific lipids and LCAT we decided to investigate the following: 1) to determine the ultracentrifugal properties of serum LCAT activity, 2) to confirm if and under what conditions complex formation between HDL and LCAT occurs, 3) to investigate formation of complexes between sonicated dispersions of phospholipid

and LCAT, and 4) to determine the properties of such phospholipid-LCAT complexes.

II. Materials and Methods

a. Preparation of Ultracentrifugal $d > 1.21$ Protein Fraction.

Blood was drawn from healthy male and female subjects and allowed to clot at room temperature for approximately 2 hours. The clot was separated from serum by centrifugation at 300 X g, for 25 minutes, at 4°C. The freshly prepared serum was raised to a salt background density of 1.21 gm/ml by addition of solid KBr. Six ml aliquots of this adjusted serum were pipetted into preparative ultracentrifuge tubes and ultracentrifuged at 114,000 X g, 16°C for 48 hours. After ultracentrifugation, the top 3 ml containing essentially all of the serum lipoproteins were removed. The bottom 3 ml were stirred, pooled and dialyzed against either 0.01 M phosphate buffer or 0.01 M Tris HCl buffer (both of the buffers were at pH 7.4 and contained 0.1 mg/ml EDTA and various amounts of NaCl). After dialysis, this ultracentrifugal $d > 1.21$ protein fraction was used as the source of LCAT activity.

b. Preparation of Lipoprotein Fractions.

Lipoprotein fractions were isolated from serum by sequential ultracentrifugal flotation. Six ml aliquots of serum were pipetted into preparative ultracentrifuge tubes and ultracentrifuged at 114,000 X g, 16°C for 18 hours. The top 1 ml, containing mostly

VLDL, was pipetted off and dialyzed against appropriate buffer for subsequent use. The next 1 ml was pipetted and discarded. The background salt solution density of the bottom 4 ml was then adjusted to d 1.063 gm/ml by addition of 2 ml of an appropriate NaBr-NaCl solution. After solubilization of the packed protein in the bottom of the tube (by gentle rocking of the tube) the mixture was ultracentrifuged at 114,000 X g, 16°C for 24 hours. The top 1 ml, containing LDL, was collected and the second 1 ml was discarded. The bottom 4 ml were adjusted to a background salt solution density of 1.21 gm/ml by addition of 2 ml of an appropriate NaBr-NaCl solution. After solubilization of the packed protein, the 6 ml mixture was ultracentrifuged at 114,000 X g, 16°C, for 24 hours. The top 1 ml, containing HDL, was collected as before.

An alternate procedure for isolation of serum lipoproteins was to utilize the total lipoprotein fraction generated by ultracentrifugation of serum at d 1.21 gm/ml during preparation of the $d > 1.21$ protein fraction. Thus, the top 1 ml, containing essentially all of the serum lipoproteins, was collected, pooled and dialyzed against an NaCl solution of d 1.063 gm/ml. Six ml aliquots of this lipoprotein solution were pipetted into preparative ultracentrifuge tubes and ultracentrifuged at 114,000 X g, 16°C, for 24 hours. The top 2 ml, containing both VLDL and LDL, were pipetted first; then the bottom 2 ml, containing the HDL, were collected dropwise by puncturing a small hole in the bottom of the ultracentrifuge tube. Separation of VLDL from LDL was achieved by subsequent ultracentrifugation of the pooled top 2 ml fraction at d 1.006 gm/ml under the

conditions already described.

c. Preparation of Sonicated Dispersions of Lipids.

Desired levels of various lipids dissolved in chloroform or absolute ethanol were introduced into sonication vials (20 ml thick wall glass tubes, M. S. E. Ltd., London). After evaporation of solvent under a gentle stream of N_2 , appropriate amounts of either 0.01 M phosphate buffer (pH 7.4, with 0.1 mg/ml EDTA) or 0.01 M Tris HCl buffer (pH 7.4, with 0.1 mg/ml EDTA) were added. The contents were sonicated for 10 minutes using an ultrasonic disintegrator (output power, 60 watts; frequency, 18,000-20,000 Hz; Model no. 3000, M. S. E. Ltd., London). During sonication the vials were cooled in an ice bath. The sonicated dispersions were usually used within several hours after preparation.

d. Preparation of Sonicated Substrates for Assay of LCAT Activity.

Substrates used for assay of LCAT activity were prepared by sonication of a mixture of egg lecithin (General Biochemicals, Chagrin Falls, Ohio) and unesterified cholesterol (Supelco, Inc., Bellefonte, Pa.) containing a small amount (usually 8000 to 50,000 dpm, 15 mCi/nmole) of H^3 -cholesterol (New England Nuclear, Boston, Mass.) in either 0.01 M phosphate buffer or 0.01 M Tris HCl buffer (both contained 0.1 mg/ml EDTA, pH 7.4). The molar proportion of lecithin to cholesterol used was 6 to 1. The lecithin concentration in the final assay medium was 0.5 mg/ml, and the cholesterol

concentration was 0.04 mg/ml.

e. Determination of LCAT Activity in Samples.

Net esterification yield of cholesteryl ester in the assay system (incubated 24 hours, at 37°C) was used, except when otherwise specified, as an approximate measure of the amount of enzyme present. Usually the assay media contained equal volumes of enzyme preparation and sonicated substrate. The percent cholesterol esterified was determined from the amount of H³-cholesterol converted to H³-cholesteryl esters.

In separate experiments, it had been shown that the amount of cholesterol esterified during a 24 hour incubation period was directly proportional to the amount of enzyme present. These results are shown in Figure 2.

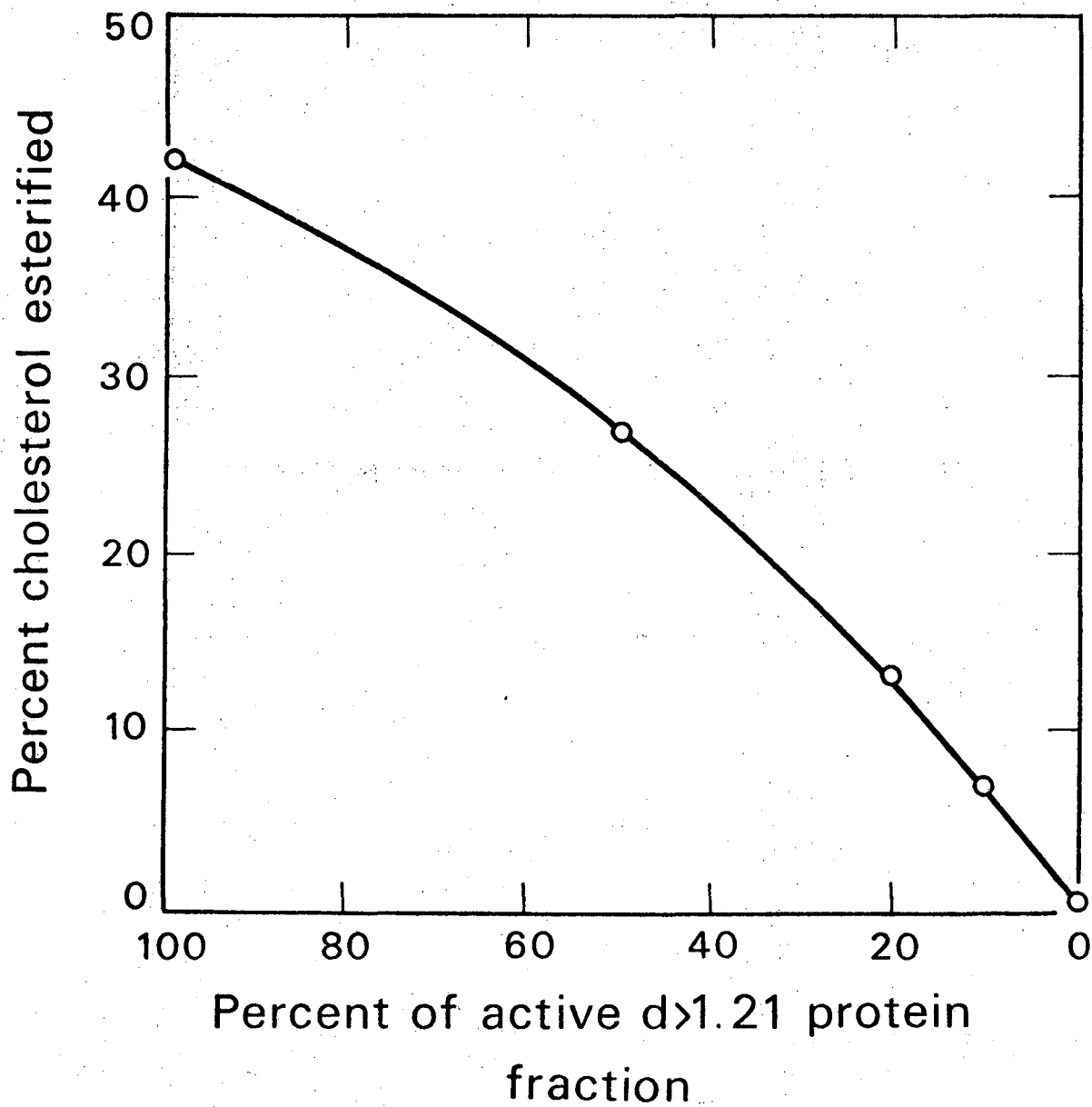
f. Lipid Analysis.

Lipid extractions were carried out either by the modified method of Sperry and Brand (112), or by a procedure (utilizing ethanol and ether) developed for extraction of samples containing sonicated dispersions of lipids (as described below). Both methods produced comparable results. The following is an outline of the ethanol-ether extraction procedure. One ml of sample, containing sonicated dispersions of lipids, was shaken vigorously with 2 ml of absolute ethanol, then 1 ml of distilled water was added, and the mixture shaken again. The final ethanol-water mixture was extracted with

FIGURE 2

EFFECT OF DILUTION OF $d > 1.21$ PROTEIN FRACTION ON % CHOLESTEROL ESTERIFIED IN ASSAY MIXTURE.

$d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) was diluted with heat inactivated (56°C for 1.5 hours) $d > 1.21$ protein fraction (in the same buffer). One ml of the mixture was incubated with 1 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) for a period of 24 hours at 37°C . The amount of cholesterol esterified was determined as described in Materials and Methods.



DBL 723-5197

20 ml of diethyl ether. The remaining aqueous phase was re-extracted with 5 ml of diethyl ether. The ether extracts thus obtained were pooled and blown down under a gentle stream of N_2 .

The lipid extracts obtained from the above procedures were dissolved in either hexane or chloroform, and chromatographed on either silicic acid columns (Bio Rad Laboratories, Richmond, Calif.) or instant thin layer chromatographic media (Gelman Instrument Co., Ann Arbor, Mich.) for separation of lipid components (usually cholesteryl esters, unesterified cholesterol and lecithin). The solvent used in silicic acid chromatography was similar to that described by Hirsch and Ahrens (56), and the solvent system used for thin layer chromatography was 5% diethyl ether in cyclohexane (v/v). Both chromatographic methods gave satisfactory and comparable results. Since the thin layer method was more rapid and gave adequate separation, it was used almost exclusively in all of the experiments.

g. Radioassay.

Lipid fractions obtained from silicic acid chromatography were collected directly into scintillation vials. After evaporation of the eluting solvent under N_2 , scintillation fluid (Omnifluor, New England Nuclear, Boston, Mass.) was added. Counting was performed in a Nuclear Chicago Mark I liquid scintillation spectrophotometer.

When lipid components were separated on instant thin layer media, the locations of the various fractions were first visualized by exposure to I_2 vapor. The regions corresponding to individual

lipid components were cut out and put into scintillation vials. After addition of scintillation fluid, counting was done in the same equipment as previously described.

All samples were counted at least to an error of less than 4% at the 95% confidence level. Quenching and efficiency of counting were determined by addition of H^3 -toluene as internal standard.

h. Protein Determinations.

The concentration of protein was determined either by the method of Lowry et al. (73), or by measurement of the absorption at 280 $m\mu$. In both cases bovine serum albumin was used as standard.

i. Molecular Sieving Chromatography.

Columns containing Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) were packed according to instructions furnished by the manufacturer. All columns were washed extensively with double distilled water and either 0.01 M phosphate buffer or 0.01 M Tris HCl buffer (both buffers were pH 7.4 and contained 0.1 mg/ml EDTA and either 0.19 M NaCl or 0.26 M NaCl) before sample application. All chromatographic procedures were done at 4°C unless otherwise stated. Flow rate was regulated by a metering pump (Instrumentation Specialties Co., Inc., Lincoln, Nebraska), and the effluent continuously monitored at 280 $m\mu$ by an ISCO recorder (Instrumentation Specialties Co., Inc., Lincoln, Nebraska).

j. Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel (7%) containing 1.7% MBA (methylene-bis-acrylamide) was prepared in glass tubes (0.6 cm X 8.0 cm) according to methods described by Davies (22). Samples (containing 50 to 100 μ g of protein) mixed with a small amount of tracking dye (bromophenol blue) and sucrose were layered on top of the gels. Electrophoresis was performed in a Hoefer electrophoretic cell (Bio Rad Laboratories, Richmond, Calif.) at 1.5 mA/tube. The buffer in the upper and lower electrodes was 0.05 M borate buffer (pH 9.0). After electrophoresis, gels were removed from the glass tubes and fixed and stained for 2 hours in a solution containing 0.25% Coomassie brilliant blue (w/v) (Colab Laboratory Inc., Chicago Heights, Ill.), 45% methanol (v/v), and 9% acetic acid (v/v). Removal of excess dye was done electrophoretically on a Canelco destaining apparatus (Canelco, Rockville, Md.). Stained gels were stored in the destaining solution.

III. Results

a. Association and Dissociation of Serum LCAT Activity with Lipoproteins.

After ultracentrifugation of human serum, adjusted to a background salt density of 1.21 gm/ml, the bulk of LCAT activity was found to be in the middle (2 ml) and bottom (3 ml) fractions (Table 7). Furthermore, when each fraction was evaluated for lipoprotein content, the top fraction contained essentially all of the serum lipoproteins while the middle and bottom fractions contained only slight to trace amounts of HDL.

From the results described in Table 7, it is evident that the

TABLE 7

DISTRIBUTION OF LCAT ACTIVITY AFTER ULTRACENTRIFUGATION OF WHOLE SERUM AT d 1.21 gm/ml (in KBr).

Whole serum was adjusted to d 1.21 gm/ml by addition of solid KBr. Six ml aliquots of the serum were pipetted into preparative ultracentrifuge tubes, and ultracentrifuged for 48 hours, at 10°C and 114,000 X g. Fractions, in volumes indicated, were collected after ultracentrifugation, and subsequently dialyzed against a 0.01 M Tris HCl buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA). Enzyme activity contained in each fraction was assayed according to procedures described in Materials and Methods.

Ultracentrifugal Fraction from Top (ml)	% of Total Enzyme Activity in Tube	Lipoprotein Content ¹
0 to 1	4.6	Most of the serum lipoproteins
1 to 3	41.9	Slight amount of HDL
3 to 6	53.4	Trace amount of HDL

¹ Demonstrated by agarose electrophoresis.

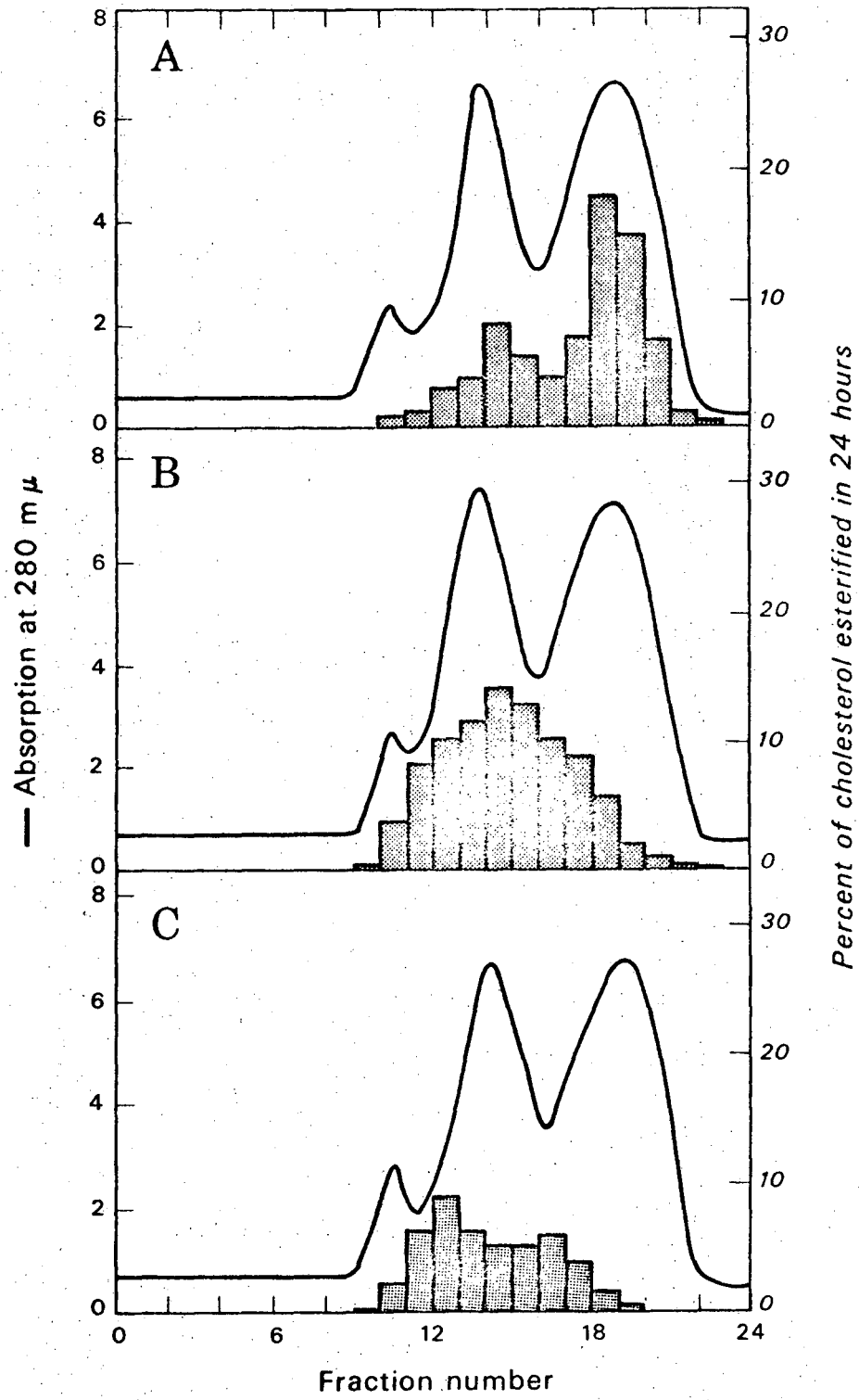
bulk of LCAT activity in serum could be separated from the lipoproteins using the ultracentrifugal procedure described here. Our results are in general agreement with those of Lossow et al. (72), Raz et al. (90) and Sugano (113). In Lossow et al.'s experiments they demonstrated that by lowering the ionic strength of the ultracentrifugal medium a significant amount of LCAT activity, associated with the HDL fraction, could be ultracentrifugally floated at $d = 1.21$ gm/ml. In order to evaluate if LCAT activity, as found in the $d > 1.21$ protein fraction, could re-associate with lipoproteins under milder conditions (i. e., low ionic strength, no ultracentrifugation, and optimal pH) the following series of experiments was performed. The $d > 1.21$ protein fraction was chromatographed on a Sephadex G-200 column, and the protein and LCAT activity profiles determined (Figure 3A). The same amount of $d > 1.21$ protein fraction was then mixed with HDL (approximately 4.0 mg/ml in the final mixture), incubated for 0.5 hours, at 37°C , and chromatographed on the same column under identical conditions (Figure 3B). In the presence of added HDL, the distribution of LCAT activity shifted from the third eluting protein peak to the second eluting protein peak (Figure 3A and 3B). Since HDL, when run by itself, was eluted in the region of the second eluting protein peak (57), it is evident that an association of LCAT with HDL did occur under the above chromatographic conditions.

In order to determine if conversion of HDL-cholesterol to cholesteryl esters has any effect in changing LCAT's affinity for

FIGURE 3**ASSOCIATION OF LCAT ACTIVITY WITH HDL. EVALUATION BY CHROMATOGRAPHY ON SEPHADEX G-200.**

Molecular sieving chromatography on a Sephadex G-200 column was carried out according to the conditions described below. Column dimensions, 2.5 cm X 100 cm; flow rate, 20 ml/hour; volume of sample, less than 2% of bed volume; volume of each fraction, 16.8 ml; direction of flow, ascending; temperature, 4°C; eluting buffer, 0.01 M Tris HCl, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA. Concentration of protein in the effluent was continuously monitored by absorption at 280 m μ , and enzyme activity in each collected fraction was determined by procedure described in Materials and Methods. Chromatogram A: sample consisted of 5 ml of d>1.21 protein fraction (47.8 mg protein/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA). Chromatogram B: sample consisted of 5 ml of d>1.21 protein fraction (47.8 mg protein/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) mixed with 1 ml HDL (24 mg/ml, in same buffer) and incubated at 37°C for 30 minutes prior to application onto column. Chromatogram C: sample was the same as chromatogram B, but was incubated for 36 hours before application onto column¹.

¹ The low LCAT activity in the collected fractions probably resulted from unavoidable prolonged storage prior to assay of enzyme activity.



the lipoproteins, the following experiment was performed. The $d > 1.21$ protein fraction was first incubated with HDL for 36 hours prior to chromatography. As indicated in Figure 3C, LCAT activity was still associated with the HDL peak even though most of the cholesterol was esterified. In this case, the distribution of LCAT activity was shifted to the higher molecular weight region of the HDL peak. Since Glomset et al. (42) have suggested that after transesterification HDL tend to aggregate, the present observation was probably due to the association of LCAT activity with the larger "product" HDL. The reason why HDL become larger after transesterification is still uncertain. Since there is a decrease in polar lipid content and an increase in apolar lipid content in these molecules after transesterification, it is possible that the change in size or aggregation of HDL may result from reorganization of lipid components within the lipoprotein molecules.

b. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin. Study Performed in Sucrose Medium of $d 1.065$ gm/ml.

The association of serum LCAT activity with its preferred substrate, HDL, under specific experimental conditions has been demonstrated in the present work as well as in a number of earlier reports (39, 72). Furthermore, sonicated dispersions of mixtures of cholesterol and lecithin have been shown to be comparable to HDL as substrates for LCAT (81, 91). Since these sonicated dispersions

of mixtures of lipids do not contain apolipoproteins and have physical and chemical characteristics quite different from native HDL, it was of interest to ascertain whether LCAT has similar affinity for such dispersions as it does for native HDL.

Since there is strong evidence indicating that the association between LCAT and HDL is sensitive to high ionic strength (72); I decided to use sucrose instead of salt (such as KBr) for density adjustment in an ultracentrifugal study on the interaction of LCAT activity with sonicated dispersions of lecithin. It was hoped that by keeping the ionic strength of the ultracentrifugal media low, a stable association between LCAT activity and sonicated dispersions of lecithin would occur.

The effect of prior incubation of the $d > 1.21$ protein fraction with sonicated dispersions of lecithin on the ultracentrifugal distribution of LCAT activity is described in Table 8. Although there was a marked reduction in overall activity in the ultracentrifugal fractions, there was a clear shift of activity towards the top fractions during ultracentrifugation. These data indicated that the enzyme was forming a complex with the lecithin dispersion, since, without preincubation with lecithin, dispersion negligible activity was found in the top fraction. The reason for the drastic reduction in overall enzyme activity in the sample preincubated with lecithin was not immediately apparent. Glomset (38) has shown that the sulphydryl group or groups in the enzyme are essential for activity. Since these groups are rather labile and can be

TABLE 8

ULTRACENTRIFUGAL ($d = 1.065$ gm/ml-sucrose) DISTRIBUTION OF LCAT ACTIVITY: EFFECT OF PREINCUBATION OF $d > 1.21$ PROTEIN FRACTION WITH LECITHIN DISPERSIONS.

Two ml of the $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) were preincubated (37°C, 0.5 hours) with 2 ml of a sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA). After preincubation, the mixture was cooled in an ice bath for approximately 10 minutes. Two ml of 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl, 0.1 mg/ml EDTA and sucrose, was added. The final volume was 6 ml and density was 1.065 gm/ml. The mixture was ultracentrifuged at 114,000 X g, 4°C for 24 hours. Three 2 ml fractions were collected after ultracentrifugation (designated as top, middle and bottom fraction) and subsequently dialyzed against the above phosphate buffer without sucrose. One ml of the sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) was added to 1 ml aliquots of each of the ultracentrifugal fractions and incubated at 37°C for 24 hours. Radioassay for extent of esterification was performed as described in Materials and Methods.

Ultracentrifugal Fraction	% Cholesterol Esterified		
	Expt. 1 (A) ¹	Expt. 2 (B)	Expt. 3 (C)
	Preincubated with lecithin		
Top	8.0	11.3	3.2
Middle	1.4	2.5	2.0
Bottom	0.9	3.0	0.9
	Control, preincubated with phosphate buffer		
Top	0.3		
Middle	9.0		
Bottom	70.1		

¹ Letters in parentheses in this and Table 9 identify donors from whom sera were obtained.

readily oxidized, it is possible that the decrease in activity in the ultracentrifugal top fraction was due to oxidation of these groups at the air-liquid interface. In an effort to restore enzyme activity in the fractions, we exposed them to mercaptoethanol prior to assay.

c. Effect of Prior Treatment with Mercaptoethanol on LCAT Activity in Ultracentrifugal (d 1.065 gm/ml-sucrose) Fractions.

Prior treatment of the ultracentrifugal fractions with 0.01 M mercaptoethanol as described in Table 9 resulted in a marked increase in the amount of LCAT activity detected in all fractions (Table 9). Although the total activity of the lecithin-preincubated fractions was still significantly lower than the total activity in the control fractions, the bulk of enzyme activity was clearly in the top fraction. The possibility that the lower activity might have resulted from removal of a factor during ultracentrifugation was evaluated by the following recombination experiment. The three fractions obtained from ultracentrifugation were recombined and assayed (Table 10). The activity of the recombined mixture¹ was still about one-half that of the recombined control mixture. This reduction in enzyme activity may possibly have been due to an inhibitory effect of the high level of lecithin present (introduced for flotation of the enzyme) in the assay medium.

¹ All fractions were pretreated with 0.01 M mercaptoethanol before recombination.

TABLE 9

EFFECT OF MERCAPTOETHANOL ON ASSAY OF LCAT ACTIVITY IN THE ULTRACENTRIFUGAL (d 1.065 gm/ml-sucrose) FRACTIONS.

All fractions were collected and prepared as described in Table 8 except for the dialysis procedure. After the fractions were collected from the ultracentrifuge tubes, they were first dialyzed for 12 hours against a 0.01 M phosphate buffer (pH 7.4) containing 0.26 M NaCl, 0.1 mg/ml EDTA and 0.01 M mercaptoethanol. The unreacted mercaptoethanol was removed by dialysis against the same phosphate buffer, but without mercaptoethanol. Enzyme assay was then performed as described in Materials and Methods.

Ultracentrifugal Fraction	% Cholesterol Esterified	
	Expt. 1 (B)	Expt. 2 (C)
	Preincubation with lecithin	
Top	43.1	33.7
Middle	10.1	15.0
Bottom	6.8	7.0
	Control, preincubated with phosphate buffer	
Top	0.3	0.2
Middle	38.1	37.5
Bottom	84.0	80.0

TABLE 10

EFFECT OF RECOMBINATION OF ULTRACENTRIFUGAL (d 1.065 gm/ml-sucrose) FRACTIONS ON LCAT ACTIVITY.

One ml of each ultracentrifugal fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) was recombined with the other two as indicated. A 1 ml aliquot of the recombined mixture was then incubated with 1 ml sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) for 24 hours at 37°C. Radioassay of enzyme activity was performed as described in Materials and Methods.

Ultracentrifugal Fraction in Final Incubation Mixture ¹	% Cholesterol Esterified
	Preincubated with lecithin
Top + Middle + Bottom	35.0
	Control, preincubated with phosphate buffer
Top + Middle + Bottom	64.3

¹ All fractions were pretreated with 0.01 M mercaptoethanol before recombination.

d. Evaluation of the Effect of Excess Lecithin on Assay of LCAT Activity.

In the experiments described above, the possible effect of high amounts of lecithin, introduced for flotation of LCAT activity¹, on the assay of enzyme activity was not considered. A series of experiments was performed to evaluate the effect of addition of different concentrations of sonicated dispersions of lecithin on LCAT activity associated with the $d > 1.21$ protein fraction. The same radioassay method was used as described in Materials and Methods. After preincubation of the $d > 1.21$ protein fraction with increasing concentrations of lecithin (Figure 4), the net esterification yield decreased, and, at a concentration of approximately 1.25 mg/ml, approached a constant value of about 60% of the value determined for the control.

From these results it is apparent that excess lecithin has an inhibitory effect on LCAT activity. Since a high amount of lecithin was also present in the ultracentrifugal ($d 1.065$ gm/ml-sucrose) top fractions, it is probable that enzyme activity was similarly inhibited in them².

e. Effect of Lecithin Concentration on Ultracentrifugal ($d 1.065$ gm/ml-sucrose) Flotation of LCAT Activity.

In all previous experiments, the final concentration of lecithin

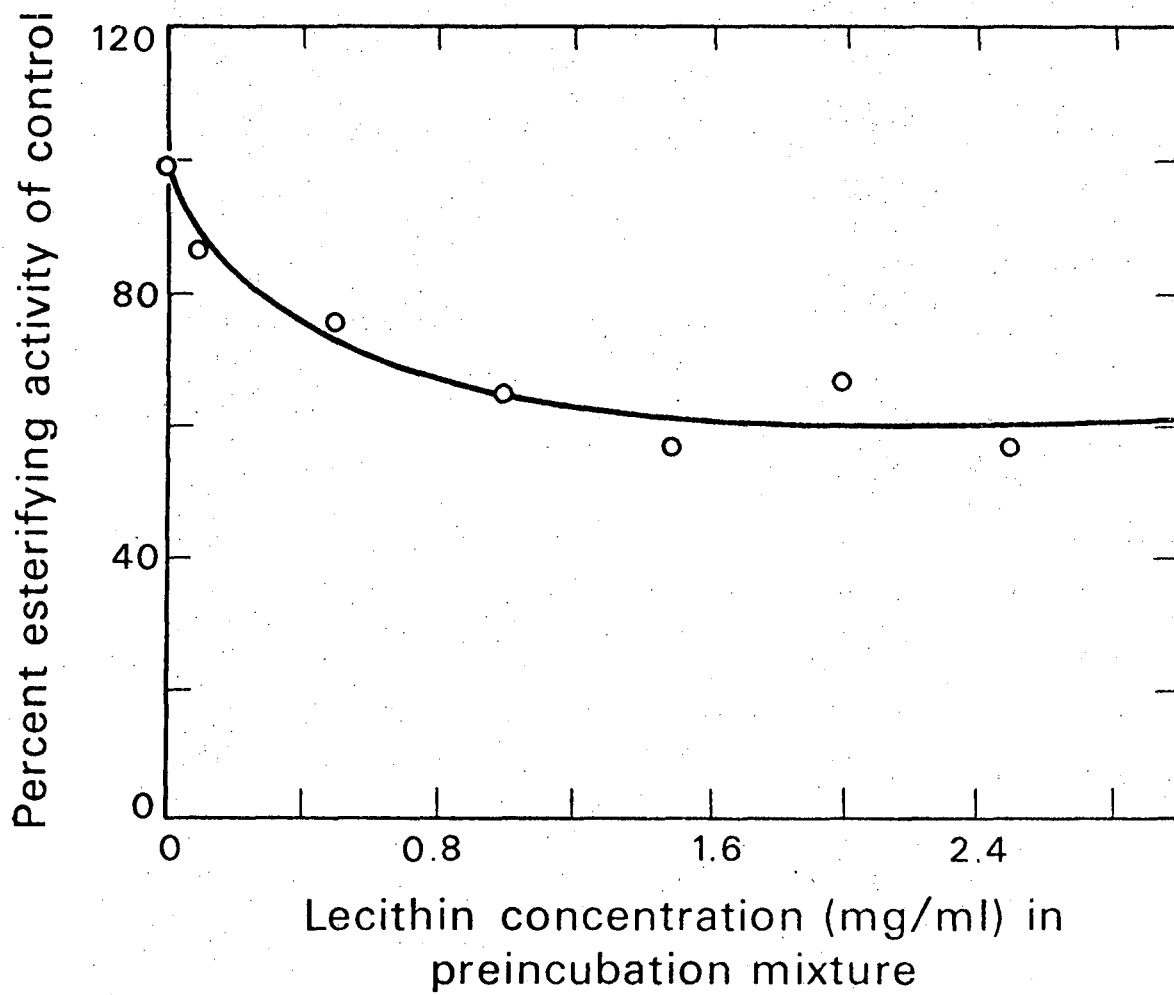
¹ By using a sonicated dispersion of lecithin containing C¹⁴-lecithin, it was demonstrated that approximately 60% of the lecithin, introduced prior to ultracentrifugation, was floated into the top fraction.

² See Appendix (page 130) for more detailed studies on the effect of mercaptoethanol and excess lecithin on assay of LCAT activity.

FIGURE 4

EFFECT OF PREINCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH VARYING CONCENTRATIONS OF LECITHIN DISPERSION ON ASSAY OF LCAT ACTIVITY.

One ml of the $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) was preincubated with 1 ml of a sonicated dispersion of lecithin (in same buffer without NaCl) at 37°C for 0.5 hours. The mixture was chilled in an ice bath for 10 minutes. A 1 ml aliquot of this mixture was added to 1 ml of sonicated substrate (in same phosphate buffer but without NaCl), and incubated at 37°C for 24 hours. In the control, 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA) was used in place of the lecithin dispersions. The ordinate is expressed as percentage of the net esterification yield determined for the control.



DBL 723-5190

in the preincubation mixtures (containing the $d > 1.21$ protein fraction plus a sonicated dispersion of lecithin) was approximately 2.5 mg/ml. In another series of experiments we evaluated the amount of activity floated into the top fraction as a function of the lecithin concentration in the preincubated mixture. In Figure 5, the LCAT activity in the top fractions is plotted against the concentration of lecithin used in the preincubation-flotation of LCAT activity. As indicated, there was an exponential-like increase of esterifying activity in the top fractions as the concentration of lecithin in the preincubated mixture was increased. The significance of this is not clear; however, one possibility is that, with the increasing concentration of lecithin, the average size of the dispersed lecithin particles may increase and hence the enzyme-dispersion complex may be more efficiently floated at the density used.

f. Factors Influencing Formation of the LCAT-Lecithin Dispersion Complex.

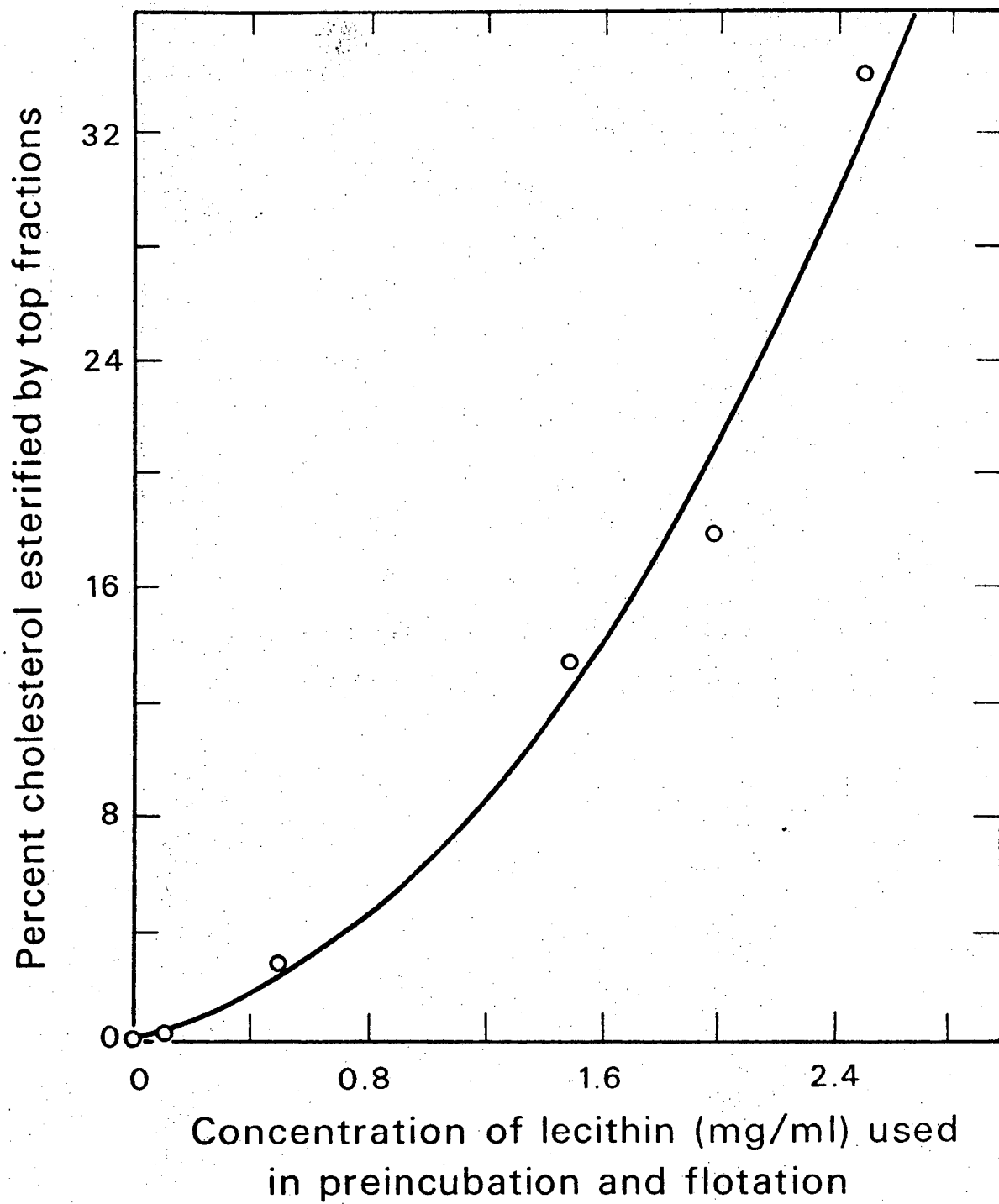
1. Effect of pH.

The influence of pH on the formation and dissociation of enzyme-lipid dispersion complexes has been amply demonstrated for phospholipase A (23) and lipoprotein lipase (16). The effect of pH on the interaction of LCAT with sonicated dispersion of lecithin was evaluated. The $d > 1.21$ protein fraction and a sonicated dispersion of lecithin were preincubated and ultracentrifuged at pH 4.1, 6.0, 7.8 and 9.6 as described in Table 11. At pH 4.1, over 80% of the

FIGURE 5

EFFECT OF LECITHIN CONCENTRATION ON ULTRACENTRIFUGAL (d 1.065 gm/ml-sucrose) FLOTATION OF LCAT ACTIVITY INTO THE TOP FRACTION.

Fractionation procedures were the same as described in Table 9. The concentrations of sonicated dispersions of lecithin used in the preincubation mixture are as designated on the abscissa. After dialysis of the ultracentrifugal top fractions as described in Table 9, enzyme activity in each fraction was assayed according to procedures described in Materials and Methods.



DBL 723-5194

TABLE 11

EFFECT OF pH ON ULTRACENTRIFUGAL ($d = 1.065$ gm/ml-sucrose) DISTRIBUTION OF LCAT ACTIVITY.

The $d > 1.21$ protein fraction and a sonicated dispersion of lecithin (5.0 mg/ml) were dialyzed separately in 0.01 M Tris maleate buffers of various pH's containing 0.19 M NaCl and 0.1 mg/ml EDTA. After dialysis, 2 ml of the $d > 1.21$ protein fraction were mixed and incubated (37°C, 0.5 hours) with 2 ml of the sonicated dispersions of lecithin at the same pH. Two ml of a sucrose solution (prepared with 0.01 M Tris maleate buffer of the same pH as the $d > 1.21$ protein fraction and lecithin dispersions) were added to raise the density of the final 6 ml mixture to 1.065 gm/ml. Ultracentrifugation, collection of fractions, dialysis of fractions, and assay of enzyme activity in each fraction were performed as described in Table 9.

pH	% of Total Cholesterol Esterified in Whole Tube ¹		
	Ultracentrifugal Fractions		
	Top	Middle	Bottom
4.1 ²	6.5	10.1	83.4
6.0	33.3	44.3	22.3
7.8	45.5	34.6	20.0
9.6	46.7	30.7	22.6

¹ This value is calculated from the % cholesterol esterified in each fraction divided by the sum of the % of cholesterol esterified in all three fractions (top, middle and bottom).

² Enzyme activity was lower in this case as compared with the others. The possible cause for this might have been denaturation and precipitation of part of the total LCAT activity when the $d > 1.21$ protein fraction was dialyzed in buffer of pH 4.1.

total LCAT activity contained in the whole tube was recovered in the bottom 2 ml fraction. As pH was raised to 6.0, 44 and 33% of the total activity contained in the whole tube was recovered in the middle and top fractions respectively. At pH 7.8 and 9.6, approximately 50% of the total activity contained in the whole tube was recovered in the top fraction while only 20% of the total activity contained in the whole tube remained in the bottom fraction. Based on these observations, the complexing of LCAT activity with sonicated dispersions of lecithin appears to occur preferably at neutral or higher pH. The nature of this pH effect is still unclear; however, a change in pH could have affected the charge characteristics of the lipid binding groups on the enzyme molecule. At the pH range used there should be no change of charge on lecithin since its pI is below 4.0 (14, 87).

Although in the present study we have shown that LCAT activity and sonicated lecithin dispersions can form complexes of $d < 1.065$ gm/ml (sucrose), the possibility that other proteins might be involved in the formation of the complexes was not excluded. Thus, the inability of LCAT to complex with the lecithin dispersions at low pH might have been due to a change in the efficiency of another protein either to enter into or promote the enzyme-lipid association.

2. Effect of Sulfhydryl Inhibition by Hydroxymercuribenzoate.

Since LCAT activity has been reported to be inhibited by sulfhydryl blocking agents (38), we investigated the effects of

hydroxymercuribenzoate on the formation of the enzyme-lecithin dispersion complex. The data in Table 12 show that exposure of the d>1.21 protein fraction to hydroxymercuribenzoate (producing a reduction in LCAT activity of approximately 70%) did not alter the amount of enzyme activity floating up with the lecithin dispersions. The affinity of the enzyme for sonicated dispersions of lecithin apparently does not depend on the presence of intact sulfhydryl group or groups which apparently are important to the esterifying action of the enzyme.

g. Dissociation of LCAT-Lecithin Dispersion Complex.

Akanuma and Glomset (4) have demonstrated that sodium taurocholate can be used to dissociate LCAT activity from a complex with HDL when the latter is attached to agarose. In this thesis, it was also demonstrated that LCAT activity could complex with HDL at low ionic strength. However, when whole serum, containing both LCAT and HDL, was ultracentrifuged in high salt, we found insignificant amounts of LCAT activity in association with the HDL fraction. Therefore, in order to dissociate the LCAT activity from the isolated enzyme-lecithin complexes as completely as possible, we exposed them to sodium taurocholate in a KBr solution of high ionic strength and subsequently subjected the mixture to ultracentrifugal procedures described in Table 13. The fractions isolated were assayed for LCAT activity, and the data are summarized in Table 13. All of the enzyme activity was found in the bottom 2 ml fraction, while most

TABLE 12

ULTRACENTRIFUGAL ($d > 1.065$ gm/ml-sucrose) DISTRIBUTION OF LCAT ACTIVITY INHIBITED WITH HYDROXYMERCURIBENZOATE.

Hydroxymercuribenzoate (Sigma Chem. Co., St. Louis, Mo.) was added to the $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) in a final concentration of 2 mM. The mixture was incubated at 37°C for 0.5 hours. The mixture was then preincubated with an equal volume of sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), ultracentrifuged, dialyzed in 0.01 M phosphate buffer containing mercaptoethanol, redialyzed in the same buffer without mercaptoethanol, and assayed as described in Table 9.

Fraction Assayed	% Cholesterol Esterified
	Hydroxymercuribenzoate treated ¹
Top	38.8
Middle	11.3
Bottom	2.7
	Control, not treated with hydroxymercuribenzoate
Top	33.7
Middle	15.0
Bottom	7.0

¹ Assay was also performed on the parent $d > 1.21$ protein fraction containing 2 mM hydroxymercuribenzoate. LCAT activity was reduced by about 70% when compared with the untreated $d > 1.21$ protein fraction. Dialysis of the inhibited $d > 1.21$ protein fraction against a 0.01 M phosphate buffer (pH 7.4) containing 0.26 M NaCl, 0.1 mg/ml EDTA and 0.01 M mercaptoethanol, restored activity to the level of the untreated $d > 1.21$ protein fraction.

TABLE 13

ULTRACENTRIFUGAL (d 1.21 gm/ml-KBr) DISTRIBUTION OF LCAT ACTIVITY OF ENZYME-LECITHIN COMPLEXES AFTER TREATMENT WITH SODIUM TAUROCHOLATE AND KBr.

Two ml of the top fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), obtained by the procedure described in Table 9 were raised to d 1.21 gm/ml by addition of KBr. Sufficient sodium taurocholate (Cal. Biochem., Los Angeles, Calif.) and mercaptoethanol were added to obtain a final concentration of 0.5% (w/v) and 0.03 M respectively. This mixture was pipetted into a preparative ultracentrifuge tube and 4 ml of a KBr solution of d 1.15 gm/ml were layered above this mixture. The sample was ultracentrifuged at 114,000 X g, 18°C for 12 hours. Three 2 ml fractions were collected, dialyzed against the above phosphate buffer containing 0.01 M mercaptoethanol, redialyzed against the same buffer containing no mercaptoethanol, and assayed as described in Materials and Methods.

Ultracentrifugal Fraction	% Cholesterol Esterified	Protein Concentration (mg/ml)
Top	0.3	0.1
Middle	0.2	0.1
Bottom	21.0	0.4

of the lecithin dispersion was floated to the top (as ascertained by visual inspection of the distribution of turbidity).

IV. Discussion

a. Nature of the LCAT Activity in the $d > 1.21$ Protein Fraction.

In the present section we demonstrated that serum LCAT activity could be sedimented away from the bulk of the lipoproteins of human serum by ultracentrifugal fractionation in a salt (KBr) medium of $d 1.21$ gm/ml. Although no extensive chemical analysis was performed on the subnatant $d > 1.21$ protein fraction, this fraction apparently contained all the serum proteins of $d > 1.21$ gm/ml along with a certain amount of apolipoproteins which split off from the native lipoproteins (particularly HDL) during ultracentrifugation (6). Among these apolipoproteins, apoLP-gln I is probably the most prominent since its association with HDL can be disrupted under a variety of experimental procedures (e. g., storage, ultracentrifugation, exposure to ether and dehydration-rehydration).

When sonicated dispersions of mixtures of cholesterol and lecithin were used as substrates for assaying LCAT activity in the $d > 1.21$ protein fraction, initial reaction rates were obtained which were similar to those when total serum lipoproteins were used as substrates (81). Since it has been shown that there are some apolipoproteins, particularly apoLP-gln I, present in the $d > 1.21$ protein fraction (6), there is reasonable basis to assume that such apolipoproteins would be incorporated into complexes with sonicated

dispersions of mixtures of cholesterol and lecithin, and hence would form structures with properties similar to serum lipoproteins. Data obtained in our laboratory (81) and in a recent report by Raz (91) support the idea that some kind of lipoprotein complex is formed during incubation of the $d > 1.21$ protein fraction with sonicated dispersions of lipid. Whether such complexes are required in order to demonstrate LCAT activity in the $d > 1.21$ protein fraction is uncertain. Questions of this nature will be discussed in detail in Chapter 3.

b. Association of LCAT Activity with HDL.

Our results confirm the findings of Lossow et al. (72) and Akanuma and Glomset (4) that, under low ionic strength, LCAT does associate with HDL. Furthermore, we have shown that the affinity of LCAT for HDL is not altered when most of the substrate cholesterol and lecithin on the lipoproteins had been converted to cholesteryl ester and lysolecithin. The reason why the enzyme still shows such high affinity for lipoprotein species rich in the products of its reaction is not clear. However, it is possible that the apolipoproteins of HDL may play a significant role in the binding of LCAT. Supporting this idea, Akanuma and Glomset (4) observed, at low ionic strength, that binding of LCAT activity occurred when delipidated HDL were substituted for native HDL as the binding agents in affinity chromatography.

The effect of high ionic strength in promoting the dissociation

of an HDL-LCAT complex has been demonstrated in the present study as well as in a number of previous reports (72, 90). The reason why high ionic strength promotes such dissociation is probably due to the disruption of ionic bonds formed between HDL and LCAT. Since, at the pH used (pH 7.4), both lecithin and probably some amino acid side groups (at pH 7.0, most of the carboxyl and amino groups in proteins are ionized) of the lipoproteins were ionized (14, 50, 87), it is not possible to interpret whether ionic binding had occurred between enzyme and phospholipid, enzyme and apolipoproteins or a combination of both.

c. Interaction of LCAT with Sonicated Dispersions of Lecithin.

It was demonstrated in this chapter of the thesis that LCAT can complex with sonicated dispersions of lecithin. Since these complexes could be ultracentrifugally floated in a sucrose medium of d 1.065 gm/ml, it is probable that their densities were less than 1.065 gm/ml. Our preliminary data obtained in other experiments indicated that LCAT also forms complexes with sonicated dispersions of mixtures of cholesterol with lecithin. These complexes could also be isolated by the ultracentrifugal flotation procedure described. The formation of such complexes appears to depend only on the amount of lecithin present and not on the molar proportions of lecithin to cholesterol in the dispersions.

Low pH appears to disrupt the association between LCAT and sonicated dispersions of lecithin. Although the basis for this

disruption is not clear, it is evident that my results are comparable to those obtained with lipoprotein lipase (16) and phospholipase A (23).

I have also demonstrated that inhibition of LCAT activity by a sulfhydryl blocking agent (hydroxymercuribenzoate) does not reduce the amount of enzyme activity floated up (at $d = 1.065$ gm/ml-sucrose) into the top fraction. This indicates that the sulfhydryl group or groups associated with the enzyme's activity apparently are not crucial for the binding of the enzyme to the lecithin dispersions.

The inhibitory effect of sonicated dispersions of lecithin on LCAT activity contained in the $d > 1.21$ protein fraction is counter to the observation of Wagner and Rogalski (114) of an activation of LCAT in whole serum by a phosphatide emulsion. Since lipoproteins were present in their incubation mixtures, and absent in our work, the results of our experiments cannot be directly compared. The exact mechanism by which sonicated dispersions of lecithin inhibit LCAT activity in the $d > 1.21$ protein fraction is still to be clarified.

The reason why prior treatment with 0.01 M mercaptoethanol restored some LCAT activity in all of the ultracentrifugal fractions is unknown. The possibility that mercaptoethanol restores enzyme activity by reducing oxidized sulfhydryl group or groups in LCAT has been advanced. However, additional data, which will be presented in the Appendix (page 130), indicate that mercaptoethanol may do

more than just maintain the enzyme's sulfhydryl group or groups intact. In this respect, substitution of other reducing compounds, such as cysteine and glutathione, had very little effect in restoring LCAT activity in the ultracentrifugal fractions.

Techniques reported by Fielding (26) for isolation of lipoprotein lipase have general features comparable to those we have applied in the isolation of LCAT. In both cases, a lipid dispersion is used and an enzyme-lipid complex is formed which is sufficiently stable to undergo flotation by ultracentrifugation. The structures of these enzymes are still unknown; however, it is quite possible that there may be a basic set of residues in these enzymes which offer similar affinities for lipid and lipoprotein surfaces.

The use of sonicated dispersions of lecithin to effect the ultracentrifugal flotation of LCAT is a potential technique in its purification¹. In other approaches to the purification of this enzyme, serum albumin has been a major contaminant which has been most difficult to remove (40). In our present work, we have substantially separated the enzyme from the bulk of the serum albumin. Subsequent fractionation by conventional procedures (e. g., column chromatography) should yield enzyme preparations of higher purity. Since it has been shown in this report that LCAT may be easily oxidized, precautions should be taken in each purification step to avoid conditions promoting oxidation. Another problem in the purification of the enzyme is the apparent increase in lability

¹ See Appendix (page 117) for purification of LCAT activity using the present technique as well as a number of others.

as the enzyme is further purified. The possibility that LCAT is stable only in the presence of other proteins is still to be evaluated¹.

¹ See Appendix (page 119) for further discussion on the possible cause of LCAT's lability during purification procedures.

CHAPTER 3.

INTERACTION OF LECITHIN: CHOLESTEROL ACYLTRANSFERASE WITH SONICATED DISPERSIONS OF LECITHIN. EVALUATION OF COMPLEX FORMATION BY ULTRACENTRIFUGAL FLOTATION IN SALT MEDIA OF d 1.065 AND d 1.21 gm/ml

I. Background and Objectives

a. Effect of Salt on the Ultracentrifugal Properties of LCAT Activity in the Presence of Sonicated Dispersions of Lecithin.

It was demonstrated in the last chapter that LCAT formed complexes with sonicated dispersions of lecithin, and that these complexes could be isolated by ultracentrifugal flotation in a sucrose medium of d 1.065 gm/ml. In these experiments, sucrose was used instead of salt (such as KBr) in order to reduce the possibility of dissociation of the complex by exposure to a medium of high ionic strength during ultracentrifugation. In this respect, association between HDL and LCAT activity was shown to be disrupted by ultracentrifugation as well as other procedures in high ionic strength in both the present thesis and a number of other reports (72, 90).

Data obtained from one of our early experiments, in which KBr was used in place of sucrose for density adjustment to d 1.065 gm/ml, showed that the amount of activity floated into the top 2 ml in the KBr solution was approximately half that in the sucrose solution (Table 14). Furthermore, increasing the preincubation period of the mixture containing the $d > 1.21$ protein fraction and sonicated dispersions of lecithin prior to ultracentrifugation did not appear to

TABLE 14

ULTRACENTRIFUGAL (d 1.065 gm/ml) DISTRIBUTION OF LCAT ACTIVITY:
KBr vs. SUCROSE.

Ultracentrifugal fractionations in both KBr and sucrose were performed as described in Table 9. The final concentration of lecithin in the preincubation mixture was 2.5 mg/ml in both fractionations.

Ultracentrifugal Fraction	% Cholesterol Esterified	
	KBr Fractionation	Sucrose Fractionation
	Preincubated with lecithin	
Top	15.4	33.7
Middle	15.4	15.0
Bottom	60.4	7.0
	Control, preincubated with phosphate buffer	
Top	0.2	0.2
Middle	0.2	37.5 ¹
Bottom	78.8	80.0

¹ The presence of a significant amount of activity observed in the control middle fraction (sucrose) was probably due to an incomplete sedimentation of the enzyme into the bottom fraction because of the high viscosity of the sucrose solution.

increase the amount of LCAT activity that could be floated under the conditions used (Table 15).

b. Nature of the LCAT Activity Not Floated in a Salt Medium (KBr) of d 1.065 gm/ml.

In all previous flotation studies a density of 1.065 gm/ml was chosen because it was considered that this density would be sufficient to float up the bulk of the sonicated dispersion of lecithin. Re-examination of the distribution of lecithin in previous experiments (page 53) showed that only 60% of the total lecithin, added during preincubation, was recovered in the $d < 1.065$ gm/ml fraction. Since, in the absence of $d > 1.21$ protein fraction, over 95% of the total lecithin was floated at d 1.065 gm/ml, the incomplete flotation observed was probably due to the formation of lipid-protein complexes of $d > 1.065$ gm/ml. In agreement with this interpretation, complex formation between proteins of the $d > 1.21$ protein fraction and sonicated dispersions of lipid yielding products of $d > 1.063$ gm/ml was observed by Nichols and Gong (81), and more recently by Raz (91). In the experiments of Nichols and Gong (81), the $d > 1.21$ protein fraction, as a source of LCAT activity, was incubated with sonicated lipid substrates for 24 hours. Following incubation, the mixture was subjected to sequential ultracentrifugal fractionation at d 1.063 and d 1.21 gm/ml. In the absence of the $d > 1.21$ protein fraction, the sonicated substrates were found almost exclusively in the $d < 1.063$ gm/ml fraction. However, in the presence of the $d > 1.21$ protein fraction, sonicated substrates were found in both

TABLE 15

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY IN KBr SOLUTION OF d 1.065 gm/ml. EFFECT OF TIME OF PREINCUBATION OF $d > 1.21$ PROTEIN FRACTION WITH LECITHIN DISPERSION.

Two ml of the $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) were preincubated with 2 ml of a sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) for the time period indicated. The mixtures were adjusted to d 1.065 gm/ml by addition of 2 ml of an appropriate KBr solution. Ultracentrifugation and assay of enzyme activity were carried out as previously described (Table 9).

Preincubation Time (hours)	% Cholesterol Esterified ¹		
	Ultracentrifugal Fraction		
	Top	Middle	Bottom
0.5	5.5 (6.2)	15.6 (17.4)	68.8 (76.5)
1.0	5.5 (5.8)	17.9 (19.1)	70.5 (75.1)
3.0	4.2 (4.8)	12.1 (13.9)	70.9 (81.3)
6.0	2.3 (2.8)	11.2 (13.5)	69.1 (83.7)
24.0	6.7 (10.8)	5.1 (8.2)	50.2 (81.0)

¹ Values in parentheses are the percentage of the sum of the % cholesterol esterified in each of the three fractions.

the $d < 1.063$ gm/ml fraction and the $d 1.063-1.21$ gm/ml fraction. Furthermore, after incubation (24 hours, 37°C), the $d 1.063-1.21$ gm/ml fraction contained more product cholesteryl esters than those in the $d < 1.063$ gm/ml fraction. Combining these results with our present observations, it is possible that sonicated dispersions of lipid, when incubated with the $d > 1.21$ protein fraction, can form lipid-protein complexes of $d 1.063-1.21$ gm/ml.

Recently, Scanu et al. (102) reported that when HDL₂ and HDL₃ apolipoproteins were sonicated with lipid mixtures containing lecithin, products resembling lipoproteins were formed. These products could be isolated ultracentrifugally into three fractions at $d < 1.063$, $d 1.063-1.21$ and $d > 1.21$ gm/ml. Furthermore, the fraction isolated at $d 1.063-1.21$ gm/ml had physical characteristics, such as hydrated density, size, shape and flotation rate, similar to HDL. In light of these findings, and the apparent presence of HDL apolipoproteins in our $d > 1.21$ protein fraction, it is probable that a similar lipid-protein complex, as described by Scanu et al. was formed during incubation of the $d > 1.21$ protein fraction with sonicated dispersions of lecithin. Moreover, since these HDL-like complexes have densities greater than 1.063 gm/ml, it is not surprising that only 60% of the total lecithin initially added into the preincubation mixture was recovered in the $d < 1.065$ gm/ml fraction.

c. Objectives of the Present Chapter.

Based on the considerations described above we extended our

ultracentrifugal flotation experiments to include an evaluation of the interaction of LCAT activity with sonicated dispersions of lecithin at $d = 1.21$ gm/ml. It was expected that the results obtained from these experiments would provide more information on the interaction of the enzyme with lecithin, as well as information leading to the characterization of the possible lipid-protein complex of $d > 1.065$ gm/ml.

II. Materials and Methods

a. General.

Procedures used in this chapter of the thesis were similar to those previously described under Materials and Methods (in Chapter 2) except for those specifically stated below.

b. Assay of LCAT Activity.

The procedure used for assay of LCAT activity was similar to that described previously except that 0.01 M mercaptoethanol was present in all assay mixtures. The effect of mercaptoethanol on the assay of enzyme activity, under the conditions used in the present research, is described in the Appendix (page 128).

c. Preparation of HDL Protein.

HDL protein was prepared by the method of Shore and Shore (105). HDL (approximately 4.0 mg/ml) were extracted with an equal volume of alcohol-ether (2:3, v/v) by gentle rotation overnight at 4°C.

The upper solvent layer was removed, and the extraction was repeated for two 15 minute periods using alcohol-ether (1:3, v/v) in the same volume as the aqueous phase. The aqueous phase containing HDL protein, was dialyzed exhaustively against double distilled water to remove any residual organic solvent.

d. Preparation of HDL Apolipoproteins.

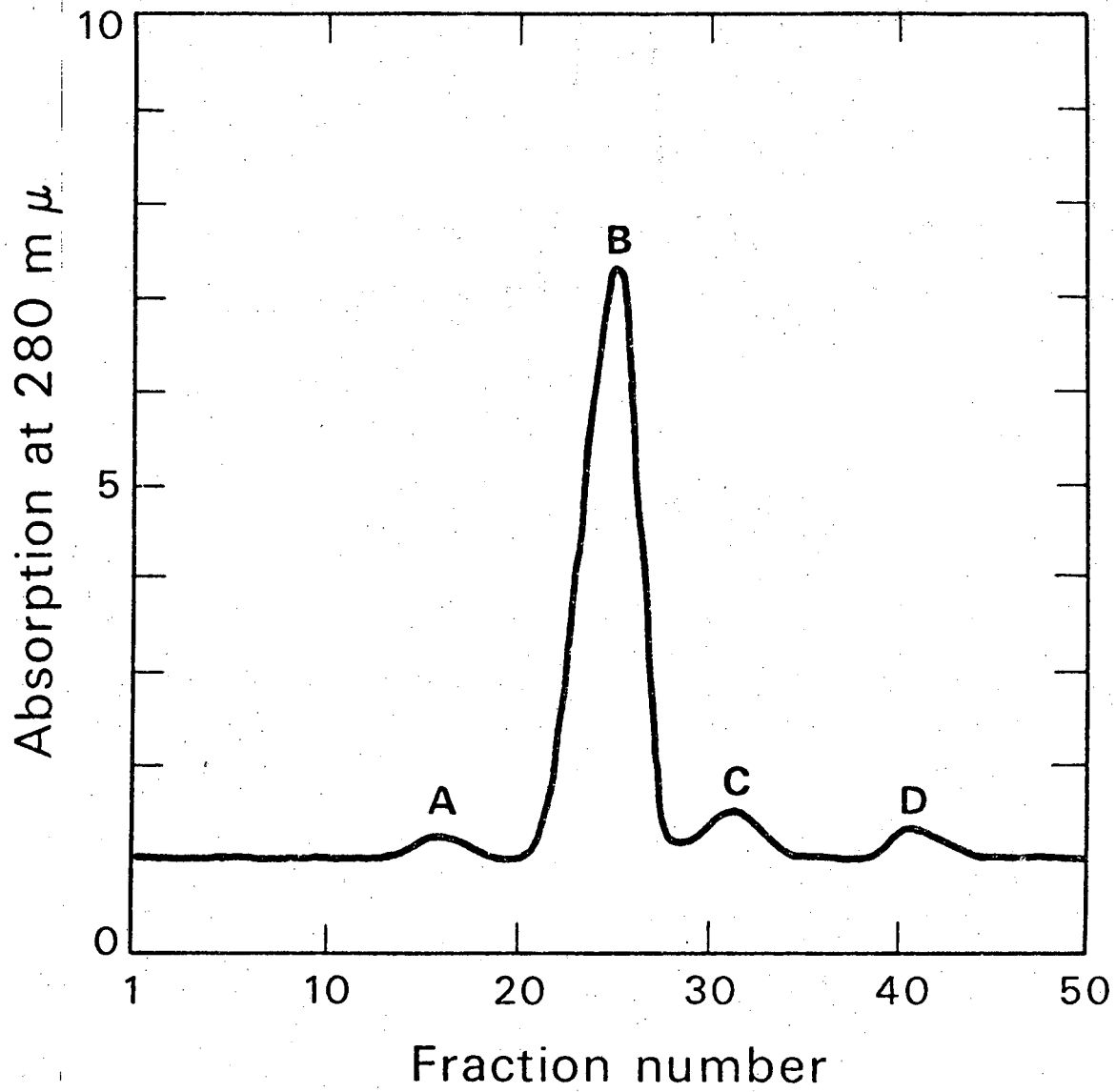
HDL protein was fractionated into its apolipoprotein components by the method of Scanu et al. (101). HDL protein was first dialyzed against 0.2 M Tris HCl buffer (pH 8.5) containing 6 M urea for 48 hours at 4°C. Twenty to 40 mg of the dialyzed HDL protein solution (in approximately 2% of the total bed volume) were applied onto a Sephadex G-200 column (2.5 cm X 100 cm), and eluted at room temperature with the same buffer system used for dialysis. The flow rate, 8 ml/hour, was controlled by a metering pump; direction of flow was ascending. Under the conditions described, the total period of chromatography was approximately 50 to 60 hours. During this period the effluent was continuously monitored for protein concentration at 280 m μ by an ISCO recorder. The fractions collected within each peak (Figure 6) were pooled and dialyzed to remove urea; first, against double distilled water and then against 0.01 M Tris HCl buffer (pH 7.4) containing 0.19 M NaCl and 0.1 mg/ml EDTA. The samples containing the individual apolipoproteins were concentrated in a Diaflow apparatus (Amicon Corp., Lexington, Mass.) using an UM-2 membrane. The concentrations of the apolipoproteins were

FIGURE 6

CHROMATOGRAPHY OF HDL PROTEIN ON SEPHADEX G-200 IN Tris HCl-6 M UREA.

Conditions of chromatography were as described in the text. Four protein eluting peaks were obtained, and are numbered as follows:

A, B, C, and D. Peak A corresponds to unresolvable HDL apolipoproteins; peak B corresponds to apoLP-gln I; peak C corresponds to apoLP-gln II; and peak D corresponds to a mixture containing apoLP-ala, apoLP-glu and apoLP-ser.



DBL 723-5193

estimated by absorption at 280 m μ using bovine serum albumin as standard.

e. Identification of the Sephadex Fractions.

Samples obtained from the chromatographic procedure described above were identified by polyacrylamide gel electrophoresis in 8 M urea. The method employed was similar to that described by Davies (22). Gels were prepared in 0.6 cm X 8.0 cm glass tubes, and the concentration of the acrylamide was 10% in the separating gel, and 2.5% in the concentrating gel. Tris glycine buffer containing 8 M urea, used in the electrodes, was prepared according to Reisfeld and Small (93). Approximately 50 μ g of protein were applied per tube, and gels were run at 2.5 mA/tube at room temperature. After removal of the gels from the glass tubes, they were fixed and stained in a solution containing 0.25% Coomassie brilliant blue (w/v), 45% methanol (v/v) and 9% acetic acid (v/v). Destaining was done electrophoretically in a solution containing 7% acetic acid (v/v) and 5% methanol (v/v) using a Canalco destaining apparatus. Figure 7 shows the positions of the various HDL apolipoproteins after electrophoresis.

III. Results

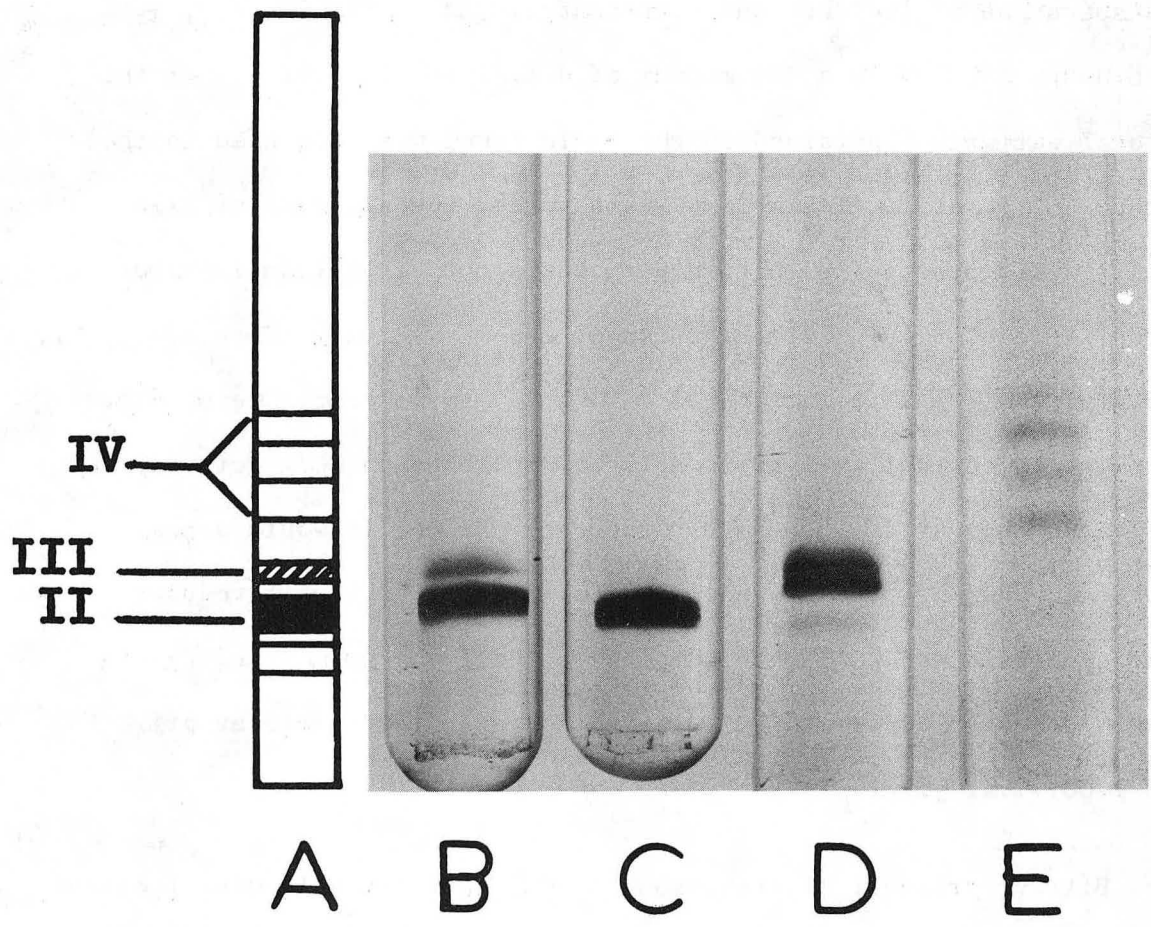
a. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin: Ultracentrifugation Performed at $d 1.21$ gm/ml (KBr).

When the $d > 1.21$ protein fraction was preincubated with sonicated

FIGURE 7

**POLYACRYLAMIDE GEL ELECTROPHORESIS OF APOLIPOPROTEINS OF HDL IN
8 M UREA.**

Conditions of electrophoresis were as described in text. A) schematic representation of total HDL protein, B) total HDL protein, C) peak B (apoLP-gln I), D) peak C (apoLP-gln II), E) peak D (mixture of apoLP-ala, apoLP-glu and apoLP-ser).



XBB 722-994

dispersions of lecithin and ultracentrifuged at 114,000 X g, for 48 hours at 10°C in a KBr medium of d 1.21 gm/ml, over 80% of the total activity (contained in the whole tube) was recovered in the top 2 ml fraction (Table 16). Furthermore, the presence of high salt during the preincubation period did not significantly alter the ultracentrifugal distribution of enzyme activity. When comparable mixtures were ultracentrifuged at d 1.065 gm/ml (KBr), we previously demonstrated that a significantly lower amount of LCAT activity was floated than at d 1.065 gm/ml (sucrose). Hence, it would appear that exposure of the preincubation mixture to high salt reduces incorporation of LCAT into complexes of $d < 1.065$ gm/ml, and favors the formation of stable LCAT-lecithin dispersion complexes of d 1.065-1.21 gm/ml.

b. Ultracentrifugal Distribution of LCAT Activity Following Incubation of the $d > 1.21$ Protein Fraction with Sonicated Dispersions of Lecithin. Effect of Ultracentrifugation in Salt Solutions (KBr) at Different Densities.

In order to explore the ultracentrifugal properties of the enzyme-lecithin complexes of d 1.065-1.21 gm/ml, aliquots of a preincubated mixture of a $d > 1.21$ protein fraction plus a sonicated dispersion of lecithin were adjusted to a series of densities (d 1.05 to 1.21 gm/ml) by addition of solid KBr and ultracentrifuged at 114,000 X g, 48 hours, at 10°C. The distribution of LCAT activity as a function of the density of the samples is shown in Figure 8.

TABLE 16

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY FOLLOWING INCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH SONICATED DISPERSIONS OF LECITHIN. ULTRACENTRIFUGATION PERFORMED AT $d 1.21$ gm/ml (KBr).

Three ml of the $d > 1.21$ protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) were preincubated with 3 ml of a sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA). After preincubation, solid KBr was added to the mixture to raise the density of the mixture to 1.21 gm/ml. Where preincubation was performed in presence of salt, KBr was added to raise the density of the mixture to 1.21 gm/ml prior to the start of the preincubation. The mixtures were ultracentrifuged at 114,000 X g, 10°C for 48 hours. Three 2 ml fractions (top, middle and bottom) were collected, dialyzed against 0.01 M Tris HCl buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), and assayed for enzyme activity as described in Materials and Methods.

Ultracentrifugal Fraction	% Cholesterol Esterified	
	Preincubated Without KBr	Preincubated With KBr
Top	36.5	32.2
Middle	2.4	2.7
Bottom	2.2	3.0

In the range of d 1.05 to d 1.10 gm/ml there was an abrupt decrease of enzyme activity in the bottom fractions; however, in the corresponding top fractions there was only a slight increase of activity. Between d 1.10 and d 1.15 gm/ml, the decrease of activity in the bottom fractions was approximately equal to the increase of activity in the top fractions. At d 1.15 to d 1.20 gm/ml both the activities in the bottom and top fractions leveled off to relatively constant values.

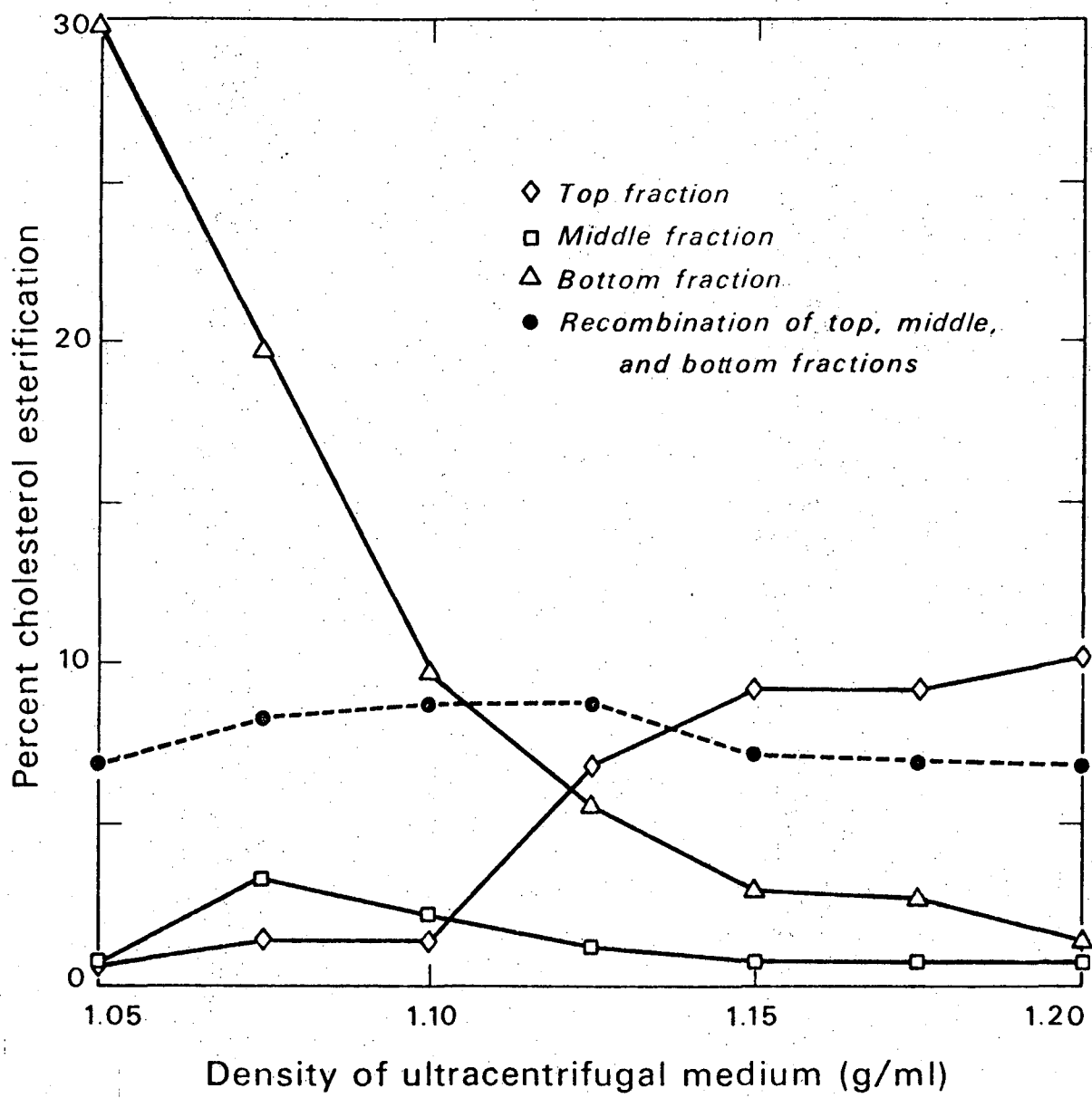
Examination of Figure 8 reveals two interesting points. First, the rapid decrease of activity in the bottom fractions between d 1.05 and d 1.10 gm/ml apparently was not due to flotation into the top fraction of LCAT activity in association with the floating lecithin dispersion. If this was the case, there would have been a concomitant increase of activity in the corresponding top fractions. Secondly, there was an unaccountable loss in recovered total activity (i. e., the sum of enzyme activity in the top, middle and bottom fractions), when the preincubation mixture was ultracentrifuged at $d > 1.10$ gm/ml. This loss of recoverable total activity could not be explained by the effect of ultracentrifugation in salt solutions of different densities, since no significant loss of total activity was observed when the same individual fractions were recombined and assayed.

In order to explain the two observations described above, we propose that a cofactor is apparently required for the transesterification reaction, and that lecithin-protein complexes, containing

FIGURE 8

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY FOLLOWING INCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH SONICATED DISPERSION OF LECITHIN. EFFECT OF ULTRACENTRIFUGATION IN SALT SOLUTIONS (KBr) AT DIFFERENT DENSITIES.

Three ml of $d > 1.21$ protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) were incubated with 3 ml of a sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA) at 37°C for 0.5 hours. After incubation, the mixture was adjusted to various densities (as indicated on the abscissa) by addition of solid KBr. The adjusted mixtures were ultracentrifuged at 114,000 X g, 10°C for 48 hours. After ultracentrifugation, three 2 ml fractions (top, middle, and bottom) were collected, dialyzed and assayed for enzyme activity as previously described (Table 16). In the recombination experiment, equal volumes of the top, middle and bottom fractions of each sample were mixed, and the mixtures were assayed for LCAT activity.



cofactor activity, of $d < 1.10$ gm/ml were formed during preincubation. Thus, the rapid decrease of activity in the bottom fractions of samples ultracentrifuged at $d 1.05$ to $d 1.10$ gm/ml was probably due to the flotation of complexes containing cofactor activity away from those containing the enzyme. At higher densities ($d > 1.10$ gm/ml) flotation of lecithin-protein complexes, containing LCAT activity, occurred since activity was clearly demonstrated in the top fractions. However, under the conditions used¹, it is possible that flotation of all of the enzyme initially present in the mixture did not occur, and hence a significant amount of LCAT activity may still remain in the bottom fraction. If the bulk of cofactor had been floated up as lecithin complexes at the higher densities, then we would expect no detectable transesterification in the bottom fractions even though a significant amount of enzyme might remain. In the absence of cofactor there would be a marked underestimation of the actual amount of LCAT activity present in the bottom fractions, and because of this, the apparent recoverable total activity of the samples ultracentrifuged at $d > 1.10$ gm/ml would be lower than of those ultracentrifuged at $d < 1.10$ gm/ml.

In view of the above discussion, the lack of LCAT activity in

¹ Since ultracentrifugal flotation of LCAT activity with HDL has been shown to be less effective in media of high ionic strength, the possibility that the association between LCAT and the lecithin dispersions is sensitive to high ionic strength is very likely. Thus, during ultracentrifugation in high salt, appreciable amounts of LCAT activity might not associate with lecithin dispersions and hence might remain in the bottom fraction.

the bottom fractions, when preincubation mixtures of the $d > 1.21$ protein fraction and lecithin dispersions were ultracentrifuged at $d > 1.15$ gm/ml, might not be due to the absence of LCAT, but to the depletion of a cofactor which was complexed with the lecithin dispersions and removed during ultracentrifugation. In support of this proposed explanation we observed no significant differences in activities when the top, middle and bottom fractions of samples ultracentrifuged at the various densities were recombined and assayed (Figure 8).

c. Effect of HDL Apolipoproteins on Assay of LCAT Activity.

Since HDL is the preferred substrate of LCAT (3), and the major apolipoproteins of HDL have been shown to form complexes of $d 1.063-1.21$ gm/ml with sonicated dispersions of lecithin (102), I decided to investigate the possibility that one of the apolipoproteins of HDL could be the cofactor proposed above. As indicated in Table 17, addition of apoLP-gln I dramatically increased the % cholesterol esterified, whereas addition of other HDL apolipoproteins had a much smaller effect. From these results, it is evident that apoLP-gln I is probably a required cofactor in the transesterification reaction catalyzed by LCAT. The manner in which this apolipoprotein promotes such activation will be investigated in later sections.

d. Effect of Duration of Ultracentrifugation ($d 1.21$ gm/ml-KBr) on the Association of LCAT Activity with Lecithin Dispersions.

TABLE 17

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY FOLLOWING INCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH SONICATED DISPERSION OF LECITHIN. EFFECT OF APOLIPOPROTEINS FROM HDL ON ASSAY OF BOTTOM FRACTION.

The 2 ml bottom fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) obtained by the method described in Table 16 was assayed. One-half ml of this fraction was mixed with 0.25 ml of apolipoprotein (0.8 mg/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA) and 0.25 ml of sonicated substrate (in 0.01 M Tris HCl, pH 7.4, containing 0.1 mg/ml EDTA). The substrate solution used contained sufficient mercaptoethanol to raise the concentration of mercaptoethanol to 0.01 M in the final assay medium. The mixtures were incubated at 37°C for 24 hours, and the percent cholesterol esterified was determined as described in Materials and Methods. In the control, 0.01 M Tris HCl buffer (pH 7.4, containing 0.1 mg/ml EDTA) was added instead of the solution containing the apolipoproteins.

Apolipoprotein Added ¹	% Cholesterol Esterified
Peak B (apoLP-gln I)	46.4
Peak C (apoLP-gln II) ²	14.3
Peak D (mixture of apoLP-ala, apoLP-ser, and apoLP-glu)	9.7
Control	3.8
Peak B alone	0.3
Peak C alone	0.2
Peak D alone	0.3

¹ See Materials and Methods for nomenclature of apolipoproteins.

² When peak C was assayed by polyacrylamide gel electrophoresis, a trace amount of apoLP-gln I was detected. Hence, value for the % cholesterol esterified was probably higher than would be determined for apoLP-gln II alone.

In order to determine the effect of duration of ultracentrifugation on the association of LCAT and lecithin dispersions, the preincubation mixture, consisting of the $d > 1.21$ protein fraction plus the sonicated dispersions of lecithin, was adjusted to $d 1.21$ gm/ml by addition of solid KBr and then ultracentrifuged for 48 and 72 hours at $114,000 \times g$, 10°C . Table 18 summarizes the distribution of activity when the fractions were assayed in the presence and absence of added cofactor, apoLP-gln I. As indicated, when assay was done with added apoLP-gln I, the percentage distribution of total activity within the tube obtained in the 48 hour ultracentrifugal period was 40%, 21% and 39% (top, middle and bottom respectively), and in the 72 hour ultracentrifugal period was 34%, 15% and 51% (top, middle and bottom respectively). If we assume that in the presence of added apoLP-gln I the amount of cholesterol esterified corresponded to the true LCAT level, then it would appear that longer periods of ultracentrifugation tended to promote more dissociation of LCAT activity from the sonicated dispersions of lecithin.

e. Ultracentrifugal ($d 1.21$ gm/ml) Flotation of Lecithin Dispersions Containing LCAT Activity. Low vs. High Ionic Strength.

Since the addition of apoLP-gln I into the regular assay medium enabled the detection of LCAT activity which previously was undetectable in certain ultracentrifugal fractions, the effectiveness of low and high ionic strength in ultracentrifugal flotation of complexes containing LCAT activity could now be examined more thoroughly.

TABLE 18

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY FOLLOWING INCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH SONICATED DISPERSIONS OF LECITHIN. EFFECT OF TIME OF ULTRACENTRIFUGATION (d 1.21 gm/ml-KBr) AND ASSAY IN THE PRESENCE OF APOLIPOPROTEIN (apoLP-gln I).

Ultracentrifugal fractions were prepared as described in Table 16. After dialysis of the fractions against 0.01 M Tris HCl buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), enzyme activity was assayed in the presence and absence of added apoLP-gln I. The composition of the final assay media was as follows: 0.5 ml ultracentrifugal fraction, 0.25 ml sonicated substrate (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA and sufficient mercaptoethanol to raise its concentration in final assay medium to 0.01 M), 0.25 ml apoLP-gln I (2.0 mg/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA) or 0.25 ml of the same buffer without the apolipoprotein. Incubation and determination of cholesterol esterified were performed as described in Materials and Methods.

Ultracentrifugal Fraction	% Cholesterol Esterified ¹			
	Ultracentrifuged for 48 Hours		Ultracentrifuged for 72 Hours	
	With apoLP-gln I	Without apoLP-gln I	With apoLP-gln I	Without apoLP-gln I
Top	49.7 (40)	25.4 (81)	41.0 (34)	19.3 (68)
Middle	26.8 (21)	2.2 (7)	17.4 (15)	1.5 (5)
Bottom	48.9 (39)	3.7 (12)	61.1 (51)	7.6 (27)

¹ Values in parentheses are the percentage of the sum of % cholesterol esterified in each of three fractions.

As in previous experiments, the $d > 1.21$ protein fraction was preincubated with a sonicated dispersion of lecithin for 0.5 hours at 37°C . The above incubation mixture was then adjusted to $d 1.21$ gm/ml either by addition of a KBr- H_2O solution or a sucrose- D_2O solution. The samples were ultracentrifuged at $114,000 \times g$, at 10°C for 60 hours instead of the regular 48 hours to ensure that density equilibrium had been reached. After collection of the fractions, and dialysis against buffer, LCAT activity was assayed in the presence of added apoLP-gln I. As indicated in Table 19, significantly higher amounts of LCAT activity were detected in the top fraction when ultracentrifugation was done at low ionic strength than at high ionic strength. The opposite was the case when activities of the bottom fractions were compared.

The results presented in this section provided no data on the effect of low vs. high ionic strength on flotation of lecithin dispersions containing cofactor (apoLP-gln I) activity. When ultracentrifugation was done in high salt only very low levels of LCAT activity could be detected in the bottom fraction when no apoLP-gln I was added to the assay medium (see results of Table 18). These results indicated that the flotation of complexes, containing apoLP-gln I, was complete under the conditions used, and the association between apoLP-gln I and lecithin dispersions was apparently stable at high ionic strength.

f. Mechanism of ApoLP-gln I Activation.

TABLE 19

ULTRACENTRIFUGAL DISTRIBUTION OF LCAT ACTIVITY FOLLOWING INCUBATION OF THE $d > 1.21$ PROTEIN FRACTION WITH SONICATED DISPERSIONS OF LECITHIN. EFFECT OF ULTRACENTRIFUGATION IN SUCROSE-D₂O (d 1.21 gm/ml) vs. KBr-H₂O (d 1.21 gm/ml).

Four ml of $d > 1.21$ protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) were preincubated with 4 ml of a sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M Tris HCl, pH 7.4, containing 0.1 mg/ml EDTA). After preincubation, 4 ml of the mixture were adjusted to d 1.21 gm/ml by addition of a sucrose-D₂O solution (2 ml) while the remaining 4 ml were adjusted to the same density by addition of a KBr-H₂O solution (2 ml). Ultracentrifugation (60 hours) and assay of LCAT activity, in presence of apoLP-gln I, were done as described in Table 18.

Ultracentrifugal Fraction	% Cholesterol Esterified ¹	
	Sucrose-D ₂ O	KBr-H ₂ O
Top	78.9 (47)	47.8 (25)
Middle	54.6 (33)	63.4 (33)
Bottom	33.3 (20)	82.6 (42)

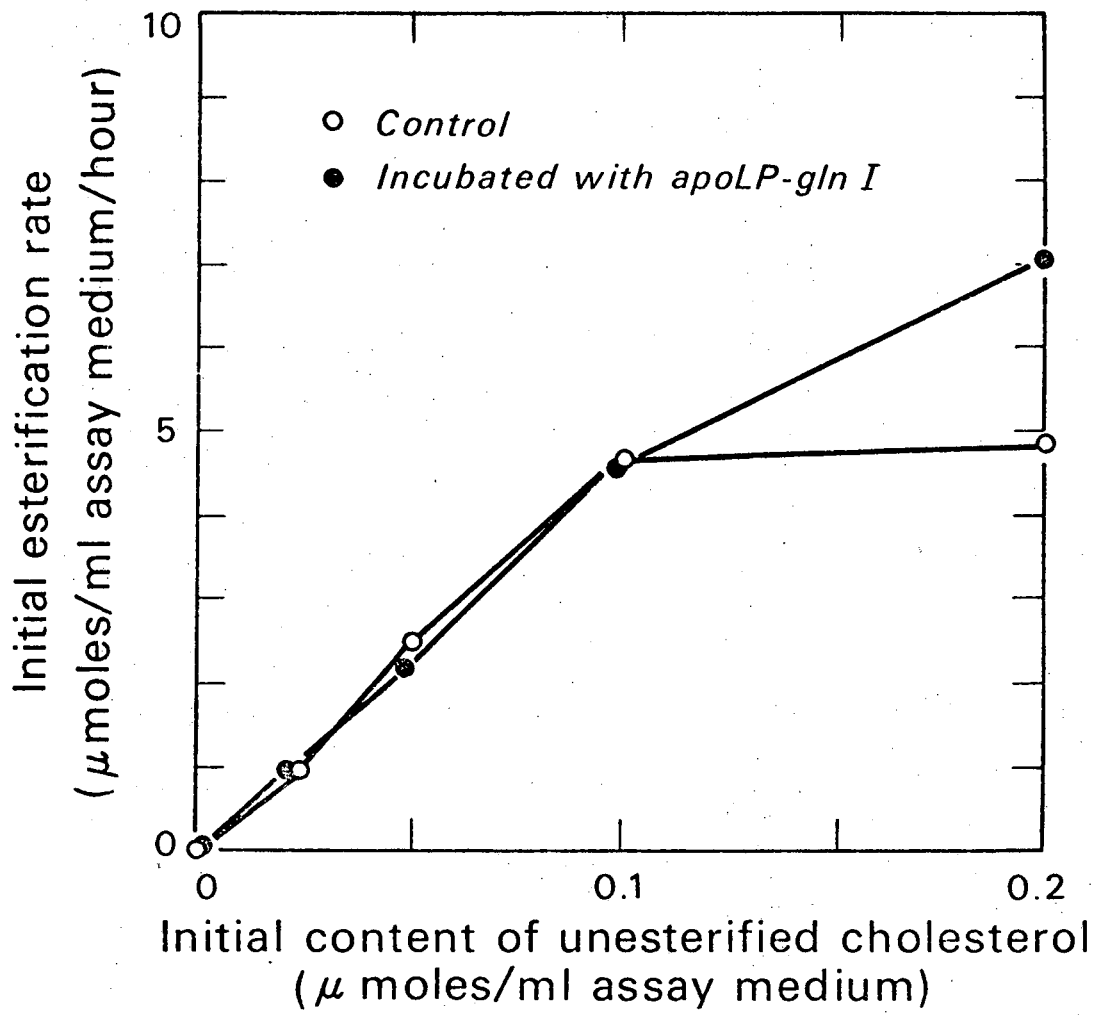
¹ Values in parentheses are the percentage of the sum of % cholesterol esterified in each of the three fractions.

I have previously noted that HDL is apparently the preferred substrate of LCAT in the bloodstream (3, 44), and that apoLP-gln I is one of the two major apolipoproteins of HDL (101, 106, 107, 108). Although apoLP-gln I has been shown as a major requirement for the transesterification reaction catalyzed by LCAT, the mechanism whereby it promotes transesterification is still not clear. This is true either when apoLP-gln I is bound to HDL or to sonicated substrates. Since apoLP-gln I has been shown to play a crucial role in the organization of sonicated dispersions of lipids into HDL-like structures (102), it is possible that apoLP-gln I might promote LCAT activity by organizing lipid dispersions into forms more reactive with LCAT. To investigate this possibility, the following experiment was performed. The $d > 1.21$ protein fraction, as a source of LCAT activity, was incubated with increasing amounts of sonicated lipid substrates in the presence and absence of added apoLP-gln I. The initial rate of reaction at each level of lipid substrate was determined, and the data are plotted as shown in Figure 9. At substrate levels up to $0.1 \mu\text{moles unesterified cholesterol/ml assay medium}^1$, the initial rates of the samples incubated with and without apoLP-gln I were rather similar. As the substrate levels were increased to $0.2 \mu\text{moles unesterified cholesterol/ml assay medium}$, the initial rate of the sample without added apoLP-gln I reached a plateau while that with

¹ The substrate used in this case actually contained both unesterified cholesterol and lecithin in the molar proportion as described in Materials and Methods (Chapter 2). Micromoles of unesterified cholesterol was used as a convenient way to designate substrate concentration.

FIGURE 9
EFFECT OF ApoLP-gln I ON THE INITIAL RATE OF ESTERIFICATION AT
DIFFERENT CONCENTRATIONS OF SONICATED SUBSTRATE.

Incubation mixtures consisted of 0.5 ml $d > 1.21$ protein fraction (in 0.01 M Tris HCl, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), 0.25 ml sonicated substrate (in 0.01 M Tris HCl, pH 7.4, containing 0.1 mg/ml EDTA and 0.04 M mercaptoethanol), and 0.25 ml apoLP-gln I (0.75 mg/ml; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA). In the control, 0.25 ml Tris HCl buffer (pH 7.4, containing 0.1 mg/ml EDTA) was substituted for the apoLP-gln I solution.



the added apoLP-gln I continued to increase linearly. This result indicated that the presence of apoLP-gln I enabled the LCAT reaction to proceed without saturation at higher levels of substrates. There are at least two possible explanations for this observation. First, the presence of apoLP-gln I may directly activate LCAT so that more transesterification can take place per unit of time. Secondly, apoLP-gln I may bind with sonicated lipid substrate dispersions, and may thereby enhance their reactivity against LCAT. If the addition of apoLP-I is responsible for converting LCAT to a more active form, an increase in the rate of esterification would probably have been observed at both high and low substrate levels. Since there was no significant difference in initial esterification rates at low substrate levels between the samples incubated with and without apoLP-gln I, the possibility that apoLP-gln I activates LCAT is unlikely.

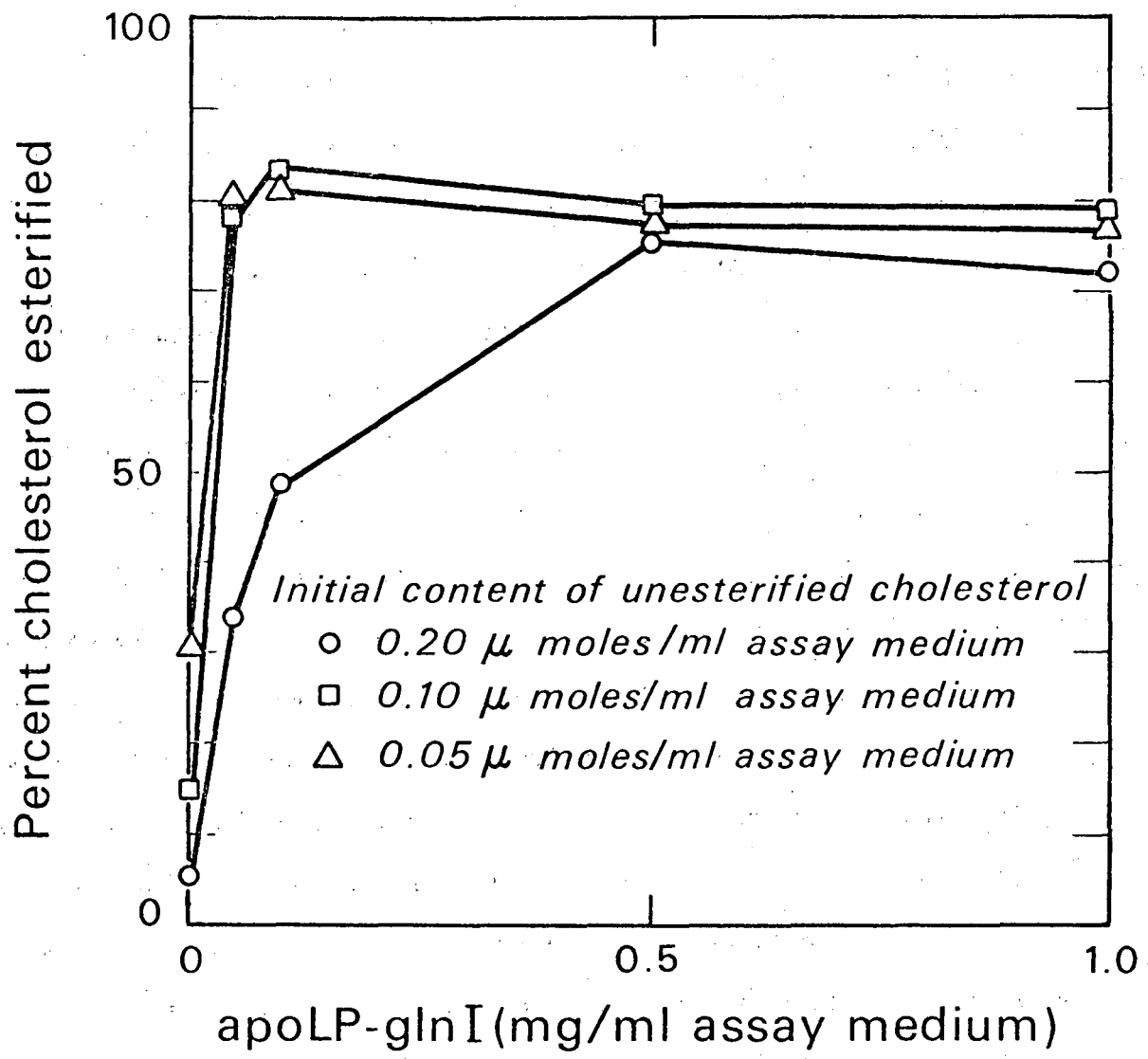
In order to further evaluate the possibility that apoLP-gln I binds with lipid dispersions and enhances their reactivity against LCAT, the following experiment was performed. The bottom fraction obtained by the preincubation and ultracentrifugal flotation procedure previously described (see Table 16) was used as a source of an essentially apoLP-gln I-free LCAT preparation. The enzyme preparation was divided into aliquots and incubated with increasing amounts of apoLP-gln I at three substrate concentrations. The % cholesterol esterified, as an indication of the substrate's reactivity against LCAT, was plotted as a function of increasing apoLP-gln I (Figure 10).

FIGURE 10

EFFECT OF ApoLP-gln I ON SUBSTRATE PROPERTIES OF SONICATED DISPERSIONS USED FOR ASSAY OF LCAT ACTIVITY.

Incubation mixtures consisted of 0.5 ml bottom fraction (obtained by the procedure described in Table 16; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), 0.25 ml sonicated substrate (in 0.01 M Tris HCl, pH 7.4, containing 0.1 mg/ml EDTA and 0.04 M mercaptoethanol), and 0.25 ml apoLP-gln I (concentration as indicated in figure; in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA). Incubation was performed at 37°C for 24 hours. Determination of the % cholesterol esterified is described in Materials and Methods.

¹ Since the maximum possible conversion of cholesterol to cholesteryl ester in the sonicated substrate is 100%, I have used the % cholesterol esterified to indicate the increase in reactivity of sonicated substrates, at each of the three substrate levels, resulting from the addition of apoLP-gln I to the assay media.



At all substrate concentrations there was minimal % cholesterol esterified with no apoLP-gln I addition. When the concentration of apoLP-gln I was increased, a rapid increase in % cholesterol esterified resulted. At higher concentrations of apoLP-gln I, the % cholesterol esterified reached a plateau. In addition to the trend described above, Figure 10 also shows that the lower the substrate level the lower the amount of apoLP-gln I required to reach maximal % cholesterol esterified. Based on the results described in Figure 10, the following are evident: 1) sonicated dispersions of lipid substrates require a certain amount of apoLP-gln I in order for the dispersion to react with LCAT (this is indicated by the rapid rise of % cholesterol esterified at all substrate concentrations when apoLP-gln I was added to the incubation mixtures); 2) the amount of apoLP-gln I required for activation of lipid dispersions is highly dependent on the concentration of lipid dispersions (thus, at higher concentrations of lipid substrate, higher amounts of apoLP-gln I are required to reach a maximum value of % cholesterol esterified); 3) once the optimal amount of apoLP-gln I required for maximum activation is reached, additional apoLP-gln I appears to slightly inhibit the % cholesterol esterified; 4) when apoLP-gln I is not added, the highest % cholesterol esterified detected was in the sample with the lowest substrate level. The latter observation probably indicates that a very small amount of apoLP-gln I was present in the original enzyme preparation, since at low concentrations of lipid dispersions in the assay system, low amounts of

0 3 0 0 3 8 0 3 0 7 7

apoLP-gln I were required for activation. Based on the results of these experiments, it is reasonable to assume that apoLP-gln I forms complexes with sonicated lipid substrate dispersions, and it is these lipid-protein complexes that probably serve as substrates for LCAT.

IV. Discussion

In the present study we have developed two interesting aspects concerning the interaction between LCAT activity and sonicated dispersions of lipids. First, we have shown that a significant amount of LCAT activity, associated with lecithin dispersions, can be floated in a KBr medium of d 1.21 but not of d 1.055 gm/ml. Secondly, we have shown that an HDL apolipoprotein, apoLP-gln I, is apparently a major requirement for activation of sonicated dispersions of substrate lipids. Without such activation no transesterification can take place even though LCAT is present in the assay medium.

a. Recapitulation of the Results on the Ultracentrifugal Flotation of LCAT Activity Using Sonicated Dispersions of Lecithin.

Based on the results of the work presented in this thesis, it is evident that LCAT activity, contained in the $d > 1.21$ protein fraction, can associate with lecithin dispersions and float, in high salt, at $d > 1.10$ gm/ml. When a preincubation mixture of $d > 1.21$ protein fraction and sonicated lecithin dispersions was ultracentrifuged at d 1.21 gm/ml in KBr, LCAT activity, associated with lecithin dispersions, could be detected in the top fraction without addition

of cofactor (apoLP-gln I) to the assay medium. On the other hand, the LCAT activity remaining in the bottom fraction, possibly associated with trace amounts of lecithin, could not be detected unless the cofactor (apoLP-gln I) was added to the assay medium. In light of these results, it is evident 1) that only part of the total LCAT activity in the $d > 1.21$ protein fraction was associated with the lecithin dispersion and floated at $d < 1.21$ gm/ml, and 2) that a significant amount of apoLP-gln I, associated with the lecithin dispersions, was floated at $d < 1.21$ gm/ml.

In contrast to the association between HDL and LCAT, the association between lecithin dispersions and LCAT is apparently more stable in high ionic strength media. The reason for this is still unknown.

So far we have omitted in our discussion results obtained from ultracentrifugal flotation studies performed at $d 1.065$ and $d 1.21$ gm/ml in sucrose instead of KBr. The reason for this is severalfold. First, sucrose tremendously increased the viscosity of the ultracentrifugal media, and the ultracentrifugal distributions of LCAT activity in association with lecithin dispersions, in such viscous media, could not be adequately compared with those obtained using KBr. Secondly, since the extent of ultracentrifugation was shown to be critical in determining the amount of dissociation of LCAT activity from lecithin dispersions, it was difficult to determine an optimal time required to reach equivalent flotation without at the same time risking the dissociation of LCAT activity from lecithin dispersions.

The results of the ultracentrifugal experiments with sucrose have provided suggestive evidence that the association of LCAT activity with lecithin dispersions is sensitive to high ionic strength. However, it is difficult to make a definitive statement, based on the current ultracentrifugal data, concerning the effect of ionic strength on the stability of the enzyme-lipid (or enzyme-lipid-cofactor) complex. In order to avoid the drawbacks arising from the present ultracentrifugal flotation technique, we are currently developing a gel filtration column system which can test the effect of ionic strength on complex stability without using the extreme conditions (high viscosity and high centrifugal force) as described in the present procedure.

b. Implication of the Present Work to Considerations of the Physiological Function of LCAT.

In this study we have demonstrated conclusively that HDL apolipoprotein, apoLP-gln I, is a crucial component in the activation of lipid dispersions for transesterification by LCAT. In agreement with our results, Fielding and Fielding (29) recently have shown that sonicated dispersions of lipids could not serve as substrates for an LCAT preparation purified 2700 fold; however, addition of HDL protein, very high density lipoprotein protein¹ and $d > 1.25$ gm/ml serum proteins into the assay medium significantly increased reactivity of these sonicated dispersions of lipids. Furthermore,

¹ The very high density lipoproteins are usually defined as those lipoproteins having a hydrated density of 1.21 to 1.25 gm/ml.

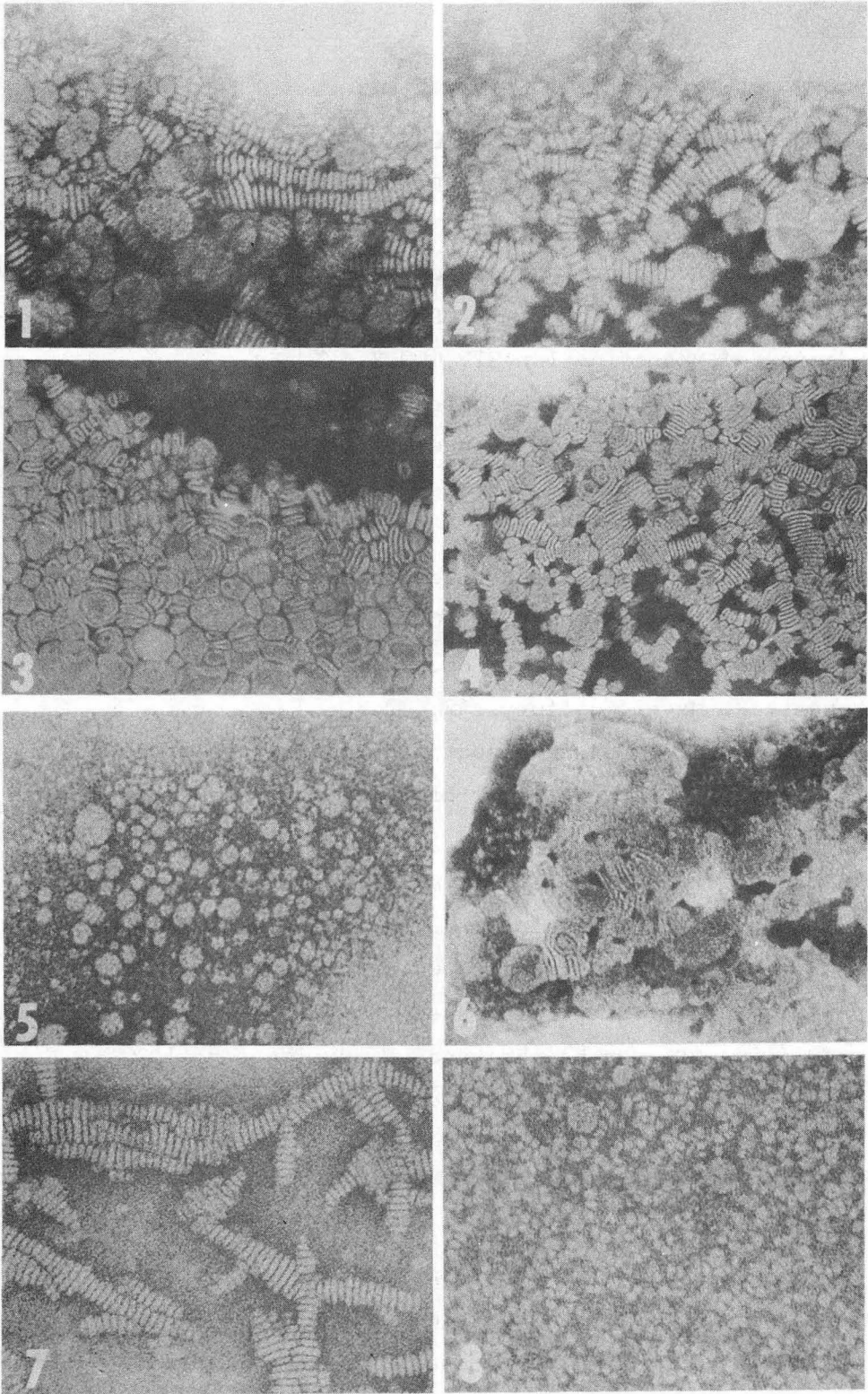
in the same study, Fielding and Fielding found that HDL₃ was a much better substrate than HDL₂. This could probably be explained by the fact that HDL₃ contain a higher proportion of apoLP-gln I than HDL₂ (6).

The mechanism whereby apoLP-gln I promotes the reactivity of either lipoproteins or dispersed lipids as substrates for transesterification is still not clear. It is well established that apoLP-gln I is one of the two major apolipoproteins in HDL (101, 106, 107, 108). Recently, Scanu et al. (102) have suggested that this apolipoprotein might be crucial in maintaining the structure of HDL, and in support of their findings they demonstrated that sonicated products of apoLP-gln I and HDL₂ lipid extract showed physical-chemical characteristics very similar to those of the native HDL₂.

It is possible that apoLP-gln I can enhance the reactivity of lipid dispersions by binding with them to form HDL-like structures. Supporting this idea, our laboratory (34) has recently obtained electron micrographs of sonicated mixtures of apoLP-gln I with various polar and apolar lipids. As indicated in Figure 11, some of them show remarkable resemblance to native HDL. Moreover, the discoidal structures obtained when apoLP-gln I was sonicated with either lecithin or mixtures of lecithin with unesterified cholesterol showed exceptional similarity to an HDL fraction obtained from LCAT deficient patients (32) (Figure 1 and Figure 11-1 and -2). When a preparation of sonicated mixtures of apoLP-gln I with lecithin and unesterified cholesterol was incubated with a $d > 1.21$ protein fraction,

FIGURE 11
ELECTRON MICROGRAPHS OF SONICATED MIXTURES OF HDL APOLIPOPROTEINS
WITH VARIOUS POLAR AND APOLAR LIPIDS (32).

1. A negatively stained sonicated mixture of apoLP-gln I (0.11 mg/ml) plus lecithin (0.16 mg/ml). Discoidal and stacked structures predominate. Magnification 212,000 X.
2. A negatively stained sonicated mixture of apoLP-gln I (0.11 mg/ml), lecithin (0.11 mg/ml) and unesterified cholesterol (0.05 mg/ml). Discoidal and stacked structures, similar to those observed in 1., predominate. Magnification, 212,000 X.
3. A negatively stained sonicated mixture of bovine serum albumin (0.11 mg/ml) plus lecithin (0.16 mg/ml). Numerous elongated structures, some of which form closed loops, as well as vesicles can be observed. Magnification, 137,000 X.
4. A negatively stained sonicated dispersion of lecithin (0.16 mg/ml). Elongated structures, many of which form closed loops, predominate. Magnification, 133,000 X.
5. A negatively stained sonicated mixture containing apoLP-gln I (0.10 mg/ml), apoLP-gln II (0.04 mg/ml), lecithin (0.09 mg/ml), unesterified cholesterol (0.02 mg/ml) and cholesteryl ester (0.10 mg/ml). Spherical particles some of which approach the dimensions of native HDL can be observed. Magnification, 189,000 X.
6. A negatively stained sonicated mixture of lecithin (0.13 mg/ml), unesterified cholesterol (0.03 mg/ml) and cholesteryl ester (0.15 mg/ml). Amorphous masses and some myelin-type figures can be observed. Magnification, 137,000 X.
7. A negatively stained preparation of the d 1.063-1.21 gm/ml fraction of a sonicated mixture containing apoLP-gln I (3.00 mg/ml), lecithin (2.77 mg/ml) and unesterified cholesterol (0.23 mg/ml). Stacked discs are the predominant structure. Magnification, 212,000 X.
8. A negatively stained preparation of the above fraction isolated after incubation (24 hours, 37°C) with LCAT activity. The disc-shaped structures seen in 7. have been transformed into smaller particles which are similar to native HDL. Magnification, 212,000 X.



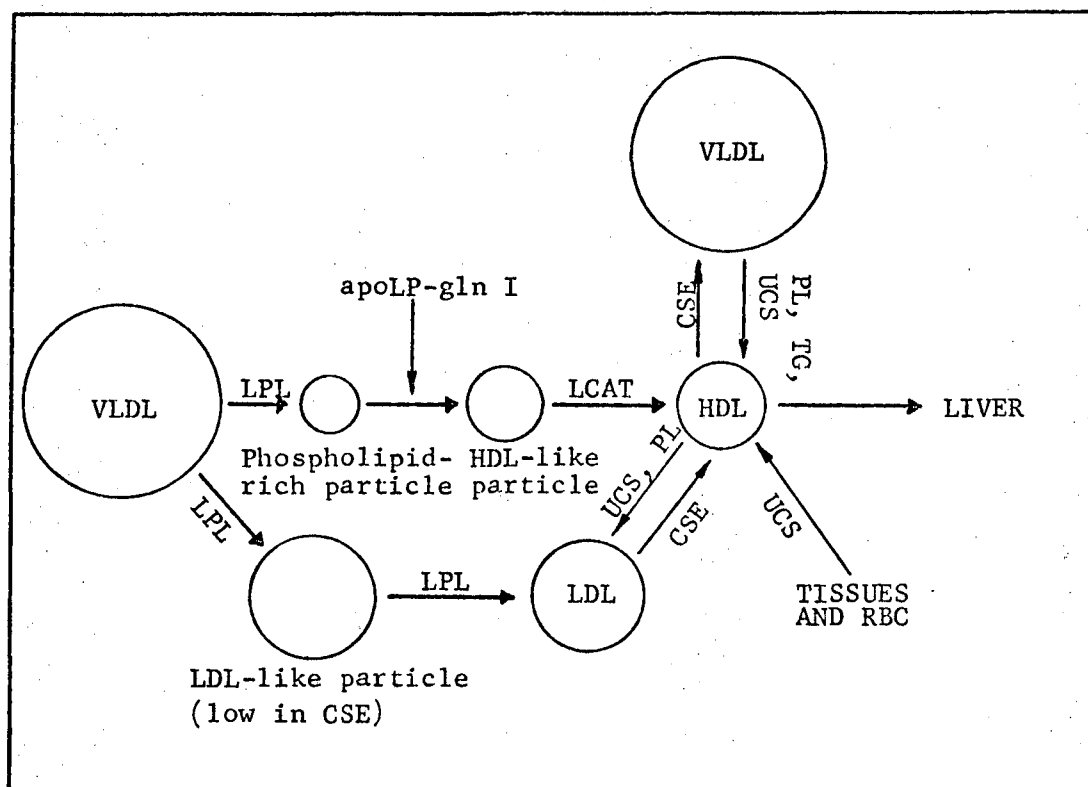
XBB 716-2618

conversion of discoidal to spherical structures resembling normal HDL resulted (Figure 11-7 and -8). From these results, it is evident that apoLP-gln I can organize lipid dispersions into structures which are similar to normal HDL, and into structures similar to naturally occurring lipoproteins which have not been subjected to transesterification by LCAT.

Although we have learned a lot about the effect of LCAT activity on lipoprotein morphology, the role this enzyme has on lipoprotein metabolism is still speculative. Schumaker and Adams (103) have suggested that LCAT may be required for removal of polar lipids (lecithin and unesterified cholesterol) from the surface of VLDL in order for the triglyceride moiety below the surface to be hydrolyzed by lipoprotein lipase. Recently, a number of investigators have provided strong evidence indicating that the hydrolysis of VLDL-triglyceride by lipoprotein lipase could lead to the conversion of VLDL to LDL-like molecules (68). Whether the high density lipoproteins are similarly produced, with the aid of LCAT activity, during VLDL metabolism is not clear.

Utilizing the results obtained in this thesis and ideas from two existing theories of LCAT function (43, 103), I have developed a scheme showing a possible role for LCAT in the metabolism of human serum lipoproteins, particularly VLDL. The general aspect of this scheme is outlined in Figure 12. VLDL are first secreted into the bloodstream from the liver. During their circulation in the vascular system, their triglycerides are hydrolyzed into diglycerides,

FIGURE 12
 PROPOSED SCHEME FOR VLDL METABOLISM BY LIPOPROTEIN LIPASE AND
 LCAT.



Abbreviations: LPL, lipoprotein lipase; LCAT, lecithin: cholesterol acyltransferase; PL, phospholipid; TG, triglyceride; UCS, unesterified cholesterol; CSE, cholesteryl ester; RBC, red blood cells; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

0 0 0 0 0 8 0 0 0 8 0

monoglycerides, and fatty acids by lipoprotein lipase present in the blood vessel walls. As additional triglycerides are hydrolyzed, and the hydrolyzed products removed from VLDL, the molar contents of phospholipid and cholesterol of these lipoproteins are increased. Consequently, because of this build-up of phospholipid and cholesterol, VLDL become less effective as substrate for lipoprotein lipase. Furthermore, because of the change in lipid composition, the native structure of VLDL could become less stable, and small lipoprotein units, rich in phospholipid (possibly containing some triglycerides, cholesterol and apolipoproteins), dissociate from the parent lipoprotein. Since the presence of excess phospholipid in triglyceride emulsions has been shown to inhibit lipoprotein lipase activity (27), the removal of phospholipid-rich lipoprotein units would probably lead to further hydrolysis of triglycerides of the remnants of VLDL. As additional triglycerides are hydrolyzed, and phospholipid-rich particles removed from these remnants of VLDL, molecules resembling LDL in size and shape may be produced. At the same time, the phospholipid-rich particles, derived from VLDL, may become activated for transesterification by LCAT by association with apoLP-gln I (this apolipoprotein can either be present as a free protein or as part of the existing HDL) in the bloodstream. As cholesteryl esters are generated, an apolar core, possibly with some pre-existing triglycerides, is formed. Organization of protein and phospholipid around this core could lead to structures resembling HDL. Further esterification of these HDL-like molecules produces additional

cholesteryl esters, and exchange of lipid components among the different classes of lipoproteins begins to take place. In addition, the reduction in unesterified cholesterol, resulting from transesterification catalyzed by LCAT, induces a net uptake of cholesterol from tissue membranes to HDL. Because of this net uptake, cholesterol from tissue membranes can be transported, using HDL as carriers, to the liver for metabolism.

The proposed scheme does not describe the details of how lipoprotein lipase and LCAT affect the interconversion of lipoproteins. I believe, however, that it intergrates much of the existing data on the interaction of LCAT and lipoproteins, and suggests several hypotheses which may be of interest to test.

APPENDIX

I. Foreword

All work presented in the Appendix section was performed prior to my demonstration that apoLP-gln I is a major cofactor required for activation of sonicated substrates for transesterification by LCAT. Hence, the studies described below utilized our initial assay system which determined the presence and extent of LCAT activity only when adequate apoLP-gln I was present.

II. Some Characteristics of LCAT Activity and Its Purification

In the following sections I have investigated the behavior of LCAT activity by molecular sieving chromatography, DEAE-cellulose chromatography and ammonium sulfate precipitation. It was hoped that by utilizing the results of these studies a logical sequence of steps could be developed for the purification of LCAT. In addition, the data obtained in these experiments have provided insight into the physical characteristics of the enzyme.

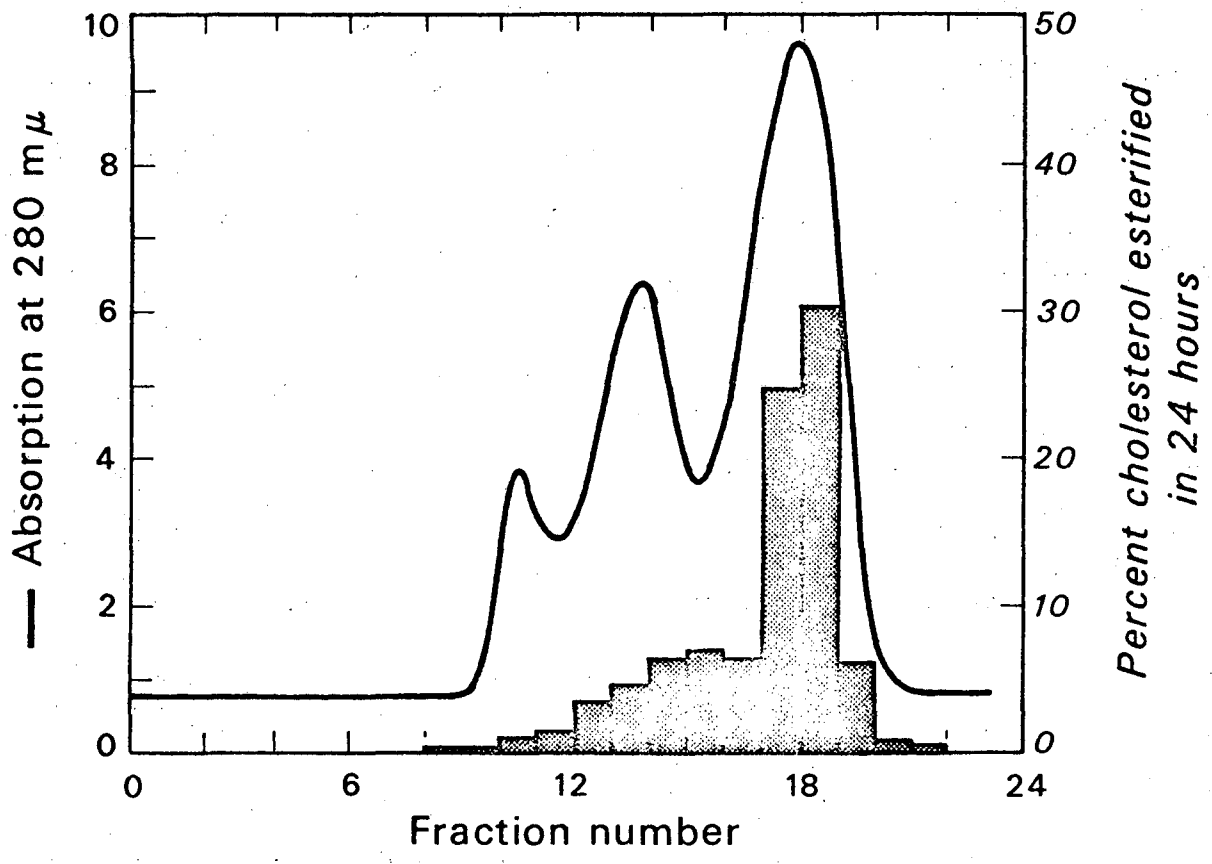
a. Molecular Sieving Chromatography of the $d > 1.21$ Protein Fraction.

A sample of the $d > 1.21$ protein fraction was chromatographed on a Sephadex G-200 column under the conditions described in Figure 13. The distribution of enzyme activity as well as the distribution of protein are shown in Figure 13. As indicated, the bulk of enzyme activity was recovered in the third protein peak, which corresponded

FIGURE 13

CHROMATOGRAPHY OF $d > 1.21$ PROTEIN FRACTION ON SEPHADEX G-200.

Five ml of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) were chromatographed on a Sephadex G-200 column under the following conditions: column dimensions, 2.5 cm X 100 cm; flow rate, 20 ml/hour; volume of each collected fraction, 16.8 ml; temperature, 4°C; direction of flow, ascending; eluting buffer, 0.01 M phosphate buffer (pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA). Protein concentration in the effluent was continuously monitored by absorption at 280 m μ . Enzyme activity in each collected fraction was determined in the presence of 0.01 M mercaptoethanol as described in Materials and Methods.



to the region where the bulk of albumin was eluted (30). In addition to the major peak, a minor peak of LCAT activity was also detected at the tail region of the second eluting protein peak. Since HDL were eluted primarily in the region of the second protein peak (57), the minor peak of LCAT activity observed in this case was probably due to complexes containing LCAT and HDL-related material, which was present in the $d > 1.21$ protein fraction. Although the column used in this experiment was not calibrated for molecular weight determination, judging from the elution profile of LCAT activity this enzyme probably has a molecular weight below 100,000 daltons.

b. DEAE-Cellulose Chromatography of the $d > 1.21$ Protein Fraction.

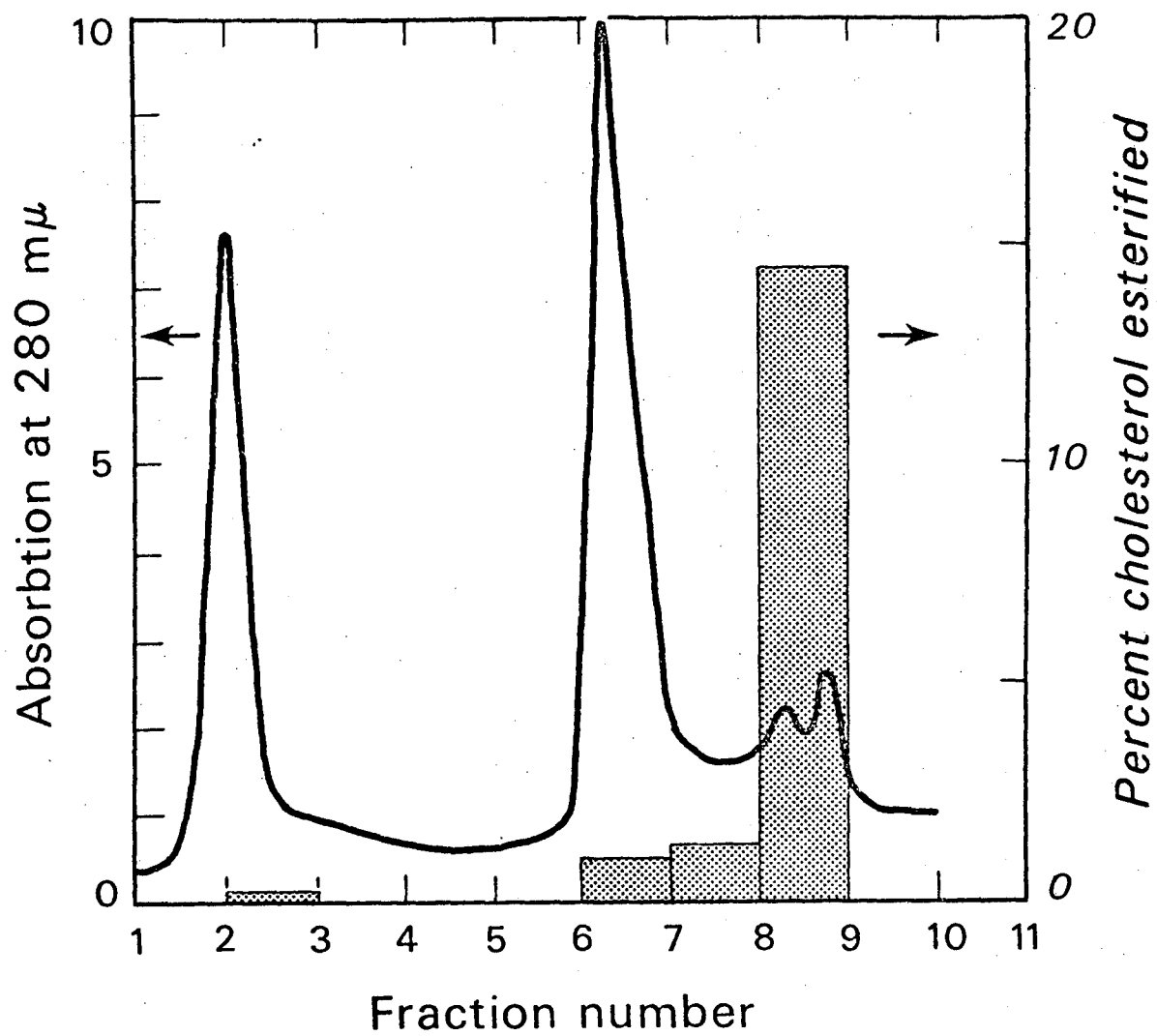
Figure 14 shows an experiment in which freshly prepared $d > 1.21$ protein fraction was chromatographed on a DEAE-cellulose column using a combined ionic and pH gradient for elution. The results clearly indicate that LCAT activity was strongly adsorbed onto the anion exchanger, and was not eluted until relatively extreme conditions (pH 4.6, 0.5 M Tris phosphate) were reached. The combined pH and ionic gradient used on DEAE-cellulose chromatography produced much better resolution than earlier experiments of Glomset and Wright (40) using a linear NaCl gradient (from 0 to 1 M NaCl). Thus, under appropriate conditions, DEAE-cellulose chromatography could be a potential technique for the purification of LCAT.

c. Ammonium Sulfate Precipitation of LCAT Activity in the $d > 1.21$ Protein Fraction.

FIGURE 14

CHROMATOGRAPHY OF $d > 1.21$ PROTEIN FRACTION ON DEAE-CELLULOSE USING A COMBINED pH AND IONIC ELUTING GRADIENT.

Four ml of $d > 1.21$ protein fraction (in 0.005 M Tris phosphate buffer, pH 8.6) were chromatographed on a DEAE-cellulose anion exchange column under the following conditions: column dimensions, 1.5 cm X 16 cm; flow rate, 160 ml/hour; volume of each collected fraction, 32 ml; temperature, 4°C; direction of flow, descending; starting buffer, 0.005 M Tris phosphate buffer (pH 8.6); limiting buffer, 0.5 M Tris phosphate buffer (pH 4.6). A nonlinear combined pH and ionic eluting gradient was produced by a gradient pump (Programmed Gradient Pump, Model 380, Instrumentation Speciality Co., Inc., Lincoln, Nebraska) using the settings of 0.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 30.0, 50.0 and 100.0 (% of limiting buffer). Time of program was 1 hour. Protein concentration in the effluent was continuously monitored by absorption at 280 m μ . The fractions collected were dialyzed against 0.01 M phosphate buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) prior to assay of LCAT activity, in presence of mercaptoethanol (0.01 M), as described in Materials and Methods.



DBL 723-5196

Data on the amount of LCAT activity precipitated from a $d > 1.21$ protein fraction at various saturation levels of ammonium sulfate are summarized in Table 20. As indicated, complete precipitation of LCAT activity was observed in the region between 30 to 50% saturation. This corresponded closely to the region where the alpha and beta globulin proteins were precipitated. Since the bulk of serum albumin did not precipitate until approximately 60% saturation, the ammonium sulfate precipitation method offers an easy and quick way of removing albumin from enzyme preparations. By using this differential precipitation technique, LCAT activity could be purified approximately 2.7 fold from the $d > 1.21$ protein fraction.

d. Purification of LCAT Activity.

Initial attempts to purify LCAT from serum were made by Glomset and Wright (40). Using a combination of ion exchange and absorption chromatography, they were able to obtain a 37 fold purification. More recently, Akanuma and Glomset (4), employing the technique of affinity chromatography, were able to further purify LCAT approximately 500 fold. In the present work, several attempts were made to purify LCAT, and they are summarized schematically in Figures 15 A, B, C and D. As indicated, they all showed only minor successes, and the best purification (approximately 500 fold) was obtained when LCAT activity was complexed to sonicated dispersions of lecithin and subsequently ultracentrifugally floated in a sucrose solution of $d 1.065$ gm/ml (Figure 15 A). The enzyme fraction obtained by this

TABLE 20

PRECIPITATION OF LCAT ACTIVITY FROM THE $d > 1.21$ PROTEIN FRACTION BY AMMONIUM SULFATE.

Two ml aliquots of $d > 1.21$ protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) were adjusted to various saturation levels by addition of solid ammonium sulfate¹.

The mixtures were rotated gently for 2 hours at 4°C to ensure complete precipitation of proteins. The mixtures were then centrifuged at 27,000 X g, 4°C for 20 minutes. The clear supernatants were removed, dialyzed against 0.01 M Tris HCl buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), and assayed for LCAT activity, in presence of 0.01 M mercaptoethanol, as described in Materials and Methods.

% of Ammonium Sulfate Saturation	% of Original Enzyme Activity Remaining in the Supernatant
0	100
30	100
40	64
50	1
60	<1
70	<1

¹ Amount of ammonium sulfate added was determined from data obtained by Dixon (24).

procedure was shown to contain (at least) 6 to 7 bands on polyacrylamide gel electrophoresis.

Since apoLP-gln I is a required cofactor when sonicated dispersions of a mixture of cholesterol and lecithin are used as substrate (see Results of Chapter 3), the low yield and sudden loss of enzyme activity encountered during fractionation might well have resulted from a separation of apoLP-gln I from the bulk of the enzyme. Thus, only slight or no LCAT activity would be detected even though a particular fraction might contain high level of the enzyme which could be detected if apoLP-gln I was added into the assay medium.

Very recently, Fielding and Fielding (29) reported a purification of LCAT by approximately 2700 fold. The techniques they used in their fractionation procedure had features similar to those described in this thesis. The highly purified LCAT was very labile, and had a half life of only 30 minutes at 4°C. Furthermore, in their work with the purified enzyme, Fielding and Fielding (29) showed that pure sonicated dispersions of lipids could not serve as substrate, and addition of HDL protein, very high density lipoprotein protein, and $d > 1.25$ gm/ml serum proteins significantly increased the reactivity of the lipid dispersions. This result corroborates our finding that apoLP-gln I is required to activate substrate lipid dispersions.

In view of the lability of LCAT and the requirement of apoLP-gln I when lipid dispersions are used as substrate, approaches to

FIGURE 15 A

OUTLINE OF A PURIFICATION SCHEME FOR LCAT: ULTRACENTRIFUGAL APPROACH.

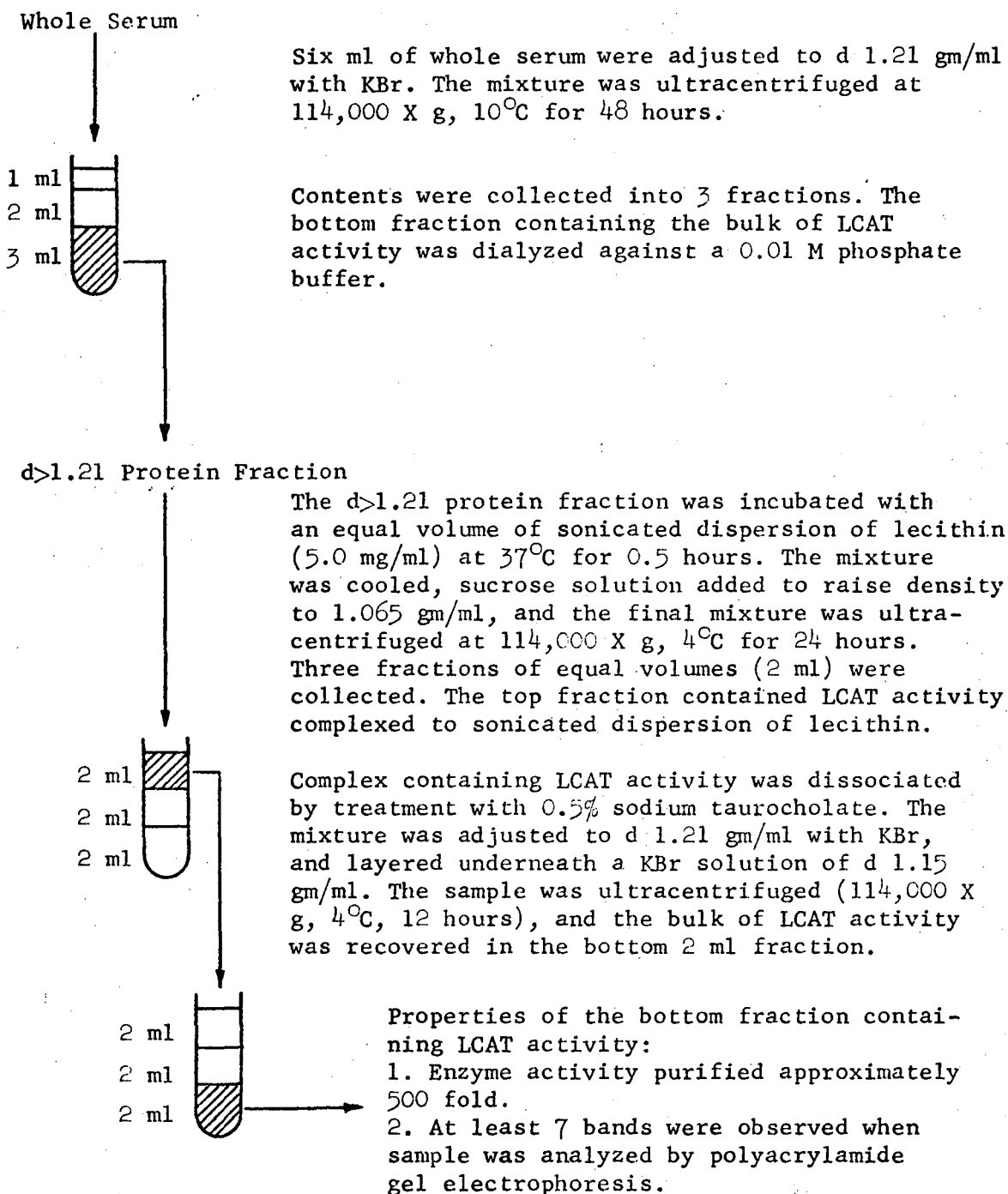


FIGURE 15 B

OUTLINE OF A PURIFICATION SCHEME FOR LCAT: PRECIPITATION PLUS

CHROMATOGRAPHIC APPROACH.

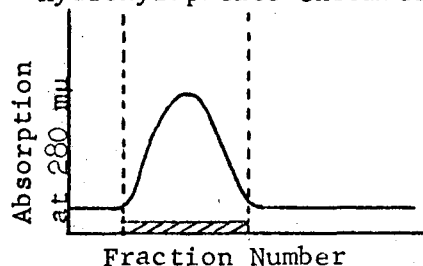
$d > 1.21$ Protein Fraction (obtained by procedure described in Figure 15 A)

The $d > 1.21$ protein fraction was differentially precipitated by addition of ammonium sulfate. The bulk of LCAT activity was precipitated between 30 to 50% ammonium sulfate saturation.

30 to 50% Ammonium Sulfate Precipitate

The ammonium sulfate precipitate, containing LCAT activity, was dialyzed against a 0.07 M phosphate buffer (pH 6.7), applied onto a hydroxylapatite column, and eluted with 0.07 M phosphate buffer (pH 6.7) under the conditions described directly below.

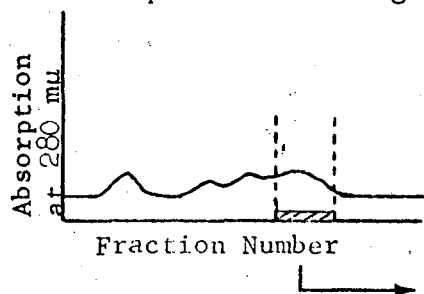
Hydroxylapatite Chromatography



Conditions of hydroxylapatite chromatography: column dimensions 2.0 cm X 40 cm; direction of flow, descending; eluting buffer, 0.07 M phosphate buffer (pH 6.7). Protein eluted in the void volume was collected as one fraction.

The void volume from the preceding step, containing LCAT activity, was concentrated, dialyzed in 0.005 M Tris phosphate buffer (pH 8.6), and applied onto a DEAE-Sephadex column. Elution was done with a combined pH and ionic gradient.

DEAE-Sephadex Chromatography (chromatographic conditions were similar to those described in Figure 16)



Properties of the fraction containing LCAT activity:

1. Enzyme activity purified approximately 20 fold
2. At least 6 to 7 bands were observed when this fraction was analyzed by polyacrylamide gel electrophoresis.

FIGURE 15 C

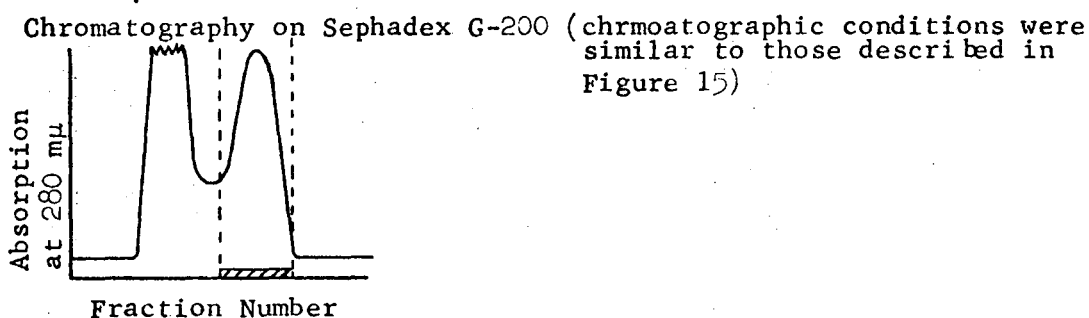
OUTLINE OF A PURIFICATION SCHEME FOR LCAT: PRECIPITATION PLUS CHROMATOGRAPHIC APPROACH.

d>1.21 Protein Fraction

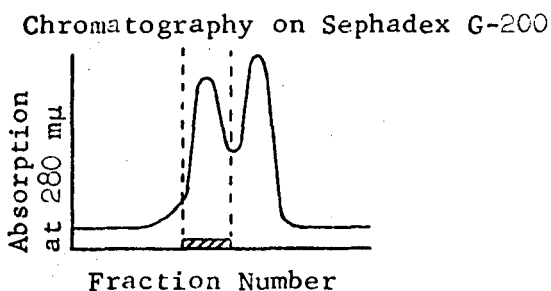
d>1.21 protein fraction and 30 to 50% ammonium sulfate precipitate were obtained as described in Figure 17 B.

30 to 50% Ammonium Sulfate Precipitate

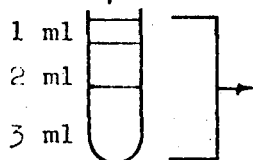
The ammonium sulfate precipitate was applied onto a Sephadex G-200 column (2.5 cm X 100 cm), and eluted with a 0.01 M phosphate buffer (pH 7.4) containing 0.19 M NaCl. The cross-hatched fraction contained the bulk of LCAT activity (see directly below).



The fraction containing LCAT activity obtained from the above step was concentrated and then incubated (37°C, 15 minutes) with HDL. The incubation mixture was then applied onto a Sephadex G-200 column (2.5 cm X 100 cm), and eluted under the same conditions as in the previous step. The cross-hatched fraction contained the bulk of LCAT activity (see directly below).



The enzyme fraction obtained from the above step was raised to d 1.21 gm/ml by addition of solid KBr. This mixture was ultracentrifuged at 114,000 X g, 4°C for 48 hours. The contents were collected into three fractions.

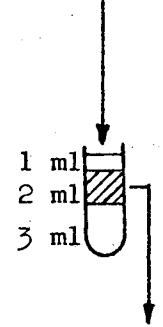


All three fractions contained slight to no LCAT activity. Subsequent re-examination of this isolation procedure indicated a marked loss of recoverable activity during the second chromatographic step. The reason for this rapid loss of activity might have been due to a separation of a cofactor from the enzyme.

FIGURE 15 D

OUTLINE OF A PURIFICATION SCHEME FOR LCAT: PRECIPITATION PLUS CHROMATOGRAPHIC APPROACH.

Whole Serum



The density of serum was adjusted to d 1.21 gm/ml by addition of KBr. The adjusted serum was ultracentrifuged at 114,000 X g, 4°C for 48 hours. Three fractions were collected. The distribution of overall LCAT activity was 4%, top; 41%, middle; and 55%, bottom. The middle fraction containing the highest enzyme activity per mg protein was isolated and used in subsequent purification steps.

Middle Fraction



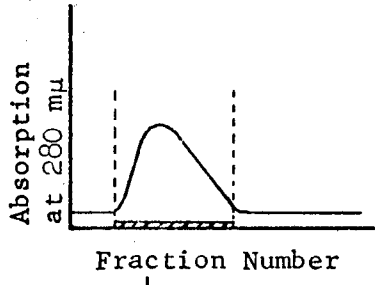
Protein of this fraction was precipitated by addition of ammonium sulfate to 50% saturation level.

0 to 50% Ammonium Sulfate Precipitate



The ammonium sulfate precipitate was dissolved and dialyzed against 0.07 M phosphate buffer (pH 6.7), and subsequently applied onto a hydroxylapatite column. LCAT activity was eluted in the void volume fraction.

Hydroxylapatite Chromatography (conditions of chromatography were as described in Figure 17 B)



Properties of the fraction containing LCAT activity:

1. Enzyme activity purified approximately 100 fold.
2. Polyacrylamide gel electrophoresis showed no resolvable protein bands.
3. Yield of LCAT activity was very low compared with other procedures.

the purification of LCAT should be designed so as to minimize these problems. Thus, if sonicated dispersions of a mixture of cholesterol with lecithin are used as substrates in the assay, the correct proportion of apoLP-gln I should be added so that the rate of transesterification is directly proportional to the enzyme level only. The lability of LCAT after it has been purified is probably the most serious problem facing further detailed studies of LCAT-substrate interaction. As suggested by Fielding and Fielding, the lability observed is probably due to aggregation of enzyme molecules. The reason why LCAT activity in the $d > 1.21$ protein fraction appears to be stable is unknown. It is possible that some factor stabilizing the enzyme is present in the $d > 1.21$ protein fraction. ApoLP-gln I appears to be a most likely candidate for such a stabilization role.

III. Interaction of LCAT Activity with Sonicated Substrate

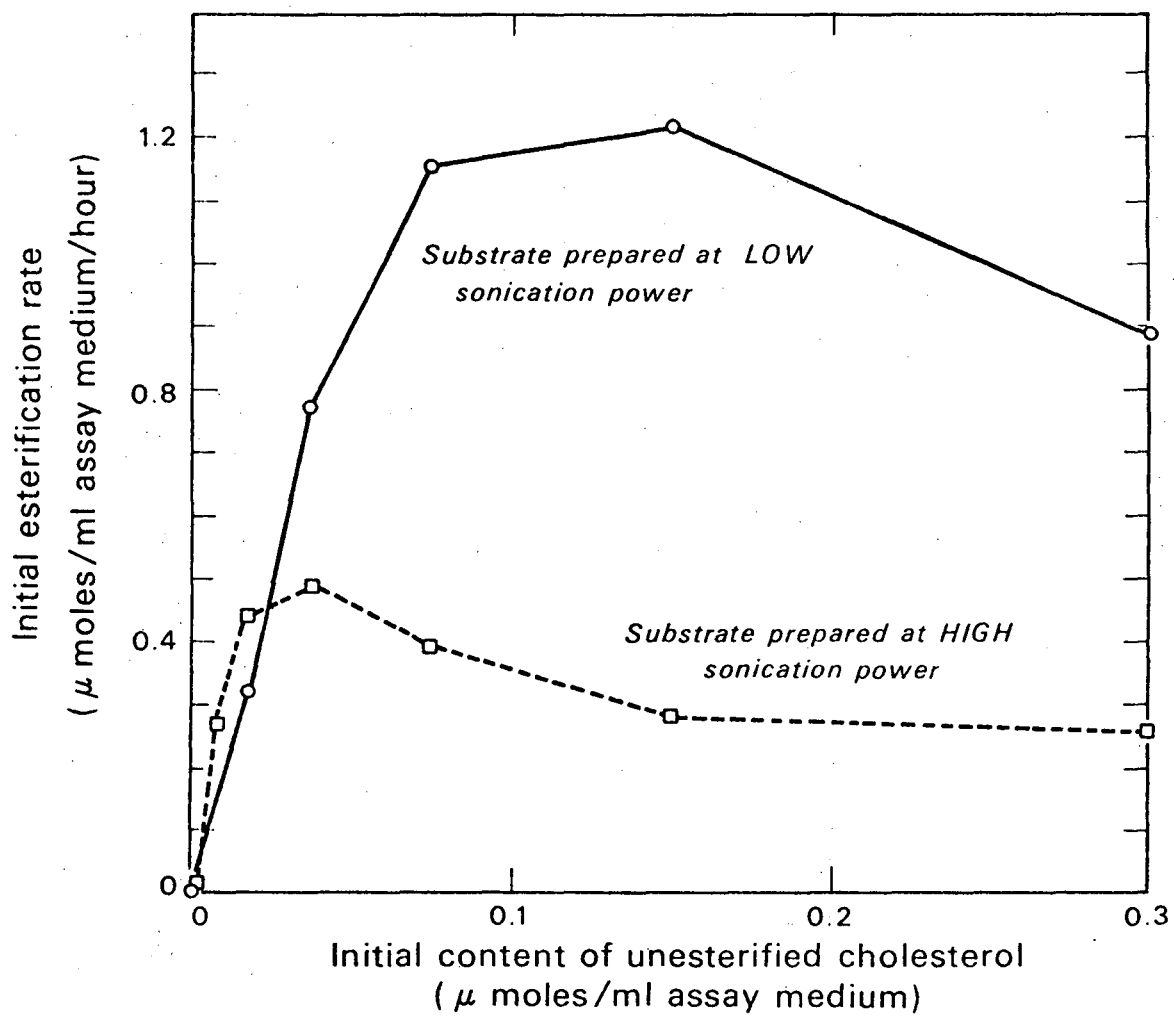
a. Effect of Size of Sonicated Dispersions on Assay of LCAT Activity Contained in the $d > 1.21$ Protein Fraction.

The effectiveness of sonicated dispersions as substrates for LCAT has been demonstrated and subsequently confirmed (81, 91). Since sonication usually produces dispersions of non-uniform sizes (8, 21, 53, 58), it was important to ascertain whether or not the size of the dispersion affected transesterification rate. Figure 16 shows the results of an experiment in which two differently prepared lipid sonicates were used as substrates. As indicated, at low levels

FIGURE 16

EXTENT OF DISPERSION OF SONICATED SUBSTRATE ON ASSAY OF LCAT ACTIVITY.

Two stock samples of sonicated dispersions of cholesterol with lecithin (lecithin, 3.6 μ moles/ml; cholesterol, 0.6 μ moles/ml; H^3 -cholesterol, 50,000 dpm/ml) in 0.01 M Tris HCl buffer (pH 7.4, containing 0.1 mg/ml EDTA) were prepared as described in Materials and Methods using two different sonication power outputs. The sample prepared at high power level (100 watts) had finer dispersions than the one prepared at low power level (60 watts). The two stock solutions of sonicated substrates were diluted with 0.01 M Tris HCl buffer (pH 7.4, containing 0.1 mg/ml EDTA) to 1/2, 1/4, 1/8, 1/16 and 1/32 of the original concentration. 0.5 ml of each of the sonicated substrates (containing 0.02 M mercaptoethanol) were mixed and incubated with 0.5 ml of the $d>1.21$ protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA). The initial esterification rates were based on the % of H^3 -cholesterol esterified 40 minutes after the start of the incubation.



of substrate concentration, the substrate dispersed at high power (100 watts) was more reactive than that dispersed at low power (60 watts). However, as the substrate concentration was increased, the substrate dispersed at low power became much more reactive than that dispersed at high power. In terms of kinetic parameters, the substrate dispersed at high power had both a smaller K_m and V_{max} ¹ than the substrate dispersed at low power. Unfortunately, without additional data the significance of K_m and V_{max} cannot be interpreted. If one assumes that the value of K_m is approximately equal to the dissociation constant of the enzyme-substrate complex, then a smaller K_m probably indicates a more efficient adsorption of LCAT or cofactor onto the lipid dispersions. The latter may result from an increase in total surface area. In the case of the smaller V_{max} , the substrate sonicated at high power may contain increased amounts of oxidized lipids (generated during sonication) which can inhibit enzyme activity. In this respect, it has been shown that prolonged sonication can disrupt the covalent structure of phospholipid (53) in addition to the oxidation of unsaturated fatty acid (61).

b. Effectiveness of Sonicated Dispersions of Mixtures of Cholesterol and Lecithin as Substrates for LCAT. Effect of Charge.

The role of surface charge of the sonicated lipid substrate on LCAT reactivity was studied. In this experiment LCAT activity was

¹ K_m and V_{max} are terms used in enzyme kinetics to designate the Michaelis constant and maximal velocity in an enzyme-substrate reaction.

tested with sonicated substrates which contained various types of charged detergents and nonpolar lipids. The results are summarized in Table 21. Addition of either anionic or cationic detergents increased the reactivity of the sonicated substrate under the conditions used for assay (as described in Table 20). On the other hand, incorporation of thiocholesterol or octadecanethiol did not affect the substrate's reactivity. From these results it appears that 1) positive or negative charges present on the lipid substrate dispersion surface do not affect the ability of LCAT to transesterify under the conditions used, and 2) the presence of detergent molecules increases the reactivity of the sonicated lipid substrate. The reason for this increase is not clear; however, it is possible that the detergent facilitates the production of smaller and more uniform lipid dispersions, which offer a greater surface for reaction with either cofactor (apoLP-gln I) or enzyme. In light of these results, the utilization of detergent to prepare substrate lipid dispersions may provide a means for producing more uniformly dispersed substrate without prolonged sonication.

c. Effect of Mercaptoethanol on Assay of LCAT Activity.

The role of mercaptoethanol in preventing oxidation of biological molecules is well documented. Glomset (38) has shown that sulphhydryl group or groups in LCAT are essential for enzyme activity. Since sonicated lipid dispersions have been shown to contain oxidized lipid products (53, 61), it is pertinent to determine whether

TABLE 21

EFFECT OF INCORPORATION OF DETERGENT AND NONPOLAR COMPOUNDS INTO SONICATED SUBSTRATES ON ASSAY OF LCAT ACTIVITY.

Substrates containing detergents or nonpolar lipids were prepared by the sonication procedure described in Materials and Methods. The concentrations of various components in the lipid dispersions were as follows: lecithin, 1.0 mg/ml; cholesterol, 0.08 mg/ml; detergent or nonpolar lipid, 10% of the molar concentration of lecithin. Volumes of various components in the incubation mixtures were: 0.5 ml of d>1.21 protein fraction (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA), 0.5 ml of sonicated substrate solution (in 0.01 M Tris HCl buffer, pH 7.4, containing 0.1 mg/ml EDTA) containing either detergent or nonpolar lipid, and 0.02 ml of 0.01 M Tris HCl buffer (pH 7.4, containing 0.1 mg/ml EDTA and 0.53 M mercaptoethanol). Percent of cholesterol esterified was determined as described in Materials and Methods.

Compound Added to Sonicated Substrates ¹	Appearance of Dispersions	% Cholesterol Esterified
Lauric Acid	Clear	78.5
Octadecanethiol	Turbid	50.2
Cholest-5-ene-3-thiol	Turbid	49.8
Octadecylamine HCl	Clear	69.0
Dodecyltrimethylammonium	Clear	72.2
Control ²	Turbid	56.1

¹ Detergents and nonpolar lipids were obtained from Eastman Organic Chemicals, Rochester, New York.

² Regular sonicated substrate was used without any detergent or nonpolar lipid added (see Materials and Methods for preparation).

addition of mercaptoethanol or other sulfhydryl containing compounds can prevent oxidation of LCAT during incubation when such oxidized products are present. In the following experiment the $d > 1.21$ protein fraction, as a source of LCAT activity, was incubated with sonicated substrate in the presence or absence of mercaptoethanol. The % cholesterol esterified was somewhat higher with mercaptoethanol in the assay (Table 22). Additional experiments are necessary to establish the significance of the observed difference. In addition to the above experiment, the possibility that LCAT might be oxidized by air during incubation was also investigated. As indicated in Table 23, when the $d > 1.21$ protein fraction was preincubated alone in air for 24 hours and then incubated with sonicated lipid substrates for another 24 hours in N_2 , the % cholesterol esterified was similar to that obtained when the preincubation was done in N_2 . Thus, if oxidation of LCAT by air had taken place, a reduction of LCAT activity should have been observed. From the results of these experiments, it would appear that appreciable oxidation of LCAT does not occur during incubation with sonicated lipid substrates.

d. Effect of Mercaptoethanol and Excess Lecithin on Assay of LCAT Activity.

As described in the main text, I had earlier demonstrated the inhibitory effect of excess lecithin of LCAT activity, and the activating effect of mercaptoethanol in restoring enzyme activity in samples containing high concentrations of lecithin (see page 50).

TABLE 22

EFFECT OF MERCAPTOETHANOL ON ASSAY OF LCAT ACTIVITY IN THE $d > 1.21$ PROTEIN FRACTION.

The $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) was assayed with and without mercaptoethanol as described in Materials and Methods.

Condition of Assay	% Cholesterol Esterified
0.01 M mercaptoethanol in incubation mixture	37
Control, no mercaptoethanol in incubation mixture	31

TABLE 23

EFFECT OF OXYGEN ON ASSAY OF LCAT ACTIVITY.

One ml of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA) was preincubated at 37°C under N_2 or air for 24 hours. After preincubation, 1 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) was added, and air was flushed out of the incubation vials by a gentle stream of N_2 . The mixtures were then incubated for 24 hours at 37°C. The percent cholesterol esterified (in absence of mercaptoethanol) was determined as described in Materials and Methods.

Condition of Preincubation	% Cholesterol Esterified
Air	43
N_2	42

In this section, experiments have been carried out to determine if the presence of excess lecithin and mercaptoethanol had any effect on the assay of LCAT activity under the conditions used for assay as described in Materials and Methods (either Chapter 2 or 3). As the results of Table 24 show, addition of mercaptoethanol and excess lecithin to the regular assay medium increased the % cholesterol esterified approximately twice that of the control. Furthermore, the increase of activity required the presence of both mercaptoethanol and excess lecithin, and in the absence of either one of the components no activation was detected.

The cause of this activation of LCAT activity was not immediately apparent. However, it is very unlikely that the preservation of sulfhydryl groups in the enzyme by mercaptoethanol was the cause, since no activation was observed in the absence of excess lecithin (Table 24).

In order to further explore the nature of this activation, we used various analogs of both lecithin and mercaptoethanol to determine if similar activation would result. As shown in Tables 25 and 26, only ethanethiol and ethanedithiol demonstrated activation comparable to that produced by mercaptoethanol. Substitution of phosphatidyl ethanolamine and phosphatidyl serine for lecithin actually inhibited activity. The inability of some sulfhydryl containing compounds (cysteine and glutathione) to induce higher activity indicated that the activation observed in the presence of mercaptoethanol was quite specific. This specificity probably

TABLE 24

ACTIVATION OF LCAT ACTIVITY BY MERCAPTOETHANOL IN PRESENCE OF SONICATED DISPERSIONS OF LECITHIN.

The volumes of components present in the assay media were: 0.25 ml of $d>1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA), 0.5 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), either 0.25 ml of sonicated dispersions of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) or 0.25 ml of 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA), and either 0.05 ml of 0.21 M mercaptoethanol (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) or 0.05 ml of 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA). The percent cholesterol esterified after 24 hour incubation at 37°C was determined as described in Materials and Methods.

Composition of Assay Mixture	% Cholesterol Esterified	
	Expt. 1	Expt. 2
$d>1.21$ Protein Fraction + Lecithin + Substrate + ME ¹	70	71
$d>1.21$ Protein Fraction + Substrate	30	31
$d>1.21$ Protein Fraction + Substrate + ME	-	37
$d>1.21$ Protein Fraction + Lecithin + Substrate	13 ⁺	-

¹ Mercaptoethanol

⁺ The lower esterification yield observed in this case agrees with the inhibitory effect of lecithin as described in Figure 4.

TABLE 25

EFFECT OF SULFHYDRYL-CONTAINING COMPOUNDS ON ACTIVATION OF LCAT ACTIVITY.

Incubation mixture contained 0.25 ml of $d>1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA), 0.5 ml of sonicated substrate solution (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), 0.25 ml of sonicated dispersions of lecithin (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) and 0.05 ml of 0.21 M sulfhydryl-containing compound (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA). Incubation was carried out at 37°C for 24 hours. Percent cholesterol esterified was determined as described in Materials and Methods.

Sulfhydryl-Containing Compound	% Cholesterol Esterified
Mercaptoethanol	76
Cysteine HCl	30
Glutathione	16
Ethanethiol	80
Ethanedithiol	80
Ethanol ¹	34
Control ²	34

¹ Ethanol was used to check steric effect as well as the role of sulfhydryl group in activation. Judging from these results the presence of ethanol appears to have no effect on LCAT activity.

² 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA) was used instead of the buffer containing 0.21 M sulfhydryl-containing compound.

TABLE 26

EFFECT OF DIFFERENT PHOSPHOLIPIDS ON ACTIVATION OF LCAT ACTIVITY.

Incubation mixtures contained 0.25 ml of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA), 0.5 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), 0.05 ml of 0.21 M mercaptoethanol (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), and 0.25 ml of sonicated dispersions of specific phospholipid (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA). Incubation and determination of percent cholesterol esterified were the same as described in Table 25.

Phospholipid	% Cholesterol Esterified
Lecithin	76
Phosphatidylethanolamine	7
Phosphatidylserine	4

involved the ability of the sulfhydryl compound to fit into a specific molecular environment as well as to provide the reducing capacity to keep the sulfhydryl group or groups of the enzyme intact.

To further evaluate the lecithin-mercaptoethanol activation, I investigated the possibility that they, in combination, may convert LCAT to a more active form or state. I preincubated the $d > 1.21$ protein fraction with sonicated dispersions of lecithin, containing 0.01 M mercaptoethanol, for varying periods of time. Substrates were then added, and the initial rates of esterification were determined (Table 27). There was a very slight increase in initial rate as preincubation time was increased. When these initial rates are compared with the control (incubated in the absence of both mercaptoethanol and lecithin), they are not significantly different from the control. Since preincubation with mercaptoethanol and lecithin did not increase initial rates, it is unlikely that the activation we observed in Table 24 was due to conversion of LCAT into a more active form or state.

It has been reported that beta mercaptopyruvate transsulfurase, in the presence of mercaptoethanol, is converted to a more active monomeric form (25). In order to explore the possibility that a similar mechanism might be involved in the activation of LCAT, the following experiment was performed. The $d > 1.21$ protein fraction was chromatographed on a Sephadex G-200 column in the presence and absence of 0.01 M mercaptoethanol. As indicated in Figure 17, both

TABLE 27

EFFECT OF PREINCUBATION OF $d > 1.21$ PROTEIN FRACTION WITH MERCAPTO-ETHANOL AND LECITHIN ON ACTIVATION OF LCAT ACTIVITY.

0.25 ml aliquots of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA) were preincubated with 0.25 ml of sonicated dispersions of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) and 0.05 ml of 0.21 M mercaptoethanol (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) for the time periods indicated below. After preincubation, 0.5 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) were added, and the mixture was incubated for 30 minutes at 37°C. Initial rates of esterification were calculated from the percent cholesterol esterified in the 30 minute period after addition of substrate. In the control, 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA) was used in place of sonicated dispersions of lecithin plus mercaptoethanol.

Time of Preincubation (minutes)	Initial Rate ¹
1	0.019
3	0.021
6	0.022
12	0.022
24	0.023
Control ²	0.024

¹ μ moles cholesterol esterified/ml assay medium/hour.

² Not preincubated.

protein and enzyme activity profiles showed no significant difference when eluted either with buffer containing mercaptoethanol or with buffer containing no mercaptoethanol. If LCAT was converted to smaller subunits in the presence of mercaptoethanol, a shift of enzyme activity towards the lower molecular weight region would have been detected. Thus, the present result provides no evidence that LCAT, in the presence of mercaptoethanol, is converted to more active subunits.

From previous experiments (Table 27) I observed no significant increase in initial rates when the $d > 1.21$ protein fraction was preincubated with mercaptoethanol and excess lecithin. In order to determine if the presence of mercaptoethanol and excess lecithin promotes higher esterification yield (24 hour incubation) by allowing more efficient utilization of substrate, I decided to follow the progress of transesterification over a period of 24 hours in the presence and absence of excess lecithin and mercaptoethanol. As indicated (Figure 18), the initial rate of esterification was higher for the control than the sample incubated with mercaptoethanol and excess lecithin. However, after one hour, the esterification rate of the control began to decrease while that of the sample with mercaptoethanol and excess lecithin remained linear for three more hours. At the 24 hour point the % cholesterol esterified of the sample incubated with excess lecithin and mercaptoethanol was twice the level of the control. Based on these results, it appears that the activation of the enzyme does not commence until sometime after

FIGURE 17

EFFECT OF MERCAPTOETHANOL ON THE CHROMATOGRAPHIC DISTRIBUTION OF LCAT ACTIVITY.

Five ml of $d > 1.21$ protein fraction (in either 0.01 M phosphate buffer, pH 7.4, containing 0.26 M NaCl, 0.01 M mercaptoethanol and 0.1 mg/ml EDTA or the same phosphate buffer containing no mercaptoethanol) were chromatographed on a Sephadex G-200 column according to the conditions described below. Column dimensions, 2.5 cm X 100 cm; flow rate, 20 ml/hour; volume of each collected fraction, 16.8 ml; direction of flow, ascending; temperature, 4°C; eluting buffer, 0.01 M phosphate buffer (pH 7.4, containing 0.26 M NaCl and 0.1 mg/ml EDTA). Protein concentration in the effluent was continuously monitored by absorption at 280 μ . Enzyme activity in each collected fraction was assayed in 0.01 M mercaptoethanol as described in Materials and Methods. Chromatogram A: sample was eluted with phosphate buffer containing no mercaptoethanol. Chromatogram B: sample was eluted with phosphate buffer containing 0.01 M mercaptoethanol.

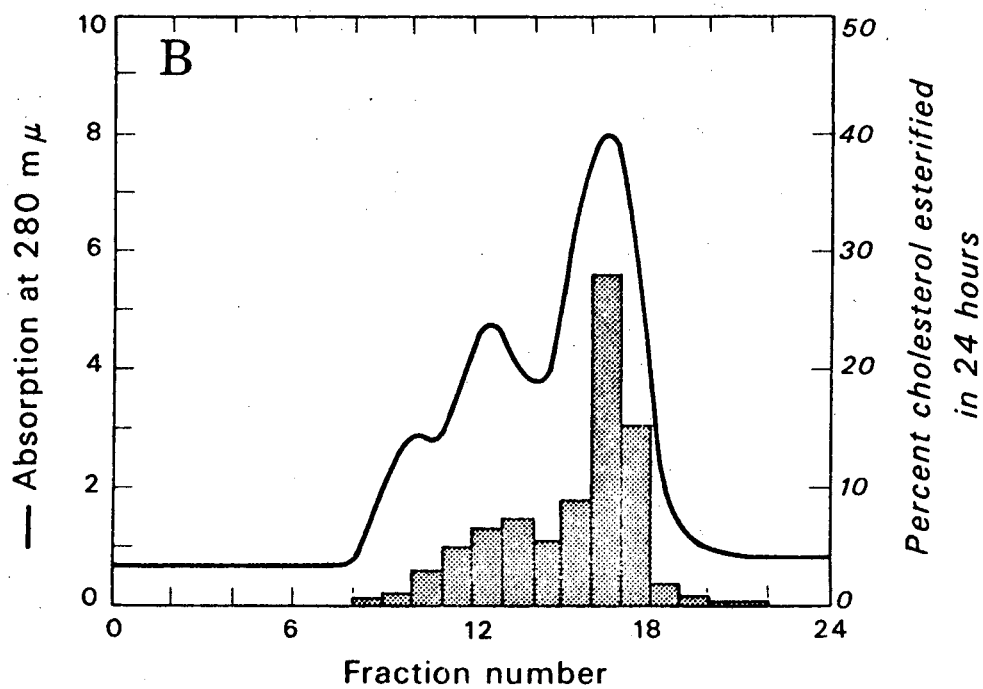
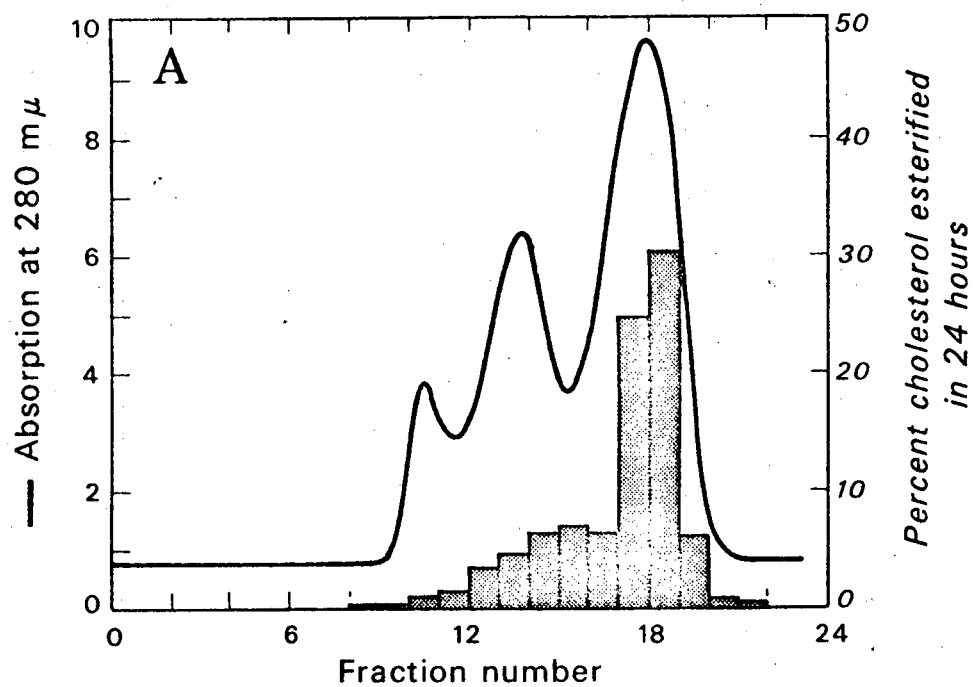
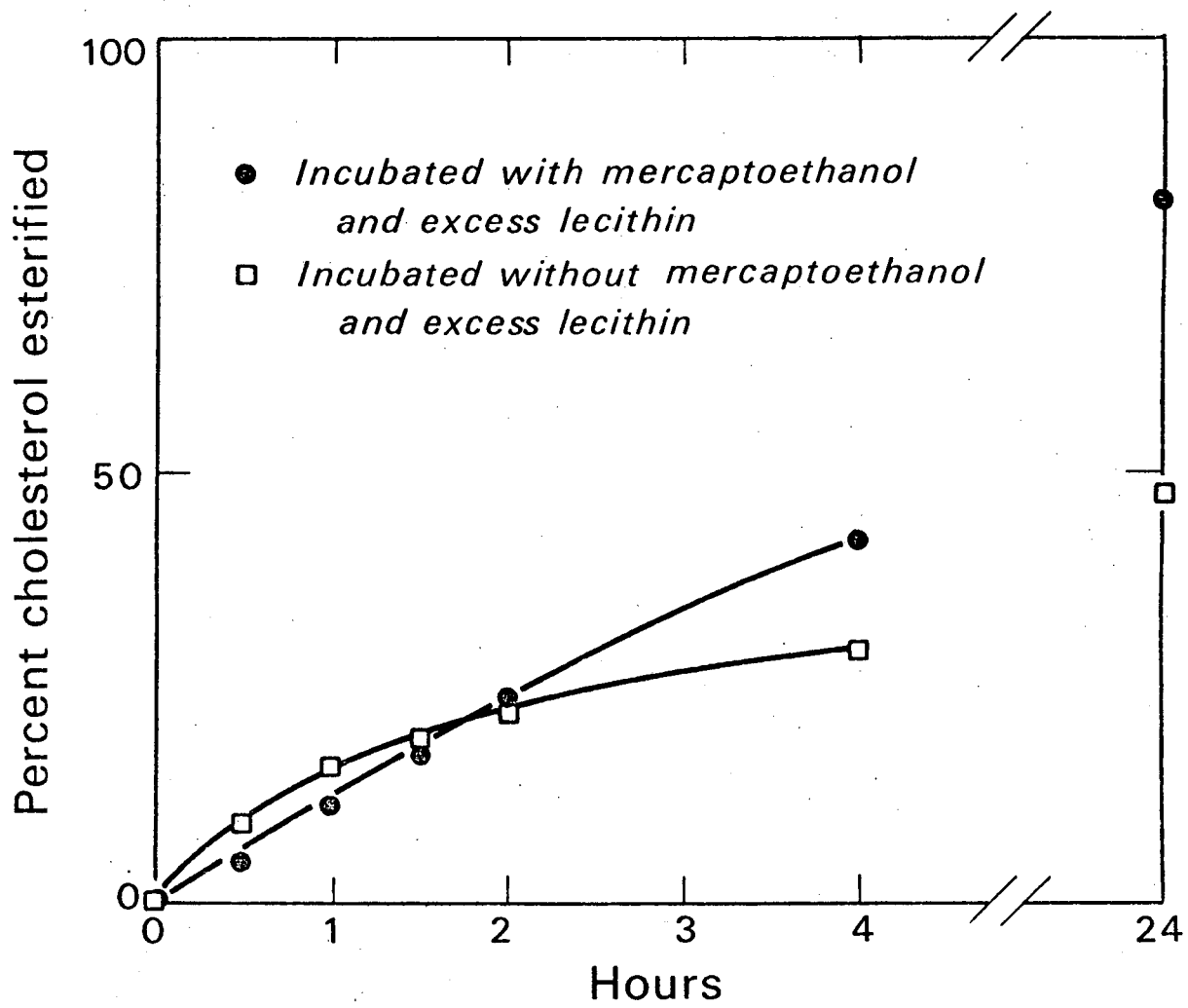


FIGURE 18

EFFECT OF ADDITIONAL LECITHIN PLUS MERCAPTOETHANOL ON THE TIME COURSE OF CHOLESTEROL ESTERIFICATION.

The incubation mixtures contained 1.75 ml of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA), 3.5 ml of sonicated substrate (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA), either 1.75 ml of sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) or 1.75 ml of the buffer, and either 0.35 ml of 0.21 M mercaptoethanol (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) or 0.35 ml of the buffer. One ml aliquots of the incubation mixtures were withdrawn at the time intervals indicated, and the percent cholesterol esterified determined as described in Materials and Methods.



DBL 723-5195

the start of the incubation.

The role of mercaptoethanol in enhancing LCAT activity in the presence of excess lecithin is still unclear. However, one of the possibilities is that it can influence the packing of lipids, and thus enable them to assume a more favorable configuration for transesterification. Furthermore, the presence of mercaptoethanol may enhance both the rate and the amount of exchange of reactants and by-products at the catalytic site, and thus produce a higher yield of esterification. Our demonstration that lipid dispersions require the prior activation by apoLP-gln I in order to serve as substrate for LCAT may provide some insight into the mechanisms whereby mercaptoethanol and excess lecithin activate transesterification. Thus, the presence of mercaptoethanol and excess lecithin may increase the ability of apoLP-gln I to bind with more lipid dispersions and as a result more substrate could be transesterified.

So far I have used only artificial substrates in the study of mercaptoethanol-lecithin activation. How the presence of mercaptoethanol and excess lecithin affect natural lipoproteins as substrates of LCAT is still unknown. In order to determine if similar activation could occur when lipoproteins were used as substrates, VLDL labeled with H^3 -cholesterol were substituted for sonicated substrates in the incubation. The results of this incubation are summarized in Table 28, and as indicated, 13% of the VLDL unesterified cholesterol was esterified in 24 hours. When mercaptoethanol was present in the incubation mixture, an increase in the % cholesterol esterified was

TABLE 28

EFFECT OF MERCAPTOETHANOL AND ADDITIONAL LECITHIN ON ASSAY OF LCAT ACTIVITY USING VLDL AS SUBSTRATE.

VLDL containing H^3 -cholesterol were prepared according to the method, described by Avigan (9). One ml of the VLDL (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA; VLDL concentration, 6.9 mg/ml; approximate radioactivity, 6000 dpm/ml) was incubated with 0.5 ml of $d > 1.21$ protein fraction (in 0.01 M phosphate buffer, pH 7.4, containing 0.52 M NaCl and 0.1 mg/ml EDTA), 0.5 ml of sonicated dispersion of lecithin (5.0 mg/ml; in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA) and 0.1 ml of 0.21 M mercaptoethanol (in 0.01 M phosphate buffer, pH 7.4, containing 0.1 mg/ml EDTA). In the controls, 0.01 M phosphate buffer (pH 7.4, containing 0.1 mg/ml EDTA) was used in place of the solution containing sonicated dispersion of lecithin or mercaptoethanol.

Composition of Assay Medium	% Cholesterol Esterified
$d > 1.21$ Protein Fraction + VLDL	13
$d > 1.21$ Protein Fraction + VLDL + Lecithin	5
$d > 1.21$ Protein Fraction + VLDL + ME ¹	25
$d > 1.21$ Protein Fraction + VLDL + Lecithin + ME	36

¹ Mercaptoethanol.

observed; with the addition of sonicated dispersions of lecithin, the % cholesterol esterified increased even more. Judging from these results the activation phenomenon is not limited to artificial sonicated substrates.

ACKNOWLEDGMENTS

I wish to thank Dr. Alexander Nichols, chairman of my thesis committee, for his guidance and encouragement throughout the years of my association with him. In addition, I would like to thank Mrs. Elaine Gong, Mrs. Patricia Blanche and Mrs. Charlie Mae Fuller for their wonderful support during the course of my thesis work. I am also grateful to all the members of the Donner Laboratory who assisted me at various stages of my research.

Outside of my immediate research group, I would like to thank Dr. John Gofman and Dr. Richard Lyman, members of my thesis committee, for their guidance in preparation of the manuscript.

I also like to thank the members of my family for their patience and support.

Financial support from the University of California, Berkeley, is gratefully acknowledged.

REFERENCES

1. Adams, G. H. and Schumaker, V. N. (1970), *Biochim. Biophys. Acta* 210, 462.
2. Afinsen, C. B., Boyle, E. and Brown, R. K. (1952), *Science* 115, 583.
3. Akanuma, Y. and Glomset, J. (1968), *J. Lipid Res.* 9, 620.
4. Akanuma, Y. and Glomset, J. (1968), *Biochem. Biophys. Res. Commun.* 32, 639.
5. Alpaupovic, P., Sanbar, S. S., Furman, R. H., Sullivan, M. L. and Walraven, S. L. (1966), *Biochem.* 5, 4044.
6. Albers, J. J. and Aladjem, F. (1971), *Biochem.* 10, 3436.
7. Anderson, N. G. and Fawcett, B. (1950), *Proc. Soc. Expt. Biol. Med.* 74, 768.
8. Attwood, D. and Saunders, L. (1965), *Biochim. Biophys. Acta* 98, 344.
9. Avigan, J. (1958), *J. Biol. Chem.* 234, 787.
10. Baker, S. P. (1957), *Circulation* 15, 889.
11. Baker, S. P., Levine, H., Turner, L. and Dubin, A. (1958), *Proc. Soc. Expt. Biol. Med.* 99, 670.
12. Bangham, A. D. and Dawson, R. M. C. (1959), *Biochem. J.* 72, 486.
13. Bangham, A. D. and Dawson, R. M. C. (1962), *Biochim. Biophys. Acta* 59, 103.
14. Bangham, A. D. (1963), *Adv. Lipid Res.* 1, 65.
15. Bier, D. M. and Havel, R. J. (1970), *J. Lipid Res.* 11, 565.
16. Bimpson, T. and Huggins, J. A. (1969), *Biochim. Biophys. Acta* 187, 447.
17. Brot, N. W., Lossow, W. J. and Chaiknoff, I. L. (1962), *J. Lipid Res.* 3, 413.
18. Brown, W. V., Levy, R. I. and Frederickson, D. S. (1969), *J. Biol. Chem.* 244, 5687.
19. Brown, W. V., Levy, R. I. and Frederickson, D. S. (1970), *Biochim. Biophys. Acta* 200, 573.

20. Brown, W. V., Levy, R. I. and Frederickson, D. S. (1970), *J. Biol. Chem.* 245, 6588.
21. Chapman, D., Fluck, D. J., Penkett, S. A. and Shipley, G. G. (1968), *Biochim. Biophys. Acta* 163, 255.
22. Davies, B. J. (1964), *Ann. N. Y. Acad. Sci.* 121, 404.
23. Dehass, G. H., Postema, N. M., Nieuwenhuizen, W. and Van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta* 159, 103.
24. Dixon, M. (1953), *Biochem. J.* 54, 457.
25. Fanshier, D. W. and Kun, E. (1962), *Biochim. Biophys. Acta* 58, 266.
26. Fielding, C. J. (1970), *Biochim. Biophys. Acta* 206, 109.
27. Fielding, C. J. (1970), *Biochim. Biophys. Acta* 218, 221.
28. Fielding, C. J., Lim, C. J. and Scanu, A. M. (1970), *Biochem. Biophys. Res. Commun.* 39, 889.
29. Fielding, C. J. and Fielding, P. E. (1971), *FEBS Letters* 15, 355.
30. Fischer, L. (1969), in *Laboratory Techniques in Biochem. and Mol. Biol.*, Work and Work, Ed., Amsterdam, Holland, North Holland Co., p 157.
31. Forte, G. M., Nichols, A. V. and Glaeser, M. (1968), *Chem. Phys. Lipids* 2, 396.
32. Forte, G. M., Norum, K. R., Glomset, J. A. and Nichols, A. V. (1971), *J. Clin. Invest.* 50, 1141.
33. Forte, G. M., Nichols, A. V. and Gong, E. (1971), unpublished observations.
34. Forte, G. M., Nichols, A. V., Gong, E. L., Lux, S. and Levy, R. I. (1971), *Biochim. Biophys. Acta* 248, 381.
35. Frederickson, D. S., Levy, R. I. and Lees, R. S. (1967), *New Engl. J. Med.* 276, 34, 94, 148, 215, and 273.
36. Gjone, E. and Norum, K. R. (1970), *Acta Med. Scand.* 187, 153.
37. Glomset, J. A., Parker, F., Tjaden, M. and Williams, R. H. (1962), *Biochim. Biophys. Acta* 58, 398.
38. Glomset, J. A. (1962), *Biochim. Biophys. Acta* 65, 128.
39. Glomset, J. A. (1963), *Biochim. Biophys. Acta* 70, 389.
40. Glomset, J. A. and Wright, J. L. (1964), *Biochim. Biophys. Acta* 89, 266.

41. Glomset, J. A. and Kaplan, D. M. (1965), *Biochim. Biophys. Acta* 98, 41.
42. Glomset, J. A., Janssen, E. T., Kennedy, R. and Dobbins, J. (1966), *J. Lipid Res.* 7, 638.
43. Glomset, J. A. (1968), *J. Lipid Res.* 9, 155.
44. Glomset, J. A. (1970), *Am. J. Clin. Nutrition* 23, 1129.
45. Glomset, J. A., Norum, K. R. and King, W. (1970), *J. Clin. Invest.* 49, 1827.
46. Goodman, D. S. (1965), *Physiol. Rev.* 45, 747.
47. Gotto, A. M., Levy, R. I. and Frederickson, D. S. (1968), *Proc. Natl. Acad. Sci. U. S.* 60, 1436.
48. Gotto, A. M. (1969), *Proc. Natl. Acad. Sci. U. S.* 64, 119.
49. Graham, D. M., Lyon, T. R., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J. and White, S. (1951), *Circulation* 4, 666.
50. Haggis, G. H., Michie, D., Muir, A. R., Roberts, K. B. and Walker, P. M. B. (1964), in *Introduction to Molecular Biology*, New York, N. Y., John Wiley and Sons Inc., p 338.
51. Hahn (1943), *Science* 98, 19.
52. Hamnstrom, B., Gjone, E. and Norum, K. R. (1969), *Brit. Med. J.* 2, 282.
53. Hauser, H. O. (1971), *Biochem. Biophys. Res. Commun.* 45, 1049.
54. Havel, R. J., Shore, V. G., Shore, B. and Bier, D. M. (1970), *Circulation Res.* 27, 595.
55. Herbert, P., Levy, R. I. and Frederickson, D. S. (1971), *J. Biol. Chem.* 246, 7068.
56. Hirsch, J. and Ahrens, E. H. (1958), *J. Biol. Chem.* 233, 311.
57. Ho, W. (1971), unpublished observations.
58. Huang, C. (1969), *Biochem.* 8, 344.
59. Kane, J. P., Richards, E. G. and Havel, R. J. (1970), *Proc. Natl. Acad. Sci. U. S.* 66, 1075.
60. Kaufmann, H. (1955), *Presse. Med.* 48, 1045.
61. Klein, R. A. (1970), *Biochim. Biophys. Acta* 210, 486.
62. Korn, E. D. (1955), *J. Biol. Chem.* 215, 15.

63. Korn, E. D. (1959), *Meth. Biochem. Anal.* 7, 145.
64. LaRosa, J. C., Levy, R. I., Herbert, P., Lux, S. E. and Frederickson, D. S. (1970), *Biochem. Biophys. Res. Commun.* 41, 57.
65. LaRosa, J. C., Levy, R. I., Brown, W. V. and Frederickson, D. S. (1971), *Am. J. Physiol.* 220, 785.
66. Lee, D. and Alaupovic, P. (1970), *Biochem.* 9, 2244.
67. Le Breton, E. and Pantaleon, J. (1947), *J. Arch. Sci. Physiol.* 1, 199.
68. Levy, R. I., Bilheimer, D. W. and Eisenberg, S. (1971), *Biochem. Soc. Symposia* 33, 3.
69. Lewis, B. (1971), *Biochem. J.* 123, 15P.
70. Lindgren, F. T., Elliott, H. A. and Gofman, J. W. (1951), *J. Phys. Chem.* 55, 80.
71. Lindgren, F. T. and Nichols, A. V. (1960), in *The Plasma Proteins*, Vol. 2, Putnam, F., Ed., New York, N. Y., Acad. Press, p 1.
72. Lossow, W. J., Shah, S. N. and Chaikoff, I. L. (1966), *Biochim. Biophys. Acta* 116, 172.
73. Lowry, W. J., Rosebrough, N. J., Farr, A. L. and Randal, R. (1951), *J. Biol. Chem.* 193, 265.
74. Margolis, S. (1969), in *Structural and Functional Aspects of Lipoprotein in Living Systems*, Tria, E. and Scanu, A. M., Ed., New York, N. Y., Acad. Press, p 369.
75. Murphy, J. R. (1962), *J. Lab. Clin. Med.* 60, 86.
76. Nichols, A. V., Rubin, L. and Lindgren, F. T. (1954), *Proc. Soc. Expt. Biol. Med.* 85, 352.
77. Nichols, A. V. and Smith, L. (1965), *J. Lipid Res.* 6, 206.
78. Nichols, A. V. (1967), *Adv. Biol. Med. Phys.* 11, 110.
79. Nichols, A. V., Coggiola, E. L., Jensen, L. C. and Yokoyama, E. H. (1968), *Biochim. Biophys. Acta* 168, 87.
80. Nichols, A. V. (1969), *Proc. Natl. Acad. Sci. U. S.* 64, 1128.
81. Nichols, A. V. and Gong, E. L. (1971), *Biochim. Biophys. Acta* 231, 175.
82. Nichols, A. V. (1971), unpublished observations.
83. Norum, K. R. and Gjone, E. (1967), *Scand. J. Clin. Lab. Invest.* 20, 231.

84. Norum, K. R. and Gjone, E. (1968), *Acta Med. Scand.* 183, 107.
85. Norum, K. R., Glomset, J. A., Nichols, A. V. and Forte, G. M. (1971), *J. Clin. Invest.* 50, 1131.
86. Otway, S. and Robinson, D. S. (1968), *Biochem. J.* 106, 677.
87. Papahajopoulos, D. (1968), *Biochim. Biophys. Acta* 163, 240.
88. Pollard, H., Scanu, A. M. and Taylor, E. W. (1969), *Proc. Natl. Acad. Sci. U. S.* 64, 304.
89. Quarfordt, S. H. and Goodman, DeW. S. (1969), *Biochim. Biophys. Acta* 176, 863.
90. Raz, A., Kummerow, F. A. and Nishida, T. (1969), *Biochim. Biophys. Acta* 176, 591.
91. Raz, A. (1971), *Biochim. Biophys. Acta* 239, 458.
92. Rehnberg, C. S. and Nichols, A. V. (1964), *Biochim. Biophys. Acta* 84, 596.
93. Reisfield, R. A. and Small, P. A. (1966), *Science* 152, 1253.
94. Robinson, D. S. and French, J. E. (1953), *Quart. J. Expt. Physiol.* 38, 233.
95. Robinson, D. S. (1963), *Adv. Lipid Res.* 1, 133.
96. Robinson, D. S. (1970), *Comp. Biochem.* 18, 51.
97. Rosenman, R. H. and Byers, S. O. (1960), *Proc. Soc. Expt. Biol. Med.* 103, 31.
98. Scanu, A. M. (1965), *Proc. Natl. Acad. Sci. U. S.* 54, 1699.
99. Scanu, A. M., Reader, W. and Edelstein, C. (1968), *Biochim. Biophys. Acta* 160, 32.
100. Scanu, A. M. (1969), in *Structural and Functional Aspects of Lipoprotein in Living Systems*, Tria, E. and Scanu, A. M., Ed., New York, N. Y., Acad. Press, p 425.
101. Scanu, A. M., Toth, J., Edelstein, C., Koga, S. and Stiller, E. (1969), *Biochem.* 8, 3309.
102. Scanu, A. M., Cump, E., Toth, J. Koga, S., Stiller, E. and Albers, L. (1970), *Biochem.* 9, 1327.
103. Schumaker, V. N. and Adams, G. H. (1970), *J. Theoret. Biol.* 26, 89.
104. Shore, B. (1955), *Proc. Soc. Expt. Biol. Med.* 88, 73.

105. Shore, V. and Shore, B. (1967), *Biochem.* 6, 1962.
106. Shore, B. and Shore, V. (1968), *Biochem.* 7, 2773.
107. Shore, V. and Shore, B. (1968), *Biochem.* 7, 3396.
108. Shore, B. and Shore, V. (1969), *Biochem.* 8, 4510.
109. Simon, J. B. and Boyer, J. L. (1970), *Biochim. Biophys. Acta* 218, 549.
110. Simon, J. B. and Scheig (1970), *New Engl. J. Med.* 283, 841.
111. Sperry, W. M. (1935), *J. Biol. Chem.* 111, 467.
112. Sperry, W. M. and Brand, F. C. (1955), *J. Biol. Chem.* 213, 69.
113. Sugano, M. (1971), *Biochem. J.* 122, 469.
114. Wagner, A. and Rogalski, L. J. (1952), *J. Lab. Clin. Med.* 40, 324.
115. Weld, C. B. (1944), *Can. Med. Assoc. J.* 51, 578.
116. Zilversmit, D. B. (1969), in *Structural and Functional Aspects of Lipoprotein in Living Systems*, Tria, E. and Scanu, A. M., Ed., New York, N. Y., Acad. Press, p 329.

LEGAL NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720