Lipid Composition of Human Serum Lipoproteins

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1. The lipid compositions of the low-density lipoproteins, the high-density lipoproteins and the ultracentrifugal residue of human serum are presented, with emphasis on certain lipoprotein classes and lipid components not previously described. 2. Except for the lipoproteins with the lowest and highest densities, there is a trend for stepwise successive increase or, respectively, decrease in the relative amounts of the main constituents of lipoproteins. 3. High-density lipoprotein-2 and high-density lipoprotein-3 have different amounts of certain lipids; high-density lipoprotein-2 has relatively more free cholesterol and sphingomyelin; high-density lipoprotein-3 has more free fatty acids, diglycerides and ceramide monohexosides. 4. All the lipoproteins contain hydrocarbons of the alkane series. The greatest amount, which averages 4.4% of total lipid extracted, is in the ultracentrifugal residue; *n*-alkanes comprise 18–50% of the hydrocarbons. 5. All the lipoproteins contain ceramide monohexosides. The highest relative contents of these glycolipids are in high-density lipoprotein-3 and in the ultracentrifugal residue. 6. The ultracentrifugal residue contains 55% of the total quantity of free fatty acids present in serum. The remaining free fatty acids are distributed among the other lipoprotein classes. 7. The choline-containing phospholipids (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) comprise about 90% of the phospholipids in all the lipoprotein classes except the low-density lipoprotein-2, which contains about 80% of these phospholipids. 8. The presence of a large amount of lysophosphatidylcholine in the ultracentrifugal residue and the successive decrease of sphingomyelin from the low-density lipoprotein-1 to the ultracentrifugal residue was confirmed. 9. The low-density lipoprotein-2 and the ultracentrifugal residue are characterized by relatively high contents of the lower glycerides.

There are indications that the two major classes of serum high-density lipoproteins, HDL₂ and HDL₃, are affected differently under diverse physiological and pathological conditions. Thus Barclay, Barclay & Skipski (1963) reported that premenopausal women have at least twice the amount of HDL₂ as have men of the same age. This is apparently related to oestrogen production (Barclay *et al.* 1965). HDL₃ is present in greater amounts in sera from men. Women with advanced cancer of the breast have selectively decreased amounts of HDL₂ compared with normal women of the same age (Barclay *et al.* 1964).

Since the exact physiological role of the high-density lipoproteins is still obscure, a relatively simple explanation for any biochemical differences may be revealed by studies of their chemical compositions. Scanu & Hughes (1962) and Levy &

Fredrickson (1965) reported that the amino acid compositions of the protein moieties of the high-density lipoproteins separated by their procedures are similar. Scanu (1966) stated that proteins of both HDL₂ and HDL₃ consist of protein sub-units, probably identical, of an average molecular weight of 21000. He suggests that the difference in the antigenic behaviour between HDL₂ and HDL₃ results from the presence of a lipid-poor protein in HDL₃.

The most complete information on the lipid composition of the high-density lipoproteins is on HDL₂ and HDL₃ combined in a single fraction (Lindgren, Nichols, Hayes, Freeman & Gofman, 1959; Lindgren & Nichols, 1960; Freeman, Lindgren & Nichols, 1963; Polonovski, Ayrault-Jarrier, Petit & Bard, 1959; Polonovski, 1961). Earlier studies of the lipid composition of lipoproteins by

Hillyard, Entenman, Feinberg & Chaikoff (1955) and Havel, Eder & Bragdon (1955), although much less complete than the above-mentioned studies, demonstrated differences in the lipid composition of HDL₂ and HDL₃, especially in the ratio of lipid to protein. Scanu (1965), who studied only a few lipid components, also reported differences in the lipid composition of HDL₂ and HDL₃ as well as differences in their lipid to protein ratios.

Our knowledge of both lipids and proteins in the lipoproteins with densities greater than 1.210 g./ml. is also limited. Freeman *et al.* (1963) reported that free fatty acids comprise 60% of the total lipid extracted from this fraction of serum from patients with xanthoma tendinosum and others with xanthoma tuberosum. Phillips (1959), Polonovski *et al.* (1959) and Polonovski (1961) studied the neutral lipids and phospholipids of lipoproteins with densities greater than 1.210 g./ml. and observed that about half of the phospholipids is lysophosphatidylcholine. These authors did not report on free fatty acids and their data on cholesterol do not agree with those of Freeman *et al.* (1963).

The purpose of the present paper is to give the lipid compositions of the two classes, HDL₂ and HDL₃, as well as the lipoproteins with densities greater than 1.210 g./ml. whose lipid composition is still a controversial matter. To present a complete picture of all the lipoprotein classes, and to follow the general trends of alterations in composition, the low-density lipoproteins ($d < 1.006$ and $d < 1.063$ g./ml.) are also included. Data on the distribution of several lipids (hydrocarbons, lower glycerides, ceramide monohexosides and phosphatidylinositol) that have not previously been reported in lipoprotein classes are presented.

MATERIALS AND METHODS

Materials. All solvents were reagent grade and were obtained from Merck and Co., Rahway, N.J., U.S.A., unless otherwise indicated. They included: chloroform, washed with water, dried with anhydrous CaCl₂ and redistilled (1% of redistilled methanol was added as a preservative); methanol and pyridine, both redistilled under anhydrous conditions; light petroleum, 'for fat determinations', redistilled, fraction collected at 42–58°; acetone, anhydrous, redistilled under anhydrous conditions in the presence of KMnO₄ and K₂CO₃; benzene, thiophen-free, redistilled; diethyl ether, anhydrous, from new bottles. The following solvents were obtained from Matheson, Coleman and Bell, Cincinnati, Ohio, U.S.A.: pentane, 99% pure; hexane, practical, with b.p. 68–69°; iso-octane (2,2,4-trimethylpentane); all these solvents were redistilled. When identification of individual hydrocarbons was performed, all redistilled solvents were further fractionated through a 3 ft. glass column packed with 3 mm. glass helices, with a 4:1 reflux ratio. Purity of such solvents was checked on gas-liquid chromatograms before use. Acetonitrile, spectro grade, was obtained from Eastman Kodak Co., Rochester,

N.Y., U.S.A. Di-isopropyl ether, from the same Company, was redistilled. Acetic acid and HCl, both of reagent grade, were obtained from Merck and Co. Aq. NH₃ (sp.gr. 0.88), reagent grade, was purchased from Fisher Scientific Co., New York, N.Y., U.S.A.

Silica gel for thin-layer chromatograms, fine (D-O type), without CaSO₄ binder (Camag, Muttenz, Switzerland, obtained through Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.), was used throughout the study. Silicic acid (100 mesh), analytical reagent, 'suitable for chromatographic analysis' (Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.), was used for column chromatography. All small particles of silicic acid were removed by extensive washing with distilled water. Only the silicic acid that settled within 1 min. was used for packing the columns.

An antioxidant, 2,6-di-*tert*-butyl-4-methylphenol, and the inhibitor 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Another inhibitor, which was used in most experiments, *N*-ethylmaleimide, was purchased from Mann Research Laboratories, New York, N.Y., U.S.A.

Oil Red O was used as a spray reagent for the detection of glycolipids and some phospholipids. It was obtained from National Aniline Division, Allied Chemical and Dye Corp., New York, N.Y., U.S.A.

The nature and the origins of the numerous lipids used as reference compounds for thin-layer chromatography and infrared spectroscopy, and for calibration of chromatographic columns or for chemical tests, were described in detail in earlier publications (Skipski, Peterson & Barclay, 1962, 1964; Skipski, Smolowe, Sullivan & Barclay, 1965b; Skipski, Barclay, Reichman & Good, 1967).

Isolation of lipoproteins. Serum was from starved normal women whose ages averaged 30 years. Lipoproteins were isolated according to the procedure of Barclay, Barclay, Terebus-Kekish, Shah & Skipski (1963). The sera were immediately used for the separation of lipoproteins in the preparative ultracentrifuge (Spinco model L, 40.3 rotor at 40000 rev./min.). The lipoprotein classes were removed sequentially with increasing densities. The procedure separates three low-density lipoprotein classes: chylomicra (often absent from normal starved subjects), lipoproteins with $d < 1.006$ g./ml. or low-density lipoproteins-2 (LDL₂), lipoproteins with $d < 1.0635$ g./ml. or low-density lipoproteins-1 (LDL₁); and three high-density classes: lipoproteins with $d < 1.125$ g./ml. or high-density lipoproteins-2 (HDL₂), lipoproteins with $d < 1.210$ g./ml. or high-density lipoproteins-3 (HDL₃) and lipoproteins with $d > 1.210$ g./ml. or the ultracentrifugal residue. The last fraction includes the complex of albumin with non-esterified fatty acid. Occasionally the high-density lipoprotein fractions, HDL₂ and HDL₃, may contain very small amounts of serum proteins. All isolated fractions, except chylomicra, were used for the quantitative lipid analyses. The terminology used throughout this paper is the same as that used by Vandenheuvel (1962).

The lipoprotein composition of the fractions obtained in the preparative ultracentrifuge was determined with the Spinco model E analytical ultracentrifuge. Lipoprotein classes LDL₂, LDL₁ and HDL₂ may contain several subclasses of lipoproteins as shown in Fig. 1. However, the total classes, without further subfractionation, were used for lipid analysis. The ultracentrifugal residue may also consist of several subclasses of lipoproteins. Separations of

Fig. 1. Lipoprotein classes and subclasses separated by preparative and analytical ultracentrifugation. The classes of lipoproteins between the solid vertical lines were used for lipid analysis. The broken lines separate the subclasses of lipoproteins observed in the analytical ultracentrifuge.

lipoprotein classes in the preparative ultracentrifuge, and subsequent dialysis to remove excess of salts, were performed in the presence of *N*-ethylmaleimide or 5,5'-dithiobis-(2-nitrobenzoic acid).

Extraction of lipids. Each class of lipoproteins isolated in the preparative ultracentrifuge was dialysed to remove excess of salts. The removal was followed by testing the diffusate with AgNO_3 . Lipoproteins were transferred quantitatively and lipids were extracted with cold chloroform-methanol (2:1, v/v). The ratio of solvent to lipoprotein solution was 20:1 (v/v). Extracts were filtered and the residues re-extracted with 5 vol. of chloroform-methanol at room temperature. Extracts were combined, evaporated in a partial vacuum under N_2 and redissolved in chloroform. Occasionally the residues were extracted a third time with an acidic solvent: 1 part of 2*N*-HCl and 99 parts of chloroform-methanol (1:1, v/v), but this did not give an appreciable yield of lipids and therefore was not used as a routine.

When large volumes of lipoprotein solutions were obtained (from 100 ml. or more of serum) the solutions were freeze-dried to about one-fifth of the original volumes before lipid extractions.

Lipid analyses. (a) Neutral lipids. Neutral lipids were separated by a two-step developmental thin-layer chromatography system (Skipki *et al.* 1965b). From 5 to 15 mg. of total lipid extracted from lipoproteins was applied across two-thirds of the bottoms of two 200 mm. $\times$ 200 mm. plates coated with a 0.5 mm. layer of silica gel. On the remaining one-third, a mixture of standards was applied. Chromatograms were developed and sprayed with Rhodamine 6G, and bands of separated lipids were marked under ultraviolet light. The lipid bands were removed and eluted (details of the method, worked out on a mixture of standards and rat liver lipid extracts, will be published subsequently). Free and total cholesterol were determined in the eluates by the method of Abell, Levy, Brodie & Kendall (1952). Free fatty acids were dissolved in a 5 ml. mixture of diethyl ether and 95% ethanol (1:1, v/v) and were titrated in the presence of Cresol Red by 0.01*N*-NaOH (prepared in 70% ethanol). Nitrogen was constantly bubbled through the sample during the titration. Triglycerides, diglycerides and monoglycerides were determined quantitatively by infrared spectrophotometry. The absorption at approx. 5.76μ was measured (characteristic of the ester group carbonyl C=O stretching

vibration according to Freeman *et al.* 1963; Shreve, Heether, Knight & Swern, 1950) by the Perkin-Elmer model 221 infrared spectrophotometer. Occasionally ester values (of triglycerides) were determined by a chemical method (Rapport & Alonzo, 1955). Hydrocarbons and minor unidentified lipids that chromatographed with neutral lipids were determined by the densitometric procedure of Blank, Schmit & Privett (1964). The determination of hydrocarbons was performed against tetracosane as a reference compound and unidentified neutral lipids were determined against 1,3-diolein as a standard source of carbon. All measurements were performed on a Photovolt densitometer.

In addition to the lipid extracts from lipoproteins, a mixture of pure reference compounds (tetracosane, cholesteryl oleate, triolein, oleic acid, 1,3-diolein, 1,2-diolein, cholesterol and 1-mono-olein) was chromatographed in each experiment. They were recovered quantitatively by the above-mentioned chemical methods in the range 95-103%. A corresponding correction factor for each compound of the reference mixture ('recovery factor') was calculated and applied to the composition of lipid extracts from lipoproteins.

(b) Column chromatography and characterization of hydrocarbons. To verify results obtained by thin-layer chromatography and to characterize hydrocarbons in more detail, lipid extracts from lipoproteins were also separated on silicic acid columns. Systems for the separation of neutral lipids on silicic acid columns described by Barron & Hanahan (1958) and phospholipid separations on silicic acid columns described by Hanahan, Dittmer & Warashina (1957) were combined and only one column was used to separate all these lipids, similar to the procedure of Zöllner & Kirsch (1960). Fig. 2 shows a typical separation. Such neutral lipids as cholesteryl esters, cholesterol, triglycerides, diglycerides and free fatty acids were analysed by the same chemical methods as the eluates from thin-layer chromatograms. The total quantities of hydrocarbons were determined by weighing the combined hydrocarbon fractions, and the detailed analysis was performed by gas-liquid chromatography with a 25C Barber-Colman Chromatograph. The separation of these total fractions was performed on a glass-coated column (6 ft. $\times$ 3 mm.) packed with 0.8% silicone oil SF96 on Anakrom (70-80 mesh), ABS type, obtained from Analab

Fig. 2. Separation of lipids extracted from HDL₂ class on a silicic acid column. The column (58 cm. high $\times$ 2.6 cm. internal diam.) contained 180 g. of silicic acid. The amount of lipids applied was 260 mg. Fractions of volume 20 ml. were collected. The composition of the eluting solvent and the number of fractions collected are shown below the lower elution curve. The lower elution curve (b) represents the weight in mg. of eluted lipids. Weight of two combined fractions is plotted for each point on the curve. The total recovery of lipids was 104.0%. The upper elution curve (a) represents the amount of different lipids determined by chemical tests: ----, represents half the total cholesterol in the two combined fractions; —, phospholipid. Each test was performed on two combined fractions. Free fatty acids were present from fraction 160 to fraction 300.

(Hamden, Conn., U.S.A.). Chromatography was temperature-programmed from 165 to 225°; a radium-argon detector was used; the pressure of argon was 28 lb./in.², and the instrument was set for 800 v and sensitivity $\times 10$. Before hydrocarbon analysis, columns were standardized by *n*-octadecane and *n*-tetracosane. Separations of *n*-alkanes from iso- and anteiso-alkanes were performed by molecular sieves (sieve 5A; Linde Air Products Co., New York, N.Y., U.S.A.) according to the method of O'Connor, Burow & Norris (1962). Individual hydrocarbons of only the *n*-alkane series were studied. For quantitative measurements the method of normalization of peaks above the base line was applied.

(c) Phospholipids. Phospholipids were analysed by quantitative thin-layer chromatography (Skipski, Peterson, Sanders & Barclay, 1963; Skipski *et al.* 1964) with the alterations described below. This method separates phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, phosphatidylinositol, phosphatidylethanolamine (together with

phosphatidylglycerol), phosphatidylserine and 'polyglycerophosphatides', which include cardiolipin and phosphatidic acid. Lipid samples were applied quantitatively with Hamilton syringes. After activation in the oven at 110–115° the plates were cooled in a desiccator and samples were applied in the Desaga-Brinkmann Application Box (model DB; Brinkmann Instruments, Westbury, N.Y., U.S.A.). Use of the application box and a dry N₂ atmosphere controlled the water content in the silica-gel layer on the plates and eliminated the necessity of adjusting the amounts of water in the developing solvents according to seasonal fluctuations of humidity in the laboratory. In samples where the amounts of neutral lipids were high and phospholipids low, the chromatograms were prewashed with acetone-light petroleum (1:3, v/v) before the development of the chromatograms for separation of phospholipids. This prewashing solvent moved all neutral lipids to the top of the chromatogram, resulting in a good separation of phospholipids with high *R_F* values when the developing solvent was

applied. Identifications of phospholipids separated on thin-layer chromatograms were performed by simultaneous chromatography of standard phospholipids with the lipoprotein-lipid extracts. Also, specific colour tests were used: ninhydrin spray for the detection of phospholipids containing free amino groups (Skipski *et al.* 1962), and the Dragendorff spray for choline-containing phospholipids (Wagner, Hörhammer & Wolff, 1961). Phosphatidylinositol was identified by microbiological assay for inositol with the yeast *Kloeckera brevis* according to the procedure of Hartree (1957). Infrared spectra of most of the phospholipids eluted from the chromatograms were compared with those of standard phospholipids as well as with those obtained by Schwarz, Dreisbach, Childs & Mastrangelo (1957), Smith & Freeman (1959) and Rouser, Kritchevsky, Heller & Lieber (1963). The phospholipid composition of the lipoprotein classes was confirmed when lipid extracts were separated on silicic acid columns followed by identification and quantitative determination of main phospholipids. Identification of phospholipids obtained from silicic acid columns was performed chromatographically on paper impregnated with silicic acid (Skipski & Rapport, 1957; Rapport, Alonzo, Graf & Skipski, 1958; Marinetti & Stotz, 1956) and by infrared spectrophotometry. Amounts of phosphatidylcholine, lysophosphatidylcholine and sphingomyelin were measured by quantitative infrared spectrophotometry. Peaks at 5.8μ were measured for phosphatidylcholine and lysophosphatidylcholine and peaks at 6.1μ for sphingomyelin. Phosphatidylinositol was measured as inositol after acid hydrolysis of this phospholipid by microbiological assay (Hartree, 1957). Phosphatidylethanolamine was measured as ethanolamine after hydrolysis according to the method of Slotta & Powers (1962).

Since the resolution and reproducibility of the separations on thin-layer chromatograms were always superior to those on silicic acid columns, the latter were used only to confirm results. When necessary to calculate the amount of phospholipid, the phosphorus value was multiplied by 25.

(d) Glycolipids. Glycolipids and 'polyglycerophosphatides' that moved with the solvent front on the above-described thin-layer chromatographic system for phospholipid separation were studied further by specially devised thin-layer chromatographic systems (Skipski *et al.* 1967). Two basically different systems were devised which separate ceramide monohexosides (cerebrosides), ceramide, cardiolipin, phosphatidic acid, phosphatidylglycerol and phosphatidylethanolamine. In both systems a two-step development was used. In system I, the first solvent was acetone-light petroleum (1:3, v/v) and the second was chloroform-methanol-acetic acid-water (80:13:8:0.3, by vol.) In system II, the first solvent was pyridine-light petroleum (3:1, v/v) and the second was chloroform-methanol-pyridine-2N-NH₃ (35:12:65:1, by vol.). Silica-gel thin-layer plates for system II were prepared with 0.1M-Na₂CO₃. Lipid extracts from different classes of lipoproteins were chromatographed with the standard lipids. Ceramide monohexosides and phospholipids were identified on the basis of the positions of spots as compared with the standard lipids in these two different thin-layer chromatographic systems. Also, specific colour tests were used for the identification of ceramide monohexosides: the orcinol test for sugars (Svennerholm, 1956), and the reagent described by Bischel & Austin (1963) for sphingolipids. For quantitative determinations the ceramide monohexosides were

detected on the chromatograms by spraying with 0.01% Oil Red O in ethanol, and eluted by the same procedure used for phospholipids (Skipski *et al.* 1964). The amounts of hexoses were determined by gas-liquid chromatography. For their determination in lipids by this procedure, an adaptation of the Penick & McCluer (1966) modification of the Sweeley & Walker (1964) procedure was employed. Our principal alterations were: the method was adjusted to the analysis of 20–30 μ g. of a single glycolipid species; samples were injected in acetonitrile or hexane; and the purification of samples by Dowex 50 (X4; H⁺ form) columns was omitted. Actual analysis was performed on a 25C Barber-Coleman gas chromatograph with the same conditions as used for hydrocarbons, but at 145°. Determinations of the hexose in known amounts of chromatographed standard ceramide monohexosides were performed concurrently with those of the lipoprotein-lipid samples. This served to check the completeness of elution from the thin-layer chromatograms, the hydrolysis of glycolipids and the methylation of hexoses. Usually 90–110% of the standard glycolipids could be recovered. Among the glycolipids that may be present in lipoproteins, only ceramide monohexosides were analysed.

Proteins. The total protein in each lipoprotein class was determined by the Lang (1958) modification of the micro-Kjeldahl procedure. The value for total nitrogen was multiplied by 6.25 to obtain total protein.

RESULTS

Fig. 1 illustrates the main classes of lipoproteins isolated by preparative ultracentrifugation and their compositional subclasses when studied in the analytical centrifuge. The lipid/protein ratio of the different classes decreased from low-density to high-density lipoproteins including the ultracentrifugal residue (Table 1). The ratio of non-phosphorus-containing lipids to phospholipids in lipids of lipoproteins decreased from the LDL₂ class to the high-density lipoproteins. HDL₂ and HDL₃ had the same ratios. In the ultracentrifugal residue the ratio increased. Thus the percentage of phospholipids in the total lipid extracted increased from the LDL₂ class to the high-density lipoproteins, followed by a notable decrease in the ultracentrifugal residue.

Table 1 also shows the quantitative distribution of serum lipids among different lipoproteins (in mg. of lipid/100ml. of serum). The main carriers of serum lipids were LDL₁ (43.1% of total serum lipids) and HDL₂ (30.5% of total serum lipids).

Fig. 3 illustrates a thin-layer chromatogram of neutral lipids isolated from the different lipoprotein classes and Table 2 gives the composition of the non-phosphorus-containing lipids. About half of the total lipids of LDL₂ were represented by triglycerides, whereas the other lipoprotein classes had relatively small amounts of triglycerides. The relative amounts of triglycerides decreased stepwise from the LDL₂ class to the ultracentrifugal residue. The ultracentrifugal residue as well as the

Table 1. *General characteristics of lipoproteins in normal young women*

Phospholipids were determined by multiplying total P present in the fraction by 25. Non-phosphorus-containing lipids were determined by subtracting phospholipids from total lipids.

	Chylo- microns	LDL ₂	LDL ₁	HDL ₂	HDL ₃	Ultra- centrifugal residue
Density characteristic (g./ml.)		< 1.006	< 1.0635	< 1.125	< 1.210	> 1.210
Lipid		92.0	79.2	47.7	31.8	1.0
Protein		8.0	20.8	52.3	68.2	99.0
Non-P-containing lipids		87.6	72.2	57.6	59.1	73.2
Phospholipids		12.4	27.8	42.4	40.9	26.8
Amount of lipids (mg./100ml. of serum)	7.6	35.1	204.8	143.5	49.5	33.4
Relative distribution of serum lipids among the lipoproteins (%)	1.6	7.4	43.1	30.5	10.4	7.0

Fig. 3. Thin-layer chromatogram of neutral lipids. Total lipid extracts from different lipoprotein classes were applied in equal quantities (approx. 300 μ g.). (1) LDL₂; (2) LDL₁; (3) HDL₂; (4) HDL₃; (5) ultracentrifugal residue; (6) chylomiera; (7) methyl palmitate; (8) mixture of standards: a, tetracosane; b, cholesteryl oleate; c, methyl oleate; d, triolein; e, oleic acid; f, 1,3-diolein; g, 1,2-diolein; h, cholesterol; i, unidentified lipid present only in lipid extracts; j, 1-mono-olein and 2-mono-olein; k, unidentified lipid present only in lipid extracts; l, phospholipids and glycolipids (at origin). The 40% H₂SO₄ spray was used for detection of spots.

LDL₂ (and the chylomicron fraction) contained relatively high amounts of lower glycerides, predominantly 1,3-diglyceride. The fraction of 'monoglycerides' possibly included some unidentified lipids (Table 2).

Of the total lipid in LDL₁, 40% was cholesterol, both free and esterified. This class was the major carrier of cholesterol of the serum lipoproteins. HDL₂ contained 28.2% of free and esterified

cholesterol. All other lipoprotein classes contained relatively less cholesterol in their lipid moieties. The ratio of esterified cholesterol to free cholesterol was lowest in the LDL₂ class; the amount of esterified cholesterol was only slightly higher than free cholesterol. All other lipoprotein classes had ratios of esterified cholesterol to free cholesterol in the range 2.3–4.2. The highest ratio was characteristic for HDL₃. In general, the ratios were higher in high-density lipoproteins than in low-density lipoproteins.

Although some free fatty acids were present in all lipoprotein classes, the greatest amount, per unit of lipid extracted, was in the ultracentrifugal residue. The relative amount decreased from 3.4% in the lipid extract of LDL₂ to 1.1% in that from LDL₁. Starting from HDL₂, it increased in lipoprotein fractions as their densities increased. HDL₃ had 3.2% and the ultracentrifugal residue 29.0% of free fatty acids in their total lipid. Fig. 4 illustrates the average absolute amounts of free fatty acids in different classes of lipoproteins expressed as m-equiv./1000ml. of serum, of which 0.3430m-equiv. was in the ultracentrifugal residue. The remainder of the total free fatty acids, 45%, was distributed among the other lipoprotein classes, and the most important carriers were the HDL₂ and LDL₁ classes.

Hydrocarbons were present in all lipoprotein classes, with the highest relative amount in the ultracentrifugal residue (Table 2). The amount of hydrocarbons in the lipoprotein classes varied greatly from subject to subject. They were present, however, even under circumstances in which all possibility of contamination had been excluded. Their chemical characterization was performed on hydrocarbon samples separated on silicic acid

Table 2. *Composition of non-phosphorus-containing lipids in lipoprotein classes in normal women*

The numbers in this Table are averages of the results obtained on a minimum of six different serum samples except those for unidentified lipids in all lipoprotein classes and hydrocarbons in LDL₁, HDL₂ and HDL₃, which were obtained on three samples. Numbers in parentheses are ranges of values observed in different samples. 'Monoglycerides' apparently contain some other lipids in addition to monoglycerides. For glycolipids (ceramide monohexosides) the numbers represent the average of the results obtained on two serum samples.

	LDL ₂	LDL ₁	HDL ₂	HDL ₃	Ultra-centrifugal residue
Total lipids (mg./100 ml. of serum)	35.1 (20.1-60.5)	204.8 (165.4-234.8)	143.5 (134.4-156.8)	49.5 (43.2-61.8)	33.4 (24.9-42.3)
Non-P-containing lipids (% of total lipid extracted)	87.6 (82.7-90.5)	72.2 (70.3-75.2)	57.6 (49.0-63.0)	59.1 (51.2-63.7)	73.2 (68.2-83.7)
Non-P-containing lipids (mg./100 ml. of serum)	30.7	130.8	82.7	29.2	24.9
Composition (% of total lipid)					
Glycerides, total	49.3	7.3	6.1	6.7	10.2
Triglycerides	45.6 (34.9-55.0)	6.2 (5.3-7.2)	4.6 (3.5-6.9)	4.4 (3.6-5.9)	3.3 (2.8-4.8)
Diglycerides	2.3 (1.8-2.5)	0.4 (0.2-0.7)	0.7 (0.4-0.9)	1.4 (1.1-2.0)	4.0 (2.6-6.4)
'Monoglycerides'	1.4 (0.6-1.9)	0.7 (0.3-1.5)	0.8 (0.6-1.4)	0.9 (0.6-1.1)	2.9 (1.0-4.4)
Cholesterol, total	20.7	39.9	28.2	24.7	13.6
Free	9.0 (7.8-10.8)	12.0 (10.1-14.4)	7.4 (6.0-9.9)	4.6 (3.4-6.6)	3.3 (2.7-4.2)
Esterified	11.7	27.9	20.8	20.1	10.3
Cholesteryl esters (as oleate)	19.8 (15.5-22.0)	47.1 (39.2-58.9)	35.1 (31.0-42.6)	33.8 (28.6-38.2)	17.3 (13.4-21.3)
Free fatty acids	3.4 (2.4-4.6)	1.1 (0.4-1.6)	1.7 (1.2-2.6)	3.2 (2.0-4.7)	29.0 (18.9-42.1)
Hydrocarbons	2.7 (1.8-4.1)	0.7 (0.3-1.4)	0.9 (0.8-1.0)	0.8 (0.6-0.9)	4.4 (1.8-10.2)
Unidentified lipids	2.0 (0.8-4.2)	2.2 (2.0-2.5)	2.2 (1.9-2.4)	2.4 (0.7-3.3)	3.6 (2.0-6.0)
Total neutral lipids recovered (% of total lipid)	86.2	70.4	53.4	51.5	67.8
Glycolipids (ceramide monohexosides) (% of total lipid)	0.3 (0.2-0.3)	0.2 (0.2-0.2)	0.2 (0.2-0.2)	1.1 (1.0-1.2)	1.5 (1.0-2.0)
Total non-P-containing lipids recovered (% of total lipid)	86.5	70.6	53.6	52.6	69.3

columns as shown on Fig. 2. Infrared spectra of isolated hydrocarbons showed the presence of $-\text{CH}_2-$ and $-\text{CH}_3$ groups; no other functional groups were observed (Fig. 5). However, the spectra from different subjects showed different relative magnitude of peaks for $-\text{CH}_2-$, $-\text{CH}_3$ and $>\text{C}(\text{CH}_3)_2$.

The gas-liquid chromatograms of hydrocarbons (Fig. 6A) showed that the *n*-alkanes formed separate well-pronounced peaks above the base line ('hill') formed by the occluded peaks of other hydrocarbons. *n*-Alkanes were distributed between carbon numbers C₁₇ and C₃₅. Hydrogenation of hydrocarbon fractions did not essentially alter the pattern of the gas-liquid chromatograms, which indicated the absence of unsaturated hydrocarbons

or their presence in very small amounts. The separation of *n*-alkanes from the other series of hydrocarbons by 5A Linde molecular sieves demonstrated the same distribution of *n*-alkane series with carbon numbers from C₁₇ to C₃₅. The amounts of *n*-alkanes in hydrocarbon fractions were from 18% to 50%, the largest peaks being in the areas between C₂₃ and C₂₉ (Fig. 6B). Gas-liquid chromatograms of the residual part of hydrocarbon fractions, after the removal of *n*-alkanes, showed that the main peaks were distributed between C₂₆ and C₃₃ (Fig. 6C). They were not characterized further but apparently consisted of different branched alkanes (iso and anteiso). The presence of small amounts of cycloalkanes is not excluded.

Among other non-phosphorus-containing lipids,

ceramide monohexosides (cerebrosides) were studied (Table 2) and were found in all lipoprotein fractions. The relative amounts were small in low-density lipoproteins as well as in HDL₂. Only the ultracentrifugal residue and HDL₃ had significant amounts. Gas-liquid chromatography revealed that they contained only glucose, and that they were ceramide monoglucosides. The nature of the fatty acids in these glycolipids was not studied.

As shown in Table 2, small amounts of unidentified lipids, which chromatographed with the neutral lipids, were present in all lipoprotein classes. The

Fig. 4. Distribution of free fatty acids in different lipoprotein classes. The amounts of free fatty acids are expressed in m-equiv./1000 ml. of serum. The total amount of free fatty acids is 0.6313 m-equiv.

Fig. 6. Gas-liquid chromatograms of hydrocarbons isolated from ultracentrifugal residue. (A) Chromatogram of total hydrocarbon fraction, showing the *n*-alkane peaks projecting above the base line of the ocluded peaks of branched-chain alkanes and possibly cyclic alkanes. About 100 μ g. of hydrocarbons was injected. (B) Chromatogram of *n*-alkanes separated from total hydrocarbon fraction by 5A Linde molecular sieves. About 50 μ g. was injected. (C) Chromatogram of the hydrocarbon residue after the removal of *n*-alkanes by molecular sieves. About 100 μ g. was injected. Peaks on the chromatogram are apparently iso-alkanes and anteiso-alkanes. Arrows *a* are the points of injection of samples. Solvent peaks are omitted. Injections were performed at 165°. Arrows *b* are the points when temperature was programmed manually to 225° at about 7°/min. Numbers on *A* and *B* indicate the carbon numbers of *n*-alkanes. Numbers on *C* estimate the position of peaks between *n*-alkanes of definite chain-length.

Fig. 5. Infrared spectrum of hydrocarbons (in KBr) isolated from ultracentrifugal residue by silicic acid column chromatography. Peaks on the spectrum were identified according to Bellamy (1959) and Colthup, Daly & Wiberley (1964).

highest amount was present in the ultracentrifugal residue.

Fig. 7 illustrates a thin-layer chromatogram of phospholipids from the different lipoprotein classes, the phospholipid compositions of which are in Table 3. The highest relative content of phospholipids was in the HDL₂ and HDL₃ classes. However, in absolute amounts the principal carriers for serum phospholipids were LDL₁ and HDL₂.

The relative amounts of sphingomyelin increased from the LDL₂ to the LDL₁ class and then there was a stepwise decrease through the high-density lipoproteins to the ultracentrifugal residue. The trend was opposite for phosphatidylcholine; the relative amounts increased from the LDL₂ to the HDL₃ class. The ultracentrifugal residue had least of all. Whereas the relative amount of lysophosphatidylcholine phosphorus was in the range 2.0–5.0% of the total lipid phosphorus in all lipoprotein fractions, in the ultracentrifugal residue it comprised 34.5% of the total phospholipid phosphorus.

The total relative amounts of choline-containing phospholipids (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) were practically the same in all lipoprotein fractions (about 90%)

except for LDL₂. Compared with the other lipoprotein classes, LDL₂ (per unit weight of phospholipids) contained approximately twice as much non-choline-containing phospholipids, comprising phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and 'polyglycerophosphatides'. All these non-choline-containing phospholipids were also present in other lipoprotein classes, but in relatively smaller quantities.

Application of thin-layer chromatographic systems especially devised for the separation of ceramide monohexosides and 'polyglycerophosphatides' along with some other acidic phospholipids (Skipski *et al.* 1967) showed that 'polyglycerophosphatides' of serum lipoproteins contain small amounts of lipid that on chromatograms behaves like phosphatidylglycerol. This phospholipid was present in all lipoprotein classes.

In studying the phospholipid composition of lipoproteins, plasmalogens were not investigated. Thus the results presented on the phosphatidyl compound actually included both phosphatidyl and plasmalogen forms of phospholipids.

DISCUSSION

In general, there was a tendency for a stepwise increase (free fatty acids, ceramide monoglucosides, proteins) or, respectively, decrease (free cholesterol, sphingomyelin) in relative amounts of the main constituents of lipoproteins from LDL₁ through HDL₂ to the HDL₃ class. Some of the minor lipid constituents (Tables 2 and 3) did not follow this trend.

The lipid moieties of the two high-density lipoproteins, HDL₂ and HDL₃, differed considerably with respect to several components: the contents of diglycerides, free fatty acids and lysophosphatidylcholine were relatively twice as high, and the content of ceramide monoglucosides was relatively five times as high in HDL₃ as in HDL₂. On the other hand HDL₂ had 1½ times as much sphingomyelin and free cholesterol per unit of lipid extract as had HDL₃. The ratio of lipid to protein was lower in HDL₃ than in HDL₂. These differences were of the same order of magnitude as those in the compositions of HDL₂ and LDL₁. Therefore, if LDL₁ is considered a separate lipoprotein class, one should also consider HDL₂ and HDL₃ as two different lipoprotein classes.

The ultracentrifugal residue and LDL₂ had their own specific lipid compositions, which differed more radically from their respective neighbours HDL₃ and LDL₁. The ultracentrifugal residue was characterized by a very high relative content of free fatty acids, lysophosphatidylcholine and hydrocarbons, whereas LDL₂ was characterized by a high content

Fig. 7. Thin-layer chromatogram of phospholipids. Total lipid extracts from different lipoprotein classes were applied in equal quantities (about 700 μ g.). (1) LDL₂; (2) LDL₁; (3) mixture of standards: phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine (from top to bottom); (4) HDL₂; (5) HDL₃; (6) ultracentrifugal residue. Identification of spots: a, solvent front (all neutral lipids, 'polyglycerophosphatides' and ceramide monohexosides); b, phosphatidylethanolamine; c, phosphatidylserine (not visible in some samples); d, phosphatidylinositol; e, phosphatidylcholine; f, sphingomyelin (with tendency to separate into two spots); g, lysophosphatidylcholine; h, origin of chromatogram. The developing solvent was chloroform-methanol-acetic acid-water (50:28:10:5, by vol.). The 40% H₂SO₄ spray was used for detection of spots.

Table 3. *Composition of phospholipids of serum lipoprotein classes in normal women*

The numbers in the Table are averages of the results obtained on a minimum of six different serum samples. Numbers in parentheses are the ranges of values observed in different samples. The phosphatidylethanolamine fraction includes small amounts of phosphatidylglycerol.

	LDL ₂	LDL ₁	HDL ₂	HDL ₃	Ultra-centrifugal residue
Phospholipids in total lipid extracted (%)	12.4 (9.5-17.3)	27.8 (24.8-29.7)	42.4 (37.0-51.0)	40.9 (36.3-48.8)	26.8 (16.3-31.8)
Phospholipids in fraction (mg./100 ml. serum)	4.4	74.0	60.3	20.3	8.5
Composition of phospholipid (% of total phosphorus)					
Origin of chromatograms	3.2 (2.5-5.1)	1.1 (0.5-1.5)	0.9 (0.3-1.5)	0.8 (0.4-1.0)	2.4 (1.2-3.5)
Sphingomyelin	14.8 (14.0-17.0)	25.9 (21.3-29.1)	14.5 (10.8-16.7)	9.2 (5.2-11.9)	5.7 (3.9-8.9)
Phosphatidylcholine	59.7 (48.7-78.5)	63.7 (54.6-73.7)	73.8 (66.1-82.5)	77.1 (70.9-84.2)	47.4 (37.4-54.1)
Lysophosphatidylcholine	5.0 (2.7-9.0)	2.7 (1.8-3.8)	2.0 (1.0-3.6)	5.4 (3.8-7.6)	34.5 (15.4-44.5)
Phosphatidylinositol	3.6 (2.5-5.4)	1.6 (1.1-2.8)	2.4 (1.5-3.0)	2.4 (1.6-3.1)	1.9 (1.1-3.7)
Phosphatidylserine	1.5 (1.3-1.7)	0.8 (0.2-1.4)	0.9 (0.3-1.2)	0.6 (0.5-0.8)	0.5 (0.3-0.8)
Phosphatidylethanolamine	4.6 (3.0-6.4)	2.2 (1.1-3.9)	3.3 (1.9-4.0)	2.5 (1.8-3.0)	4.2 (2.6-5.5)
Polyglycerophosphatides	7.6 (5.0-9.6)	2.0 (1.2-3.3)	2.2 (1.5-2.9)	2.0 (1.5-3.0)	3.4 (2.4-5.8)
Phosphatidylcholine and lysophosphatidylcholine	64.7	66.4	75.8	82.5	81.9
Choline-containing phospholipids	79.5	92.3	90.3	91.7	87.6
Non-choline-containing phospholipids	17.3	6.6	8.8	7.5	10.0

of triglycerides and of non-choline-containing phospholipids.

According to our results, the relative amounts of free fatty acids in the ultracentrifugal residue were approximately half those reported by Freeman *et al.* (1963), who measured the free fatty acids by quantitative infrared spectrophotometry in ultracentrifugal residues obtained from two patients with xanthoma tendinosum or xanthoma tuberosum. Differences in the type of samples (normal versus pathological) and the methods of measurement employed may explain the variance. The differences may also reflect the natural fluctuation of the free fatty acid content of serum.

We obtained an average total absolute amount of 0.6313 m-equiv. of free fatty acids/1000 ml. of serum. This value is in good agreement with those obtained by others for the free fatty acid content in starved human subjects, expressed in m-equiv./1000 ml. of serum or plasma: Svanborg, Svennerholm, Dorrien & Soomägi (1958), 0.690 in plasma; Hollister & Wright (1965), 0.408 in plasma; Rifkind (1965), 0.550 in plasma; Balasse & Conard (1962),

0.532 in plasma; Munkner (1959), 0.562 in plasma; Nestel (1964), 0.420 in plasma; Carlson & Orö (1963), 0.720 in plasma; Carlsten *et al.* (1962), 0.790 in plasma; Castelli, Nickerson, Newell & Rutstein (1966), 0.697 in serum; Dole (1956), 0.315-1.210 in serum.

Unexpectedly, only 55% of all the free fatty acids present in serum was associated with the ultracentrifugal residue, presumably as a complex of albumin with non-esterified fatty acid. The remaining 45% was distributed among other lipoprotein classes, mainly in HDL₂ and LDL₁. It is not likely that the free fatty acids present in different isolated lipoprotein classes, other than the ultracentrifugal residue, result from a displacement of equilibrium between the complex of albumin with non-esterified fatty acid of the ultracentrifugal residue toward the relatively greater binding by other lipoprotein classes because of the high ionic strength of solutions used for the separation of the lipoproteins (Gofman *et al.* 1954). If this were so, the ratio of fatty acids to proteins should be higher in HDL₃ than in LDL₂ since HDL₃ was isolated in

the presence of a high salt concentration. Actually this ratio was much higher in LDL₂ separated at the density of serum. Therefore it seems improbable that the observed distribution of free fatty acids among different lipoprotein classes is an artifact resulting from the isolation procedure used.

Free fatty acids are considered to be the primary form in which lipids are transported from adipose tissues to most organs for metabolic purposes (Gordon & Cherkas, 1956; Gordon, Cherkas & Gates, 1957; Fredrickson & Gordon, 1958a,b). However, it is not clear whether such a role applies only to the free fatty acids present in the ultracentrifugal residue in the form of the complex of albumin with non-esterified fatty acid or whether it also applies to the free fatty acids present in the other lipoprotein classes.

The presence of hydrocarbons, including the alkane series, in living matter has been known for some time (Deuel, 1951; Nagy, Modzeleski & Murphy, 1965). Only recently was attention drawn to the fact that hydrocarbons are present in the tissues of higher animals and humans. Thus Nicholas & Bombaugh (1965) reported the presence of saturated branched-chain hydrocarbons ranging from C₁₀ to C₃₀ in acetone extracts of ox brain. They also indicated the presence of *n*-alkenes in such extracts. Gazzarrini & Nagy (1966) demonstrated *n*-alkanes in human femoral arterial tissues and plaques. Probably some alkenes were also present. Skipski *et al.* (1965a) observed hydrocarbons on thin-layer chromatograms of lipid extracts from livers of normal rats, and Riley, Hokama & Kratz (1958) reported the presence of hydrocarbons in liver from patients who had died from cancer. Boitnott & Margolis (1966a,b) reported that lesions observed in abdominal lymph nodes, spleen and portal triads of the liver in human biopsy and autopsy material were characterized by large oil droplets, mainly alkanes, in the cells.

It was therefore not surprising to find some hydrocarbons in human serum (Skipski *et al.* 1965b; Rose & Liber, 1966). In the present paper we have partially characterized the chemical nature of these serum hydrocarbons and their content in different lipoproteins. The distribution pattern (high relative amounts in the ultracentrifugal residue and in LDL₂, with low amounts in LDL₁, HDL₂ and HDL₃) indicates that the hydrocarbons may not just be solubilized in lipid moieties of lipoproteins, but may be transported from the intestines to different tissues. [The content of hydrocarbons in the chylomicron fraction was also relatively high (Fig. 3).] Probably, to some degree at least, *n*-alkanes are oxidized by animal tissues and converted into fatty acids because, when McCarthy (1964) administered [¹⁴C]octadecane or [¹⁴C]-hexadecane to goats, rats and chickens, the radio-

activity was recovered in fatty acids of the same chain length as the hydrocarbons and practically no radioactivity was recovered in isolated hydrocarbons. However, the absolute amounts of the *n*-alkanes administered were extremely small, and it is impossible to estimate the capacity of these animals to oxidize *n*-alkanes. Hydrocarbons in large quantity (15–20% of total food intake) are toxic and cause death of weanling rats (Akiya & Shimizu, 1965). At present no information is available on the ability of higher animals to metabolize the branched-chain hydrocarbons. It is difficult to evaluate the significance of the presence of alkanes in blood or tissues from either the physiological or the pathological point of view. Detailed studies of the nature and the amounts of hydrocarbons consumed, as natural ingredients of food, as additives from food processing or as medicines, as well as the amounts and compositions of the hydrocarbons present in tissues, are important in elucidating the possible role of hydrocarbons in the human.

The presence of glycolipids in plasma was suspected early. Kirk (1938) and Erickson, Souders, Shepherd, Teague & Williams (1940) measured the differences in the reducing power of lipid extracts before and after hydrolysis. The differences were attributed to ceramide monohexoside (cerebroside) sugars released by hydrolysis. Svennerholm & Svennerholm (1956, 1958) attempted to isolate cerebroside from plasma and to devise a quantitative method for their determination. However, only when chromatographic techniques were applied by Svennerholm & Svennerholm (1962, 1963a,b) and Polonovski & Petit (1963a,b) were they able to prove that ceramide monohexosides were present in plasma and serum. In addition, the presence of ceramide dihexosides, ceramide trihexosides and aminoglycolipids was demonstrated. Svennerholm & Svennerholm (1963a,b) reported that ceramide monohexosides accounted for 40–50% of the total neutral glycolipid present in serum.

The present study demonstrates that ceramide monohexosides were present in all the lipoprotein classes, with the relatively greatest quantities in HDL₃ and the ultracentrifugal residue. The ceramide monohexosides in the lipoproteins contain only glucose. Thus they are ceramide monoglucosides.

Our values for the percentage of triglycerides in the different lipoprotein fractions were lower than those reported by Freeman *et al.* (1963). Several possibilities account for the differences: our samples were exclusively from starved young women, the method for separating lipoproteins differed, and Freeman *et al.* (1963) did not separate the lower glycerides from triglycerides in the lipid extracts and quite possibly the lower glycerides contributed

to the values they reported for triglycerides. Our results are quite similar to those reported by Polonovski *et al.* (1959), although theirs were obtained on lipoprotein fractions not identical with those we used.

The values for the relative amounts of free and esterified cholesterol in lipoproteins with densities between 1.006g./ml. (LDL₂) and 1.210g./ml. (HDL₃) agree with those of Freeman *et al.* (1963). The relative amounts of total cholesterol are the same in the ultracentrifugal residues. However, the distribution differed. According to Freeman *et al.* (1963) the ultracentrifugal residue contains several times as much free cholesterol as esterified cholesterol. We found, as did Polonovski (1961), that the ultracentrifugal residue contained more esterified than free cholesterol. This difference might be because Freeman *et al.* (1963) studied abnormal subjects.

Our high values for lysophosphatidylcholine in the ultracentrifugal residue agree with those of Phillips (1959) and Polonovski (1960). Switzer & Eder (1965) also reported high values for this phospholipid in plasma from man and the rat. The values for lysophosphatidylcholine content in the ultracentrifugal residue vary somewhat (ours are the lowest reported), but all the reports indicate unusually high amounts of this phospholipid in the ultracentrifugal residue.

Our findings, which confirm reported data on the stepwise decrease of the relative amounts of sphingomyelin from the LDL₁ class to the high-density lipoproteins (Lindgren & Nichols, 1960; Freeman *et al.* 1963; Smith, 1960) are in greater detail since we studied HDL₂ and HDL₃ separately and included the ultracentrifugal residue.

The present study is the first to characterize in detail the lipid moieties of the two major high-density lipoproteins, HDL₂ and HDL₃, and shows that they differ considerably. The information on the distribution of many lipids such as lower glycerides, ceramide monohexosides, hydrocarbons and several phospholipids present in serum in relatively small amounts, has not been reported previously.

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