Degradation and Reassembly of a Human Serum High-Density Lipoprotein. Evidence for Differences in Lipid Affinity among Three Classes of Polypeptide Chains*

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ABSTRACT: Turbid aqueous suspensions of lipid extracts from human serum high-density lipoprotein of *d* 1.063–1.125 (HDL_2) were converted into clear solutions when sonicated in the presence of the lipid-free protein moiety (apo HDL_2). This was due to the formation of a major lipoprotein complex isolated in the density range of 1.063–1.21 g/cm^3. Incorporation of the nonpolar lipids (cholesterol esters and triglycerides) into this water-soluble complex required the presence of both protein and phospholipids and energy. The reconstituted lipoprotein proved very similar to, although not identical with, native HDL_2 on the basis of chemical, immunological, ultracentrifugal, circular dichroic, and electron microscopic studies. Two other minor lipoproteins were also produced by sonication: one floating at *d* 1.063 and the other containing only phospholipids sedimenting at *d* 1.21. The two species could also be distinguished on the basis of their polypeptide moieties. When the three polypeptide classes: III, IV, and V, obtained from apo HDL_2 by gel filtration, were separately sonicated in the presence of HDL_2 lipids under identical conditions, three distinct lipoprotein species were obtained. III produced the smallest and heaviest particles of $F_{(1.21)}$ = 2.0–2.2 and average diameter of 120 Å; IV and V produced larger species of $F_{(1.21)}$ = 1.0–6.0 (average diameter 180 Å) and = $S_{f(1.063)}$ 4–6 (average diameter, 350 Å), respectively. The results indicate that a product structurally similar to HDL_2 can be reassembled *in vitro* starting from its separate protein and lipid constituents. They also provide evidence that each of the polypeptide chains of apo HDL_2 has a distinct affinity for lipids and can incorporate independently polar and nonpolar lipids. The significance of the reassembly data to the overall structure of HDL_2 was not established. The speculative view that HDL_2, as routinely isolated from the ultracentrifuge, is in fact a mixture of three distinct lipoprotein species appeared to receive support from the results of the partial degradation of HDL_2 by ethyl ether. The applications and versatility of the reassembly technique were discussed especially in view of the demonstration that purified lipids from a commercial source could replace HDL_2 lipids.

Earlier studies from this laboratory showed that the protein moiety of human serum high-density lipoprotein (HDL),[1] prepared in an essentially lipid-free form (apo HDL) by treatment with organic solvents (Scanu *et al.*, 1958; Scanu, 1966), retains its capacity to bind lipids both *in vitro* (Scanu and Hughes, 1960) and *in vivo* (Scanu, 1965). In subsequent work (Scanu, 1967; Sohdi and Gould, 1967), it was shown that there was a direct interaction between apo HDL and phospholipids, or phospholipids and free cholesterol, whereas the uptake of cholesterol esters and triglycerides was minimal (Sodhi and Gould, 1967). More recently, the problem of the reassembly of HDL from its constituents was further analyzed by Hirz and Scanu (1969), who, by subjecting mixtures of apo HDL and HDL lipids to sonic irradiation at 40°, were able to restore a complex closely resembling a native high-density lipoprotein. Stimulated by such observations and by the recent discovery that apo HDL is made of chemically distinct polypeptide chains (Shore and Shore, 1968; Scanu *et al.*, 1969) we planned to investigate, in greater detail, the mechanism of HDL reassembly with the hope of learning more about HDL structure. An account of such investigations is the object of the present report. It will also include studies on the partial degradation of HDL since the data obtained are complementary to those on reassembly.

Materials and Methods

Preparation of HDL_2 and Apo HDL_2. HDL_2 was separated from freshly drawn sera of healthy male, 24-hr fasted, Caucasian human donors (Rh^+, group A) as described previously (Scanu, 1966). Apo HDL_2, in an essentially lipid-free form, was prepared by extraction of HDL with 3:2 ethanol–ether at −10° (Scanu, 1966).

Fractionation of Apo HDL_2 by Gel Filtration. Apo HDL_2, dissolved in 0.01 M Tris buffer (pH 8.4)–8 M urea was separated in Sephadex G-200 columns at 15° according to the conditions described previously (Scanu *et al.*, 1969). Fractions III, IV,

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[1] The abbreviations used in the text are as follows: HDL_2, serum high-density lipoprotein of *d* 1.063–1.125; apo HDL_2, protein moiety of HDL_2 after extraction with ethanol–ether; L, whole lipid extract from HDL_2; III, IV, and V refer to the three classes of proteins separated from apo HDL_2 by gel filtration (Scanu *et al.*, 1969).

and V, each of which contains a chemically distinct class of polypeptides (Scanu *et al.*, 1969)—I and II appear to represent mixtures of these—were dialyzed against 0.01 M Tris buffer (pH 8.4)–10^{-3} M EDTA, to remove the urea, and then concentrated by vacuum dialysis at 4° to a final concentration of 1–2 mg/ml. The lack of cross-contamination among these three fractions was checked by polyacrylamide gel electrophoresis in 8 M urea and, occasionally, by immunoprecipitation and amino acid analysis. The details of these techniques are given elsewhere (Scanu *et al.*, 1969).

Preparation and Storage of HDL_2 Lipids. The 3:2 ethanol–ether extracts from HDL_2 were cleared of any precipitate by centrifugation at 4° (3000 rpm, Sorvall refrigerated centrifuge) and then brought to dryness by flash evaporation. The dry materials were dissolved in hexane (10–20 mg/ml) and stored at −20° under N_2. In certain experiments the phospholipids of HDL_2 were separated from the nonpolar lipids by column chromatography as described before (Scanu, 1966). Further separation of individual phospholipids or neutral lipids was carried out by thin-layer chromatography (see below).

Reassembly Experiments. After evaporation of the hexane phase by N_2, the lipids were suspended in 0.02 M EDTA (pH 7.4) to a final concentration of 10–50 mg/ml and the suspensions were sonicated briefly in a Branson Sonifier (Heat Systems Co., Melville, L. I., N. Y.) (75 W, 15 sec, 40°) to obtain an even lipid dispersion. Aliquots of the desired lipid concentration were diluted to a final volume of 8 ml with 0.02 M EDTA (pH 7.4) and transferred into an aluminum cooling cell, 7 cm long × 2 cm diameter (Heat Systems Co.), designed for fast dissipation of heat. To the lipid emulsion was then added sufficient dry apo HDL to obtain the final lipid–protein weight ratio of 1:1. Protein or lipid above this ratio remained unbound (see Results). After a gentle hand shaking of the final mixture to ensure mixing of the constituents, sonication was carried out in a Branson Sonifier using the standard microtip assembly at 40° for a total period of 3 min (three 1-min sonication periods with two 2-min intervals). Bubbling of N_2 through the mixture during sonication was initially employed to prevent possible peroxidation, but was, however, found to be unnecessary, and was omitted in subsequent studies. Sonic irradiation of separate preparations of HDL_2, apo HDL_2, or HDL_2 lipids was carried out under the same conditions as those for lipid–protein mixtures. Aliquots of the resulting products were dialyzed against NaCl–$NaBr_2$ d 1.21 g/cm^3 solutions and then analyzed in a Spinco Model E analytical ultracentrifuge.

Fractionation of Lipid–Protein Mixtures after Sonic Irradiation. Three fractions (1 ml) were separated by preparative ultracentrifugation (Spinco 40.3 rotor, 40,000 rpm, 15°, 36 hr): $d < 1.063$, d 1.063–1.21, and $d > 1.21$. Density adjustments were made with solid NaCl and NaBr as previously described (Scanu *et al.*, 1969). In some instances the $d < 1.063$ fraction was diluted and further separated into $d < 1.006$ and d 1.006–1.063 fractions by preparative ultracentrifugation.

Analyses of Sonicated Mixtures after Ultracentrifugal Fractionation. A. BEFORE DELIPIDATION. Each fraction was studied by analytical polyacrylamide gel electrophoresis in 8 M urea (Scanu *et al.*, 1969) and then analyzed for protein and lipid composition (analytical ultracentrifugation and electron microscopy). The fractions were also tested by immunodiffusion techniques (Ouchterlony and immunoelectrophoresis) with antisera prepared in the rabbit against human apo HDL, and its Sephadex fractions III, IV, and V (Scanu *et al.*, 1969). Circular dichroic spectra of all of these products were also recorded.

B. AFTER DELIPIDATION. All fractions were delipidated by 3:2 ethanol–ether (Scanu, 1966) except that the extraction procedure was, in most instances, carried out in smaller (10 ml) conical centrifuge tubes when handling specimens with a protein content of 0.5–1 mg/ml. The lipid extracts were analyzed by a combination of thin-layer chromatography and chemical procedures (see below). The lipid-free proteins were, after analysis by polyacrylamide gel electrophoresis, hydrolyzed by 6 N HCl (see, for conditions, Scanu *et al.*, 1969) and the amino acid content of the hydrolysates was determined in a Beckman Model 120C amino acid analyzer equipped with a scale expander. Tryptophan and cystine were determined as described previously (Scanu, 1966).

Studies of HDL_2 after Degradation by Ethyl Ether. To ensure reproducibility the following conditions were carefully followed. Pyrex round-bottom centrifuge tubes (50-ml capacity) were partially filled with 30 ml of ethyl ether and kept at 4.° HDL_2 containing 10 mg of protein in 2 ml of 0.15 M NaCl–0.001 M EDTA was added dropwise, and the tubes were then filled completely with ethyl ether. After stoppering, the tubes were rotated in a Multi-Purpose Rotator Model 150V (Scientific Industries, Inc., Springfield, Mass.) at approximately 15–20 rpm for 12 hr at 4.° This was followed by a centrifugation step (3000 rpm at 4°) to allow for a clear separation between the aqueous and other phases. The latter was removed by aspiration and the yellow clear lipoprotein solution was washed twice with ethyl ether at 4.° After the two washings, any trace of organic solvent was removed by N_2. The resulting product was analyzed at d 1.21 g/cm^3 in a Spinco Model E analytical ultracentrifuge and then fractionated by preparative ultracentrifugation into three fractions: d <1.063, d 1.063–1.21, and $d > 1.21$. These fractions were then analyzed before and after complete delipidation as indicated for the fractions obtained from the experiments of reassembly.

Separation of HDL_2 and Ether-Treated HDL_2 by Gel Filtration. The experiments were carried out at 10° in Sepadex G-200 columns (2 × 100 cm) using 0.1 M Tris (pH 8.4) 0.001 M EDTA as the equilibrating and eluting buffer. An ascending flow of 10–12 ml/hr was used with monitoring of effluent at 280 mm in an ISCO recorder (Instrumentation Specialties Co., Inc., Lincoln, Neb.). The peaks were concentrated by vacuum dialysis at 4° and then either analyzed or rechromatographed under the same conditions.

Fractionation of HDL_2 was also carried out by filtration through Sephadex 4B (Pharmacia, Uppsala, Sweden) columns (2.5 × 40 cm) using 0.05 M Tris (pH 7.4)–10^{-3} M EDTA at a flow rate of about 10 ml/hr.

Analytical Procedures. Flotational studies were carried out in a Spinco Model E analytical ultracentrifuge using a Spinco An-D rotor and double-sector cells to allow for the simultaneous analysis of solvent and lipoprotein solution. Runs were carried out at 42,040 rpm, 20°. Flotational rates were determined from schlieren photographs magnified (× 20) in a Nikon Model 6C (Nikon Co., Japan) microcomparator.

Electron microscopy was performed in a RCA EMU-3G unit using a pointed filament, an accelerating voltage of 50 kV, a 50-U objective aperture, and a maximum instrument

TABLE I: Properties of Three Ultracentrifugal Fractions from a Sonicated Mixture of Apo HDL_2 + L.

		Lipid (% weight[a]) in Ultracentrifugation Fractions (1 ml)				
				Cholesterol		
Density (g/cm³)	Appearance	Protein	Phospholipid	Ester	Free	Triglycerides
<1.063	Slightly turbid	18.2	30.8	33.0	6.8	11.2
1.063–1.21	Clear	52.8	33.2	10.0	2.0	2.0
>1.21	Clear	96.0	4.0			

[a] Values are the average of two determinations.

magnification of ×30,000. Magnification was determined by reference to a carbon grating replica of 2160 lines/mm. Particle sizes were determined directly from negatives on a Nikon microcomparator. Before microscopy the samples were extensively dialyzed against 0.01 M phosphate buffer (pH 8.6)–10^{-3} M EDTA. Ultra-thin, carbon-coated, fenestrated formvar grids were employed. The negative stain was 2% phosphotungstic acid in 0.001 M phosphate buffer, (pH 7.0).

Studies by the technique of *circular dichroism* were carried out in a Cary Model 6001 spectropolarimeter with circular dichroism attachment, as described previously (Scanu and Hirz, 1968; Scanu, 1969).

Immunological testing of the various lipoprote fractions was carried out by double diffusion techniques in agarose gels (Scanu, 1966). The antisera used against HDL, apo HDL, fractions III, IV, and V were prepared in the laboratory (Scanu *et al.*, 1969).

The technique for the determination of total protein, total cholesterol, and phospholipids were as described previously (Scanu, 1966). Separation of free and ester cholesterol was carried out by silica gel thin-layer chromatography and so was the separation of the various phospholipid fractions (Scanu, 1966). These fractions, after identification by iodine vapor, were scraped off the thin-layer plates and quantified chemically.

All organic solvents were freshly distilled before use.

Results

Studies on Reassembly of Apo HDL_2 and Subfractions. apo HDL_2 + L. After sonic irradiation (total sonication time: 3 min) the mixture, which was turbid, became clear yellow, similar in appearance to native HDL_2. As shown previously with HDL_3 (Hirz and Scanu, 1969), turbidity persisted when HDL_2–L was sonicated in the absence of apo HDL_2. Such a solubilizing effect by apo HDL_2 was not observed with human serum albumin, α_1- and α_2-globulin, γ-globulin, cytochrome *c*, lysozyme, and myoglobin at least in terms of the commercial preparations used (Nutritional Biochemicals, Cleveland, Ohio; Mann Research Laboratories, Orangeburg, N. J.).

In the analytical ultracentrifuge the sonicated apo HDL_2 + L mixture examined at *d* 1.21 g/cm³ at 20° exhibited a main component (about 80% of total) of $F_{1.21}$ = 2.0, and two minor components, one in the low-density range ($F_{1.21}$ = 20) and the other of density greater than 1.21. Similar results were obtained by preparative ultracentrifugation. In terms of protein content, 70–80% of the starting material was in the top 1 ml of the *d* 1.063–1.21 and gave a single component in the analytical ultracentrifuge (Figure 1C); 10–15% was in the $d > 1.21$ fraction, and about 5% in the top of the $d < 1.063$ fraction. The chemical composition of these fractions was distinct (Table I) indicating not only differences in protein:lipid but also in cholesterol:phospholipid ratios—see top 1 ml of $d < 1.063$ and $d > 1.21$. These two fractions, however, had similar cholesterol free:ester ratio and the phospholipid distribution (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and serine, lysophosphatidylcholine) described for HDL (Scanu, 1966). The *d* 1.21 bottom 1 ml had only a small amount of lipids, almost exclusively lecithin, with traces of cholesterol.

By polyacrylamide gel electrophoresis in 8 M urea, the *d* 1.063 fraction did not penetrate the gel. After delipidation

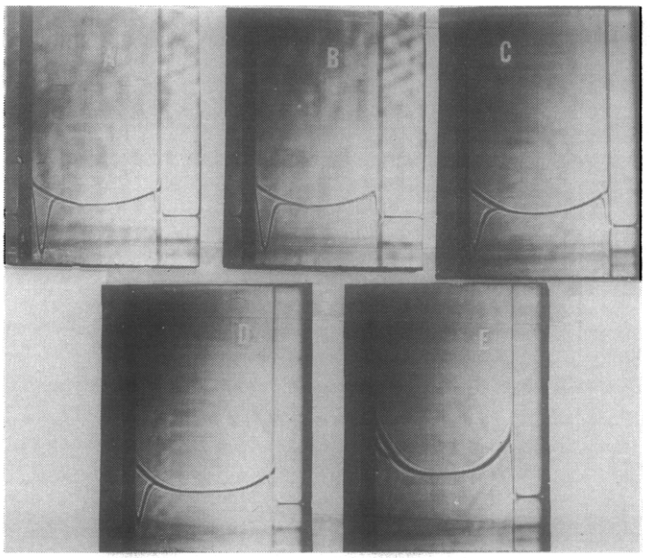

FIGURE 1: Schlieren optics ultracentrifugal patterns of various HDL_2 products before and after reassembly. (A) HDL_2, 4 mg/ml; (B) HDL_2 sonicated, 4 mg/ml; (C) Apo HDL_2 + L (*d* 1.063–1.21) 3.2 mg/ml of protein; (D) III + L (*d* 1.063–1.21) 1.7 mg/ml; (E) IV + L (*d* 1.063–1.21) 0.46 mg/ml. All specimens were dialyzed against *d* 1.21 NaCl–$NaBr_2$ solutions before analysis: speed, 42,040 rpm; AN-D rotor with double-sector cells; *T*, 20°. All pictures were taken 40 min after rotor had reached full speed. Flotation is from left to right.

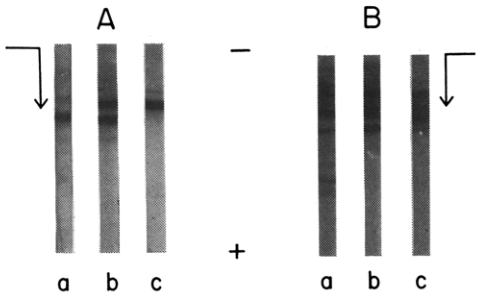

FIGURE 2: Polyacrylamide gel electrophoresis (8 M urea) of three ultracentrifugal fractions from sonicated apo HDL_2 + L mixtures: (A) after 3-min sonication; (B) after 6-min; (a) $d < 1.063$; (b) d 1.063–1.21; (c) $d > 1.21$. All fractions were studied after extraction with 3:2 ethanol–ether. The electrophoretic conditions were described before (Scanu *et al.*, 1969).

with ethanol ether (Figure 2A), however, several bands with distinct mobility were observed. The d 1.21 top (2A,b) showed two major bands of about equal intensity. The d 1.21 bottom exhibited only a major band (Figure 2A,B) while a faster component was either absent or only seen in trace amounts. The results were identical before and after delipidation.When these band patterns are compared with that of apo HDL_2, previously described (Scanu *et al.*, 1969) and represented in Figure 3, it can be concluded that the polypeptide chains corresponding to Sephadex peaks III and IV are about equally represented in the d 1.21 top, peak III was the almost exclusive component of the d 1.21 bottom, and d 1.063 top contained traces of III, but was predominantly IV and V. The correspondence of the observed bands with the Sephadex fractions previously described was supported by immunological studies using specific antisera and also by the result of the amino acid analyses.

Electron microscopy showed that there was a significant

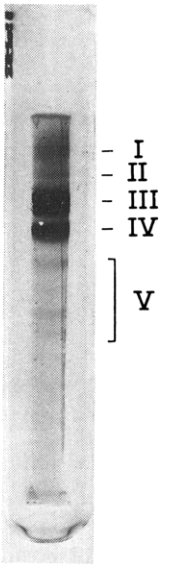

FIGURE 3: Polyacrylamide gel electrophoresis in 8 M urea of apo HDL_2. The correspondence of these bands with fractions obtained by gel filtration (Scanu *et al.*, 1969) is indicated.

TABLE II: Summary of Electron Microscopic Findings in the Relipidated Products from Apo HDL_2 and Its Subfractions III and IV.

Materials		Particle Size, (Å) Range	Number Average
HDL_2		60–135	90
HDL_2 sonicated		70–170	110
Apo HDL_2 + L	$d < 1.063$	150–400	250
	d 1.063–1.21	65–180	110
	$d > 1.21$	50–120	70
III + L	$d < 1.063$	120–410	200
	d 1.063–1.21	60–160	120
	$d > 1.21$	40–140	90
IV + L	$d < 1.063$	180–600	300
	d 1.063–1.21	70–280	180
	$d > 1.21$	50–150	100

difference in number-average particle size among the three ultracentrifugal fractions studied only before delipidation (Table II). The $d < 1.063$ contained the largest particles (range 150–400 Å; number average 250 Å) and $d > 1.21$ the smallest (range 50–120 Å; number average 70 Å). The d 1.21 top had particles in the size range of 65–180 Å; number average 110 Å (Figure 4B). These particles were slightly larger than those observed with HDL_2 (Table II and Figure 4A) but about equal in size to sonicated HDL_2 (Table II, see also later).

Further similarity between HDL_2 and the d 1.063–1.21 top from sonicated apo HDL_2 + L mixture was observed by the technique of circular dichroism. The two products gave indistinguishable patterns (0.01 M Tris (pH 8.4)– 10^{-3} M EDTA, 27°) and, as previously described (Scanu and Hirz, 1968), exhibited two negative bands at 222 and 208 nm and a positive peak at 198 nm. The molar ellipticity values in 10^{-4} deg-cm^2/dmole were $[\theta]_{222} = 2.0$–2.2, $[\theta]_{208} = 1.9$–2.1, and $[\theta]_{198} = 3.8$–4.2. When the spectra were recorded as a function of temperature in the range of 20 and 80,° the course of the thermal transition of both HDL_2 and the reconstituted lipoprotein, based on the changes of the 222-nm band, was similar and markedly distinct from apo HDL_2 (Figure 5). In all of the above experiments, protein and lipid in the sonicated mixtures were in the weight ratio of 1:1. If apo HDL_2 exceeded this ratio, it was recovered in the $d > 1.21$ bottom as a mixture of III and IV (no V was detected). In turn, any excess lipid was recovered in the $d < 1.006$ top fraction.

The effect of the length of sonication time on the reassembly experiments was also examined. At 1-min sonication the apo HDL_2 + L mixture, although significantly clearer than the starting one, was still opalescent. This was due to unbound lipids that floated at $d < 1.006$ and led to lower recoveries of d 1.21 top and bottom fractions. The polyacrylamide gel pattern of the d 1.21 top exhibited both peaks III and IV, whereas the bottom 1.21 ml had essentially only III. With longer periods of sonication (above 3 min) the pro-

TABLE III: Lipid–Protein Composition of Three Ultracentrifugal Fractions from Sonicated Mixtures of Apo HDL_2 Subfractions and Lipids.

Apo HDL_2 Subfractions	*d* 1.063–1.21 (top 1 ml) (% weight[a])				
		Lipid			
	Protein	Phospholipid	Cholesterol Ester	Cholesterol Free	Glyceride
III	50.1	30.0	11.8	2.0	6.2
IV	45.0	27.8	17.2	4.0	6.0
V	*b*	*b*	*b*	*b*	*b*

[a] The values are the average of two determinations. [b] None detected.

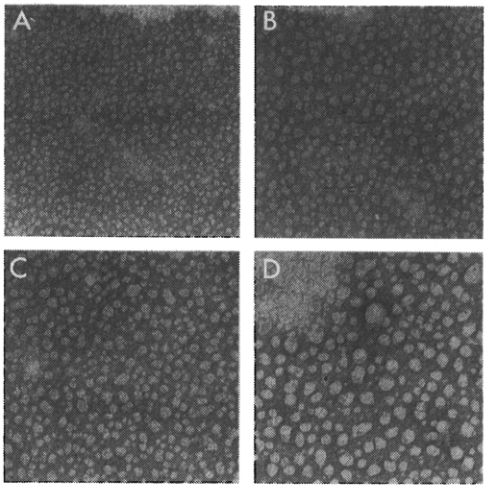

FIGURE 4: Electron micrographs of HDL_2 (A); apo HDL_2 + L, *d* 1.21 top (B); III + L, *d* 1.21 top (C); IV + L, *d* 1.21 top (D). For experimental conditions, see text. Final magnification = 60,000 ×.

tein–lipid mixtures remained perfectly clear: there was a tendency for IV to decrease in relation to III in the *d* 1.21 top and to progressively increase in the $d < 1.063$ fraction. Concomitantly, more III sank into the *d* 1.21 bottom. The patterns remained unchanged when the same products were analyzed after 1 to 2 weeks at 4°. Patterns after 6-min sonication are shown in Figure 2B. Additional bands preceding III, probably representing aggregation, were seen.

Relipidation of Apo HDL_2 with Non-HDL Lipids. The experiments were carried out with purified preparations of phosphatidylcholine (egg) (Sylvana Co., Millburn, N. J.), free cholesterol, cholesterol oleate, and triolein (Applied Science Laboratories, State College, Pa.). Sonication of apo HDL_2 with phosphatidylcholine (1:1 weight ratio) for 3 min at either 4 or 40° led to formation of a protein–phospholipid complex floating at *d* 1.24 with properties very similar to those described for a corresponding product prepared in the laboratory by a different relipidation technique (Scanu, 1967). III and IV were the polypeptide chains of this complex. Apo HDL_2 produced also solubilization (sonication 3 min, 40°) of aqueous suspensions of phosphatidylcholine, cholesterol free and ester and triolein added in proportions similar to their counterparts in HDL_2. The high-density lipoprotein recovered in the *d* 1.21 top ml had again III and IV as its protein constituents. A more detailed investigation of this and other systems containing varying mixtures of artificial lipids will be reported elsewhere.

Relipidation of III, IV, and V. Sonic irradiation of these fractions was carried out in the presence of whole lipid extracts from HDL_2 under the experimental conditions described in Methods. Fractions III or IV produced a complete solubilization of the HDL_2 lipid suspension. The solubilization time was more rapid (within 2 min) with fraction IV than with fraction III (within 3 min). In all protein–lipid weight ratios used (0.1–2:1), fraction V failed to produce complete clearing of the lipid suspension; this appeared slightly opalescent in contrast to the starting mixture which was frankly turbid. The lipid–protein composition of the top ml of the *d* 1.063–1.21 fractions obtained from the relipidation of III and IV and V are shown in Table III. Clear differences in protein:lipid ratio and lipid distribution were noted between fractions III and IV, the latter having more lipid with a prevalence of neutral lipids. On the other hand, no material floating between *d* 1.063 and 1.21 was noted with sonicated fraction V + L mixtures. About 90% of fraction V was recovered in the *d* 1.063 top of $S_{f(1.063)} = 4$–6 (% weight composition: protein, 20; phospholipid, 24; cholesterol esters, 39; cholesterol free, 7; glyceride, 10); the remainder was in the *d* 1.21 bottom. In the sonicated mixtures containing either III or IV, about 1–2% was recovered in the *d* 1.063 top ml (because of low yield no accurate chemical analysis was performed). The remaining 10–20% was in the *d* 1.21 bottom ml and contained 3–4% lipid.

By polyacrylamide gel electrophoresis, the relipidated fractions III and IV maintained their distinct mobilities (see Scanu *et al.*, 1969, and Figure 3). This was also the case of the ultracentrifugal *d* 1.21 top and bottom fractions whether studied before or after delipidation with ethanol–ether. In the *d* 1.063 top ml, only a minute amount of either III or IV

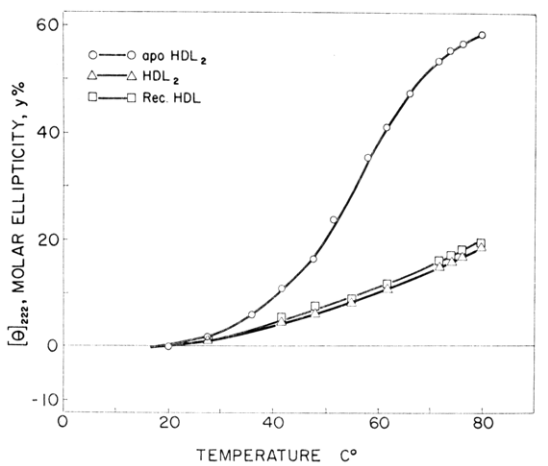

FIGURE 5: Thermal dependence of denaturation of HDL_2, HDL_2, and reconstituted HDL as assessed by circular dichroic analyses. The graph is a representation of the per cent change of the 222-nm band as a function of temperature.

TABLE IV: Graded Relipidation of Fractions III and IV from Apo HDL_2.

Constituents in the Mixture[a]			Polypeptides in Ultracentifugally Separated Fractions[c]		
A	B	C	$d < 1.063$	1.063–1.21	>1.21
III	Lipids[b]	IV	*III*, IV	III, *IV*	III
III	Lipids	V	*III*, *V*	III, *V*	III
IV	Lipids	III	IV	IV, *III*	III
IV	Lipids	V	IV, V	IV, *V*	

[a] A + B were sonicated for 3 min at 40°. The resulting product $(A + B)_{son}$ was then mixed with C *without* sonication and the mixture kept for 12 hr at 4° before separation in the preparative ultracentrifuge. Identification of proteins after delipidation was carried out by polyacrylamide gel electrophoresis in 8 M urea and by amino acid analysis. [b] The lipids were a total extract from HDL_2. [c] Components in italic type were only present in small amounts with respect to the major component.

TABLE V: Lipid–Protein Composition of Ultracentrifugal Fractions from Ethyl Ether Treated HDL_2.[a]

Product Density (g/cm^3)	Protein	Lipids (% weight)		
		Phospholipid	Total Cholesterol	Triglycerides
<1.063	20.0	35.6	30.4[b]	14.0
1.063–1.21	51.6	29.5	15.5[c]	3.4
>1.21	96.0	4.0		

[a] All percentages based on weight. [b] Per cent cholesterol esters: 78.6. [c] Per cent cholesterol esters: 82.0.

was observed after delipidation. In terms of the sonicated V + L mixtures, the top <1.063 fraction, when analyzed before delipidation, failed to penetrate the gel. After treatment with 3:2 ethanol–ether the characteristic four band pattern of peak V (see Figure 3) was observed. The same pattern was found in the $d < 1.063$ bottom when V was added in excess to the system.

In the analytical ultracentrifuge the *d* 1.21 top from sonicated III + L mixtures exhibited a single flotational component with $F_{1.21} = 2.0$–2.2 (Figure 1D). On the other hand, the corresponding fraction from IV + L mixture gave a broad floating component (Figure 1E) with $F_{1.21}$ values between 1.0 and 6. No studies on the analytical ultracentrifuge were conducted with the IV + L mixture after fractionation in the preparative ultracentrifuge.

Electron microscopy revealed a significant difference in size distribution between reassembled products from III and IV (Table II), the former exhibiting a striking similarity with apo HDL_2 + L. Electron micrography of these products (*d* 1.21 top) are shown in Figure 4.

Graded Relipidation of III and IV. These experiments were designed to determine whether the high-density lipoprotein species obtained from the reassembly experiments with III or IV, are capable of further incorporating IV or III, respectively. The experimental design was as follows: III or IV were sonicated in the presence of HDL_2 lipids (weight ratio 1:1) for 3 min at 40°. After cooling to 4°, either III or IV was added without sonication (weight ratio, III:IV, 1:1) and the resulting mixtures $(III+L)_{son}$ +IV or $(IV+L)_{son}$ + III kept at 4° for 12 hr. They were then separated in the preparative ultracentrifuge at $d < 1.063$, *d* 1.063–1.21, and > 1.21 and the fractions obtained were analyzed as indicated previously. In some experiments, peak V was also used. The results of such experiments (Table IV) indicate that when a lipoprotein complex is formed by the sonication procedure, further addition of protein, although in small amounts, is possible with very little energy requirement. Examination of Table IV again indicates the tendency by III, IV, and V to distribute specifically in given ultracentrifugal fractions. If the initial sonication step was omitted, however, no significant incorporation of either cholesterol esters or triglycerides was observed.

Effect of Sonication on HDL_2. As observed previously with HDL_3 (Hirz and Scanu, 1969), sonication (3 min) did not change the appearance or spectral properties (ultraviolet absorption spectroscopy and circular dichroism) of HDL_2 although differences in average size distribution were observed by electron microscopy (Table II). When HDL_2, before and after sonication, was separated into $d < 1.063$, *d* 1.063–1.21, and $d > 1.21$ fractions, more significant differences between the two products were noted. The $d < 1.063$ fraction was only found in sonicated HDL_2 (10–15% of the starting material) and gave a IV–V pattern after delipidation, fraction V being predominant. In the *d* 1.21 top fraction, both fractions III and IV were present in apparently similar proportions as in apo HDL_2 (Figure 3) with no V. About 10–20% of the starting material was recovered in the *d* 1.21 bottom with sonicated HDL_2, in contrast to 1–2% observed with HDL_2; the sonicated specimen had peak III almost exclusively. The differences in distribution of electrophoretic band patterns in HDL_2 before and after sonication were corroborated by amino acid analysis. When the time dependence of sonication on HDL_2 was studied (1–5 min at 40°), it was found that by prolonging sonic irradiation, although the solutions remained perfectly clear, there was a tendency for the $d < 1.006$ fraction to increase (the peptides were predominantly IV and V), and for III to increase in the *d* 1.21 bottom. These reactions were not reversed after a maximum observation period of 2 weeks during which the specimens were kept at 4°.

Effect of Sonication on Apo HDL_2. Sonic irradiation under the standard conditions described in Methods appeared to favor solubilization of apo HDL_2. Solutions of apo HDL_2 (2–5 mg in 10^{-2} M (Tris pH 8.6)– 10^{-3} M EDTA), compared before and after sonication, showed no significant differences at least in terms of the parameters employed (ultraviolet absorption spectroscopy, circular dichroism, and polyacrylamide gel electrophoresis). The observations were the same regardless of the time of sonication (1–5 min) at 40°.

Studies on Degradation of HDL_2. EFFECT OF ETHYL ETHER ON HDL_2. Under the experimental conditions employed, ether

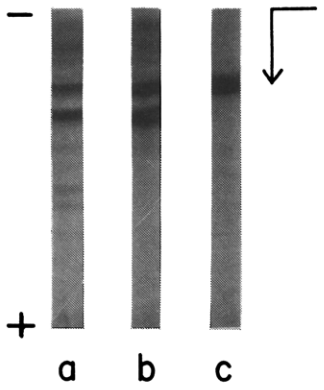

FIGURE 6: Polyacrylamide gel electrophoresis of ultracentrifugal fractions from HDL_2 treated with ethyl ether. Before analysis all fractions were extracted with 3:2 ethanol–ether. The conditions of electrophoresis were the same as in Figure 2: (a) $d < 1.063$; (b) d 1.063–1.21; (c) $d > 1.21$.

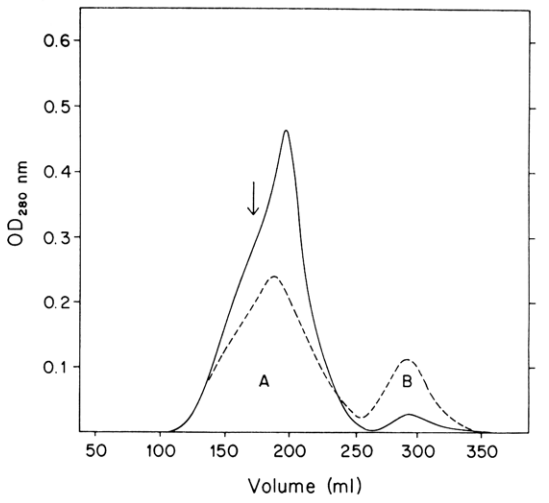

FIGURE 7: Fractionation of HDL_2 by Sephadex G-200. Column dimensions, 2 × 100 cm; eluting buffer, 0.1 M Tris (pH 8.4)–0.01 M EDTA; ascending flow rate, 10–12 ml/hr; T, 10°. The arrow indicates the void volume.

extracted only about 2% of lipids, both cholesterol and phospholipids. When the resulting ether-treated product was fractionated by ultracentrifugation, marked structural consequences were noted. In contrast to untreated HDL_2 which, upon recentrifugation, was essentially all recovered in the d 1.21 top (about 1–2% of protein was found in the d 1.21 bottom), ether treatment caused about 50% decrease of the d 1.063–1.21 fraction, appearance of a fraction floating at d 1.063 (about 10% of the starting material) and a significant portion (30–40%) essentially free of lipids, in the d 1.21 bottom. As indicated by the chemical analysis (Table V) the floating fractions at 1.063 and 1.21 differed not only in protein:lipid ratio but also in lipid distribution, the lipid fraction showing a greater percentage of cholesterol than the heavier one.

By polyacrylamide gel electrophoresis, the ultracentrifugal fractions examined after further delipidation by ethanol–ether, showed again significant changes. As shown in Figure 6, the d 1.063 top was characterized by the presence of IV and V (III was not constantly seen), the d 1.21 top by III and IV in about equivalent amounts, whereas $d > 1.21$ contained almost exclusively peak III. The interpretation of the band pattern was again consistent with the results of the immunological studies and amino acid analysis. By prolonging ether extraction up to 48 hr the effects on HDL_2 were only slightly changes. These consisted of a further decrease of the $d > 1.21$ top fraction with a proportional increase of $d < 1.063$ and $d > 1.21$. Polyacrylamide gel electrophoresis indicated the following patterns: predominance of IV + V in $d < 1.063$; predominance of IV over III in $d > 1.21$ and almost exclusive presence of III in $d > 1.21$ bottom.

Separation of HDL_2 before and after Ether Treatment by Gel Filtration. By Sephadex G-200, HDL_2, before ether treatment, exhibited two distinct components, the major one (A) partially eluted in the void volume and a second one (B) representing about 5% of all eluted material (Figure 6). A third peak, representing about 1% of the total eluted protein was seen occasionally but not identified. Recoveries were in the order of 90%. Fraction A had essentially the same chemical composition as HDL_2 whereas B contained only 4% phospholipids and trace of cholesterol. After delipidation, the proteins of A exhibited, by polyacrylamide gel electrophoresis, the band patterns of peaks III and IV in approximately the same distribution as in apo HDL_2. On the other hand, B showed exclusively a peak III band pattern. The interpretation of the polyacrylamide gel pattern was corroborated by immunology and amino acid analysis.

Fractionation of HDL_2, that had been extracted with ether, by Sephadex G-200, produced a decrease of A with a relative increase of peak B (Figure 7). This exhibited the same characteristics as the corresponding product obtained by gel filtration of untreated HDL_2. Either treatment thus appeared to increase the amount of lipid-poor apoprotein, an observation in keeping with the results obtained by ultracentrifugation.

Sepharose 2B experiments (these were limited to HDL_2) gave results very similar to those with Sephadex G-200 and therefore were not pursued further.

Discussion

Previous work from this laboratory has shown that when the lipid moiety of human serum HDL_3 is sonicated in the presence of lipid-free HDL_3 protein, the resulting mixture, initially turbid, becomes clear due to the formation of a water-soluble lipid–protein complex which as a whole closely resembled the starting lipoprotein (Hirz and Scanu, 1969). The current studies have now extended such observations to HDL_2 protein, and demonstrated in addition, that three distinct lipoprotein species are produced in a proportion largely dependent on the conditions of sonication. The analysis of such lipoproteins indicated that the two classes floating in the d 1.063 and 1.21 top had all the lipid components of HDL_2, whereas the heavier fraction ($d > 1.21$) composed of about 95% protein, contained essentially only phospholipids. It is further interesting that each lipoprotein species could also be distinguished on the basis of their protein moiety (see Figure 8). For instance, with 3-min sonication, IV and V were the predominant polypeptides of the d 1.063 top, III and IV those of the d 1.21 top whereas the d 1.21 bottom contained III almost exclusively. Upon increasing the time of sonication there was a tendency for III to increase in the d 1.21 bottom, with the largest percentage

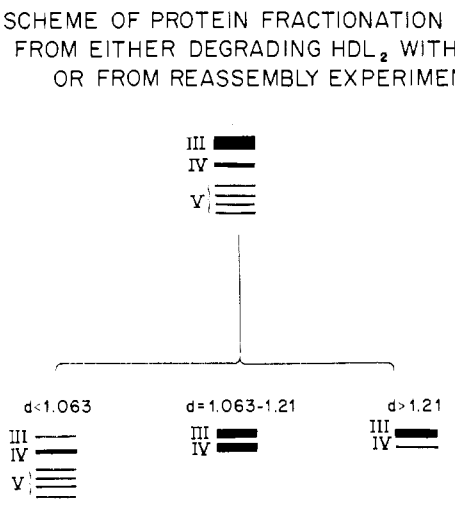

FIGURE 8: Scheme of fractionation of polypeptide chains of apo HDL_2 achieved in the reassembly experiments employing apo HDL_2 + L.

of IV in the *d* 1.063 top. It is thus apparent that the three classes of polypeptide chains previously described in apo HDL_6 (Scanu *et al.*, 1969) have a different affinity for lipids, a conclusion the receives support from the reassembly experiments using individual III, IV, and V. In every instance the results of such studies showed that (1) free cholesterol, ester cholesterol, and glycerides could be incorporated into distinct lipid–protein complexes as indicated by chemical, ultracentrifugal, and electron microscopic data; (2) the process required the presence of both protein and phospholipids in the system; and (3) energy, provided in the form of thermal and sonic irradiation. However, the possibility remains that free radicals may have formed during sonication (Lindstrom and Lamm, 1951; Peacocke and Pritchard, 1968) and may have contributed to the reassembly phenomenon. The energy for the incorporation of neutral lipids into the water-soluble lipid–protein complex contrasts with low energy requirement observed with the reaction involving only protein and phospholipids (Scanu, 1967). We interpret this observation to mean that nonpolar lipids must overcome some resistance to penetration by the protein–phospholipid polar surface of the lipoprotein to occupy a core location away from the aqueous environment. This observation may not necessarily apply to circulating plasma where current evidence suggests that cholesterol esters in HDL derive from an intramolecular process through the activity of the enzyme lecithin–cholesterol acyl transferase (Glomset *et al.*, 1966).

Of interest were the experiments on HDL_2 degradation showing that ether treatment causes dissociation of this lipoprotein into species similar to those formed during the reassembly experiments. It was surprising that ether, which extracted little lipid from HDL_2, had a profound structural effect on the HDL_2 causing it to dissociate into products distinct in protein:lipid ratio, lipid distribution, and nature of the protein moiety. Such results, which are in general agreement with those by Hayashi *et al.* (1959), suggest that HDL_6, as we isolate it in the ultracentrifuge, is in fact a mixture of distinct lipid–protein complexes, although they give little insight as to how they are assembled. Some speculative thoughts appear, however, permissible on the basis of the reassembly studies. The idea, for instance, that III is an essential and specific constituent of HDL_2 appears supported by the following findings: (1) it represents 70–80% of whole apo HDL_2 (Scanu *et al.*, 1969); (2) the reassembled III + L produced a high-density lipoprotein closely resembling that of native HDL in all the physical and chemical parameters examined. (3) III appeared size limiting since in its absence lipoprotein particles formed during reassembly were of a significantly larger diameter than in HDL_2. In contrast, upon sonication with lipids, IV formed a rather unusual high-density lipoprotein, markedly heterogeneous in size and density (see Figures 1 and 4, and Table II), or had the tendency to dissociate from ether-treated or sonicated HDL_2 as a protein–lipid complex of $d < 1.063$. In no instance was IV, in contrast to III, either encountered or a predominant component of the *d* 1.21 bottom. This was also the case with V which, like IV, had, upon removal from HDL_2, a preferred association with a low-density lipid–protein complex.

Based on the experimental evidence obtained in the current studies, we would favor the hypothesis that III is the "structural" protein constituent of HDL_2 contributing, with phospholipids, to the surface coat of this lipoprotein which has as a nonpolar core, cholesterol esters and triglycerides. On the other hand, IV and V would be "functional" elements, probably involved in the interaction of HDL_2 with the other plasma lipoproteins. As far as IV is concerned, this alleged functional role is not clear. With regard to V, its derivation from exchange processes between high and very low-density lipoproteins is suggested by the fact that this polypeptide class is present in both lipoproteins (to be published). It is of interest to point out that either IV or V could be added to the reassembled sonicated III + L complex to give a final lipoprotein very similar in protein distribution to that observed in ultracentrifugally isolated HDL_2. Support for the structural role of III in HDL comes also from the observation tha tthe reassembled IV + L, by itself distinct from any described high-density lipoproteins, binds III only to a limited extent, at least in the absence of esternal energy source (sonication and temperature). If proteins of type III are in fact essential structural elements of HDL_6, able by their special arrangement at the surface of the molecule to control the overall size of the lipoprotein, then marked structural alteration of the latter would be expected upon partial or total removal of these polypeptide chains. Coroboration for such speculations comes from the degradation experiments: an increase in the time of either sonication or ether extraction produced a progressive loss of III from HDL_2 and a parallel increase of a low-density complex, probably originating from the reduction of polar groups and the coalescence of lipids into relatively more hydrophobic particles with preferential affinity for IV or V. Regardless of the exact nature of this phenomenon, the molecular details of which obviously remain to be established, one is impressed by the high degree of structural reorganization possible in HDL, which allows for lipid to remain "soluble" in an aqueous environment. A better understanding of these phenomena is likely to derive from the isolation and characterization (now in progress) of the various polypeptide chain members of III, IV, and V, and from the definition of their specific binding properties for lipids.

We would like to stress that the reassembly technique

developed in the current studies, or any similar methodology, appears to provide a very useful tool for the study of the structure and function of plasma high-density lipoprotein and even allows for some fractionation of its polypeptide chains. Since the natural lipids of HDL may be replaced effectively by artificial mixtures, it would be possible in the future to introduce appropriate markers to define whether native high-density lipoproteins and their reassembled counterpart have identical biological behavior. One must be aware, however, of some of the drawbacks of the sonication method in reassembly studies. Our results have clearly indicated that sonic irradiation, under the experimental conditions employed, causes some modifications of intact HDL_2 as expressed by a slight increase of its median particle size (see Table II) and some protein loss (10–15%) from the complex. We are not presently in a position to define the extent of the structural differences between "native" and sonicated products precisely or even to assess whether the term "native" can be properly applied to a lipoprotein after its isolation from plasma. If we rely, however, on the chemical, immunological, ultracentrifugal, and circular dichroic data obtained in the current studies and on the nuclear magnetic resonance results to be reported elsewhere (R. B. Leslie and A. Scanu, to be published) then we may conclude that the two products (intact and reassembled) are at least strikingly similar. It remains for future studies (chemical modification of intact and reassembled complexes, response to proteolytic and lipolytic enzymes, deuterium exchange studies, etc.) to determine how close such structural similarity is and whether the correlation also extends to their functional behavior (*i.e.*, participation in the activity of the enzymes lipoprotein lipase and lecithin–cholesterol acyl transferase). The information obtained should improve our understanding of the role of high-density lipoproteins in fat transport.

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