Title
RELIPIBATION OF HUMAN PLASMA APOLIPOPROTEINS WITH PHOSPHOLIPIDS

Permalink
https://escholarship.org/uc/item/54t8f426

Author
Verdery Iii., Roy Burton

Publication Date
1975-12-01
RELIPIDATION OF HUMAN PLASMA APOLIPOPROTEINS WITH PHOSPHOLIPIDS

DONNER LABORATORY

Roy Burton Verdery, III

Ph.D. thesis

December 1975

Prepared for the U. S. Energy Research and Development Administration under Contract 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.
For

Mom and Dad
Table of Contents

MOTIVATION .................................................. 1
INTRODUCTION ............................................... 3
STATEMENT OF PURPOSE ................................. 20

METHODS AND MATERIALS
1. Apolipoprotein Preparation ......................... 23
2. Phospholipid Purity and Measurement ............... 34
3. Preparation of Lecithin Vesicles .................... 37
4. Preparative Ultracentrifugation ....................... 45
5. Fluorescence ............................................ 46
6. Circular Dichroism ..................................... 52
7. Electron Microscopy ................................... 59
8. Gradient Gel Electrophoresis ......................... 65

RESULTS AND DISCUSSION
I  Relipidation of A-I with Phospholipids ............... 68
Ia Relipidation of A-I with Lecithin: Ultracentrifugal
   Analysis .............................................. 70
Ib Relipidation of A-I with Lysolecithin:
   Ultracentrifugal Analysis .......................... 73
Ic Effect of Lysolecithin on the Relipidation of A-I with
   Lecithin: Ultracentrifugal Analysis ............... 78
Id Effect of Lysolecithin on Lecithin Vesicles in the
   Absence of A-I: Ultracentrifugal Analysis .......... 85
Ie Summary of Ultracentrifugal Results: Relipidation of
   A-I with Lecithin and Lysolecithin ................. 88
If Relipidation of A-I with Lecithin: Results of
   Gradient Gel Electrophoresis ....................... 92
Ig Structure of Complexes of A-I and Lecithin:
   Electron Microscopy ................................ 100
Ih Effect of Lysolecithin on the Structure of Complexes
   of A-I and Lecithin: Electron Microscopy .......... 105
II Structure of Mixed Micelles of Lecithin and
   Lysolecithin: Electron Microscopy .................. 118
Ij Summary of Electron Microscopy: Use of a
   3-Component Diagram ................................ 122
Ik Effect of Lysolecithin on the Conformation of A-I:
   Fluorescence and Circular Dichroism Studies ....... 129
II Relipidation of A-II with Phospholipids .............. 138
IIa Relipidation of A-II with Lecithin:
Gradient Gel Electrophoresis ................. 139
IIb Structure of Complexes of A-II and Lecithin:
Electron Microscopy .................. 147
IIc Effect of Lysolecithin on the Conformation of A-II:
Circular Dichroism Studies ................. 153
III Relipidation of C-III with Phospholipids .............. 159
IIIa Relipidation of C-III with Lecithin:
Results of Gradient Gel Electrophoresis .......... 161
IIlb Effect of Lysolecithin on the Conformation of C-III:
Fluorescence and Circular Dichroism Studies ........ 166
SUMMARY AND CONCLUSIONS .................. 174

APPENDICES

1. Guanidine Denaturation of A-I and the Effect
   of Lysolecithin ..................... 182
2. Arrangement of Lipids and Proteins in Serum High Density
   Lipoproteins: A Proposed Model ............ 191
3. Crosslinking of HDL Apoproteins in situ: Implications
   with Regard to HDL Structure ............ 206

BIBLIOGRAPHY .................. 210
ACKNOWLEDGEMENTS ................ 220
Relipidation of Human Plasma Apolipoproteins with Phospholipids

Roy Burton Verdery, III

Abstract

Lipoproteins are the soluble complexes of lipid and protein which transport otherwise insoluble lipids throughout the body. The protein components of lipoproteins, the apolipoproteins, solubilize and stabilize the transported lipids. The physical-chemistry of the interaction of lipids with these apolipoproteins is a subject of current interest and research. The results of such studies are important to our understanding of both plasma lipoprotein metabolism and lipid-protein interactions in general.

The relipidation of three apoproteins from human plasma lipoproteins with two phospholipids was investigated. Apolipoproteins from high density lipoproteins and very low density lipoproteins were relipidated with lecithin, lysolecithin, or mixtures of these phospholipids. The physical chemical properties of the resulting complexes were investigated using a variety of biophysical techniques. The density of the particles was studied by preparative ultracentrifugation. The molecular weights of these particles was estimated by polyacrylamide gradient gel electrophoresis. Electron microscopy was used to investigate the shapes of particles in mixtures of these apolipoproteins and phospholipids. Circular dichroism and fluorometry were used to determine the effect of lysolecithin on the conformation of A-I, A-II, and C-III.

Relipidated complexes were formed with all of these apolipoproteins.
These particles had specific shapes and molecular weights which depended for the most part on the ratios of the components in mixtures of the apolipoproteins and phospholipids. Several distinct classes of particles with particular shapes and molecular weights were observed. Small spheres 10-20 nm in diameter with molecular weights of 100,000-200,000, particles which appeared as rouleaux with molecular weights of 300,000-400,000, or vesicles with attached protein with molecular weights greater than 900,000 were observed in specific mixtures containing phospholipids and specific amounts of A-I, A-II, or C-III.

Some of the particles formed in these mixtures of apolipoproteins and phospholipids had similarities to abnormal high density lipoproteins observed in patients with specific diseases. Particles containing A-I and phospholipids resembled the abnormal HDL found in patients with lecithin:cholesterol acyltransferase deficiency. This enzyme deficiency results in accumulation of lecithin and cholesterol in plasma and peripheral tissues. Particles containing A-II and phospholipids resembled the abnormal high density lipoproteins found in patients with Tangier disease, a disease characterized by low concentrations of plasma high density lipoproteins. Formation of such complexes, resembling lipoproteins found in vivo, in relatively simple model systems provided insights into relationships between the concentration of lipids in a human plasma and the occurrence of specific lipoproteins.
MOTIVATION

This thesis deals with relipidation of apolipoproteins and characterization of synthetic lipid-protein complexes. The work was motivated by an interest in lipid-protein interactions in general, and specifically by an interest in the relationship between structure and composition of lipid-protein complexes.

Lipids and proteins are ubiquitous compounds found in all independently living organisms. Their role in the structure of cells as mediated by their role as components of the plasma membrane is a subject of intense study. The relationship between structure, composition, and cellular metabolism is complex even in a relatively simple cell such as an erythrocyte.

Lipoproteins contain fewer components than cells or cell membranes, yet they exhibit a variety of structures differing in both size and shape. The studies described in this thesis developed from observations that mixing lecithin and apoproteins from high density lipoproteins in the presence of lysolecithin caused formation of lipid-protein complexes. Complexes with various compositions, sizes and shapes could be formed in this way. Some of these reassembled lipoproteins resembled the abnormal high density lipoproteins from patients deficient in the enzyme lecithin:cholesterol acyltransferase, while others had compositional and structural properties similar to high density lipoproteins from patients with Tangier disease. It was therefore possible to gain some insight into the relationship
between abnormal lipoprotein composition and abnormal lipoprotein structure seen in these diseases by studying this model system.

The two major subjects addressed in this thesis are: the relationship between composition and structure of synthetic lipoproteins, and the influence of various factors on the formation of such complexes.
INTRODUCTION

Plasma lipoproteins are soluble complexes of protein and lipid and serve the general role of transporting otherwise insoluble lipids. Biologically important lipids can be limited to "aliphatic compounds with chain lengths 12 carbons or longer and aromatic compounds containing at least three fused rings" (1). Several families of amphipatic lipids, with both polar and apolar regions, are important in biological systems and particularly in lipoproteins. These families include: phospholipids, cholesterol and its esters, and triglycerides. When mixed with water these lipids form swollen lamellae, crystals, or droplets depending on physical-chemical properties of their polar and apolar moieties (1). All are relatively insoluble in water except for a subgroup of the phospholipids, the lysophospholipids which form soluble micelles in water (2). Proteins are polymers of amino acids with properties which depend primarily on the linear order of their constituent amino acids (3). When mixed with water, proteins assume characteristic folded structures (4). Since amino acids vary in their polarity (5), the three dimensional arrangement of amino acids in a protein, as dictated by its folded structure, affects the polar and apolar characteristics of the protein (6). The three dimensional arrangement of polar and apolar amino acids in the helical regions of apoproteins from lipoproteins has been suggested to be important in the lipid-binding characteristics of these apoproteins (7).
Human plasma lipoproteins can be divided into four major classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (8). These classes differ in size, density, and charge, and can be identified and separated by techniques based on these differences. Thus, analytic ultracentrifugation (9), gel permeation chromatography (10), rate zonal centrifugation (11), preparative ultracentrifugation (12), density gradient centrifugation (13), affinity chromatography (10), electrophoresis (15), and isoelectric focusing (16), have been used to fractionate and/or prepare plasma lipoproteins.

An operational definition of the various lipoprotein classes, based on preparative ultracentrifugation, will be used in this thesis. Chylomicrons and VLDL float during centrifugation in a solution of mean density $1.006 \text{ g/ml}$; LDL float during centrifugation in $1.063 \text{ g/ml}$; and HDL float in $1.21 \text{ g/ml}$. HDL can be separated into two subclasses, HDL$_2$ and HDL$_3$, which float and sink respectively in $1.125 \text{ g/ml}$. In addition, two very high density lipoprotein (VHDL) classes can be defined. VHDL$_1$ float in $1.25 \text{ g/ml}$, and VHDL$_2$ include all lipoproteins which do not float in $1.25 \text{ g/ml}$. The details of preparative centrifugation will be discussed in the Methods and Materials section.

Each of the major lipoprotein classes as defined above is heterogeneous. Thus, a preparation of lipoproteins of any major class consists of subclasses of lipoproteins with different compositions and physical properties. Density gradient ultracentrifugation (13) and gel permeation chromatography (10) have been used to subfractionate
each class. Plasma from healthy subjects generally contains all classes of lipoproteins, and the protein and lipid composition of each class is similar from person to person (17). Table I shows the approximate composition of each lipoprotein class.

Various abnormalities in lipoprotein metabolism have been described. The most prevalent are those characterized by elevated plasma concentrations of otherwise normal lipoproteins. Chylomicrons, VLDL, and LDL, although apparently normal in all other respects, are elevated in the plasma of certain patients. These simple hyperlipoproteinemias are thought to be genetically determined (28,29,30,31).

Three other genetically determined lipoprotein abnormalities have been reported. Patients with abetalipoproteinemia have virtually no chylomicrons, VLDL, or LDL because they do not synthesize the B apolipoprotein common to these lipoproteins (32). Patients with Tangier disease have negligible plasma concentrations of HDL. The HDL present are abnormally small and have an unusual protein composition (33,34). This disease is thought to be due to a deficiency in the synthesis of the A-I apoprotein of HDL (33). Patients with lecithin:cholesterol acyltransferase deficiency have a variety of lipoprotein abnormalities which appear to be directly related to the absence of this enzyme (35). The HDL fraction of these patients contains two species of abnormal lipoproteins: low molecular weight spherical particles, and high molecular weight particles which stack in rouleaux (36). The LDL fraction contains a vesicular subspecies in addition to normally appearing LDL (36). The lipoprotein abnormalities in lecithin:cholesterol
### TABLE I

**LIPID AND PROTEIN COMPOSITION OF THE MAJOR CLASSES OF HUMAN PLASMA LIPOPROTEINS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>6</td>
<td>12</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>3.5</td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Other phospholipids</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Unesterified cholesterol</strong></td>
<td>1-3</td>
<td>5-7</td>
<td>8-9</td>
<td>2-3</td>
</tr>
<tr>
<td><strong>Cholesteryl esters</strong></td>
<td>2-6</td>
<td>13-17</td>
<td>36-40</td>
<td>15-17</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>80-87</td>
<td>47-50</td>
<td>7-12</td>
<td>5-8</td>
</tr>
<tr>
<td><strong>Apolipoproteins</strong> <strong>(major)</strong></td>
<td>2-3</td>
<td>7-15</td>
<td>20-25</td>
<td>47-55</td>
</tr>
<tr>
<td>A-I</td>
<td>P</td>
<td>?</td>
<td>A</td>
<td>P(major)</td>
</tr>
<tr>
<td>A-II</td>
<td>P</td>
<td>?</td>
<td>A</td>
<td>P(major)</td>
</tr>
<tr>
<td>B</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>C-I</td>
<td>P</td>
<td>P</td>
<td>A</td>
<td>P(minor)</td>
</tr>
<tr>
<td>C-II</td>
<td>P</td>
<td>P</td>
<td>A</td>
<td>P(minor)</td>
</tr>
<tr>
<td>C-III</td>
<td>P</td>
<td>P</td>
<td>A</td>
<td>P(minor)</td>
</tr>
</tbody>
</table>

*Numbers indicate weight percent of component (17). P indicates the presence and A, the absence, of a particular apolipoprotein.

** A-I is a major apoprotein of HDL (18) with carboxyl-terminal glutamine, amino-terminal aspartic acid and molecular weight 28,331 (19). A-II is a major apoprotein of HDL (18) with carboxyl-terminal glutamine, amino-terminal pyrrolidone carboxylic acid, and molecular weight 17,380 (20). B is the major apoprotein of LDL with carboxyl-terminal serine, amino-terminal glutamic acid, and molecular weight 250,000 (21,22). C-I is a major apoprotein of VLDL (23) with carboxyl-terminal serine, amino-terminal threonine, and molecular weight 6,550 (24). C-II is a major apoprotein of VLDL (23) with carboxyl-terminal glutamic acid, amino-terminal threonine, and molecular weight 10,515 (25). C-III is a major apoprotein of VLDL (23) with carboxyl-terminal alanine, amino-terminal serine, and molecular weight 8,760 (26). The protein composition of chylomicrons is from reference (27).
acyltransferase deficiency occur in association with reduced cholesteryl ester and lysolecithin and increased lecithin and cholesterol concentrations in the plasma.

In addition to the primary hyperlipoproteinemias apparently due to altered lipoprotein metabolism, secondary hyperlipoproteinemias have also been described. Cholestasis due to obstructive liver disease is characterized by increased plasma cholesterol and phospholipids and the presence of abnormal LDL and HDL. The abnormal LDL appear as flattened vesicles (37) while the abnormal HDL stack as rouleaux (38). These lipoproteins have compositions and structures different from normal. The origin of these abnormal lipoproteins is not clear. The work of Small (39) would suggest that the abnormal lipoproteins seen in cholestasis are due to an interaction of bile salts with plasma lipids and are not due to some alteration in cellular processes involved in lipoprotein synthesis.

Normally, chylomicrons and VLDL are assembled in the Golgi apparatus of cells of the liver and intestinal mucosa, and the assembled lipoproteins reach the extracellular fluid by exocytosis (41). The origins of LDL and HDL are less well known. LDL are thought to be produced, in part, as products of chylomicron and VLDL degradation by lipoprotein lipase. Lipoprotein lipase hydrolyses the triglyceride of these lipoproteins, releasing unesterified fatty acids for further metabolism in adipose and other tissues (42). Lipoprotein remnants have been detected during VLDL breakdown after release of lipoprotein lipase into the plasma by heparin injection (43).
In a normal individual, these remnants would be transformed into LDL by a process not yet understood (44).

HDL are thought to originate from precursor or nascent HDL, produced in the liver, which take on their final structure when acted upon by plasma LCAT. Evidence to support the synthesis of HDL precursors by the liver comes from the work of Hamilton (45). In studies with perfused rat liver he observed secretion of lipoproteins which under electron microscopy stacked as rouleaux. When acted upon by LCAT, these nascent HDL transformed into apparently normal HDL. The transformation to normal HDL was accompanied by the production of cholesteryl esters by lecithin:cholesterol acyltransferase. In addition, exchange of protein and lipid between HDL and VLDL or chylomicrons (46,47), and exchange of lipid between HDL and blood cells or peripheral tissue (48), may contribute to HDL composition and structure.

As described in the previous paragraph, synthesis and metabolism of the various classes of plasma lipoproteins in healthy subjects is associated with changes in their size, shape, and composition. Changes in size and shape apparently accompany changes in composition, suggesting that size, shape, and composition are interrelated. The independent contribution of each component to the size and shape of lipoproteins is still largely unknown. Physical-chemical properties of each component place restrictions on the possible ways one component may be located relative to another. For example, apolar moieties might be expected to associate and be separate from polar moieties.

The importance of such physical-chemical properties in determining
lipoprotein structure relative to cellular processes involved in lipoprotein synthesis is unclear. In the case of HDL, cellular processes apparently do not determine the final structure. Rather, in this case, the final structure appears to be determined by the action of LCAT, which produces apolar cholesteryl esters and therefore changes the composition, and thereby the physical properties of nascent particles.

To gain insight into possible roles of each component in the structure and metabolism of lipoproteins, properties of complexes resulting from the recombination of lipids and apoproteins from lipoproteins have been extensively studied. These studies have depended on development of methods for isolating the various components and on methods for effecting their recombination. Only recently, for example, have separated apolipoproteins become available for recombination studies.

Studies on relipidation of apolipoproteins have raised questions bearing on the problem of the interrelation of composition and structure. It has been found that certain lipids interact with apolipoproteins only when other specific lipids are present and only under certain conditions. For example, cholesterol combines with apolipoproteins only in the presence of phospholipids. Cholesteryl esters can be recombined with apolipoproteins but also only in the presence of phospholipids and only during sonication. In addition, some apolipoproteins such as A-I have been refractory to any significant relipidation without sonication. Considering the above observations, the mechanism by which an apolipoprotein is lipidated in vivo is a most interesting problem.
Relipidation experiments have therefore been conducted to obtain clues to answer such questions as: What is the interrelation between lipoprotein structure and composition? How might an apolipoprotein be combined with lipid in vivo or in vitro? And, what structural and compositional characteristics permit apolipoproteins to be components of lipoproteins?

Relipidation of apolipoproteins or synthesis of lipoproteins in vitro was first studied by Scanu and coworkers (49,50,51). De-lipidated protein from HDL (apo-HDL), prepared by extracting HDL with ethanol-ether mixtures, was used in these studies. Apo-HDL prepared in this way contained less than 1% lipids. After iodination with $^{131}$I, $^{131}$I-labeled apo-HDL was incubated (10 min, 24°C) with whole serum, HDL, LDL, VLDL, or emulsions of triglyceride or coconut oil. Apo-HDL bound to lipoproteins and formed complexes which floated in the HDL density range and which were indistinguishable by electrophoresis from normal HDL. Incubating apo-HDL with triglyceride or coconut oil, however, only resulted in formation of complexes of density < 1.063 g/ml. In the presence of mixtures of HDL and other lipoproteins it was observed that apo-HDL had a specificity for association with HDL. Observations that the rates of clearance of $^{131}$I-HDL and $^{131}$I-apo-HDL were similar, led to the additional conclusion that the properties of the HDL protein component were not substantially altered by delipidation.

In 1967, Sodhi et al. (52) reported that apo-HDL can be relipidated in a two-phase system. Lipids were dissolved in petroleum ether, mixed with an equal volume of buffer containing apo-HDL, and rotated
(16 hr, 4°C). The aqueous portion was removed and analyzed for protein and lipid content. Plasma phospholipids readily recombined with apo-HDL but cholesterol did so only in the presence of phospholipid. When total plasma lipids were used, triglyceride, cholesterol, and phospholipid were incorporated into the relipidated complex. No incorporation of cholesteryl ester was observed.

Fleischer et al. (54) and Scanu (55) used phospholipids dispersed by dialysis of a butanol-cholate-phospholipid solution (56) or by sonication, respectively, to relipidate apo-HDL. The apo-HDL used by Fleischer et al. was delipidated with ethanol and retained some lipid (about 5% of the phospholipid and 7% of the cholesterol). Mixtures of apo-HDL and dispersed phospholipid were incubated at room temperature (25°C) for periods of time varying from 30 min to 16 hr. Relipidation of the apo-HDL was noted to cause inhibition of the lecithin-requiring mitochondrial enzyme, β-hydroxybutyrateapodehydrogenase in the presence of apo-HDL. The relipidated complex was further studied by gel filtration, flotation at 1.21 g/ml, electrophoresis, and analytic ultracentrifugation. The Stokes' radius of the complex was intermediate between that of apo-HDL and dispersed phospholipid. The complex floated at 1.24 g/ml, and migrated on starch gel electrophoresis to a position distinct from HDL, apo-HDL, or phospholipid without added protein. Optical rotatory dispersion and infrared spectroscopic measurements showed that the spectroscopic properties of the apolipoprotein in the relipidated complex were similar to those of the protein in native HDL. A study by Chapman et al. (57) of the nuclear magnetic
resonance spectra of complexes of apo-HDL relipidated with phospholipid by the method of Scanu (55) suggested that the phospholipids in these complexes were somewhat "looser" than in native HDL.

Sodhi and Gould (58) showed formation of a lipid-protein complex during incubation of apo-HDL with plasma lipids dissolved in Tween 20. Lipids extracted from serum were dissolved in Tween 20 and apo-HDL was added. The mixture was then subjected to preparative ultracentrifugation. The complex formed of apo-HDL and plasma lipids floated in the HDL density range, between 1.063 and 1.21 g/ml, and contained most of the phospholipid in the mixture. The other lipids were reported to be present in the complex although quantitative results were not presented.

Camejo et al. (59) studied the relipidation of apo-HDL and individual apolipoproteins in a two-phase system consisting of hexane and an aqueous buffer. Proteins were prepared by electrophoresis and identified indirectly by assuming that the most abundant apoprotein of HDL was A-I and the next most abundant was A-II. Equal volumes of protein in buffer, and HDL lipids in redistilled hexane (weight ratio lipid:protein 2:1), were mixed and shaken (12 hr, 22°C). The aqueous phase was separated and analyzed for protein and lipid. Under these conditions, apo-HDL, A-I, and A-II bound only phospholipid and no cholesterol, triglyceride, or cholesteryl ester. Apo-HDL, A-I, and A-II were indistinguishable in their affinity for phospholipid. Albumin, peptides from HDL other than A-I and A-II, and trypsin-treated apo-HDL bound no lipid in this two-phase system.

In 1970, Scanu and coworkers (60,61) showed that cosonication of
apo-HDL and HDL lipids resulted in formation of a lipid-protein complex containing all of the HDL lipids. Prior to this, no procedure had caused incorporation of cholesteryl esters into a relipidated complex. Lipid and protein (1:1 by weight) were sonicated (3×1 min 40°C) in an aqueous buffer. It was subsequently shown (62) that a temperature of 40°C was necessary because reassembly required that the cholesteryl ester acyl chains be melted. The resulting suspension was sequentially fractionated by preparative ultracentrifugation at densities of 1.063 and 1.21 g/ml. The fraction recovered in the HDL density range was analyzed for composition, for flotation rate by analytic ultracentrifugation, and for size by electron microscopy. Reassembly of HDL₂ and HDL₃ by this technique resulted in complexes with properties nearly identical to the starting lipoproteins although there was some tendency for formation of particles slightly larger than native HDL₂ or HDL₃.

The relipidation of the individual apolipoproteins when sonicated with total HDL lipids was also investigated (60). A-I formed complexes with the same lipid composition and size as complexes formed by apo-HDL. A-II, on the other hand, formed complexes somewhat larger (7-28 nm as compared with 6-18 nm) which contained a greater proportion of cholesteryl esters.

In 1971, Forte et al. (63) studied by electron microscopy products formed by cosonicating HDL apoproteins with lipid. A-I, A-II, or albumin, dissolved in a low ionic strength buffer, was added to lipids previously dried under nitrogen giving a mixture containing about 50% protein by weight. The mixture was sonicated at 40°C for 5 min, a
procedure similar to that employed by Hirz et al. (61). Either A-I or A-II, when sonicated with lecithin or lecithin plus cholesterol, produced rouleaux which were interpreted as stacks of disc-shaped particles. When cholesteryl esters were included in the lipid mixture, spherical particles, 5-20 nm in diameter, which resembled HDL, were seen. Similar spherical particles were formed from discs, containing cholesterol plus lecithin, during incubation with a d > 1.21 fraction from plasma which contained lecithin:cholesterol acyltransferase. When the above lipid mixtures were sonicated without added protein or with albumin, only vesicular structures could be seen.

The circular dichroism spectra of A-I, A-II, and apo-HDL after cosonication with lipid were studied by Lux et al. (64). Protein was mixed with lecithin or lecithin plus cholesteryl ester, 1:1 by weight, sonicated by the method of Hirz et al. (61), and separated by preparative ultracentrifugation. The circular dichroism spectrum of the protein in the d 1.063-1.21 fraction was analyzed by several methods to estimate the helical content of the protein. Apo-HDL as well as the separated apolipoproteins became more helical during relipidation. Cosonication of the apolipoproteins with a mixture of lecithin plus cholesteryl esters induced a greater change in the helical content of the apolipoproteins than did lecithin alone. Under all conditions of relipidation, A-I had a greater helical content than A-II, and apo-HDL had an intermediate amount of helix.

Kruski et al. (65) studied products formed by cosoninating apo-HDL, containing both A-I and A-II, with lecithins having definite
acyl chain lengths, and with other phospholipids containing a naturally occurring mixture of acyl chains. The phospholipids were separately sonicated (15 sec, 25°C), incubated (45 min, 40-50°C), mixed with protein, and sonicated again (3×1 min, 40°C). The resulting suspension was separated by isopycnic density gradient ultracentrifugation or gel permeation chromatography, and the fractions were analyzed for protein and lipid composition. Products formed by cosoninating apo-HDL₃ with lecithins with acyl chains of 10–18 carbons, egg lecithin, phosphatidyl ethanolamine, or sphingomyelin, banded into three fractions. A nearly lipid-free protein fraction, a lipid-protein complex fraction, and a protein-free phospholipid fraction were found. After apo-HDL₃ was cosonicated with di-C₈-lecithin, one band was seen on density gradient ultracentrifugation, but two fractions were eluted from the gel column, indicating formation of a lipid-protein complex which apparently dissociated during chromatography. Phosphatidyl serine or phosphatidyl inositol when sonicated with apo-HDL₃ formed single complexes, and no free lipid or protein was seen. The complexes formed by sonication of lysolecithin with apo-HDL₃ separated into three bands when subjected to density gradient ultracentrifugation: two lipid-protein complexes with different lipid:protein ratios, and a band of nearly lipid-free protein. In all the above studies the fraction which was nearly lipid-free, if present, contained only A-I; A-II was always found in a lipid-protein complex.

Relipidation of the C apolipoproteins from VLDL has been studied by several groups. Forte et al. (66) showed that during sonication
of the C apolipoproteins with lipid, various complexes were formed which could be distinguished by electron microscopy. Heterogeneous round particles 18-45 nm in diameter, myelin forms, rouleaux of about 10 nm period, and small spherical particles 8-10 nm in diameter were seen. On preparative ultracentrifugation, the large round particles and myelin forms were predominantly found in the d 1.006-1.063 fraction; small spherical particles and rouleaux were found in the d 1.063-1.21 fraction. Formation of complexes between the C apolipoproteins and lecithin during incubation has also been shown to inhibit the activity of β-hydroxybutyrateapodehydrogenase (67,68). Circular dichroism measurements have shown that all of the C apolipoproteins apparently become more helical on relipidation (67,69,70).

Relipidation of C-III has been particularly well studied. This apolipoprotein combines with lecithin without sonication when the lecithin is at a temperature above its liquid crystal phase transition (71,72). Titration of lecithin vesicles with C-III has been followed by electron microscopy (73,74). A change in the lipid:protein weight ratio was accompanied by changes in the sizes and shapes of the complexes. As the amount of protein increased, dispersed vesicles changed into rouleaux and subsequently into extended sections of myelin forms. These electron microscopic results as well as hydrodynamic and light scattering studies (74) led to the suggestion that the apolipoprotein was bound to the vesicle. The various forms seen in the electron micrographs were interpreted as resulting from fusion of the vesicles during preparation (negative staining and dehydration) for
electron microscopy (73,74). Fluorescence studies of the graded re-lipidation of C-III with lecithin were interpreted as showing that one molecule of C-III binds up to 80 lecithin molecules (67).

Relipidation of A-I and A-II without sonication has recently been extensively studied. Assman et al. (75) studied the relipidation of A-I, A-II, and apo-HDL during incubation with dispersions of lecithin and sphingomyelin. They concluded that A-II readily interacts with both phospholipids, but that A-I does not. A-I in the presence of A-II, however, was found to be readily relipidated. Stein et al. (76) showed that A-I and sphingomyelin formed rouleaux when incubated together and suggested that an interaction between this phospholipid and apolipoprotein took place without sonication.

Nichols et al. (77) showed that the presence of lysolecithin in incubated mixtures of apo-HDL and lecithin facilitated the formation of a lipid-protein complex which floated in the d 1.063-1.21 range. The basis for this facilitation was further studied by Verdery et al. (78) who suggested that lysolecithin "primed" the apolipoprotein permitting further interaction with lecithin. These studies make up part of this thesis. In this way, lysolecithin was implicated as a possible factor in the organization of lipids and proteins in lipoproteins. Lysolecithin is a product of the plasma lecithin:cholesterol acyltransferase reaction (79). It is a component occurring naturally in lipoproteins (17). In high concentrations, lysolecithin disrupts cell membranes (80), and interestingly is present in relatively large amounts in atherosclerotic plaques (81). Lysolecithin therefore has been suggested as a factor in the development of atherosclerosis (82).
A novel approach to studying the interaction of A-I and A-II with lysolecithin and other phospholipids during incubation was developed by Rosseneu et al. (83,84). They determined the heat of combination of lysolecithin and various lecithins with these apolipoproteins. The results were interpreted as showing that A-I and A-II can combine with these phospholipids to certain maximum lipid:protein ratios. Association constants for the reaction, protein + lipid ⇌ complex, were measured by this technique. This group also showed that A-I mixed with A-II behaved during relipidation as if a complex of molar ratio 1:1 A-I:A-II had been formed (85). The heats of combination of mixtures of A-I and A-II with lecithin were not due simply to addition of the heats of recombination of each apolipoprotein with lecithin. The deviation from additivity was maximum at a 1:1 mole ratio A-I:A-II.

Another approach to the study of relipidation of A-I and A-II with lysolecithin and other detergents has been explored recently by Reynolds et al. (86,87,88,89). They investigated the binding of these lipids to A-I and A-II by equilibrium dialysis. In this way, A-I and A-II were shown to have discrete high-affinity sites which bind lysolecithin, detergents, or alkanes. Few other proteins are known to possess such sites. At high levels of detergent binding, a conformational change was induced in the apolipoproteins; and substantially more ligand could be cooperatively bound to the protein after this conformational change. An analogy was drawn by this group between cooperative binding of detergents and cooperative binding of lecithins. Since the binding of a few detergent molecules induced a
change in the protein which allowed other ligands to bind, it was suggested that there might be a "synergistic" interaction between the lipids in a lipoprotein. The few molecules of lysolecithin present in an HDL molecule might be responsible for a change in the apolipoproteins which would facilitate the binding of other lipids. This suggestion, that the presence of a few lysolecithin molecules might affect binding of other lipid, is similar to the suggestion of Nichols et al. (77) that lysolecithin "facilitates" lecithin binding to apo-HDL and to the suggestion of Verdery et al. (78) that lysolecithin "primed" the apolipoprotein, permitting subsequent interaction with lecithin.

As apparent in the literature reviewed above, various techniques have been developed to effect relipidation of apolipoproteins. De-lipidated apolipoproteins have been recombined with lipid by incubation with serum, by mixing with lipids in a two-phase system, by sonication with lipids, and by incubation in the presence or absence of a detergent lysolecithin. The resulting synthetic lipoproteins have been characterized by many different methods, and appear to have some similarities to lipoproteins from plasma of normal and abnormal patients. Generally, however, these studies were performed on complexes, produced from starting mixtures of lipids and apolipoproteins, exhibiting little variation in composition. There have been few studies to assess the influence of variation in the relative amounts of lipoprotein components on the properties of synthetic complexes of lipids and apolipoproteins. Considerable variation in composition, however, occurs naturally and is associated with interesting changes in lipoprotein structure.
STATEMENT OF PURPOSE

The studies described in this thesis were undertaken (1) to develop methods for relipidation of apolipoproteins with various amounts of lipids, (2) to characterize the complexes formed, and (3) to study the effect of such relipidation on the conformation of the apolipoproteins. Although studies have been reported on relipidated apolipoproteins, they generally considered only a limited range of compositional variation. Thus the goal of this thesis was to systematically study the effect of varying composition on the physical-chemical properties of the relipidated complexes. In order to accomplish this, it was necessary to develop methods for relipidating the apolipoproteins in a controllable fashion, and to characterize the complexes formed.

As previously described, the most important lipids in lipoproteins are phospholipids, cholesterol, cholesteryl esters, and triglycerides. Incorporation of cholesterol or its esters, or triglycerides into lipoprotein complexes has been observed only in the presence of phospholipids. The phospholipid, lecithin, was therefore chosen as the principal lipid for these studies both because of its important role in the structure and stability of native lipoproteins, and because it can be readily dispersed into uniform single bilayer vesicles which provide a well-defined lipid dispersion for investigation of relipidation.

The phospholipid detergent, lysolecithin, was also employed in
these studies, since it had been shown to facilitate formation of lipid-protein complexes in mixtures of lecithin and apolipoproteins, and also forms well-defined micellar dispersions. The major apoprotein of HDL, A-I, interacts with lecithin much more readily in the presence of lysolecithin than in its absence. The presence of lysolecithin was therefore used to facilitate the graded relipidation of A-I with lecithin. The physical-chemical basis for this facilitation was investigated by studying the effect of lysolecithin on the conformation of A-I.

A-II, the other major HDL apoprotein, and C-III, a major apoprotein of VLDL, interact with lecithin spontaneously and do not require lysolecithin for their relipidation. In order to understand the bases for these differences between A-I and A-II or C-III, experiments were designed to compare various aspects of the relipidation of these apolipoproteins. The effect of lysolecithin on the conformation of A-II and C-III was also studied to determine if these apolipoproteins were affected by this phospholipid in a specific manner which might account for their observed relipidation properties.

The composition of complexes formed in a model system consisting of a single apolipoprotein and lecithin or lysolecithin is relatively simple compared to the composition of native lipoproteins. However, similarities have been observed between complexes with this limited composition and abnormal lipoproteins which occur in certain disease states. In the studies described in this thesis, a special attempt was made to determine which mixtures of lipids and
apolipoproteins would form complexes which show structural characteristics of abnormal lipoproteins or HDL precursors.

In summary, three apolipoproteins were used in the relipidation experiments: A-I and A-II from HDL, and C-III, a major apoprotein of VLDL. As indicated above, the lipids used were the phospholipids, lecithin and lysolecithin. Several different kinds of measurements were performed. Preparative ultracentrifugation was used to demonstrate formation of lipid-protein complexes, particularly with A-I. Electron microscopy was used to characterize the size and shape of the relipidated complexes. Gradient gel electrophoresis was used to estimate the molecular weight of these complexes. Fluorescence and circular dichroism were used to assess the effect of relipidation on the conformation of these apolipoproteins.
METHODS AND MATERIALS

1. Apolipoprotein Preparation

HDL were isolated by sequential preparative ultracentrifugation in NaBr-NaCl solutions at densities of 1.063 g/ml and 1.21 g/ml (see section on preparative ultracentrifugation). Isolated HDL were re-centrifuged at 1.21 g/ml to remove any residual albumin. A-I and A-II were prepared from HDL by several different techniques based on chromatography on either Sephadex G-200 (90) or DEAE (18,23).

In order to separate the apolipoproteins on Sephadex G-200, the HDL were first delipidated by extraction with methanol-chloroform-ether (91). The resulting dry apo-HDL was dissolved in a buffer of 6M urea, 0.1% mercaptoethanol, 0.2M Tris-HCl, pH 8.0, applied to a Sephadex G-200 (Pharmacia, Piscataway, NJ) glass bead column (90), and eluted at 4°C with the same buffer. Fractions predominantly containing A-I were subjected to polyacrylamide gel electrophoresis on 7% polyacrylamide disc gels in the presence of 6M urea according to the method of Kane (92). Those fractions showing only a single A-I band were pooled and concentrated by ultrafiltration using an Amicon UM-2 filter (Amicon, Lexington, MA). Protein concentrations were determined by the method of Lowry et al. (93) using albumin standards and were not corrected for possible variations in chromogenicity.

For chromatography on DEAE, HDL were delipidated by the methanol-chloroform-ether extraction either directly or following a fractionation procedure involving incubation of the HDL with guanidine HCl. In the
latter procedure, HDL were incubated in 6M guanidine HCl (3 hr, 37°C). The resulting mixture was dialysed to 1.21 g/ml NaBr-NaCl, and ultracentrifuged (40.3 rotor, 114,000 × g, 24 hr, 15°C). The top 1 ml, enriched in lipid and A-II, was delipidated by chloroform-methanol-ether extraction, or by incubation with tetramethylurea (92). Delipidation with tetramethylurea gave a higher yield of protein. The bottom 1 ml, enriched in A-I, was nearly lipid-free (94) and was used without further treatment.

For fractionation on DEAE, 20-30 mg of apo-HDL was dissolved in (or dialysed against) a buffer (7M urea, 0.005M Tris-HCl, pH 8.0), and applied to a 0.9 cm × 40 cm column packed with DEAE cellulose (Whatman, Maidstone, Kent, England) which had previously been equilibrated with the same buffer. The apolipoproteins were eluted at 5°C with a non-linear gradient formed by pumping elution buffer at 16 ml/hr from a 250 ml reservoir into which limiting buffer flowed. Elution was begun with the reservoir filled with starting buffer (7M urea, 0.005M Tris-HCl, pH 8.0) into which flowed the first limiting buffer (7M urea, 0.1M Tris-HCl, pH 8.4). After 20 hr, a second limiting buffer (7M urea, 0.2M Tris-HCl, pH 8.2), replaced the first, and was used for an additional 10 hr. After 30 hrs elution with a gradient-forming buffer system, the second limiting buffer (7M urea, 0.2M Tris-HCl, pH 8.2) was used directly to elute the apolipoproteins. Fractions were collected every 12 min and the optical density at 280 nm of each fraction was measured on a Cary spectrophotometer to monitor the protein concentration. Fractions corresponding to a particular elution peak were pooled, dialysed to 0.005M NH₄HCO₃, concentrated by
ultrafiltration using an Amicon UM-2 filter (Amicon, Lexington, MA), and analysed by polyacrylamide gel electrophoresis (92). Protein concentrations were determined by the method of Lowry et al. (93).

Figure 1 shows the elution pattern of a typical DEAE chromatographic run and Figure 2 shows results of polyacrylamide gel electrophoresis of aliquots of pooled fractions corresponding to specific peaks. These results are similar to those reported by Shore et al. (18,23). The peaks shown in Figure 1 can be identified using the polyacrylamide gel electrophoresis results. Peak 1 is C-II, peak 2 is A-II, peaks 3 and 4 are A-I, and peaks 6 and 7 are C-III. Peak 5 is apparently a mixture of several apolipoproteins and probably includes the arginine-rich peptide (95). Observation of two peaks containing A-I is consistent with reports of microheterogeneity of this apolipoprotein (96). The two peaks containing C-III probably correspond to C-III differing in the number of attached sialic acid residues as previously reported (97).

Figures 3 and 4 show results of DEAE chromatography of the two fractions obtained after treating HDL with guanidine HCl as described above. In the top fraction, obtained after ultracentrifugation of the guanidine-treated HDL at a density of 1.21 g/ml, A-II and C-III are the major apolipoproteins present and only a small amount of A-I can be detected (Figure 3). In the bottom fraction, the A-I peak predominates (Figure 4), and the A-II and C-III peaks are relatively diminished. These results illustrate the enrichment of the top fraction in A-II and C-III, and the bottom fraction in A-I. In apo-HDL, only about 20% of the apoprotein is A-II (98). When fractionated
FIGURE 1  DEAE chromatography of apo-HDL. HDL from human plasma were delipidated by chloroform-methanol-ether extraction. Apo-lipoproteins applied to the column were eluted with a non-linear gradient of Tris HCl in the presence of 7M urea as described in the text. Fractions corresponding to the numbered peaks were pooled and analyzed by polyacrylamide gel electrophoresis.
Figure 1
FIGURE 2 Polyacrylamide gel electrophoresis of pooled fractions corresponding to peaks from DEAE chromatography of apo-HDL shown in Figure 1. Electrophoresis was performed on 7% polyacrylamide in the presence of urea by the method of Kane (92). Peaks were identified with particular apolipoproteins as described in the text.
Peak number

XBB 7511-8338

Figure 2
FIGURE 3: DEAE chromatography of a delipidated top fraction obtained after ultracentrifugation of HDL treated with guanidine HCl. HDL from human plasma were incubated (3 hr, 37°C) in 6M guanidine HCl. After dialysis to a background salt density of 1.21 g/ml, the mixture was subjected to ultracentrifugation (114,000×g, 24 hr, 37°C). The top 1 ml fraction was delipidated by chloroform–methanol–ether extraction and subjected to DEAE chromatography. The optical density (____) and conductivity (----) of each fraction are shown.
FIGURE 4  DEAE chromatography of a bottom fraction obtained after ultracentrifugation of HDL treated with guanidine HCl. HDL from human plasma were incubated (3 hr, 37°C) in 6M guanidine HCl. After dialysis to a background salt density of 1.21 g/ml, the mixture was subjected to ultracentrifugation (114,000×g, 24 hr, 37°C). The bottom 1 ml fraction was subjected to DEAE chromatography without further delipidation. The optical density (----) and conductivity (-----) of each fraction are shown.
on DEAE as described above, preparation of A-II directly from total apo-HDL is not as efficient as preparation of A-II from the enriched fraction obtained by ultracentrifugation of HDL treated with guanidine HCl.

2. Phospholipid Purity and Measurement

Phospholipids, egg lecithin (General Biochemicals, Chagrin Falls, OH) and lysolecithin from egg (Supelco, Inc., Bellefonte, PA, or Analabs, North Haven, CT) were used in the relipidation studies. Solutions of these lipids with known concentrations were made by drying the phospholipids under nitrogen to a constant weight and suspending them in known volumes. In some experiments requiring measurement of low concentrations of lecithin, (see section on Results and Discussion), a small amount of \( ^{14} \text{C} \)lecithin (algal, New England Nuclear, Boston, MA) was added to give a solution of known specific activity. Lecithin concentrations were then measured by radioassay using Aquasol (New England Nuclear, Boston, MA) as the scintillation cocktail. Lecithin and lysolecithin appeared as single spots on thin layer chromatography, indicating no major contaminants. The chromatogram on silica gel H (Supelco, Bellefonte, PA) was developed with chloroform:methanol:water (60:30:10 by volume), and the lipids were visualized by exposure to iodine vapor (Figure 5). To check the position of lysolecithin on the chromatogram, a sample of lecithin was treated with snake venom (Crotalus adamanteus, Sigma, St. Louis, MO). Snake venom contains phospholipase A which hydrolyses the fatty acid ester in position 2 of lecithin forming lysolecithin and free fatty acids (99). 1 mg of
FIGURE 5 Thin layer chromatogram of lecithin and lysolecithin. Lane 1, lecithin; lane 2, lecithin after treatment with snake venom; lane 3, lysolecithin. The chromatogram on silica gel H was developed with chloroform:methanol:water (60:30:10 by volume). The lipids were visualized by exposure to iodine vapor.
Figure 5

Lane number

XBB 7511-8337
lecithin was dissolved in 3 ml of diethyl ether to which 0.5 mg of snake venom in 0.1 ml of 5mM CaCl₂ was added. After incubation (18 hr, 23°C), the ether was removed, dried, and the residue was applied to a chromatographic plate along with the samples of lecithin and lysolecithin. The lecithin treated with snake venom showed a single spot whose position was used to locate lysolecithin on the chromatogram.

3. Preparation of Lecithin Vesicles

Single bilayer vesicles of lecithin were used for relipidation studies involving this lipid. When simply mixed with water, lecithin forms multilayered structures with limited permeability (100). By use of single bilayer vesicles, interaction of lecithin with other components in the solution was not limited by the rate of diffusion through lecithin multilayers. In addition, by use of such vesicles, relipidation was investigated in mixtures where the sizes and shapes of the particles in the lecithin dispersion were known. Under conditions of sonication, lecithin forms small vesicles, generally consisting of one closed bilayer containing trapped buffer (101). However, prolonged sonication of mixtures of lecithin and water has been shown to cause formation of lysolecithin (102). In this thesis, vesicles were formed either with short-term sonication or without sonication (103,104), thus avoiding the formation of lysolecithin.

Two specific methods were used to prepare vesicles. Following a modification of the procedure of Huang (101), egg lecithin was mixed with (¹⁴C)lecithin and buffer (0.01M Tris-HCl, 0.1M NaCl, 0.01% EDTA) and
sonicated (3 × 10 min, 25°C) using a 60 watt ultrasonic disintegrator (No. 7100, MSE, London, England) operating at 18-29 kHz. The sonicated lecithin dispersion was ultracentrifuged (114,000 × g, 1 hr, 15°C) to remove any large lecithin aggregates and any titanium from the disintegrator probe. To obtain vesicles of uniform size, the supernatant was applied to a 2.5 cm × 40 cm column packed with Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The column was equilibrated and eluted with the same buffer used to form the lecithin dispersion. Fractions were collected at 20 min intervals and the concentration of lecithin in each fraction was measured by radioassay as described above. Figure 6 shows the elution profile of a sonicated dispersion of lecithin.

The second method for preparation of lecithin vesicles followed the procedure of Batzri et al. (103). 0.1 ml of a 10 mg/ml solution of lecithin in absolute ethanol was injected into 4.9 ml of the appropriate buffer using a 100 µl Hamilton syringe. The concentration of lecithin in the resulting dispersion was 0.2 mg/ml in a buffer containing 2% ethanol. The vesicles formed were used without further fractionation.

Electron microscopy of vesicles prepared by the method of Batzri et al. (103) showed primarily vesicles of about 25 nm diameter and virtually no aggregates (Figure 7(a)). Analysis of such an unsonicated dispersion by chromatography on Sepharose 4B as described above, however, showed the presence of both large and small particles (Figure 8). Also, after concentrating unfracionated solutions of
FIGURE 6 Gel chromatography of lecithin vesicles. Egg lecithin, with a small amount of added ($^{14}$C)lecithin, was mixed with eluting buffer, sonicated (3 x 10 min, 25°C), and fractionated on a 2.5 x 40 cm Sepharose 4B column. Concentration of lecithin in the eluate was measured by radioassay of ($^{14}$C)lecithin in aliquots of each fraction.
FIGURE 7 Electron micrographs of lecithin vesicles and myelin figures. (a), vesicles formed by injecting an ethanolic solution of lecithin into water. (b), vesicles from the second peak eluted during gel chromatography of a sonicated lecithin dispersion. (c), myelin figures formed by concentrating vesicles by ultrafiltration. (d), myelin figures from the second peak eluted during gel chromatography of a sonicated lecithin dispersion.
FIGURE 8 Gel chromatography of lecithin vesicles. Egg lecithin, with a small amount of added (\(^{14}\)C)lecithin was dissolved in absolute ethanol. The ethanolic solution was injected into eluting buffer, and fractionated on a 2.5\(\times\)40 cm Sepharose 4B column. Concentrations of lecithin was measured by radioassay of (\(^{14}\)C)lecithin in aliquots of each fraction.
such vesicles by ultrafiltration using an Amicon PM30 filter, electron microscopy showed predominantly myelin figures and aggregates of lecithin (Figure 7(c)). These observations suggest that additional handling of vesicles during gel chromatography or ultrafiltration tended to promote aggregation.

Electron microscopy of vesicles prepared and fractionated by the method of Huang showed the presence of both vesicles, about 25 nm in diameter, and myelin figures, in the same column fraction (Figures 7(b) and 7(c)). In order to prepare these vesicles for electron microscopy, however, it was necessary to dialyse them to 0.12M NH₄-acetate. The myelin figures were therefore encountered only after dialysis.

4. Preparative Ultracentrifugation

Sequential preparative ultracentrifugation was used for isolating HDL and separating relipidated apolipoproteins according to density (105). Plasma or mixtures of lipid and apolipoproteins in 0.19M NaCl were adjusted to a mean background density of 1.063 g/ml by adding a NaBr-NaCl solution (0.19M NaCl). The mixture was ultracentrifuged (40.3 rotor, 114,000 × g, 24 hr, 15°C) and the top and second 1 ml fractions were removed with a transfer pipette. These fractions were designated d 1.063 0-1 and d 1.063 1-2, respectively. Additional NaBr-NaCl solution was then added to give a mean density of 1.21 g/ml and the mixture was again ultracentrifuged (40.3 rotor, 114,000×g, 24 hr, 15°C). Six 1 ml fractions were removed from the top to the bottom of the ultracentrifuge tube using a transfer pipette and were designated d 1.21 0-1, d 1.21 1-2, etc. When plasma was fractionated, the d 1.21 0-1 fraction
was used as the source of HDL. When relipidated apolipoproteins were fractionated, measurements of lipid and protein content were made on each fraction. Subsequently, results of these measurements were added and the amounts of lipid and protein present in three density ranges, d < 1.063, d 1.063-1.21, and d > 1.21, were reported. The protein and lipid reported in the d < 1.063 range was in the d 1.063 0-1 fraction. The protein and lipid in the d 1.063-1.21 range was in the d 1.063 1-2 and d 1.21 0-1 fractions. The protein and lipid in the d > 1.21 range was in all of the fractions d 1.21 1-2 through d 1.21 5-6.

In the course of preparative ultracentrifugation, the background salt redistributed and a density gradient was established (Figure 9). In addition, under the usual conditions of time and ultracentrifugal field, equilibrium was not reached for particles the size and density of HDL. Thus separations of HDL and relipidated apolipoproteins of different densities were not complete and fractions did not correspond to sharply defined density ranges.

5. Fluorescence

Spectrofluorometry of the apolipoproteins before and after relipidation was performed on an Aminco-Bowman spectrophotofluorometer equipped with a constant-temperature cell which maintained the samples at 23°C. Relative fluorescence intensity and the wavelength of the fluorescence emission peak were measured. Intensity was measured on an arbitrary scale. Wavelength calibration of the monochrometers of the spectrophotofluorometer was reported to be accurate to about 2 nm and measurements of emission peak wavelength were repeatable to within 1 nm.
FIGURE 9 Density gradient produced in a solution of NaCl-NaBr during ultracentrifugation (114,000 x g, 24 hr, 15°C). The starting density was 1.21 g/ml throughout the tube. The densities of 1 ml fractions, removed from the top after ultracentrifugation and designated d 1.21 0-1 etc., were measured by refractometry. Results from two separate samples are presented.
Figure 9
Protein fluorescence is mostly due to fluorescence of the tyrosine and tryptophan residues of the protein. In proteins containing both tryptophan and tyrosine, the more intense tryptophan fluorescence usually masks any tyrosine fluorescence (106). The emission peak wavelength of tryptophan is dependent on the dielectric constant of its environment and the emission peak wavelength shifts from about 330 nm to about 350 nm when this amino acid is transferred from a nonpolar solvent to water (107). Denaturation of proteins with urea or guanidine HCl often cause a shift in the fluorescence peak toward longer wavelengths because denaturation changes the polarity of the environments of the tryptophans in the protein (108).

Fluorescence intensity can be described as a function of experimental factors by the following equation: \( I = I_o \phi (1 - e^{-C \varepsilon}) \), where \( I \) is the intensity of the emission, \( I_o \) is the intensity of the source at the wavelength of absorption, \( C \) is the concentration of the fluorophore, \( \varepsilon \) is the extinction coefficient of the fluorophore at the absorption wavelength, \( \ell \) is the path length of the sample cell, and \( \phi \) is the quantum yield. This equation may be approximated by: \( I = I_o \phi C \varepsilon \). Thus, for sufficiently small \( C \), \( I \) is linearly related to \( C \). A linear calibration curve for \( I \) versus \( C \) can therefore be expected for some range of \( C \), and can be used to accurately estimate \( C \) from determinations of \( I \). Calibration curves of intensity versus concentration of A-I under two different experimental conditions are shown in Figure 10. This graph shows both the linearity and repeatability of the fluorescence measurement of low protein concentrations. The
FIGURE 10 Dependence of fluorescence intensity on concentration of A-I. The fluorescence intensity of both A-I and A-I plus lysolecithin (molar ratio lysolecithin:A-I 240:1) is linear over the indicated range of protein concentration. Results from duplicate dilutions of A-I are shown and indicate the magnitude of variation encountered in this measurement.
Figure 10

Fluorescence intensity (arbitrary units) vs. Protein concentration (mg/ml)

- A-I
- Lysolecithin: A-I (molar ratio 240:1)
quantum yield, $\phi$, is affected by fluorescence quenching due to various factors (109). By changing the quenching of the fluorescence of tryptophan and tyrosine residues, denaturation of proteins with urea or guanidine HCl is also associated with changes in the fluorescence emission intensity (108).

Figures 11, 12, and 13 show fluorescence spectra of A-I, A-II, and C-III. The emission peak wavelength of A-I is at about 330 nm, corresponding to tryptophan in an apolar environment. Fluorescence intensity of A-II is much less than that of A-I, and the emission peak wavelength, 300 nm, corresponds to the emission peak wavelength of tyrosine. Absence of any measurable tryptophan fluorescence in A-II is consistent with the reported absence of that amino acid in A-II (20). The low fluorescence intensity of A-II makes it difficult to use fluorometry for estimating A-II concentration or for investigating the relipidation of A-II. The emission peak wavelength of C-III is about 350 nm. This corresponds with the emission peak wavelength of tryptophan in a polar environment. Thus the tryptophans of C-III appear to be located in a polar environment while the tryptophans of A-I appear to be located in an apolar environment. This suggests that, when delipidated and dissolved in water, the regions near the tryptophans of these two apolipoproteins are folded differently.

6. Circular Dichroism

Ultraviolet circular dichroism spectra of apolipoproteins and relipidated apolipoproteins were obtained at 23°C using a Cary 6002. Spectra were recorded as observed ellipticity in degrees, $\theta_\lambda$, at
FIGURE 11 Fluorescence activation and emission spectra of A-I from human plasma HDL. The emission peak wavelength (332 nm) is the emission peak wavelength of tryptophan in an apolar environment.
Figure 11

Fluorescence intensity (arbitrary units) vs. Wavelength (nm)

- Activation
- Emission
FIGURE 12. Fluorescence activation and emission spectra of A-II from human plasma HDL. The emission peak wavelength (300 nm) is the emission peak wavelength of tryosine.
Figure 12

Fluorescence intensity (arbitrary units)

Wavelength (nm)

Activation
Emission
FIGURE 13 Fluorescence activation and emission spectra of C-III from human plasma VLDL. The emission peak wavelength (350 nm) is the emission peak wavelength of tryptophan in a polar environment.
Figure 13
wavelength $\lambda$. After background subtraction, spectra were plotted as mean residue ellipticity $[\theta]_\lambda$. Where $[\theta]_\lambda = \frac{(\text{mean residue weight})\theta_\lambda}{10^4c}$, $\lambda$ is the path length of the sample cell in cm, and $C$ is the concentration of the protein in g/ml. Values of mean residue weight, calculated from the amino acid composition, and used in the results reported in this thesis, were: A-I, 112.1; A-II, 116.6; C-III, 111.0.

Ultraviolet circular dichroism spectra of proteins are related to their secondary structures (110). Various methods for determining the helical content of proteins from their circular dichroism spectra have been reported (111,112). A comparison was made by Lux et al. (64) of several different methods for computing the helical content of A-I and A-II using a computer program to fit curves to the complete ultraviolet circular dichroism spectrum. Calculated helical content varied up to 10% between methods. The method used in this thesis for calculating helical content of apolipoproteins and relipidated apolipoproteins is that of Greenfield et al. (111): $\% \text{ helix} = \frac{[\theta]_{208-4000}}{29,000}$.

Using results reported in this thesis (see Results and Discussion), the helical content of delipidated A-I and A-II may be calculated to be 50% and 34%, respectively. This compares well with the ranges reported by Lux et al. of 54-57% for A-I and 33-38% for A-II. Reported spectra vary up to 10% (64,78,118).

7. Electron Microscopy

Electron microscopy was performed on samples prepared in 0.005M $\text{NH}_4\text{HCO}_3$ or dialysed to 0.12M $\text{NH}_4$-acetate. No significant differences were seen between these two methods for preparing samples. Samples
(about 1 mg/ml lipid plus protein, about 50 μl) were mixed 1:1 by volume with 2% or 4% Na-phosphotungstate (pH 7.4). 30 sec after mixing, a small drop (about 5 μl) was applied to a formvar-coated, carbon-backed grid. 30 sec later, most of the drop was removed by absorption onto filter paper leaving only a thin film of negatively stained sample. The grid was immediately examined using a JEM 200B electron microscope.

Special consideration was given to the focus. As illustrated in Figure 14, as the focus was varied, the phase grain seen in the background changed. As the phase grain size changed, the contrast changed and was a minimum when the phase grain was smallest. An attempt was made to make all micrographs with minimum phase grain. This choice of focus was made for two reasons: (1) edges of particles are not artificially sharpened under these conditions, and (2) small particles do not appear to have substructure due to defocus effects. Lack of contrast seen in micrographs made with minimum phase grain was considered to be offset by increased accuracy in interpretation of micrographs with the resulting minimum background granularity.

The possibility of misinterpretation of micrographs due to artefacts was minimized by a subjective procedure based on repetition, Figure 15 shows some artefacts which look like lipoproteins which were observed on grids to which no lipoproteins were applied. These artefacts were probably due to contamination of the formvar surface, severe underfocussing, or defects formed during drying of the negative stain. It must be emphasized, however, that these micrographs are of worst possible cases and that the field photographed was not
FIGURE 14  Electron micrographs of a mixture of lysolecithin and A-I showing the effect of variation of focus. The defocus interval was about 1500 Å. (a) shows granularity due to underfocusing. (e) shows granularity due to overfocusing.
FIGURE 15 Electron micrographs of control grids without lipoproteins illustrating the problem of possible artefacts. (a) and (b), 4% Na-phosphotungstate mixed 1:1 by volume with 0.005M NaHCO₃. (c)-(f), 4% Na-phosphotungstate mixed 1:1 by volume with 0.12M NH₄-acetate. Misinterpretation of results reported in this thesis due to possible artefacts was minimized by taking micrographs of several fields on at least two different grids.
representative of the whole grid. To avoid confusion due to such artefacts, at least two different grids were made with each lipoprotein preparation and were photographed in at least two different regions. The micrographs reproduced in this thesis are representative of all of those made of a particular preparation.

8. Gradient Gel Electrophoresis

Gradient gel electrophoresis was carried out on slab gels having a 4-30% gradient in polyacrylamide concentration (Pharmacia, Piscataway, NJ). Samples prepared in 0.005M NH₄HCO₃ were applied to a gel and electrophoresed (16-18 hr, 10°C) in buffer (0.09M Tris HCl, 0.08M borate, 0.003M EDTA, pH 8.35). Gels were stained with 1% amido black and were electrophoretically destained.

The migration distance of particles on a gradient gel is approximately proportional to the inverse logarithm of their molecular weights (114,115). A molecular weight scale was therefore established for each gel using reference proteins of known molecular weights (Figure 16). Molecular weights of lipoproteins or relipidated apolipoproteins were estimated using such a scale, established with reference proteins run simultaneously on the same gel. In this way, estimates of the molecular weight range of HDL₂ and HDL₃ were made. The range of molecular weights in a preparation of HDL₂ was 230,000-380,000 and in a preparation of HDL₃ was 150,000-230,000. These ranges correspond well to values of reported molecular weights of 334,000 for HDL₂ and 184,000 for HDL₃ obtained by other methods (116).
FIGURE 16  Relation of migration distance to molecular weight of reference proteins in gradient gel electrophoresis. The migration distance is inversely proportional to the logarithm of the molecular weight. Such calibration curves were used to estimate weights of lipoproteins and synthetic lipid-protein complexes which were simultaneously run with reference proteins on the same slab gel.
Figure 16

Migration distance (cm) vs. Molecular weight (log scale)

- Ovalbumin
- Albumin
- Catalase
- Apoferritin
- Thyroglobulin
RESULTS AND DISCUSSION

I. Relipidation of A-I with Phospholipids

As reviewed in the Introduction, the interaction of A-I and lecithin has been studied by several groups in a variety of ways. Incorporation of A-I and lecithin into lipid-protein complexes has been accomplished by use of two-phase mixtures, sonication, and incubation. Incubation of mixtures of A-I and phospholipids was used predominantly in the relipidation studies reported in this thesis in order to approximate the physiological conditions of lipoprotein synthesis and metabolism. Several groups have previously studied the binding of A-I and lecithin during incubation (75,77,83). The results are at variance. Some indicated that A-I readily bound lecithin (83) while others found that little binding took place during incubation without sonication (75,77). Nichols et al. showed that mixtures of A-I and lecithin, which do not readily form complexes during incubation, will interact and form complexes in the presence of lysolecithin (77). Further work on formation of complexes in mixtures of lecithin, lysolecithin, and A-I was undertaken, as described in this thesis, to more completely characterize the effect of lysolecithin on the relipidation of A-I with lecithin.

In order to study the properties of the complexes formed by incubating A-I with lecithin, lysolecithin, or both, mixtures of A-I and these phospholipids were prepared and subjected to preparative ultracentrifugation, gradient gel electrophoresis, electron microscopy,
fluorometry, or circular dichroism spectrometry. In all cases involving incubation with lecithin, lecithin in the form of single bilayer vesicles was used. All incubations were carried out at room temperature (23°C) for sufficient time to allow the mixtures to reach equilibrium. In studies involving mixtures of lecithin, lysolecithin, and A-I, A-I and lysolecithin were mixed first and allowed to interact at room temperature for 15 min before the lecithin vesicles were added.
Ia. Relipidation of A-I with Lecithin: Ultracentrifugal Analysis

Lecithin vesicles, prepared by the method of Huang (103), were mixed with A-I in various molar ratios and incubated (1 hr, 23°C). The mixtures were then fractionated by preparative ultracentrifugation. The distribution of (14C)lecithin and A-I in the three density ranges, d < 1.063, d 1.063-1.21, and d > 1.21, is presented in Table II. With increasing lecithin, the relative amount of A-I in the d > 1.21 fraction decreased, while an increase of A-I in the d < 1.063 fraction was observed. In the HDL density range, d 1.063-1.21, little lecithin or A-I was found for any molar ratio lecithin:A-I.

Some (14C)lecithin was observed in the d > 1.21 range. The amount was significantly greater than that found in the control containing only lecithin vesicles, and varied from 1 to 6 molecules of lecithin per A-I molecule. This observation suggests that a lipid-protein complex containing a small number of lecithin molecules formed spontaneously during incubation of lecithin vesicles with A-I. Results of recent studies by David et al (117) support this suggestion. The observation that A-I may have a high affinity for a small number of lecithin molecules is consistent with the observation that it is difficult to remove all of the phospholipid from HDL by solvent extraction as usually performed (49,53).

As the amount of lecithin was increased from a molar ratio lecithin:A-I of 10:1 to a molar ratio of 1000:1, the amount of A-I in the d < 1.063 range increased from 0 to 100%. Therefore, under the incubation conditions used in these studies, it appeared as if A-I and lecithin predominantly formed complexes with densities less than
1.063 g/ml. There apparently was little tendency for A-I and lecithin to form complexes with densities in the d 1.063-1.21 range, although A-I recombines with lecithin during sonication to form reassembled lipoproteins which float in this range (60).

The reasons for these observations are not completely clear. It has been suggested that A-I aggregates in solution and forms multimers which are not readily relipidated (118). The observation that a lower pH facilitates relipidation of A-I during incubation (119) tends to support this suggestion since the aggregation of apolipoproteins has been shown to be affected by pH (120). Subsequent sections will describe the effect of lysolecithin on the interaction of A-I and lecithin. Under the same pH conditions (pH about 8.4) used in the studies of the interaction of lecithin and A-I described above, lysolecithin apparently enabled formation of complexes of lecithin, lysolecithin, and A-I to occur. Further studies of the effect of lysolecithin on A-I are therefore necessary to understand the process of relipidation in mixtures of lecithin, lysolecithin, and A-I.
TABLE II

DISTRIBUTION OF A-I AND (14C)LECITHIN AFTER ULTRACENTRIFUGAL FRACTIONATION OF MIXTURES OF LECITHIN VESICLES AND A-I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I and (14C)lecithin*</th>
<th>Molar ratio of mixture**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10:1</td>
</tr>
<tr>
<td>d &lt; 1.063</td>
<td>A-I</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(14C)lecithin</td>
<td>84</td>
</tr>
<tr>
<td>d 1.063-1.21</td>
<td>A-I</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(14C)lecithin</td>
<td>6</td>
</tr>
<tr>
<td>d &gt; 1.21</td>
<td>A-I</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(14C)lecithin</td>
<td>10</td>
</tr>
</tbody>
</table>

*Percent of recovered A-I or (14C)lecithin.

**Molar ratio lecithin:A-I.
Ib. Relipidation of A-I with Lysolecithin: Ultracentrifugal Analysis

Lysolecithin micelles, formed by dissolving lysolecithin in water, were mixed with A-I in various molar ratios and incubated (1 hr, 23°C). The mixtures were fractionated by preparative ultracentrifugation. The distribution of A-I in the three density ranges \( d < 1.063 \), \( d = 1.063-1.21 \), and \( d > 1.21 \) is shown in Table III. At a low lysolecithin:A-I molar ratio, 34:1, the A-I sedimented at 1.21 g/ml as it did with no added lysolecithin. This material might have included some complexes of A-I and lysolecithin with densities greater than 1.21 g/ml. However, at a higher ratio, 170:1, some of the apolipoprotein was recovered in the \( d = 1.063-1.21 \) fraction. This is clear evidence that a lipid-protein complex, containing lysolecithin and A-I, formed during incubation at room temperature.

The results presented in Table III did not show by themselves whether any interaction took place between lysolecithin and A-I when mixed in a 34:1 molar ratio lysolecithin:A-I. Furthermore, observation of protein in both the \( d = 1.063-1.21 \) and \( d > 1.21 \) fractions after ultracentrifugation of a mixture with a molar ratio lysolecithin:A-I of 170:1, did not indicate whether two classes of complexes with different average densities were present or whether one class with an average density near 1.21 g/ml predominated.

More detailed results of ultracentrifugal fractionation of incubated mixtures of lysolecithin and A-I are presented in Table IV. As shown in this table, there was a substantially smaller proportion of A-I in the bottom fraction of a mixture of lysolecithin and A-I with a molar ratio lysolecithin:A-I of 34:1 than in the bottom
fraction after ultracentrifugation of A-I without added lysolecithin. This difference in the proportions of A-I in the bottom fractions was probably due to formation of complexes of lysolecithin and A-I which had densities less than free A-I. No A-I was seen floating into the top fraction, however, probably because insufficient lysolecithin was present in this mixture to form complexes with densities less than 1.21 g/ml. The observed differences in ultracentrifugal distribution were probably due to differences between the complexes and free A-I in their rates of sedimentation. Therefore it was probable that complexes of lysolecithin and A-I had formed in mixtures with a molar ratio lysolecithin:A-I of 34:1.

The detailed results of ultracentrifugal fractionation of a mixture of lysolecithin and A-I with a molar ratio lysolecithin:A-I of 170:1 do not show any A-I sedimenting to the bottom. It appears as if all of the A-I was incorporated into floating complexes. These results contrast those of David et al. (117) who observed free A-I in mixtures of lysolecithin and A-I which were analysed by electrophoresis. From the studies described here, it appears as if the A-I detected in each ultracentrifugal fraction had been incorporated into complexes, with densities near 1.21 g/ml, which did not completely reach ultracentrifugal equilibrium under the experimental conditions of ultracentrifugal force and time.

Studies by other workers have also shown that lysolecithin and A-I form complexes. The studies of Rosseneu et al. (84) on the enthalpy of mixing A-I and lysolecithin were interpreted as showing that A-I binds lysolecithin to a maximum molar ratio lysolecithin:A-I
of about 100:1. Equilibrium dialysis experiments by Haberland et al. (89) support this conclusion and suggest that the mechanism of binding lysolecithin to A-I does not involve the simple incorporation of A-I into lysolecithin micelles. These results are consistent with the preparative ultracentrifuge results reported above.
# TABLE III

DISTRIBUTION OF A-I AFTER ULTRACENTRIFUGAL FRACTIONATION OF MIXTURES OF LYSOLECITHIN AND A-I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Molar ratio of mixture&lt;sup&gt;**&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no lysolecithin</td>
<td>34:1</td>
</tr>
<tr>
<td>d &lt; 1.063</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d 1.063-1.21</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>d &gt; 1.21</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>*</sup>Percent of recovered A-I.

<sup>**</sup>Molar ratio lysolecithin:A-I.
TABLE IV
DETAILS OF A-I DISTRIBUTION AFTER ULTRACENTRIFUGATION OF MIXTURES OF LYSOLECITHIN AND A-I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I*</th>
<th>Molar ratio of mixture **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no lysolecithin</td>
<td>34:1</td>
</tr>
<tr>
<td>d 1.21-0-1***</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d 1.21 1-2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>d 1.21 2-3</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>d 1.21 3-4</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>d 1.21 4-5</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>d 1.21 5-6</td>
<td>62</td>
<td>37</td>
</tr>
</tbody>
</table>

*Percent of recovered A-I.
**Molar ratio lysolecithin:A-I.
***d 1.21 0-1 is the first 1 ml fraction from the top of a 6 ml centrifuge tube after ultracentrifugation (114,000 × g, 24 hr, 17°C) at 1.21 g/ml. Succeeding fractions are labelled analogously.
Ic. Effect of Lysolecithin on the Relipidation of A-I with Lecithin: Ultracentrifugal Analysis

For a preliminary investigation of the effect of lysolecithin on the interaction of lecithin and A-I, lecithin vesicles were added to mixtures of A-I and lysolecithin and the resulting mixture was fractionated by preparative ultracentrifugation. As previously described, A-I and lysolecithin were mixed in various molar ratios and incubated (15 min, 23°C). Vesicles prepared by the method of Huang (101) were subsequently added, and the mixture was further incubated (30 min, 23°C). Results of ultracentrifugal fractionation of these mixtures of lecithin, lysolecithin, and A-I are presented in Tables V and VI.

Table V shows the results of ultracentrifugal fractionation of mixtures of lecithin, lysolecithin, and A-I, with a molar ratio lysolecithin:A-I of 34:1. When small amounts of lecithin were present, almost all of the lecithin appeared in the d > 1.21 fraction. With increasing amounts of lecithin, a decreasing proportion was found in the d > 1.21 fraction, and an increasing proportion appeared in the HDL density range, d 1.063-1.21. With amounts of lecithin greater than that in a mixture of molar ratio lecithin:lysolecithin:A-I 34:34:1, both lecithin and A-I appeared in substantial quantities in the d < 1.063 fraction. These observations contrast those made on mixtures of A-I and lecithin (Table II) which showed no significant formation of lipid-protein complexes which floated in the HDL density range and which showed formation of complexes which floated in the d < 1.063 fraction only when large amounts of lecithin were present.
To investigate the effect of varying the amount of lysolecithin on the formation of complexes of A-I and lecithin, a similar study was made with mixtures having a molar ratio lysolecithin:A-I of 170:1 (Table VI). With small amounts of added lecithin, molar ratio lecithin:lysolecithin:A-I 17:170:1, most of the apolipoprotein and \(^{14}C\)lecithin was found in the d 1.063-1.21 fraction. In the absence of added lecithin, a mixture of lysolecithin and A-I with molar ratio lysolecithin:A-I 170:1 formed complexes which floated into the d 1.063-1.21 fraction (Table III). The small amount of lecithin in the 17:170:1 mixture was apparently incorporated into these preformed complexes of lysolecithin and A-I. The added lecithin apparently caused no dissociation of these complexes. The lecithin vesicles therefore probably were disrupted and the lecithin was distributed among the various complexes of lysolecithin and A-I.

Increasing the amount of lecithin in the final mixture led to an increase in the proportion of lecithin and A-I in the d 1.063-1.21 range. The proportion of lecithin observed in this density range was maximal when the mixture had a molar ratio lecithin:lysolecithin: A-I of 57:170:1. However, in a mixture containing a greater amount of lecithin, molar ratio 170:170:1, the proportion of lecithin in the d 1.063-1.21 range was less, and a larger relative amount of lecithin was observed in the d < 1.063 range. In mixtures with still larger amounts of lecithin, almost all of the A-I and lecithin was in the d < 1.063 fraction. These observations are similar to those made on mixtures of lecithin, lysolecithin and A-I with a molar ratio lysolecithin:A-I of 34:1. In both cases, when lecithin was added to
mixtures of lysolecithin and A-I in amounts greater than a molar ratio lecithin:lysolecithin of 1:1, low density complexes were formed which floated into the d < 1.063 fraction.

From the above, it was not possible to discern if more than one class of complexes was present in any mixture of lecithin, lysolecithin, and A-I. To gain insight into this question, detailed results of ultracentrifugal fractionation of incubated mixtures of lecithin, lysolecithin, and A-I with a molar ratio lecithin:lysolecithin:A-I of 17:170:1 are presented in Table VII. Both the A-I and (14C)lecithin decreased in concentration toward the bottom of the centrifuge tube. There was no indication that a dense sedimenting complex might be present. The complexes in the starting mixtures might have included subclasses, but, if so, they could not be distinguished by these preparative ultracentrifugation experiments.

Similar examination of the detailed results (not shown) of preparative ultracentrifugation of mixtures of lecithin, lysolecithin, and A-I with a molar ratio lysolecithin:A-I of 34:1 also suggests that no distinct subclasses of the lipid-protein complexes were formed. In these studies there were no complexes which sedimented to the bottom during ultracentrifugation at 1.21 g/ml. In addition, when the ratio of lecithin:A-I in each fraction was calculated, it was found that this ratio did not vary throughout the tube. Thus, it appeared that the complexes in each ultracentrifugal subfraction might have differed only in lysolecithin content if they differed at all in composition. More likely, the density of these complexes was near 1.21 g/ml and their observed ultracentrifugal distribution was
due to the ultracentrifugal conditions of force and time (114,000 × g, 24 hr) which did not allow them to come to isopycnic equilibrium in a solution with a background density of 1.21 g/ml.

These results are similar to those of Nichols et al. (77) who showed formation of complexes which floated into the d 1.063-1.21 fraction when A-I was incubated with lecithin vesicles containing lysolecithin. In that study, various molar ratios of lecithin:lysolecithin were investigated. Molar ratios lecithin:lysolecithin of 1:3, 1:1, and 3:1 were used while the molar ratio phospholipid:A-I was held constant at about 35:1. In all mixtures containing lysolecithin, formation of complexes which floated in the d 1.063-1.21 fraction was observed. Prior to these studies, the relipidation of A-I with lecithin and formation of complexes which could be isolated ultracentrifugally, had been observed only during sonication (60).
TABLE V

DISTRIBUTION OF A-I AND (\textsuperscript{14}C)LECITHIN AFTER ULTRACENTRIFUGAL FRACTIONATION OF MIXTURES OF LECITHIN VESICLES, LYSOLECITHIN, AND A-I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I and (\textsuperscript{14}C)lecithin*</th>
<th>Molar ratio of mixture**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3:34:1</td>
</tr>
<tr>
<td>d &lt; 1.063</td>
<td>A-I</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(\textsuperscript{14}C)lecithin</td>
<td>4</td>
</tr>
<tr>
<td>d 1.063-1.21</td>
<td>A-I</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(\textsuperscript{14}C)lecithin</td>
<td>15</td>
</tr>
<tr>
<td>d &gt; 1.21</td>
<td>A-I</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>(\textsuperscript{14}C)lecithin</td>
<td>81</td>
</tr>
</tbody>
</table>

*Percent of recovered A-I or (\textsuperscript{14}C)lecithin.

**Molar ratio lecithin:lysolecithin:A-I.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I and ((^{14})C)lecithin*</th>
<th>Molar ratio of mixture**</th>
</tr>
</thead>
<tbody>
<tr>
<td>d &lt; 1.063</td>
<td>A-I</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>((^{14})C)lecithin</td>
<td>1</td>
</tr>
<tr>
<td>d 1.063-1.21</td>
<td>A-I</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>((^{14})C)lecithin</td>
<td>65</td>
</tr>
<tr>
<td>d &gt; 1.21</td>
<td>A-I</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>((^{14})C)lecithin</td>
<td>34</td>
</tr>
</tbody>
</table>

*Percent of recovered A-I or (\(^{14}\)C)lecithin.
**Molar ratio lecithin:lysolecithin:A-I.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of A-I</th>
<th>Distribution of (^{14}\text{C})\text{lecithin}^*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>d 1.21 0-1**</td>
<td>66</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>d 1.21 1-2</td>
<td>17</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>d 1.21 2-3</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>d 1.21 3-4</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>d 1.21 4-5</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>d 1.21 5-6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Percent of recovered A-I or \(^{14}\text{C})\text{lecithin}.

** d 1.21 0-1 is the first 1 ml fraction from the top of a 6 ml centrifuge tube after ultracentrifugation (114,000 × g, 24 hr, 17°C) at 1.21 g/ml. Succeeding fractions are labelled analogously.
Id. Effect of Lysolecithin on Lecithin Vesicles in the Absence of A-I: Ultracentrifugal Analysis

In the previous section it was shown that lysolecithin interacted with A-I and lecithin and formed complexes of these components which floated in the \( d \leq 1.063 \) density range. In order to evaluate the effect of lysolecithin on the ultracentrifugal properties of lecithin vesicles in the absence of A-I, mixtures of lecithin and lysolecithin were subjected to preparative ultracentrifugation. Vesicles prepared by the method of Huang (101) were mixed with various amounts of lysolecithin and incubated (30 min, \( 23^\circ \mathrm{C} \)) before being subjected to preparative ultracentrifugation. The results of ultracentrifugal fractionation are presented in Table VIII which shows the distribution of \( ^{14}\text{C}\text{lecithin} \) among the fractions \( d < 1.063 \), \( d = 1.063-1.21 \), and \( d > 1.21 \). At low ratios of lecithin:lysolecithin, almost all of the \( ^{14}\text{C}\text{lecithin} \) was found in the \( d = 1.063-1.21 \) fraction. Lysolecithin alone would also have been found in this density range. With increasing amounts of lecithin, a decreasing proportion of \( ^{14}\text{C}\text{lecithin} \) was found in the \( d = 1.063-1.21 \) fraction, and an increasing proportion was found in the \( d < 1.063 \) fraction. Lecithin without added lysolecithin was found in the \( d < 1.063 \) fraction.

These results are consistent with reported densities of 1.085 g/ml for lysolecithin (121) and 1.01 g/ml for lecithin vesicles (101). Mixtures of lecithin and lysolecithin have been reported to form mixed micelles (1) and it appears that the densities of these mixed micelles are intermediate between the densities of isolated lecithin and lysolecithin. Therefore, complexes occurring in mixtures predominantly
containing lysolecithin floated in the d 1.063-1.21 fraction; and complexes occurring in mixtures predominantly containing lecithin floated in the d < 1.063 fraction.
TABLE VIII

DISTRIBUTION OF $^{14}$CLECITHIN AFTER ULTRACENTRIFUGAL FRACTION OF MIXTURES OF LECITHIN AND LYSOLECITHIN

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Distribution of $^{14}$Clecithin*</th>
<th>Molar ratio of mixture**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no lysolecithin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>3:1</td>
</tr>
<tr>
<td>d &lt; 1.063</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>d 1.063-1.21</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>d &gt; 1.21</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Percent of recovered $^{14}$Clecithin.

**Molar ratio lecithin:lysolecithin.
Ie. Summary of Ultracentrifugal Results: Relipidation of A-I with Lecithin and Lysolecithin

A summary of the results of preparative ultracentrifugation of mixtures of A-I with lecithin, lysolecithin, or both is presented in Table IX. Except for mixtures of A-I and lecithin, all of the mixtures of lecithin, lysolecithin, and A-I formed complexes with densities intermediate between the densities of the components. Mixtures of lecithin and A-I apparently formed complexes either with densities near those of isolated A-I or with densities near those of isolated lecithin. Complexes with intermediate densities were not observed in mixtures of lecithin and A-I in the absence of lysolecithin. In the presence of lysolecithin, on the other hand, complexes were observed in all three density ranges. As discussed previously, there was no indication that more than one distinct class of complexes was present in these mixtures of lecithin, lysolecithin, and A-I. Thus it appeared that lysolecithin affected the A-I, the lecithin, or both in such a way that complexes of various intermediate densities could readily form.

Lysolecithin facilitated the formation of complexes which floated in the d 1.063-1.21 fraction since such complexes were observed only in mixtures containing lysolecithin. However, lysolecithin itself floated into this density range as did complexes of lysolecithin and A-I or lecithin and lysolecithin. It might therefore be surmised that the A-I and (14C)lecithin which were found together in the d 1.063-1.21 fraction were in distinct particles and that there was no formation of mixed micelles of lecithin, lysolecithin, and A-I. Careful examination of the data presented in the preceding sections, however, shows both
that A-I affected the distribution of $^{14}$C-lecithin and that lecithin affected the distribution of A-I. For example, in a mixture of lecithin and lysolecithin with a molar ratio lecithin:lysolecithin of 1:1, only 9% of the $^{14}$C-lecithin was found in the d 1.063-1.21 fraction. However, in a mixture of lecithin, lysolecithin, and A-I, with a molar ratio lecithin:lysolecithin:A-I of 34:34:1, although the lecithin:lysolecithin ratio was still 1:1, 64% of the $^{14}$C-lecithin was present in the d 1.063-1.21 fraction. In a similar way it can be noted that in a mixture containing only lysolecithin and A-I with a molar ratio lysolecithin:A-I of 34:1, 100% of the A-I was found in the d < 1.21 fraction, while in a mixture with a molar ratio lecithin:lysolecithin:A-I of 34:34:1, 58% of the A-I was found in the d 1.063-1.21 fraction.

These observations, as well as the observation that all of the subfractions of the mixture with a molar ratio lecithin:lysolecithin:A-I 17:170:1 (Table VII) contained the same molar ratio lecithin:A-I, lead to the conclusion that mixtures of lecithin, lysolecithin, and A-I formed mixed micelles containing all three components. Since it also appears that the average densities of these complexes are intermediate between the densities of their components, and that no distinct groups of complexes could be separated by preparative ultracentrifugation, it is probable that the composition of any mixed micelle approximates the average composition of the mixture in which it was formed. This conclusion will be used, along with information about the shape of mixed micelles of lecithin, lysolecithin, and A-I, to draw conclusions about
the probable molecular weights of complexes in these mixtures (See Appendix 3).

Several questions regarding the nature of the complexes found in mixtures of lecithin, lysolecithin, and A-I, and the process by which they were formed can be asked at this point: (1) is there more than one class of complexes in mixtures containing lecithin, lysolecithin, and A-I? (2) what is the average molecular weight of the complexes in each mixture? (3) since relipidated apolipoproteins have been reported to have a variety of possible shapes (66), what are the shapes of the complexes in each mixture of lecithin, lysolecithin, and A-I? (4) what did lysolecithin do to the A-I or the lecithin vesicles to facilitate formation of complexes containing both lecithin and A-I which floated in the d 1.063-1.21 range? (5) what is the minimum amount of lysolecithin that is needed for formation of the complexes which float in the d 1.063-1.21 range, and is this least amount related to the amount of A-I or the amount of lecithin present in the mixture, or is it related to the ratio of lecithin:A-I?

Answers to these questions were sought by a variety of means. Gradient gel electrophoresis was used to estimate the molecular weight and the number of subclasses of complexes. The shapes, and molecular weights of the lipid-protein complexes present in mixtures of lecithin, lysolecithin, and A-I were assessed by electron microscopy. The effect of lysolecithin on A-I was assessed by spectroscopic means: fluorescence and circular dichroism. The results of these studies are presented in the following sections.
### TABLE IX

**SUMMARY OF RESULTS OF ULTRACENTRIFUGAL FRACTIONATIONS OF MIXTURES OF LECITHIN, LYSOLECITHIN, AND A-I**

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Results of fractionations **&lt;br&gt;**Ultracentrifugal fraction</th>
<th>d &lt; 1.063</th>
<th>d 1.063-1.21</th>
<th>d &gt; 1.21</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I (no added lipid)</td>
<td>A</td>
<td>A</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Lysolecithin (no added A-I)</td>
<td>A</td>
<td>P</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Lecithin (no added A-I)</td>
<td>P</td>
<td>A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Lysolecithin plus lecithin</td>
<td>P</td>
<td>P</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>A-I plus lysolecithin</td>
<td>A</td>
<td>P</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>A-I plus lecithin</td>
<td>P</td>
<td>A</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>A-I plus lysolecithin plus lecithin</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td></td>
</tr>
</tbody>
</table>

*Results of fractionation of mixtures at all mole ratios of lecithin, lysolecithin, and A-I are presented.

**P indicates the presence and A indicates the absence of significant amounts (see text) of A-I or phospholipid in a particular fraction for any mixture of the indicated composition.
If. Relipidation of A-I with Lecithin: Results of Gradient Gel Electrophoresis

In order to estimate the molecular weight and the number of subclasses of complexes formed by incubating mixtures of A-I and lecithin vesicles, gradient gel electrophoresis was performed. Lecithin vesicles, prepared by the method of Batzri et al. (103) were mixed with A-I in various molar ratios. The resulting mixtures were then incubated (1 hr, 23°C) and subsequently subjected to electrophoresis on a polyacrylamide gradient gel. Figures 17 and 18 show the results of two such electrophoresis runs. After incubating vesicles with various amounts of A-I (molar ratios lecithin:A-I 18:1, 37:1, and 74:1), complexes were observed on the gradient gel in two distinctly different molecular weight ranges (Figure 17). In each lane, bands were observed in a very high molecular weight range, > 900,000, and triangular streaks were observed with a minimum molecular weight near 60,000. Figure 18 shows the results of subjecting A-I with no added lecithin to gradient gel electrophoresis. In this case, only a triangular streak with a similar minimum molecular weight near 60,000 could be seen. It therefore appears that the triangular streaks observed in the incubated mixtures of A-I and lecithin vesicles were A-I combined with little or no lecithin.

To compare the above results of incubating lecithin and A-I with the results of cosonicating A-I and lecithin, a mixture of lecithin and A-I, molar ratio lecithin:A-I 37:1 was subjected to sonication (3 x 1 min, 37°C) using a 60 watt ultrasonic disintegrator (No. 7100, MSE, London, England) operating at 18-20 kHz. After sonication, the
A mixture was briefly ultracentrifuged (114,000 × g, 1 hr, 15°C) to remove any large aggregates and any titanium from the disintegrator probe. The resulting sonicated mixture was subjected to electrophoresis on a gradient gel simultaneously with incubated mixtures and reference proteins (Figure 17, Lane 4). Gradient gel electrophoresis showed that sonication produced several classes of complexes which were not the same as those produced by incubation. In addition to a triangular streak with minimum molecular weight near 60,000, two major bands were observed in the lane which contained the sonicated mixture. One band had a range of molecular weights from 100,000-160,000, and the other had a range of molecular weights 260,000-400,000.

Comparison of the results of gradient gel electrophoresis with the results of preparative ultracentrifugation of incubated mixtures of lecithin and A-I (Table II), suggests that the triangular streak should be identified with the material in the d > 1.21 fraction. In both cases, these complexes probably consisted primarily of A-I with little lecithin. The complexes with molecular weights > 900,000, on the other hand, probably corresponded to the complexes in the d < 1.063 fraction. The molecular weight of a single bilayer vesicle is about 2,000,000 (101). Therefore, if the molecular weight calibration of the gradient gel results is accurate, the very high molecular weight complexes are not simply vesicles with attached A-I, but must have some other structure.

Several distinct bands with molecular weights > 900,000 were observed by gradient gel electrophoresis of incubated mixtures of
lecithin and A-I. This indicates that the d < 1.063 fraction obtained by preparative ultracentrifugation of such an incubated mixture probably contained several distinct subclasses. The triangular streak observed in these incubated mixtures of lecithin and A-I is also an indication of heterogeneity. Thus, the lipid protein complexes in the d > 1.21 fraction, obtained by preparative ultracentrifugation of a mixture of lecithin and A-I, probably contained many subclasses with different molecular weights. The minimum molecular weight of the subspecies in the triangular streak, 60,000, was near the molecular weight of a dimer of A-I (57,000). This is consistent with the identification of this streak as a complex of A-I with little lecithin. A-I has been shown to aggregate (118), and the species, observed in gradient gel electrophoresis of A-I with no added lecithin, with molecular weights greater than 60,000 might correspond to multimers of this apolipoprotein.

Several investigators have reported that A-I is at least a dimer in aqueous solution (85,118,122), and this is also consistent with the gradient gel electrophoresis observations reported here.

In the sonicated sample, the complexes with a range of molecular weight 260,000-400,000 migrated to a position similar to the position where abnormal high molecular weight HDL from lecithin:cholesterol acyltransferase deficient patients migrate (122). These abnormal HDL have been shown to stack as rouleaux on electron microscopy (36). In addition, particles found in sonicated mixtures of lecithin and A-I have been shown to stack as rouleaux on electron microscopy (63). Thus it is probable that the 260,000-400,000 molecular weight particles are the particles which have been observed to stack as rouleaux.
The 100,000-160,000 molecular weight complexes in the sonicated mixture of lecithin and A-I cannot be readily identified. However, as will be shown later, they were similar in molecular weight to complexes of lecithin and A-II (Section IIb), which appeared to be small spheres with radii of about 4-6 nm. If the complexes of lecithin and A-I had had the same size, 4-6 nm, they would have been similar in size, shape, and composition to the low molecular weight subspecies of abnormal HDL seen in lecithin:cholesterol acyltransferase deficient patients (63).

The bands seen on electrophoresis of the sonicated mixture of lecithin and A-I were associated with particles with molecular weights between 100,000 and 400,000 which probably would have been found in the d 1.063-1.21 fraction (60). The absence of bands in this molecular weight range on electrophoresis of incubated mixtures of lecithin and A-I, therefore, probably corresponded to the absence of lipid-protein complexes which would float in the d 1.063-1.21 range. As will be shown in other sections, the presence of lipid-protein complexes with molecular weights between 100,000 and 400,000 is correlated with the presence of rouleaux in electron micrographs of these mixtures. The presence of rouleaux, in turn, is correlated with the presence of complexes which float in the d 1.063-1.21 fraction.
FIGURE 17 Gradient gel electrophoresis of mixtures of lecithin vesicles and A-I. Lane 1, reference proteins. Lanes 2-4, incubated mixtures of lecithin and A-I: lane 2, molar ratio lecithin:A-I 18:1; lane 3, molar ratio 37:1; lane 4, molar ratio 74:1. Lane 5, sonicated (3 × 1 min) mixture of lecithin and A-I, molar ratio 37:1. Lane 6, reference proteins.
Thyroglobulin (MW 670,000)
Apoferritin (MW 480,000)
Catatase (MW 235,000)
Albumin (MW 77,000)
Ovalbumin (MW 45,000)

Figure 17
FIGURE 18  Gradient gel electrophoresis of mixtures of lecithin vesicles and A-I. Lane 1, reference proteins. Lane 2, incubated mixture of lecithin and A-I, molar ratio lecithin:A-I 37:1. Lane 3, A-I with no added lecithin.
Thyroglobulin (MW 670,000)
Apoferitin (MW 480,000)
Catalase (MW 235,000)
Albumin (MW 77,000)
Ovalbumin (MW 45,000)

Figure 18
Ig. Structure of Complexes of A-I and Lecithin: Electron Microscopy

Approximate molecular weights, number of subclasses, and shapes of complexes which were formed by incubating lecithin with A-I, were investigated by electron microscopy. Vesicles, prepared by the method of Batzri et al. (103) were mixed with A-I in various molar ratios. After incubation (1 hr, 23°C), aliquots of the mixtures were mixed with 4% Na-phosphotungstate and examined immediately in the electron microscope.

Figure 19 shows electron micrographs of particles seen in such mixtures of A-I and lecithin. In the absence of lecithin (Figure 19(a)), the A-I appeared as fibers and aggregates of various sizes and shapes. In a mixture with a molar ratio lecithin:A-I of 20:1 (Figure 19(b)), round particles, 5-10 nm in diameter, and filaments were seen. In a mixture with a molar ratio of 50:1, (Figure 19(c)), larger particles, 10-20 nm in major dimension, were observed; while in a mixture with a molar ratio of 100:1 (Figure 19(d)), most of the particles were greater than 20 nm across. The particles in both the 50:1 and 100:1 mixtures were irregularly shaped. In mixtures with a molar ratio of 200:1 (Figure 19(e)), vesicular particles were almost exclusively present.

The variety of shapes of the complexes seen in mixtures of A-I and lecithin was unexpected. Micrographs were therefore made of many mixtures and those presented are representative of the repeated studies. A-I had previously been reported not to bind appreciably with lecithin without sonication (34, 78). The micrographs in Figure 19, however, show definite variations in structure of particles present in mixtures of
lecithin and A-I. These changes were repeatable and depended on the molar ratio lecithin:A-I in the incubation mixtures. They suggested that an interaction between lecithin and A-I took place during incubation.

The results of preparative ultracentrifugation and gradient gel electrophoresis of mixtures of A-I and lecithin vesicles showed that both large low-density particles and small high-density particles were present in these mixtures. Therefore only two classes of particles, one, resembling isolated vesicles, and another, resembling isolated A-I were expected to be seen by electron microscopy. The results of electron microscopy, however, showed continuous changes in the size and shape of the particles formed by incubating mixtures of lecithin and A-I. While it may be possible to divide the particles seen in the micrographs in Figure 19 into two classes, small round particles about 10 nm in diameter, and large amorphous particles about 20-25 nm in diameter, two distinctly different groups of particles are not evident in the micrographs.

The apparent differences in the results of electron microscopy and the results of preparative ultracentrifugation or gradient gel electrophoresis may be due to modification of the complexes during preparation for electron microscopy (negative staining and dehydration). On the other hand, it may be that the techniques of preparative ultracentrifugation and gradient gel electrophoresis caused dissociation of the complexes. During preparative ultracentrifugation, complexes in a mixture of lecithin and A-I were exposed to high concentrations of NaBr which might have cancelled any ionic forces holding them
together. In the course of gradient gel electrophoresis, complexes in such a mixture were exposed to the large surface area of the polyacrylamide gel matrix, and this exposure might have caused dissociation. In any case, the results of electron microscopy are consistent with the formation of complexes in incubated mixtures of lecithin and A-I.
FIGURE 19  Electron micrographs of mixtures of lecithin vesicles and A-I. Vesicles and A-I were mixed, incubated (30 min, 23°C), negatively stained with Na-phosphotung state, and examined immediately. (a), A-I with no added lecithin. (b)-(e), mixtures of lecithin and A-I: (b), molar ratio lecithin:A-I 20:1; (c), molar ratio 50:1; (d), molar ratio 100:1; (e), molar ratio 200:1.
Ih. Effect of Lysolecithin on the Structure of Complexes of A-I and Lecithin: Electron Microscopy

Electron microscopy was also used to investigate the number of subclasses, shapes, and molecular weights of complexes in mixtures of lecithin, lysolecithin, and A-I. Lysolecithin was mixed with various amounts of A-I and incubated (15 min, 23°C). Subsequently, lecithin vesicles, prepared by the method of Huang (101) were added. The mixture was further incubated (45 min, 23°C), and an aliquot was mixed with 4% Na-phosphotungstate and examined immediately by electron microscopy.

Figure 20 shows the results of electron microscopy of mixtures of lecithin, lysolecithin, and A-I with a molar ratio lysolecithin:A-I of 34:1. With no added lecithin, small round or ellipsoidal particles, 5-10 nm in diameter, as well as some filaments could be seen (Figure 20(a)). In a mixture with a molar ratio lecithin:lysolecithin:A-I of 34:34:1, similar round particles and filaments were also seen (Figure 20(b)). In a mixture with a molar ratio of 170:34:1 (Figure 20(c)), three types of particles, round particles 10-15 nm in diameter, hexagonally packed particles 25-30 nm from center to center, and elongated particles, 5 nm thick and 35-40 nm in length, were observed. In a mixture with a molar ratio of 340:34:1 (Figure 20(d)), rouleaux about 30 nm wide with a 5 nm repeat distance predominated, although some round particles 25-30 nm in diameter could also be observed. In a mixture with a molar ratio of 640:34:1, large hexagonally packed particles with a 25-30 nm center-to-center distance predominated, although there were a few rouleaux present (Figure 20(e)).
Different particle shapes, depending on the amount of lecithin, can be noted in the micrographs of mixtures of lecithin, lysolecithin, and A-I with a molar ratio lysolecithin:A-I of 34:1. Filaments, small round or ellipsoidal particles 5-10 nm in diameter, rouleaux with a 5 nm repeat distance, large round particles 25-30 nm in diameter, and hexagonally packed particles were observed in this order as the amount of lecithin in the mixtures was increased. Table X lists these particles and the mixtures in which they were found. By comparing this table with Table V, which shows the results of ultracentrifugal fractionation of similar mixtures, it was possible to correlate the particles seen by electron microscopy with the complexes separated by preparative ultracentrifugation. To make this correlation, it was assumed that the particles seen by electron microscopy would have been found in the fraction containing the majority of the complexes. Thus, filaments and small round or ellipsoidal particles 5-10 nm in diameter were probably present in the \( d > 1.21 \) fraction; rouleaux and small round particles 5-15 nm in diameter were probably present in the \( d 1.063-1.21 \) fraction; and large round particles 25-30 nm in diameter as well as hexagonally packed particles were probably present in the \( d < 1.063 \) fraction.

These observations correlating the shapes with the densities of complexes of apolipoproteins and phospholipids are similar to observations by other investigators (63, 66). In particular, rouleaux have usually been observed in the \( d 1.063-1.21 \) fraction.

To evaluate the effect of varying the molar ratio of lysolecithin: A-I on the shapes of complexes formed in mixtures of lecithin, lysolecithin, and A-I, other mixtures with different amounts of lysolecithin
were prepared and examined by electron microscopy. Figure 21 shows the results of electron microscopy of mixtures of lecithin, lysolecithin, and A-I with a molar ratio lysolecithin:A-I of 170:1. With no added lecithin (Figure 21(a)), round particles with a diameter of 4-6 nm predominated. No ellipsoidal particles were observed in this mixture in contrast to their presence in the mixture of lysolecithin and A-I with a molar ratio lysolecithin:A-I of 34:1 (Figure 20(a)). In micrographs of mixtures of molar ratio lecithin:lysolecithin:A-I of 17:170:1, filaments and round particles 10-20 nm in diameter could be seen (Figure 21(b)). In mixtures with a molar ratio of 170:170:1 (Figure 21(c)), round particles, 10-20 nm in diameter and hexagonally packed particles about 30 nm from center to center could be seen. A few distinct vesicles could be seen among the hexagonally packed particles. Figure 21(d) shows an electron micrograph of a mixture with a molar ratio of 1000:170:1. Dispersed vesicles, 25-30 nm in diameter, hexagonally packed particles with a 25-30 nm center-to-center distance, and some rouleaux with a 5 nm repeat distance were apparently present in such mixtures.

Similar to the results on mixtures with a molar ratio lysolecithin:A-I of 34:1, different particle shapes, depending on the amount of lecithin, could be noted in micrographs of mixtures of lecithin, lysolecithin, and A-I with a molar ratio lysolecithin:A-I of 170:1. Filaments, small round particles 4-6 nm in diameter, larger round particles 10-20 nm in diameter, rouleaux, large round particles 25-30 nm in diameter, and hexagonally packed particles were observed in this order with increasing proportions of lecithin in the mixtures.
Table XI lists these particles and the mixtures in which they were found. By comparing this table with Table VI, which shows the results of preparative ultracentrifugal fractionation of similar mixtures, it is possible to correlate the particles observed by electron microscopy with the complexes separated by preparative ultracentrifugation, by assuming that the particles observed by electron microscopy would have been found in the ultracentrifugal fraction containing the largest amount of material.

The comparison gives results similar to those made on mixtures with a molar ratio lysolecithin:A-I of 34:1. The d > 1.21 fraction probably contained the complexes which appeared on electron microscopy as filaments and small round particles 4-20 nm in diameter. The d 1.063-1.21 fraction probably contained the complexes which appeared as small round particles 4-20 nm in diameter and rouleaux with a 5 nm repeat distance. The d < 1.063 fraction probably contained the complexes which appeared as large round particles 25-30 nm in diameter and hexagonally packed particles.

In both series of mixtures with ratios lysolecithin:A-I of 34:1 and 170:1, it appeared to be possible to directly correlate the kinds of particles observed by electron microscopy with the complexes separated by preparative ultracentrifugation. Therefore the particles observed by electron microscopy were probably representative of the particles in the mixtures of lecithin, lysolecithin, and A-I. Electron microscopy, in addition, showed that several classes of particles were present in any mixture, and that, in general, these classes were distinct from the classes of particles seen in micrographs of the
starting vesicles and A-I. Clear dependence of the occurrence of a particular class of particles on the amount of lecithin in a mixture of lecithin, lysolecithin, and A-I was also observed. It therefore seems possible to generalize that the shape of complexes in a particular mixture of lecithin, lysolecithin, and A-I is directly related to the ratio of these components.

Rouleaux are easily identified shapes; and to better understand the compositional requirements for formation of particles of a particular shape, an attempt was made to find mixtures with ratios of lecithin:lysolecithin:A-I which would be associated with the presence of rouleaux. Figure 22 shows micrographs of mixtures with a molar ratio lecithin:lysolecithin of 10:1 containing various amounts of A-I. With molar ratios lecithin:lysolecithin:A-I of 113:11:1, 340:34:1, and 1000:100:1 (Figures 22(b), (c), and (d), respectively), rouleaux were clearly present. In mixtures with a molar ratio of 8:0.8:1 (Figure 22(a)), however, only round particles about 10 nm in diameter could be found. These results supported the hypothesis that the shape of particles in a mixture of lecithin:lysolecithin:A-I depended on the ratios of these components. This dependence, however, appeared to be complex since rouleaux were only found in those mixtures where both the total lipid:A-I and the lecithin:lysolecithin ratios were in a certain range.
FIGURE 20  Electron micrographs of mixtures of lecithin vesicles, lysolecithin, and A-I. Lysolecithin and A-I were mixed and incubated (15 min, 23°C). Vesicles were subsequently added and the mixture further incubated (15 min, 23°C). Samples were negatively stained with Na-phosphotungstate and examined immediately. (a), molar ratio lecithin:lysolecithin: A-I 0:34:1; (b), molar ratio 34:34:1; (c), molar ratio 170:34:1; (d), molar ratio 340:34:1; (e), molar ratio 640:34:1. In all cases the molar ratio lysolecithin:A-I was 34:1.
TABLE X

SUMMARY OF RESULTS OF ELECTRON MICROSCOPY OF MIXTURES OF LECITHIN, LYSOLECITHIN, AND A-I (MOLAR RATIO LYSOLECITHIN:A-I 34:1)

<table>
<thead>
<tr>
<th>Mixture*</th>
<th>Results of electron microscopy**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particles observed</td>
</tr>
<tr>
<td></td>
<td>filaments small, round</td>
</tr>
<tr>
<td>0:34:1</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>(5-10 nm)</td>
</tr>
<tr>
<td>34:34:1</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>(5-10 nm)</td>
</tr>
<tr>
<td>170:34:1</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>(10-15 nm)</td>
</tr>
<tr>
<td>340:34:1</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>(30-40 nm)</td>
</tr>
<tr>
<td>640:34:1</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>(30-60 nm)</td>
</tr>
</tbody>
</table>

*Molar ratio lecithin:lysolecithin:A-I.

**P indicates the presence and A indicates the absence of a particular species of the particle. Numbers in parentheses are measurements of the largest dimension of the particles.

***Rouleaux having a 5 nm repeat distance.
FIGURE 21 Electron micrographs of mixtures of lecithin vesicles, lysolecithin, and A-I. Lysolecithin and A-I were mixed and incubated (15 min, 23°C). Vesicles were subsequently added and the mixture was further incubated (15 min, 23°C). Samples were negatively stained with Na-phosphotungstate and examined immediately. (a), molar ratio lecithin:lysolecithin:A-I 0:170:1; (b), molar ratio 17:170:1; (c), molar ratio 170:170:1; (d), molar ratio 1000:170:1. In all cases the molar ratio lysolecithin:A-I was 170:1.
Figure 21
<table>
<thead>
<tr>
<th>Mixture *</th>
<th>Results of electron microscopy **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>filaments</td>
</tr>
<tr>
<td>0:170:1</td>
<td>A</td>
</tr>
<tr>
<td>17:170:1</td>
<td>P</td>
</tr>
<tr>
<td>170:170:1</td>
<td>A</td>
</tr>
<tr>
<td>1000:170:1</td>
<td>A</td>
</tr>
</tbody>
</table>

* Molar ratio lecithin:lysolecithin:A-I.

** P indicates the presence and A indicates the absence of a particular species of particle. Numbers in parentheses are measurements of the largest dimension of the particles.

*** Rouleaux having a 5 nm repeat distance.
FIGURE 22 Electron micrographs of mixtures of lecithin vesicles, lysolecithin, and A-I. Lysolecithin and A-I were mixed and incubated (15 min, 23°C). Vesicles were subsequently added and the mixture was further incubated (15 min, 23°C). Samples were negatively stained with Na-phosphotungstate and examined immediately. (a), molar ratio lecithin:lysolecithin:A-I 8:0.8:1; (b), molar ratio 113:11:1; (c), molar ratio 340:34:1; (d), molar ratio 1000:100:1. In all cases, the molar ratio lecithin:lysolecithin was 10:1.
Figure 22
II. Structure of Mixed Micelles of Lecithin and Lysolecithin: Electron Microscopy

In the preceding section it was shown that the shapes of particles in mixtures of lecithin, lysolecithin, and A-I depended on both the molar ratios of lecithin:lysolecithin and lysolecithin:A-I. In order to independently study the shapes of particles in mixtures of lecithin and lysolecithin, in the absence of A-I, vesicles were prepared by the method of Huang (101), mixed with various amounts of lysolecithin and incubated (1 hr, 23°C). Aliquots were mixed with 4% Na-phosphotungstate (1:1 by volume), and examined immediately by electron microscopy.

Figure 23 shows the particles seen in such mixtures. Lysolecithin with no added lecithin (Figure 23(a)) appeared as small round particles about 6 nm in diameter. This diameter corresponds to that of a sphere of lysolecithin with a molecular weight of about 90,000, in good agreement with previous reports on the size of lysolecithin micelles (121). In a mixture of lecithin and lysolecithin with a molar ratio lecithin:lysolecithin of 1:10 (Figure 23(b)), slightly larger, ellipsoidal particles which aligned in rows could be seen. In a mixture with a molar ratio of 1:1, round particles about 10 nm in diameter were present as well as some myelin figures (Figure 23(c)). In some micrographs (not shown) of mixtures with this molar ratio, myelin figures which were not continuous, but which looked like pieces of cut onion, could be seen. In a mixture with a molar ratio of 10:1, vesicles and aggregates with irregular myelin forms were present (Figure 23(d)); while, with no added lysolecithin, only vesicles and regular myelin forms could be found (Figure 23(e)).
The mixture with a molar ratio lecithin:lysolecithin of 10:1 showed little tendency to form particles smaller than vesicles; while in the mixtures with more lysolecithin, smaller particles could generally be found. The presence of lysolecithin in amounts great enough to disrupt the regular bilayer structure of lecithin vesicles might be the reason only small round particles, and few rouleaux were found in the mixtures of lecithin, lysolecithin and A-I with a molar ratio lysolecithin:A-I of 170:1 and a molar ratio lecithin:lysolecithin of less than 5:1 (Table XI).
FIGURE 23 Electron micrographs of mixtures of lecithin vesicles and lysolecithin. Vesicles and lysolecithin were mixed and incubated (30 min, 23°C). Samples were negatively stained with Na-phosphotungstate and examined immediately. (a), lysolecithin with no added lecithin. (b)-(c), mixtures of lecithin and lysolecithin: (b), molar ratio lecithin:lysolecithin 1:10; (c), molar ratio 1:1; (d), molar ratio 10:1. (e), lecithin with no added lysolecithin.
Figure 23
Summary of Electron Microscopy; Use of a 3-Component Diagram

In the studies presented in the previous sections, A-I was incubated with various amounts of lecithin, lysolecithin, or both lecithin and lysolecithin. Complexes with various shapes were observed in these mixtures by electron microscopy. The shapes of these complexes correlated well with the results of preparative ultracentrifugation of similar mixtures. As previously described, the shapes of particles present in a particular mixture of lecithin, lysolecithin, and A-I depended in a complex fashion on the ratios of the components. Both the ratios lecithin:lysolecithin and lysolecithin:A-I affected the shapes of complexes formed in any mixture. In order to summarize the results of electron microscopy it was necessary to consider the relative proportions of all components in a particular mixture. The triangular diagram, Figure 24, was therefore constructed. Its use was based on a consideration of the work of Small (1).

The summary diagram, Figure 24, was constructed by the following method. The proportions of components in mixtures of lecithin, lysolecithin, and A-I were expressed as weight percent. Each mixture of lecithin, lysolecithin, and A-I was then located as a point on the diagram, considering the vertices of the triangle to represent samples containing only one component. Lines were drawn, connecting specific points, enclosing regions which represented mixtures containing particular kinds of complexes.

Several regions could be identified on the 3-component diagram (Figure 24). These regions corresponded to mixtures containing the same kinds of particles. Region I corresponded to mixtures
containing vesicles, myelin figures, or hexagonally packed particles with a 25-30 nm center-to-center distance. Vesicles, hexagonally packed particles, and myelin figures tended to appear together and were considered to be varieties of the same class of lamellar lecithin particles because myelin figures can be changed into vesicles by sonic dispersion (101). Some micrographs of concentrated solutions of vesicles showed them to be hexagonally packed in a way similar to the particles which were hexagonally packed in the mixtures of lecithin, lysolecithin, and A-I. These particles, vesicles, myelin figures, and hexagonally packed particles with a 25-30 nm center-to-center distance will collectively be referred to as vesicles. Region II corresponded to mixtures containing vesicles and particles which appeared as rouleaux with a 5 nm repeat distance. Region III corresponded to mixtures containing vesicles and small particles 10-20 nm in diameter. Mixtures of lecithin and A-I with no added lysolecithin were included in region III on the basis of the gradient gel electrophoresis results as well as the results of electron microscopy. Region IV corresponded to mixtures which contained particles appearing as rouleaux with a 5 nm repeat distance and small particles 10-20 nm in diameter. Region V corresponded to mixtures exhibiting only small particles 6-20 nm in diameter; and region VI corresponded to mixtures showing filaments and small particles 10-20 nm in diameter.

This diagram shows that particular particles were consistently observed in micrographs of mixtures with similar compositions. Points corresponding to mixtures which contained particles appearing as rouleaux or other specific shapes, occurred in particular regions and
were not haphazardly scattered on the diagram. In addition, regions corresponding to mixtures containing two kinds of particles such as filaments and small spheres appeared to be surrounded by the region representing mixtures containing only small spheres. Each region was apparently continuous within certain compositional ranges. These observations attest to the repeatability of the results of electron microscopy and support the conclusion that the particles observed had reached equilibrium in the mixtures investigated. Thus, this diagram should allow prediction of the shapes of particles in mixtures of known composition which had not been previously examined.

It is interesting to note that Region III in Figure 24, which represents mixtures of lecithin and A-I with no added lysolecithin, occurs next to Region IV which represents mixtures of lecithin, lysolecithin, and A-I which contained particles which appeared as rouleaux. Mixtures represented by points within Region III would probably appear as rouleaux if sonicated (63). Thus, the earlier statement, that regions representing particular particles are apparently continuous, would still be true if the 3-component diagram, Figure 24, were modified by the inclusion of sonicated mixtures. In this representation, the effect of sonication would be to simply remove the line between regions III and IV.

Three additional points, calculated from the work of Nichols et al (78), are also shown in Figure 24. The mixture corresponding to the point falling within the region characteristic of mixtures showing filaments and small round particles (Region VI) showed round particles in the
published micrograph. Electron micrographs of the other two mixtures, also indicated on Figure 24, showed small spheres and rouleaux. Although these points do not fall within the marked boundary corresponding to the boundary compositions of mixtures with rouleaux, they fall in a region for which there were no micrographs made in the course of this thesis. They therefore agree with the other results summarized in Figure 24 and suggest simply, that the boundary marking the region corresponding to mixtures which contain rouleaux should be redrawn as shown.

The 3-component diagram in Figure 24 resembles a section of a quaternary phase diagram of mixtures of lecithin, lysolecithin, A-I and water. This diagram would correspond to such a section if the weight percent of water had been kept constant. The weight percent of the water in the starting mixtures, however, was not kept constant, although it was consistently approximately 99.8 ± 0.1%. Further, the particles summarized in Figure 24 were actually seen dehydrated and negatively stained on electron microscope grids where the water content was virtually zero. However, the good correlation between particle shape and the ultracentrifugal fraction containing most of the complexes suggests that the dehydration occurring in the course of preparation for electron microscopy did not alter any qualitative properties of the mixtures of lecithin, lysolecithin, and A-I. One may therefore come to the cautious conclusion that Figure 24 approximates a section of a phase diagram of lecithin, lysolecithin, A-I, and water.

Two other observations may be made in support of the hypothesis that Figure 24 represents part of such a phase diagram. The first is
that the particles shown were repeatably observed in each mixture, and that there was a consistent interrelation between composition of the mixtures and the shapes of particles that they contained. Thus it is probable that the particles occurring together were in equilibrium as required for a phase diagram. Secondly, the Gibbs' phase rule was not violated by this hypothesis. No point on the phase diagram shows the simultaneous presence of more than three different classes of particles as required by the Gibbs' phase rule for three-component mixtures with temperature and pressure variations.

In conclusion, it appears that a description of the particles in incubated mixtures of lecithin, lysolecithin, and A-I can be made using an equilibrium phase diagram. Such a diagram shows the interrelation between the structures of particles and the composition of a mixture. It also enables one to predict which particles would be observed in a mixture of known composition.
FIGURE 24 Phase diagram summarizing results of electron microscopy of mixtures of lecithin, lysolecithin, and A-I. This phase diagram approximates a plane in a quaternary phase diagram of water, lecithin, lysolecithin, and A-I. The vertices represent solutions containing only lecithin, lysolecithin, or A-I. Points within the triangle represent mixtures with specific weight ratios lecithin:lysolecithin:A-I. Numbered regions correspond to mixtures containing complexes which have specific shapes on electron microscopy. The dashed line shows how Region IV would be enlarged if the data of Nichols et al (77) had been used in constructing this diagram.
Figure 24
Ik. Effect of Lysolecithin on the Conformation of A-I: Fluorescence and Circular Dichroism Studies

As described in the preceding sections, complexes of lecithin, lysolecithin, and A-I with various densities and shapes were apparently formed in mixtures of these components. Ultracentrifugal fractionation studies showed that complexes which floated in the d 1.063-1.21 range were present in mixtures of lecithin, lysolecithin, and A-I but were mostly absent in mixtures of lecithin and A-I containing no lysolecithin. Similarly, electron microscopy showed that particles of certain shapes were only seen in mixtures containing lysolecithin, but were not seen in mixtures containing no lysolecithin.

The reasons for these differences are not completely clear. Studies by other workers on the interaction of lysolecithin with proteins such as glucagon and casein, showed that lysolecithin has the ability to induce changes in protein conformation (123,124). These studies suggested that the stimulating effect of lysolecithin on the relipidation of A-I in mixtures of lecithin, lysolecithin, and A-I may have been a consequence of conformational changes induced in A-I by lysolecithin. Spectroscopic measurements, fluorescence and circular dichroism, were therefore performed to determine the effect of lysolecithin on the fluorescence of A-I and to ascertain if lysolecithin induced changes in the conformation of A-I.

Lysolecithin and A-I were mixed in various molar ratios and incubated (60 min, 23°C). Figure 25 shows the fluorescence intensity and emission peak wavelength of A-I mixed with various amounts of lysolecithin. The fluorescence intensity increased with increasing
amounts of lysolecithin and reached a maximum when the molar ratio lysolecithin:A-I was between 100:1 and 200:1. The emission peak wavelength, 331 nm, was constant and corresponded to the peak wavelength of tryptophan in an apolar solvent (107). These results suggest that some interaction occurred between A-I and lysolecithin in aqueous solution. Since changes in the fluorescence intensity occurred in mixtures with a molar ratio lysolecithin:A-I as low as 10:1, the earlier tentative conclusion based on results of preparative ultracentrifugation that lysolecithin and A-I interacted in mixtures with a molar ratio of 34:1 was substantiated by the fluorescence results.

Changes in the circular dichroism spectrum of A-I in the presence of lysolecithin are shown in Figures 26 and 27. The increase in negative ellipticity at 208 nm as shown in Figure 26 can be interpreted as showing an increase from about 50% to about 70% in the helical content of A-I on addition of lysolecithin (111). Comparing the mean residue ellipticity at 208 nm with the amount of added lysolecithin (Figure 27) shows that the ellipticity decreased with increasing amounts of lysolecithin and reached a minimum when the molar ratio lysolecithin:A-I was between 200:1 and 300:1. These changes in ellipticity support the conclusion that an interaction between lysolecithin and A-I occurs in aqueous solution.

These observations on the changes in fluorescence and circular dichroism spectra do not disprove the hypothesis that lysolecithin induces a conformation in A-I which facilitates the further binding of lecithin. Since a change in the conformation of A-I was observed even with low amounts of added lysolecithin, any binding of lecithin,
observed in the studies described in the previous sections, must have taken place after some change in the conformation of A-I. Thus the hypothesis that a conformational change was necessary for lecithin binding is not provable by these spectroscopic observations, although the hypothesis is supported by them.

The maximum helical content induced in A-I by addition of lysolecithin was about 70%. This is the same as the helical content induced in A-I by sonication with lecithin (64). The similarity of these two observations suggests that the interaction of lysolecithin with A-I during incubation was somehow similar to the interaction of lecithin with A-I during sonication. The role of lysolecithin in the formation of complexes in mixtures of lecithin, lysolecithin, and A-I may therefore be the formation of complexes of lysolecithin and A-I without sonication which bind additional amounts of lecithin, and which have properties similar to complexes formed during cosonication of lecithin and A-I.

This idea is consistent with the recent data of Reynolds et al. (86,87,88,89) which have been interpreted as showing that small amounts of detergents react with apolipoproteins, particularly A-I and A-II, creating nucleation sites for cooperative binding of other apolar or amphipathic compounds molecules. The facilitation of lecithin binding by detergents was described by them as a "synergistic" effect by detergents on phospholipid binding.
FIGURE 25 Effect of lysolecithin on the fluorescence of A-I. Mixtures of various molar ratios lysolecithin:A-I were prepared, incubated (60 min, 23°C), and examined fluorometrically. Dependence of fluorescence intensity (closed circles) and emission peak wavelength (open circles) on the molar ratio lysolecithin:A-I is shown.
Fluorescence peak wavelength (nm)

Fluorescence intensity (arbitrary units)

Molar ratio (lysolecithin: A-I)

Figure 25
FIGURE 26 Circular dichroism spectra of mixtures of lysolecithin and A-I. Lysolecithin was mixed with A-I and incubated (60 min, 23°C). The spectra were obtained on a Cary 6002. Spectra of mixtures with various molar ratios lysolecithin:A-I are presented.
Molar ratio
lysolecithin: A-I

Mean residue ellipticity (deg-cm²/dmole)

Wavelength (nm)
FIGURE 27. Effect of lysolecithin on the circular dichroism spectrum of A-I. Mixtures of lysolecithin and A-I with various molar ratios were prepared and incubated (60 min, 23°C). The circular dichroism spectra were obtained on a Cary 6002. The dependence of the mean residue ellipticity on the molar ratio lysolecithin:A-I is shown. The continuous decrease in ellipticity can be interpreted as indicating a continuous increase in helical content of the apolipoprotein (see text).
Figure 27

Mean residue ellipticity (deg-cm²/dmole) at 208 nm

Molar ratio (lysolecithin: A-I)
II. Relipidation of A-II with Phospholipids

The preceding sections described experiments on the relipidation of one of the HDL apoproteins, A-I. The other major HDL apoprotein, A-II, has been reported to differ markedly from A-I in its ability to recombine with phospholipids (34). Whereas A-I was reported to interact with phospholipids only during sonication or in the presence of lysolecithin (34, 60, 77), A-II was reported to readily combine with phospholipids forming complexes which could be ultracentrifugally isolated (34). The relipidation of A-II was investigated by the means described in the previous sections, in order to compare the physical-chemical properties of the complexes formed during relipidation of these two apolipoproteins.

In order to study the complexes formed by incubating A-II with lecithin or lysolecithin, mixtures of A-II and these phospholipids were prepared, incubated, and subjected to gradient gel electrophoresis, electron microscopy, or circular dichroism spectrometry. In all experiments involving incubation of A-II with lecithin, lecithin was used in the form of single bilayer vesicles.
IIa. Relipidation of A-II with Lecithin: Gradient Gel Electrophoresis

Electrophoresis of mixtures of lecithin and A-II on polyacrylamide gradient slab gels was used to estimate the number and molecular weights of subclasses of particles in such mixtures. Lecithin vesicles, prepared by the method of Batzri et al. (103) were mixed with A-II in various molar ratios. The resulting mixtures were incubated (1 hr, 23°C), and subsequently subjected to gradient gel electrophoresis. Figures 28 and 29 show the results of two electrophoresis runs.

After incubating lecithin and A-II in various molar ratios, (molar ratios lecithin:A-II of 11:1, 44:1, and 88:1), several classes of particles with various ranges of molecular weights were observed on the gradient gel. With a molar ratio lecithin:A-II of 11:1, two major classes and one minor class were evident (Figure 28, Lane 2). A triangular band of particles with minimum molecular weights of about 40,000, and a broad band of particles with a range of molecular weights 110,000-160,000 were especially prominent. A smaller band of particles with molecular weights 180,000-220,000 was also seen in this mixture. With greater amounts of lecithin, the triangular band disappeared, and a high molecular weight class of complexes with molecular weights greater than 900,000 appeared (Figure 28, Lanes 4 and 5). In these mixtures, with molar ratios of 44:1 and 88:1, two intermediate molecular weight classes of complexes, with molecular weights 120,000-175,000 and 180,000-220,000, were also present. The class with molecular weights 120,000-175,000 decreased with increasing lecithin,
while the amount of the other class, with molecular weights 180,000-220,000, was maximal when the molar ratio was 44:1 and decreased with either higher or lower amounts of lecithin.

Figure 29 shows the results of gradient gel electrophoresis of a sample containing A-II with no added lecithin and a sample with a molar ratio lecithin:A-II of 23:1. In the absence of lecithin, A-II appeared as a streak with a range of molecular weights 40,000-80,000. This suggests that the streak seen in the mixture with a molar ratio of 11:1 (Figure 28, Lane 2) corresponded to A-II with little or no lecithin. A-II has been reported to be present in a dimer (molecular weight 34,000) in aqueous solutions (118). The 40,000-80,000 range of molecular weights of A-II particles suggests that dimers, trimers, and tetramers were present. In the mixture with a molar ratio of 23:1, a class of particles with a range of molecular weights 120,000-180,000 predominated.

In summary, the distribution of particles within specific ranges of molecular weights changed with increasing amounts of lecithin in mixtures of lecithin and A-II. As the amount of lecithin was increased, the number of particles with molecular weights 40,000-80,000 diminished. The number of particles with molecular weights 120,000-180,000 first increased, then decreased, and was maximum in a mixture with a molar ratio of 23:1. The number of particles with molecular weights 180,000-220,000 first increased, then decreased, and was maximum in a mixture with a molar ratio of 44:1. The number of particles with molecular weights greater than 900,000 progressively decreased with increasing amounts of lecithin.
The increase in molecular weight of the predominant class of particles in mixtures of lecithin and A-II, suggests that a simple relationship exists between such classes. It appears as if the relipidation of A-II occurs step-wise with formation of particular classes of complexes with different lecithin:A-II ratios. The following scheme of equilibrium reactions might explain the gradient gel electrophoresis observations:

\[ 4(\text{A-II}) + 92 (\text{lecithin}) \overset{\dagger}{\longrightarrow} \text{complex (140,000 MW, molar ratio 23:1)} \]

\[ \text{complex (140,000 MW)} + 80 (\text{lecithin}) \overset{\dagger}{\longrightarrow} \text{complex (200,000 MW, molar ratio 43:1)} \]

\[ \text{complex (200,000 MW)} + 1,000 (\text{lecithin}) \overset{\dagger}{\longrightarrow} \text{complex (2,000,000 MW)} \]

Complexes containing 4 molecules of A-II were considered in this system of reactions in order to account for the appearance of a complex with a molar ratio of 23:1 and a molecular weight of 120,000-180,000. The complexes with a molecular weight of about 2,000,000 would appear to be a vesicle with 4 molecules of A-II attached. In a mixture with any ratio of lecithin:A-II, the complexes present would depend on the molar ratio and the equilibrium constants between the various complexes.

The complexes with a range of molecular weights 120,000-160,000 are similar in molecular weight to the low molecular weight particles observed in sonicated mixtures of lecithin and A-I (Section If). As will be shown in the next section, these particles would probably appear as small round particles 4-6 nm in diameter upon electron microscopy.
The results of gradient gel electrophoresis are consistent with the results of Assman et al. (94) and show that A-II is spontaneously relipidated when incubated with lecithin vesicles. These results also agree with those of David et al. (117) who showed that various classes of complexes can be observed in incubated mixtures of lecithin and A-II. This latter group, however, did not try to correlate the changes in molecular weight of the predominant class with the amount of lecithin present in the mixture.
FIGURE 28 Gradient gel electrophoresis of mixtures of lecithin vesicles and A-II. Lane 1, reference proteins. Lanes 2-5, mixtures of lecithin and A-II: lane 2, molar ratio lecithin:A-II 11:1; lane 3, blank; lane 4, molar ratio 44:1; lane 5, molar ratio 88:1. Lane 6, reference proteins.
Reference proteins:

- Thyroglobulin (MW 670,000)
- Apoferritin (MW 480,000)
- Catalase (MW 235,000)
- Albumin (MW 77,000)
- Ovalbumin (MW 45,000)

Lane number

Approximate molecular weight

Figure 28
FIGURE 29  Gradient gel electrophoresis of mixtures of lecithin vesicles and A-II. Lane 1, A-II with no added lecithin. Lane 2, incubated mixture of lecithin and A-II, molar ratio lecithin:A-II 23:1. Lane 3, reference proteins.
Reference proteins

- Thyroglobulin (MW 670,000)
- Apoferritin (MW 480,000)
- Catalase (MW 235,000)
- Albumin (MW 77,000)
- Ovalbumin (MW 45,000)
II b. Structure of Complexes of A-II and Lecithin: Electron Microscopy

Electron microscopy was used to investigate the subclasses of particles in mixtures of lecithin and A-II. The number of subclasses, and the approximate molecular weights and shapes of particles in these subclasses were studied. Vesicles prepared by the method of Batzri et al. (103) were mixed with A-II in various molar ratios, and 0.005M NH₄HCO₃ to give a constant lipid concentration of 0.1 mg/ml. The resulting mixtures were incubated (1 hr, 23°C), mixed 1:1 by volume with 4% Na-phosphotungstate, and immediately examined by electron microscopy.

Figure 30 shows the results of electron microscopy of such mixtures of lecithin and A-II. In the absence of lecithin, A-II appeared as fibers of different sizes and shapes (Figure 30(b)). In electron micrographs of mixtures with a molar ratio lecithin:A-II of 23:1, distinct particles, 6-8 nm in diameter, could be seen (Figure 30(c)). Very few lecithin vesicles could be found in a mixture with this molar ratio. Figure 30(a) shows lecithin vesicles without added A-II at a similar concentration for comparison. In a mixture with a molar ratio of 77:1 (Figure 30(d)), larger round particles 10-14 nm in diameter were observed. Rouleaux with a 5 nm repeat distance, 25-50 nm wide, and some single bilayer vesicles were seen in micrographs of mixtures with a molar ratio of 115:1 (Figure 30(e)); while in a mixture with a molar ratio of 154:1, rouleaux with a 5 nm repeat distance, 26-100 nm wide predominated (Figure 30(f)). In a mixture with a higher ratio lecithin:A-II of 230:1, only dispersed
vesicular structures could be seen.

By assuming that the predominant class of particles seen in gradient gel electrophoresis of mixtures of lecithin and A-II corresponded to the predominant species observed by electron microscopy, it is possible to correlate the results of electron microscopy with the results of gradient gel electrophoresis. The small round particles 6-8 nm in diameter therefore were probably similar to the particles observed by gradient gel electrophoresis with molecular weights 120,000-180,000. The round particles 10-14 nm in diameter were probably similar to the particles with molecular weights 180,000-220,000. The particles which appeared as rouleaux were seen in mixtures with molar ratios greater than any studied by gradient gel electrophoresis. However, from the results of gradient gel electrophoresis, mixtures with molar ratios greater than 88:1 predominantly contained particles with molecular weights greater than 900,000. Therefore rouleaux probably occurred in mixtures containing particles with these very high molecular weights.

The small round particles 6-8 nm in diameter (Figure 30(c)) were similar in size to the low molecular weight subspecies of HDL seen in patients with lecithin:cholesterol acyltransferase deficiency (36) and to HDL from patients with Tangier disease (34). Since HDL found in patients with Tangier disease are primarily composed of A-II and phospholipid (33), the small particles 6-8 nm in diameter observed here were chemically and structurally similar to Tangier HDL. The apoprotein in the small HDL from patients with lecithin:cholesterol acyltransferase deficiency has been reported to contain mostly A-I (125). Formation of small particles containing A-I and phospholipid
was discussed previously (Section If) and, as previously mentioned, if they had the same size, 6-8 nm, as the particles observed in these studies with lecithin and A-II, then they were chemically and structurally similar to the small molecular weight subspecies of HDL from lecithin:cholesterol acyltransferase deficient patients.

It is not clear where the A-II was located in the rouleaux with a 5 nm repeat distance. The mixtures with molar ratios of 115:1 and 154:1 originally contained vesicles about 20-40 nm in diameter. The rouleaux formed in these mixtures, however, had different ranges of widths, 26-50 nm and 26-100 nm, respectively. It may be that the A-II was located in such a way as to account for this difference. Assuming that the rouleaux were stacks of disc-shaped particles (36), then if the protein had been between the discs, a larger proportion of lecithin would probably not have led to formation of wider rouleaux. However, if the protein had been around the perimeter of each disc, covering the hydrophobic chains of the lecithin at the edges of a piece of bilayer, increasing the proportion of lecithin would have been expected to lead to larger discs since the surface area of a disc, and hence the amount of lecithin in a disc, increases as the square of the circumference.

The 5 nm repeat distance of most of the observed rouleaux was approximately the thickness of a bilayer (126), so the repeating units of these rouleaux may have had a bilayer arrangement of the lecithin. It is not clear from the present studies whether this structure was derived from vesicles with attached A-II which aggregated and collapsed to form rouleaux, or whether this structure came from
small particles such as those seen in the mixture with a molar ratio lecithin:A-II of 77:1. Rouleaux were observed when lecithin vesicles were added to a mixture with a molar ratio of 77:1 which electron microscopy had shown to predominantly contain particles 10–14 nm in diameter. This suggests that rouleaux formation is a complex process dependent mainly on the final lipid:apolipoprotein ratio and independent of the shape of stoichiometry of the starting components. This suggestion is consistent with the observations on rouleaux formation in mixtures of lecithin, lysolecithin, and A-I (Section Ih).
FIGURE 30  Electron micrographs of mixtures of lecithin vesicles and A-II. Lecithin vesicles and A-II were mixed and incubated (30 min, 23°C). Samples were negatively stained with Na-phosphotungstate, and examined immediately. (a), vesicles with no added A-II. (b), A-II with no added vesicles. (c)-(g), mixtures of vesicles and A-II: (c), molar ratio lecithin:A-II 23:1; (d), molar ratio 77:1; (e), molar ratio 115:1; (f), molar ratio 154:1; (g), molar ratio 230:1.
Figure 30
IIC. Effect of Lysolecithin on the Conformation of A-II: Circular Dichroism Studies

As shown in Section IIk, lysolecithin induced a conformational change in A-I, causing an increase in helical content equal to that caused by sonication with lecithin (64). Sonication of A-II with lecithin had also been shown to cause an increase in helical content (64). The effect of lysolecithin on the conformation of A-II, however, had not been studied. Therefore, to further compare the relipidation of A-I and A-II, circular dichroism spectra of mixtures of A-II with various molar ratios of lysolecithin were obtained.

Figure 31 shows the circular dichroism spectra of A-II in the presence of various amounts of lysolecithin. The increase in negative ellipticity at 208 nm can be interpreted as showing an increase from about 34% to about 76% in the helical content of A-II on addition of lysolecithin. Comparing the mean residue ellipticity at 208 nm with molar ratio lysolecithin:A-II in the mixture (Figure 32) shows that the ellipticity decreased with increasing amounts of lysolecithin and reached a minimum when the molar ratio was about 100:1. These changes in mean residue ellipticity suggest that an interaction between lysolecithin and A-II occurs in aqueous solution and that an A-II molecule will bind at least 100 lysolecithin molecules.

Sonication of A-II with an equal weight of lecithin has been shown to cause an increase from 35% to 50% in the helical content of A-II (64). On the other hand, incubation of A-II with lysolecithin caused a greater increase in helical content (to about 76%). Lysolecithin and lecithin apparently interact in different ways with A-II,
whereas they interact in similar ways with A-I, causing identical changes in helical content.
FIGURE 31 Circular dichroism spectra of mixtures of lysolecithin and A-II. Lysolecithin was mixed with A-II and incubated (60 min, 23°C). The spectra were obtained on a Cary 6002. Spectra of mixtures with various molar ratios lysolecithin:A-II are presented.
Figure 31
FIGURE 32 Effect of lysolecithin on the circular dichroism spectrum of A-II. Mixtures of lysolecithin and A-II with various molar ratios were prepared and incubated (60 min, 23°C). The circular dichroism spectra were obtained on a Cary 6002. The dependence of the mean residue ellipticity at 208 nm on the molar ratio lysolecithin:A-II is shown. The continuous decrease in ellipticity can be interpreted as indicating a continuous increase in helical content of the apolipoprotein (see text).
Figure 32

Molar ratio (lysolecithin: A-II) vs.
Mean Residue Ellipticity (deg-cm²/dmole)
at 208 nm

-5x10³
-10x10³
-15x10³
-20x10³
-25x10³

0
100
200
300
III. Relipidation of C-III with Phospholipids

Studies on the relipidation of A-I and A-II with phospholipids were reported in the previous sections. C-III, one of the major apoproteins of VLDL, has also been reported to interact with phospholipids during incubation without sonication (67,73). Preparative ultracentrifugation showed that complexes formed by incubation of mixtures of lecithin and C-III, with molar ratios lecithin:C-III of 14:1, floated into the d 1.063-1.21 range (67). An electron microscopic study of the interaction of C-III with single bilayer vesicles prepared by the method of Huang (101) has also been reported (73). As observed in this study, various classes of lipid-protein complexes were formed in incubated mixtures of lecithin vesicles and C-III. In mixtures with a molar ratio lecithin:C-III of 290:1, vesicles were predominantly observed. With increasing amounts of C-III, particles which stacked as rouleaux with a 10 nm repeat distance were predominantly observed, and in mixtures with a molar ratio of 36:1, extended sections of lamellae were seen. These electron microscopic studies, as well as light scattering (74) and ultracentrifugation studies were interpreted as showing that C-III, in mixtures with a high ratio of lecithin:C-III, attached to the lecithin vesicles and formed rouleaux during preparation for electron microscopy.

In order to further investigate the interaction of C-III and phospholipids and to compare the relipidation of C-III with the relipidation of A-I and A-II, mixtures of lecithin or lysolecithin and C-III were prepared and analysed by gradient gel electrophoresis,
and fluorescence or circular dichroism spectroscopy. Incubations involving lecithin used single bilayer lecithin vesicles and were conducted at room temperature (23°C).
IIIa. Relipidation of C-III with Lecithin: Results of Gradient Gel Electrophoresis

In order to estimate the number and approximate molecular weights of the classes of lipid-protein complexes present in incubated mixtures of lecithin and C-III, gradient gel electrophoresis was performed. Lecithin vesicles, prepared by the method of Batzri et al. (103), were mixed with C-III in various molar ratios, incubated (1 hr, 23°C), and the resulting complexes were subjected to polyacrylamide gradient gel electrophoresis. Figure 33 shows the results of such an electrophoresis experiment.

In all mixtures, no distinct classes of complexes were observed. In a mixture with a molar ratio lecithin:C-III of 7:1 (Figure 33, Lane 2), a broad band of particles with molecular weights 200,000-500,000 could be seen. In addition, a smaller amount of protein was present in complexes with molecular weights greater than 900,000. With increasing amounts of lecithin (Figure 33, Lanes 3 and 4), the intensity of staining in the region corresponding to 200,000-560,000 molecular weight particles decreased, and there was an increase in the intensity of the high molecular weight region.

In order to compare the particles formed by incubation with the particles formed by sonication, a mixture of lecithin and C-III with a molar ratio of 14:1 was subjected to sonication (3 x 1 min, 37°C) using a 60 watt ultrasonic disintegrator (No. 7100, MSE, London, England) operating at 18-20 kHz. After sonication, the mixture was briefly ultracentrifuged (114,000 x g, 1 hr, 15°C) to remove any large aggregates and any titanium from the disintegrator
probe. The resulting mixture was also subjected to electrophoresis on the gradient gel (Figure 33, Lane 5). The particles observed in the sonicated mixture had lower molecular weights than those in the incubated mixtures. The range of molecular weights was 66,000-270,000. No distinct classes of particles were observed.

Comparison of the results of gradient gel electrophoresis of incubated mixtures of lecithin and C-III with the micrographs of Hoff et al (73) suggested that the particles of molecular weights 200,000-560,000 were the particles which made up the rouleaux seen in a mixture with a molar ratio of 36:1. These particles had approximately the same molecular weights as the large particles seen in a sonicated mixture of A-I and lecithin (Section II). On electron microscopy, these large particles of A-I and lecithin also appeared as rouleaux. The high molecular weight subspecies of HDL from patients with lecithin:cholesterol acyltransferase deficiency, which also appear as rouleaux on electron microscopy (36), migrate to a position on a gradient gel similar to that of the particles in incubated mixtures of lecithin and C-III (122). It is therefore likely that the particles with molecular weights 200,000-560,000 formed by incubating lecithin and C-III were the particles observed by Hoff et al (73) which appeared as rouleaux and extended lamellae with a 5 nm repeat distance. This observation contrasts the conclusion reached by Morrisett et al (74) that the observed rouleaux and lamellae were formed in the course of negative staining and dehydration of vesicles with attached C-III. Instead, it seems that these
rouleaux and lamellae were formed from particles with molecular weights 200,000-560,000 which were definitely smaller than vesicles.

Sonication of mixtures of lecithin and C-III formed particles smaller than those formed during incubation. These particles had molecular weights in the same range as those formed by incubation of lecithin and A-II, or the small particles formed by sonication of lecithin and A-I. However, the particles formed by sonicating lecithin and C-III did not form distinct bands on a gradient gel and apparently had a wide range of molecular weights.

Sonicated or incubated mixtures of lecithin and C-III appear to form classes of particles of similar molecular weights as incubated mixtures of A-I or A-II and lecithin. Particles with molecular weights about 140,000 are formed during sonication of mixtures of C-III and lecithin, although they occur spontaneously during incubation of A-II and lecithin. Particles with molecular weights about 300,000 are formed spontaneously during incubation of mixtures of C-III and lecithin, and particles of similar molecular weight are formed during cosonication of A-I and lecithin.
FIGURE 33 Gradient gel electrophoresis of mixtures of lecithin vesicles and C-III. Lane 1, reference proteins. Lanes 2–4, incubated mixtures of lecithin and C-III: lane 2, molar ratio lecithin:C-III 7.5:1; lane 3, molar ratio 15:1; lane 4, molar ratio 30:1. Lane 5, sonicated (3 × 1 min) mixture of lecithin and C-III, molar ratio 15:1. Lane 6, reference proteins. Molecular weights of reference proteins are in parentheses.
Thyroglobulin
(MW 670,000)

Apo ferritin
(MW 480,000)

Catalase
(MW 235,000)

Albumin
(MW 77,000)

Ovalbumin
(MW 45,000)

Figure 33
IIIb. Effect of Lysolecithin on the Conformation of C-III: Fluorescence and Circular Dichroism Studies

As was shown in previous sections, lysolecithin induced conformational changes in both A-I and A-II. Interestingly, relipidation of A-II with lysolecithin caused a greater increase in helical content than did relipidation of A-II with lecithin. On the other hand, lysolecithin and lecithin apparently caused similar changes in the conformation of A-I. In order to further compare the relipidation of these apolipoproteins, the relipidation of C-III with lysolecithin was studied by fluorescence and circular dichroism spectrometry. Mixtures of lysolecithin and C-III with various molar ratios were prepared and incubated (1 hr, 23°C). The fluorescence emission peak intensity and wavelength and the circular dichroism spectra of the resulting mixtures were then determined.

Figure 34 shows the fluorescence intensity and emission peak wavelength of C-III mixed with various amounts of lysolecithin. The fluorescence intensity increased with increasing amounts of lysolecithin and reached a maximum when the molar ratio lysolecithin:C-III was about 200:1. The emission peak wavelength decreased from 348 nm to 331 nm with increasing amounts of lysolecithin and reached a minimum when the molar ratio was about 100:1. This change in emission peak wavelength corresponded to the expected change in emission peak wavelength of tryptophan on transfer from a polar to a relatively apolar environment. These fluorescence changes indicate that an interaction between C-III and lysolecithin took place during incubation. The changes in emission intensity are similar to those
observed during interaction of A-I with lysolecithin and suggest some similarity between the relipidation of C-III and A-I with lysolecithin.

Changes induced in the circular dichroism spectrum of C-III by lysolecithin are shown in Figures 35 and 36. The increase in negative ellipticity at 208 nm as shown in Figure 35 can be interpreted as showing an increase from about 26% to about 76% in the helical content of C-III on addition of lysolecithin. Comparing the mean residue ellipticity at 208 nm with the amount of added lysolecithin (Figure 36) shows that the ellipticity decreased with increasing amounts of lysolecithin and did not reach a minimum even in a mixture with a molar ratio lysolecithin:C-III of 500:1.

The maximum helical content induced in C-III by lysolecithin was about 76%. Previously, Morrisett et al (74) had shown that lecithin induced a maximum helical content of about 54%. The helical content of both A-II and C-III was increased to a greater extent by interaction with lysolecithin than by interaction with lecithin. With each apolipoprotein, A-I, A-II, or C-III, lysolecithin induced comparable maximum helical content: 70%, 76%, and 76%. Thus, in contrast to the interaction of these apolipoproteins with lecithin, interaction with lysolecithin induced similar helical contents in these apolipoproteins.
FIGURE 34 Effect of lysolecithin on the fluorescence of C-III.
Mixtures of lysolecithin and C-III with various molar ratios were prepared, incubated (60 min, 23°C), and examined fluorometrically. Dependence of fluorescence intensity (closed circles) and emission peak wavelength (open circles) on the molar ratio lysolecithin:C-III is shown.
Fluorescence peak wavelength (nm) vs. Molar ratio (lysolecithin: C-III)

Fluorescence intensity (arbitrary units) vs.

Figure 34
FIGURE 35  Circular dichroism spectra of mixtures of lysolecithin and C-III. Lysolecithin was mixed with C-III and incubated (60 min, 23°C). The spectra were obtained on a Cary 6002. Spectra of mixtures with various molar ratios lysolecithin:C-III are presented.
Molar ratio
lysolecithin: C-III

Molar ellipticity (deg-cm²/dmole)

Wavelength (nm)

Figure 35
FIGURE 36  Effect of lysolecithin on the circular dichroism spectrum of C-III. Mixtures of lysolecithin and C-III with various molar ratios were prepared and incubated (60 min, 23°C). The circular dichroism spectra were obtained on a Cary 6002. The dependence of the mean residue ellipticity at 208 nm on the molar ratio lysolecithin:C-III is shown. The continuous decrease in ellipticity can be interpreted as indicating a continuous increase in helical content of the apolipoprotein (see text).
Figure 36

Molar ratio (lysolecithin: C-III)

Mean residue ellipticity (deg-cm²/dmole)

at 208 nm

-5 × 10⁻⁴
-3 × 10⁻⁴
-1.5 × 10⁻⁴
-0.5 × 10⁻⁴
0

100
200
300
400
500
SUMMARY AND CONCLUSIONS

This thesis focussed on the relipidation of three major apolipoproteins with two phospholipids, lecithin and lysolecithin. Preparative ultracentrifugation, electron microscopy, and gradient gel electrophoresis were used to characterize the complexes formed. Circular dichroism and fluorescence were used to gain insight into the effect of lysolecithin on the conformation of these apolipoproteins.

Lecithin apparently relipidated A-I during incubation without sonication, forming two different complexes at all ratios of A-I and lecithin. One complex had a low lipid:protein ratio and the other complex had a high lipid:protein ratio. The complex with the low lipid:protein ratio apparently had a molecular weight less than 120,000; the other had a high molecular weight, greater than 900,000. Electron microscopy, however, indicated that various classes of complexes with different sizes were formed in each mixture of lecithin and A-I. These micrographic results were difficult to interpret and may have been due in part to artefacts of negative staining and dehydration.

In the presence of lysolecithin, A-I was relipidated with lecithin in a markedly different way than in the absence of lysolecithin. Based on the results of preparative ultracentrifugation it appeared as if only one class of complexes was formed during incubation of mixtures of lecithin, lysolecithin, and A-I. In no mixture did there appear to be distinct classes with different densities. By electron microscopy, particles of specific shapes could
be discerned in specific mixtures of lecithin, lysolecithin, and A-I. These results were summarized by means of a ternary phase diagram showing the relation of mixture composition to the shapes of the mixed micelles seen in a mixture with a particular ratio lecithin: lysolecithin:A-I.

An hypothesis to explain the effect of lysolecithin on the relipidation of A-I was developed. It was suggested that lysolecithin primed the apolipoprotein and induced a conformation allowing it to further interact with lecithin. The results of the fluorescence and circular dichroism measurements were consistent with this hypothesis but did not prove it.

Relipidation of A-II with lecithin during incubation without sonication differed from relipidation of A-I with lecithin. By gradient gel electrophoresis it was seen that several complexes of different molecular weights were formed in incubated mixtures of A-II and lecithin. One of these complexes had a molecular weight intermediate to the molecular weights of the two complexes formed by A-I and lecithin. Electron microscopy showed particles in mixtures of A-II and lecithin which had shapes similar to those observed in mixtures of lecithin, lysolecithin, and A-I. Specific shapes such as small or large spheres, rouleaux, or vesicles were seen in specific mixtures with particular mole ratios lecithin:A-II. It therefore appeared as if A-II behaved in a way similar to A-I which had been complexed with lysolecithin.

With increasing lecithin, the predominant species seen in electron
micrographs of mixtures of lecithin and A-II gradually changed. Although it was suggested that a set of equilibrium reactions could explain these changes, the variation in particle shape with the ratio lecithin:A-II might also have been described by a phase diagram similar to that used to describe mixtures of lecithin, lysolecithin, and A-I.

Relipidation of C-III with lecithin during incubation without sonication appeared to be different from both relipidation of A-I and relipidation of A-II. Although, by electron microscopy, C-III, when mixed with lecithin vesicles, formed complexes which had shapes similar to those seen in mixtures of A-II and lecithin vesicles (13), gradient gel electrophoresis showed that mixtures of C-III and lecithin vesicles contained a range of particles with continuously varying molecular weights. This was clearly in contrast to the results of relipidating both A-I and A-II which apparently formed classes of particles with quite distinct molecular weights.

Sonication of mixtures of A-I or C-III and lecithin resulted in formation of particles different from those formed during incubation. A-I formed two distinct classes of particles similar in molecular weight to those formed by incubating A-II and lecithin. During sonication with lecithin, C-III formed a wide range of particles as it did during incubation, but with a lower average molecular weight. The results of sonication of A-I and lecithin are particularly interesting as it appears that sonication caused formation of particles of the same shape as were formed during incubation of A-I with
lecithin in the presence of lysolecithin (63). The mode of action of
lysolecithin thus appeared to be similar to that of sonication.

Fluorescence and circular dichroism measurements on A-I, A-II,
and C-III after relipidation with lysolecithin showed unexpected
similarities among these apolipoproteins. Fluorescence measurements
showed some changes in the environment of the tryptophans of A-I
during relipidation with lysolecithin which resulted in increased
average quantum yield. The cause of this increase might have been
any of several factors (109) and was not further investigated.
Fluorescence measurements on C-III after relipidation with lyso­
lecithin showed that the tryptophans of this apolipoprotein apparent­
ly moved into a more apolar environment during relipidation. Circular
dichroism measurements on all three apolipoproteins showed that each
became more helical on interaction with lysolecithin. Surprisingly
each apolipoprotein increased in helical content to about the same
maximum, 75%. As previously reported, phospholipids or phospholipids
plus cholesteryl ester also cause an increase in helicity, though not
to the same extent, in each apolipoprotein (64). Apparently the
mechanism of lysolecithin-apolipoprotein interaction is more similar
among the different apolipoproteins than is the interaction of other
lipids with these apolipoproteins.

Although A-I is relatively refractory to relipidation with phos­
pholipids, and A-II and C-III are relipidated spontaneously with
phospholipids during incubation at room temperature, the products of
relipidation of these three apolipoproteins were similar in size and
shape. Small particles 10-20 nm in diameter with molecular weights 100,000-200,000, particles which appeared as rouleaux with molecular weights 300,000-400,000, or vesicles with attached protein with molecular weights greater than 900,000 were observed in specific mixtures containing A-I, A-II, or C-III and phospholipids. Ultracentrifugal fractionation and gradient gel electrophoresis studies confirmed that particles which form these structures are present in aqueous solution and are not artefacts of the preparative procedures for electron microscopy.

Proteins such as albumin which do not form relipidated complexes with phospholipids have not been reported to form particles such as those described above. The ability to form such particles, then, appears to be a property of the apolipoproteins. Although there are differences in the primary and secondary structures of these apolipoproteins, they apparently possess properties in common which direct the formation of lipid-protein complexes of the specific sizes and shapes described in this thesis.

There remain some differences among these apolipoproteins. Most notable is that each requires a slightly different technique to effect formation of a specific complex with phospholipids. C-III requires sonication to cause formation of the small particles described above. Particles containing A-II and lecithin which formed rouleaux appeared to have molecular weights higher than those containing C-III and lecithin which had the same structure on electron microscopy. The presence of lysolecithin, in mixtures of A-I
and lecithin, facilitated formation of all of the classes of complex.

As earlier noted, lysolecithin induced a similar maximal helical content in each apolipoprotein studied. The helical content of A-I was increased from 50% to 70%. The helical content of A-II was increased from 34% to 76%. The helical content of C-III was increased from 26% to 76%. In contrast, sonication with lecithin increased their helical contents to 70%, 50%, and 54%, respectively. The interaction of glucagon with lysolecithin has also been shown to cause an increase in the helical content of this protein (123). However, the helical content of glucagon, calculated from the published spectra, only changed from 5% to 21% on interaction with lysolecithin.

It seems as if the increase in helical content to about 75%, during interaction with lysolecithin, is a characteristic peculiar to apolipoproteins. This characteristic may be due to the alternation of pairs of polar and apolar amino acid residues as observed in apolipoproteins, which would give helical regions of an apolipoprotein amphipathic properties allowing it to interact with amphipathic phospholipids such as lecithin or lysolecithin. In glucagon, pairs of polar and apolar amino acids do not alternate. The observed similarities between the apolipoproteins on interaction with lysolecithin might be interpreted as demonstrating their similar amphipathic properties. It may be that these amphipathic properties are the determining factors, unique to apolipoproteins, which direct the formation of specific lipid-protein complexes.
Although the final helical contents of A-I, A-II, and C-III when complexed with lysolecithin are similar, the helical contents of these apolipoproteins in solutions containing no phospholipids are quite different. A-I has considerably greater helical content than A-II and C-III in the absence of lipid. It may be that this is part of the reason that A-I is relatively refractory to relipidation. The high content of amphipathic helix in A-I may promote the formation of multimers of A-I in solution which are refractory to relipidation. An agent which causes a dissociation of A-I multimers might therefore facilitate relipidation of this apolipoprotein. Lysolecithin may be capable of playing this role and, based on the work in this thesis, does facilitate formation of lipid-protein complexes. Sonication, which also facilitates formation of such complexes, might also be expected to disaggregate A-I. The effect of the increase in helical content observed during interaction of A-I with lysolecithin on the formation of relipidated complexes containing A-I needs further study.

Mixtures of apolipoproteins and phospholipids formed mixed micelles of different compositions and structures. The compositions studied in the course of the research on this thesis were limited to simple binary or ternary mixtures of apolipoproteins and phospholipids. Lipoproteins found in plasma have many more components. Nonetheless, in the relatively simple model systems studied, important similarities to naturally occurring HDL were observed. In mixtures of lecithin, lysolecithin, and A-I, lipid-protein complexes similar in shape, molecular weight, and composition to either species of
abnormal HDL from patients with lecithin:cholesterol acyltransferase deficiency were observed. In mixtures of lecithin and A-II, small lipid-protein complexes similar in shape, molecular weight, and composition to the abnormal HDL form patients with Tangier disease were observed. Extensions of these studies to include mixtures with a greater variety of components should yield important information on interrelations among the various subspecies of lipoproteins observed in vivo.
APPENDICES

1. Guanidine Denaturation of A-I and the Effect of Lysolecithin

To investigate the effect of lysolecithin on the stability of A-I conformation, denaturation of A-I and a mixture of lysolecithin and A-I by guanidine-HCl was investigated. Figure 37 shows the fluorescence intensity and emission peak wavelength of A-I without added lysolecithin in solutions with various guanidine-HCl concentrations. With increasing concentrations of guanidine-HCl the fluorescence intensity decreased slightly until a concentration of about 1.2M guanidine-HCl was reached. At this guanidine-HCl concentration there was an abrupt change in the fluorescence intensity. With still greater concentrations, the fluorescence intensity continued to slowly decrease. In solutions of about 1.2M guanidine-HCl, a change in the emission peak wavelength occurred. Below 1.2M guanidine-HCl, the peak wavelength was about 331 nm and above this concentration, the peak wavelength was near 349 nm. These changes in the fluorescence spectrum of A-I occurred within 15 min of mixing A-I with guanidine-HCl and were stable for 72 hr.

Figure 38 shows the effect of guanidine on the fluorescence intensity and emission peak wavelength of a mixture of A-I and lysolecithin with a molar ratio lysolecithin:A-I of 170:1. With increasing guanidine-HCl concentration, the fluorescence intensity initially increased and then abruptly decreased near 3M guanidine-HCl. At greater guanidine-HCl concentrations, the intensity remained constant at a level less than the intensity observed without added guanidine. At about 3M, the peak emission wavelength also shifted from about 331 nm to about 350 nm.
Comparing the effects of guanidine-HCl on A-I fluorescence and on the fluorescence of A-I mixed with lysolecithin shows that even low concentrations of guanidine-HCl caused some changes in the fluorescence properties of the protein. This change in the properties of A-I with low guanidine-HCl concentrations, however, was different if the A-I was mixed with lysolecithin and supports the observation that lysolecithin and A-I formed a complex in which A-I exhibited different molecular properties (see Section I). At sufficiently high concentrations of guanidine-HCl there was an abrupt change in the fluorescence properties of the A-I both in the presence and in the absence of lysolecithin. At such a guanidine-HCl concentration, the fluorescence intensity abruptly decreased and there was a shift in the emission peak wavelength which corresponded to a shift of tryptophan residues from a nonpolar to a polar environment. Concerted changes in fluorescence intensity and emission peak wavelength have been reported to occur in other proteins on denaturation (108). This is consistent with the observation that high concentrations of guanidine cause unfolding of most proteins (106). Without added lysolecithin, however, the abrupt change in protein conformation takes place near 1M guanidine and with added lysolecithin, near 3M. Lysolecithin apparently forms a complex with A-I and stabilizes the structure of A-I in addition to increasing its helicity.

A plot of fluorescence intensity versus the time after addition of guanidine-HCl to mixture of A-I and lysolecithin with a molar ratio of 170:1 is presented in Figure 39. Denaturation of A-I in
This mixture apparently was not instantaneous and was not complete until 24 hours after addition of guanidine-HCl. As described in the section on methods, guanidine-HCl was used to effect a partial separation of A-I and A-II by incubating intact HDL in a 6M solution for 3 hours, less than the time for complete denaturation of A-I. It may be surmised that the enrichment was due to differences in the rates of denaturation of A-I and A-II in guanidine and to differences in the rates of recombination of A-I and A-II with lipid.
FIGURE 37 Fluorescence intensity and emission peak wavelength of A-I in solutions containing various concentrations of guanidine-HCl. The changes in fluorescence intensity and emission peak wavelength at about 1.2M guanidine-HCl shows the denaturation of A-I at this concentration.
Figure 37
FIGURE 38  Fluorescence intensity and emission peak wavelength complexes of A-I and lysolecithin, molar ratio lysolecithin:A-I 170:1, in solutions containing various concentrations of guanidine-HCl. The changes in fluorescence intensity and emission peak wavelength at about 3M guanidine-HCl shows the denaturation of A-I at this concentration.
Figure 38

Fluorescence intensity (arbitrary units) vs. Guanidine concentration (M)

Fluorescence peak wavelength (nm)
FIGURE 39 Dependence of fluorescence emission wavelength on time after addition of guanidine-HCl to mixtures of lysolecithin and A-I with molar ratios lysolecithin:A-I of 170:1. Changes in fluorescence emission wavelength produced by the presence of 4.0M guanidine-HCl (dashed line) and 3.2M guanidine-HCl (solid line) are both presented.
2. Arrangement of Lipid and Protein in Serum High Density Lipoproteins: A Proposed Model

In the following publication, an approach to formulating models of lipoprotein structure is developed. The basic problem addressed is: given the size, shape, and composition of a lipoprotein, what restrictions are placed on possible models, considering that the components must fit into a volume of known shape? This problem has been alluded to by other workers who have described lipoproteins as having an apolar lipid core surrounded by polar lipids and proteins. Many variations on this simple description have been proposed. However, no one has previously described explicitly how each of the components of a lipoprotein might be arranged on the "polar surface" or in the "apolar core".

The proposed model was constructed using compositional data readily available in the literature. An attempt was made to make the conclusions insensitive to the reported variability of composition of HDL. New data are constantly being reported which should be incorporated into any reassessment of the model. In fact, the ability of this model to withstand reassessment based on new data will be the final criterion of its correctness.

Recent reports on the molar ratios of A-I:A-II in various subclasses of HDL are at variance with the ratios used (A-I or A-II are called apoLP-Gln I and apoLP-Gln II respectively in the following paper). It seems clear that the A-I:A-II ratio decreases as the size of the HDL in various subclasses decreases. In addition, some workers have claimed that HDL containing only A-I can be isolated in the HDL\textsubscript{2} molecular weight range. HDL\textsubscript{2} containing no A-II, would probably contain 5 A-I
molecules per lipoprotein. The total length of amphipathic helix per lipoprotein would then be 1530 Å, and this could be easily accommodated in the area on the surface calculated as available for protein. (See Table 3 in this publication). Similarly, it can be shown that change in the ratio of A-I:A-II from 3:1 as used in the calculations in the paper to 2:2 would lead to no serious modifications of the results.

A good model should suggest means to test its implications. One clear point which may be testable is the suggestion that the ester regions of the cholesteryl ester molecules of HDL$_2$ are located in the interior of the lipoprotein at a particular radius from the center. Such an hypothesis is clearly distinct from the hypothesis that the cholesteryl ester is randomly oriented in the HDL core as it might be in an oil droplet. Small-angle x-ray scattering studies of subclasses of HDL with carefully defined sizes should show a region of different electron density corresponding to the location of these ester linkages if the model is correct. Similarly, it would be possible to test the conclusion that the cholesteryl esters in HDL$_3$ are in a folded conformation. Since this conformation requires the more electron-dense region to be near the surface, it could be distinguished from the possible alternative that the cholesteryl esters are arranged in an extended conformation with their major axes at an angle and not simply perpendicular to the surface. Alternatively, the model could be used as the basis for detailed calculations of an expected electron density profile which might then be tested by small angle x-ray scattering.

Another conclusion basic to the presented model is that the surface of either HDL$_2$ or HDL$_3$ is mostly covered with protein and there is not enough lipid too keep the various apoproteins on the surface from
close proximity with each other. Appendix 3, which presents preliminary results on the ability of the apolipoproteins on HDL to be cross-linked with dimethyl suberimidate, supports the conclusion that the apoproteins must be in close proximity on the surface of HDL. In addition, these results support the assumption that A-I and A-II are both on the surface of some human plasma HDL particles.
ARRANGEMENT OF LIPID AND PROTEIN IN HUMAN SERUM HIGH DENSITY LIPOPROTEINS: A PROPOSED MODEL

Roy B. VERDERY III and Alex V. NICHOLS
Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA. 94720, USA

Received July 15, 1974, accepted September 27, 1974

Based on size and compositional data, models for the two major density subclasses of human serum high density lipoproteins have been developed. Descriptions of both the surface structure of the high density lipoproteins and the structure of their apolar core regions are integral parts of these models. The surface is described as a mosaic of amphiphilic lipid and helical protein regions. The conformation and orientation of the various apolar lipid components as restricted by the surface organization is discussed.

I. Introduction

The exact arrangement of the lipid and protein in human serum high density lipoproteins (HDL) is unknown, although in a widely held model the phospholipid polar heads and the protein are in an outer hydrophilic layer; and the cholesteryl esters, triglycerides, and the acyl chains of the phospholipids are in a hydrophobic core. Evidence for such a model comes primarily from small angle X-ray scattering [1].

In this paper we will present simple calculations on the size, area, volume, and composition of high density lipoproteins which lend support to a spherical model of HDL having a hydrophobic core and a hydrophilic surface of protein and phospholipid. We will show that space-filling considerations support the hypothesis that proteins in association with lipoproteins partially fold into helices to form polar and apolar faces suitable for interacting simultaneously with a polar environment and an apolar inner core [2]. In addition, we will suggest a model describing a probable location for the apolar lipid in relation to the helical apolipoproteins on the surface.

II. Composition of high density lipoproteins

HDL can be divided on the basis of density into two classes, HDL$_2$ and HDL$_3$. 
Table 1
Description of HDL density classes.

<table>
<thead>
<tr>
<th></th>
<th>HDL2</th>
<th>HDL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter(^{a})</td>
<td>100 A</td>
<td>80 A</td>
</tr>
<tr>
<td>Area(^{b})</td>
<td>3.14 x 10^4 A²</td>
<td>2.01 x 10^4 A²</td>
</tr>
<tr>
<td>Volume(^{b})</td>
<td>5.24 x 10^5 A³</td>
<td>2.68 x 10^5 A³</td>
</tr>
<tr>
<td>Average density(^{c})</td>
<td>1.09 g/ml</td>
<td>1.14 g/ml</td>
</tr>
<tr>
<td>Molecular weight(^{d})</td>
<td>3.20 x 10^5</td>
<td>1.75 x 10^5</td>
</tr>
<tr>
<td>Molecular weight(^{e})</td>
<td>3.44 x 10^5</td>
<td>1.84 x 10^5</td>
</tr>
</tbody>
</table>

**Composition wt %\(^{d}\)**

<table>
<thead>
<tr>
<th></th>
<th>HDL2</th>
<th>HDL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

**Number of components per HDL\(^{e}\)**

<table>
<thead>
<tr>
<th></th>
<th>HDL2</th>
<th>HDL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>5.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>147</td>
<td>59</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>44</td>
<td>19</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>90</td>
<td>37</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^{a}\) Diameters were chosen from the range reported by Forte and Nichols [16] to agree with the molecular weights reported by Scanu [1].

\(^{b}\) Calculated from diameter.

\(^{c}\) From ref. [3].

\(^{d}\) From ref. [1].

\(^{e}\) Numbers of components were calculated on the basis of the following average molecular weights: triglyceride, 850; cholesteryl ester, 650; phospholipid, 775; cholesterol, 387; protein, 2.4 x 10^4.

The average protein molecular weight corresponds to either 2:1 or 3:1 apoLP-Gln I*apoLP—Gln II* molar ratios, assuming 2.85 x 10^4 and 1.7 x 10^4 for the molecular weights of apoLP—Gln I and apoLP—Gln II, respectively.

1.063—1.125 g/ml and 1.125—1.21 g/ml, respectively [3]. Average molecular weight, size, and composition have been reported for each class by various investigators. Table 1, derived primarily from a review by Scanu [1], summarizes information describing HDL₂ and HDL₃ and is a reasonable compromise of diverse reports. Reported values of the parameters summarized in table 1 range approximately 20% greater or 20% less than recorded here.

The calculated numbers of protein molecules in HDL₂ and HDL₃ suggest that HDL₂ have 4 molecules of apoLP—Gln I and 2 molecules of apoLP—Gln II while

\(^{*}\) ApoLP—Gln I, with carboxyl terminal glutamine, amino terminal aspartic acid and molecular weight of 2.85 x 10^4 (4) and apoLP—Gln II, with carboxyl terminal glutamine, amino terminal pyrrolidone carboxylic acid, and molecular weight of 1.7 x 10^4 (5) are the major proteins found in human serum high density lipoproteins.
HDL₃ have 3 molecules of apoLP–Gln I and 1 molecule of apoLP–Gln II. These numbers of protein molecules are consistent with the reported weight ratio of apoLP–Gln I to apoLP–Gln II of 3.4 in HDL₂ and 4.6 in HDL₃ [6]. Other choices of protein composition of the HDL species are not consistent with these weight ratios and the molecular weights of the apolipoproteins. Very low density lipoprotein apoproteins are found in association with the HDL fraction; however, they contribute less than 5% of the protein weight in HDL and would not change the conclusions presented here.

III. Surface structure

Several components of HDL have potential amphiphilic character suitable for surface location: the phospholipids, unesterified cholesterol and proteins. Using the calculated number of phospholipids and assuming the unesterified cholesterol is interdigitated among the phospholipids and also contributing to the surface area, the area of the HDL surface available for protein can be calculated assuming the protein occupies that part of the surface not covered by polar lipid (table 2).

Table 2 shows that most of the surface of HDL is probably covered by apolipoproteins. These apolipoproteins assume conformations which will allow them to interact simultaneously with the aqueous medium, with the apolar core of the lipoprotein, and possibly with each other. Since the numbers and molecular weights of the proteins in HDL are known, the possible conformations which the proteins might assume must be consistent with the area on the surface which is covered by protein. Circular dichroism determinations of HDL have been interpreted as indicating the average protein conformation in HDL is about 70% α helix, 11% β, and

<table>
<thead>
<tr>
<th>Surface area covered by protein</th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total surface areaᵃ</td>
<td>3.14 x 10⁴ A²</td>
<td>2.01 x 10⁴ A²</td>
</tr>
<tr>
<td>Lipid covered areaᵇ</td>
<td>0.84–1.08 x 10⁴ A²</td>
<td>0.34–0.44 x 10⁴ A²</td>
</tr>
<tr>
<td>(26–34% of total)</td>
<td>(17–22% of total)</td>
<td></td>
</tr>
<tr>
<td>Area available for proteinᶜ</td>
<td>2.06–2.30 x 10⁴ A²</td>
<td>1.57–1.67 x 10⁴ A²</td>
</tr>
<tr>
<td>(66–77% of total)</td>
<td>(78–83% of total)</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Calculated from diameter.
b Area of the lipoprotein surface covered by phospholipid and cholesterol was calculated assuming 35 Å for the area covered by a cholesterol molecule [7] and 47–63 Å for the area covered by a phospholipid molecule. The range of values for the area covered by a phospholipid molecule is the range reported by Levine and Wilkins [7] for the area occupied by a lecithin molecule in bilayers containing various amounts of lecithin and cholesterol.
c Difference between total and lipid covered areas.
19% disordered [7]. Considering the helical regions of the apolipoproteins, we will discuss two forms of the apolipoproteins at the HDL surface embedded in the surface lipids: spherical globular proteins containing folded helices, and proteins with the helical regions extended across the surface of the lipoprotein.

If the proteins at the lipoprotein surface are considered to be globular, apoLP-Gln I can occupy about $1.24 \times 10^3 \text{ A}^2$ and apoLP-Gln II about $0.92 \times 10^3 \text{ A}^2$. Surface area covered by a globular protein (specific volume 0.74 ml/g) was estimated by using the following formula:

$$\text{Area} = \pi \left( \frac{3}{4\pi} (0.74) \frac{M}{N} \right)^{2/3}$$

(1)

where $M$ is the molecular weight, and $N$ is Avogadro's number. Based on 4 apoLP-Gln I and 2 apoLP-Gln II in HDL$_2$, the protein could cover only about 22% of the surface, compared with 66–77% calculated as available for surface coverage by protein; and based on 3 apoLP-Gln I and 1 apoLP-Gln II in HDL$_3$, the protein could cover only about 23% of the surface, compared with 78–83% calculated as available. If the proteins are associated in globular complexes consisting of several subunits, then only a smaller fraction of the available surface could be covered. Thus it appears that there is not enough protein to cover the area calculated as available to protein if all of the protein is in a globular conformation.

If all regions of apolipoprotein in α-helical conformation exhibit one polar and one apolar face as suggested by Segrest et al. [2] for apoLP-Gln II and by Baker et al. [4] for apoLP-Gln I, the surface of the lipoprotein might be considered as a mosaic of phospholipid and protein with certain regions covered by phospholipid and others covered with the amphiphilic helical regions of the apolipoproteins. The non-helical portions of the protein may either extend outside or be under the hydrophilic surface of the lipoprotein or simply separate the helical regions. Enzymatic digestion [8] and succinylation [9] experiments support an exposed location since no portions of the protein are inaccessible. These non-helical portions of the apolipoprotein then, are visualized as extending from the surface into the surrounding medium or lying on top of the surface.

Considering the helical regions to be those portions of the apolipoproteins entering into the surface structure of HDL, we have calculated the total surface area which could be covered by the helical regions and compared it with the previously calculated area available for protein (table 3). Helical regions of apolipoprotein were considered as cylinders whose length is proportional to the percent α-helix and the number of amino acids in the protein. This cylinder was estimated to have a diameter of 12–18 A by considering a CPK model of a portion of apoLP-Gln II (residues 7 to 31) and the area occupied by such a cylinder was calculated as the product of length and diameter.

The agreement between the area available for protein and the area which could be covered by the equivalent cylinder model for the helical regions shows that there is enough helical apolipoprotein to cover the surface of HDL not covered by polar
Table 3
Area covered by helical regions of apolipoprotein.

<table>
<thead>
<tr>
<th></th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of proteins (see text)</td>
<td>4 apoLP-Gln I</td>
<td>3 apoLP-Gln I</td>
</tr>
<tr>
<td></td>
<td>2 apoLP-Gln II</td>
<td>1 apoLP-Gln II</td>
</tr>
<tr>
<td>Total length of helical regions⁶</td>
<td>1507 A</td>
<td>1060 A</td>
</tr>
<tr>
<td>Area covered by equivalent cylinder (see text)</td>
<td>1.81–2.71 × 10⁴ A²</td>
<td>1.27–1.91 × 10⁴ A²</td>
</tr>
<tr>
<td>Area available for protein (from table 2)</td>
<td>2.06–2.30 × 10⁴ A²</td>
<td>1.57–1.67 × 10⁴ A²</td>
</tr>
</tbody>
</table>

⁶ Length of helical protein was based on the following data: apoLP-Gln I, 246 amino acids [4], 83% helix when relipidated [7]; apoLP-Gln II, 154 amino acids [5], 61% helix when relipidated [7]. The length of an α helix was assumed to be 1.5 Å per residue in helical conformation.

lipid. Protein helices are quite rigid and would not be expected to bend enough to accommodate the 40–50 Å radius of HDL. However, in apoLP-Gln I and apoLP-Gln II, there appear to be enough proline residues to break the helical structure into sections small enough to fit piece-wise around a sphere with a 40–50 Å radius.

The estimates (table 2) of the proportion of HDL surface which can be covered by polar lipid have shown that more than 60% of the surface is probably covered by protein. HDL₃ in particular can have only a small portion (about 20%) of their surface covered by lipid. Hence protein–protein interactions between helical regions, non-helical regions, or between helical and non-helical regions are thus highly probable and would be expected to play a significant role in HDL₃ structure. Although details of the interaction are not known, recent studies [10] have shown that apoLP-Gln II markedly affects the lipid binding properties of apoLP-Gln I. Studies in our laboratory on the susceptibility of HDL to degradation by guanidine-HCl also point to protein-protein interaction as an important factor in HDL stability.

IV. Core structure

Based on the above model where the surface of HDL is considered to be divided into areas of polar lipid and helical protein, a volume filling model for the whole HDL can be described. It is convenient to divide the HDL into three regions: a polar surface, including the protein and the polar part of the phospholipids; an outer hydrophobic core, extending a distance of 20 Å from the polar surface to the ends of the phospholipid chains; and an inner hydrophobic core from the ends of the phospholipid acyl chains to the center of the lipoprotein. The volumes of these regions can be estimated based on the numbers and volumes of the various components (table 4).
Table 4
Volumes of surface and core regions of HDL.

<table>
<thead>
<tr>
<th>Volume</th>
<th>HDL2</th>
<th>HDL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume</td>
<td>$5.24 \times 10^5 , \text{Å}^3$</td>
<td>$3.68 \times 10^5 , \text{Å}^3$</td>
</tr>
<tr>
<td>Polar surface volume$^a$</td>
<td>$2.23 \times 10^5 , \text{Å}^3$</td>
<td>$1.48 \times 10^5 , \text{Å}^3$</td>
</tr>
<tr>
<td>Outer hydrophobic core volume$^b$</td>
<td>$2.59 \times 10^5 , \text{Å}^3$</td>
<td>$1.15 \times 10^5 , \text{Å}^3$</td>
</tr>
<tr>
<td>Inner hydrophobic core volume$^c$</td>
<td>$0.42 \times 10^5 , \text{Å}^3$</td>
<td>$0.05 \times 10^5 , \text{Å}^3$</td>
</tr>
</tbody>
</table>

$^a$ Protein volume + volume of phospholipid polar heads. Protein volume was estimated assuming 0.74 ml/g for the partial specific volume. The volume of the phospholipid polar heads was estimated assuming $340 \, \text{Å}^3$ for the volume of a polar head group [18].

$^b$ Total volume - polar surface volume - inner hydrophobic core volume.

$^c$ $V = \left( \frac{3}{4\pi} \left( \text{total volume} - \text{polar surface volume} \right) \right)^{1/3} - 20 \, \text{Å}^3$.

Fig. 1. The space under a helical protein region and between the acyl chains of phospholipid molecules (a) available to accommodate apolar lipid, and (b) filled with the sterol moiety of a cholesteryl ester molecule. The components are drawn from projections of CPK models.
Table 5
Calculated volume available for apolar lipid.

<table>
<thead>
<tr>
<th></th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer hydrophobic core volume (from table 4)</td>
<td>$2.59 \times 10^5 \text{Å}^3$</td>
<td>$1.15 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Phospholipid acyl chain volume a</td>
<td>$1.40 \times 10^5 \text{Å}^3$</td>
<td>$0.56 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Cholesterol volume b</td>
<td>$0.27 \times 10^5 \text{Å}^3$</td>
<td>$0.11 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Volume available for apolar lipid c</td>
<td>$0.92 \times 10^5 \text{Å}^3$</td>
<td>$0.48 \times 10^5 \text{Å}^3$</td>
</tr>
</tbody>
</table>

a The volume of the acyl chains of a phospholipid molecule was assumed to be $930 \text{Å}^3$ [18].
b The volume of a cholesterol molecule was assumed to be $600 \text{Å}^3$.
c (outer hydrophobic core volume) – (phospholipid acyl chain volume + cholesterol volume).

The outer hydrophobic core contains the phospholipid acyl chains, the cholesterol, and the side chains of the hydrophobic amino acids. The hydrophobic amino acid side chains (1–6 Å in length) are not as long as the acyl chains of the phospholipids (about 20 Å in length); and therefore under each protein there is an available hydrophobic region (fig. 1). We propose that this region can accommodate some of the apolar lipid of the HDL. The volume available for apolar lipid under the protein and between the phospholipid acyl chains can be estimated by subtracting the volume of the phospholipid acyl chains plus the cholesterol from the total volume of the outer hydrophobic core (table 5).

The triglyceride and the cholesteryl ester of HDL would thus be accommodated within the inner hydrophobic core and the volume of the outer hydrophobic core available for apolar lipid. A comparison of the total volumes of the triglyceride and cholesteryl ester molecules actually present in HDL with the calculated volumes of

Table 6
Volumes associated with apolar lipid.

<table>
<thead>
<tr>
<th></th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride volume a</td>
<td>$0.33 \times 10^5 \text{Å}^3$</td>
<td>$0.11 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Cholesteryl ester volume b</td>
<td>$1.00 \times 10^5 \text{Å}^3$</td>
<td>$0.40 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Total apolar lipid volume</td>
<td>$1.33 \times 10^5 \text{Å}^3$</td>
<td>$0.51 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Volume within outer hydrophobic core available for apolar lipid</td>
<td>$0.92 \times 10^5 \text{Å}^3$</td>
<td>$0.48 \times 10^5 \text{Å}^3$</td>
</tr>
<tr>
<td>Inner hydrophobic core volume</td>
<td>$0.42 \times 10^5 \text{Å}^3$</td>
<td>$0.05 \times 10^5 \text{Å}^3$</td>
</tr>
</tbody>
</table>

a Assuming $1600 \text{Å}^3$ for the volume of a triglyceride molecule.
b Assuming $1090 \text{Å}^3$ for the volume of a cholesteryl ester molecule.
Fig. 2. The apolar lipids, cholesteryl ester and triglyceride, in possible conformations and orientations with respect to the polar lipoprotein surface. The folded compact conformation of cholesteryl ester and the extended "tuning fork" conformation of triglyceride are shown. The figures are drawn from projections of CPK models.

the regions into which they must be packed is presented in Table 6. It is readily seen that in HDL3 almost all of the apolar lipid (> 94%) is readily accommodated within the outer hydrophobic core, that is, under the protein helices and between the acyl chains of the phospholipids; but, in contrast, in HDL2, the outer hydrophobic core can accommodate about 64% of the apolar lipid and hence the inner hydrophobic core of HDL2 must accommodate a substantial portion of the apolar lipid.

The packing of the apolar lipids into the core regions of HDL is constrained by the geometry of the core regions and the possible conformations of the lipids. The apolar lipids can adopt a variety of conformations and orientations with respect to the HDL surface (fig. 2). The cholesteryl esters, if extended, can be oriented with the sterol moiety either toward or away from the surface, or the acyl chain of the cholesteryl ester can be folded over the sterol moiety giving a compact conformation. Similarly, the triglyceride can be either in a compact conformation with all of the acyl chains pointing in one direction, or in an extended conformation with one chain pointing opposite to the other two. Since there are at least three times as many cholesteryl ester molecules as triglyceride molecules in HDL, we will concentrate exclusively on the cholesteryl esters and discuss possible packing arrangements for these molecules in the HDL species.

The packing of cholesteryl ester into a hydrophobic core of HDL2 has been considered by Muller et al. [11] who have suggested that the sterol moieties of the cholesteryl esters in HDL2 are in the center-most region, the inner hydrophobic...
core as described here. They point out, however, that their X-ray scattering data do not have sufficient resolution to specify the arrangement of the lipid. Smith and Green [12] have recently reported energy transfer data comparing quenching of protein tryptophan fluorescence by cholesterol and cholesteryl ester analogs. If HDL protein is located on the surface of the lipoprotein, as in the model discussed here, the data of Smith and Green [12] are not consistent with the Müller et al. [11] suggestion since a central location of the sterol moiety of the cholesteryl ester would result in less energy transfer than was observed in these fluorescence studies.

Applying our volume-filling model, we calculate that the volume of the inner hydrophobic core of HDL$_2$ (about $0.42 \times 10^5$ A$^3$) is close to the calculated total volume of the HDL$_2$ cholesteryl ester acyl chains ($0.46 \times 10^5$ A$^3$) and is definitely smaller than the total volume of the corresponding sterol moieties ($0.54 \times 10^5$ A$^3$). It is therefore likely that in HDL$_2$, the sterol moieties of the cholesteryl esters are in the outer hydrophobic core and their associated acyl chains fill the inner hydrophobic core.

The packing of apolar lipid into the hydrophobic core regions of HDL$_3$ must be different from the packing into the core regions of HDL$_2$ since the volume of the inner hydrophobic core of HDL$_3$ ($0.05 \times 10^5$ A$^3$) is not at all sufficient to contain the acyl chains of the HDL$_3$ cholesteryl esters (total volume = $0.18 \times 10^5$ A$^3$). If all of the acyl chains of the cholesteryl esters were fully extended, 37 chains would have to traverse the inner hydrophobic core. This is an impossible packing problem since the inner hydrophobic core is not large enough (radius $\sim 10$–11 Å) to accommodate more than about 10 of the chains. However, if the acyl chains of the cholesteryl esters were folded over the sterol moieties giving a compact conformation to the cholesteryl esters, it would be possible to pack most of the apolar lipid of an HDL$_3$ into the outer hydrophobic core (i.e., under the helical protein regions and between the acyl chains of the phospholipid). These considerations suggest that one of the major structural differences between HDL$_2$ and HDL$_3$ might be the conformation of their cholesteryl esters. In HDL$_2$ the cholesteryl esters would be predominately in a compact, folded conformation, and in HDL$_2$, in an extended conformation.

HDL$_3$ (but not HDL$_2$) is known to serve as a substrate for lecithin: cholesterol acyltransferase (LCAT) [13], and the compact conformation of cholesteryl ester in HDL$_3$ might directly result from LCAT activity. At the HDL$_3$ surface, substrate cholesterol molecules are probably situated between phospholipids with the cholesteryl hydroxyl groups near the phospholipid acyl ester bonds. Transesterification of cholesterol in situ could therefore lead to product cholesteryl esters initially in the compact folded conformation.

The HDL model presented here indicates that triglyceride and cholesteryl ester may play similar structural roles in HDL since both are considered to lie beneath the helical protein regions and between the phospholipid acyl chains and thus they might be interchangeable. Supporting this are observations that during incubation of mixtures of lipoproteins with LCAT, triglyceride transfers from very low density
Fig. 3. Cross sections of an HDL2, and an HDL3, A and B, respectively. In these schematic representations, the various components are drawings of projections of CPK models and illustrate the proposed packing of the lipids and proteins into HDL in the model discussed in the text. The proportions of lipids and proteins are not accurate although surface perimeters are approximately divided into lipid regions and protein regions in the correct proportion. The non-helical regions of the proteins have not been depicted.
lipoproteins (VLDL) to HDL and cholesteryl ester transfers from HDL to VLDL [14]. This interchangeability has also been observed in lipoprotein reassembly studies using HDL apoproteins and phospholipids where either cholesteryl ester or triglyceride are appropriate apolar lipids for the reconstitutions of spherical high density particles; in the absence of triglyceride or cholesteryl ester, discs and other forms are observed [15].

V. Summary and conclusions

The proposed model (fig. 3) specifies the positions of the proteins and each of the lipid components. The proteins are arranged such that their helical portions are on the surface surrounded by phospholipids. Under the proteins are triglycerides and the sterol moieties of the cholesteryl esters. The phospholipids, cholesteryl esters, and triglycerides have acyl chains extended toward the center of the spherical HDL and, in HDL2, the inner hydrophobic core is filled with the acyl chains of the cholesteryl esters. The unesterified cholesterol molecules are intercalated between the phospholipids in the manner characteristic of mixed phospholipid-cholesterol bilayers.

We have discussed compositional and geometric restrictions on the packing of lipids and proteins into spherical lipoproteins. We have considered both the division of the surface area between amphiphilic proteins and lipids, and the packing of apolar lipids into the interior. Our calculations suggest that the apolipoproteins on the surface of HDL are probably not in a spherical globular conformation because such a conformation is not consistent with the size and composition of HDL. In contrast, models of HDL with the helical regions of the apolipoprotein extended across and embedded in the surface are consistent with the known size and composition. The proposed surface structure leads to models for HDL2 and HDL3 with specific arrangements for the apolar lipids in the HDL interior. The models describe the locations of each of the components of HDL and suggest that the conformation of the cholesteryl esters is different in HDL2 and HDL3. While it is improbable that all of the details of the proposed arrangement of lipid and protein in HDL are correct, the models are sufficiently specific to be further tested.

Acknowledgements

This work was supported in part by National Institutes of Health Research Grant HL 10878, USPHS Training Grant 5 TO1 GM00829, and the U.S. Atomic Energy Commission.
References

[16] T. Forte and A.V. Nichols, Advan. Lipid Res. 10 (1972) 1
3. Crosslinking of HDL Apoproteins in situ: Implications with Regard to HDL Structure

The model calculations presented in Appendix 2 suggested that apoproteins must cover most of the surface of HDL. It was calculated that only 26%-34% of the surface of HDL₂ could be covered by lipid; while only 17%-22% of the surface of HDL₃ could be covered by lipid. Thus it appears that there is not enough lipid to keep the apoproteins on a particular HDL particle from close proximity to each other.

In order to test this conclusion, an attempt was made to crosslink the apoproteins on the surface of HDL. Crosslinking was carried out using the bifunctional reagent dimethyl suberimidate, which has about 10 Å between reactive groups. HDL were crosslinked by the method of Davies et al (128) and delipidated by mixing with tetramethyl urea (92). The crosslinked apolipoproteins were analyzed by electrophoresis on a 7.5% polyacrylamide slab gel containing sodium dodecyl sulfate (SDS) (129).

Figure 40 shows the results of electrophoresis of crosslinked HDL and crosslinked A-I. Lanes 1 and 4 show the electrophoresis pattern of A-I without crosslinking. A single major band is seen in these lanes. Lanes 2 and 3 show the pattern of crosslinked A-I. High molecular weight products of crosslinking A-I are clearly seen. This shows that A-I was probably present as multimers in the reaction mixture.

Lanes 6-9 show the electrophoresis pattern of HDL without crosslinking. Several bands, including a prominent band near that of isolated A-I are evident. Lanes 11-13 show the pattern of electrophoresis
of crosslinked HDL. Several bands, not present in the pattern from uncrosslinked HDL, are present. These bands represent high molecular weight products of crosslinking the apoproteins of HDL. Their formation by crosslinking HDL with dimethyl suberimidate suggests that the apoproteins on HDL are closer than 10 Å to each other. Analytic ultracentrifugation and electron microscopy showed no major changes in the structure or size of HDL caused by such crosslinking. The crosslinked apoproteins were therefore probably on the same HDL particle.

Several bands in the electrophoresis pattern of crosslinked HDL appear similar to bands in the pattern of crosslinked A-I. It therefore appears that the A-I molecules in HDL can be crosslinked together. One band present in the crosslinked HDL pattern is clearly not evident in crosslinked A-I. This band probably represents a dimer consisting of an A-I molecule crosslinked with an A-II molecule.

Dimethyl suberimidate crosslinking therefore shows that the apoproteins on the surface of a HDL particle are closer to each other than 10 Å. This evidence supports the model of the surface structure of HDL as described in Appendix 2. In addition, formation of dimers of A-I and A-II show that these apoproteins are both on the same HDL molecule.

Since these experiments were begun, a report containing the same basic conclusions has appeared (130).
Figure 40  SDS polyacrylamide slab gel electrophoresis of A-I, and HDL before and after crosslinking with dimethyl suberimidate. There was no gradient of polyacrylamide concentration which was uniformly 7.5%. Lane 1, A-I without crosslinking agent. Lanes 2 and 3, A-I after crosslinking with dimethyl suberimidate. Lane 4, A-I without crosslinking. Lane 5, blank. Lanes 6-9, HDL without crosslinking. Lane 10, blank. Lanes 10-13, HDL after crosslinking with dimethyl suberimidate.
Figure 40
BIBLIOGRAPHY


70 Studies on the conformation of C-II have not been conducted.


94 Nichols, A. V. (1975) private communication.


ACKNOWLEDGEMENTS

I would like to thank each member of Dr. Nichols' research group: Elaine Gong, Pat Blanche, and Charlie Mae Fuller for their various contributions. Each significantly aided my research activities.

Discussions with Drs. Bob Glaeser and Trudy Forte on electron microscopy, and the help of Bob Nordhausen and Francis Taylor in making my micrographs are gratefully acknowledged. Discussions with Dr. N. Keith Freeman were very valuable. Many thanks are due to Dr. Ken Applegate in Dr. John Glomset's lab for running the gradient gels and to Peter Sybert for his help in the crosslinking experiments.

Dr. Alex Nichols' kindness and help were essential for completion of this thesis.

This work was supported in part by USPHS Training Grant 5 TO1 GM00829, NIH Research Grant HL 10878, and the U. S. Energy Research and Development Administration.