

The isolation of lipoproteins from human plasma by ultracentrifugation in zonal rotors

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ABSTRACT The major classes of lipoproteins were isolated from human plasma by ultracentrifugation in continuous density gradients using the Ti-14 and Ti-15 zonal rotors. Chylomicrons + VLDL, LDL, and HDL were separated from each other and from the more dense residual proteins (albumin fraction) of plasma by rate-zonal flotation in NaBr gradients in the density range 1.0–1.4. The chylomicron-VLDL fraction was subfractionated into constituent chylomicrons and VLDL by zonal ultracentrifugation in NaBr gradients in the density range 1.0–1.1. Plasma lipoproteins were analyzed for composition of lipids and content of protein, for electrophoretic mobility on paper, and for antigenic determinants by immunoelectrophoresis and immunodiffusion. Flotation constants (S_f) of the LDL and HDL were calculated from measurements made in the analytical ultracentrifuge. Lipoproteins isolated from plasma by zonal ultracentrifugation were identical by these criteria to lipoproteins isolated by the usual procedure of sequential ultracentrifugation in solvents of increasing density. The procedure of zonal ultracentrifugation is rapid, quantitative, and less laborious than sequential techniques. Lipoproteins isolated by zonal ultracentrifugation are relatively uncontaminated by other proteins and extensive washing is therefore unnecessary. Zonal ultracentrifugation is more than a preparative method for the plasma lipoproteins; it is also an analytical procedure in that a record is obtained of the distribution and quantity of the lipoprotein within the continuous density gradient.

SUPPLEMENTARY KEY WORDS continuous density gradient · lipoprotein protein · lipoprotein lipids · chylomicrons

THE APPLICATION of rate-zonal flotation in the ultracentrifuge to the isolation of lipoproteins from the plasma of man and animals was reported recently (1). The rotors which had been used in those separations were the B14 and B15 titanium zonal rotors obtained

from the Oak Ridge National Laboratories, Oak Ridge, Tenn. Rapid and quantitative isolation from plasma of the VLDL-chylomicron fraction and of the LDL was attained with either the B14 or B15 rotors. The maximal force generated by either of these rotors (2), however, was not sufficient under our experimental conditions to resolve the HDL adequately from the more abundant residual proteins of human plasma. Zonal rotors capable of generating greater centrifugal forces have since become available. The Ti-14 and Ti-15¹ rotors are titanium zonal rotors whose design has been modified from the prototype B14 and B15 titanium zonal rotors and which can produce greater maximal centrifugal forces than can the B-series rotors. At its rated speed of 35,000 rpm, 121,000 *g* will be generated by the Ti-15 rotor at its maximal radius (radius = 8.89 cm; path length = 7.50 cm). At its rated speed of 48,000 rpm, 172,000 *g* will be generated by the Ti-14 rotor at its maximal radius (radius = 6.67 cm; path length = 5.30 cm). A procedure has now been developed for use with the Ti-14 and Ti-15 rotors which permits the isolation of the HDL as well as the less dense classes of lipoproteins. As reported in earlier communications from this laboratory (1), the least dense fraction isolated by zonal ultracentrifugation from plasma contained both chylomicrons and VLDL. This fraction, from normal human fasting serum, has been separated into three components, defined opera-

Abbreviations: VLDL, very low density lipoprotein, *d* < 1.006; LDL, low density lipoprotein, *d* 1.006–1.063; HDL, high density lipoprotein, *d* 1.063–1.210; *d*, density; TG, triglyceride; C, free cholesterol; CE, cholesteryl esters; C_T, total cholesterol; PL, phospholipid; EDTA, ethylenediaminetetraacetic acid.

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¹ The Ti-14 and Ti-15 rotors were obtained from the Spinco Division of Beckman Instruments, Palo Alto, Calif.

tionally as chylomicrons, VLDL, and an albumin-containing contaminant. It is, therefore, now possible to isolate all of the major classes of lipoproteins from human plasma by the procedures of zonal ultracentrifugation described in this manuscript. Preliminary reports of this work have been published (3, 4).

METHODS

Centrifugations with the Ti-14 and Ti-15 zonal rotors were carried out in a Spinco Model L2-65B ultracentrifuge. The B14 titanium rotor was used with a modified Spinco Model L ultracentrifuge (2). Centrifugations with the Ti-15 rotor were carried out routinely at 35,000 rpm. Centrifugations with the Ti-14 rotor were carried out at either 48,000 rpm (172,000 *g*) or 44,500 rpm (147,000 *g*). The lower velocity is recommended when the density of the gradient at the periphery of the rotor is 1.40. The procedures for zonal ultracentrifugation were similar generally to those described previously (1). Continuous linear density gradients (NaBr, *d* 1.00–1.40) were pumped directly into rotors, without an overlay of water, at a rate of 35–45 ml/min using a Beckman gradient pump (Model 141). The rotors were filled with either 600 ml (Ti-14) or 1600 ml (Ti-15) of gradient volume. For the separation of the VLDL from the chylomicrons by rate-zonal flotation in the B14 or Ti-14 rotor, continuous linear density gradients in the range *d* 1.00–1.10 (NaBr) were utilized. All solutions contained 350 μ M EDTA. A sample of plasma whose density had been adjusted to 1.40 with solid NaBr was injected into the rotor at its periphery after the gradient had been pumped into the rotor. Blood, obtained from apparently healthy human adult male volunteers after an overnight fast or a light breakfast, was treated with 7 USP units of heparin/ml, or in certain cases, with EDTA (1 mg/ml of blood). Plasma separated from the blood was centrifuged immediately or stored overnight in the refrigerator at 4–5°C. CaCl₂ (final concentration, 0.005 M) was added to the EDTA-plasma and the fibrin clot was removed after incubation at room temperature for 30 min. The volume of plasma injected into the rotor was followed by a sufficient volume of high density solution to fill the rotor completely. The gradient material and sample were introduced into the rotor at room temperature (23°C). Since large volumes of sample (up to 130 ml in this report) were centrifuged in the Ti-15 rotor, as much as 60 ml of the light end of the gradient was displaced. Centrifugation was carried out at 13°C for various times and velocities; the specific details are described in the legends for the figures and tables. The rotor contents were collected and monitored as reported previously (1); 25-ml fractions were collected, except in

certain experiments with the Ti-14 rotor, when 10-ml fractions were collected.

Lipids in the lipoproteins were analyzed in one of two ways. Aliquots of the lipoprotein fractions were precipitated with Ba(OH)₂–ZnSO₄ and were extracted with CHCl₃–CH₃OH 2:1 (v/v) (1). Separate aliquots were analyzed for protein (5). Alternatively, the lipoprotein fractions were dialyzed and concentrated at 13°C through membrane filters (Diaflo Ultrafiltration Cell, Model 400, with an XM-50 membrane; Amicon Corp., Lexington, Mass.) to a volume small enough to be extracted conveniently with 20 volumes (25–50 ml) of CHCl₃–CH₃OH 2:1 (v/v). Additional dialysis was accomplished by diluting the concentrated lipoprotein in 0.9% NaCl–350 μ M EDTA and passing the diluted fraction through membrane filters until the desired salt concentration (*d* 1.01) was attained. The advantages and disadvantages of the methods of lipid extraction were discussed previously (1).

Cholesterol and phospholipid in the washed CHCl₃–CH₃OH extracts of the Ba(OH)₂–ZnSO₄ precipitates were estimated by the methods of Sperry and Webb (6) and King (7), respectively. The CHCl₃–CH₃OH extracts of the lipoproteins which had been concentrated by membrane filtration were washed with 0.02% MgCl₂ (8) and placed on columns which contained 5.0 g of silicic acid in a minimal volume of CHCl₃. Total cholesterol was measured in the CHCl₃-eluates from the silicic acid columns (9); individual lipid classes present in the CHCl₃-eluates were then separated by thin-layer chromatography on silica gel (9). The bands of lipids were eluted from the plates with chloroform and analyzed for triglyceride (10), cholesterol (6), or cholesteryl esters. Lipid-soluble phosphorus was estimated in the CH₃OH-eluates from the silicic acid columns (7). Protein in the fractions concentrated by membrane filtration was measured by the procedure of Lowry, Rosebrough, Farr, and Randall (5). Samples which contained an appreciable amount of lipid produced slightly turbid solutions. The turbidity was removed easily by extraction of the solution with 4 ml of diethyl ether after the color had been developed with the phenol reagent. When the contents of the standard bovine serum albumin tube were treated similarly with ether, color development was not affected. There was no loss of lipoprotein protein during membrane filtration and dialysis as judged by this assay.

After dialysis and concentration by membrane filtration, the lipoproteins were characterized further by their electrophoretic mobilities and immunochemical properties, and by measurement of flotation constants in the analytical ultracentrifuge. Electrophoresis of the lipoproteins was accomplished in Veronal-albumin buffer using Whatman No. 1 paper strips (11). The strips were stained for lipid with Oil Red O after electrophoresis

(12). The immunochemical purity of the lipoproteins was evaluated by the methods of double immunodiffusion and immunoelectrophoresis described by Ouchterlony (13). Immunodiffusion and immunoelectrophoresis were carried out on microscope slides using 1% agarose in 0.05 M Veronal buffer, pH 8.2; 5 μ l of antigen and 25 μ l of antiserum (50 μ l for immunoelectrophoresis) were placed in the appropriate wells. The rabbit antisera to various human plasma proteins were obtained from Behring Diagnostics, Inc., Woodbury, N.Y. It was possible to detect as little as 0.05 mg/ml of VLDL or LDL protein with antiserum to β -lipoprotein, and 0.07 mg/ml of HDL protein with antiserum to α -lipoprotein; 0.07 mg/ml of total serum proteins was barely detectable when reacted with antiserum to albumin. The flotation constants at 23.5°C of the LDL ($S_{f, 1.063}$) and the HDL ($F_{1.210}$) were calculated from moving boundary measurements obtained with the Spinco Model E analytical ultracentrifuge (14, 15).

RESULTS

A series of experiments was carried out with the Ti-14 zonal rotor to determine the effect of duration of centrifugation on flotation of the plasma lipoproteins in the d 1.00–1.40 NaBr gradient. The chylomicron-VLDL and LDL fractions were resolved quite rapidly and, as seen in Fig. 1, were separated from each other and from the remaining plasma proteins in only 45 min. The lowest density fraction can be assumed to be primarily

VLDL, since the plasma was collected after an overnight fast or a light breakfast. The HDL started to appear as a distinct peak after 12 hr of centrifugation, but at this time the chylomicron-VLDL and LDL fractions began to merge, and satisfactory isolation of any particular class of lipoprotein was not possible. The HDL was separated completely from the residual plasma proteins after 24 hr or more of ultracentrifugation, but the chylomicron-VLDL and LDL were merged into a single peak. It is evident from these observations that one centrifugation in this gradient will not be sufficient to effect simultaneous isolation of all classes of lipoproteins from serum. Clearly, however, a single centrifugation is most useful for special requirements. Thus, the chylomicron-VLDL and LDL can be separated from the remainder of serum constituents in a short time; alternatively, the HDL can be obtained without the necessity of isolating all other components.

If the distribution of the lipoproteins in the gradient as estimated by absorption at 280 nm is compared with the distribution of protein measured chemically, certain discrepancies are seen. The "absorption" of the chylomicron-VLDL peak to a large extent results from light scattering by the turbid solution. The chemical measurement of protein content represents more adequately the degree of separation of the HDL from the residue proteins; the absorption at 280 nm was increased by the specific batch of reagent grade NaBr used for the gradient in proportion to the concentration of salt in solution; this increase varied from batch to batch. The optical

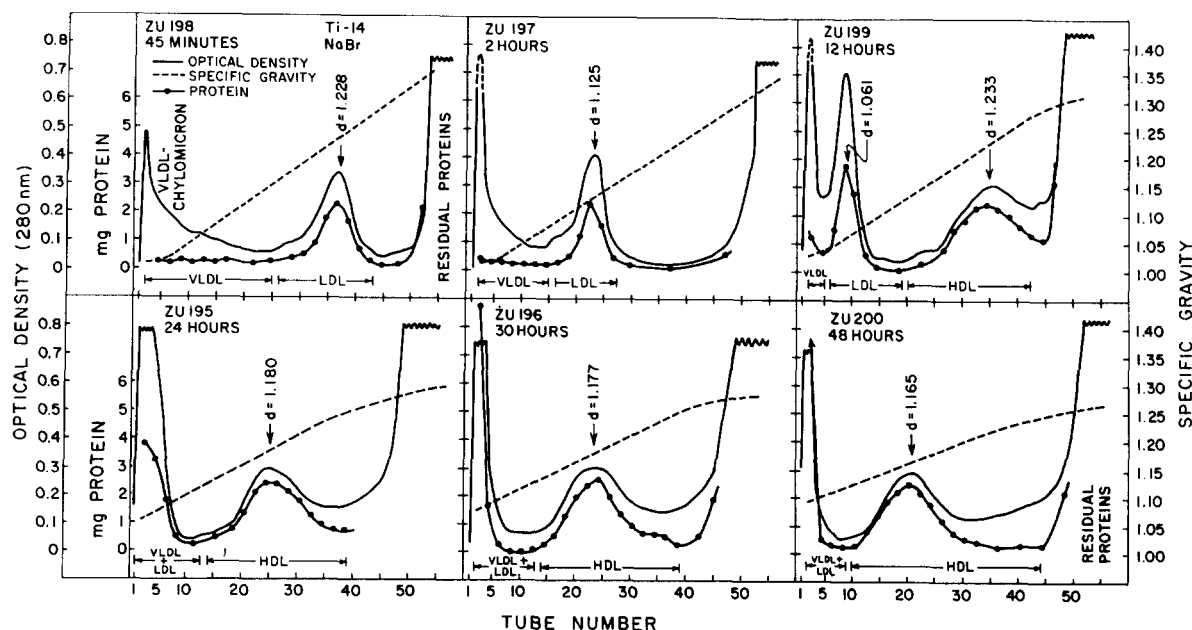


FIG. 1. Effect of time on the rate-zonal flotation of plasma lipoproteins in the Ti-14 zonal rotor. The gradient material was NaBr in the density range 1.00–1.40. The concentrated salt solution contained 557 g/l; 20 ml of plasma from the same pooled sample was centrifuged in each experiment. The centrifugations were carried out for various periods of time at 48,000 rpm in the Ti-14 rotor.

density at 280 nm of two solutions (d 1.4) made from two separate batches of NaBr (Mallinckrodt, AR) was 0.12 and 0.24, respectively. In a few preliminary experiments, the absorption was reduced further if the sample of plasma was dialyzed overnight against a solution of NaBr (d 1.4) containing 0.001 M EDTA before zonal ultracentrifugation. The amount of absorption at 280 nm in the area between the HDL and residual proteins is therefore misleading as a criterion for the completeness of separation, but does serve as a semiquantitative estimate.

Table 1 shows the chemical analyses of the isolated lipoproteins (Fig. 1). The data show clearly that the content of protein, total cholesterol, and phospholipid of the lipoproteins remained relatively constant during centrifugations lasting up to 48 hr. The ratios of mg C_T/mg PL for the LDL and HDL (1.51 and 0.67, respectively) agree with those reported by Havel, Eder, and Bragdon (16) for lipoproteins isolated by the usual sequential procedures and with those reported earlier by us for lipoproteins isolated by zonal ultracentrifugation (B15, sucrose-KBr, d 1.00–1.28) (1).

A series of experiments similar to those just described for the Ti-14 rotor were carried out with the Ti-15 rotor. The results of these experiments are shown in Fig. 2. The chylomicron-VLDL and LDL were well separated from each other and from the remaining plasma proteins after 4 hr of centrifugation; shorter periods of centrifugation were not examined because these separations were achieved already in 2 hr using the slower B15 titanium zonal rotor and the more viscous sucrose-KBr gradient.

All major classes of lipoproteins were identified after 18 hr of centrifugation in the d 1.00–1.40 NaBr gradient; the resolution was, however, not sufficient for preparative work because merger of the chylomicron-VLDL and LDL had started and the HDL was not completely separated from the residual proteins. The "lipoprotein profile" obtained at 18 hr may be of value for the initial comparison of plasma lipoproteins of man and other species and for diagnostic applications in the clinic. After 24 hr of centrifugation, the merger of the chylomicron-VLDL and LDL had progressed further, and the HDL was now separated more clearly; after 48 hr of centrifugation, the chylomicron-VLDL and LDL were combined into a single peak and the HDL was separated from the residual proteins. The resolution of the lipoprotein classes attained with the Ti-15 is similar to that described for the Ti-14, except that larger volumes of plasma may be centrifuged in the Ti-15 rotor. The chemical analyses of the lipoprotein fractions for cholesterol, phospholipid, and protein were in agreement with those described for lipoproteins isolated with the Ti-14 rotor.

Since the isolation from plasma of the major classes of lipoproteins could not be achieved simultaneously with a single centrifugation in either the Ti-14 or Ti-15 rotor in this gradient, a procedure requiring two sequential steps was devised. In this procedure, the chylomicron-VLDL and LDL were separated and removed from the rotor prior to continued centrifugation and isolation of the HDL from the residual proteins. It is necessary in the first step that the chylomicron-VLDL and LDL be

TABLE 1 COMPOSITION OF PLASMA LIPOPROTEINS AFTER ZONAL ULTRACENTRIFUGATION*

Fraction†	Hours of Centrifugation						Mean
	3/4	2	12	24	30	48	
	mg/fraction‡						
I. Cholesterol (Total)							
1. VLDL-chylomicron	6.00	6.86	8.39	—	—	—	7.08
2. LDL	24.88	27.27	24.51	—	—	—	25.55
3. (1 + 2)	(30.88)§	(34.13)	(32.90)	30.37	34.08	31.61	32.33
4. HDL	—	—	6.36	7.06	6.78	7.06	6.81
II. Phospholipid							
1. VLDL-chylomicron	7.55	8.24	8.02	—	—	—	7.94
2. LDL	17.64	15.33	17.83	—	—	—	16.93
3. (1 + 2)	(25.19)	(23.57)	(25.85)	—	23.85	21.67	24.03
4. HDL	—	—	9.65	10.46	10.71	9.98	10.20
III. Protein							
1. VLDL-chylomicron	6.12	3.53	3.85	—	—	—	4.50
2. LDL	18.66	13.05	18.56	—	—	—	16.75
3. (1 + 2)	(24.78)	(16.58)	(22.41)	20.09	22.09	22.13	21.34
4. HDL	—	—	33.45	36.06	32.27	36.65	34.60

* Lipids were isolated from lipoproteins precipitated with Ba(OH)₂-ZnSO₄. See text for details. The total recovery of phospholipid in the lipoproteins was 70.6 ± 2.6% of that present in the original plasma when the plasma was extracted with CHCl₃-CH₃OH 2:1 (v/v) (8); the comparable recovery of cholesterol was 74.4 ± 1.8%.

† The lipoprotein fractions are those defined in Fig. 1.

‡ Same as mg per original 20-ml sample of plasma. Molecular weights used in calculation were cholesterol, 387; phospholipid, 750; and triglyceride, 850.

§ The parentheses indicate that this value is the arithmetic sum of VLDL + LDL, rather than an independent chemical measurement.

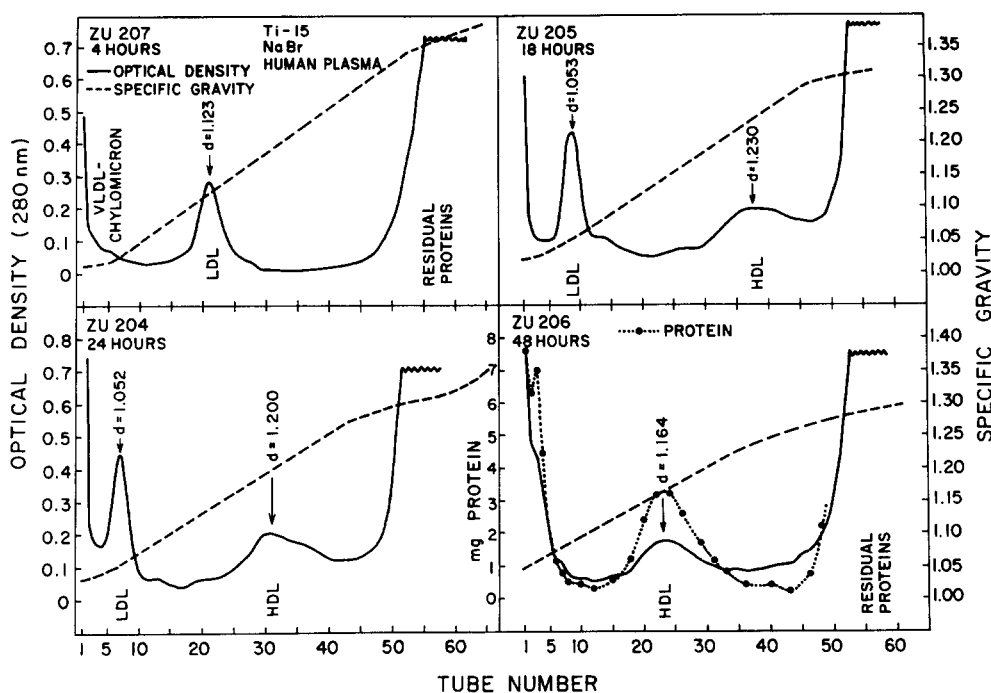


FIG. 2. Effect of time on the rate-zonal flotation of plasma lipoproteins in the Ti-15 zonal rotor. The gradient material was NaBr in the density range 1.00–1.40; 30 ml of plasma from the same pooled sample was centrifuged in each experiment. The centrifugations were carried out for various periods of time at 35,000 rpm in the Ti-15 rotor.

separated from each other and from the remaining plasma proteins and that the lipoproteins be contained in the smallest possible volume of gradient. The small volume of gradient is convenient since it is necessary to remove chylomicron-VLDL and LDL and then reintroduce the gradient into the rotor before proceeding to the second step. The only requirement for the second step is that the duration of centrifugation be sufficient to obtain satisfactory separation of the HDL from the residual proteins. The length of time selected for step I was 2 hr for the Ti-14 rotor and 5 hr for the Ti-15 rotor. Although periods longer than 5 hr for step I (Ti-15) would have resulted in smaller volumes of gradient, longer periods would have been inconvenient to the investigator when one considers that 2.0–2.5 hr are required to set up steps I and II independent of duration of centrifugation. Centrifugation for 24 hr (Step II) with either rotor resulted in satisfactory separation of the HDL from the residual plasma proteins. In practice, the “two-step” procedure was carried out as follows: The first step was set up, as described in the methods section, using the d 1.00–1.40 NaBr gradient. After 2 (Ti-14) or 5 (Ti-15) hr of centrifugation, the rotors were decelerated to 3000 rpm and 300 or 625 ml of gradient, respectively, containing the chylomicron-VLDL and LDL were displaced from the rotor and collected in 25-ml fractions. A volume of gradient equal to that displaced (d 1.00–1.18 or d 1.00–1.15 for the Ti-14 and Ti-15 rotors, respectively) was then pumped back into the rotor, heavy

end first, through the core. Centrifugation was reinstated for an additional 24 hr and the isolated HDL and residual plasma proteins were collected.² The results of such centrifugations are shown in Fig. 3 (Ti-14) and Fig. 4 (Ti-15). The preparative isolation of the plasma lipoproteins appears to be quite satisfactory by these procedures. The lipoprotein fractions were analyzed for content of lipid and protein and the data obtained from one of these centrifugations are presented in Table 2. Similar results were obtained in all four centrifugations. The distribution of the plasma lipids and proteins among the various classes of lipoproteins is presented in Table 3; the distribution of these lipids and protein within a particular class of lipoprotein is presented in Table 4. These data are in agreement with published values for plasma lipoproteins isolated by sequential procedures.

The flotation constants for the LDL (fraction B, ZU 253, Fig. 3) and the HDL (fraction D, ZU 254, Fig. 3) were calculated from moving boundary measurements made in the analytical ultracentrifuge (Fig. 5). The mean $S_{f(1.063)}$ of the LDL isolated by zonal ultracentrifugation was 6.5; simultaneously, the mean S_f of the d 1.006–1.063 fraction isolated by sequential procedures (14) was 6.2. The mean $F_{(NaBr, 1.21)}$ of the HDL isolated by zonal ultracentrifugation was calculated to be

² It was observed recently that when distilled water containing EDTA was substituted for the volume of gradient returned to the rotor, resolution of the HDL was achieved similar to that depicted in Figs. 3 and 4.

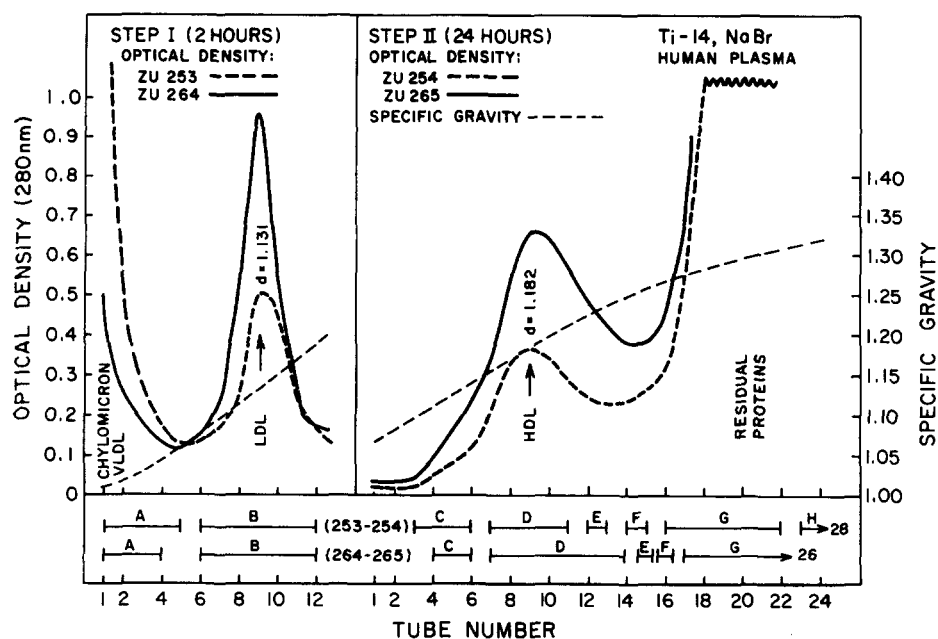


FIG. 3. "Two-step" isolation of lipoproteins from plasma in the Ti-14 zonal rotor. Step I: The procedure was identical to that described in the legend for Fig. 1; 20 ml of plasma was centrifuged in ZU 253 and 50 ml in ZU 264. Centrifugation was carried out for 2 hr at 44,500 rpm in the Ti-14 rotor, after which time the chylomicron-VLDL and LDL were collected as described in the text by displacement with d 1.40 solution. Gradient (~ 300 ml) in the density range 1.00–1.18 was then pumped into the rotor through the core, heavy end of the gradient first, to replace the volume which had been lost and to remove from the rotor the excess d 1.40 solution which had been used originally to displace the VLDL and LDL. The d 1.18 solution was prepared by diluting the stock d 1.40 solution. Step II: ZU 254 is the continuation of ZU 253, and ZU 265 is the continuation of ZU 264. Centrifugation was carried out for 24 hr at 44,500 rpm, after which time the fractions were collected as described in the text.

3.4; the mean F under identical conditions of the d 1.125–1.210 lipoprotein isolated by sequential procedures (15) was 3.6.

In all centrifugations in zonal rotors, a small shoulder was observed at the lighter end of the density gradient

on the large peak of HDL. This small shoulder, which may represent the HDL₂, was analyzed independently of the main HDL peak (Figs. 3 and 4). The lipid composition of the "HDL₂" and "HDL₃" from one experiment (Fig. 4, ZU 259, fractions D and E, respectively) are

TABLE 2 COMPOSITION OF PLASMA LIPOPROTEINS ISOLATED BY ZONAL ULTRACENTRIFUGATION

Fraction*	Protein	Cholesterol†			PL	TG	C _T /PL	CE/C
		C _T	C	CE				
		mg‡						
A	19.74	15.29	5.68	7.62	17.94	32.29	0.85	1.34
B	9.13	14.58	4.04	9.47	10.84	6.34	1.34	2.34
C	53.56	81.08	16.09	49.81	52.26	9.03	1.55	3.10
D	6.79	2.95	0.69	2.20	5.37	0.48	0.55	3.19
E	71.68	14.69	2.11	11.22	28.66	1.71	0.51	5.32
F	3.40	0.23	0.07	0.12	0.86	0.07	0.27	1.71
G	11.12	0.16	0.08	0.07	0.51	0.09	0.31	0.88
H	4307.00	0.57	0.35	0.15	12.86	0.80	0.04	0.43
Total	4482.42	129.55	29.11	80.67	129.30	50.81	1.00	2.77
Plasma‡	—	138.66	31.19	78.91	130.31	48.45	1.06	2.53
Recovery§ %	—	93.4	93.3	102.2	99.2	104.9	—	—

* The lipoprotein fractions are those defined in Fig. 4, ZU 258–259. Lipoproteins were extracted with CHCl₃–CH₃OH 2:1 after concentration through membrane filters. See text for details.

† Mg of cholesterol in free or esterified form. Total cholesterol is the result of an independent analysis, not the sum of free + esterified cholesterol.

‡ Total lipid or protein/50 ml of plasma, which was the volume of sample injected into the zonal rotor (i.e., same as total/fraction).

§ (Mg lipid recovered in zonal rotor)/(mg lipid/50 ml plasma) × 100.

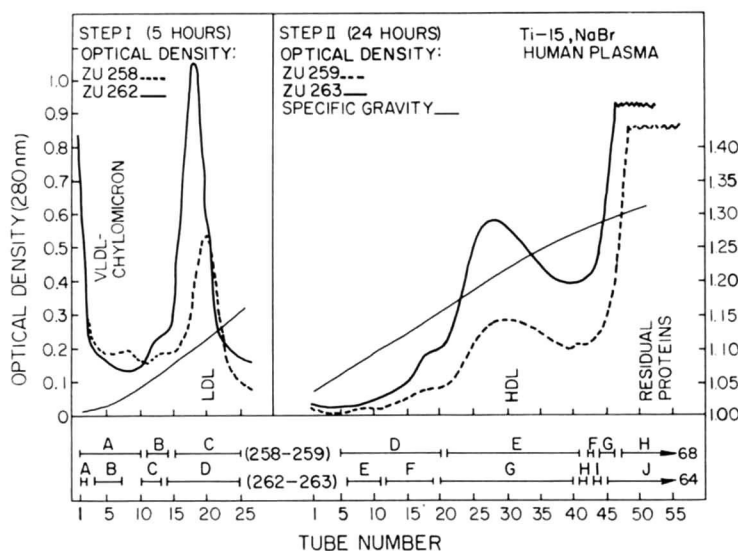


FIG. 4. "Two-step" isolation of lipoproteins from plasma in the Ti-15 zonal rotor. The procedures for isolation of the lipoproteins were similar to those described in the legend for Fig. 3. Step I: 30 ml plasma was centrifuged in ZU 258 and 100 ml in ZU 262. Centrifugation was carried out for 5 hr at 35,000 rpm in the Ti-15 rotor. After displacement of the chylomicron-VLDL and LDL, gradient (625 ml) in the density range 1.00-1.15 was pumped into the rotor. Step II: ZU 259 is the continuation of ZU 258, and ZU 263 is the continuation of ZU 262. Centrifugation was carried out for 24 hr at 35,000 rpm, after which time the fractions were collected as described in the text.

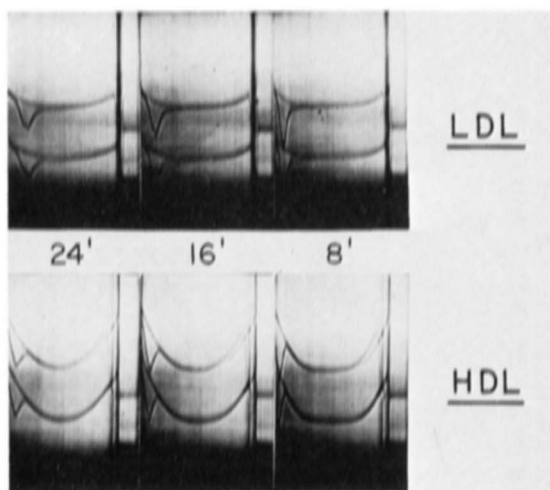


FIG. 5. Analytical ultracentrifugation of LDL and HDL. Centrifugation was carried out in the AN-D rotor at 52,640 rpm in the Spinco Model E ultracentrifuge at 23.5°C. **LDL**: The LDL was contained in a solution of NaCl (d 1.063) which was 350 μ M with respect to EDTA. 1.3 mg of protein/ml (d 1.006-1.063) isolated with the Spinco No. 40 angle-head rotor by sequential techniques (14) was added to the wedge-window cell (upper pattern in each photograph). The regular double sector cell contained 1.3 mg/ml of the LDL protein isolated by zonal ultracentrifugation (fraction B, ZU 253, Fig. 3). **HDL**: The HDL was contained in a solution of NaBr (d 1.210) which was 350 μ M with respect to EDTA. 2.5 mg of protein/ml (d 1.125-1.210) isolated by sequential procedures (15) was added to the wedge-window cell. The regular double sector cell contained 2.5 mg/ml of the HDL protein isolated by zonal ultracentrifugation (fraction D, ZU 254, Fig. 3). Estimates of the flotation constants ($S_{f,1.063}$ and $F_{1.21}$) were calculated from measurements of the distance of migration of peaks during the period 8-32 min.

compared in Table 2. The composition of these sub-fractions differed in that the HDL₃ had a higher ratio of CE/C and protein/PL than did the HDL₂, although the ratio C_T/PL was similar in both groups (Table 5). The $F_{(1.210)}$ at 23.5°C of fraction D (ZU 259) calculated from a single measurement made in the analytical ultracentrifuge was 7.6. It is possible, therefore, that the HDL₂ and HDL₃ are separable from one another by zonal ultracentrifugation.

Lipoproteins isolated by the two-step centrifugation (Fig. 6) were examined by electrophoresis on paper (Fig. 7A). The chylomicron-VLDL fraction appeared to contain two components, one which remained at the origin and one which moved to the "pre- β " position; the LDL migrated as a β -globulin and the HDL as an α -globulin. Each fraction produced a precipitin reaction when examined by immunodiffusion against specific antisera (Fig. 8). The HDL₂ (fraction C) and the HDL₃ (fraction D) reacted strongly with anti- α -lipoprotein; the precipitin band seemed to be identical to that formed with whole plasma. The HDL₃ also reacted slightly with antiserum to human albumin. A faint reaction between the HDL₂ (fraction C) and antiserum to β -lipoprotein was observed in this case and has been observed in three other of seven additional HDL₂ fractions which were examined by immunodiffusion. The LDL reacted only with the antiserum to human β -lipoprotein and formed a band of identity with whole plasma (1), although such is not clear from Fig. 8. The chylomicron-VLDL (frac-

TABLE 3 DISTRIBUTION OF LIPIDS AND PROTEIN AMONG CLASSES OF PLASMA LIPOPROTEINS*

Experiment	Fraction	C _T	PL	TG†	Protein‡
% (w/w)§					
I. VLDL-chylomicron					
262	A, B (Fig. 4)	4.2	7.5	54.9	3.3
264	A (Fig. 3)	4.1	6.0	53.1	4.8
258	A (Fig. 4)	11.8	13.9	63.7	12.0
253	A (Fig. 3)	14.5	19.7	77.0	9.5
	Mean	8.7	11.8	62.2	7.4
	d < 1.006	7.6	13.1	68.5	7.8
II. LDL					
262	C, D	77.3	52.6	35.5	42.9
264	B	74.8	50.3	37.2	42.4
258	B, C	73.8	48.8	30.3	38.2
253	B	68.7	42.8	15.6	37.0
	Mean	73.6	48.6	29.7	40.1
	d 1.006–1.063	72.8	50.9	26.0	42.3
III. HDL					
263	E–H (Fig. 4)	17.8	30.6	9.6	53.8
265	C–E (Fig. 3)	20.3	34.1	9.7	52.8
259	D–F (Fig. 4)	13.8	27.0	4.5	49.8
254	C–F (Fig. 3)	16.4	33.7	5.5	53.4
	Mean	17.1	31.3	7.3	52.5
	d 1.063–1.210	19.6	36.0	5.5	49.8
IV. Residual proteins					
263	I, J	0.7	9.4	—	—
265	F, G	0.8	9.6	—	—
259	G, H	0.6	10.3	1.5	—
254	G, H	0.4	3.8	1.8	—
	Mean	0.6	8.3	1.6	—

* Lipoproteins were extracted with CHCl₃–CH₃OH 2:1 after concentration through membrane filters. Refer to text for details. ZU 262–265 contained aliquots of the same sample of pooled plasma.

† TG was not measured in the residual proteins in ZU 263 and 265; the distribution of TG in these experiments represents that in the lipoproteins only. The quantity of TG in the residual proteins is small and does not alter appreciably the percent distribution.

‡ The percentage of protein is calculated for the lipoproteins only. The protein present in the lipoprotein is about 4–5% of the total protein of plasma (see Table 2).

§ Each value represents that percentage of the total plasma component, e.g., phospholipid, found in a particular fraction.

|| These lipoproteins were isolated by the usual sequential techniques in angle-head rotors (16).

tion A) reacted with antisera to both β -lipoprotein and albumin. When analyzed by immunoelectrophoresis, the chylomicron-VLDL fraction formed a precipitin arc in the presence of antisera to albumin and β -lipoproteins (Fig. 9); an additional precipitin arc, which may correspond to γ -globulin, was formed in the presence of antiserum to human plasma proteins.

The chylomicron-VLDL fraction had been treated as a single component in earlier publications from this laboratory (1, 4). It appears possible, however, to subfractionate this component by zonal ultracentrifugation. The chylomicron-VLDL fraction, after isolation in zonal rotors, was reduced in volume to about 30 ml by ultrafiltration and was recentrifuged in a NaBr gradient, d 1.00–1.10, for 30 min at 30,000 rpm in either the B14

TABLE 4 COMPOSITION OF LIPOPROTEIN CLASSES

Experiment	TG	C	CE	PL	Protein
% (w/w)*					
I. VLDL-chylomicron					
ZU 253	57	4	11	17	10
ZU 258	37	6	15	20	22
ZU 262	55	5	9	20	11
ZU 264	52	6	10	15	17
Mean	51	5	11	18	15
d < 1.006†	54	4	9	18	15
Literature‡	55	8	5	22	10
II. LDL					
ZU 253	6	8	37	24	25
ZU 258	6	8	38	24	24
ZU 262	6	9	39	23	23
ZU 264	6	9	40	21	24
Mean	6	8	39	23	24
d 1.006–1.063†	7	9	31	24	29
Literature‡	11	8	37	22	21
III. HDL					
ZU 254	4	3	16	28	49
ZU 259	2	2	16	24	56
ZU 263	3	2	20	22	53
ZU 265	3	3	21	24	49
Mean	3	3	18	24	52
d 1.063–1.210†	2	2	18	26	51
Literature‡	6	3	15	26	50

* (Mg individual component)/(mg TG + C + CE + PL + Protein) \times 100 = %. FFA was not included in the calculation because it was small in all cases (<1%). Protein was calculated as mg of bovine serum albumin. CE was calculated as cholesteryl oleate.

† These lipoproteins were isolated by the usual sequential techniques in angle-head rotors (16).

‡ Ref. 17.

TABLE 5 COMPARISON OF HDL₂ AND HDL₃*

Ratio (w/w)	HDL ₂	HDL ₃	P†
CE/C‡	2.90 \pm 0.15	4.63 \pm 0.41	<0.01
Protein/PL	1.49 \pm 0.11	2.24 \pm 0.16	<0.01
C _T /PL	0.65 \pm 0.05	0.56 \pm 0.05	<0.30

* The HDL₂ analyzed were fractions C (ZU 254 and ZU 265, Fig. 3), D (ZU 259, Fig. 4), and F (ZU 263, Fig. 4). The HDL₃ analyzed were fractions D (ZU 254 and ZU 265, Fig. 3), E (ZU 259, Fig. 4), and G (ZU 263, Fig. 4).

† Significance of differences between HDL₂ and HDL₃. All values in table are means \pm SEM.

‡ Mg of cholesterol, whether free or esterified.

(titanium) or Ti-14 rotor. The chylomicron-VLDL was separated into three fractions (Fig. 10). The first peak was located at the light end of the gradient after the brief centrifugation. The second peak varied somewhat in position and volume with the different samples of plasma. The third peak remained at the sample zone at the periphery of the rotor. The least dense peak was assumed to be the chylomicron fraction, while the intermediate peak was designated as the VLDL. Designation of these subfractions as chylomicrons or VLDL is based on the observation that, when analyzed by electro-

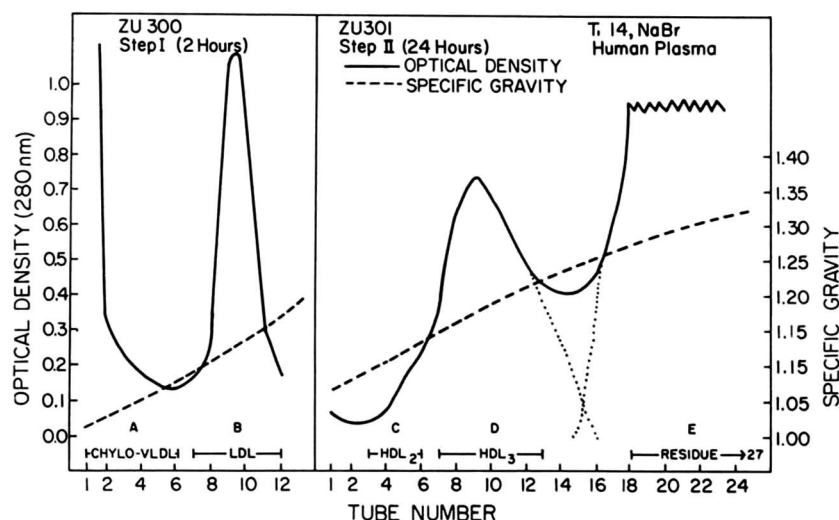


FIG. 6. "Two-step" isolation of plasma lipoproteins. 55 ml of human plasma was centrifuged in the Ti-14 rotor. Refer to legend for Fig. 3 for details.

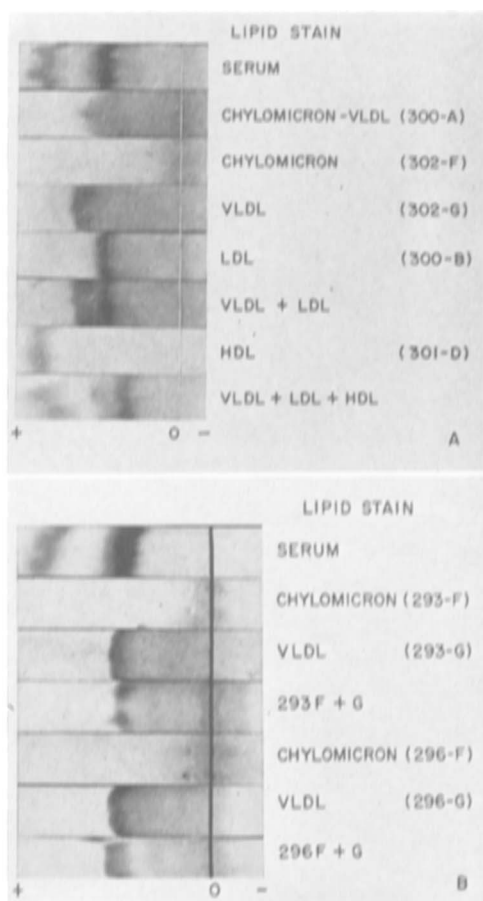


FIG. 7. Paper electrophoresis of serum lipoproteins. A. Fractions analyzed were those from ZU 300, 301, and 302 (Figs. 6 and 10). B. Fractions analyzed were those from ZU 293 and 296 (Fig. 10). Electrophoresis was carried out using Whatman No. 1 paper strips in an LKB or Gelman (Gelman Instrument Co., Ann Arbor, Mich.) horizontal apparatus for 16 hr at 4 v/cm at room temperature in Veronal-albumin buffer, pH 8.6 (11). The strips were stained for lipid with Oil Red O after electrophoresis (12).

phoresis on paper, the lightest fraction remained at the origin whereas the middle fraction migrated as a "pre- β " globulin (Fig. 7, A and B). Furthermore, when examined by immunodiffusion, the chylomicron fraction did not form precipitin lines with antisera to albumin, β -lipoprotein, α -lipoprotein, or α -acid glycoprotein; the VLDL reacted only with antisera to β -lipoprotein (Fig. 8). On immunoelectrophoresis, the VLDL formed precipitin arcs with antiserum to β -lipoprotein and to whole plasma but not with antiserum to albumin and to α -lipoprotein. The chylomicrons did not form precipitin arcs with antiserum to β -lipoprotein and to whole plasma (Fig. 9). The third fraction (designated as "albumin," Fig. 10), probably represents the contamination of the original chylomicron-VLDL fraction with the heavier serum proteins. This "albumin" or "contaminant" fraction reacted strongly with antisera to albumin when examined by immunodiffusion (Fig. 8) or immunoelectrophoresis (Fig. 9). When this contaminant fraction was reacted with antisera to human plasma proteins, an additional precipitin arc was observed in the region associated commonly with γ -globulins. The albumin fractions of ZU 293 and 296 reacted neither with antiserum to β -lipoprotein nor with antiserum to plasma proteins to form a precipitin arc corresponding to the β -lipoprotein; the albumin fraction of ZU 302, which reacted positively with these two antisera, would have been expected to do so since the fractional cut was made to obtain uncontaminated VLDL. It is curious that the chylomicron-VLDL fraction (as in Fig. 6) appeared to be contaminated to a small extent with plasma proteins, but that the heavier LDL and most of the HDL did not react with antiserum to albumin and could not have been contaminated to any significant extent. It is of interest

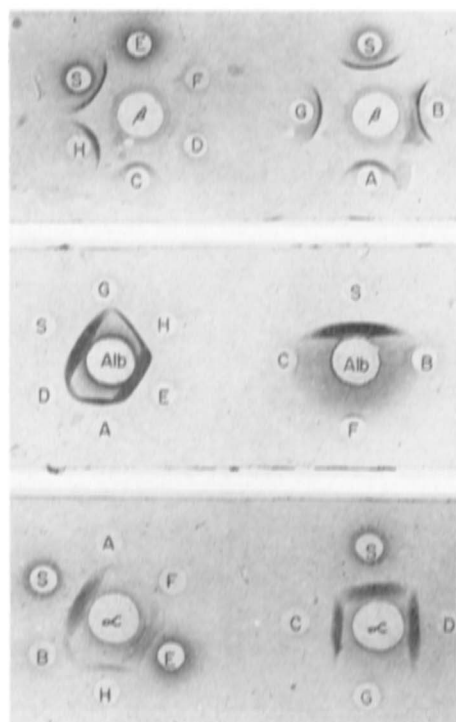


FIG. 8. Analysis of lipoproteins by double immunodiffusion. Samples of antigen were placed in the outer wells and samples of antiserum in the center well. Incubation was for 24 hr at room temperature. The antisera are designated β (anti-human β -lipoproteins), α (anti-human α -lipoprotein), or *Alb* (anti-human albumin). The antigens are labeled as follows: *S* refers to whole plasma, *A-E* to the corresponding fractions from ZU 300-301 (Fig. 6), and *F-H* to those fractions from ZU 302 (Fig. 10). The designation and concentration of protein (mg/ml) of each fraction analyzed was as follows: *A*, chylomicron-VLDL, 0.11; *B*, LDL, 2.7; *C*, HDL₂, 1.3; *D*, HDL₃, 14.5; *E*, residue, 210.0; *F*, chylomicron, 0.14; *G*, VLDL, 0.3; *H*, "albumin" contaminant, 0.6; and *S*, serum, 105.0. 5.0 μ l of each of the above antigens was used. When reacted against antiserum to albumin, 5.3 mg/ml each of *S* and *E* were added. When reacted against antiserum to α -lipoprotein, 0.7 mg/ml of fraction *D* was added to the well. The slides were washed, dried and then stained for protein with Buffalo Black.

that the position and the peak of the VLDL in the gradient varied with each of three different centrifugations and sera. Although all the samples of plasma were collected after an overnight fast or a light breakfast, differences in the VLDL may reflect the past dietary history of the individual and should be investigated carefully.

The lipid composition of the chylomicrons and VLDL isolated by zonal ultracentrifugation is presented in Table 6. The percentage distribution of the various lipids in the VLDL isolated by zonal ultracentrifugation is in agreement with data reported in the literature for lipoproteins isolated by the usual sequential methods (18-21). The percentage of TG appears to be lower and the percentage of PL appears to be higher in chylomicrons isolated by zonal ultracentrifugation than in chylomicrons which had been isolated by sequential

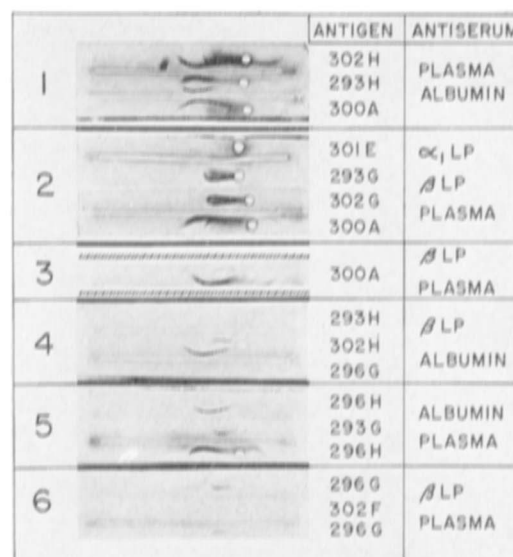


FIG. 9. Immuno-electrophoresis of plasma lipoproteins. The antigens correspond to the lipoprotein fractions in Figs. 6 and 10. *A* is the chylomicron-VLDL (ZU 300) and *E* is the residue fraction of ZU 301. *F* refers to the chylomicron fraction, *G* to the VLDL, and *H* to the albumin-containing contaminant (Fig. 10). Electrophoresis was carried out for 1 hr at 250 v. The concentrations of antigens used were identical to those described in the legend for Fig. 8. After the antisera were placed in the troughs, the slides were incubated for 24 hr at room temperature. The slides were then washed, dried, and stained for protein with Buffalo Black (slides 3-6) or were first stained for lipid with Oil Red O and then with Buffalo Black (slides 1-2).

techniques and which had been reported earlier in the literature. The reason for these differences is unknown; it is conceivable that these differences derive from the fact that the plasma samples for our experiments were obtained from men after an overnight fast, whereas the literature values were obtained from subjects given fatty meals or were obtained from hyperlipemic patients. In one experiment in which plasma was obtained from two individuals after a reasonably fatty luncheon, the lipid composition of the chylomicrons isolated by zonal ultracentrifugation was 83, 10, 4, and 3% for TG, PL, CE, and C, respectively, in agreement with values reported in the literature (Table 6).

DISCUSSION

In our experience, the most satisfactory procedure for the ultracentrifugal isolation of the three major classes of plasma lipoproteins in the Ti-14 zonal rotor was the two-step separation in the linear gradient (d 1.0-1.4) described in this report. Lower gradient ranges (d 1.0-1.2, 1.0-1.3) were less satisfactory for single-step resolution of the HDL than a gradient in the range 1.0-1.4. It is possible that the HDL can be isolated more rapidly and completely from the residual plasma proteins if a

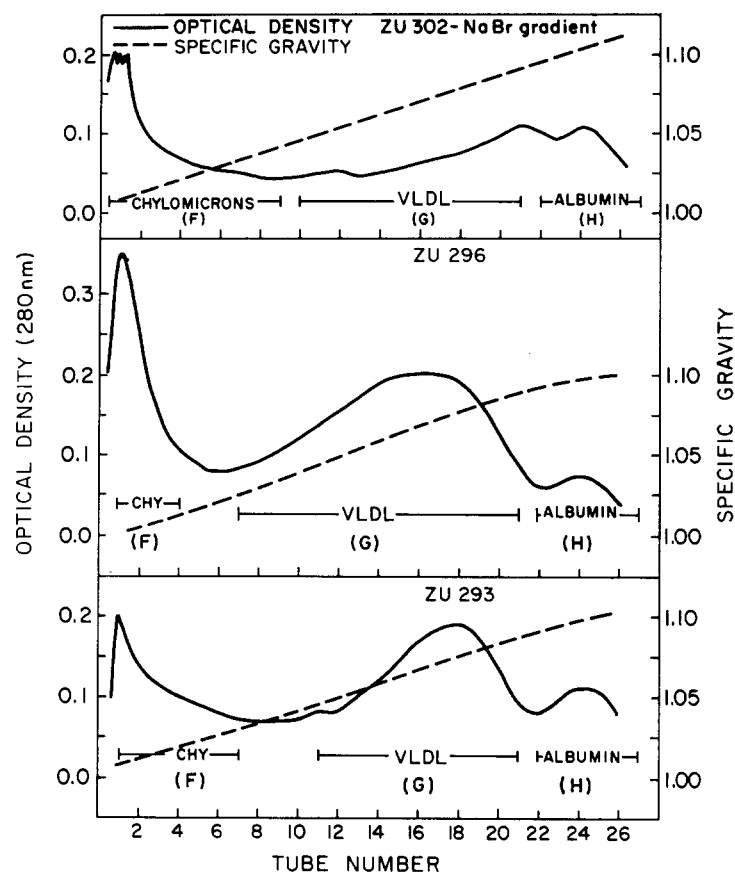


FIG. 10. Separation of chylomicrons and VLDL in the zonal rotor. Centrifugations were carried out in the B14 titanium zonal rotor at 30,000 rpm for 30 min in a linear gradient, NaBr, d 1.0–1.1. The sample in each experiment was the chylomicron–VLDL fraction which had been concentrated to 30–40 ml and adjusted to a density of 1.10 with solid NaBr. The sample for ZU 302 was obtained from Step I of ZU 300 (Fig. 6). The samples for ZU 293 and 296 were the chylomicron–VLDL fractions from two separate 80-ml batches of pooled plasma.

gradient steeper than 1.0–1.4 were employed. In one centrifugation, we attempted to use a NaBr gradient in the density range 1.0–1.5. Even when the Ti-14 rotor was operated at the derated velocity of 43,000 rpm, the force exerted on the walls of the rotor at the periphery was sufficient to cause expulsion of some of the rotor contents through the joints; when the velocity was reduced to 40,000 rpm, the separation of the HDL in the d 1.0–1.5 gradient was not improved over that obtained with the d 1.0–1.4 gradient at maximal rotor velocity. The two-step method in the Ti-14 and Ti-15 rotors has, in our hands, proved to be more appropriate for separation of all three major classes of lipoproteins than any single-step technique. Simultaneous and complete separation of the VLDL, LDL, and HDL in salt gradients, in the more viscous salt–sucrose gradients, or in the initially discontinuous gradients constructed with water overlays, has not yet been accomplished even though more centrifugal force was generated in Ti-series rotors than in the prototype B-series rotors. Viikari, Haahti, Helelä, Juva, and Nikkari (22) reported recently the

simultaneous isolation of all the classes of plasma lipoproteins in a single centrifugation; the sample of plasma was centrifuged for 18 hr at 35,000 rpm in a modified B15-type rotor,³ using a 300-ml overlay of 0.15 M NaCl (d 1.005) and an NaCl–KBr gradient (d 1.005–1.220). Their results were similar to ZU 205 (Fig. 2), in which plasma was centrifuged for 18 hr at 35,000 rpm in the Ti-15 rotor in the NaBr gradient, d 1.0–1.4. In both cases, the HDL was separated incompletely from the residual proteins, although the “HDL₂” was more conspicuous in the procedure reported by Viikari and his associates.

Some of the advantages and disadvantages of the procedure of rate-zonal flotation for the isolation of plasma lipoproteins have been discussed previously (1). Whether isolated by zonal procedures or by the usual sequential methods in angle-head rotors, the corresponding lipoproteins exhibit similar chemical, physical, and immuno-

³ Manufactured by Measuring and Scientific Equipment Ltd., London, England.

TABLE 6 LIPID COMPOSITION OF CHYLOMICRONS AND VLDL ISOLATED BY ZONAL ULTRACENTRIFUGATION

Fraction	% of Total Lipid*				Reference
	C	CE	PL	TG	
I. Chylomicrons					
ZU 293 (overnight fast)	5	10	14	71	—
296 (overnight fast)	3	4	16	77	—
302 (overnight fast)	2	8	18	72	—
288 (fed)	3	4	10	83	—
—	5	6	8	81	(18)
—	2	5	7	86	(19)
—	2, 3	4, 7	4, 7	90, 83	(20)
—	—	—	3-10	—	(21)
II. VLDL					
ZU 293	6	16	23	55	—
296	6	12	22	60	—
302	5	15	26	54	—
—	8	12	21	59	(18)
—	8	14	21	57	(19)
—	4, 5, 5	12, 15, 15	12, 15, 15	72, 65, 65	(20)
—	—	—	20	—	(21)

* (Mg lipid/mg C + CE + PL + TG) × 100. The weights of lipid were calculated using the molecular weights listed in Tables 1 and 4.

logical properties. The isolation of lipoproteins can be accomplished with greater ease and rapidity by rate-zonal flotation than by sequential techniques. Zonal procedures allow one to fractionate relatively large volumes of plasma (up to 150 ml in the Ti-15 rotor) and, simultaneously, to obtain an analytical description of the quantity of the lipoprotein present in the plasma and the position of the lipoprotein in the density gradient. It is not necessary that isopycnic equilibrium of a lipoprotein in a gradient be achieved in order to effect separation of the lipoprotein from serum. Rather, as described in this report, the separation depends primarily on the flotation rate of the lipoprotein. It is possible, of course, that lipoproteins reach isopycnic equilibrium in zonal rotors, and this was achieved for the LDL in a sucrose-KBr density gradient (1). Isopycnic equilibrium was not attained for the LDL in the NaBr gradient (d 1.0-1.4), since the gradient shifted slightly during prolonged centrifugation (Figs. 1 and 2). Isopycnic equilibrium might have been achieved for the LDL had a less steep NaBr gradient been utilized. The isopycnic position of the HDL may, no doubt, also be determined provided the gradient is stable.

The lipoproteins isolated by zonal ultracentrifugation are in a native state and are relatively free of contamination by other proteins. Most of the HDL can be isolated free of contamination by a single centrifugation of 24-48 hr duration. About 90% (tubes 3-16, ZU 301, Fig. 6) of the HDL protein was obtained free of detectable contamination with albumin, as judged by immunodiffusion. In four of eight experiments, the HDL₂ protein was found by immunodiffusion to be contaminated with

β protein to the extent of 3-4%. In contrast to the zonal procedure, several centrifugations are necessary to isolate the HDL by sequential techniques; repeated centrifugations are then required to wash the contaminating proteins from the HDL and this often may be accompanied by loss of lipoprotein. The LDL can be isolated by zonal ultracentrifugation in less than 1 hr, apparently uncontaminated by other proteins. The chylomicrons, VLDL, and LDL isolated by sequential techniques must be washed several times by recentrifugation to remove contaminating albumin. The chylomicron-VLDL isolated by zonal ultracentrifugation is also contaminated with albumin, but the albumin can be removed when the fraction is partitioned into the chylomicrons and VLDL. One might not have expected the chylomicron-VLDL to be contaminated with albumin, since the fraction had to migrate through a large volume of protein-free gradient (600 or 1600 ml) and since the heavier lipoproteins, such as LDL, were not contaminated. Conceivably, the chylomicron-VLDL, because of their high proportion of lipid and large surface area, may adsorb appreciable amounts of contaminating albumin and other dense proteins.

We would suggest from the data presented here, and from earlier reports from this laboratory, that the isolation of the lipoproteins from plasma by rate-zonal flotation in the ultracentrifuge is a most promising procedure for preparative requirements and analytical measurements. The potential of the procedure is such that it may find wide application in experimental and clinical problems of the metabolism of the plasma lipoproteins.

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