LIPOPROTEIN STRUCTURE: APOPROTEIN INTERACTIONS IN HUMAN PLASMA HIGH DENSITY LIPOPROTEINS

By

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DEDICATION

I dedicate this dissertation to my best friend William A. Patterson and the Krenu Construction Company.

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KEY TO SYMBOLS AND ABBREVIATIONS NOT DEFINED IN TEXT

Å	-	angstrom
Apo	-	apoprotein
CD	-	circular dichroism spectroscopy
Ci	-	curie
dpm	-	disintegrations per min.
IR	-	infrared spectroscopy
LCAT	-	Lecithin cholesterol acyl transferase
leu	-	leucine
MW	-	molecular weight
NMR	-	nuclear magnetic resonance spectroscopy
ORD	-	Optical rotory dispersion
val	-	valine

Abstract of Dissertation Presented to the Graduate Council
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Chairman: Melvin Fried
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The modes of association of the major apoprotein species of human plasma high density lipoprotein (HDL) was investigated using bifunctional crosslinking reagents. How these associations help to govern the structure of the HDL subclass, HDL_2 and HDL_3, was studied by observing both apoprotein and lipid exchange between HDL particles.

Crosslinking of HDL with a 5 to 10-fold molar excess of difluorodinitrobenzene resulted in the production of high molecular weight crosslinked products with the major product shown to be the combination of the two major apoprotein species ApoA-1 and ApoA-2. The composition of this product was confirmed by amino acid analysis.

Crosslinking with other bifunctional reagents indicates specificity of initial reaction sites as well as some freedom of movement of the apoproteins within the particle. However, the crosslinking patterns obtained with the HDL subclasses were identical, suggesting specific structural properties of HDL_2 and HDL_3.

Apoproteins, labeled with ^{125}I, were shown to exchange between and among the HDL subclasses. This bidirectional exchange process was

inhibited by crosslinking with bifunctional reagents and appeared to depend upon collision complex formation. By the utilization of Sepharose bound HDL it could be shown that both free apoprotein molecules, as well as subunits consisting of lipid-apoprotein combinations, could exchange between HDL_2 and HDL_3. The exchange appears to involve a conformational change in the apoproteins during the transfer process.

Radioactive cholesterol, cholesterol palmitate, and phosphatidyl choline, bound to HDL after adsorption to celite particles, could be shown to undergo rapid exchange between HDL particles. This exchange also appears to depend on collision complex formation but was not inhibited by chemical crosslinking of the apoproteins.

The rate of these exchange processes is significant in the lifetime of the lipoprotein particles in vivo: 2.5%/hour for apoprotein exchange and >50%/hour for lipid exchange.

It was concluded that ApoA-1 and ApoA-2 must lie very close to one another in the intact lipoprotein particle. The reproducibility of the crosslinking experiments on HDL obtained from several donors suggests that in the intact HDL particle the apoprotein components may be found in a rather fixed spatial orientation to one another. It is also suggested that there is a dynamic relationship between HDL_2 and HDL_3. Even though these two subclasses can exist as stable separate entities, when they are present together in solution, significant interaction between particles may occur. Exchange of lipids and apoproteins occurs between HDL_2-HDL_2 and HDL_3-HDL_3, as well as between HDL_2 and HDL_3 molecules.

It is suggested that the interconversion of HDL_2 and HDL_3 may be determined by the availability of certain lipids.

INTRODUCTION

Plasma Lipoproteins

Plasma lipoproteins play an important role in mammalian energy metabolism and also serve as the major transport particles for many physiologically important molecular species. It has been suggested that they are causally related to atherosclerosis since they are the carriers for those substances deposited in the atheromatous plaque. Circulatory system pathology found in hyperlipoproteinemics may result from quite small alterations in lipoprotein structure; differences that may be due to altered protein-protein or protein-lipid interaction.

Human serum contains four major types of lipoproteins: chylomicrons (CHYL), very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Although these four classes appear to differ in both lipid and protein composition as well as function, there are distinct interrelationships between the particles. The presence of one class directly influences the other classes (1).

Chylomicrons, formed in the intestinal mucosa and released into the blood from the major lymphatic duct, function as the carrier of many lipid types. Lypolytic enzymes remove lipids from these particles as they circulate. VLDL and LDL appear to be closely related as LDL may be the result of VLDL delipidation (2). LDL functions as the major cholesterol carrier in blood. HDL can be divided into two

subgroups; HDL_2 and HDL_3. HDL may be important in cholesterol deposition disease (3).

The isolation of lipoproteins from serum or plasma is relatively simple and may be accomplished by flotation in the ultracentrifuge. As the density (d) of serum lipoproteins ranges from approximately 0.95 gm/cm^3 to 1.21 gm/cm^3, centrifugation of serum, the density of which has been raised to 1.21 gm/cm^3 by the addition of salt, will result in the lipoproteins floating to the top of the centrifuge tube while the other proteins sink (4). Each lipoprotein class is characterized by its own density range for isolation: CHYL and VLDL < 1.006, LDL 1.006 - 1.063, and HDL 1.063 - 1.21. By stepwise increases in density (4), or by density gradient centrifugation (5), these lipoprotein classes may be prepared in relatively pure form. Although other methods such as molecular sieve chromotography (6) and chemical precipitation (7) have been described, the ultracentrifugal floatation method remains the most widely used.

The protein and lipid compositions of these lipoprotein classes have been studied and the following have been reported (1,8) (all percentages on a dry weight basis).

HDL

HDL, whose molecular weight ranges from 180,000 to 350,000, consists of approximately 50% protein, 22% phospholipids, 3% free cholesterol, 14% cholesterol esters, and 8% triglycerides. Of the phospholipid portion, 70% is phosphatidylcholine, 14% is spingomyelin, 5% phosphatidyl serine and phosphatidyl ethanolamine, and the remainder other minor phospholipids. The protein portion of the HDL particle

consists of two major polypeptides, ApoA-1 (ApoGln-I) and ApoA-2 (ApoGln-II). The amino acid sequences of both polypeptides are known (9,10,11) and ApoA-2 is known to be a dimer of two identical subunits connected by a single disulfide bridge at position 6 (10). Another group of smaller proteins, designated ApoC or the C-peptides, comprises approximately 5% of the total HDL protein. At least three C-peptides, C-I, C-II, and C-III, are recognized in HDL (2). The protein of HDL contains 3-4% covalently bound carbohydrate including glucosamine, fructose, galactose, mannose, and sialic acid (8). In addition to the C-peptides, HDL also may contain the "thin-line" protein and "arginine rich" protein as minor components (1).

LDL

LDL particles are larger than HDL with molecular weights ranging from 2 to 3 million. They also contain a larger percentage of lipid than HDL (75-78%); consisting of 36-42% cholesterol esters, 21-23% phospholipids, 8-9% free cholesterol, and 6-8% triglycerides. Other lipids may be present in smaller amounts. The proteins make up 20-22% of the particle. The major polypeptide is denoted apoprotein B (ApoB), a large protein of molecular weight 225,000. There may also be very small amounts of the C-peptides described for HDL. Again, 3-5% carbohydrate is found covalently associated with the protein.

VLDL

VLDL particles cover a wide range of densities and molecular weights ($3 - 128 \times 10^6$) with at least two maxima at densities of 0.980 and 0.958 gm/cm^3. They contain 90% lipid with an average composition of 50-60% triglycerides, 10-12% free cholesterol, 4-6% cholesterol

esters, and 18-20% phospholipids. Protein represents only 8-12% of which 40% is apoprotein B and the remaining 60% is divided among the three C-peptides described for HDL. A small portion of ApoA may also be present and it has been demonstrated that different fractions may contain different amounts of apoproteins (2).

Chylomicrons

Chyl contain mostly lipid with only 1-2% protein. These very large particles have molecular weights of 5 - 4300 x 10^8. The protein consists of all types of previously described apoproteins found in the other lipoprotein classes. It has been shown that human lymph chylomicrons contain approximately 66% ApoC, 22% ApoB, and 12% ApoA. The lipids are predominately triglycerides (80%) with only about 8% phospholipids and 5% cholesterol (1,2).

It is important to note the overlapping protein composition in all of these classes. It is presumed that the specific protein composition of each is important in determining its total lipid content and the specific type of particle that is formed. These proteins should play a very important role in determining the function of the particle as well (1).

Apoprotein Structure and Function

ApoC

ApoC-I has been sequenced (12) and is a single polypeptide chain of 57 amino acid residues (MW 8000). ApoC-I is known to function in lipid binding as well as LCAT activation (13). Lipoprotein lipase also appears to be activated by ApoC-I (14).

ApoC-II is the largest of the C-peptides with 100 amino acid residues and a molecular weight of about 12,500 (15). It also binds lipids and is a potent activator of lipoprotein lipase (16).

ApoC-III, a single polypeptide of 79 amino acid residues has also been sequenced (17). Attached to threonine-74, ApoC-III contains an oligosaccharide moiety consisting of galactose, galactosamine, and sialic acid (17). Although no direct physiological role is yet known, ApoC-III has been used often for the study of lipid-protein interactions (18-21).

ApoB

The characterization of ApoB has led to many conflicting statements concerning the number and size of the polypeptide subunits. Due to technical problems associated with isolation of a soluble apoprotein, a variety of physical methods have indicated molecular weights ranging from 8,000 to 275,000 (22). However, most investigators support the idea of a large (275,000) molecular weight peptide. These problems have also hindered studies on specific functions of ApoB and as yet no definite physiological role other than lipid binding has been discovered.

ApoA

ApoA is a term used to describe the two most extensively studied apolipoproteins, ApoA-1 and ApoA-2. ApoA-1, a single polypeptide of molecular weight 28,330, contains 245 amino acid residues and has been sequenced (8,23,24). Although the binding of phospholipids by ApoA-1 has been studied by several investigators, conflicting results on lipid binding in the absence of ApoA-2 have been reported (25,26,27). These

differences in the extent of lipid binding by ApoA-1 may be due to self-association of the polypeptides that may mask lipid binding sites (28). Self-association has been demonstrated by both ultracentrifugal and chemical techniques (1).

ApoA-2 is a polypeptide dimer of two identical chains with a molecular weight of 17,500 (29). Each chain contains 77 amino acid residues. The disulfide bond occurs between the single cysteine residues that occur at position 6 (29). The disulfide linked dimer appears to be characteristic of only man and chimpanzee; the dimer form apparently is not crucial for ApoA-2's lipid carrying role (1). ApoA-2 does bind lipid strongly and may increase the binding capacity of ApoA-1 as well (2).

Although lipid binding is the only role as yet assigned to ApoA-2, ApoA-1 appears to have several functions other than lipid binding in the HDL particle. ApoA-1 serves as an activator of LCAT (30), may be an acceptor of cholesterol and cholesterol ester during VLDL catabolism (2), and appears to have some function in regulating the lipid content of membranes (31,32).

Lipoprotein Structure

The study of lipoprotein structure has been approached utilizing three basic types of techniques: 1) spectroscopic methods (IR, CD, ORD, X-ray, neutron scattering, NMR, and fluorescence); 2) physical techniques (Ultracentrifugation and electron microscopy); and 3) chemical methods (chemical modification, amino acid analysis, bifunctional reagents, reassembly). The actual composition of lipoprotein particles has been fairly well described, as indicated above, and only in the

case of LDL is there significant disagreement on Apoprotein size and composition (1,8). However, when three-dimensional structure is considered, little is known at present regarding specific protein-protein or protein-lipid spatial relationships or interactions in intact lipoprotein particles.

VLDL and Chyl have proved difficult to study due to the significant turbidity of their solutions. This fact has limited the use of spectroscopic methods and as a result only the techniques of ultracentrifugation, electron microscopy, and gel filtration have been used. This, coupled with the fact that these classes consist of a spectrum of a wide variety of sizes with differing compositions, has prevented all but the most basic of structural studies. Ultracentrifugation techniques indicate the following: Chyl - MW 5-4300 x 10^8 daltons, diameter 1200-11000 Å, hydrated density 0.93gm/cm^3. VLDL- MW 3-128 x 10^6 daltons, diameter 300-700 Å, hydrated density 0.93 - 1.0gm/cm^3 (1). The only structural determinations made thus far for these particles are based on the surface activity of the protein components which seems to indicate that some portion of the proteins are located on the surface of the particle (2). The information now available has led to the suggestion of a lipid-core model for the triglyceride rich Chyl and VLDL particles. This model assumes that the surface of the particle is occupied by phospholipids, cholesterol, and proteins, while the central core consists of the less polar and more hydrophobic triglycerides and cholesterol esters (33). The quantities of protein, phospholipid and cholesterol in these particles appear to be sufficient to cover the surface of the particles as predicted (34).

LDL structure has been studied by many of the different techniques mentioned earlier (2,8,35,36,37,38). Data from electron microscopy and X-ray diffraction indicate a spherical particle with neither well defined nor significant subunit structure (2). Information from analytical ultracentrifugation, as well as X-ray and electron microscopy, give the following information: MW 2.3-2.7 x 10^6 daltons, hydrated density 1.028-1.034gm/cm^3, diameter 216-220 Å. In distinction to Chyl and VLDL, LDL is rather uniform in size and composition. The protein component appears to have about 25% α-helix, 37% β-structure, and 37% random coil as determined by CD (38). Although this structure appears to be temperature dependant, a recent report indicates the CD spectrum may be greatly complicated by the lipids present in the particle (39). Both NMR and fluorescence studies indicate that the protein, although near the surface of the LDL particle, is highly associated with the lipid components of the molecule (36,37). It has also been suggested that the core of LDL is mostly composed of hydrocarbon chains while the outer layer of the particle is sparsely occupied by proteins that emerge from the lipid core (35); a model similar to the lipid-core model for VLDL. Other investigators, utilizing electron microscopy and X-ray scattering, have suggested a significant degree of subunit structure that includes many protein subunits (20-60 with MW 8,000 or 27,000) arranged with icosahedral symmetry. The protein subunits give a symmetrical appearance with the surface area occupied by both protein and phospholipid (40,41). These subunit models do not take into consideration recent findings that indicate the major protein component consists of only two polypeptides of molecular weight 250,000 or greater (42).

HDL has been studied most extensively, probably because it is the easiest of the particles to work with. As noted above, its protein composition is well known and the major polypeptides have been sequenced. HDL can be divided into two subclasses, HDL_2 and HDL_3, which appear to differ mainly in their molecular weights ($HDL_2 \tilde{=}$ 184,000) (1). X-ray studies have indicated that HDL consists of two regions of differing electron densities: an outer shell and an inner core. The inner core has a radius of approximately 43 Å in HDL_2 (37 Å in HDL_3) while the outer shell radius is 14 Å (11 Å in HDL_3). The electron density of the outer shell indicates that is consists of polar lipid headgroups and proteins (43). The size of a fully extended phospholipid headgroup is about 11 Å, a value that fits in well with the X-ray data. Fluorescence techniques yield information on porcine HDL_3 consistent with a peripheral location of the protein component, with the suggestion that as much as 80% of the protein is located at or near the surface (37). This hypothesis is further supported by the finding of Scanu that 90% of the lysine residues of the apoprotein components of human HDL is accessible to succinylating agents (2). This would indicate that most of the protein is near the surface as there are many lysines rather evenly distributed throughout the protein chains.

CD of the protein portions of HDL indicate 70% α-helix, 11% β-structure, and 19% random coil (38). The removal or alteration of lipid components greatly affects the structure of HDL apoproteins. Delipidation lowers the helicity to 52%. Delipidation followed by relipidation with egg phosphatidyl choline changes α-helicity to 64%

while the addition of cholesterol further increases the helicity to 70% (38). These observations indicate strong lipid-protein interactions.

Two basic models of HDL that are consistent with spectroscopic and chemical data have been proposed. Assman, et al. and Assman and Brewer (22,44) have suggested a model similar to the membrane structure proposed by Singer and Nicolson (45) in which the HDL protein is depicted as an "iceberg" floating in a "sea of lipid." This model takes into account the fact that portions of the helical regions of the apoproteins are two-sided; that is, one side of the helix is polar while the other is non-polar (46). This amphipathic organization of the helix could allow simultaneous interaction of the helix with lipid, protein, and the aqueous medium. Whether or not the helices are oriented perpendicular or parallel to the surface of the particle is the essential difference between this model of Assman, et al. and Assman and Brewer (22,44) and that of Jackson, et al. (25). Jackson has suggested that the long axes of the helical regions are oriented perpendicularly to the fatty acyl chains of the phospholipids. In this model, carbon atoms 2-4 of the fatty acyl chains could interact with the non-polar side of the helix, allowing the hydrophyllic portions to interact with the medium or other protein chains. There is evidence to suggest that little electrostatic interaction occurs between phospholipid polar headgroups and the charged amino acid residues of intact lipoproteins (22,47,48).

Little is known of the protein-protein interactions that may occur in intact lipoproteins. The only studies reported have been carried out on human HDL apoproteins <u>in vitro</u>. It has been shown that if one mixes the HDL apoproteins A-1 and A-2 together in equimolar amounts,

they appear to associate into an aggregate of molecular weight 46,000 (28). This A-1:A-2 complex will bind less lipid than would be expected, indicating that some of the lipid binding sites are blocked by protein-protein interaction. Other studies indicate that in the absence of lipids and other apoproteins, both A-1 and A-2 will self-associate (49-53). Although such studies help to understand the types of interactions that may occur, only work done on the intact particle can shed light upon the actual associations and interactions that govern the final structure and function of the lipoproteins.

Recent evidence from Friedberg and Reynolds (54) suggests that A-1 and A-2 always exist in a molar ratio of 2 A-1 polypeptides to 1 A-2 polypeptide dimer in human HDL. Their study also suggests that this ratio of 2:1 is constant from individual to individual as well as from subclass to subclass (HDL_2 to HDL_3). This would indicate that perhaps, for ApoA-1 and ApoA-2, a specific polypeptide composition is found in HDL_2 (4 A-1 + 2 A-2) as well as in HDL_3 (2 A-1 + 1 A-2).

It is evident that much is yet unknown concerning the structure of lipoproteins and that experimental evidence derived from studies utilizing intact particles and techniques that allow precise definition of spatial orientation is needed.

Alternative Classification of Lipoproteins

It should be noted at this point that another method of classification for plasma lipoproteins has been suggested by Kostner and Alaupovic (55). Based on immunochemical evidence, they have suggested that lipoproteins occur in separate families, the composition of which is determined by the presence of specific apoproteins. The three

families they describe, LP-A, LP-B, and LP-C, represent particles containing ApoA-1 and/or ApoA-2 (LP-A), ApoB (LP-B), and ApoC-I-III (LP-C). The important difference between the two methods of classification is that, according to Kostner and Alaupovic, a physically defined density class, such as HDL, may contain not only particles with just ApoA-1 and ApoA-2 but also particles with only ApoB and only ApoC. One should realize, however, that HDL isolated as a density class will contain mostly LP-A and LP-C with a very small amount of LP-B (2%). This LP-B is only found associated with the HDL_2 density subclass suggesting the possibility of contamination by remnant LDL particles. Even the presence of particles containing only LP-C could be an artifact resulting from the generation of incomplete or altered particles during the extensive ultracentrifugation required for the isolation of the HDL density class. In any case, the actual difference between HDL as a density class and LP-A as described by these investigators would only be the presence of the small amount of C peptides generally believed to be associated with the HDL molecule. Although the possibility of lipoprotein families is a question that merits consideration, the results of the experiments described herein would apply to either model of the lipoprotein particles.

Exchange Reactions of Lipoproteins

Plasma lipoproteins are known to be a very dynamic population of macromolecules. They are continuously being synthesized and degraded at a high rate. The amount of any individual lipoprotein class present at a given time is determined by the balance between synthesis and catabolism. The individual components of lipoproteins, however, are

not degraded or synthesized at the same rate. This is due to the fact that both lipid and protein components can be exchanged between individual lipoprotein molecules as well as between different classes of lipoproteins (1).

Lipid Exchange

Phospholipids appear to exchange between all classes of serum lipoproteins at rates dependent upon their relative concentrations in the individual classes (56,57). When labeled phospholipids are incorporated into an individual class of lipoproteins and this class is allowed to interact with other lipoprotein classes *in vivo* or *in vitro*, an exchange reaction with equilibrium, reached in 4 to 5 hours, is observed (58). This exchange is independant of any protein exchange and occurs at significantly different rates for different phospholipid types. Lysolecithin exchanges most rapidly, followed by lecithin and sphingomyelin (57,59). No significant differences are observed for subclasses of an individual phospholipid class (59). This exchange can be increased *in vitro* by the presence of a phospholipid exchange protein (60).

Cholesterol is known to exchange rapidly between all classes of human serum lipoproteins (61). This rapid exchange occurs both *in vivo* and *in vitro* with equilibrium attained after 2 to 6 hours (62,63). Cholesterol ester exchange between plasma lipoproteins *in vivo* and *in vitro* has been both suggested and disputed (62,64,65). There is evidence that seems to indicate that cholesterol esters can be transferred from LDL to other lipoproteins *in vitro* (66).

The exchange of other lipid types has been reported for human as well as other animal lipoproteins. Triglycerides exchange between different classes of lipoproteins (67) as do unesterified fatty acids (68) and α-tocopherol (69)..

Protein Exchange

Although the exchange of lipids does not appear to depend upon the simultaneous exchange of protein, it has been shown that apoprotein exchange does occur. Only ApoC of HDL and VLDL has been shown to exchange *in vitro* and *in vivo* (70,71). This exchange is bidirectional and not like the apparent transfer of protein from VLDL to LDL (2).

Mechanisms of Exchange

The movement of either lipid or protein from one lipoprotein class to another can represent an exchange, a transfer, or the combination of the two processes. It is evident that both processes depend upon the lipoproteins existing in a dynamic state.

The *in vitro* exchange of lipids has been the most studied and the process appears to be a physicochemical one (72). Exchange could occur by at least two mechanisms. First, it has been suggested that exchange occurs when individual lipid molecules escape the lipoprotein particle and enter the aqueous medium. They are then picked up by other lipoproteins or membranes (73,74). This model for exchange lacks strong evidence in its support but may be the mechanism for certain specific lipid types (74).

An alternate mechanism involves the formation of collision complexes between lipoproteins (75). This would allow for diffusion of lipid and/or protein molecules between the particles and at the same time

would not require the thermodynamically unfavorable dissociation of hydrophobic molecules into an aqueous medium.

As both mechanisms have been supported with experimental evidence, it is important that each be considered as a possible explanation for any exchange reaction. Experiments that will help distinguish between the two mechanisms would include the observation of the effect of factors that influence collision rates between molecules, i.e. temperature, dilution, etc.

Although only lipid transfer mechanisms have been postulated, the same types of models should be considered when observing protein transfer or exchange.

Experimental Rational

In studying either protein structure or protein-protein interactions occurrences of natural crosslinking due to disulfide bridges, peptide linkages between lysine ϵ-NH_2 and glutamic or asparatic acid γ or β-COOH, or carbohydrate bridges have been valuable in understanding the final active structure that is being considered, e.g., insulin, ribonuclease, collagen. As such crosslinks are often not found where one might like to find them, the introduction of stable covalent bridges or crosslinks can aid in the study of protein structure (See Ref. (76) for a recent review of bifunctional reagents). Bifunctional reagents are capable of producing three types of crosslinked products: intramolecularly crosslinked proteins, intermolecularly crosslinked homopolymers (identical subunits), and intermolecularly crosslinked protein complexes (nonidentical subunits or different proteins). Such reagents have been used in a variety of ways

to determine such things as the spatial geometry of membrane components (77-81), the mapping of ribosome proteins (82), and the subunit structure of oligomeric proteins (83).

There are two basic types of crosslinking reagents: those that are cleavable and those that are not. The advantage of cleavable crosslinkers is that one may isolate the crosslinked product, cleave the newly formed bridges, and identify the proteins that were crosslinked. This technique is particularly useful when studying the crosslinking of multicomponent systems such as membranes.

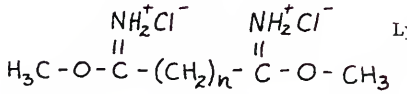
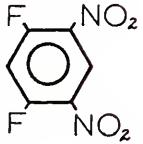
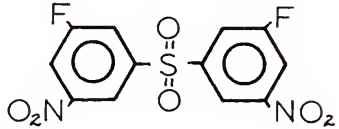
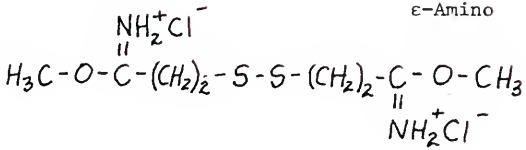
Table 1 lists a selection of different types of crosslinkers and gives certain specific characteristics. The selection of reagents with differing lengths, degrees of hydrophobic character, or reactive group specificity can yield products that differ because of the specific structure or physical environment of the proteins that are crosslinked. For example, in studying the human erythrocyte membrane, Moxley and Tsai were able to produce different crosslinking patterns by varying only the length of the bifunctional reagent used (81).

As all lipoprotein particles are thought to contain two or more polypeptide chains and since the structure and function of these particles may depend to a great extent upon specific protein composition and/or protein-protein interactions, investigation with bifunctional reagents could prove to be a useful method to define some of these interactions.

Because the function and characteristics of individual lipoprotein classes differ, and these differences ultimately must be related to structure, studies dealing with the interactions and associations of

Table 1.

Bifunctional Crosslinking Reagents

	REAGENT	STRUCTURE	REACTS WITH	LENGTH	CLEAVED
1.	Imidoesters (i.e. dimethyl suberimidate)		Lysine, Tyrosine	Variable	--
2.	Dinitro Difluoro-Benzene		Lysine, Tyrosine	5-6 Å	--
3.	Difluoro Dinitro-Diphenyl sulfone		Lysine, Tyrosine	9-10 Å	--
4.	N, N-Di (Bromoacetyl) Phenylhydrazine		SH, ε-Amino (High pH)		Pd + HOAc
5.	Dimethyl Dithiobis propionimidate Dihydrochoride		ε-Amino		β Mercapto-ethanol

the characteristic apoproteins of a given lipoprotein class should prove of value in understanding how these characteristics are determined by the structure.

HDL, the best characterized of the lipoproteins, was used in the experiments described in this report. Its small size, stable structure, ease of isolation and handling, as well as its variety of polypeptides made it the model of choice. Although no definitive physiological role has yet been assigned to HDL, certain other characteristics make this an interesting model for study. C-peptides such as are found in HDL are known activators of LCAT and lipoprotein lipase, and Apo-1 is known to activate LCAT specific for certain substrates (1). Reynolds and Simon (28) have reported altered lipid binding upon associations of the apoproteins of HDL. Perhaps this interaction is a mechanism for regulation of lipid binding. As the different lipoprotein classes appear closely related and even share many of the same components, both lipid and protein, the actual association of the different protein components may play a very important role in regulating both structure and function for the different lipoprotein particles.

Research Objectives

The purpose of this research was to gain insight into the structure of intact serum lipoprotein particles specifically, the high density lipoprotein particle. This was approached by attempting to understand the interactions and associations of the individual apoprotein components of these macromolecules. To aid in organizing the information gathered, the following objectives were set forth:

1. To determine if bifunctional reagents are capable of inducing crosslinks between individual apoproteins in intact HDL

2. To characterize and identify any crosslinked products produced

3. To evaluate the result of the use of crosslinking reagents of differing length and hydrophobic or hydrophyllic character

4. To determine and explain any possible differences in the crosslinking patterns produced from HDL_2 vs. HDL_3

5. To discover if the apoproteins and lipids of intact HDL exchange between the separate HDL subclasses

6. To investigate any possible effects of chemical crosslinking on such exchange processes

7. To gain a greater understanding of the relationships and structures of the two HDL subclasses, HDL_2 and HDL_3.

MATERIALS AND METHODS

Materials

Chemicals

1,5-difloro, 2,4-dinitrobenzene (DFDNB) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co., St. Louis, Missouri. 4,4'-difluoro 3,3'-dinitrodiphenylsulfone (DFDNPS); dimethyl suberimidate dihydrochlorida (DMA); and dimethyl 3,3'-dithiobispropionimidate dihydrocholoride (DTBP) were purchased from Pierce, Rockford, Illinois. Reagents for acrylamide gel electrophoresis were obtained from BioRad Laboratories, Los Angeles, California. All other chemicals used were reagent grade and purchased from common suppliers.

Radioisotopes and Scintillation Materials

[^{125}I]-NaI; [choline-methyl-^{14}C]-phosphatidyl choline, specific activity 50mCi/mmole; [11-12 ^{3}H]-cholesterol, specific activity 60Ci/mmole; [11-12 ^{3}H]-cholesterol palmitate, specific activity 294mCi/mmole; Aquasol-2; and mini-vials were all purchased from New England Nuclear, Boston, Massachusetts.

Blood

Whole human blood was obtained fresh from healthy male donors or as outdated blood units from the Civitan Regional Blood Center, Gainesville, Florida. In no instances were the results of any repeated experiments different depending upon the source of blood.

Methods

Isolation of Lipoproteins

Whole blood was centrifuged for 20 min at 2000 RPM using an IEC Model SBV centrifuge to remove red cells. The clear serum or plasma was removed by decanting and the density then raised to 1.063gm/cm^3 by the addition of solid KBr. This solution was centrifuged for 20-24 hrs. at 42,000 using a Beckman Ti60 rotor in a Beckman model L2-65B ultracentrifuge. After centrifugation, the top 3-5 ml containing CHYL, VLDL, and LDL were removed by aspiration and discarded. The remaining solution was adjusted to density = 1.21gm/cm^3 by the addition of more solid KBr. The serum was again centrifuged for 24 hrs. at 42,000 RPM as above. The top 2-3 ml of each tube were then removed using a Pasteur pipet and pooled. When total HDL (HDL_t) was desired, the pooled lipoprotein solution was further purified by another centrifugation step. This was carried out as above after the crude HDL solution was adjusted to density = 1.063gm/cm^3 by the addition of 0.15M NaCl containing 10^{-5}M EDTA and 0.02% NaN_3. Each tube was underlayered with 5-10 ml of KBr solution of density = 1.21gm/cm^3 before centrifugation. After centrifugation, the top 5 ml of each tube (LDL contamination) was removed by aspiration and discarded. The yellow band in the tube located above the 1.21 density layer was removed using a Pasteur pipet and saved as purified total HDL. This HDL solution was immediately dialyzed against 2 x 4 liters of 0.15M NaCl containing 10^{-5}M EDTA and 0.02% NaN_3, and stored at 4°C until use.

When the separate HDL subclasses, HDL_2 and HDL_3, were desired, the following procedure was used. After the three ultracentrifugation

steps described above, the total HDL was dialyzed against a KBr-NaCl solution of density = 1.125gm/cm^3 containing 0.15M NaCl, 10^{-5}M EDTA, and 0.02% NaN_3. The solution was placed in centrifuge tubes and underlayered with 5 ml. of KBr solution, density = 1.21gm/cm^3; then centrifuged for 36-48 hrs. at 45,000 RPM using a Ti60 rotor. This step resulted in the formation of two separate yellow bands; one at the top of the tube, and one just above the 1.21 plug. The top band was taken as HDL_2 and the lower as HDL_3. The bands were removed using a Pasteur pipet and the region between the bands discarded. The HDL_2 and HDL_3 solutions were then dialyzed against 2 x 4 liters of 0.15M NaCl containing 10^{-5}M EDTA and 0.02% NaN_3, and stored at 4°C until use. The purity of HDL preparations was checked by SDS gel electrophoresis (see next section for reference and details). In no preparation did the contamination by other proteins (usually serum albumin) exceed 1-2% and usually no contamination could be seen at all unless the gels were greatly overloaded.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out using a Model 300B or 301 BioRad Electrophoresis Cell powered by a BioRad Model 400 Power Supply. Two types of gel formulation systems were used. First, gels were prepared using the procedure of Lammli (84). The acrylamide concentration of the separating gel was adjusted to 10-12% by alteration of the ratio of acrylamide to water during gel formulation. SDS concentration was maintained at 0.1% and for 5 x 100mm gels electrophoresis was carried out at 1.5ma/gel. Bromophenol Blue was used as a tracking dye and electrophoresis continued until the dye band was approximately

1cm from the bottom of the gel. Gels were fixed in 12.5% TCA: 40% ethanol: 7% acetic acid, stained with 0.05% Coomassie Blue in 10% ethanol: 7% acetic acid, and destained using a BioRad Model 172A diffusion destainer. Gels were stored in 7% acetic acid.

For reasons to be discussed later, the gel system was changed during the course of the work to that of Fairbanks, et al. (85). A 7.8% acrylamide gel with 1.0% SDS was prepared and run at 8ma/gel for 5 x 100mm gels. Pyronin Y was used as a tracking dye. After electrophoresis the gels were fixed in 40% propanol: 7.5% acetic, and stained with 0.05% Coomassie Blue in 40% propanol: 7.5% acetic acid. After destaining as above the gels were stored in 7.5% acetic acid.

Molecular weights were determined from the recorded scans using added molecular weight markers as well as the location of the apoproteins of known molecular weights.

Crosslinking reactions

The desired amount of protein solution to be crosslinked was added to a 13 x 100mm glass tube and 10X concentrated buffer added until a final concentration of 0.025M buffer was achieved. Crosslinker dissolved in ethanol, water, or acetone (1-5mg/ml) was then added with rapid mixing to attain the desired molar ratio of crosslinker to protein. The amount of organic solvent added never exceeded 5% of the total volume of the reaction mixture. For specific crosslinkers the following buffer and solvent systems were used: DFDNB - 0.025M Na_2CO_3 buffer, pH 8.8, ethanol: DFDNPS - 0.025M Na_2CO_3 buffer pH 8.8, acetone; DMA, DMS, DTBP - 0.025M Na_2HPO_4 buffer pH 7.5, water. The reaction mixtures were incubated for at least 2 hrs. at room temperature unless otherwise

indicated. The reaction mixtures were used directly without further treatment as it could been shown that only very small amounts of the free hydrolysis products of the crosslinkers were formed.

For labeling with FDNB, the monofunctional analog of DFDNB, the conditions described for DFDNB were used.

Amino acid analysis of specific crosslinked products

To determine the amino acid content of a specific crosslinked product it was first necessary to separate that product from all the other products of the crosslinking reaction. This was achieved by preparative gel electrophoresis of fluorescent labeled proteins. Using a BioRad Model 301 gel system and 10% Lammli gels as described above, 0.5 to 1.0 ml of HDL solution containing 1-3mg/ml protein, that had been made fluorescent by reaction with fluorescamine (86), was applied to the top of each gel. Electrophoresis was carried out at 6 ma/gel until sufficient resolution of the separate bands was achieved. The bands were easily visualized using a long wave UV light and after electrophoresis were sliced from the gel using a razor blade. Slices from duplicate gels were combined, crushed with a glass rod, and mixed with 5 to 10 ml of 0.1% SDS solution. The mixture was incubated at 37°C with shaking for 12 hrs., filtered, and the procedure repeated. The combined solutions were filtered through a .45μ Millipore filter and dialyzed against 3 x 4 liters of H_2O. The dialysis bag was agitated to suspend the fine precipitate and the suspension lyophyllized. The powdered residue was dissolved in 5 ml of 6N HCl with 10μl of β-mercaptoethanol and 10μl of 0.5% phenol. The solution was sealed in glass ampules in vacuo and then hydrolyzed for 24 hrs. at 110°C. The

hydrolyzate was dried by lyophyllization, redissolved in 0.2M citrate buffer pH 2.2, and standard amino acid analysis run on a Beckman Model 120C amino acid analyzer. Standard citrate buffers and a ninhydrin detection system were used.

Calculation of the amino acid composition of the proteins analyzed was carried out using the formula below.

$$\frac{\text{residues of leu or val predicted/molecule}}{\text{mmoles leu or val}} \times \text{mmoles AA} = \frac{\text{residues AA}}{\text{molecule}}$$

As the amino acid composition was used as a method of verification of predicted composition, this type of calculation proved most useful.

Delipidation of lipoproteins

Ten ml of HDL solution to be delipidated were added with stirring to 250 ml of chloroform:methanol (2:1) at -10°C. The turbid solution was allowed to stand at -10°C for 30 min, 250 ml of cold ethyl ether were added and the solution filtered on a Buchner funnel through Whatman #1 filter paper. The filtrate was washed with 250 ml of cold ethyl ether and dried with a stream of N_2. The precipitate was removed from the paper and stored at -10°C under N_2 until use. This method is a modification of the procedure of Lux et al. (87).

Gel filtration chromatography

Sephadex G-200 was allowed to swell in water and then poured into a 2.5 x 100cm glass column to a height of 90cm. It was equilibrated with 0.1% SDS, 0.02M tris-HCl, 10mM NaN_3, 1mM EDTA buffer pH 8.0, 30mg of delipidated protein dissolved in this buffer was added to the column. An ascending flow rate of 10ml/hr was used and 150 drop fractions collected. The OD_{280} was monitored using an ISCO UA-2 UV monitor. The column was stopped after 120ml of buffer had passed through the column.

Iodination of lipoproteins

Five ml of the sample to be labeled were dialyzed against 0.5M glycine buffer pH 9.4, containing 0.15M NaCl, 10^{-5}M EDTA, and 0.02% NaN_3; then labeled using the iodine monochloride (IM) method of MacFarlane (88). The iodine carrier solution (0.5ml) containing 250μCi of [^{125}I]-NaI was added to the HDL solution with rapid mixing. After 10 min. the unreacted iodine and glycine buffer were removed by gel filtration chromotography using Sephadex G-25 equilibrated with 0.15M NaCl containing 10^{-5}M EDTA and 0.02% NaN_3. Protein solutions with a specific activity of 5,000 to 10,000cpm/μg were routinely obtained using this method. Unlabeled controls were treated as above except for the addition of the [^{125}I]-iodine carrier solution. It could be shown that approximately 99% of the radioactivity was associated with the apoproteins while only 1% was found in the lipid fraction.

Incorporation of labeled lipid into HDL

Fifty mg of celite (50μ particles, Johns-Mansville Co.) were mixed with 1.0ml of chloroform to which 50-100μl of labeled lipid dissolved in toluene-ethanol (1:1) had been added. After mixing, the solvent volume was reduced with a stream of N_2 and the remainder of the solvent removed in vacuo. Two ml of the HDL solution to be labeled was added with gentle mixing and the suspension incubated for the desired time at 37°C. The celite was kept in suspension by occasional swirling of the tubes. After incubation was complete the suspension was filtered through a 0.45μ Millipore filter to remove the celite. All incubations were carried out for 2 hrs. unless otherwise indicated.

Exchange reactions - Incubation of HDL_2 and HDL_3 mixtures

The desired amounts of labeled and unlabeled HDL_2 and HDL_3 in solution were added directly to 5/8" x 3" cellulose nitrate centrifuge tubes and incubated for the time and temperature indicated. SDS-PAGE of controls was used as a method to detect any possible deterioration of the HDL during incubation. Unless otherwise indicated, for all incubations described herein the following volumes and concentrations were used: controls - 200µl containing 300µg of HDL protein, mixtures - 400µl containing 300µg labeled HDL protein and 300µg cold HDL protein. For the chaotropic ion experiments, solid KBr was added directly to the solution to attain the desired concentration of Br^- ion.

Ultracentrifugation of HDL_2 - HDL_3 mixtures

The reseparation of labeled and unlabeled HDL_2 - HDL_3 mixtures was achieved in the following manner. As the mixture to be resolved had already been placed directly into centrifuge tubes, sufficient NaBr solution of density = 1.3288gm/cm^3 was added to the mixture to raise the density to 1.125gm/cm^3. NaBr-NaCl solution (density = 1.125gm/cm^3) containing 0.15M NaCl, 10^{-5}M EDTA, and 0.02% NaN_3 was added to give a final volume of 10.0ml. The solutions were then underlayered with 1.0ml of NaBr-NaCl, density = 1.21gm/cm^3. The tubes were capped and placed in a Beckman Ti50 rotor for centrifugation at 42,000 RPM for 44-48 hrs.

Gradient Fractionation

After ultracentrifugation all gradients were fractionated using an ISCO Model 183 gradient fractionator modified to collect fractions from the bottom of the tube. Before fractionation each gradient was overlayered with 1.0ml of 0.15M NaCl solution. 30 drop (0.6ml) fractions were collected for all experiments.

Activation of Sepharose by Cyanogen Bromide

Ten ml of packed and well washed Sepharose 4B were placed in a 150ml beaker, 20ml of H_2O were added, the pH adjusted to 11.0 with 1M NaOH, and the temperature adjusted to 20°C by the addition of a small amount of ice. Finely divided CNBr (200mg) were added with constant stirring and the reaction allowed to proceed until cessation of proton release was observed by monitoring the pH. The pH was maintained at 11 during the reaction by the addition of 1M NaOH when needed and the temperature was adjusted by the addition of ice. When the reaction was complete, after approximately 15-20 min., 100ml of ground ice were added to the mixture. To Sepharose-ice slurry was then placed on a coarse glass filter and the Sepharose washed with several volumes of ice-cold water followed by 200ml of ice-cold 0.1M $NaHCO_3$: 0.5M NaCl buffer pH 8.0. The washed "activated" Sepharose was used immediately according to the following procedure.

Coupling of HDL to "Activated" Sepharose

Ten ml of "activated" Sepharose prepared as above or obtained from Pharmacia were mixed with 10-15ml of 0.1M $NaHCO_3$:0.5M NaCl buffer pH 8.0 in a 100ml beaker. HDL solution (5-10ml of 1-5mg/ml) was added and the mixture stirred gently for 2-3 hours at 4°C. The mixture was then filtered on a glass filter and washed for 30 min. with several volumes of 1M ethanolamine. The Sepharose-HDL (S-HDL) was then washed with 4 x 100ml of 0.1M NaOAc:0.5M NaCl buffer pH 4.0; 4 x 100ml of 0.1M $NaHCO_3$:0.5 NaCl buffer pH 8.0; and finally with 2 x 100ml of 0.15M NaCl: 10^{-5}M EDTA:0.025% NaN_3. The Sepharose prepared in this manner was stored wet in the final wash solution at 4°C.

Exchange reactions using Sepharose-bound HDL

A measured portion of packed Sepharose-HDL labeled with [^{125}I] or radioactive lipid was pipeted into 13 x 100mm glass tubes and an equal volume of 0.15M NaCl was added. Cold HDL was added in solution and the mixture swirled to suspend the gel. After incubation for the desired time, with occasional swirling to keep the gel suspended, the mixture was filtered to remove the gel. The filtrate was then counted in Aquasol-2 in a liquid scintillation counter for lipid exchange experiments, counted in the gamma counter for peptide exchange experiments, or subjected to ultracentrifugation or SDS-PAGE. For lipid exchange experiments the following incubation mixtures and times were used: 25µl of Sepharose-HDL_3 (3mg HDL/ml gel), 200µl of 0.15M NaCl, 50µl of HDL_3 (4mg/ml) or HDL_2 (3mg/ml) incubated for 15 min. at 37°C. For peptide exchange experiments larger amounts of gel were used due to the lower levels of HDL binding to the gel (100µg HDL/ml gel). For most experiments the conditions used were: 400µl Sepharose-[^{125}I]-HDL (HDL_2 or HDL_3), 200µl HDL (HDL_2 or HDL_3) in solution, 200µl 0.15M MaCl. Incubations were carried out for 5 hours at 37°C.

Determination of radioactivity

All counting of [^{125}I] was done using a Nuclear Chicago Model 8725 manual gamma counter. The counting efficiency was approximately 63% based on a comparison with the efficiency of counting of a Packard Auto-Gamma Scintillation Spectrophotometer Model 5130 (76% efficiency). All samples were counted in 13 x 100mm glass tubes using the same volume of solution in each tube (0.6ml).

For the measurement of [^{14}C] and [^{3}H], liquid scintillation spectroscopy was used. The sample was added to 5.0ml of Aquasol-2 and the

mixture counted in "mini-vials" purchased from New England Nuclear. All scintillation counting was carried out using a Beckman Liquid Scintillation Spectrophotometer. The counting efficiency for [^{14}C] was calculated to be 92% while for [^{3}H] the efficiency was 46%.

All scintillation counting values were corrected for background and counting efficiency by coincidence counting with commercially available standards.

RESULTS AND DISCUSSION

Isolation and Purity of Lipoprotein Preparations

As the procedure for isolation of HDL is well established (4,6), the two factors of importance considered were the purity of HDL_T preparations with respect to contamination by other proteins, and the effectiveness of separation during the preparation of HDL subclasses, HDL_2 and HDL_3.

Figures 1a and 1b show the scan of SDS gels of a typical preparation of HDL_T. It can be seen that the only major polypeptide bands observed have molecular weights that correspond to the major apoproteins known for HDL. As expected, the ApoA-2 peak (MW 17,500) is split into its monomer form upon the addition of β-mercaptoethanol (Fig. 1b). The peak is reduced in size due to a significant loss of low molecular weight proteins during the fixation and staining of the gels (89).

The purity of HDL_2 and HDL_3 preparations was checked by recentrifugation of the separate subclasses at the density of $1.125 gm/cm^3$ used during isolation. Under these conditions HDL_3 should sink toward the bottom of the centrifuge tube while HDL_2 should float at the top of the solution. Figure 2 shows the fractionation patterns after this recentrifugation. It can clearly be seen that both HDL_2 and HDL_3 preparations are pure by the physical criterion of ultracentrifugal mobility. All preparations of HDL_2 were shown to be pure by this method. HDL_3 preparations, however, occasionally showed some slight contamination by HDL_2.

Figure 1. SDS-PAGE of SDS solubilized apoproteins of HDL_T.

A. Pattern obtained in the absence of reducing agent.

B. Pattern observed when 5% β-mercaptoethanol is included in the preparation.

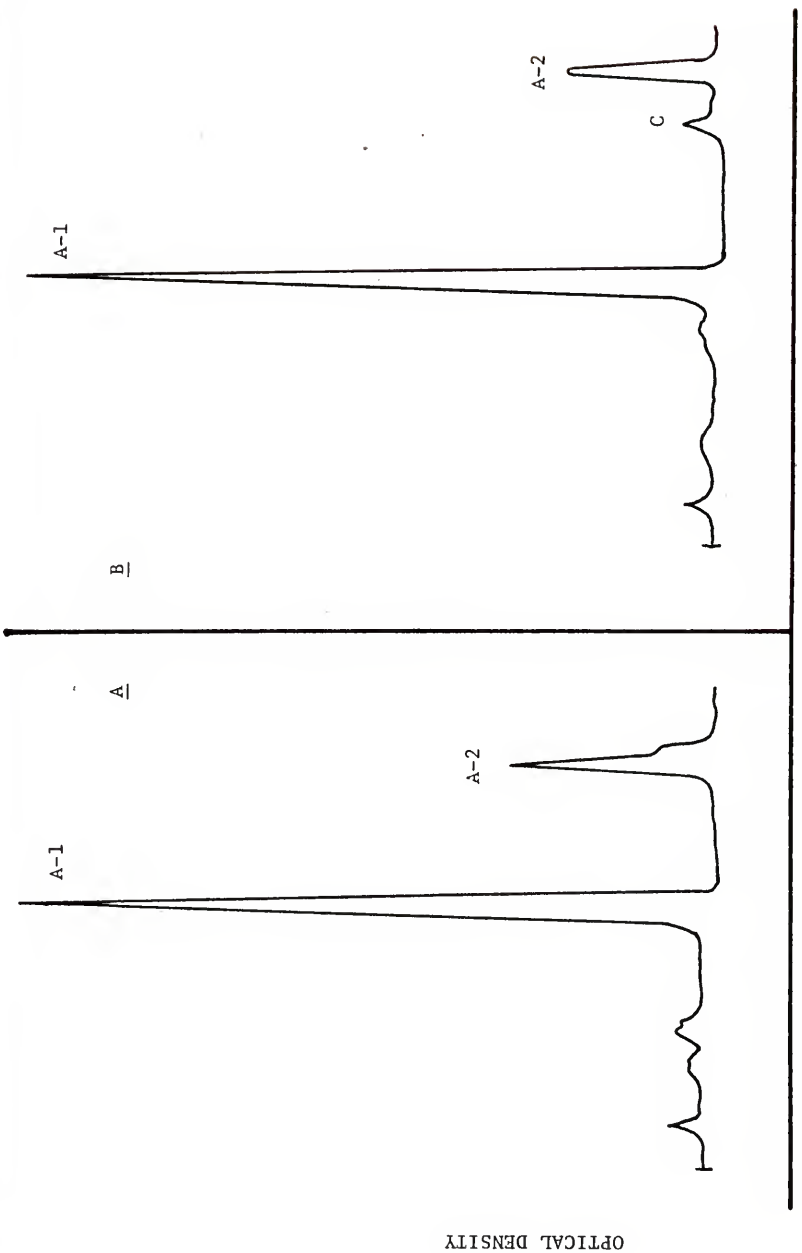
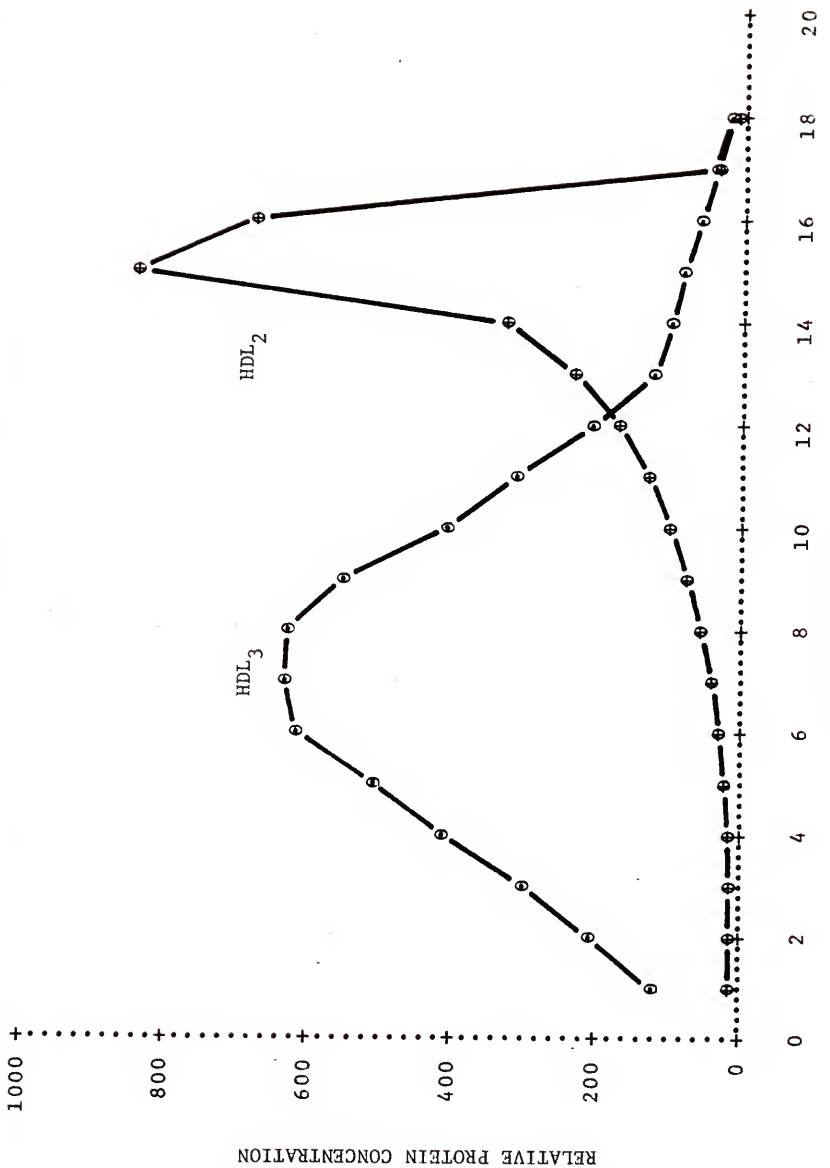

A-1
A-2
A
B
A-1
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 2. Distribution of protein after recentrifugation of purified HDL_2 (Θ) or HDL_3 (Φ) at a density of 1.125gm/cm^3.


HDL2
HDL3
RELATIVE PROTEIN CONCENTRATION
FRACTION NUMBER
0
200
400
600
800
1000
0
2
4
6
8
10
12
14
16
18
20

This contamination never represented more than 5% of the total protein present, and, when observed, an additional centrifugation step was used to remove it. This HDL_2 contamination did not appear to be the result of HDL_3 dimer formation; as once removed, no new contaminant appeared under any conditions of treatment.

Crosslinking of Total HDL (HDL_T) with DFDNB

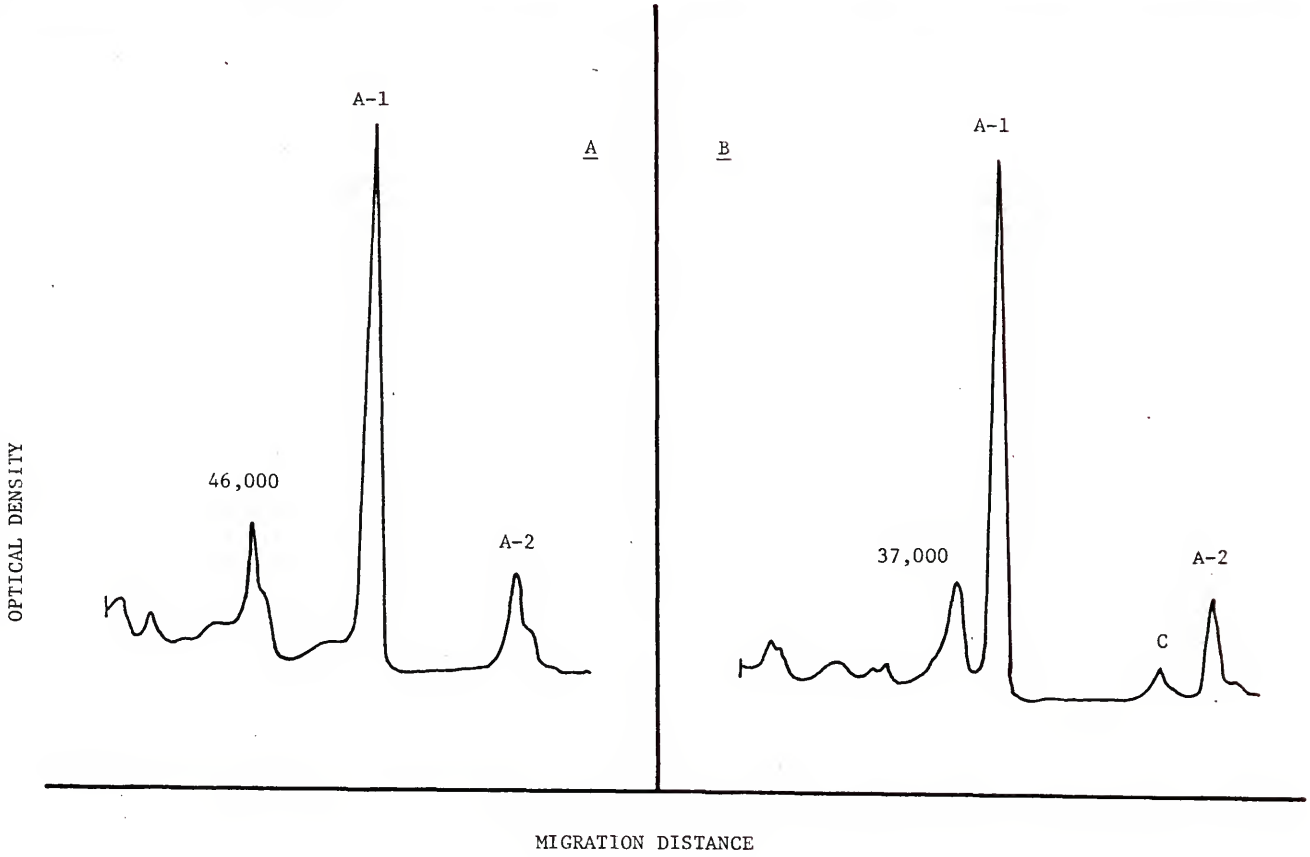
The selection of DFDNB as the initial crosslinking reagent to be evaluated was based on several factors. First, it has been widely used in previous crosslinking studies involving a variety of protein types (76). Second, its monofunctional analog FDNB has also been widely used (76). Third, it was believed that its hydrophobic nature would aid in its ability to penetrate the lipoprotein particles. Finally, pure DFDNB and FDNB are available from commercial chemical laboratories at low cost.

Figure 3 shows the gel patterns observed when a 2mg/ml protein solution of HDL_T is crosslinked with a 5 fold molar excess of DFDNB and then run on SDS-PAGE. Figure 3a shows the pattern obtained in the absence of any reducing agent while 3b is observed in the presence of 1% β-mercaptoethanol. Although a variety of high molecular weight crosslinked products are produced (Figure 3a), the first product to appear and in the highest quantity is a polypeptide complex with an apparent molecular weight of approximately 46,000. In the presence of β-mercaptoethanol this major peak is shifted to an apparent molecular weight of 37,000. Considering only ApoA-1 (MW 28,330) and ApoA-2 (dimer MW 17,500) the appearance of a crosslinked product with a molecular weight of 46,000 could only be the result of one A-1 linked

Figure 3. SDS-PAGE (10% Lammli gels) of SDS solubilized apoproteins of HDL_T crosslinked with a 5-fold molar excess of DFDNB.

(A) DFDNB crosslinked HDL_T, no reducing agent.

(B) DFDNB crosslinked HDL_T, with 5% β-mercaptoethanol.


A-1
A
46,000
A-2
B
A-1
37,000
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

to one A-2 unit. This conclusion is supported by Figure 3b which shows that the addition of the disulfide cleaving agent, β-mercaptoethanol, results in the loss of half of the A-2 dimer leaving a product consisting of one A-1 linked to one A-2 monomer. The molecular weight of such a complex would be approximately 36,000.

As the estimation of molecular weights by SDS gel electrophoresis can result in considerable error when considering proteins which may have both inter- and intra-peptide crosslinks (90), additional evidence is desirable before one can conclude that an A-1···A-2 crosslinked product is actually produced. To approach this problem it is necessary to separate the individual crosslinked products so they can be characterized. Gel filtration chromotography using Sephadex G-200 containing 1.0% SDS was attempted. Although this procedure was capable of a reasonable separation of ApoA-1 from ApoA-2, the crosslinked products were eluted as a broad, even band; each fraction containing a mixture of similar molecular weight products. This procedure was not capable of sufficient purification of any single product. The problem was solved using the experimental approach diagrammed in Figure 4. The procedure is detailed in the Methods section of this dissertation. The results of the amino acid analysis of the 46,000 dalton crosslinked product is shown in Table 2. Comparison to the predicted values for sleected amino acids for an A-1···A-2 complex indicates the 46,000 dalton product is likely a complex of one A-1 and one A-2. Certain amino acid values were not shown because they were components of the gel buffer system, gave unexpected high or low values for purified A-1 or A-2, or were destroyed during hydrolysis. That both A-1 and A-2

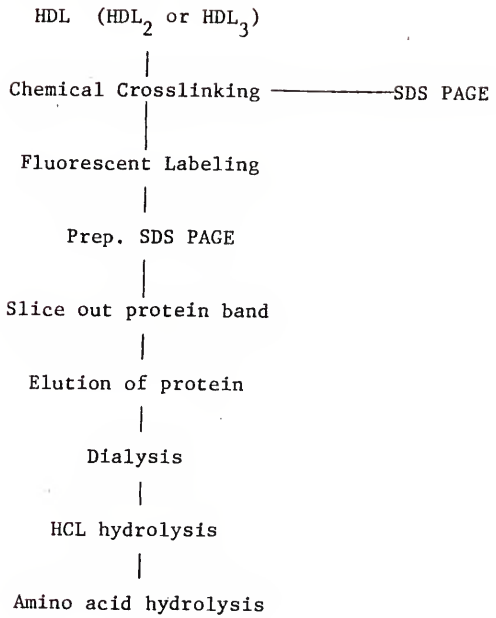


Figure 4. Experimental approach to amino acid analysis of crosslinked products

Table 2.

Amino Acid Analysis of the 46,000 Dalton Crosslinked Product from HDL_T Crosslinked with DFDNB

Amino Acid	Observed[a]	A-1:A-2 Predicted[a]
Lysine	36.3	39.0
Histidine	7.0	5.0
Arginine	16.8	16.0
Alanine	31.2	31.0
Valine	25.7	25.0
Leucine	55.0	55.0
Tyrosine	15.0	15.0
Phenylalanine	15.2	14.0
Aspartic acid	30.1	27.0
Threonine	25.0	26.0
Isoleucine	1.8	2.0
Methionine	4.2	5.0

[a]All values expressed as residues per mole of protein based on the value for leucine.

are components of this complex is suggested by two observations. First, the shift in molecular weight upon addition of reducing agent could only occur if ApoA-2 was present as no disulfide bonds occur in Apo-1. Second, there is no histidine in ApoA-2 or isoleucine in ApoA-1, but both occur in the 46,000 dalton product. As ApoA-1 contains 5 histidine residues/molecules and ApoA-2 dimer contains 2 isoleucine residues/molecule, these must be the source of the histidine and isoleucine found in the complex. Although the C-peptides contain both isoleucine and histidine, the low total amount of C-peptides in HDL excludes these polypeptides from a significant contribution to the composition of any major crosslinked product.

Effect of Crosslinking on HDL Structure

In discussing the meaning of the results of crosslinking experiments it is important to establish that the introduction of these crosslinks does not significantly alter the structure of the macromolecular complex that is being studied. In the case of HDL, it could be shown that crosslinked HDL retains the physical properties of native HDL and will migrate in the analytical ultracentrifuge as a single peak essentially identical to native HDL. Although even very high levels of crosslinking (100-fold molar excess) do not appear to disrupt the particles, all conclusions presented are based on data obtained at much lower levels of crosslinking.

Crosslinking of HDL_2 and HDL_3 with DFDNB

Although the peptide composition of the two HDL subclasses, HDL_2 and HDL_3, appears to be the same, the amount of total protein is different (1). HDL_2 should contain approximately 160,000 daltons of

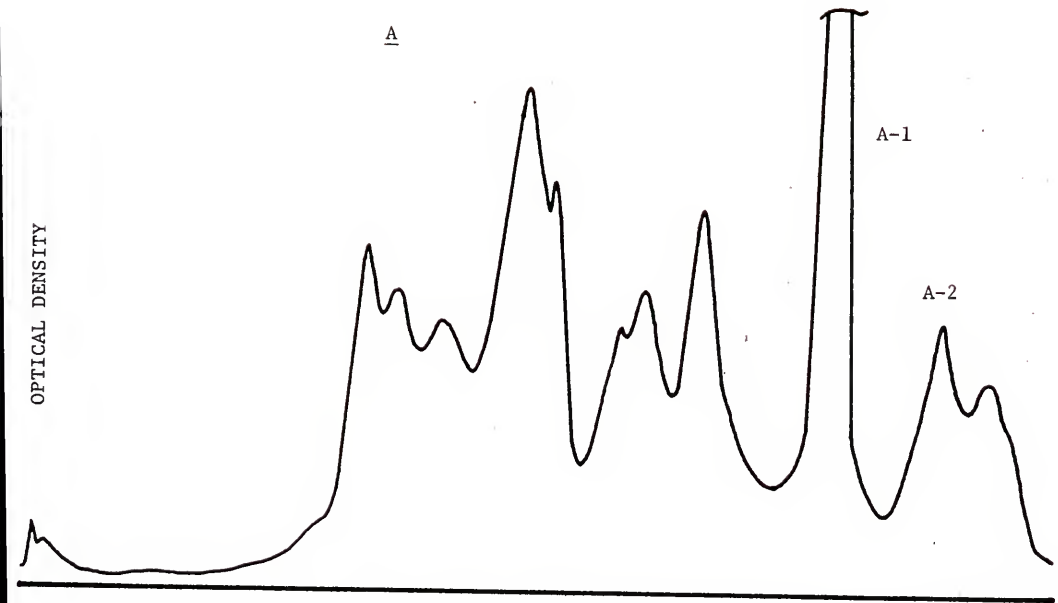
protein/molecule while HDL_3 should only contain 80,000 daltons/molecule. Under conditions of increasing amounts of crosslinker, one might expect to observe larger molecular weight crosslinked products for HDL_2 as compared to HDL_3. However, using the Lammli gel system with molar ratios of crosslinker higher than 5 to 10-fold, one observes only a smear of stain at the top of the gel for each subclass. This phenomenon has been noted for other types of crosslinkers (91). For this reason a different gel system was used. Fairbanks, et al. have noted that an SDS concentration of 1%, rather than the 0.1% found in the Lammli system, is necessary to dissociate membrane protein complexes (85). Figure 5 shows the comparison of the pattern obtained for HDL_T crosslinked with a 20-fold molar excess of DFDNB and then run on the two gel systems (5a-Lammli, 5b-Fairbanks). The 1% SDS in the Fairbanks gel system is apparently sufficient to cause total dissociation of even highly crosslinked lipoprotein particles.

Figures 6 (HDL_2) and 7 (HDL_3) demonstrate that for both HDL_2 and HDL_3 the crosslinking patterns are essentially the same. It is interesting to note that no crosslinked products of apparent molecular weight products occur even if the level of crosslinker is increased up to 100-fold. For this reason no separation of HDL_2 and HDL_3 was carried out for other crosslinking experiments; instead, HDL_T was used.

Crosslinking of HDL_T with DMS and DFDNDPS

To investigate the hypothesis that the hydrophobic nature and length of the bifunctional reagent used should have some effect on the extent and type of crosslinking produced, a comparison of the gel patterns obtained was made after DMS or DFDNDPS was used to crosslink HDL_T. These

Figure 5. Comparison of the SDS-PAGE methods of Fairbanks (A) and Lammli (B) for the separation of highly crosslinked HDL_T (25x DFDNB).


A
A-1
A-2
OPTICAL DENSITY
MIGRATION DISTANCE

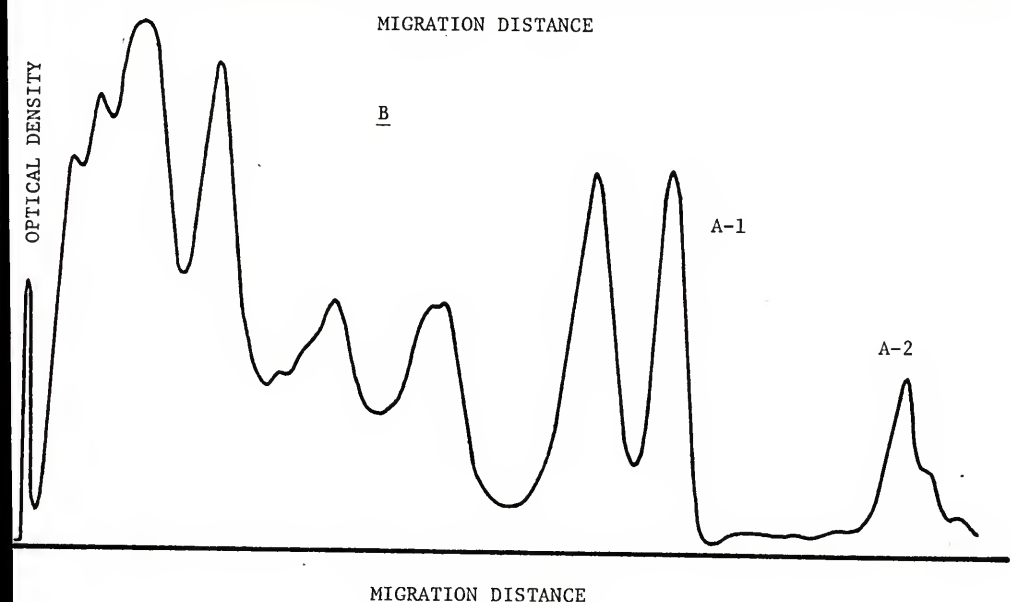

B
A-1
A-2
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 6. SDS-PAGE (7.8% Fairbanks gels) of SDS solubilized HDL_2 crosslinked with a 10-fold molar excess of DFDNB.

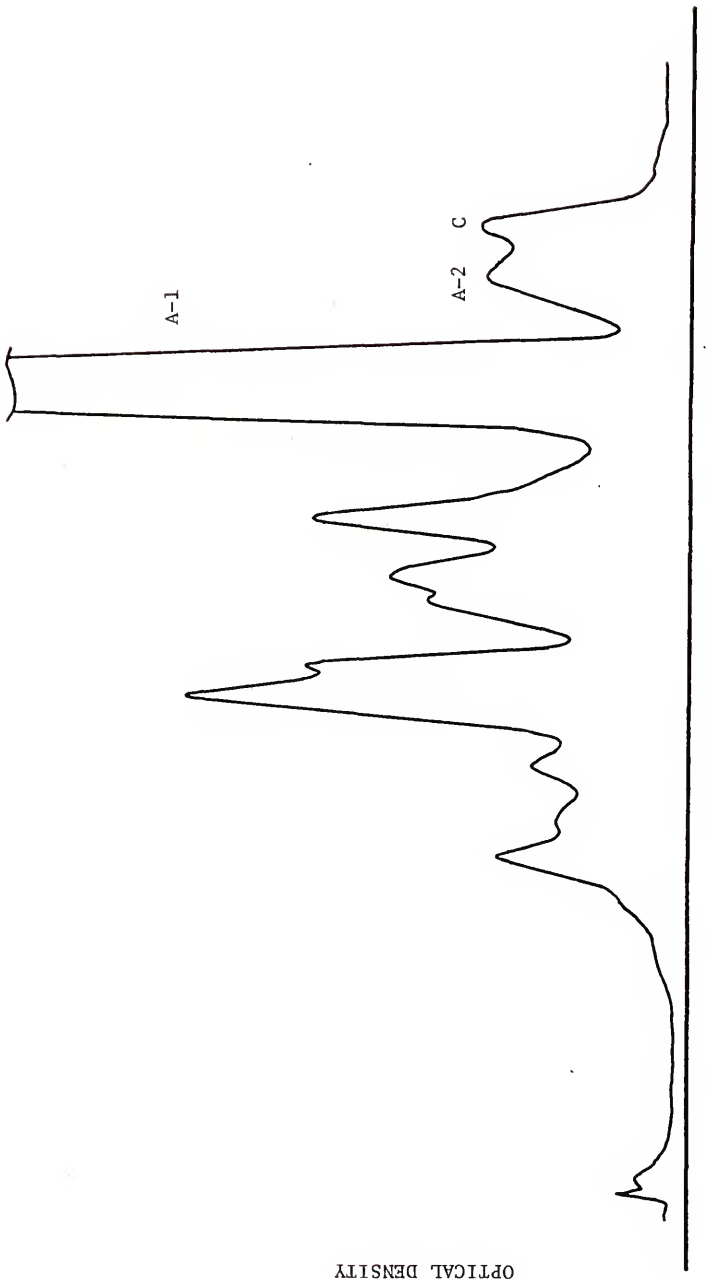
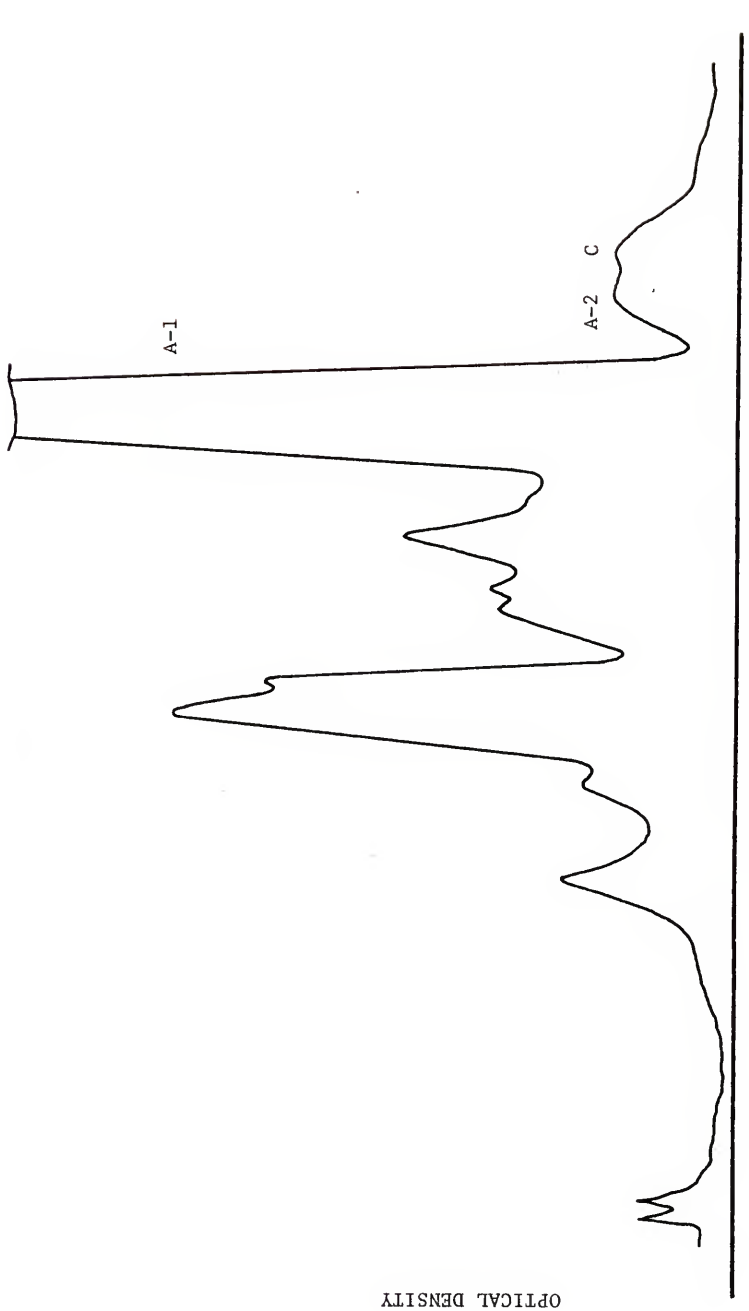

A-1
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 7. SDS-PAGE (7.8% Fairbanks gels) of SDS solubilized HDL_3 crosslinked with a 10-fold molar excess of DFDNB.


A-1
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

two reagents have similar distances between the functional groups but differ greatly in hydrophobic character. DMS is water soluble and carries a positively charged imido group while DFDNDPS is quite insoluble in water and must be added in an acetone solution. At low levels of crosslinker (10-fold molar excess) these two reagents appear to generate significantly different initial crosslinked products (Figures 8-11). Two major differences between DMS and DFDNDPS crosslinking can be seen. First, the total amount of crosslinking is smaller for the hydrophyllic reagent DMS. Second, the initial site of reaction for DMS appears to differ from DFDNDPS with a 65,000 dalton product appearing first. At higher molar ratios of these reagents the patterns become very similar with all different products appearing in similar amounts in each case.

The initial products produced with DFDNDPS crosslinking are essentially the same as those formed with DFDNB even though the former reagent is twice as long as the latter.

Temperature Dependence of Crosslinking

When the crosslinking of HDL_T with a 10-fold molar excess of DFDNB is carried out at different temperatures ranging from 4° to 70°C, no significant differences in the crosslinking patterns are produced. The number and relative amounts of the separate crosslinked products remains similar at all temperatures studied. The only difference observed is a reduced amount of crosslinking at the very high temperatures, 60-70°C. It should be noted, however, that the rate of formation of hydrolysis products of this bifunctional reagent increases significantly at these elevated temperatures. This would lead to

Figure 8. SDS-PAGE (10% Lammli gels) of SDS solubilized HDL_T crosslinked with a 5-fold molar excess of DMS. No reducing agent present.

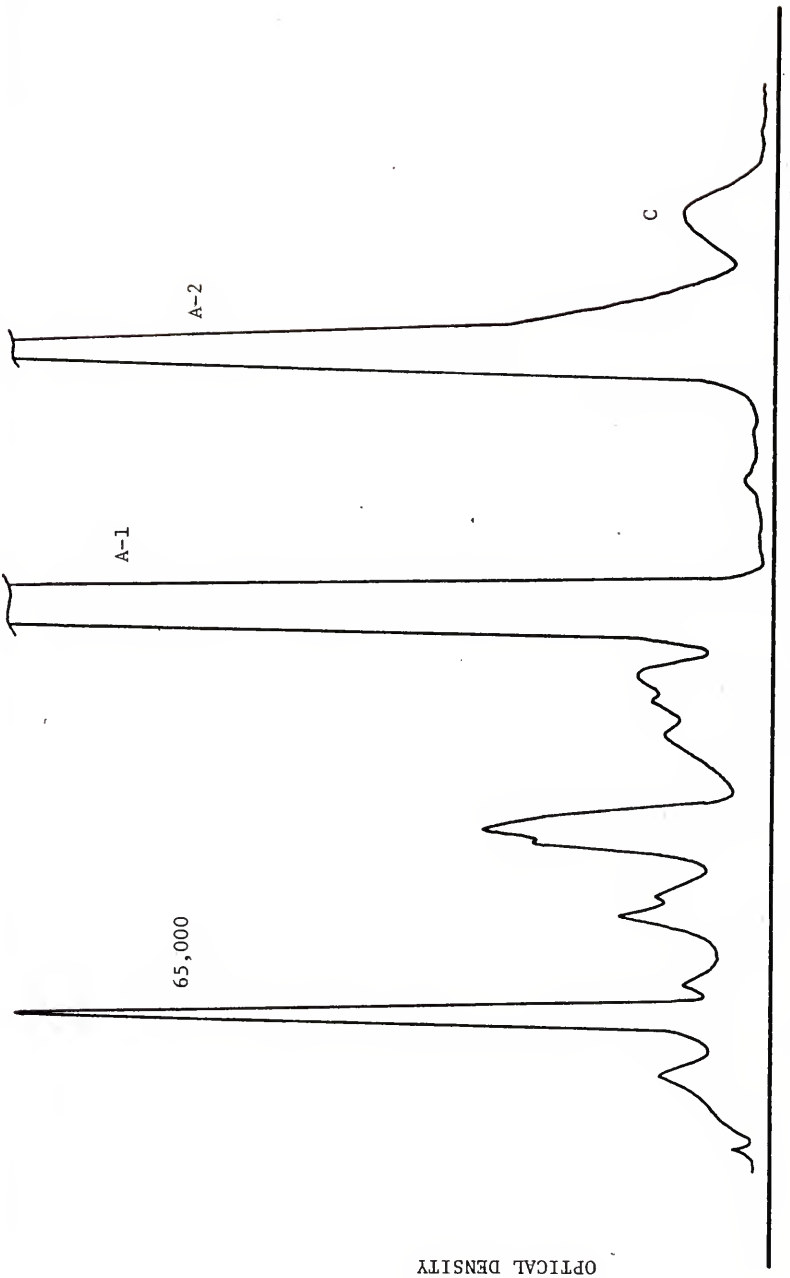

65,000
A-1
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 9. SDS-PAGE (10% Lammli gels) of SDS solubilized HDL_T crosslinked with a 5-fold molar excess of DMS. 5% β-mercaptoethanol included.

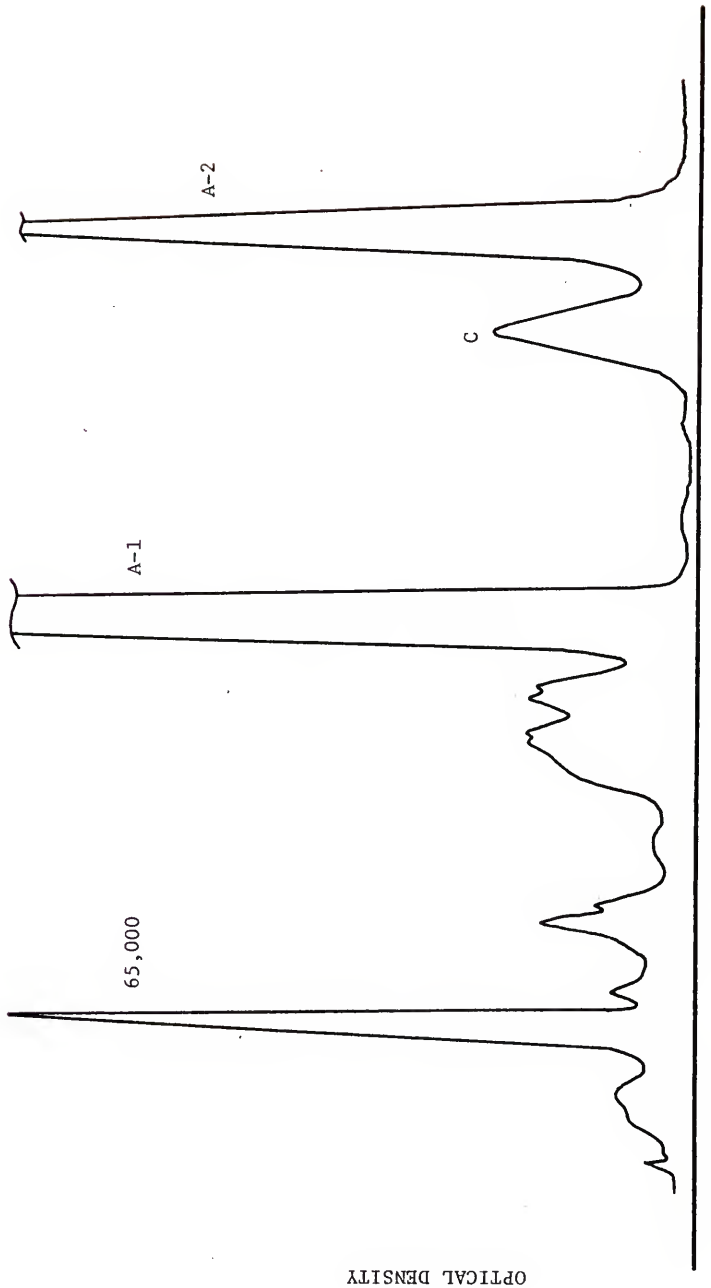

65,000
A-1
C
A-2
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 10. SDS-PAGE (10% Lammli gels) of SDS solubilized HDL_T crosslinked with a 5-fold molar excess of DFDNDPS. No reducing agent present.

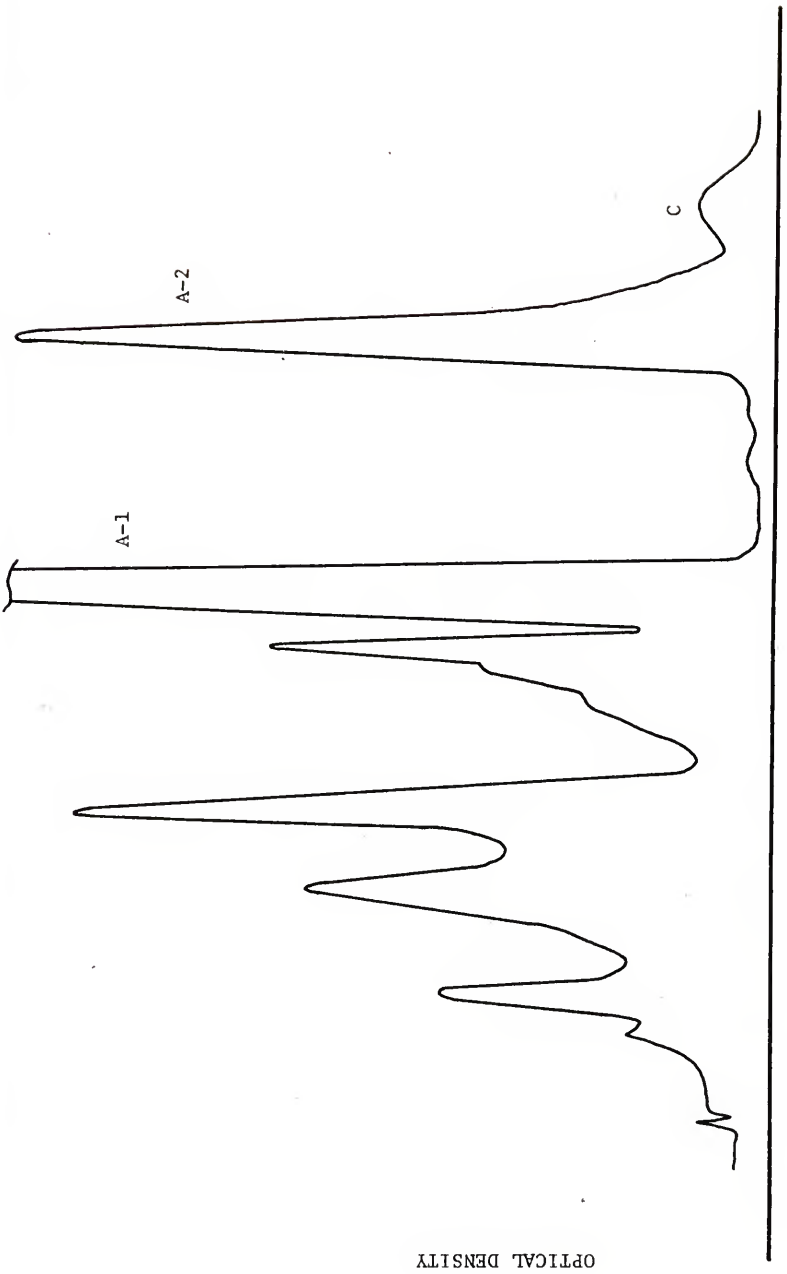
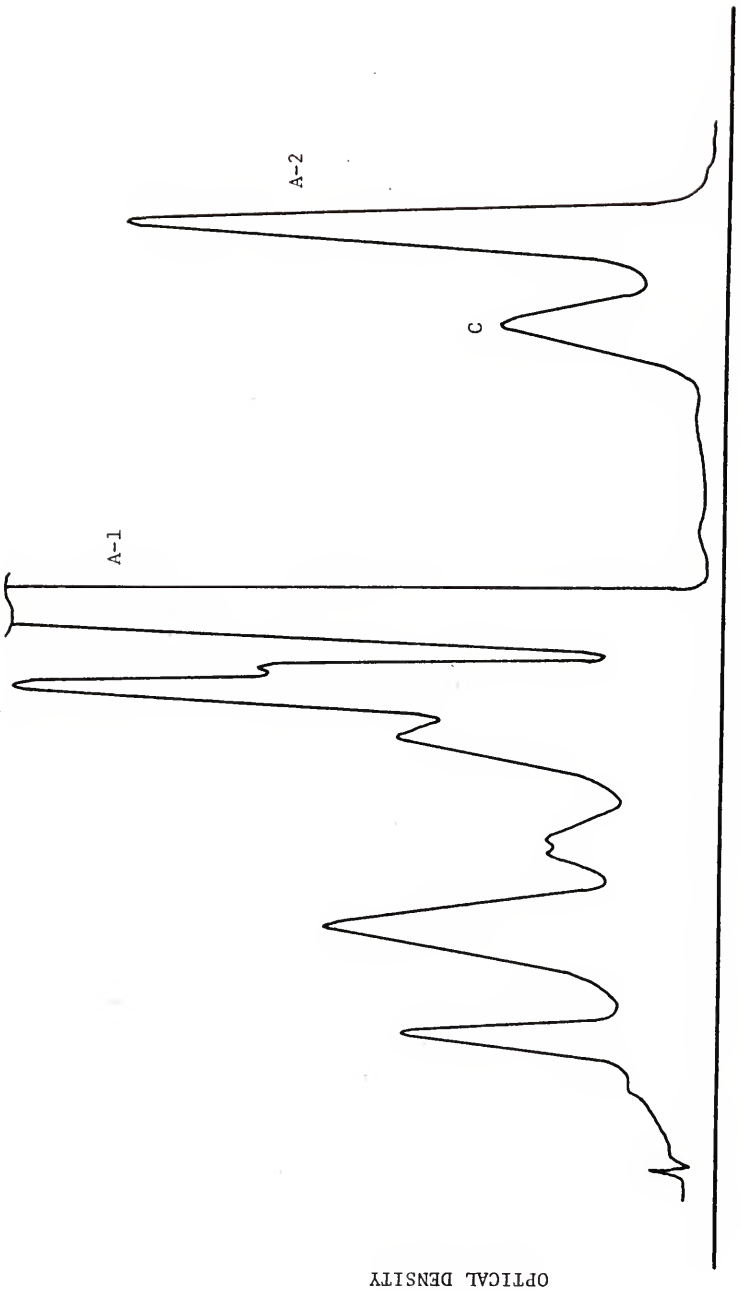

A-1
A-2
C
OPTICAL DENSITY
MIGRATION DISTANCE

Figure 11. SDS-PAGE (10% Lammli gels) of SDS solubilized HDL_T crosslinked with a 5-fold molar excess of DFDNDPS. 5% β-mercaptoethanol included.


A-1
A-2
C
MIGRATION DISTANCE
OPTICAL DENSITY

decreased availability of reagent as well as an increased probability of monofunctional labeling.

Interpretation of Crosslinking Results

From the data in this section we conclude that at least some of the two major apoprotein components must lie very close to one another in the intact lipoprotein particle since DFDNB will only crosslink groups about 5A apart. If each HDL particle contains both A-1 and A-2 molecules, a number of possible crosslinking products could result. From our findings with low levels of DFDNB (5-10 fold molar excess) several observations can be made. First, the major crosslinked product appears to comprise one A-1 plus one A-2; second, there is no indication of any A-2 intramolecular crosslinking which might be expected with a molecule already crosslinked with a disulfide bridge. There also seems to be little significant crosslinking-reactive association of two A-1 molecules as suggested by Scanu (92). Since both A-1 and A-2 contain numerous reactive residues fairly evenly distributed throughout their sequence (1), any close association of the chains should make multiple sites available for possible crosslinking.

Experiments using higher molar ratios of crosslinker (20-100X) do show that most possible combinations of A-1 and A-2 are formed. Still, even at these high concentrations, no A-2 intramolecular crosslinked molecules are produced. The C-peptides do not appear to be involved in the crosslinking reactions. At high levels of reagent the amount of unreacted C-peptide does not appear to decrease when compared to controls. This suggests little association of C-peptides with A-1 or A-2.

The formation of most of the possible products at high crosslinker concentrations seems to indicate either a significant degree of mobility of the apoproteins or significant alteration of the native structure of the particle. Two factors suggest that although some mobility exists, a high degree of specific association of the apoproteins occurs in the intact HDL particle. First, the decreased crosslinking observed at high temperature could be due to increased mobility of the polypeptide chains. Second, the failure of A-2 to form intramolecular crosslinks suggests a stable conformation that remains fairly fixed even at these elevated temperatures. The reproducibility of the crosslinking experiments with respect to order of formation and ratios of products formed, even when HDL is obtained from varied sources, suggests that in the intact HDL particle the apoprotein components may be found in a rather fixed spatial orientation to one another.

That the crosslinking patterns for HDL_2 and HDL_3 do not differ also argues for a fixed association of 2A-1 and 1A-2 apoproteins. Even though HDL_2 may have two HDL_3 protein subunits (54), these do not appear to interact. This would lead one to speculate that HDL_2 might be a dimer of 2-HDL_3 molecules. This idea will be discussed in detail later in this work.

An additional argument in favor of fixed associations with flexibility of the apoproteins comes from the experiments using the two reagents DMS and DFDNDPS. These two reagents consistently yield initial crosslinking products that differ. This may occur due to different initial sites of reaction for these reagents. The initial products do not differ when one compares DFDNB and DFDNDPS, although the latter is twice as long as DFDNB. This again suggests some flexibility in the associations of the apoproteins.

From these observations one might suggest a model in which the apoproteins lie in a fixed, flexible conformation held in place in part by protein-protein, lipid-protein, and lipid-lipid interactions. The ability to bind lipids may in turn be governed by these interactions; the flexibility allowing for differing degrees of binding of specific "functional" lipids while certain "structural" lipids remain fixed.

Apoprotein Exchange Between HDL_2 and HDL_3

If the ratio of A-1 to A-2 dimer is 2:1, in both HDL_2 and HDL_3 as suggested by Friedberg and Reynolds (54), then the difference between HDL_2 and HDL_3 may represent the addition of one A-1:A-2 unit, which consists of two A-1 plus one A-2 peptide dimer. Because our cross-linking data suggests a degree of flexibility as well as specific interaction within an A-1:A-2 unit but the two units do not appear to cross-link in intact HDL_2, it is necessary to consider possible modes of association of the apoproteins of the two proposed units in HDL_2.

To investigate this possible association, as well as the relationship between the apoproteins of the different subclasses of HDL, we have examined the exchange of labeled apoproteins between HDL_2 and HDL_3, in the presence and absence of bifunctional crosslinking reagents.

Figures 12 and 13 show the ultracentrifugal fractionation patterns obtained for controls (300µg HDL protein in 200µl) of [^{125}I]-labeled HDL_3 and [^{125}I]-labeled HDL_2 incubated in the absence of any other protein. The results are expressed as dpm per fraction as it was found that the radioactivity and protein concentration curves coincided (the specific activities of control fractions were constant throughout the

Figure 12. Exchange of apoproteins between [^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊕ = [^{125}I]-HDL_3 control; ⊖ = [^{125}I]-HDL_3 incubated with cold HDL_2.

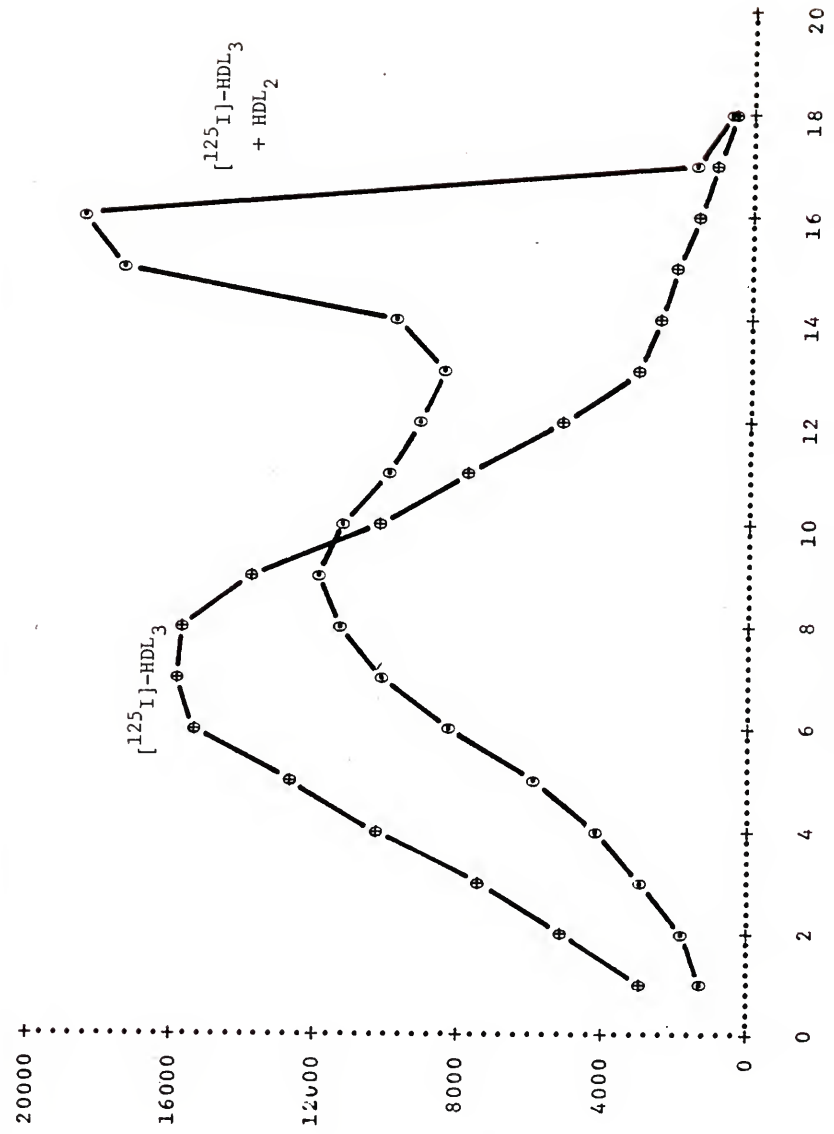
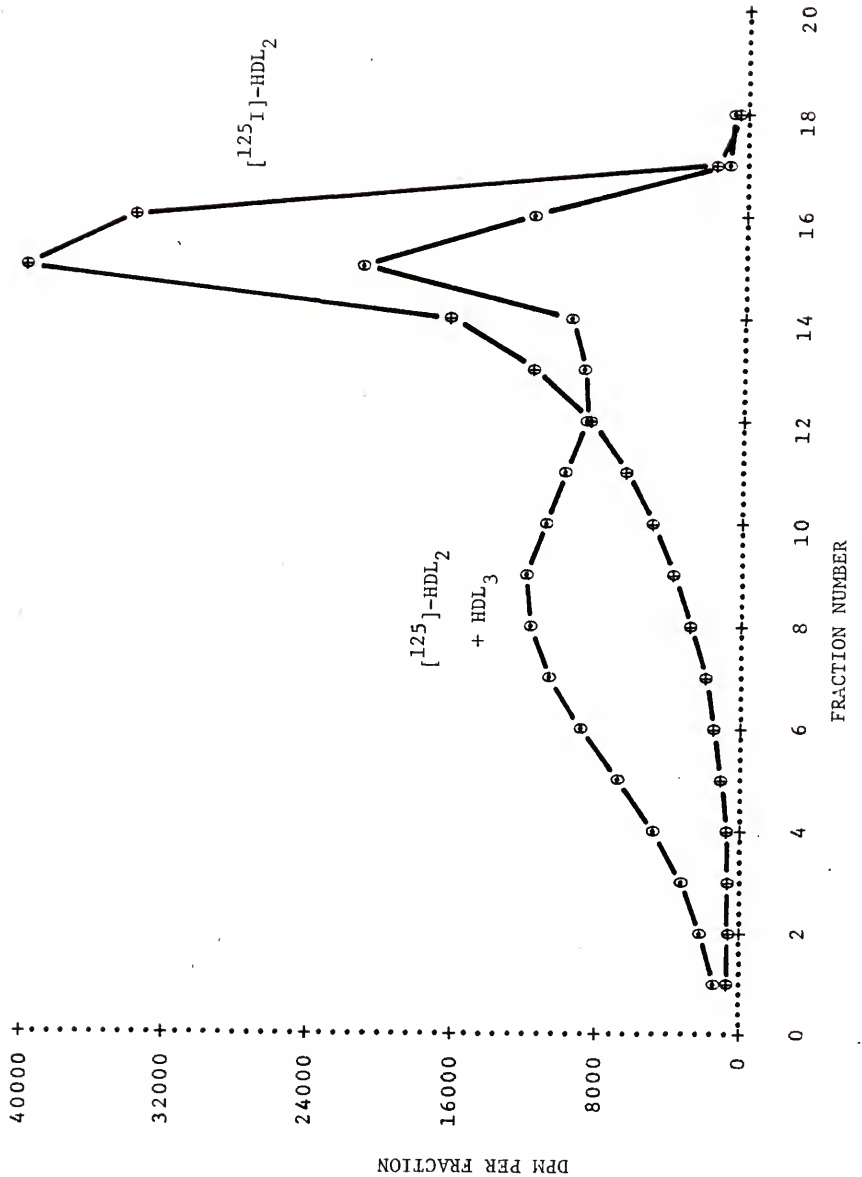

[125I]-HDL3
+ HDL2
[125I]-HDL3
DPM PER FRACTION
FRACTION NUMBER
0
4000
8000
12000
16000
20000
0
2
4
6
8
10
12
14
16
18
20

Figure 13. Exchange of apoproteins between [^{125}I]-HDL_2 and HDL_3.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊕ = [^{125}I]-HDL_2 control; ⊖ = [^{125}I]-HDL_2 incubated with cold HDL_3.


40000
32000
24000
16000
8000
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER
[125I]-HDL2
[125I]-HDL2
+ HDL3

tube). Figures 12 and 13 also illustrate the results obtained after mixing equal amounts (300µg protein in 200µl) of [^{125}I]-labeled HDL_3 and cold HDL_2 (Figure 12) or [^{125}I]-labeled HDL_2 and cold HDL_3 and incubating the mixture for 7 hrs. at 37°C (Figure 13). In Figure 12 it can clearly be seen that a transfer of label from HDL_3 to HDL_2 has occurred. Further, the specific activity of the HDL_3 fraction was significantly decreased when compared to controls. In a typical experiment, the values for a selected fraction in the HDL_3 region of the tube (fraction 6) were: [^{125}I]-HDL_3 control = 2850 dpm/µg; [^{125}I]-HDL_3 incubated with cold HDL_2 = 1680 dpm/µg. Similar results were obtained when labeled HDL_2 was incubated with cold HDL_3 (Figure 13). However, due to the breadth of the HDL_3 peak as compared with the HDL_2 fractions, the results are not as easily visualized.

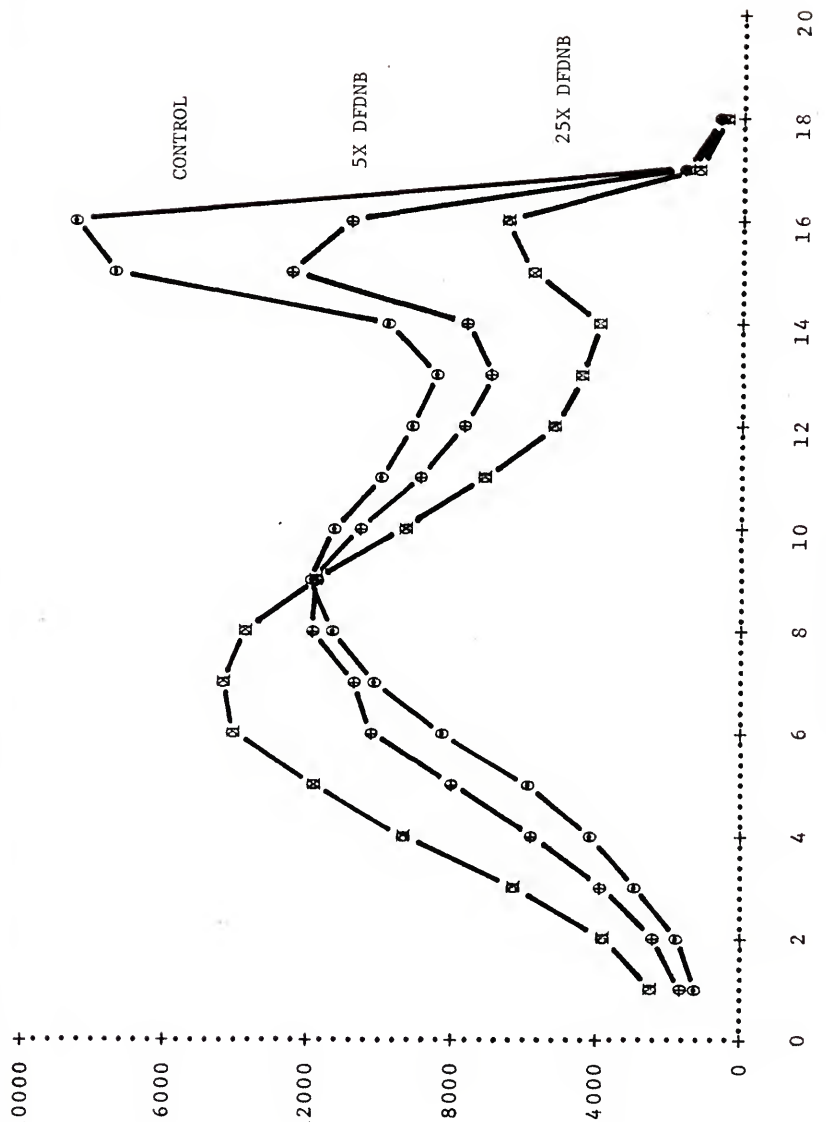
Effect of Crosslinking and Monofunctional Labeling on Apoprotein Exchange

If the labeled HDL_3 is crosslinked with DFDNB before incubation with HDL_2, the transfer of labeled protein is reduced depending upon the extent of crosslinking. Figure 14 shows the three fractionation patterns obtained with : [^{125}I]-HDL_3 incubated with HDL_2 (control); [^{125}I]-HDL_3 crosslinked with a 5-fold molar excess of DFDNB and then incubated with HDL_2; and [^{125}I]-HDL_3 incubated with HDL_2 following crosslinking of the HDL_3 with a 25-fold molar excess of DFDNB. It can be seen that increased crosslinking results in decreased exchange.

To rule out the possibility that the monofunctional substitution of the apoproteins with the crosslinking reagent was the cause of this decrease in exchange, FDNB, a monofunctional analog of DFDNB, was used to modify the proteins. Reaction with concentrations of FDNB, comparable

Figure 14. Effect of crosslinking on the apoprotein exchange between [^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊖ = [^{125}I]-HDL_3 incubated with cold HDL_2; ⊕ = [^{125}I]-HDL_3 crosslinked with a 5-fold molar excess of DFDNB followed by incubation with HDL_2; ⊠ = [^{125}I]-HDL_3 crosslinked with a 25-fold molar excess of DFDNB then incubated with HDL_2.


CONTROL
5X DFDNB
25X DFDNB
DPM PER FRACTION
20000
16000
12000
8000
4000
0
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

to amounts of DFDNB that greatly inhibited exchange, had little effect on the exchange process (Figure 15).

When HDL_2 is crosslinked before incubation with labeled HDL_3, the exchange process is also inhibited (Figure 16). The reverse also holds true; that is for crosslinked HDL_3 incubated with labeled HDL_2, the exchange is reduced.

Effect of Temperature and Dilution on Peptide Exchange

Both low temperature and dilution have an effect upon the rate of protein exchange in HDL. If the incubation is carried out at 4°C, very little exchange will occur in the time period in which significant exchange occurs at 37°C (Figure 17). Using the amounts of HDL_2 and HDL_3 described in the Methods section, but diluting this mixture with a 10-fold excess (5ml) of 0.15M NaCl, the rate of exchange at 37°C is also significantly reduced (Figure 18). These two observations are consistent with a mechanism of exchange involving a collisional complex.

It is this reduction in exchange rates that permits one to employ the 48 hour centrifugation required for reseparation of the HDL_2 and HDL_3 mixtures. When equal amounts of labeled HDL_3 and cold HDL_2 are mixed, then immediately diluted, cooled to 4°C, and placed in the ultracentrifuge, essentially no exchange occurs, even after 48 hrs.

Rate of Peptide Exchange

Figure 19 shows a time course plot for the exchange of apoproteins from [^{125}I]-HDL_3 into HDL_2. The radioactivity represents the total transferred into the HDL_2 region of the centrifuge tube after subtraction of the amount in those fractions attributed to HDL_3. Estimation

Figure 15. Effect of monofunctional labeling with FDNB on the exchange of apoproteins between [^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊖ = [^{125}I]-HDL_3 reacted with a 25-fold molar excess of FDNB; ⊕ = [^{125}I]-HDL_3 reacted with a 25-fold molar excess of FDNB followed by incubation with HDL_2.

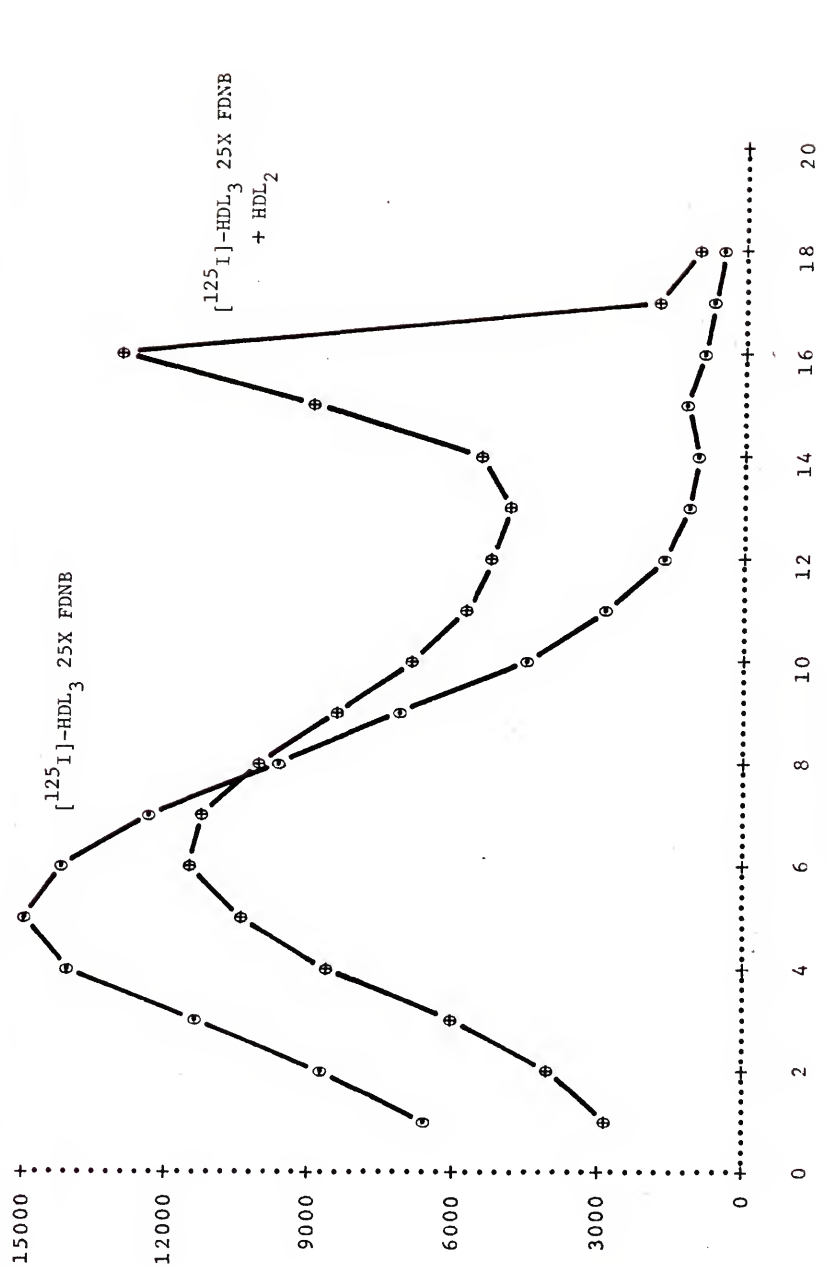

[125I]-HDL3 25X FDNB
[125I]-HDL3 25X FDNB + HDL2
DPM PER FRACTION
FRACTION NUMBER
15000
12000
9000
6000
3000
0
0
2
4
6
8
10
12
14
16
18
20

Figure 16. Effect of crosslinking the unlabeled HDL subclass on the exchange of apoproteins between [^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊖ = [^{125}I]-HDL_3 control; ⊕ = [^{125}I]-HDL_3 incubated with HDL_2 crosslinked with a 25-fold molar excess of DFDNB.

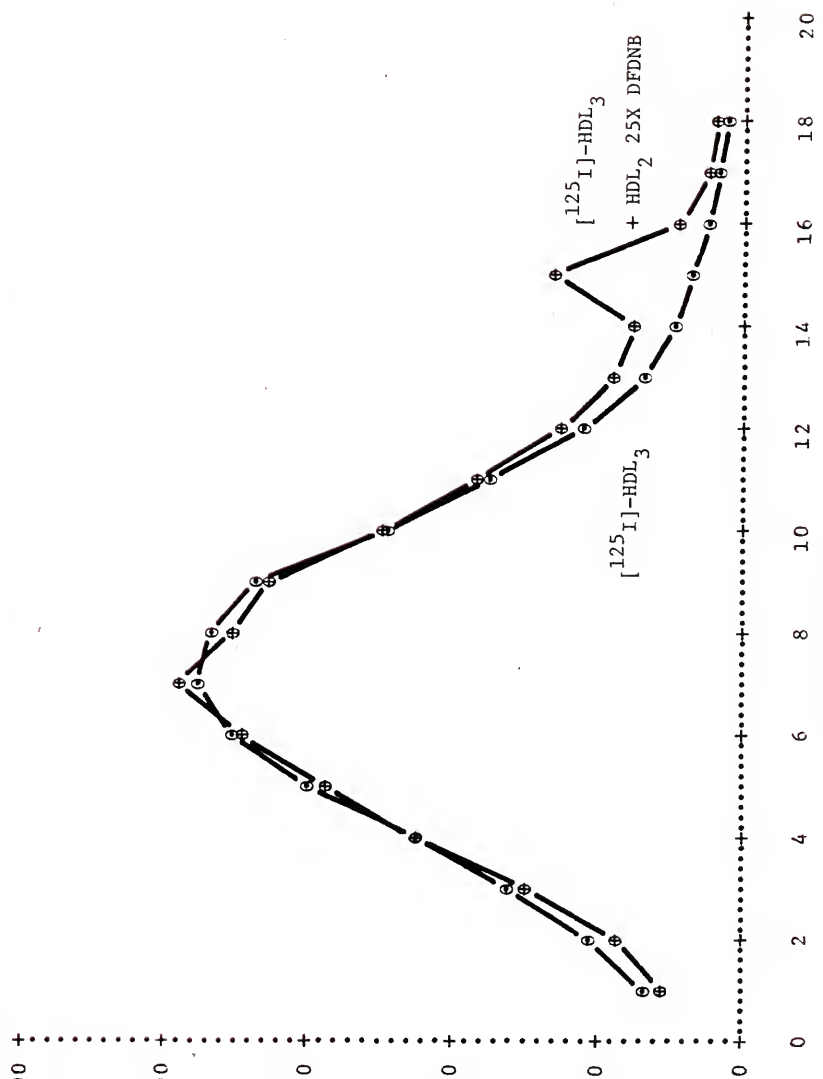

20000
16000
12000
8000
4000
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER
[125I]-HDL3
[125I]-HDL3
+ HDL2 25X DFDNB

Figure 17. Effect of temperature on the apoprotein exchange between $[^{125}I]$-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊖ = $[^{125}I]$-HDL_3 control; ⊕ = $[^{125}I]$-HDL_3 incubated with HDL_2 at 4°C. Compare to Figure 12.

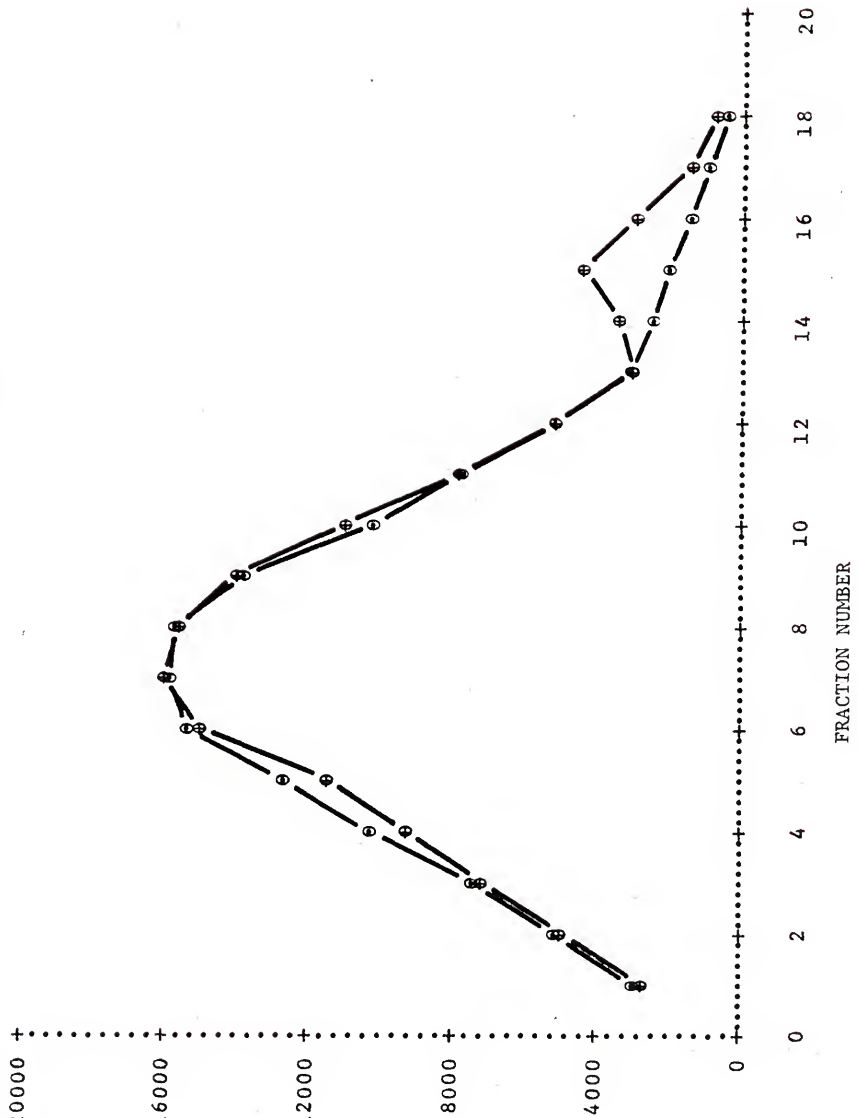

DPM PER FRACTION
20000
16000
12000
8000
4000
0
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Figure 18. Effect of temperature and dilution on the apoprotein exchange between [^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. Θ = [^{125}I]-HDL_3 and HDL_2 were incubated at 4°C after dilution with 5ml of 0.15M NaCl.

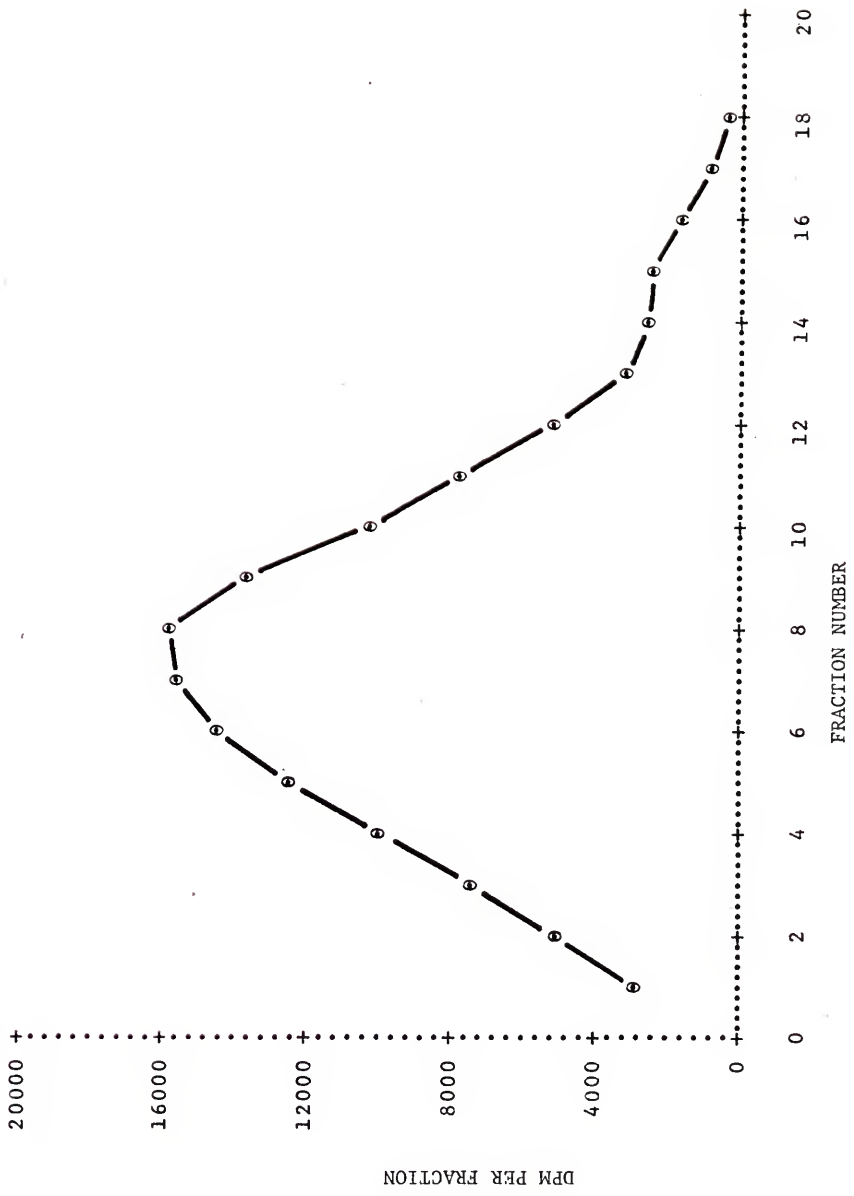
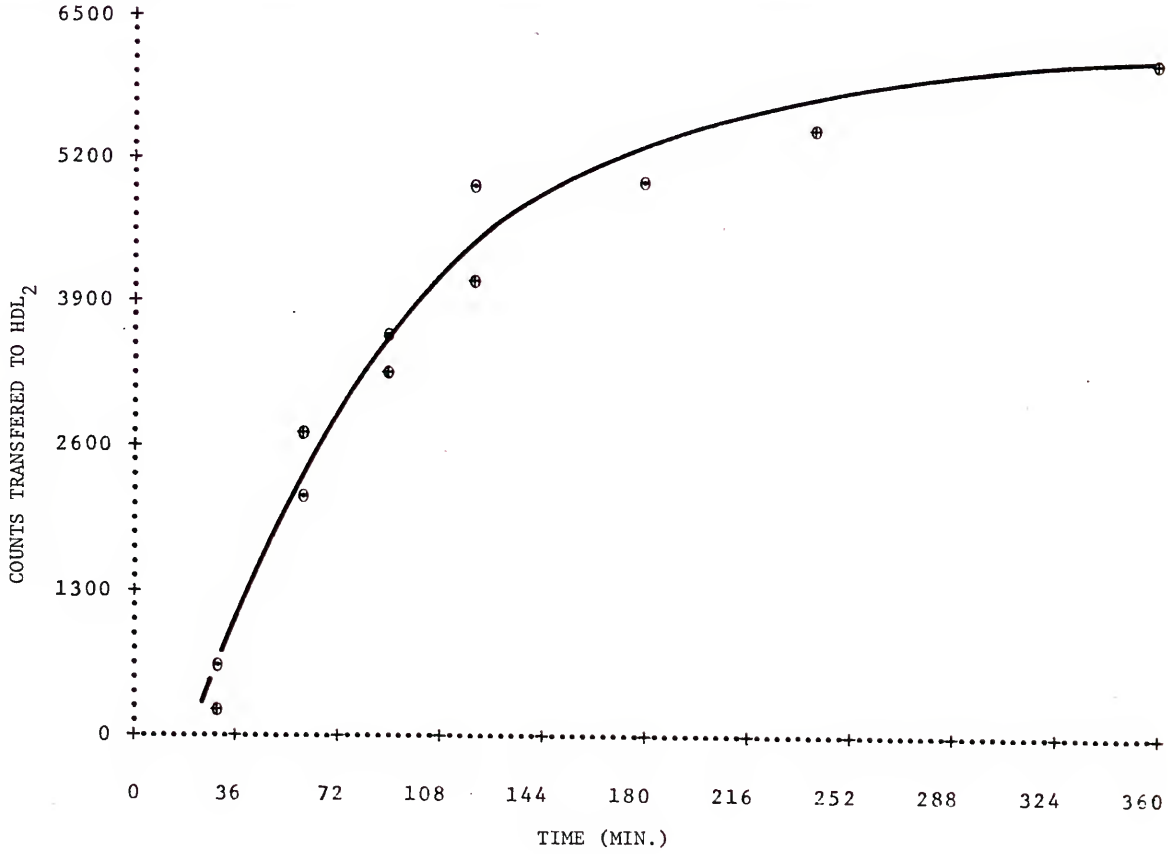

20000
16000
12000
8000
4000
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Figure 19. Time course for the exchange of [^{125}I]-labeled apoproteins from [^{125}I]-HDL_3 into HDL_2. 3mg HDL protein/ml each subclass.


6500
5200
3900
2600
1300
0
COUNTS TRANSFERED TO HDL2
0
36
72
108
144
180
216
252
288
324
360
TIME (MIN.)

of the initial slope at 2300 dpm/hr out of a total of 90,000 dpm gives an exchange rate of approximately 2.5%/hr. These calculations are based upon two separate experiments utilizing concentrations of HDL_2 and HDL_3 comparable to plasma levels (3mg/ml each subclass). The rate can be altered by varying the initial concentrations of either or both subclasses.

Effect of Bromide Ion on Protein Exchange

If the chaotropic Br^- ion is included in the incubation mixture at 37°C, the amount of exchange in a given time period is greater when compared to controls with no Br^-. Figure 20 shows the fractionation pattern obtained when 2M KBr is included in the 7 hour incubation solution as compared to controls. A significant increase in exchange can be seen. As chaotropic ions are known to effect protein conformation and association (93), these factors may be involved in the exchange process.

SDS-Polyacrylamide Gel Analysis of Exchanged Proteins

Gel electrophoresis of the recipient HDL subclass, into which labeled apoproteins have been transferred, indicates that ApoA-1, ApoA-2, and the C-peptides are all exchanged. Figure 21 shows the radioactivity pattern obtained when HDL_2, into which labeled apoproteins have been transferred by incubation with $[^{125}I]$-HDL_3, is run on SDS-PAGE. The radioactive bands were identified by staining and scanning duplicate gels.

If the labeled HDL subclass is subjected to limited crosslinking with a 10-fold molar excess of DFDNB before mixing and incubation

Figure 20. Effect of KBr on the exchange of apoproteins between $[^{125}I]$-HDL_3 and HDL_2.

Distribution of label after enctrifugation to separate HDL_2 and HDL_3. ⊖ = $[^{125}I]$-HDL_3 incubated with HDL_2; ⊕ = $[^{125}I]$-HDL_3 incubated with HDL_2 in the presence of 2M KBr.

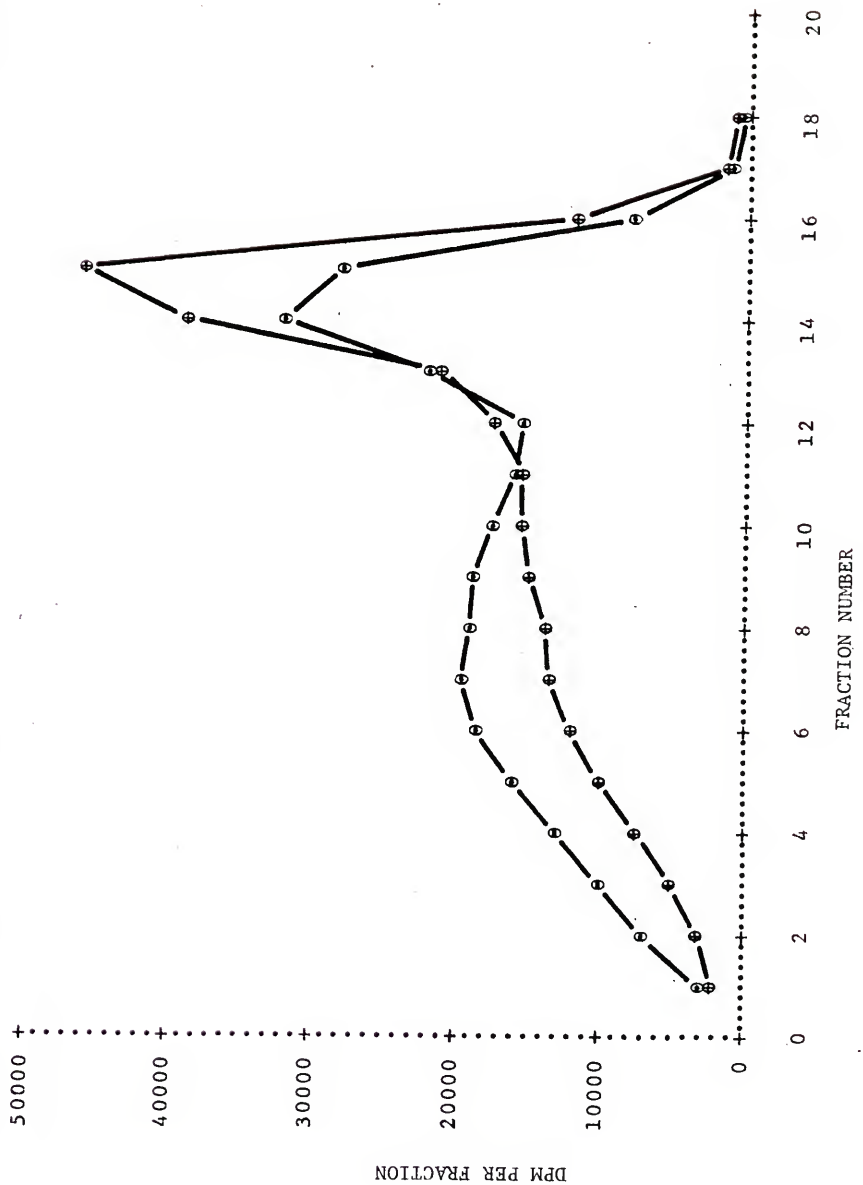
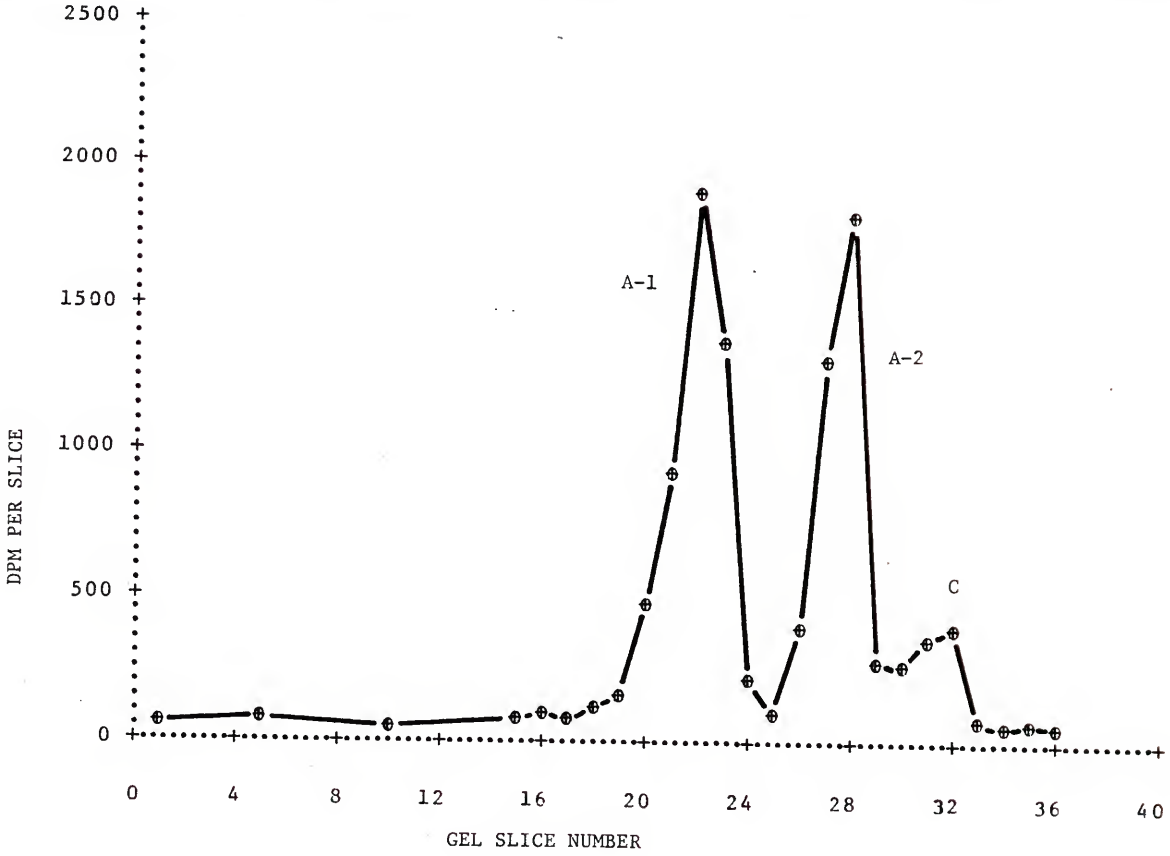

50000
40000
30000
20000
10000
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER

Figure 21. Polyacrylamide gel analysis of exchanged apoproteins.

SDS-PAGE of HDL_2 after incubation with [^{125}I]-HDL_3 followed by reseparation. The gel was sliced into 2mm slices and the slices counted.


2500
2000
1500
1000
500
0
DPM PER SLICE
A-1
A-2
C
0
4
8
12
16
20
24
28
32
36
40
GEL SLICE NUMBER

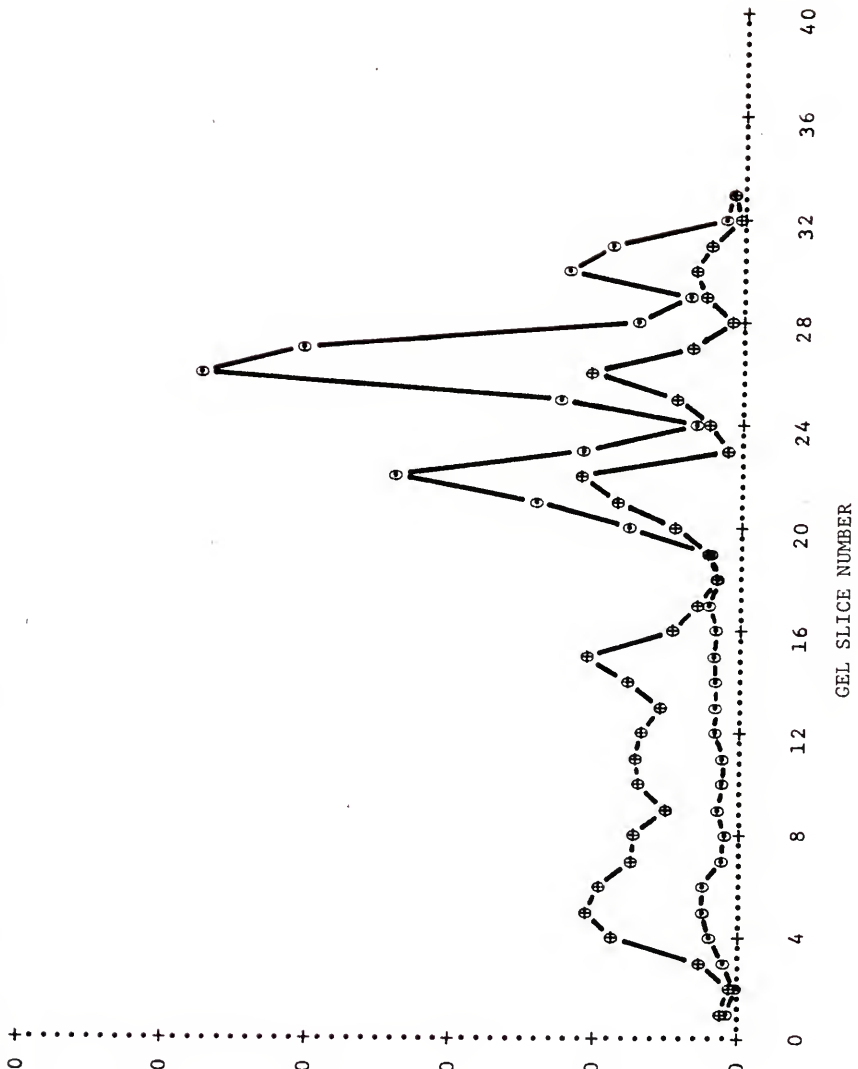
with cold HDL, the labeled apoproteins that are exchanged are those that were not crosslinked. Figure 22 shows the gel pattern obtained for crosslinked [^{125}I]-HDL_3 alone, and for HDL_2 that was labeled by exchange of apoproteins from crosslinked [^{125}I]-HDL_3. Although a significant portion of the HDL_3 apoproteins were crosslinked into higher molecular weight products, only uncrosslinked A-1 and A-2 appear to be exchanged.

Interpretations of Protein Exchange Experiments

The transfer of labeled apoprotein from one HDL subclass to another does not proceed with a loss of protein content of the originally labeled fraction. The specific activity of that fraction decreases but the protein content remains unchanged. This is consistent with an exchange process in which there is a one to one exchange of protein of one subclass with the other. Such exchange could occur by the transfer of single polypeptide apoproteins, i.e., A-1 or A-2; or by the exchange of complexes of apoproteins such as 2A-1:1A-2. These experimental results do not permit us to rule out either of these possibilities. It might be suggested that since crosslinking inhibits the exchange, single polypeptide chains are involved. However, the crosslinking may also prevent a conformational change that might be necessary for the exchange process. That a conformational change might be involved is suggested by the fact that when high concentrations of the chaotropic ion, Br^-, are included in the incubation mixture, increases in the rates of exchange are observed. An actual exchange involving a conformation change is also suggested by the finding that crosslinking the unlabeled subclass also greatly inhibits the exchange

Figure 22. Polyacrylamide gel analysis of exchanged apoproteins: Effect of crosslinking with DFDNB.

SDS-PAGE of HDL_2 reisolated after incubation with $[^{125}I]$-HDL_3 crosslinked with DFDNB. ⊕ = $[^{125}I]$-HDL_3 crosslinked with a 25-fold molar excess of DFDNB; ⊖ = HDL_2 after incubation with crosslinked $[^{125}I]$-HDL_3.


400
320
240
160
80
0
DPM PER SLICE
0
4
8
12
16
20
24
28
32
36
40
GEL SLICE NUMBER

process. If a simple transfer of peptide into the recipient class occurred, one would not expect crosslinking to have much effect.

If HDL_2 is made up of two identical subunits as suggested by Friedberg and Reynolds (54), the transfer of label might be visualized in terms of the exchange of HDL_3 for one of the HDL_2 subunits. Although this is a reasonable possibility, one must again consider the possibility of a conformational change to explain the results of the crosslinking experiments.

Even though the two HDL subclasses can exist as stable separate entities, when they are present together in solution, significant interaction between particles may occur. Exchange of apoproteins could possibly occur between HDL_2-HDL_2 or HDL_3-HDL_3, as well as between HDL_2 and HDL_3 molecules. If collisional complexes cause exchange and single polypeptides are capable of exchange, then one would expect HDL_3-HDL_3 exchange to occur. Collisional complex formation should also result in the rapid exchange of lipids of all types due to their small size and the fusion required for peptide exchange.

Lipid Exchange Between HDL_2 and HDL_3 in Solution

When HDL_3 is labeled with [^{3}H]-cholesterol, [^{3}H]-cholesterol palmitate, or [^{14}C]-phosphatidyl choline, then incubated with cold HDL_2, as with peptide exchange; label is transferred to HDL_2. However, even under conditions of low temperature, dilution, and no incubation, total exchange occurs during the ultracentrifugal reseparation of HDL_2 and HDL_3. This rapid movement prevents the study of the exchange using the experimental approach that was possible for peptide exchange. For this reason an alternative approach was developed.

Binding of Labeled HDL to Sepharose

The rapid rate of lipid exchange places one requirement on the method employed to study this exchange: the separation of HDL_3 and HDL_2 must be done very quickly. As use of the ultracentrifuge requires at least 24 hours even at 60,000 rpm, it was decided to approach the problem by immobilizing one of the subclasses by covalently binding it to Sepharose. This would allow separation by filtration; a process that would take less than one minute.

When [^{125}I]-HDL_3 was reacted with commercially obtained CNBr-activated Sepharose 4B (Pharmacia) under the coupling conditions described in the Methods sections, a Sepharose preparation containing approximately 0.5mg protein per ml of packed Sepharose was obtained. To determine what percentage of the protein was bound to the Sepharose, a portion of the gel was washed with 1% SDS solution. Under these conditions any protein chains not covalently bound to the gel should be washed off. Table 3 shows the results of such an experiment. Using the Pharmacia CNBr-activated Sepharose, no more than 30% of the label could be removed with SDS. Assuming only 3 polypeptide chains/HDL_3 molecule, this indicates that an average of two are linked to the Sepharose. To obtain a preparation with a lower level of binding, it was necessary to activate our own Sepharose 4B using the conditions described in the Methods section. Under these conditions, preparations with over 50% of the protein removable with SDS could be obtained. The same conditions also gave acceptable levels of binding for HDL_2 preparations (20-40% bound). For all peptide exchange experiments, preparations with low levels of binding were used. For lipid exchange

Table 3.

Effect of Amount of CNBr Activation on the Amount of Binding of [^{125}I]-HDL_3 by Sepharose 4B

SAMPLE	TOTAL DPM	DPM of Filtrate	% BOUND
SEPHAROSE 4B-HDL_3 (CNBr Activated-Pharmacia)	14,500	3,920	73%
SEPHAROSE 4B-HDL_3 (CNBr Activated-Pharmacia)	12,500	4,000	68%
SEPHAROSE 3B-HDL_3 (Limited Activation - See Methods Section)	19,590	10,970	44%

experiments, where higher levels of peptide-gel linking were permissible, the CNBr-activated Sepharose from Pharmacia was used.

Peptide Exchange Using Sepharose-Bound HDL

To test the validity of the Sepharose method we repeated the peptide exchange experiments and compared the results to those obtained using the ultracentrifugal separation. Table 4 gives the data for peptide exchange obtained under a variety of conditions similar to those previously described. Several important observations can be made. First, both cold and dilution reduce the amount of exchange that occurred in a given time period. Second, crosslinking of the HDL subclass on the Sepharose or in solution reduces the exchange. Finally, exchange can occur between Sepharose-HDL_3 and HDL_3, as well as between Sepharose-HDL_2 and HDL_2 in solution. These results suggest that the binding to Sepharose does not greatly affect the exchange process.

Because this method allows the study of the interaction of like molecules (i.e. HDL_3-HDL_3) as well as different types of particles, several experiments that could yield information regarding the possibility of subunit exchange became possible.

Ultracentrifugal Analysis of Peptide Exchange Using Sepharose-Bound HDL

If HDL_2 is bound to Sepharose and then incubated with HDL_3 in solution, one would expect different products in solution depending upon the possibility of subunit exchange. Figure 23 shows the ultracentrifugal fractionation pattern obtained after HDL_3 was incubated with [^{125}I]-HDL_2 bound to Sepharose. It can be seen that both labeled HDL_2 and HDL_3 particles are formed during the incubation. This could

Table 4.

Peptide Exchange Using Sepharose-Bound [^{125}I]-HDL (S-[^{125}I]-HDL). Effect of Dilution, Temperature, and Crosslinking on Peptide Exchange.

400µl of S-[^{125}I]-HDL (S-HDL_2 = 28,000 total counts, S-HDL_3 = 44,000 total counts) was mixed with 150µg cold HDL and incubated for 3.5 hours at the indicated temperature. After incubation the mixture was filtered, washed with 200µl 0.15M NaCl, and counted for 20 sec.

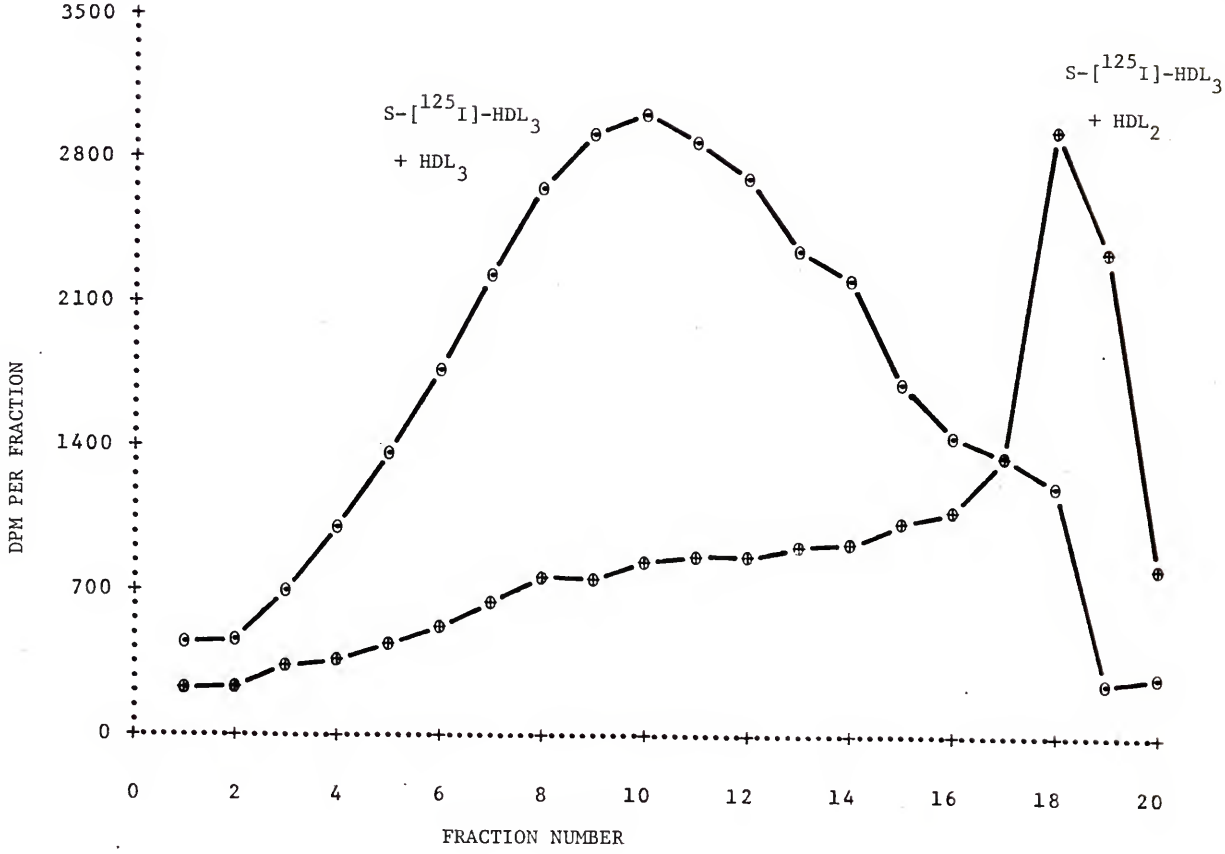
S-HDL	COLD HDL	SPECIAL CONDITIONS	COUNTS FILTRATE	Δ COUNTS
$S\text{-}HDL_2$	-	Control, 37° (Incubation temp.)	742	0
$S\text{-}HDL_2$	HDL_3	- , 37°	6,025	5,283
$S\text{-}HDL_2$	HDL_2	- , 37°	4,157	3,415
$S\text{-}HDL_2$	HDL_3	Cold HDL_3 C.L.‡ 50x DFDNB, 37°	4,499	3,757
$S\text{-}HDL_2$	HDL_2	Cold HDL_2 C.L. 50x DFDNB, 37°	3,390	2,648
$S\text{-}HDL_2$	HDL_3	$S\text{-}HDL_2$ C.L. 50x DFDNB, 37°	2,448	1,706
$S\text{-}HDL_2$	HDL_2	$S\text{-}HDL_2$ C.L. 50x DFDNB, 37°	2,439	1,697
$S\text{-}HDL_2$	HDL_3	Dilute RXN. 1ml 0.15M NaCl, 37°	4,440	3,698
$S\text{-}HDL_2$	HDL_3	Incubate 4°C	1,414	672
$S\text{-}HDL_3$	-	Control, 37°	148	0
$S\text{-}HDL_3$	HDL_3	- , 37°	3,568	3,420

$S\text{-}HDL_3$	HDL_2	- , 37°	2,026	1,878
$S\text{-}HDL_3$	HDL_3	Cold HDL_3, C.L. 50x DFDNB, 37°	1,276	1,128
$S\text{-}HDL_3$	HDL_2	Cold HDL_2, C.L. 50x DFDNB, 37°	650	502
$S\text{-}HDL_3$	HDL_3	$S\text{-}HDL_3$ C.L. 50x DFDNB, 37°	373	225
$S\text{-}HDL_3$	HDL_2	$S\text{-}HDL_3$ C.L. 50x DFDNB, 37°	372	224
$S\text{-}HDL_3$	HDL_3	Dilute RXN. 1ml 0.15M NaCl, 37°	2,270	2,122
$S\text{-}HDL_3$	HDL_2	Incubate 4°C	578	430

ǂ C.L. = Crosslinked

Figure 23. Subunit exchange between S-[^{125}I]-HDL_2 and HDL_3.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊖ = HDL_2 incubated with S-[^{125}I]-HDL_2; ⊕ = HDL_3 incubated with S-[^{125}I]-HDL_2.


3500
2800
2100
1400
700
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER
S-[125I]-HDL3
+ HDL3
S-[125I]-HDL3
+ HDL2

only occur if one of the unbound subunits of HDL_2 was combined with an HDL_3 molecule to form an HDL_2 molecule in solution. If cold HDL_2 is incubated with Sepharose bound [^{125}I]-HDL_2, only labeled HDL_2 is found in solution (Figure 24). If subunit exchange occurs, one would also expect both HDL_2 and HDL_3 to be formed when HDL_2 is incubated with [^{125}I]-HDL_3 bound to Sepharose. Figure 24 shows this to be the case. Figure 24 also shows that HDL_3 incubated with bound [^{125}I]-HDL_3 only yields labeled HDL_3 in solution. This could only occur by the exchange of single apoprotein molecules as at least one of the HDL_3 peptides must be covalently bound to the Sepharose. This would obviously prevent the exchange of a whole subunit.

SDS-PAGE Analysis of Exchanged Peptides Using Sepharose-Bound HDL

To show that all the different types of apoproteins were available for exchange, [^{125}I]-HDL_2 and [^{125}I]-HDL_3 bound to Sepharose were washed with 1% SDS and the wash solution subjected to SDS-PAGE. Figure 25 shows the radioactivity pattern obtained after slicing and counting the gels. ApoA-1, ApoA-2, and the C-peptides are all capable of being removed and available for exchange. This experiment also indicates that there is no preferential binding of any single apoprotein species to the Sepharose as the ratios obtained are essentially the same as those found in native HDL.

That all types of apoproteins are exchanged is indicated when one does SDS-PAGE of either HDL_2 or HDL_3 after incubation with [^{125}I]-HDL_3 bound to Sepharose (Figure 26). SDS was added to solubilize the apoproteins of the HDL particles found in the filtrate after incubation and

Figure 24. Subunit exchange between S-[^{125}I]-HDL_3 and HDL_2.

Distribution of label after centrifugation to separate HDL_2 and HDL_3. ⊕ = HDL_3 incubated with S-[^{125}I]-HDL_3; ⊖ = HDL_2 incubated with S-[^{125}I]-HDL_3.

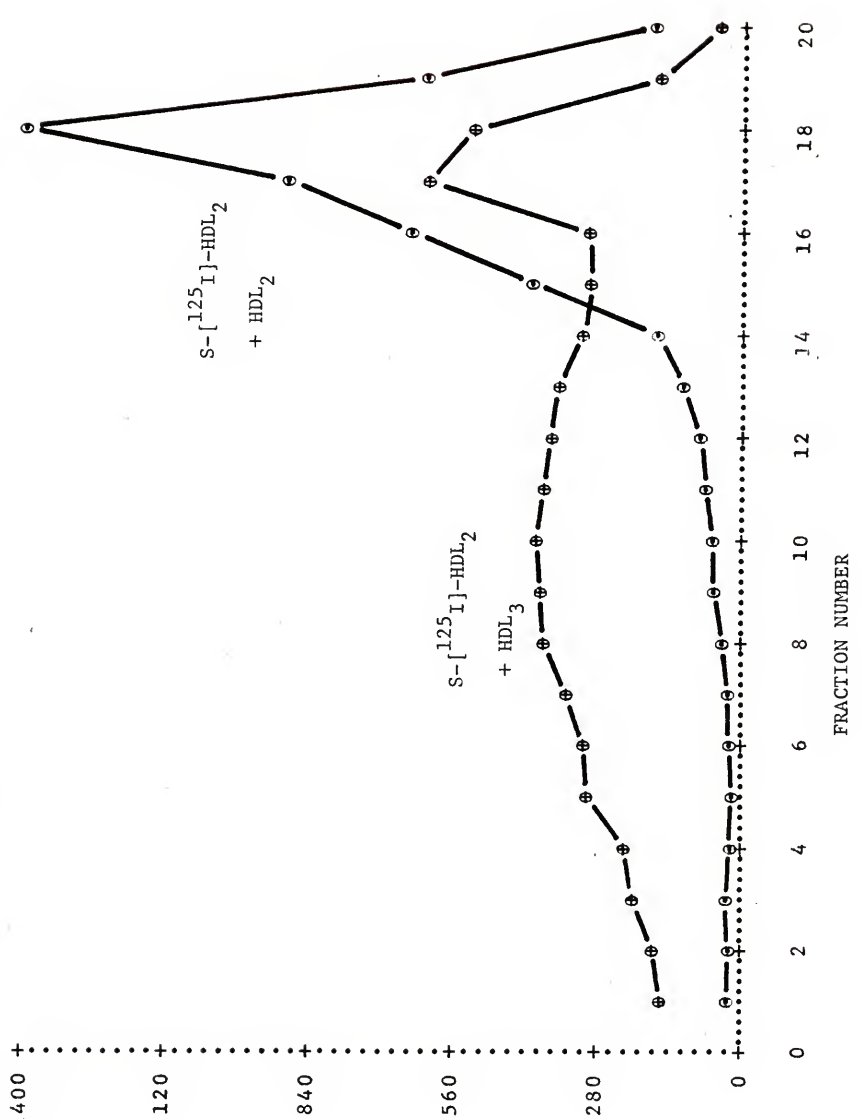

1400
1120
840
560
280
0
DPM PER FRACTION
0
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER
S-[125I]-HDL2
+ HDL2
S-[125I]-HDL2
+ HDL3

Figure 25. Polyacrylamide gel analysis of available apoproteins from S-HDL_3.

SDS-PAGE of SDS wash of S-HDL_3. Peptides labeled with [^{125}I]. The gel was sliced into 1mm slices and the slices counted.

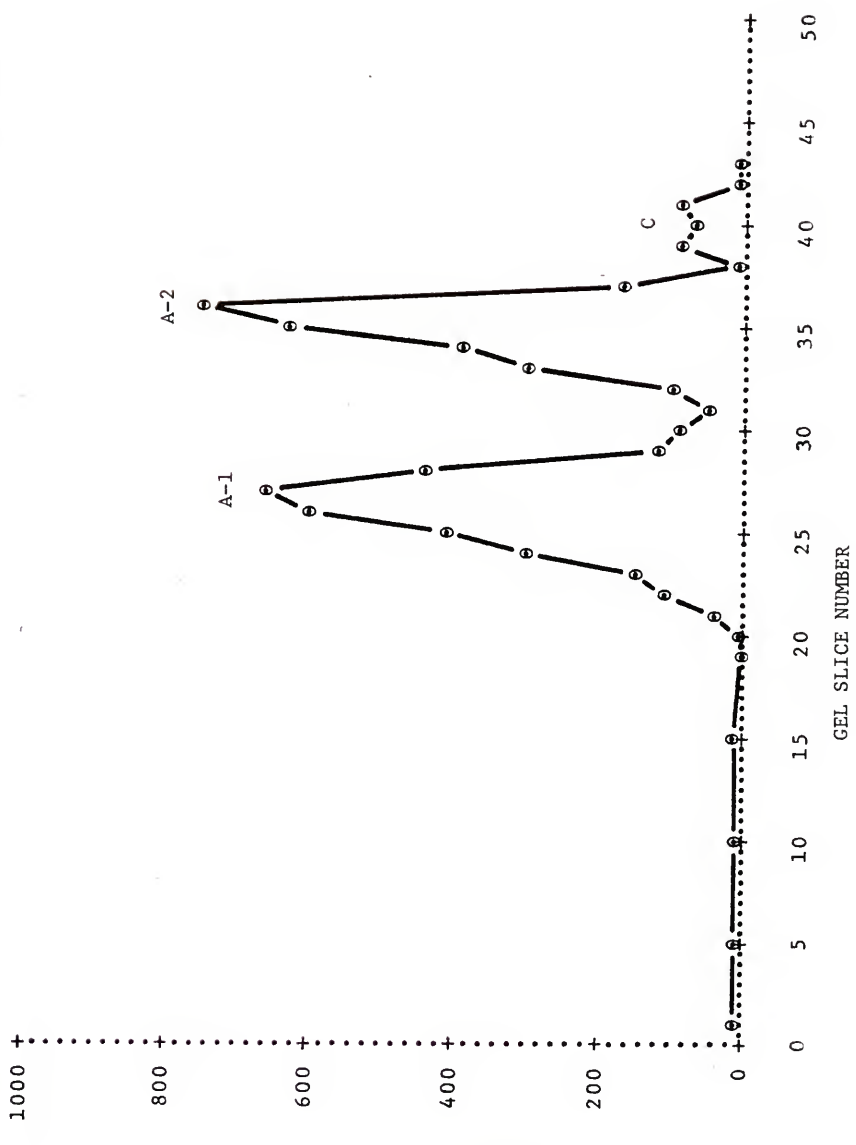
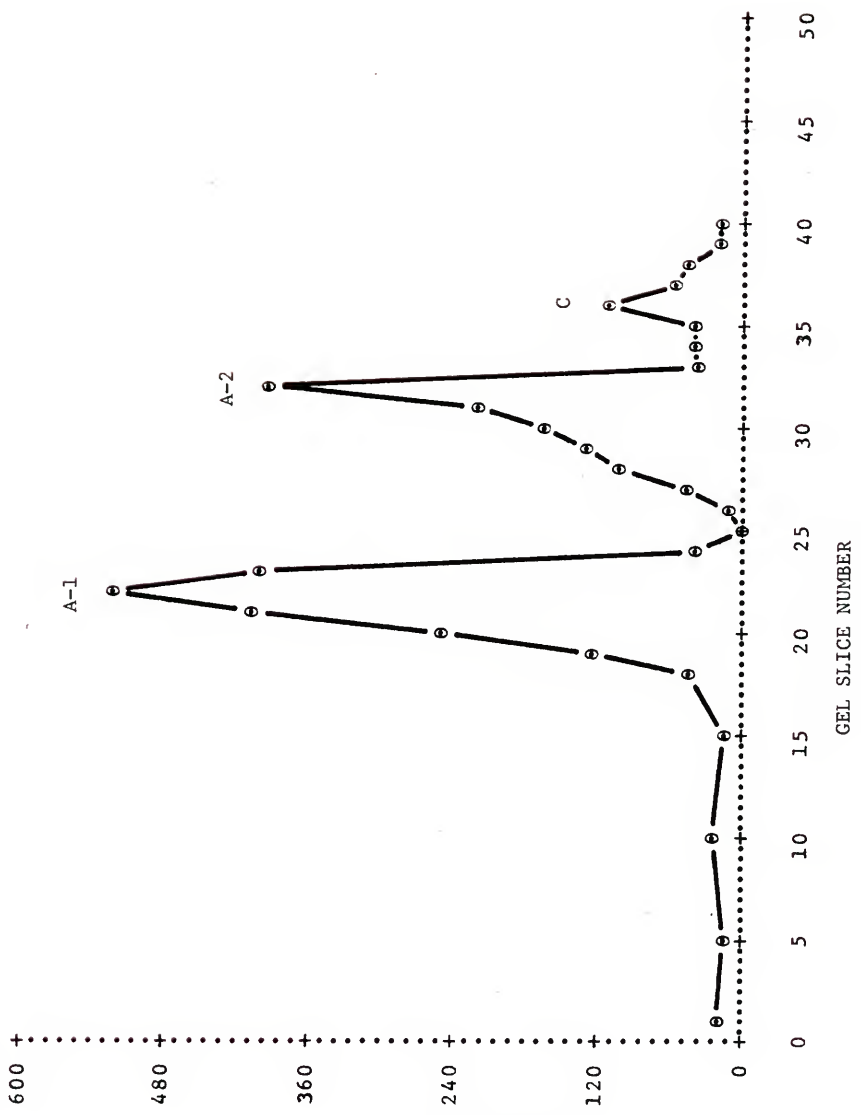

DPM PER SLICE
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
45
50
GEL SLICE NUMBER
A-1
A-2
C

Figure 26. Polyacrylamide gel analysis of apoproteins exchanged from S-[^{125}I]-HDL_3.

SDS-PAGE of HDL_3 into which labeled apoproteins have been transferred by incubation with S-[^{125}I]-HDL_3. The gels were sliced into 1mm slices and the slices counted.


600
480
360
240
120
0
DPM PER SLICE
0
5
10
15
20
25
30
35
40
45
50
GEL SLICE NUMBER
A-1
A-2
C

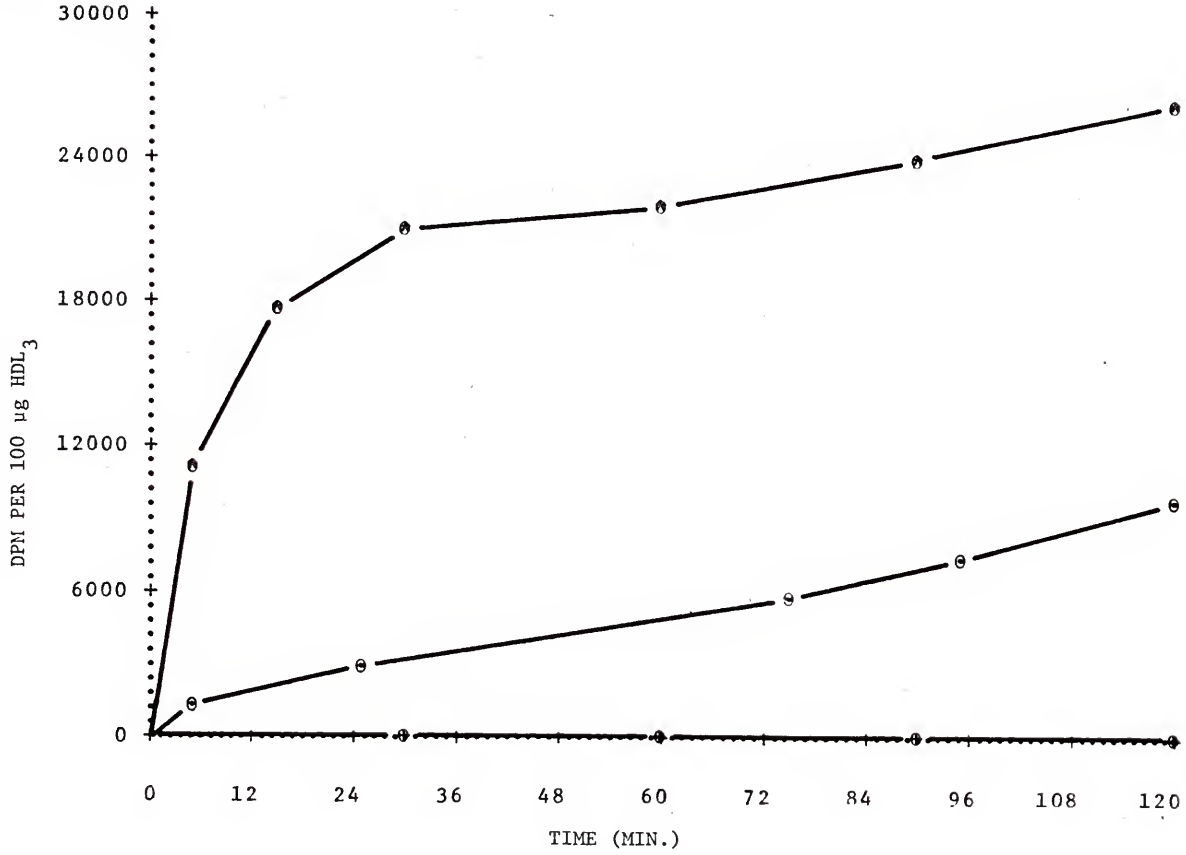
removal of Sepharose. Again, all three types of labeled apoproteins are present. The only possible source of label is by exchange with labeled HDL bound to the Sepharose.

Incorporation of Labeled Lipid into HDL_2 and HDL_3

To study lipid exchange in lipoproteins it is first necessary to incorporate labeled lipid into one of the lipoprotein subclasses. As most lipids are quite insoluble in aqueous solutions and because one must be able to separate any free lipid from the labeled HDL, it is desirable to place the lipid on a solid support that can be easily separated from the solution. Because the physical state of the lipids appears to be important in determining a lipid binding protein's ability to incorporate them (94), the lipid was first adsorbed to celite particles before mixing with the HDL to be labeled. This procedure proved very satisfactory for the incorporation of cholesterol, cholesterol palmitate, and phosphatdyl choline into HDL_2 or HDL_3. Figure 27 shows the time course for uptake of labeled lipids from celite by HDL_3 under the conditions described in the Methods section. It should be noted that the leveling off of the curves represents the uptake of 90% or more of the label available, not a saturation of the HDL by the lipid. Low levels of labeling were used to try and achieve binding of fewer than one labeled lipid molecule per HDL molecule. This was done to prevent such major alterations in the lipoprotein particle's structure as might occur with increased lipid binding. Calculations of the number of lipid molecules bound per HDL molecule gave the following results for the preparations used for all the experiments described: $[^3H]$-cholesterol-HDL_3 = 3700 HDL molecules/$[^3H]$ cholesterol molecule,

Figure 27. Uptake of labeled lipid by HDL_3.

Time course for the uptake of labeled lipid from celite particles. Θ = uptake of [^{3}H]-cholesterol palmitate by HDL_3; ⊕ = uptake of [^{14}C]-phosphatidyl choline by HDL_3; ⊗ = [^{14}C]-phosphatidyl choline uptake by control buffer.


30000
24000
18000
12000
6000
0
DPM PER 100 µg HDL3
0
12
24
36
48
60
72
84
96
108
120
TIME (MIN.)

[^{3}H]-cholesterol palmitate-HDL_3 = 5.0 HDL molecules/[^{3}H]cholesterol/ molecule, [^{14}C]-phosphatidyl choline-HDL_3 = 35 HDL molecules/[^{14}C]-phosphatidyl choline molecule. These preparations of labeled HDL were bound to CNBr-activated Sepharose 4B (Pharmacia) and the resulting products were used for all Sepharose-HDL lipid exchange experiments described.

To measure the amount of label available for exchange and not bound to the HDL irreversibly, aliquots of the Sepharose-HDL_3 labeled with lipid were washed with 1% SDS solution, the Sepharose removed by filtration, and the filtrate counted in Aquasol (Table 5). Essentially all of the label is removed with SDS while washing with buffer has no effect. This fact, in addition to the results obtained with SDS removal of labeled peptides, suggests that stable intact particles are bound to the gel and that non-covalently bound materials can be removed only when the lipoprotein particles are disrupted with detergent.

Ultracentrifugation of HDL labeled with radioactive lipid at d = 1.125gm/cm^{3} shows that the label followed the lipoprotein pattern produced when the peptides were labeled and indicated no free label in the solution.

Lipid Exchange Between HDL_3 and Sepharose-HDL_3

To determine the extent of bulk transfer of lipid by subunit exchange from HDL_2, lipid exchange between HDL_3 molecules was studied. Because it had been previously demonstrated that lipids will exchange, experiments designed to help to understand the mechanism of exchange were carried out. If lipid labeled, Sepharose-bound HDL_3 (S-HDL_3^*) was incubated for 30 min with varying amounts of HDL_3 in solution (Figures 28,29). All three lipid types exhibit this behavior suggesting

Table 5.

Removal of Labeled Lipid from Sepharose-Bound HDL_3 ($S\text{-}HDL_3^*$) By SDS Wash

100µl of $S\text{-}HDL_3^*$ was washed with 1ml of 1% SDS, filtered, and the filtrate counted in 5ml of Aquasol.

Labeled $S\text{-}HDL_3^*$	DPM Before SDS	DPM SDS wash
S-[^{3}H]-Cholesterol-HDL_3	29,388	27,265
S-[^{3}H]-Cholesterol palmitate-HDL_3	21,020	19,912
S-[^{14}C]-Phosphatidyl choline-HDL_3	9,870	9,117

Figure 28. Effect of increasing amounts of cold HDL_3 on lipid exchange.

25µl S-HDL_3^* (3µg/HDL protein/µl gel) + 100µl 0.15M NaCl containing cold HDL_3. ⊖ = [^{3}H]-cholesterol-S-HDL_3; ⊕ = [^{3}H]-cholesterol palmitate-S-HDL_3.

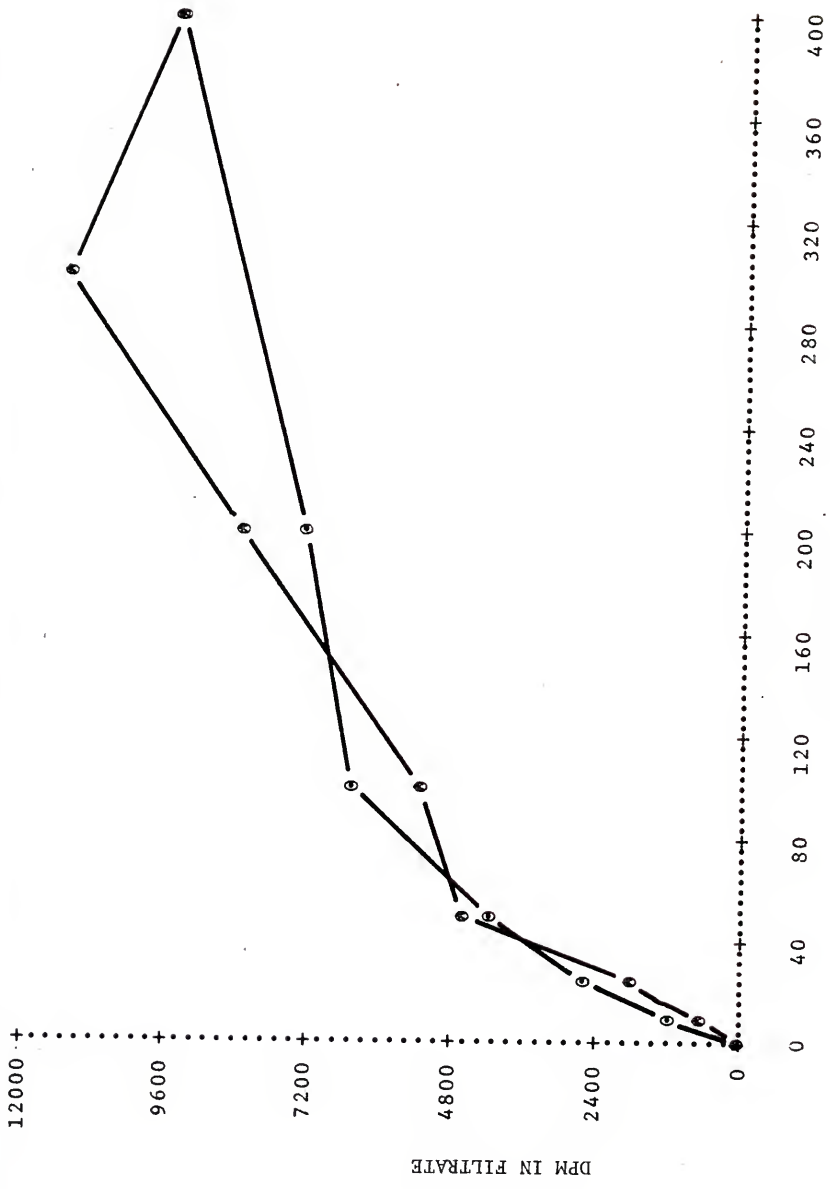
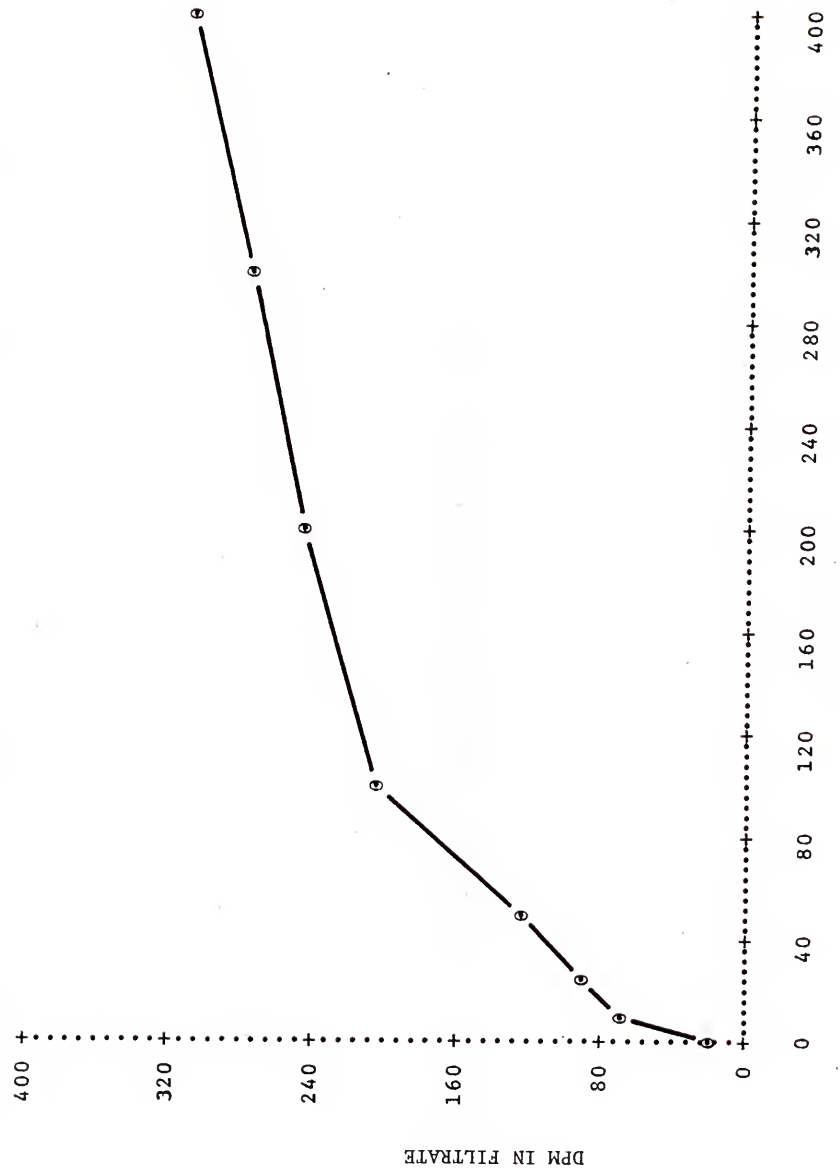

12000
9600
7200
4800
2400
0
DPM IN FILTRATE
0
40
80
120
160
200
240
280
320
360
400
µg HDL3 ADDED

Figure 29. Effect of increasing amounts of cold HDL_3 on lipid exchange.

$[^{14}C]$-phosphatidyl choline exchange from S-HDL_3^*. 25µl S-HDL_3^* (3µg HDL_3 protein/µl gel) + 100µl 0.15M NaCl containing cold HDL_3.


DPM IN FILTRATE
400
320
240
160
80
0
0
40
80
120
160
200
240
280
320
360
400
µg HDL3 ADDED

that collisional complex formation is required for lipid exchange as it appears to be for protein exchange.

To try to establish the relative rates of exchange of these lipid types, incubations were carried out at various times and the amount of label transferred plotted as a function of time of incubation (Figures 30,31). It appears that at the concentrations of HDL used, cholesterol and cholesterol palmitate both exchange very rapidly with half times of less than 30 min. Higher concentrations of free HDL result in even faster exchange rates. In the case of phosphatidyl choline, an initially rapid exchange with a short half-life (<30 min) is followed by a slower exchange process. Exchange of cholesterol and cholesterol palmitate after 24 hrs. results in exchange with total counts transferred not much greater than the values obtained for 2 hrs. However, for phosphatidyl choline, the 24 hr. value is more than twice the 2 hr. value.

Effect of Temperature and Dilution on Lipid Exchange

If separate batches of S-HDL_3^* and HDL_3 are mixed and incubated for equal times at different temperatures, the amount of exchange is decreased at lower temperatures for cholesterol and cholesterol ester (Table 6). Dilution (increasing the volume) of the incubation solution also appears to decrease the exchange (Table 6). These observations are consistent with collision complex formation.

Effect of Saturating Cold Lipid on Labeled Lipid Exchange

If the mechanism of exchange depended upon the dissociation of lipid from the HDL particle into solution and then subsequent reassociation by other lipoprotein molecules, saturation of the aqueous medium

Figure 30. Rate of lipid exchange between S-HDL_3^* and HDL_3.

Time course for lipid exchange between S-HDL_3^* and cold HDL_3. 25µl S-HDL_3^* (3µg HDL protein/µl gel) + 100µl 0.15M NaCl containing 100µl HDL_3. ⊖ = [^{3}H]-cholesterol-S-HDL_3; ⊕ = [^{3}H]-cholesterol palmitate-S-HDL_3.

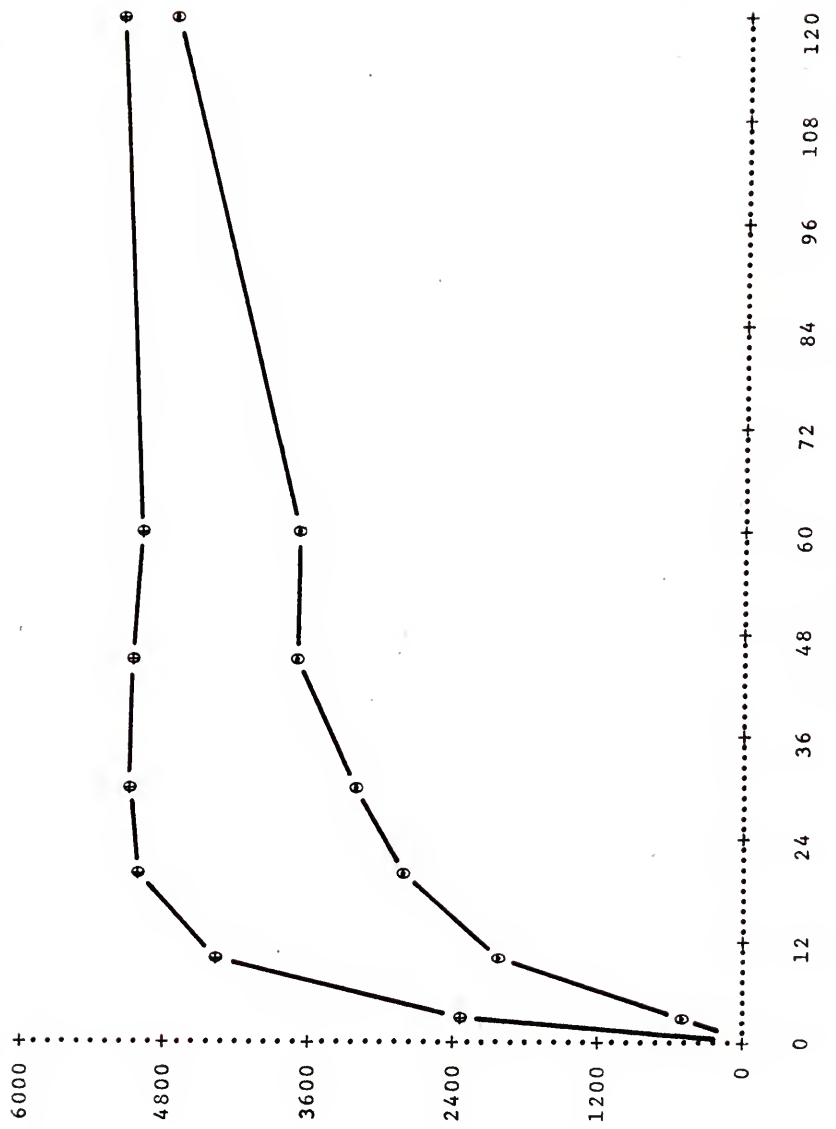

6000
4800
3600
2400
1200
0
DPM IN FILTRATE
0
12
24
36
48
60
72
84
96
108
120
TIME (MIN.)

Figure 31. Time course for lipid exchange between S-HDL_3^* and HDL_3. [^{14}C]-phosphatidyl choline--HDL_3^* exchange to cold HDL_3. 25µl S-HDL_3^* (3µg HDL protein/ 1 gel) + 100µl 0.15M NaCl containing 100µg HDL_3.

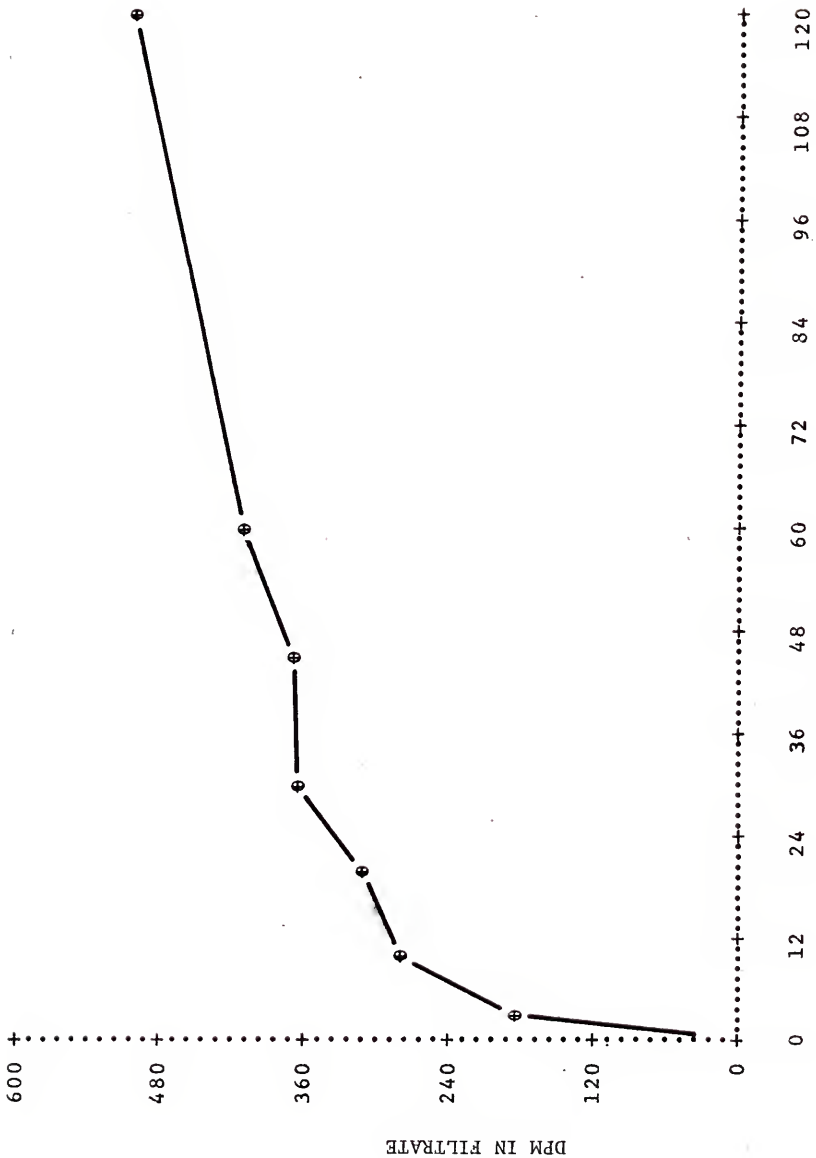

DPM IN FILTRATE
600
480
360
240
120
0
0
12
24
36
48
60
72
84
96
108
120
TIME (MIN.)

Table 6.

Effect of Temperature and Dilution on Lipid Exchange Between S-HDL_3^* and HDL_3.

25µl S-HDL_3^* (3mg HDL Protein/1 gel) + 150µl 0.15M NaCl containing 100µg HDL_3. Incubations were carried out for 15 min.

SAMPLE	CONDITIONS	DPM FILTRATE
[^{3}H]-Cholesterol	4° Incubation	3,157
"	20° Incubation	5,473
"	37° Incubation	6,815
"	37° Incubation, Diluted with 3ml of 0.15M NaCl	1,344
[^{3}H]-Cholesterol palmitate	4° Incubation	7,733
"	20° Incubation	8,580
"	37° Incubation	9,614
"	37° Incubation, Diluted with 3ml of 0.15M NaCl	4,352
[^{14}C]-Phosphatidyl choline	4° Incubation	286
"	20° Incubation	283
"	37° Incubation	273
"	37° Incubation, Diluted with 3ml of 0.15M NaCl	147

with unlabeled lipid of the same type being transferred should greatly decrease the rate of transfer. Table 7 shows the results of such an experiment. The amount of cold lipid added to the solution was approximately 100 times greater than the total contained in all the lipoprotein particles present. The lipid was added dissolved in a small amount of organic solvent. The majority of the lipid precipitated indicating that saturating conditions were reached. Even under these rather extreme conditions, the amount of exchange observed was equal to the controls containing only bound lipid. This strongly suggests that dissociation into the medium is not a significant factor in the exchange process.

Effect of Crosslinking on Lipid Exchange

When either the labeled or cold HDL is crosslinked with a 25-fold molar excess of DFDNB before mixing, no significant reduction in exchange appears to occur (Table 8). This observation is not unexpected as fusion of lipids during collision complex formation would not be influenced greatly by protein crosslinking.

Effect of Added Organic Solvent on Exchange of Lipids and Proteins

When both lipid and apoprotein exchange is studied in the presence of increasing amounts of ethanol in the solution, the rates of exchange increase significantly (Figures 32,33). These increases appear to be the result of factors other than the total disruption of the particles as control experiments indicate that labeled lipid is not removed from $S\text{-}HDL_3^*$ by the solvent alone.

Table 7.

Effect of Saturating Cold Lipid on Lipid Exchange Between S-HDL_3^* and HDL_3.

50 l S-HDL_3 (3 g HDL Protein/ l gel) + 300 l 0.15M NaCl containing 100 l HDL_3. 15 min. incubations.

SAMPLE	CONDITIONS	DPM FILTRATE
[^{3}H]-Cholesterol	Buffer Only	62
"	Buffer + Saturating Cholesterol	619
"	HDL_3	5,719
"	HDL_3 + Saturating Cholesterol	6,662
[^{3}H]-Cholesterol palmitate	Buffer Only	316
"	Buffer + Saturating Cholesterol palmitate	291
"	HDL_3	10,581
"	HDL_3 + Saturating Cholesterol palmitate	10,432
[^{14}C]-Phosphatidyl choline	Buffer Only	29
"	Buffer + Saturating Phosphatidyl choline	51
"	HDL_3	384
"	HDL_3 + Saturating Phosphatidyl choline	388

Table 8.

Effect of Crosslinking on Lipid Exchange Between S-HDL_3^* and HDL_3.

25µl S-HDL_3^* (3µg HDL Protein/µl Gel) + 150µl 0.15M NaCl containing 100µg HDL_3. All crosslinking (C.L.) 50x molar excess DFDNB. 15 min. incubations.

SAMPLE	CONDITIONS	DPM FILTRATE
[^{3}H]-Cholesterol	HDL_3	10,611
"	HDL_3 - C.L. DFDNB	9,075
"	HDL_3, S-HDL_3^*-C.L. DFDNB	9,069
[^{3}H]-Cholesterol palmitate	HDL_3	17,821
"	HDL_3 - C.L. DFDNB	17,124
"	HDL_3, S-HDL_3^*-C.L. DFDNB	18,323
[^{14}C]-Phosphatidyl choline	HDL_3	421
"	HDL_3 - C.L. DFDNB	386
"	HDL_3, S-HDL_3^*-C.L. DFDNB	394

Figure 32. Effect of ethanol on the exchange of lipids from S-HDL_3^* to HDL_3.

Duplicate incubations with S-HDL_3^* were carried out in the presence of increasing amounts of ethanol. ⊖ = [^{3}H]-cholesterol S-HDL_3^* + HDL_3; ⊕ = [^{3}H]-cholesterol palmitate-S-HDL_3 + HDL_3; ⊠ = [^{3}H]-cholesterol palmitate-S-HDL_3 control; ⊗ = [^{3}H]-cholesterol-S-HDL_3 control.

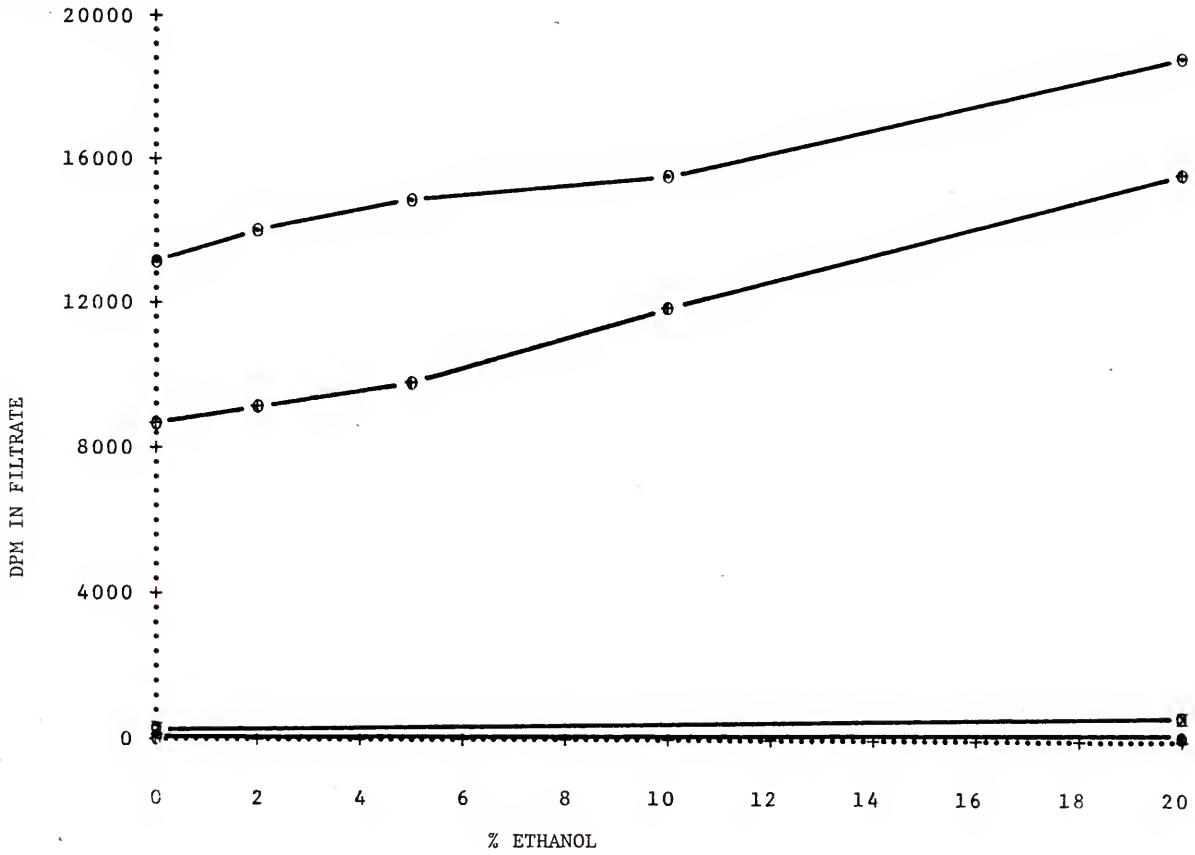
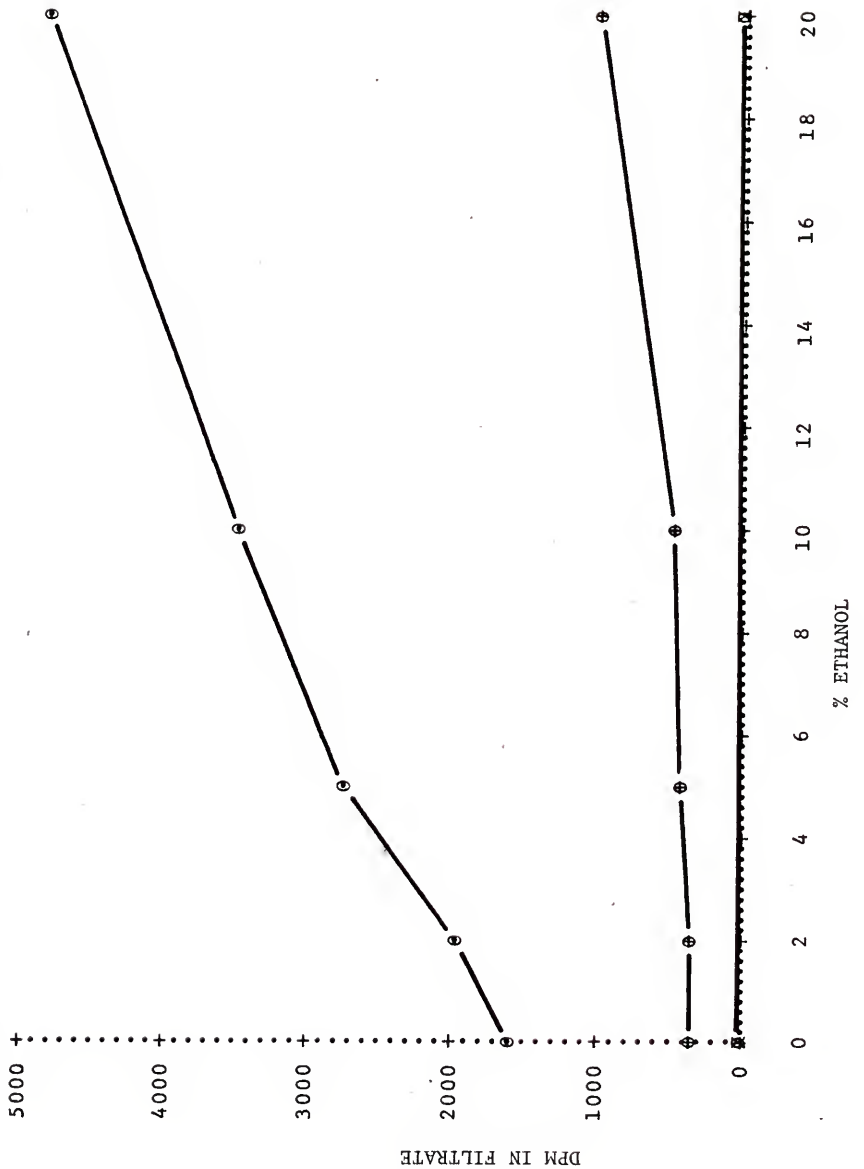

20000
16000
12000
8000
4000
0
DPM IN FILTRATE
0
2
4
6
8
10
12
14
16
18
20
% ETHANOL

Figure 33. Effect of ethanol on the exchange of lipid and protein from S-HDL_3 to HDL_3.

Duplicate incubations with S-HDL_3^* or S-[^{125}I]-HDL_3 were performed in the presence of increasing amounts of ethanao. ⊖ = [^{125}I]-S-HDL_3 + HDL_2; ⊕ = [^{14}C]-phosphatidyl choline-S-HDL_3 + HDL_3; ⊠ = [^{14}C]-phosphatidyl choline-S-HDL_3 control.


DPM IN FILTRATE
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
18
20
% ETHANOL

Interpretations of Sepharose-HDL Peptide and Lipid Exchange Experiments

The data obtained in the Sepharose-bound HDL experiments compares well with that obtained using the ultracentrifugal separation procedure. Low temperature, dilution, and crosslinking all reduce peptide exchange in both systems. In the gel system, however, the observed reduction is not as great as that seen in the non-bound system. This may be ascribed to the fact that the amount of HDL bound to the gel (100-200 µg/ml) is much less than 2-3mg/ml, the concentration of HDL at which the original method was used. To observe significant exchange in a similar time period, the concentration of cold HDL added far exceeds that of the labeled and bound subclass. This condition pulls the exchange process in the direction of removal of label from the bound HDL. To obtain levels of binding comparable to those possible in solution (3mg/ml), highly activated Sepharose is needed. Unfortunately, this level of activation also results in multiple binding to each HDL molecule and subsequently reduced amounts of free peptide are available for exchange.

A very interesting observation, not possible with the solution exchange procedure, is that one can demonstrate exchange between individual HDL_3 molecules and between HDL_2 molecules. $S\text{-}HDL_2 \rightarrow HDL_2$ exchange could occur by both subunit exchange and exchange involving the movement of single polypeptide chains. However, $S\text{-}HDL_3 \rightarrow HDL_3$ exchange could only occur by transfer of single apoprotein species. An actual exchange is again strongly suggested by the ultracentrifugal analysis of $S\text{-}HDL_3 \rightarrow HDL_3$ exchange products which shows only HDL_3 molecules are produced. If only a transfer of protein occurred, the resulting particles

would have a higher density than native HDL_3 molecules and would sink to the bottom ov the centrifuge tube. The results of this experiment indicate a normal distribution pattern for HDL_3.

The production of free HDL_2 when HDL_3 is incubated with S-HDL_2 indicates that subunit exchange has occurred. The only possible source of free HDL_2 is from the combination of an HDL_3 molecule and one of the unbound subunits of the S-HDL_2. The reverse experiment using S-HDL_3 and free HDL_2 results in the formation of free HDL_3; a possibility only if a subunit is transferred from HDL_2 in solution to S-HDL_3, resulting in free HDL_3 molecules and bound HDL_2 molecules. Subunit exchange would have to involve both A-1 and A-2 apoproteins but single peptide exchange between S-HDL_3 and HDL_3 could be specific for a particular apoprotein. However, Figures 25 and 26 show that this is not the case. This is interesting in view of the fact that A-1 and A-2 appear to differ significantly in their ability to bind lipids. The results of this experiment also suggest that a dynamic association exists between A-1 and A-2. If one of the members of the A-1:A-2 unit is bound to the Sepharose, a dissociation of those labeled apoproteins that are free, followed by a reassociation with unlabeled peptides must occur. Since the particles do not appear to be disrupted during the exchange process, a significant degree of flexibility must exist within the intact particle.

The results of the lipid exchange experiments support the suggestion that collision complex must occur for the exchange process to occur. As with the peptide exchange, lower temperature, lower concentrations of free HDL, and dilution of the reaction mixtures, all reduce

the rate of exchange appreciably. The conclusion is also strongly supported by the experiments using saturating levels of cold lipid. If dissociation of the lipid molecule into the medium was required, surely increasing the amount of free lipid would have a great effect on exchange.

The rates of lipid exchange are consistent with their size and mobility in the intact particles. A fusion of the contact surface of the collision complex would result in a fast exchange of lipids and would also provide a hydrophobic environment for movement and rearrangement of the apoproteins and interior lipid molecules. The exchange of phospholipids appears to follow two processes. A very rapid exchange of approximately one-fourth of the label, followed by a much slower exchange of the remaining label; this could be explained by assuming the slowly exchanging phospholipid was strongly associated with the protein while the more rapidly exchanging fraction was free phospholipid. The lack of effect of crosslinking is not unexpected as the incubation time only included the fast exchange portion.

By decreasing solvent polarity with the addition of ethanol, one might expect to cause a disruption of the HDL particle. This does not appear to be the case, but the rate of exchange is increased. This could be due to an overall decrease in the strength of interaction of both protein and lipid components. Such a decrease, although reducing the interactions, still is not sufficient to break apart the particle. This finding suggests that the HDL molecules are held together by strong forces, capable of withstanding conditions which would greatly affect certain other types of molecular aggregations.

CONCLUSIONS AND SPECULATIONS

From the data in these experiments we may reach the following major conclusions:

1) Bifunctional crosslinking reagents of both hydrophobic and hydrophillic character are capable of forming protein-protein cross-links between the major apoprotein components of human HDL.

2) DFDNB crosslinking suggests that ApoA-1 and ApoA-2 must, at least part of the time, lie in close proximity in intact HDL_2 and HDL_3.

3) HDL_2 appears to contain two subunit complexes that are not crosslinked with DFDNB, DMS, or DFDNDPS under conditions tested.

4) The separate apoprotein molecules can exchange between and among the HDL subclasses HDL_2 and HDL_3 at a rate rapid enough to be of importance in the biological lifetime of the particles.

5) Subunits of HDL_2 can exchange with HDL_3 resulting in a new HDL_2 particle containing the original HDL_3.

6) Crosslinking with bifunctional reagents apparently prevents a conformational change necessary for exchange of the apoproteins.

7) The three major lipid types of HDL all exchange among and between the HDL subclasses.

8) Both lipid and protein exchange appear to involve formation of collision complexes between the lipoprotein particles.

9) There exists a dynamic relationship between HDL_2 and HDL_3. Even though these two subclasses can exist as stable separate entities,

when they are present together in solution, significant interaction between particles may occur.

The results of the experiments of Friedberg and Reynolds along with the evidence presented here suggest that a "lipid binding unit" of 2 ApoA-1 + 1 ApoA-2 molecules form the basis for the structure of both HDL_2 and HDL_3. However, the combination of two HDL_3 molecules does not produce an HDL_2 molecule. There are two possible explanations; first, the extra lipid present in HDL_2 (60% lipid, 40% protein) could come from membranes or other lipoprotein molecules by lipid binding to HDL_3 molecules. The interaction of a possibly unstable lipid rich HDL_3 molecule with another HDL_3 molecule could result in the formation of an HDL_2 molecule. HDL_3 molecules alone in solution would not have a source of extra lipid and would thus remain as HDL_3 molecules only. Since HDL_2 molecules in solution would have no place in which to deposit this extra lipid, they could not produce HDL_3 molecules. An alternative explanation would involve the alteration of apoprotein interactions and conformation into a form capable of increased lipid binding. Again, this change may be only possible in the presence of other lipoproteins or cell membranes. Since the exact nature of such subtle apoprotein interaction may govern lipid binding, we suggest that further studies should be undertaken to determine the exact location of chemical cross-links.

Although the exact role of HDL is not known, we suggest that it might serve its major function as a transport vehicle for cholesterol and cholesterol esters. The ease with which HDL adsorbs these lipids from celite particles would seem to indicate that any readily available

lipid of this type would be rapidly picked up by HDL in the circulatory system. This idea could accommodate an interconversion of HDL_3 and HDL_2 by interaction with a lipid source. A source containing phospholipids as well as cholesterol would be required; again other plasma lipoproteins or cell membranes come to mind. The phospholipid would serve to maintain apoprotein structure and perhaps interaction. This model suggests experiments that could determine the effect of different lipid sources on HDL_3 - HDL_2 interconversion and exchange. Such a model might be diagrammed as shown in Figure 34. In both HDL_2 and HDL_3 the protein covers the surface of the particles. However, when HDL_3 is placed in the presence of a lipid source, extra cholesterol ester and triglyceride could be accommodated by altering the conformation of the protein in such a way that when two HDL_2 molecules fuse, the surface of the new particle is covered with protein and additional inner space for neutral lipid is made available. The larger HDL_2 particle formed might also require small increases in phospholipid or cholesterol but the major difference would be increased neutral lipid content.

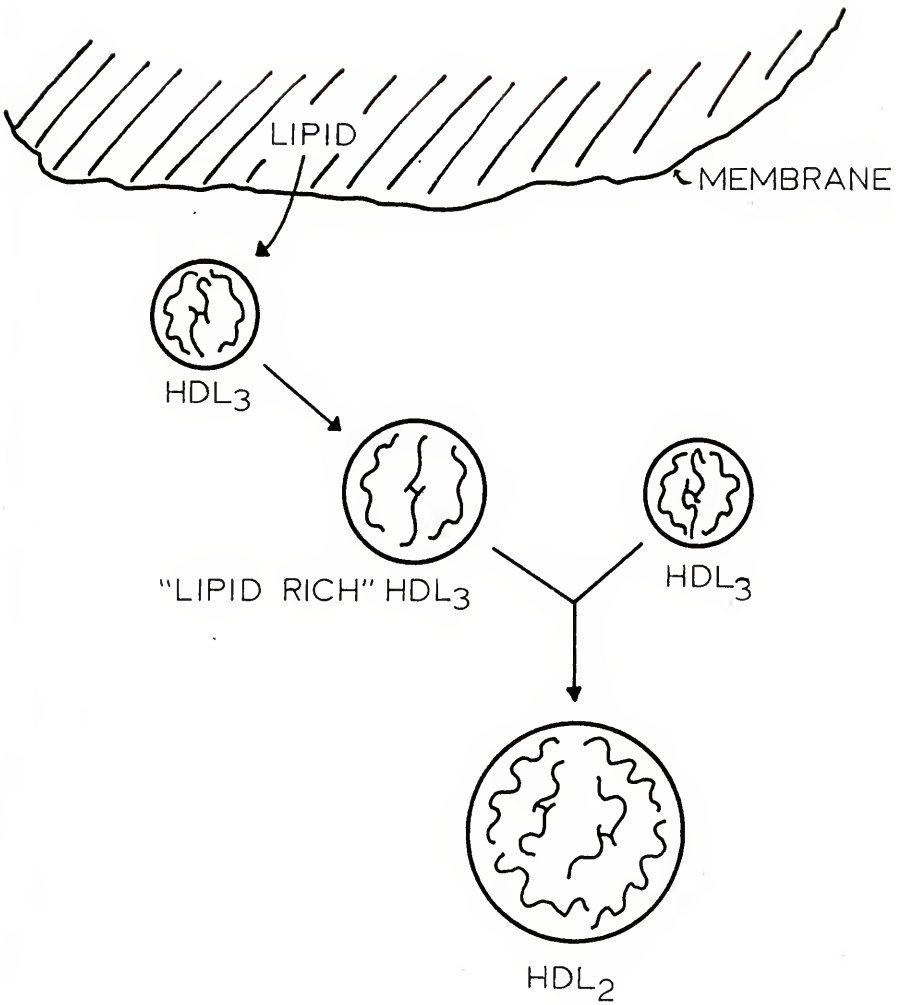


Figure 34. Model for HDL_3-HDL_2 Interconversion.

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BIOGRAPHICAL SKETCH

Thomas Ellis Grow was born in Pensacola, Florida, on January 6, 1945, the son of Captain Harold B. Grow and Beatrice D. Grow. He completed his primary and secondary education in Pensacola and graduated from Escambia High School in 1962. He then attended Pensacola Junior College from 1962 to 1964 and received an A.S. degree in 1964. After one semester at Florida State University he moved to California where he worked as a research assistant at Scripps Clinic and Research Foundation. In 1967 he returned to Pensacola where he completed his B.S. degree in Biology in 1969 at the University of West Florida. He entered the Master's degree program in Marine Biology in 1969 and while working toward this degree was employed as a research biologist by the Environmental Protection Agency's Pesticide Research Laboratory at Sabine Island, Gulf Breeze, Florida. From 1970 to 1973 he was employed as a teacher at the Pensacola School of Liberal Arts. He left Pensacola in 1973 to enter the University of Florida's Department of Biochemistry to work for a Ph.D. degree. He received a Ph.D. in Biochemistry in June of 1977.

I certify that I have read this study and that, in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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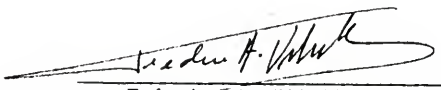

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This dissertation was submitted to the Graduate Faculty of the Department of Biochemistry in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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