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ON THE STRUCTURE OF HUMAN SERUM

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FOOTNOTES

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- 4 Abbreviations: LDL = low-density lipoproteins within the density range of 1.019-1.063g; HDL = high-density lipoproteins of d 1.063-1.21g; VHDL = very high density lipoproteins of $d > 1.21$. Apo LDL and apo HDL refers to the delipidated protein moiety of LDL and HDL, respectively.

ON THE STRUCTURE OF HUMAN SERUM
LOW- AND HIGH-DENSITY LIPOPROTEINS¹

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An analysis is presented on the current information regarding the structure of human serum low- and high-density lipoproteins (LDL and HDL). Studies on their protein moieties are outlined with the presentation centered on their polypeptide composition and properties. Re-assembly studies employing these polypeptides and natural or synthetic lipids are discussed with particular emphasis on the more recent results obtained by physical, chemical and immunological techniques. Recently proposed structural models of LDL and HDL are critically analyzed in light of the more recent acquisitions in the field.

Serum lipoproteins represent a well-recognized class of particles whose function appears to primarily reside in the transport of lipids. An important characteristic of these complexes is their water-solubility despite a relatively large content in lipids, a fact which all the proposed structural models have attempted to clarify. According to the more commonly adopted ultracentrifugal

criteria⁴, several classes of lipoproteins have been identified and found to differ in protein-lipid ratio, hydrated density, size, shape and molecular weight. This class heterogeneity is compounded by the multiplicity of polypeptides which have been isolated from these lipoproteins. Although these polypeptides are commonly referred to as "apolipoproteins", their actual structural relation to serum lipoproteins has not been unequivocally established in all instances. This is mainly a reflection of the current structural uncertainties regarding serum lipoproteins, uncertainties which also extend to the area of their functions.

The purpose of this discussion is to provide an up-to-date summary on the status of our knowledge on the structure of two of the major lipoprotein classes of human serum LDL and HDL. The analysis will not be exhaustive but limited to contributions with direct bearing on structure. This selectiveness finds justification in the fact that recent reviews on the subject have either appeared (Schumaker and Adams 1969; Gotto 1969; Scanu and Wisdom 1972) or will soon be published (Scanu 1972_{a,b})

EXPERIMENTAL DATA ON LDL

The intact particle

Its average concentration (Hatch and Lees 1968) in normal medium age male subjects is 369 ± 81 mg/100ml serum. Females of the corresponding age group exhibit a somewhat reduced concentration (303 ± 36 mg/100ml). Data on the chemical composition of LDL are reported in Table 1. This particle contains about 20 percent protein

TABLE 1

Composition of Human Serum LDL

Component	Percent by Weight
Protein	21
Phospholipid ⁺⁺	22
* Percent distribution: Lysolecithin, 2.7; sphingomyelin, 25.9; phosphatidylcholine, 63.7; phosphatidylinositol, 1.6; phosphatidylserine, 0.8; phosphatidylethanolamine, 2.2; and polyglycerophosphatides, 2.0.	
Cholesterol	8
Cholesterol esters ^{**}	37
Glycerides ^{***}	11
Nonesterified fatty acids ^{****}	1

+ Percent fatty acid composition: 16:0, 32.1; 16:1, 1.6; 18:0, 15.9; 18:1, 12.0; 18:2, 20; 20:4, 6.7.

** Percent fatty acid composition: 16:0, 10.8; 16:1, 3.3; 18:0, 1.3; 18:1, 19.3; 18:2, 51.9; 20:4, 5.8.

*** Percent fatty acid composition: 16:0, 24.9; 16:1, 4.2; 18:0, 4.8; 18:1, 35.6; 18:2, 17.2; 20:4, 1.4.

**** All the above data were taken from the review by Scanu & Kruski (1972).

TABLE 2

Major Physical Properties of Human Serum LDL

Flotation rate (S_f (1.063))	0-20
Medium density range (g/ml)*	1.006-1.063
Hydrated density (g/ml)	1.035
Molecular weight (10^6)**	2.30
Diameter (Å) ***	170-260
Electrophoretic behaviour****	β migration

* Several sub-classes of these lipoproteins can be isolated by ultracentrifugation at intermediate densities. The more recent structural studies have been conducted on LDL of d 1.019-1.063 or cuts thereof.

** Values of $2.6-2.7 \times 10^6$ have recently been reported by Fischer et al. (1971).

*** Determined by either electron microscopy (Forte & Nichols 1972) or by small-angle x-ray scattering (Mateu et al. 1971; Mateu et al. 1972).

**** By free boundary or on various supporting media.

by weight (2-3% is contributed by carbohydrate), the remainder being made of lipid (Table 1). Cholesterol esters and phospholipids and in particular phosphatidylcholine are the most abundant lipid species with an ester composition showing a predominance of palmitate, oleate and linoleate.

In terms of physical properties, the LDL particles have been shown to have an hydrated density of 1.035g/ml and a molecular weight of $2.2-2.3 \times 10^6$, although a higher figure (2.7×10^6) has been recently reported (Table 2). These molecular weight figures obtained by ultracentrifugation are in fairly good agreement with those calculated from electron micrographs of negatively-stained LDL showing spherical particles with an average diameter of 193Å. Such particles have been described to either have a granular surface (Gotto et al. 1968), to contain a strand-like organization (Forté & Nichols 1972) or to have a fine substructure interpreted to represent globular subunits of about 50 Å diameter (Pollard et al. 1969). The presence of subunits has also been indicated by isodensity maps constructed from electron micrographs of LDL (Pollard & Devy 1971), an observation compatible with the recent small angle x-ray scattering studies conducted on particles in solution. The electron density of the aqueous medium was progressively increased by the addition of NaBr and at each density, different lipoprotein concentrations were studied (Mateu et al. 1972). Even the most concentrated solutions appeared homogeneous and isotropic, a fact taken to suggest a highly organized internal structure. A feature common to all x-ray

diffraction diagrams was the presence of a diffuse band around $S=4.5 \text{ \AA}^{-1}$, characteristic of the liquid-like organization of the hydrocarbon chains (Luzzati 1968). Furthermore, all of the curves exhibited a few bands, separated by low minima, leading to the formulation of an ordered structure whose features will be discussed in the section on Models.

The techniques of optical rotatory dispersion and circular dichroism applied to solutions of LDL have provided spectra of not unequivocal interpretation. What appears firmly established is that LDL in aqueous solutions represents a metastable structure influenced by lipid content of the particle (Dearborn & Wetlaufer 1969), lipoprotein concentration (Scanu et al. 1969a) and temperature (Dearborn & Wetlaufer 1969; Scanu et al. 1969a). Three basic structures have been described -- α -helix, antiparallel β -sheet and unordered structure -- with relative proportions dependent upon nature of particle and experimental conditions used. In spite of early claims invoking a very high percentage of β -structure in LDL (Gotto 1969; Gotto et al. 1968), it now appears that, under physiological conditions of pH and temperature, such a particle has a predominance of α -helix and unordered structure (Dearborn & Wetlaufer 1969; Scanu et al. 1969), an interpretation in keeping with the x-ray findings (Mateu et al. 1971; Mateu et al. 1972) and the substructure model proposed by Pollard et al. (1969).

The nuclear-magnetic resonance studies by Leslie et al. (1969) and Steim et al. (1968) have led to suggest that the protein has

TABLE 3

Significant Information Derived From
Physical Studies on LDL

Technique	Information derived from the study
Electron microscopy	Spherical particles, average diameter 193 Å (Forte & Nichols 1971; Pollard <u>et al.</u> 1969). Presence of substructure suggested but not yet widely accepted (Pollard <u>et al.</u> 1969).
Small-angle x-ray scattering	Presence of a diffuse band around $S = 4.5 \text{ Å}^{-1}$, in addition to multiple diffraction bands separated by low minima. Spherical model postulated (Mateu <u>et al.</u> 1972).
Optical rotatory dispersion and circular dichroism	Spectra found dependent on temperature, lipid content of particle and lipoprotein concentration (Dearborn & Wetlaufer 1969; Scanu <u>et al.</u> 1969a). Existence of at least three basic structures (α -helix, antiparallel β - and unordered structure) has been postulated with proportions dependent on experimental conditions (Gotto 1969; Dearborn & Wetlaufer 1969; Scanu <u>et al.</u> 1969a).
Nuclear magnetic resonance	No evidence for protein-lipid interactions obtained. However, studies were not quantitative (Leslie <u>et al.</u> 1969; Steim <u>et al.</u> 1968).
Electron spin resonance (spin label)	Two types of signals observed: narrow and broad, the latter indicative of areas in the molecule with constrained motion and possibly relating to protein-lipid interactions (Gotto & Kom 1969a,b).

little influence on the structure of the lipid moiety. The conclusion was primarily based on the fact that the lipid signals originating from the intact complex were indistinguishable from those of the extracted lipids. However, there are reservations against such conclusions owing to the fact that the technique is not quantitative and relatively small areas of the molecule with close lipid-protein interactions may have passed undetected. Such assumption appears to receive support from the electron spin resonance spectra of LDL particles, spin labeled in their protein moiety, exhibiting both narrow and broad signals, the latter indicative of a constrained region(s) in the molecule and probably relating to protein-lipid interactions (Gotto & Kon 1969a, b). Fluorescence spectroscopy has seen little application in the study of LDL structure although information on the subject is expected to be forthcoming (Table 3).

From the chemical standpoint (Table 4), modification of some of the functional groups in LDL has been used to help in defining the surface property of this particle. In the first extensive investigation on the subject (Margolis 1969), three types of reactive groups were observed, some with complete side chain reactivity in the native lipoproteins (ϵ -amino groups), some with limited reactivity in both native and delipidated LDL (histidine and tyrosine) and others with limited reactivity in native LDL but increased reactivity after delipidation (disulfide groups). The succinylation reaction has indicated that about 90% of the ϵ -amino groups are accessible to modification to yield a product with morphological, hydrodynamic and

TABLE 4

Significant information derived from
chemical and enzymatic studies on LDL

Chemical modification	Information Obtained
Acetylation, succinylation, maleylation, amidination, reduction and carboxymethylation, citroconylation (Gotto 1969; Margolis 1969; Aggerbeck 1962)	<p>About 90% ϵ-amino groups are accessible to chemical modification.</p> <p>Chemical modifications affect the immunological reactivity of LDL, but much less its morphological, hydrodynamic and optical properties.</p> <p>Chemical modification such as succinylation and citroconylation increase the hydrophilic properties of the apoprotein so that it dissolves more readily in water following delipidation.</p>
Enzymatic modification	
Neuraminidase (Margolis 1969)	Presence of sialic acid at the surface of the molecule.
Trypsin (Margolis 1969)	Only partial proteolysis obtained. The nature of the peptides released not clearly defined.
Phospholipase A ₂ (Nishida 1968; Aggerbeck & Scanu 1971)	LDL retains hydrodynamic, immunological and morphological properties of the native particles.
Phospholipase C (Pollard <u>et al.</u> 1969)	Induces prompt instability of LDL.

TABLE 5

Approximate number of lipid molecules
per LDL particle

Lipid species	Assumed MW	Moles of lipid/ mole LDL*
Phosphatidylcholine	807	400
Sphingomyelin	748	175
Lysophosphatidylcholine	541	25
Phosphatidylethanolamine	747	15
Phosphatidylinositol	866	9
Phosphatidylserine	791	9
Cholesterol esters (as linoleate)	649	1311
Free cholesterol	387	475
Triglycerides (as triolein)	884	286

* Assuming MW = 2.3×10^6

optical properties of the native molecule, although exhibiting some changes in immunological reactivity probably relating to changes in surface charge. Implicit in this type of experimentation is the assumption that a ready reactivity of a functional group relates to its surface position in the molecule. This, of course, may be an oversimplification and independent ways of testing surface topology ought to be carried out. A practical corollary of these studies was that the more hydrophylic properties induced by chemical modification permitted a more readily solubilization of the LDL protein following delipidation.

Enzyme probes have seen rather limited application to the study of the structure of LDL. The ready release of sialic acid by neuraminidase has led to the suggestion that this sugar occupies the surface of the molecule (Margolis 1969). It has also been shown that proteolytic enzymes produce only partial hydrolysis of LDL, yielding particles having an increased tendency to aggregate, increased flotational rates and lipid extractibility. The nature of the peptide(s) released by proteolysis has not been clearly defined (Margolis 1969). According to Rudman et al. (1970), trypsin would release less than 10 percent of the protein moiety as small molecular weight peptides, leaving a protein core with a molecular weight of 15,000 to 20,000 in the presence of sodium dodecylsulfate. Because of the different experimental conditions, it is not possible to compare all of these sets of results. However, it is evident that this area is in need of clarification. In terms of lipolytic

enzymes, phospholipase C has been shown to cause rapid instability of LDL (Pollard et al. 1969), whereas phospholipase A₂-treated particles retain stability in the presence of albumin even after essential total hydrolysis of the hydrolyzable phospholipids (Nishida 1968; Aggerbeck & Scanu 1970). Such particles also retain the hydrodynamic, immunological, and morphological properties of native LDL, leading to the suggestion that the 2-ester linkage in phospholipids is not critical for LDL stability (Aggerbeck & Scanu 1971).

The lipid moiety

From the MW figure of 2.3×10^6 and from data in Table 1, the moles of lipid per mole of LDL can be calculated (Table 5). Because of the number of assumptions inherent in the computations, the values ought to be considered only approximate. Very little is presently known on the molecular organization of these lipids. A bilayer arrangement has recently been suggested from x-ray studies of LDL particles in solution (see STRUCTURAL MODELS section), but this is in need of further experimental corroboration.

The protein moiety

Several methods are available for successfully preparing the LDL protein in an essentially lipid-free form (Gotto 1969; Margolis 1969), but its solubilization in aqueous solutions is presently far from standardized since no stringent solubility criteria have been established. Methods of solubilization have utilized sodium dodecyl (Granda & Scanu 1966) or decylsulfate (Gotto 1969; Gotto et al.

1968), non-ionic detergents (Helenius & Simon 1971), urea and detergent (Shore & Shore 1967; Day & Levy 1968), guanidine hydrochloride alone (Dawson & Smith 1972), guanidine hydrochloride and detergent (Kane et al. 1970), titration at pH 11.5 (Pollard et al. 1969), and succinylation (Scanu et al. 1968a). The mechanism for solubilization has not been clearly established, although it is known that the agents employed are likely to prevent protein aggregation and promote denaturation.

It has been shown that removal of lipids from LDL induces marked changes in the morphology (Gotto 1969; Gotto et al. 1968) and optical properties of the protein moiety (Gotto 1969; Scanu et al. 1969a). The conformation of delipidated apo LDL is more readily influenced than the native complex by solvent perturbation or temperature. This has been interpreted to mean that lipids have some stabilizing role in the secondary and tertiary structure of the protein (Dearborn & Wetlaufer 1969; Scanu et al. 1969a). The circular dichroic spectra of apo LDL recorded in the presence of sodium dodecyl (Scanu et al. 1969a) or decylsulphate (Gotto 1969) appear more difficult to interpret, although the data seem to suggest strongly that these anionic detergents promote an α -helical conformation (Scanu et al. 1969a; Lux et al. 1972). The lack of standardization in preparing a water-soluble, lipid-free LDL protein is probably responsible for the various molecular weights, extending from 27,000 (Pollard et al. 1969) to 246,000 (Dawson & Smith 1972), reported thus far. The data appear to reflect the nature of the

dissociating agent and the experimental conditions employed. At present there is no unequivocal way of resolving the conflicting reports.

On the assumption that apo LDL has a multi-unit structure, isolation of the subunits has been attempted by chromatographic procedures (Kane et al. 1970; Shore & Shore 1969). No definitive results have been obtained, for, when heterogeneity was observed, no unequivocal distinction could be made between intrinsic and extrinsic protein components that might be members of distinct lipoprotein classes. Contributions to this rather complex area are expected from the comparative analysis of the polypeptide composition of LDL particles isolated within narrow density ranges, as well from the analysis of genetic variants affecting the LDL protein. In this respect, studies on human hypo- β -lipoproteinemia (Aggerbeck et al. 1971) and a β -lipoproteinemia (Kayden 1972) have contributed no significant information about LDL structure. However, it is likely that new variants will be uncovered in the future.

EXPERIMENTAL DATA ON HIGH-DENSITY LIPOPROTEINS

The intact particle

In medium-age normal male subjects, the concentration of HDL₂ ($d=1.063-1.125\text{g/ml}$) is $53\pm 48\text{mg}/100\text{ ml}$ as compared to HDL₃ ($d=1.125-1.21\text{g/ml}$), the concentration of which is $222\pm 31\text{mg}/100\text{ ml}$. In females, HDL₂ has a concentration of $172\pm 81\text{mg/ml}$ compared to HDL₃, which has a concentration of $264\pm 59\text{mg}/100\text{ ml}$. The reasons for the sex-dependent

TABLE 6

Composition of Human Serum HDL

Component	HDL ₂	HDL ₃	VHDL ₁
	Percent Distribution		
Protein	43	56	63
Phospholipid ⁺⁺	29	23	28
<p>* Percent distribution (average for the three sub-classes): Lysolecithin: 4.0; Sphingomyelin: 12.0; Phosphatidylcholine: 74; Phosphatidylinositol: 3; Phosphatidylserine: 1; Phosphatidylethanolamine: 3.0; Polyglycerophosphatides: 2.0.</p>			
Cholesterol	5	3	<1
Cholesterol esters ^{**}	17	12	3
Glycerides ^{***}	5	4	5
Non-esterified fatty acids ^{****}	1	2	<1

+ Percent fatty acid composition (HDL₂ + HDL₃): 16:0, 30.2; 16:1, 1.4; 18:0, 16.0; 18:1, 12.4; 18:2, 20.8; 20:4, 8.5.

** Percent fatty acid composition: 16:0, 10.7; 16:1, 3.2; 18:0, 1.2; 18:1, 18.9; 18:2, 52.2; 20:4, 6.3.

*** Percent fatty acid composition: 16:0, 24.5; 16:1, 4.0; 18:0, 4.6; 18:1, 35.9; 18:2, 17.1; 20:4, 1.3.

**** The above data were derived from the review article by Scanu and Kruski (1972).

TABLE 7
Major Physical Properties of HDL Sub-classes

Parameter	HDL ₂	HDL ₃	VHDL ₁
Flotation rate ($S_{f1.21}$)	4-9	0-4	--
Medium density range	1.063-1.125	1.125-1.21	1.21-1.25
Molecular weight (10^5)*	3.60	1.75	1.54
Diameter**	90x365	40x130	74
Electrophoretic behaviour	$\alpha 1$	$\alpha 1$	pre-alb?

* Determined by ultracentrifugation (Scanu & Wisdom 1972; Scanu 1972a).
 Lower figures have recently been reported.

** Determined by hydrodynamic parameters (Scanu & Wisdom 1972; Scanu 1972a).
 The estimates imply that HDL represent asymmetrical particles. This,
 however, has not been clearly established (see text).

distribution of the two major HDL sub-classes have not been clarified. A third HDL sub-class, called VHDL, has been described. The possibility that it is an artifact has not been ruled out; its presence in the circulation in small concentrations (about 20mg/100 ml) has been reported. HDL₂, HDL₃, and VHDL differ in their chemical composition (Table 6), particularly in the ratio of protein to total lipids. The relatively low content of cholesterol esters in VHDL should be noted. The three sub-classes of HDL also differ in their physical properties (Table 7). Based on ultracentrifuge data, various figures for the molecular weights of HDL₂ and HDL₃ have been reported (Scanu & Wisdom 1972; Scanu 1972a). Recent estimates are 265,000 to 236,000 for HDL₂, and 170,000 to 148,000 for HDL₃. The discrepancy in the figures cannot be explained except as an expression of differences in the methods of preparation of the particles, and in the assumptions made in the estimates of molecular weight. By hydrodynamic criteria, both HDL₂ and HDL₃ have been thought to represent asymmetrical particles (Table 7) of the prolate ellipsoid type. However, electron micrographs do not clearly support these conclusions, for they indicate that the particles are spherical with diameters of 95-100 Å for HDL₂ and 70-75 Å for HDL₃ (Forte & Nichols 1972). These values are in fairly good agreement with the small-angle x-ray scattering data (see below).

Forte et al. (1968, 1971) first described the presence of a substructure in both HDL₂ and HDL₃. In the earlier study (Forte et al. 1968), they interpreted their data to indicate an electron-

dense central core, surrounded in a ring-like fashion by 4 to 5 globular subunits, each with a diameter of 35-50 Å. More recently (Forte & Nichols 1972; Forte et al. 1971), they estimated a subunit diameter of 40-50 Å for HDL₂ and 35-40 Å for HDL₃. They also detected occasional elongated subunits, with dimensions of 40-50x80-100 Å in HDL₂ and 35-40x70-75 Å in HDL₃. A subunit structure in both HDL₂ and HDL₃ has also been observed in this laboratory in electron micrographs of negatively stained preparations. However, an unequivocal interpretation has not yet been obtained owing to the variability of patterns observed and the possible artifact nature of some of the images.

Some information on HDL structure has recently been obtained by means of small-angle x-ray scattering (see Table 8), a technique which has proven valuable in the study of macromolecules in solution. The scattering curves of HDL₂ have been interpreted to indicate a symmetrical particle with a radius of gyration of 46 Å (Shipley et al. 1972). The position and intensities of the subsidiary maxima were not consistent with those of a uniformly electron dense sphere but rather with a step-model radial electron density distribution consistent with a predominantly surface location for HDL₂ protein and phospholipids head groups with a hydrophobic core being located in the interior of the particle. Furthermore, a diffuse band near $s = 4.5 \text{ Å}^{-1}$ has been observed, which is indicative of unordered liquid paraffin chains.

TABLE 8

Significant Information Derived From
Physical Studies of HDL Sub-classes

Technique	Information derived from the study
Electron microscopy (Forte & Nichols 1972; Forte <u>et al.</u> 1968; Forte <u>et al.</u> 1971).	Differences in size between HDL ₂ and HDL ₃ . A substructure in each particle has been recognized. Its significance not yet clearly defined.
Small-angle x-ray scattering (Shipley <u>et al.</u> 1972).	Presence of a diffuse band around $S = 4.5 \text{ \AA}^{-1}$. Also, multiple diffraction bands separated by low minima. Spherical model postulated.
Optical rotatory dispersion, circular dichroism and infrared spectroscopy (Scanu & Wisdom 1972; Scanu 1972a,b; Lux <u>et al.</u> 1972).	HDL ₂ and HDL ₃ have a predominant α -helical conformation, the remainder being represented by unordered structure.
Nuclear magnetic resonance (Leslie <u>et al.</u> 1969; Steim <u>et al.</u> 1968).	Absence of apolar interaction favoring the concept of a "micellar" structure. However, conclusion is not unequivocal.
Fluorescence spectroscopy (Hart <u>et al.</u> 1970)	Indication of three apolar binding sites in the molecule.
Electron spin resonance (spin label) (Gotto & Kon 1969a,b; Barratt <u>et al.</u> 1971).	Presence in HDL of areas with restricted or relatively unrestricted motion of the spin labeled probe employed.

The techniques of optical rotatory dispersion and circular dichroism, when applied to intact HDL₂ or HDL₃, have given spectra compatible with a relatively high content of α -helix (60-70 percent), the remainder consisting of an unordered structure (Scanu & Wisdom 1971; Scanu 1972a,b) and perhaps some β -structure (Lux et al. 1972). Similar structural conclusions have been derived from the analysis of infrared spectra. An additional information derived from circular dichroic studies, where spectra of HDL₂ or HDL₃ were recorded as a function of medium perturbation and temperature, was that lipids play a stabilizing role in the conformation of HDL protein. This was particularly evident from comparative analyses carried out between HDL and apo HDL preparations (Scanu & Wisdom 1972; Scanu 1972a,b).

Studies by the technique of nuclear magnetic resonance have thus far provided a rather modest information on the structure of HDL (Chapman et al. 1969; Steim et al. 1968). The data obtained seem to indicate that hydrophobic interactions do not play a role in HDL structure. However, such conclusions ought to be accepted with reservation because of the limitations of the technique which were pointed out in the discussion on LDL. Fluorescence spectroscopy has indicated the presence of three apolar binding sites in the molecule (Hart et al. 1970) and furthermore electron spin resonance studies, using spin labels, have shown that the probes employed have some restrictions in motion probably related to interaction of the protein with lipids (Gotto & Kon 1969a,b; Gotto et al. 1969; Barratt et al. 1971).

TABLE 9

Significant information derived from
chemical and enzymatic studies on LDL

Chemical modification	Information obtained
Acetylation, succinylation (Scanu 1972 <u>a,b</u> ; Scanu <u>et al.</u> 1968 <u>a</u>)	More than 90% of the ϵ -amino groups are accessible to modification. The modified particles retain chemical, ultracentrifugal and morphological properties of the untreated products.
Reduction and carboxymethylation (unpublished)	All disulfides accessible to modification. The particles retain properties of native HDL.
Enzymatic modification	
Trypsin (Camejo 1969; Wisdom & Scanu 1972)	Surface localization of a large percentage of HDL protein (Camejo 1969). Fraction III more readily hydrolyzable than IV (Wisdom & Scanu 1972). Particles treated with trypsin lose stability and become more sensible to organic solvents extraction.
Phospholipase A ₂ (Camejo 1969; Ashworth & Green 1963)	Attacks more than 95% of the choline and ethanolamine phospholipids; yet HDL exhibits essentially the same hydrodynamic behaviour as the untreated lipoproteins
Phospholipase C (Camejo 1969; Ashworth & Green 1962)	Causes prompt HDL instability; mechanism not clearly established.

The most significant chemical and enzymatic information obtained in studies with either HDL₂ or HDL₃ is summarized in Table 9. As a whole the structural information which deals with surface topology suggests that both protein and phospholipids are readily accessible to chemical modification since they are probably at or near the surface. The recent observation that the disulfide of fraction IV is readily accessible to reduction (Wisdom & Scanu 1972) adds a new important handle to the study of HDL structure by the incorporation of suitable probes for spectroscopic studies. The introduction of heavy atoms for x-ray studies may also be considered provided the lipoprotein molecule retains stability after this modification.

The lipid moiety

From the data in Tables 6 and 7 and the molecular weights in Table 5, the moles of lipids/mole of HDL may be calculated (Table 10). Both HDL₂ and HDL₃ have approximately equal ratio of phosphatidylcholine and cholesterol ester molecules, and relatively less free cholesterol. In VHDL₁, lecithins are the predominant species, with a relatively lower abundance of ester and free cholesterol. The structural role of sphingomyelin is totally unknown at present. The internal organization of these lipid molecules within each HDL sub-class is not presently understood except that some information about their long-range organization has been derived from the recent x-ray data (Shipley et al. 1972).

TABLE 10
Approximate Number of Lipid Molecules
per HDL Particle

Lipid species*	Moles of lipid/mole particle		
	HDL ₂	HDL ₃	VHDL ₁
Phosphatidyl choline	97	38	38
Sphingomyelin	21	5	7
Lysophosphatidylcholine	4	4	3
Phosphatidylethanolamine	5	1	2
Phosphatidylinositol	3	1	5
Phosphatidylserine	1	0	
Cholesterol esters (as linoleate)	90	32	8
Free cholesterol	50	13	1
Triglycerides (as triolein)	18	8	8

* The assumed molecular weights are reported in Table 5.

The protein moiety

Since the early observations (Scanu et al. 1958), numerous techniques for delipidation have been applied to HDL to yield a product soluble in aqueous buffers under appropriate conditions of pH and ionic strength (Schumaker & Adams 1969; Scanu & Wisdom 1972; Gotto 1969; Scanu 1972a,b). Refinements of the technique (Scanu & Edelstein 1971) and an extensive review on the subject have recently been published (Scanu 1971). In terms of the essentially lipid-free protein, many laboratories have contributed to the present status of the knowledge on its physical, chemical and immunological characteristics. Since there is no established nomenclature, the non-committal operational terminology adopted in this laboratory will be used in referring to the various polypeptides thus far isolated from human serum HDL¹ (Scanu et al. 1969b). We suspect, based on the preliminary evidence in this laboratory, that additional HDL polypeptides will be isolated in the near future.

In approaching this problem of isolation of HDL polypeptides, it is useful to remember that, upon lipid removal, they tend to aggregate with each other and that such aggregation can be prevented by the appropriate use of dissociating and denaturing agents such as

¹ Nomenclature: Fraction III = apo LP-Gln I or apo A-1 (Kostner & Alaupovic 1971); Fraction IV = apo LP-Gln-II or apo A-ii; Fraction V = apo C (Alaupovic et al. 1972); Fraction III also corresponds to R-Thr of Shore and Shore (1969). This terminology should be abandoned since it is now established that glutamine (Kostner & Alaupovic 1971; Edelstein et al. 1972) and not threonine is the COOH terminal residue in this polypeptide.

TABLE II
Properties of Human Serum Polypeptides

Sephadex Fractions	Alternative nomenclature	Behaviour DEAE chromatograph	Characteristics in amino acid composition	NH ₂ -Terminal	COOH-Terminal	Carbohydrate Content	MW 10 ⁴
I	--	under investigation	--	--	--	--	5 to 5.5. Influenced by disulfide cleavage
III	Apo LP-A-I Apo LP-Gln-I	Gives 2 major components, III _a and III _b . (Edelstein et al. 1972)	No clear distinction between III _a and III _b . No isoleucine ² , cysteine or cysteine.	Aspartic	Glutamine ³	No sialic acid or glucosamine. Neutral sugars?	2.7-2.8 (Scanu et al. 1969b; Edelstein et al. 1972; Scanu et al. 1971). Lower molecular weights have been reported (Shore & Shore 1968, 1969).
IV	Apo LP-A-II Apo LP-Gln-II	Single component (Edelstein et al. 1972; Brewer et al. 1972; Edelstein & Scanu 1972)	No histidine, arginine or tryptophane.	Pyrrolidone carboxylic acid (Brewer et al. 1972; Edelstein & Scanu 1972)	Glutamine	Same as above.	Dimer=1.7-1.8 (Scanu et al. 1971). Monomer (obtained by cleavage of the single disulfide bridge)=0.8-0.9 (Scanu et al. 1971). The two chains are very similar or identical (Scanu et al. 1972a; Brewer et al. 1972; Edelstein & Scanu 1972)
V	Apo C (Alaupovic et al. 1972)	Four major components and minor ones	Composition similar to the D ₁ , D ₂ , D ₃ and D ₄ from apo VLDL (Brown et al. 1969)	Same as fractions D ₁ , D ₂ , D ₃ and D ₄ of apo VLDL by Brown et al. 1969.			0.7-1.0 (Scanu et al. 1969b)

1. This fraction was believed to represent an aggregate of III and IV and possibly V. However, it now appears that it contains a high molecular weight component whose relation to fractions III and IV is under investigation.
2. Variants containing 1 mole isoleucine/mole protein have been described.
3. Previously believed to be threonine (Shore & Shore 1969)

urea or guanidine hydrochloride or nonionic and/or anionic surfactants (Triton X-100, Nonidet, Tween 20, sodium dodecylsulphate, etc.). Furthermore, these polypeptides have been shown to differ from each other in molecular weight and/or charge and thus chromatographic and electrophoretic techniques alone or in combination can be used for their successful isolation and purification (Schumaker & Adams 1969; Scanu & Wisdom 1972; Gotto 1969; Scanu 1972a,b). Once isolation is achieved, microanalytical techniques such as polyacrylamide gel in either urea or sodium dodecylsulphate have proven useful in assessment of purity, particularly if employed together with immunological methods owing to the demonstrated antigenic specificity of the various HDL polypeptides (Schumaker & Adams 1969; Alaupovic et al. 1972).

In this laboratory, the customary first step is to subject the lipid-free apo HDL₂ or apo HDL₃ to gel filtration in the presence of 8M urea or 6M urea guanidine hydrochloride. Generally, four fractions are obtained in the proportions indicated in Figure 1. Fraction II is either present in very small amounts or may be absent. The degree of resolution of fractions I, III, IV, and V depends on the experimental conditions employed, such as column dimensions, molarity of urea or guanidine hydrochloride, flow rate, and protein load. As a second step, DEAE ion exchange column chromatography affords both purification and further sub-fractionation of the Sephadex component, yielding final products that are homogeneous by polyacrylamide gel electrophoresis in sodium dodecylsulphate (Edelstein

et al. 1972; Scanu et al. 1972a), and preparative and analytical isoelectric focusing (Scanu et al. 1972b). Alternative fractionation procedures have been described (Rudman et al. 1970; Shore & Shore 1968, 1969) which, according to their proponents, are capable of yielding pure HDL polypeptides.

The present status of knowledge on the HDL polypeptides is shown in Table 11. In general, one is impressed by the degree of chemical heterogeneity of the various HDL polypeptides, which occur in different proportions and also have marked differences in molecular weights. Significant advances have been made in the study of the two major polypeptides, III (also called apo LP-Gln-I or apo A-I) and IV (apo LP-Gln-II or apo A-II). It is now established that fraction III, which represents about 75% of the apo HDL mass (Scanu et al. 1969b) is made of single polypeptide chains having no cysteine or cystine (Shore & Shore 1968, 1969; Edelstein et al. 1972), and having NH₂-terminal aspartic acid and glutamine as the COOH-terminal residue (Kostner & Alaupovic 1971; Edelstein et al. 1972). According to recent studies and contrary to previous reports (Shore & Shore 1968, 1969), the molecular weight of III is about 27,000 (Scanu et al. 1969b; Scanu et al. 1971), with a large percentage of α -helix (60-70%), random coil and no (Scanu & Wisdom 1972; Scanu 1972a,b) or a small amount (Lux et al. 1972) of anti-parallel β -structure. A preliminary undocumented report on the sequence of the first 39 amino acid residues from the NH₂-terminus has recently appeared. The reported sequence (Brewer et al. 1972): NH₂-Asp-Glu-Pro-Pro-

Gln-Ser-Pro-Trp-Asp-Arg-Val-Gln-Asp-Leu-Ala-Pro-Val-Tyr-Val-Asp-Val-Leu-Arg-Asp-Ser-Gly-Arg-Asp-Tyr-Val-Ser-Glu-Phe-Glu-Gly-Arg-Ala-Leu-Gly. . . . indicates that most of the proline residues are near the NH₂-terminus suggesting that this polypeptide segment does not contain a significant α -helical structure.

Fraction IV has proved to be a most interesting polypeptide with a rather unusual structure. Recent studies have shown that the molecule is a dimer made of two monomers with identical molecular weight (about 8.500) linked together by a single disulfide bridge (Scanu et al. 1971). The two chains are chemical identical (Brewer et al. 1972) or nearly identical (Scanu et al. 1972b) with pyrrolidone carboxylic acid (PCA) on the NH₂-terminus (Brewer et al. 1972; Edelstein & Scanu 1972) and glutamine as the COOH-terminus (Shore & Shore 1968, 1969; Scanu et al. 1972a; Brewer et al. 1972). The sequence of the IV protomer has just been announced (Brewer et al. 1972):

PCA-Ala-Lys-Glu-Pro-Cys-Val-Glu-Ser-Leu-Val-Ser-Gln-Tyr-Phe-Gln-Thr-Val-Thr-Asp-Tyr-Gly-Lys-Asp-Leu-Met-Glu-Lys-Val-Lys-Ser-Pro-Glu-Leu-Gln-Ala-Gln-Ala-Lys-Ser-Tyr-Phe-Glu-Lys-Ser-Lys-Glu-Gln-Leu-Thr-Pro-Leu-Ile-Lys-Lys-Ala-Gly-Thr-Glu-Leu-Val-Asn-Phe-Leu-Ser-Tyr-Phe-Val-Glu-Leu-Gly-Thr-Gln-Pro-Ala-Thr-Gln-C $\begin{matrix} = O \\ \backslash \\ OH \end{matrix}$

According to this study, IV is made of two chemically identical protomers linked by a single disulfide bridge in position 6 from the NH₂-terminal. This disulfide appears to have important structural significance since, as assessed by circular dichroism, its cleavage favors α -helix random coil transition reversed by re-oxidation (Scanu 1970).

Regarding the lesser abundant components, fraction I comprises polypeptide(s) of about 50,000 MW still in the process of identification. The fraction V can be resolved by DEAE column chromatography into at least three chemically-distinct polypeptides identical with the low-molecular weight polypeptides described in very low-density lipoproteins (Brewer et al. 1969; Albers & Scanu 1971). Other minor components are still in the process of characterization. Fraction V, which appears to correspond to apo C immunologically and chemically, is easily detachable from HDL, for instance, by brief sonication (Scanu et al. 1970). The absence of fraction V also characterizes the re-assembled HDL obtained by sonication of apo HDL and HDL lipids (Scanu et al. 1970).

The re-assembled products

The lipid-free apo HDL has been shown to retain binding capacity for lipids and when mixed with whole serum, to associate essentially only with its parent lipoprotein (Scanu & Hughes 1960). The recognition of such particle specificity has stimulated a number of studies to elucidate its mechanism. Since extensive reviews on the subject have been published (Scanu 1971, 1972a), this discussion will be limited to an outline (Table 12) of the most significant developments in the field. It now appears to be accepted that apo HDL (III and IV in particular) and phospholipids represent the primary complex which permit the further addition of free cholesterol, cholesterol esters and triglycerides.

TABLE 12

Outline of Developments in Re-assembly Studies¹

-
1. Apo HDL and phospholipids form a "primary complex" which appear in the electron microscope as discs of 50-55 Å diameter. Both fractions III and IV are part of the complex.
 2. Formation of the primary complex is essential for further incorporation of free cholesterol and cholesterol esters.
 3. Cholesterol esters are important structural components of HDL. These lipids appear to give the molecule its globular shape as well as stabilization.
 4. Incorporation of cholesterol esters require apo HDL, phospholipids, sonic irradiation and a minimum temperature of 40°C.
 5. The temperature of incorporation of cholesterol esters in HDL is that at which these esters undergo crystalline→liquid crystalline transitions.
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¹ The data are derived from work cited in reviewing by Scanu and Wisdom (1972); Scanu (1972a,b); and Scanu (1971).

Means have been developed for the incorporation of cholesteryl esters in the molecule. This has been shown to require approximate *appropriate* experimental conditions including temperature. Particles re-assembled partially (apo HDL plus phospholipids) and totally re-assembled particles (minimum components = apo HDL, phospholipids and cholesterol esters) have been studied by a number of physical techniques which have shown that only those re-assembled complexes containing cholesterol esters are those with structural properties at least very similar to the native particles. These conclusions are based upon results obtained by preparative and analytical ultracentrifugation, circular dichroism, electron spin resonance spectroscopy and electron microscopy. In spite of these similarities, it is not yet possible to state unequivocally that HDL can be re-assembled in vitro from its constituents, for the actual structure of HDL, which is the essential term of comparison, is still undefined (see below). Of particular interest has been the demonstration that the enzyme lecithin-cholesterol acyl transferase can be effectively utilized in re-assembly studies by promoting the esterification of free cholesterol from lecithin (Ho & Nichols 1971). In partially re-constituted complexes the actual molecular details are such reaction have not yet been established.

STRUCTURAL MODELS

Low-density lipoproteins

Based on the interpretation of electron micrographs of

negatively-stained preparations of LDL and on the results of hydrodynamic data on LDL and apo LDL, Pollard et al. (1969) have postulated that LDL has a sub-structure with 20 subunits arranged in icosahedral symmetry (Fig. 2, upper panel). Such a model has been supported by isodensity maps extracted from electron micrographs of LDL (Pollard & Devi 1971) and the available chemical information and the results of the enzymatic studies, using phospholipase A and C (Pollard et al. 1969; Margolis 1969; Nishida 1968; Aggerbeck & Scanu 1971). It is also compatible with the recent results obtained with the technique of small-angle x-ray scattering which were conducted on aqueous solutions of LDL using NaBr to progressively raise the electron density of the medium (Mateu et al. 1971, 1972). All solutions studied were homogeneous and isotropic giving a diffuse band around $S = 4.5 \text{ \AA}^{-1}$, characteristic of the liquid-like organization of the hydrocarbon chains (Iuzzati 1968). Further evidence for homogeneity came from the presence of a few diffraction bands separated by low minima, this indicating that the particles in solution had a highly ordered structure, at least at low resolution (about $33 \text{ \AA}$) and that they were very probably spherically symmetric. The electron density distribution exhibiting a low-density shell (hydrocarbon chains?) and high density peaks on both sides of this minimum (protein and phospholipids?) was strongly suggestive of a lipid bilayer as shown in the schematic drawing in the lower panel of Fig. 2. This conclusion is at variance with all the previously suggested models of LDL (Schumaker & Adams 1969; Scanu & Wisdom 1972). The

definition of the molecular details of these models must obviously depend on future higher resolution studies. In the meantime the usefulness of the technique of small-angle x-ray scattering in the analysis of LDL is apparent and more work in this direction is likely to be done. As discussed by Mateu et al. (1972), the postulate of a lipid bilayer in LDL is a novel concept in the field of serum lipoproteins. Such concept would appear to be peculiar for LDL, since the studies on HDL (see below) favor a structure having a lipid core surrounded by protein. It is implicit in the model shown in Fig. 2 that the cholesterol ester molecules interact with some of the LDL protein, behaving as if the latter were the polar head of these lipids. This type of organization and the distribution of phospholipids would be consistent with the concept of a mosaic surface structure (Oncley et al. 1950) and also with the model proposed by Pollard et al. (1969). It would also satisfy the solubility requirements of serum lipoproteins with most of the protein and polar lipid occupying the surface and with the non-polar lipids at the inner core. It is evident that a new working hypothesis regarding the LDL structure has emerged from the x-ray studies. The validity of such a hypothesis can only be assessed by direct experimental testing possibly applying independent techniques. It is by such future studies that it would hopefully be possible to go from hypothetical models to an actual structure at the molecular level.

High density lipoproteins

Human HDL is among the better characterized serum lipoproteins. Significant advances have been made possible through the systematic application to the intact particle of a number of spectroscopic techniques, the analysis of the water-soluble, lipid-free apoprotein and the investigation of the various re-assembly products taking advantage of the marked avidity of this apoprotein for lipids. Very recently the details of the primary structure of one of the major HDL polypeptides were unraveled; similar information on the other polypeptides is likely to be forthcoming. Although these techniques have provided some understanding on the nature, conformation and interactions among the individual HDL components, thus far the details on the overall structure rest on electron microscopy and small-angle x-ray scattering.

Electron micrography of negatively-stained preparations of HDL have been interpreted as representing a subunit structure with 4-5 subunits, each having a diameter of 35-50 Å, arranged in a ring-like fashion around a central core (Forte & Nichols 1972; Forte et al. 1971). The nature of the postulated subunits was not established. They were said to represent lipoproteins each with its own complement of protein, phospholipids and neutral lipids (Forte et al. 1968). Scale model analyses have led to questions about the likelihood of such a proposed sub-structure (Leslie 1971) which also appears to be incompatible with the recently published x-ray observations by

Shipley et al. (1972). As in the case of LDL (Mateau et al. 1971, 1972), small-angle x-ray scattering studies were carried out on HDL in aqueous solutions in order to probe their overall size and internal structure on the assumption that this particle has an ordered organization. Such studies have not only validated the assumptions but also provided important parameters (i.e., particle diameter, radius of gyration, thickness of outer layer and inner core) as shown in the schematic model presented in Fig. 3. This model, derived from the analysis of scattering curves, indicates a symmetrical particle having a step-model radial electron density distribution with an electron-deficient core and an electron-rich shell. This would be consistent with an interior location of the hydrocarbon chains and an outer shell consisting of protein and of phospholipids head groups, this in turn is compatible with the water-solubility of the particle. The model is in obvious need of more architectural details, which cannot be obtained at the level of resolution of the x-ray studies carried out thus far. However, the valuable information already obtained provides an important basis for future experimentation.

PERSPECTIVES

Research on plasma lipoproteins is currently moving at a considerable pace as shown by the numerous data obtained during the past 4-5 years. The problems relating to plasma lipoprotein structure have not come within experimental reach although they require

increasingly more complex and specialized techniques. The recent demonstration that x-ray methods can be applied successfully to the study of plasma lipoproteins in solution is an important development from the structural standpoint. This is likely to stimulate interest in studies directed at the crystallization of the intact particles and of their components and will permit precise three-dimensional analyses at a high degree of resolution. At the same time, it is expected that a more detailed probing of the intact particles will be possible because of the many developments in spectroscopic techniques. Particularly promising techniques include C^{13} or P^{31} -nuclear magnetic resonance, and electron spin resonance utilizing suitable probes in the protein, in the lipid moiety, or in both. Important structural information is also likely to be derived from the re-assembly studies which have already seen a useful application in the area of HDL. This type of approach should be helpful in answering questions related to the structural relevance of each constituent to overall particle structure. The answer also ought to provide a more precise basis for defining the various classes of plasma lipoproteins. In spite of the obvious complexity of the problem, the many recent developments in the field appear to justify a feeling of confidence that the structure of plasma lipoproteins will be resolved in the nondistant future.

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Figure Legends

Fig. 1 Scheme of fractionation of the polypeptides from human serum HDL. The data are derived from the work by Scanu et al. (1969b); Edelstein et al. (1972) and Scanu et al. (1972).

Fig. 2 Schematic models of LDL derived from electron microscopy (upper panel) and small-angle x-ray scattering (lower panel).

Fig. 3 Schematic models of HDL₂ derived from small-angle x-ray scattering studies.

GEL FILTRATION

	% weight
I	1-2
II	0-0.1
III	70-75
IV	20-25
V	3-5

*Polyacrylamide gel-sodium dodecyl sulphate electrophoresis

□ Hydrophilic shell
■ Hydrophobic core