Isolation of plasma lipoproteins by zonal ultracentrifugation in the B14 and B15 titanium rotors

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ABSTRACT Lipoproteins were isolated from plasma of man, dog, rabbit, rat, and chicken by ultracentrifugation in continuous density gradients using the B14 titanium and B15 titanium zonal rotors. Both the VLDL and the LDL of human plasma were separated easily from the HDL and from the other more plentiful plasma proteins by centrifugation for only 1 or 2 hr in the B14 or B15 rotor, respectively. Satisfactory separation of the HDL from the more dense plasma proteins was not achieved with these rotors. The human LDL achieved isopycnic equilibrium (d 1.04) on prolonged periods (> 24 hr) of centrifugation in a sucrose–KBr density gradient. The pattern of distribution of cholesterol and phospholipid throughout the density gradient coincided with the pattern of distribution of the lipoprotein-protein measured spectrophotometrically or chemically. The concentration of cholesterol and phospholipid in the lipoproteins isolated by zonal ultracentrifugation agreed with analyses reported for lipoproteins isolated by sequential centrifugation in solutions of increasing density. The lipoproteins isolated by zonal ultracentrifugation were characterized further by their electrophoretic behavior. The fractions which were identified as the LDL (d 1.04–1.05) from all species migrated on paper as a β-globulin; the LDL from plasma of dogs contained an additional component which has been designated as an α_2-globulin. The fractions which were identified as the HDL from all species migrated as an α_1-globulin.

Reaction of human LDL with either rabbit antihuman β-lipoprotein or rabbit antihuman serum resulted in a single immunodiffusion band. The $S_{f,1.063}$ of the human LDL was calculated to be 6.0. When plasma from humans or rabbits was centrifuged in the B15 rotor, the HDL was not visible as a distinct peak and was not separable from the bulk of the more dense plasma proteins; when plasma from dogs or chickens was centrifuged under identical conditions, the HDL was clearly detectable. Even though the mean density of the HDL from dogs or chickens was not different from that of man or rabbits, the visibility of this lipoprotein in dogs and chickens was probably due to its high concentration in the plasma of these species. When plasma from the rat was centrifuged under similar conditions, the HDL was also clearly in evidence. Although rat plasma contained a relatively small concentration of HDL, the lipoprotein had a lower mean density than did the HDL of the other species and was therefore more easily separable from the dense plasma proteins.

The procedure of zonal ultracentrifugation for the isolation of lipoproteins by flotation is simultaneously preparative and analytical and should find useful application in the investigation of the soluble lipoproteins from plasma and tissues.

SUPPLEMENTARY KEY WORDS human · dog · chicken · rabbit · rat continuous density gradient · lipoprotein protein · lipoprotein lipids

THE STRUCTURE and function of the serum lipoproteins have been the subject of recent excellent reviews (1, 2). The variety of methods which have been utilized in the past to isolate lipoproteins from serum have been discussed in a recent review by Hatch and Lees (3). Clearly, any technique which will increase the ease of isolation of the lipoproteins from the blood would be welcome to the investigator. Perhaps the most successful and detailed separations of the serum lipoproteins were obtained by ultracentrifugation of the serum in solutions of increasing solvent density. In practice, the procedures which are now generally employed for the preparative

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; C, cholesterol; PL, phospholipid; EDTA, ethylenediamine-tetraacetic acid.

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isolation of the serum lipoproteins by ultracentrifugation involve lengthy, tedious, and sequential adjustments of density and repeated centrifugations at various solvent densities. The fractions so obtained by these procedures are classes of lipoproteins with densities between finite limits. It would require a minimum of three centrifugations in angle-head rotors at 100,000 *g* for 16–20 hr each to obtain all the major lipoprotein fractions even in a state of relative impurity. Further purification of the lipoproteins requires additional lengthy centrifugations.

A second centrifugal method involves flotation of the lipoprotein in a continuous density gradient (4, 5). The major requirement for such a procedure is that the gradient be stable in the centrifugal field relative to the rates of flotation of the lipoprotein molecules. The value of these continuous gradients has, in the past, been limited by several factors. These procedures, which were carried out in tubes in angle-head or swinging-bucket rotors, were not useful as preparative techniques because of the obvious limitation of capacity. A similar limitation applies to the technique of sequential density adjustments. A second disadvantage is that the tubes must be filled and unloaded while the rotor is at rest; under these conditions, diffusion may distort both the gradient and the position of the lipoproteins in the gradient. The zonal rotors overcome many of the basic disadvantages of angle-head or swinging-bucket rotors for a continuous density gradient. Density gradient separation carried out in the zonal ultracentrifuge is a most promising procedure which, in recent years, has been used for the isolation of particulate and macromolecular substances by rate-zonal sedimendation (6, 7). Large (or small) samples may be placed in the zonal rotors, stable gradients can be formed, and the rotor can be filled or unloaded while it is revolving at speeds sufficient to stabilize and maintain the zones. Theoretically, if one has a rotor with sufficient path length and which is capable of generating sufficient centrifugal force, it should be possible to separate and isolate all of the plasma lipoproteins in a single centrifugation, provided that appropriate materials are used for the density gradients and that the gradient has the appropriate range. Thus zonal ultracentrifugal procedures offer the possibility of techniques that are simultaneously analytical and preparative. We, therefore, examined the possibility of isolating and characterizing the lipoproteins from human plasma and from plasma of experimental animals by rate-zonal flotation in the ultracentrifuge. Preliminary reports of this work have been presented (8–10).

EXPERIMENTAL PROCEDURE

All centrifugations were carried out in a Spinco Model L ultracentrifuge which had been adapted to accept zonal rotors with removable seals (11, 12). The rotors which have been used in the experiments reported here are the B14 and B15 titanium zonal rotors.[1] The characteristics of these rotors have been described in detail by Anderson and his colleagues (12). The rotors were filled, and the contents were removed in a manner analogous to that used for rate zonal sedimentation runs except that the samples were added at the periphery of the rotor for rate-zonal flotation (Fig. 1). During the interval when the rotor was loaded or unloaded, its velocity was maintained at 3,000 rpm. In the majority of the experiments reported here, the density gradient was constructed as follows (specific details are described in the legends for the figures). Various quantities of distilled water ("overlay") were placed in the rotor which was then accelerated to 3,000 rpm. The remaining rotor volume was filled with the appropriate gradient solution at the rate of 35 ml/min, using a peristaltic action pump. Continuous gradients in the range of densities of 1.0–1.4 were constructed empirically to yield a final gradient which was linear with volume. The initial density gradient was formed with two similar vessels connected at the base (13). The gradient materials used were aqueous solutions of a mixture of NaCl and KBr, a mixture of sucrose and KBr, and NaBr; only analytical grade chemicals were used to prepare the gradients. Unless indicated otherwise, all solutions were 35 μM with respect to ethylenediamine-tetraacetic acid[2] (EDTA). When the rotor was almost completely filled, the sample was injected with a hypodermic syringe into the rotor at its periphery, and about 10 ml of high density solution was added to insure that the entire sample was within the rotor and that the rotor was completely filled. Centrifugation (15°C) was carried out at 37,000 rpm (B14) or 27,000 rpm (B15) for various periods of time. When the experiment had been completed, the rotor was decelerated to 3,000 rpm, and the contents of the rotor were collected at a rate of 35 ml/min by displacement with the particular solution of high density. Generally, the rotor contents were monitored by measurement of absorption at 280 nm in a Beckman DU spectrophotometer equipped with a quartz flow-through cell (0.2 cm light path), a Gilford optical density conver-

[1] These rotors were obtained from the Oak Ridge National Laboratories, Oak Ridge, Tennessee.

[2] When EDTA was omitted from the solutions with which the gradients were constructed, the apparent buoyant density of the LDL was in the range d 1.06–1.08 rather than d 1.04. The increase in buoyant density was accompanied by an increase in the absorption of the lipoprotein at 280 nm and by an increase in electrophoretic mobility on paper. These changes in physical properties were eliminated when the LDL was isolated in the presence of 35 μM EDTA. This concentration of EDTA, however, may permit some peroxidation of lipid to occur, and therefore gradient media which are 350 μM with respect to EDTA are now used.

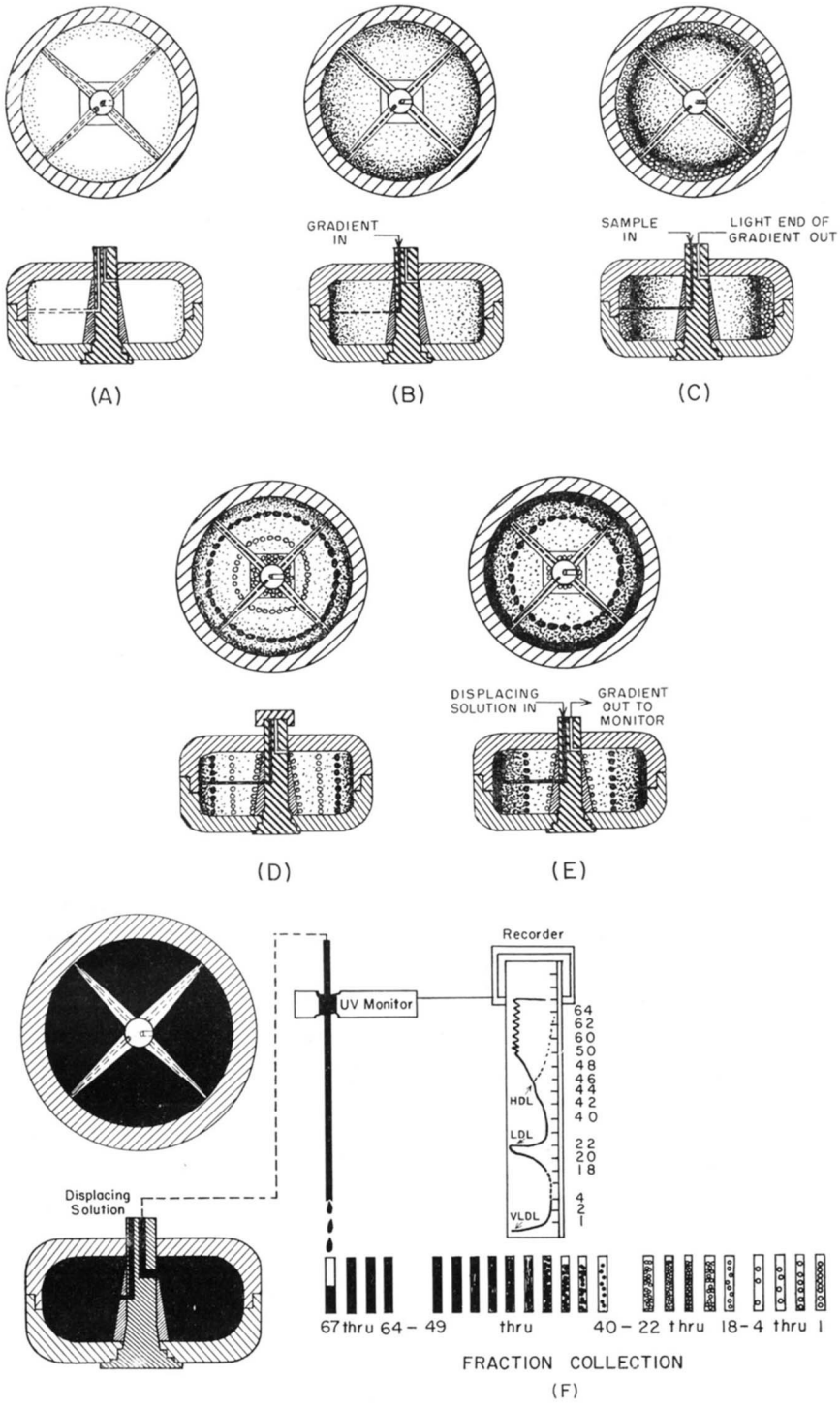

FIG. 1. Loading and unloading of the B15 zonal rotor for isolation of lipoproteins by flotation. Top and side views of the rotor during various stages of centrifugation are shown. The rotor in *A* was filled partially with the overlay of water when desired. In *B*, the entire gradient has been pumped into the rotor through lines leading to the edge of the rotor. The light end of the gradient was pumped in first, such that the light end was at the core and the heavy end at the periphery of the rotor. In *C*, the sample and wash have been introduced through the lines leading to the periphery of the rotor. Steps *B* and *C* were carried out at 3,000 rpm. At this point, the static seal was removed, and a cap was placed on the rotor. The rotor was then accelerated to operational speeds in a vacuum to achieve the separation indicated in *D*. After deceleration of the rotor to 3,000 rpm, the rotor contents were displaced (*E*, *F*) by pumping solutions of high density to the periphery of the rotor and collecting through the core. The fractions collected from the core were monitored by absorption at 280 nm (*F*).

ter, and a chart recorder. 67 fractions (B15), or 26 fractions (B14), each of 25 ml of effluent, were collected. The absorption of the individual 25-ml fractions at 280 nm was measured with a Beckman DB spectrophotometer (1.0 cm light path). The specific gravity of the various fractions was measured with a specific gravity balance ("Speegrav," wide range; A. S. Aloe Co., St. Louis, Mo.).

The samples for centrifugation were either fresh heparinized plasma (approximately 7 USP units/ml of blood) or the total lipoproteins derived from the plasma. The specific gravity of the samples of plasma was adjusted to that of the maximal density of the particular gradient employed. Blood was obtained from experimental animals that were maintained on standard laboratory chow ad lib. Blood was obtained from normal human volunteers after an overnight fast or following a light breakfast. Outdated blood-bank blood was used in some experiments. Samples of the total lipoproteins with d < 1.210 were obtained by centrifugation of plasma (adjusted to a density of 1.210 with KBr) in a Spinco 40 rotor for 24 hr at 105,000 *g*. The lipoproteins floating in the uppermost 1.0–1.5 ml were removed. These lipoproteins were then separated further by zonal ultracentrifugation after adjustment of the solution density to the desired value.

Lipids were extracted from fractions of the rotor effluent after precipitation of the lipoproteins. The protein present in 20-ml aliquots of individual or combined fractions was precipitated with 5 ml each of 0.15 N $Ba(OH)_2$ and 0.15 N $ZnSO_4$. The precipitate was washed twice with 20 ml of distilled water, and the lipids were extracted from the precipitate with three 20-ml portions of $CHCl_3$–CH_3OH 2:1 (v/v). The combined extracts were washed with 0.02% $MgCl_2$ and were dried in vacuo at 50°C. The volume of the washed extracts was adjusted to 20 ml with $CHCl_3$, and aliquots were taken for estimation of lipid soluble phosphorus (14) and total cholesterol (15). In some cases, the neutral lipids were separated from the phospholipids on silicic acid columns prior to chemical analyses.

The procedure for precipitation of the lipoproteins with $Ba(OH)_2$–$ZnSO_4$ was selected since it was a convenient and rapid method for the analysis of numerous samples obtained from the zonal rotors. This method eliminated the necessity for dialysis and concentration of the samples prior to extraction of the lipids by a modified Folch procedure (16). The method was most useful for the large numbers of samples which had to be analyzed, and it allowed us to correlate the distribution of lipid in the gradient with that of protein, which was measured either by absorption at 280 nm or by chemical analysis (17, 18). The precipitation procedure with $Ba(OH)_2$–$ZnSO_4$ should, however, not be used for quantitative analysis of the lipoprotein lipids other than for their percentage composition. Using this method, about 35 fractions (B15) were analyzed for cholesterol and phospholipid in each centrifugation; recovery of cholesterol and phospholipid in the gradient after centrifugation was 75.6 ± 3.5% (eight centrifugations) of that amount present originally in the plasma or d < 1.210 lipoproteins. Aliquots of the d < 1.210 lipoproteins, equal to 0.5 ml plasma, which had been isolated in angle-head rotors, were diluted to 20 ml with solutions whose density varied from 1.00 to 1.28; the recovery of cholesterol and phospholipid in these fractions after precipitation with $Ba(OH)_2$–$ZnSO_4$ and extraction with $CHCl_3$–CH_3OH was 88.0 ± 0.9% (duplicate analyses at d 1.00, 1.05, 1.15, and 1.28, respectively). When aliquots of the rotor contents were extracted directly with $CHCl_3$–CH_3OH 2:1 (v/v), essentially 100% recovery of cholesterol and phospholipid was obtained (Table 1). The percentage of the total cholesterol or phospholipid appearing within any particular lipoprotein was similar with both procedures. Furthermore, the ratio of cholesterol to phospholipid in each lipoprotein class was essentially the same using either method and was similar to those values reported by Havel, Eder, and Bragdon (19) for lipoproteins isolated by the sequential procedure. Since complete recovery of cholesterol and phospholipid was obtained after zonal ultracentrifugation, it is probable that this precipitation procedure does not alter the content or composition of the lipid moieties of the lipoproteins.

For identification of the fractions by electrophoresis, aliquots were first dialyzed for 24 hr against 50 volumes of 0.9% NaCl containing 0.001 M EDTA, and then they were concentrated by filtration through a Diaflo UM-1 or XM-50 membrane (Amicon Corp., Lexington, Mass.). In later work, the concentration and dialysis of the lipoproteins were carried out simultaneously in the ultrafiltration cell. In most cases, quantitative recovery of fractions from the membrane filter was not attempted, and therefore the position to which the protein migrated during electrophoresis is the only comparison which should be made. It must be stated clearly, however, that quantitative recovery of lipoprotein lipids was obtainable when the fractions were concentrated by ultrafiltration and when the lipids were extracted with a minimum of 20 volumes $CHCl_3$-CH_3OH 2:1 (v/v). Paper electrophoresis of selected fractions of the rotor effluent was carried out using Whatman 3MM paper strips (3 × 41 cm) in an LKB or Gelman (Gelman Instrument Company, Ann Arbor, Mich.) horizontal apparatus for 16 hr at 10 v/cm at room temperature. Veronal buffer (0.05 M, I = 0.125, pH 8.6, 0.001 M EDTA) was used. After electrophoresis, the strips were stained for protein with Amido Black 10B or Naphthol

TABLE 1 DISTRIBUTION OF CHOLESTEROL (C) AND PHOSPHOLIPID (PL) IN LIPOPROTEINS ISOLATED FROM HUMAN PLASMA BY ZONAL ULTRACENTRIFUGATION

	Method of Isolation					
	BZ†			CM‡		
Fraction*	C	PL	C/PL§	C	PL	C/PL§
	μmoles/fraction			*μmoles/fraction*		
A	9.49	4.89	1.00	15.00	8.88	0.87
B	171.21	50.21	1.76	234.26	61.77	1.95
C	2.90	1.02	1.46	2.02	2.15	0.48
D	37.61	27.17	0.71	48.69	47.73	0.53
E	16.97	17.57	0.50	31.60	35.37	0.46
Total	238.18	100.86		331.57	155.90	
% Recovery‖	67.3	65.1		93.6	100.6	

* Refer to Fig. 14, ZU 237.

† The fractions were precipitated with $Ba(OH)_2$–$ZnSO_4$ prior to extraction. Refer to text for details.

‡ The fractions were extracted with $CHCl_3$–CH_3OH by a modified Folch procedure (16). Refer to text for details.

§ Ratio by weight, C/PL = (μmoles C) (mol wt C)/(μmoles PL) (mol wt PL).

‖ Total μmoles lipid recovered/μmoles lipid in sample of plasma × 100.

blue-black. After electrophoresis in the Veronal buffer containing 1% bovine serum albumin (20), the strips were stained for lipid with Oil Red 0 (21). The immunochemical purity of the human LDL was examined with the double immunodiffusion technique described by Ouchterlony (22). Antiserum to human β-lipoprotein produced in rabbits was obtained from Behring Diagnostics, Inc., Woodbury, N.Y. This antiserum did not react with the HDL isolated by zonal ultracentrifugation (23). The flotation constant ($S_{f,\ 1.063}$) of the human LDL was calculated from measurements obtained with the Spinco Model E analytical ultracentrifuge (24).

RESULTS

In our earliest experiments, the density gradients for the zonal rotors were constructed with solutions of NaCl–KBr because this mixture had been utilized successfully for the separation of plasma lipoproteins in angle-head rotors by the usual sequential techniques. The gradient (d 1.0–1.3) was introduced into the rotor behind an overlay of 320 ml (B15) or 150 ml (B14) of distilled water. The VLDL[3] and LDL were isolated from human plasma after centrifugation of the plasma for 2 and 6 hr in the B15 rotors (Fig. 2) or for 1 hr in the B14 rotor (Fig. 3). Under these conditions, the HDL could not be separated from the plasma proteins. When plasma was centrifuged in the B15 for longer periods of time (>24 hr), the HDL appeared as a shoulder on the peak of the residual plasma proteins, while the VLDL and the LDL tended to merge near the core of the rotor. Even with longer periods (48 hr) of centrifugation, complete separation of the HDL from the residual plasma proteins was not achieved.

To circumvent some of these problems, a steeper linear density gradient (d 1.0–1.4 NaBr) was constructed in the rotor. After centrifugation of human plasma for 5 hr in the B15 rotor, the VLDL and LDL were collected; the displaced gradient was replaced with fresh gradient which was pumped, heavy end first, back into the rotor through its core. Centrifugation was then reinstated at 27,000 rpm for an additional 24 hr (Fig. 4). The HDL was, however separated incompletely from the residual plasma proteins and appeared as a shoulder on the protein peak. Similar results were obtained with the B14 rotor in which step 1 lasted for 2 hr and step 2 for 24 hr. The greater velocity of the B14 was still inadequate for complete separation of the HDL from the residual proteins, even though the absorption of the HDL at 280 nm was more prominent than that observed with the B15 rotor. We recently obtained two new rotors[4] which are modeled after the B14 and B15 prototypes, but which can generate a maximum of 172,000 *g* (Ti14) or 121,000 *g* (Ti15) under normal operating conditions. In preliminary experiments with the Ti14 and Ti15 rotors, it was observed that the VLDL, LDL, and HDL were separated completely from the residual proteins using the d 1.0–1.4 NaBr gradient and the two-step procedure described above (23). We have examined also the potential of the B15 aluminum rotor, which is identical with the B15-Ti rotor in design, but whose maximal velocity is limited to 20,000 rpm; the VLDL and LDL were separated readily from plasma using the NaCl–KBr gradient, but even partial separation of the HDL was not achieved with this rotor.

[3] The "absorption" at 280 nm of the VLDL to a large extent must be the result of light scattering. The term VLDL as used in the manuscript actually includes chylomicrons and VLDL which were present in the samples of plasma.

[4] The Ti14 and Ti15 rotors were obtained from the Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.

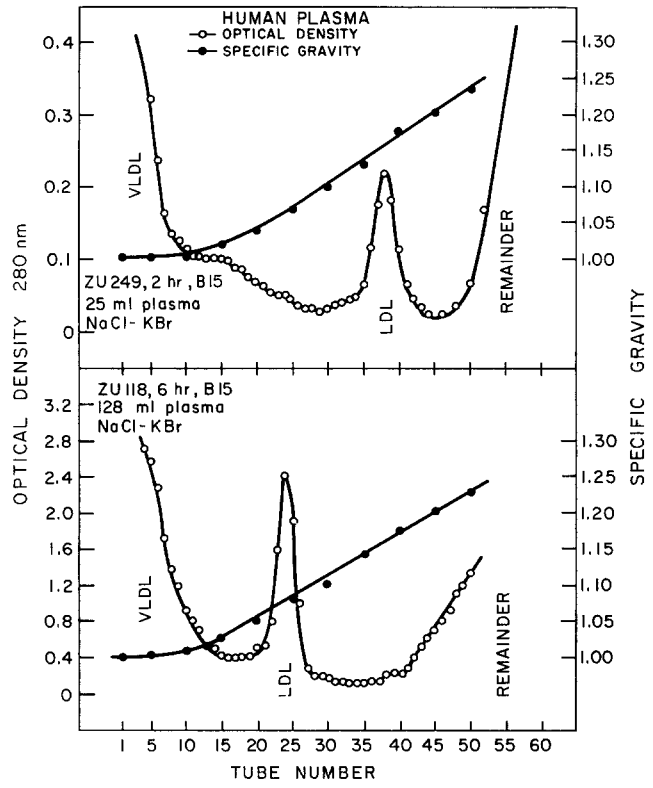

FIG. 2. The gradient material was NaCl–KBr in the density range 1.00–1.30. The concentrated salt solution (d 1.30) contained 324 g KBr and 140 g NaCl per liter. The samples were 25 ml of fresh human male plasma (ZU 249) or 128 ml of outdated blood-bank plasma (ZU 118). The specific gravity of the samples was adjusted to 1.30 with solid KBr before they were injected into the system. An overlay of 320 ml of distilled water was placed in the rotor. This was followed, in order, by 1200 ml of gradient, sample (in 147 ml total volume), and 5 ml of wash. Centrifugations were carried out for 2 or 6 hr at 27,000 rpm in the B15 rotor.

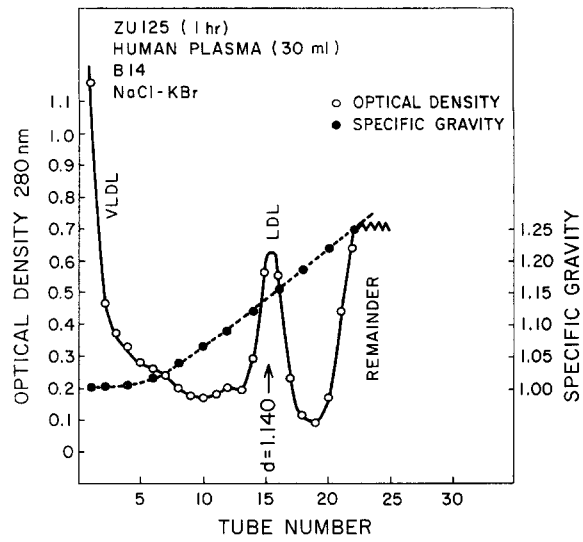

FIG. 3. The gradient material was NaCl–KBr in the density range 1.00–1.30 (see Fig. 2). The sample was 30 ml outdated blood-bank plasma. The specific gravity of the sample was adjusted to 1.30 with KBr before injection into the system. An overlay of 150 ml of distilled water was used, followed by 470 ml of gradient, sample, and 5 ml of high density solution to wash the sample into the rotor. Centrifugation was for 1 hr at 37,000 rpm in the B14 rotor.

We have utilized a mixture of sucrose–KBr 2:1 (w/w) for the construction of linear density gradients in order to retard the rate of diffusion of the gradient and to decrease the rate of flotation of the lipoprotein during centrifugation. It was possible under these conditions to separate the VLDL and LDL as previously and, in addition, to achieve isopycnic equilibrium of the LDL fraction. The effect of the duration of centrifugation on the rate-zonal flotation of plasma lipoproteins was studied in the B14 rotor using the sucrose–KBr gradient in the density range 1.00–1.28 (Fig. 5). The VLDL and the LDL were separated from the bulk of the serum proteins after only 3 hr of centrifugation. With increasing length of centrifugation, the LDL migrated toward the less dense end of the gradient. The LDL, after 24 hr of centrifugation, appeared to have reached its isopycnic position in the gradient, d 1.04, in agreement with the mean density reported in the literature for the LDL or β-lipoprotein (25). As the centrifugation continued, the LDL merged with the VLDL, and the HDL appeared as a shoulder on the bulk of the serum proteins. Similar time-sequence studies were carried out using the B15 rotor. The results of these experiments may be seen in Fig. 6. When centrifuged in a sucrose–KBr gradient (d 1.00–1.20), the VLDL and LDL were separated from the bulk of the serum proteins in 6 hr. In this particular gradient the HDL was not isolated satisfactorily from the residual serum proteins even after 72 hr of centrifugation. The density of the LDL peak was 1.083, 1.042, 1.037, and 1.035 after 6, 24, 48, and 72 hr of centrifugation, respectively. It can be presumed that isopycnic equilibrium (d 1.04) of the LDL peak was reached after 24 hr of centrifugation. The same equilibrium density was attained for the LDL with either the B14 or B15 rotors.

The effect of sample volume on the rate-zonal flotation of the lipoproteins can be seen in Fig. 7. The VLDL and LDL from 6, 12, and 25 ml of plasma were isolated in the B14 rotor after centrifugation for 3 hr. The total absorption of the LDL at 280 nm was directly proportional to sample volume. Satisfactory separation of the VLDL and LDL from the bulk of the serum proteins and from the remaining lipids of the HDL and albumin fraction was obtained with as much as 50 ml of plasma in the B14 or 150 ml in the B15 zonal rotor.

The distribution of cholesterol and phospholipid in the fractions obtained from the B15 zonal rotor after centrifugation of human plasma corresponds to the distribution of absorption at 280 nm (Fig. 8). An additional peak of lipid which corresponds to the HDL in

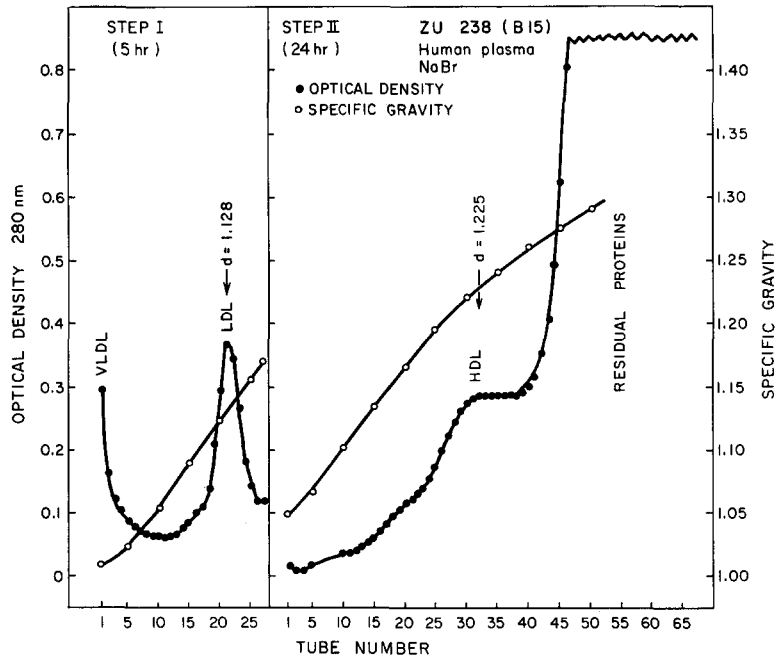

FIG. 4. The rotor was filled with 1,600 ml of a linear gradient constructed with NaBr in the density range 1.00–1.40. The concentrated salt solution (d 1.40) contained 557 g NaBr per liter. The sample was 36 ml of human plasma. The specific gravity of the sample was adjusted to 1.40 with NaBr. The sample was injected into the rotor followed by approximately 10 ml of high density solution to fill the rotor. Centrifugation was for 5 or 24 hr, respectively, in the B15 rotor at 27,000 rpm. Refer to the text for additional details.

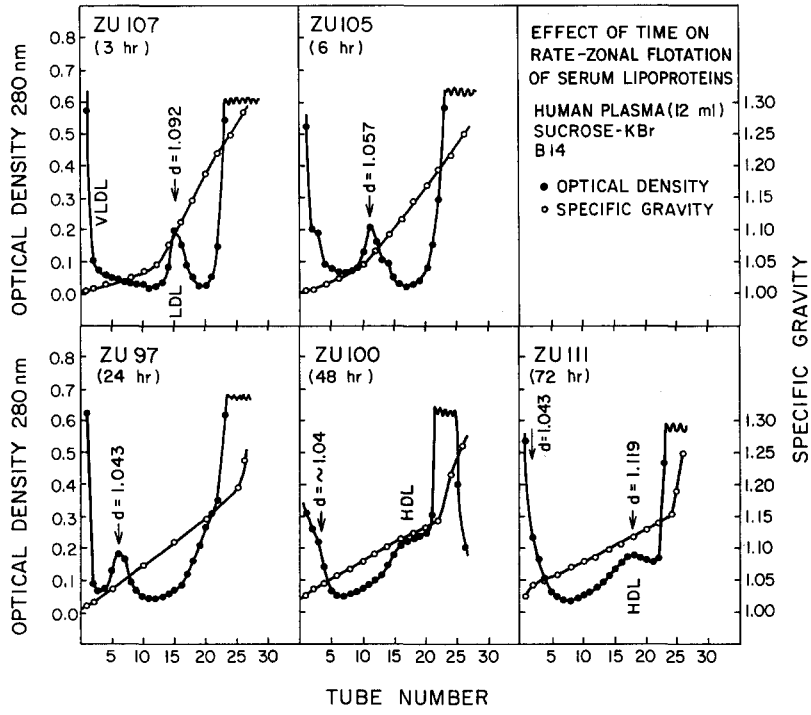

FIG. 5. The gradient material was sucrose–KBr (2:1, w/w) in the density range 1.00–1.28. The concentrated solution (d 1.28) contained 414 g sucrose and 207 g KBr per liter. The samples were 12 ml each of fresh heparinized (7 USP units/ml) plasma from normal human males. The plasma was adjusted to a specific gravity of 1.28 with solid KBr before it was injected into the rotor. The overlay was 200 ml of distilled water; 430 ml of gradient solution was pumped in, followed by the sample and enough high density solution to fill the rotor. Centrifugation was carried out for various periods of time at 37,000 rpm in the B14 rotor. ZU 105 and ZU 107 were from the same plasma sample; ZU 97 and ZU 100 were from a second sample, and ZU 111 from a third sample.

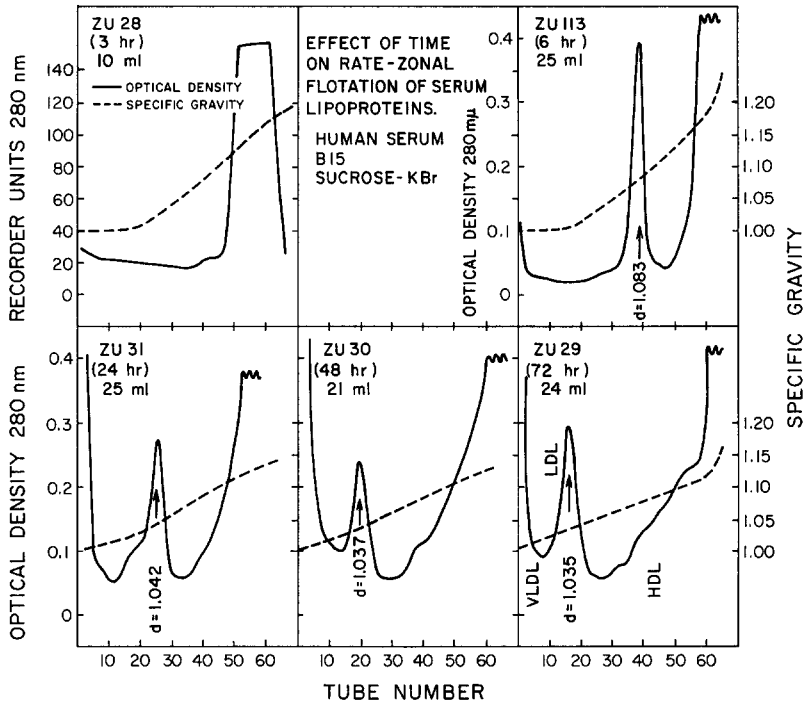

FIG. 6. The gradient material was sucrose–KBr (2:1, w/w) in the density range 1.00–1.20. The concentrated solution (d 1.20) contained 284 g sucrose and 142 g KBr per liter. The samples were serum from normal human males; 10, 24, 21, 25, and 25 ml of serum were used in ZU 28, 29, 30, 31, and 113, respectively. The specific gravity of the sample was not adjusted before injection into the system. In all experiments, an overlay of 470 ml of distilled water was placed in the rotor. This was followed, in order, by 1166 ml of gradient, sample, and sufficient high density solution to fill the rotor. The centrifugations were carried out for various periods of time at 27,000 rpm in the B15 rotor.

the region of higher density can also be seen. The various lipoprotein fractions from this zonal separation were characterized by their electrophoretic behavior, as shown in Figs. 9 and 10.

The LDL from the plasma of the human male (Fig. 8, ZU 95, tubes 18–26, d 1.035–1.062) migrated as a β-globulin; fractions 41–46 appeared to be relatively pure HDL since only one component, which migrated as an α_1-globulin, was visible. Fractions 47–49 contained HDL but were contaminated with plasma proteins of higher density. It was of interest to compare the distribution of the lipoproteins in the density gradient when the samples were either plasma or the total lipoproteins with d < 1.210 derived from the same sample of plasma. It may be seen in Fig. 11 that the distribution of the VLDL and LDL was identical with that obtained after centrifugation of whole plasma; the position of the HDL

TABLE 2 RECOVERY OF LIPID IN THE LDL ON REPEATED ZONAL ULTRACENTRIFUGATION

Experiment (Tubes)	Cholesterol		Phospholipid	
	μmoles/ sample	*% recovery*	*μmoles/ sample*	*% recovery*
ZU44 (15–25)	226.8	—	69.1	—
ZU45 (9–19)	76.9	94.3	25.5	102.6
ZU46 (15–25)	79.7	97.7	22.6	90.9

Refer to the text and Fig. 13 for experimental details. The volume of tubes 15–25 (ZU44) inclusive was 278 ml. Of this total volume, 100 ml (containing 81.6 and 24.9 μmoles of cholesterol and phospholipid respectively) were used in both ZU45 and ZU46 as the sample to be centrifuged.

TABLE 3 DISTRIBUTION OF CHOLESTEROL AND PHOSPHOLIPID IN PLASMA LIPOPROTEINS ISOLATED BY ZONAL ULTRACENTRIFUGATION

Species	VLDL		LDL		HDL	
	C	PL	C	PL	C	PL
	*% of total C or PL**					
Human plasma	8.8	12.7	68.5	41.7	22.7	45.5
Human lipoproteins (d < 1.210)	7.8	10.5	70.1	48.4	22.2	41.1
Human lipoproteins (d < 1.210)	1.9	5.4	66.4	44.4	32.0	50.2
Canine plasma	0.9	2.1	13.4	11.4	85.6	86.5
Canine lipoproteins (d < 1.210)	1.5	2.5	16.9	16.3	81.6	81.2
Rabbit plasma	14.4	10.8	22.3	11.1	63.3	78.1

* μmoles C or PL in each lipoprotein fraction/μmoles C or PL recovered from zonal rotor × 100.

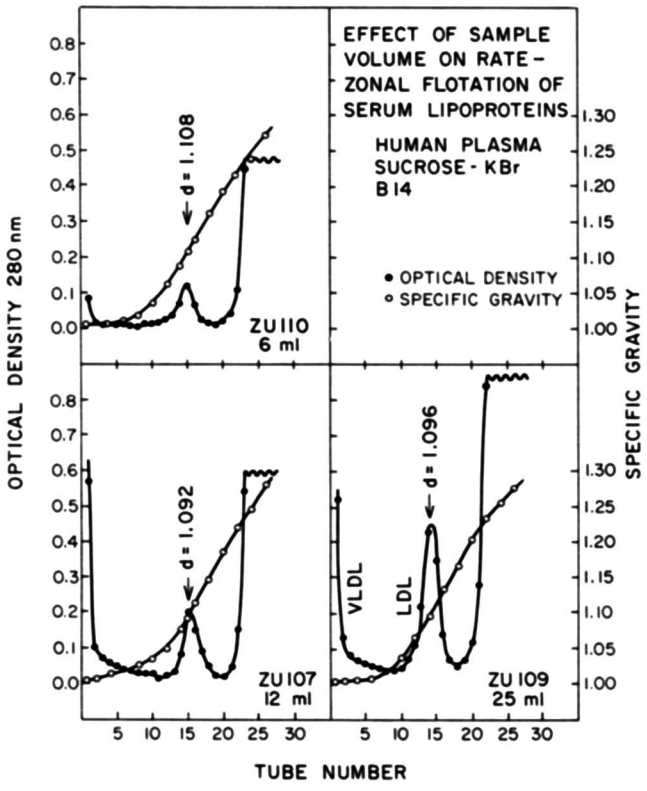

FIG. 7. The gradient material was sucrose–KBr in the density range 1.00–1.28. The samples were various volumes of fresh heparinized (7 USP units/ml) plasma from normal human males. The plasma was adjusted to a specific gravity of 1.28 with solid KBr before it was injected into the rotor. Conditions were otherwise identical with that described in Fig. 5. All centrifugations were for 3 hr. ZU 109 and ZU 110 were from the same plasma sample.

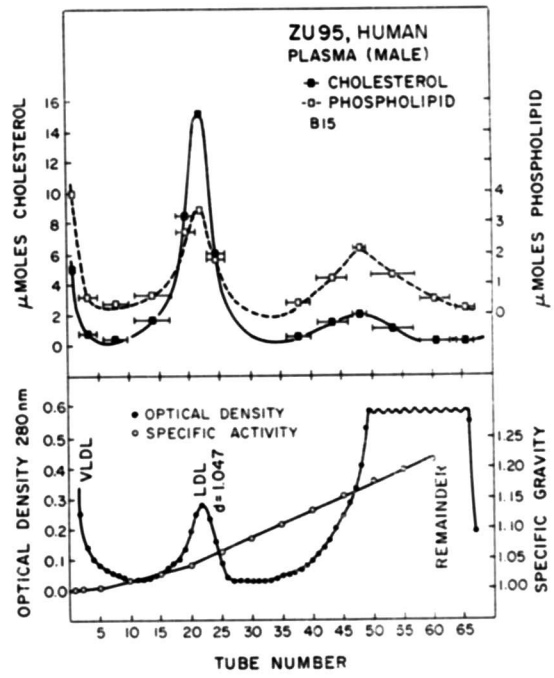

FIG. 8. The gradient material was sucrose–KBr (see Fig. 5). The sample was 25 ml of pooled human plasma (adult males). The plasma was adjusted to a specific gravity of 1.28 with solid KBr before it was injected into the rotor. The overlay was 420 ml of distilled water; 1,200 ml of gradient solution was pumped in followed by the sample and about 10 ml of solution of d 1.28 to fill the rotor. Centrifugation was carried out for 24 hr at 27,000 rpm in the B15 rotor.

was, however, now apparent (peak at d 1.17). The shoulder at the position of higher density was shown by electrophoretic analysis to be a result of contamination of the HDL with the more dense plasma proteins (albumin, etc.) which had not been removed in the preliminary centrifugation. The total lipoproteins with

TABLE 4 COMPARISON OF LIPOPROTEIN-LIPIDS ISOLATED BY SEQUENTIAL OR ZONAL ULTRACENTRIFUGATION

	Lipoprotein Class				
	d < 1.063		d > 1.063		
Species	C	PL	C	PL	Reference
	% of total C or PL				
Rabbit	36.7	21.9	63.3	78.1	*
	58.6	29.6	41.5	70.4	(19)
	37.0	16.9	63.1	83.1	(26)
Dog	16.4	16.2	83.6	83.9	*
	8.6	8.2	91.4	91.8	(19)
	9.8	6.3	90.2	93.6	(26)
Human	77.6	56.7	22.4	43.3	*
	74.1	47.4	25.9	52.6	(19)
	64.4	39.9	35.6	60.1	(26)

* Data are average values recalculated from Table 3. The d < 1.063 fraction includes both VLDL and LDL.

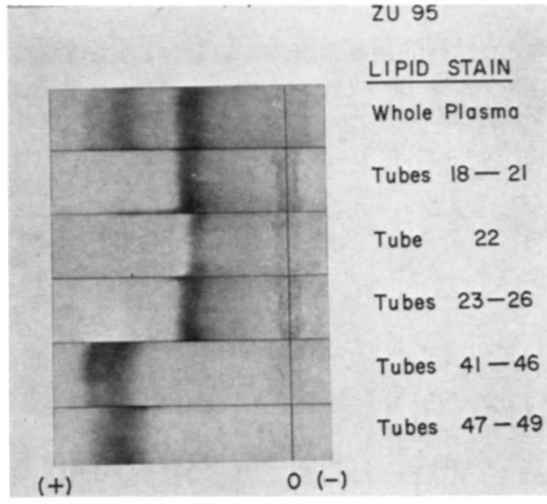

FIG. 9. Paper electrophoresis of human plasma lipoproteins Fractions obtained after centrifugation (ZU 95, Fig. 8) were separated by electrophoresis and stained for lipid.

d < 1.210 also were isolated with the B14 rotor from the plasma of a human male. After centrifugation for 24 hr in the sucrose–KBr gradient, the LDL, which migrated as a β-lipoprotein, had a mean density of 1.04, and the HDL, which migrated as an α-lipoprotein, had a mean density of 1.14. We have not included studies on the

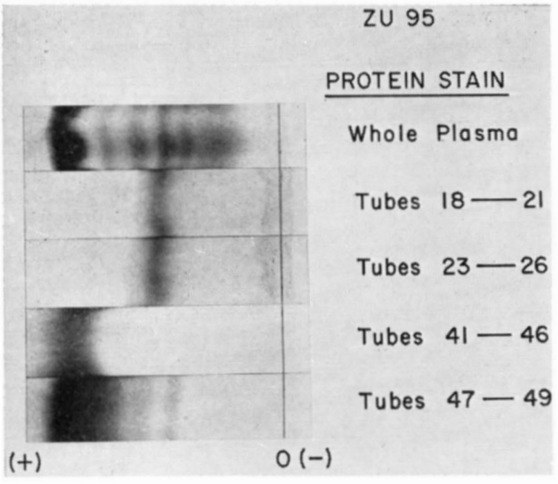

FIG. 10. Paper electrophoresis of human plasma lipoproteins. Fractions obtained after centrifugation (ZU 95, Fig. 8) were separated by electrophoresis and stained for protein.

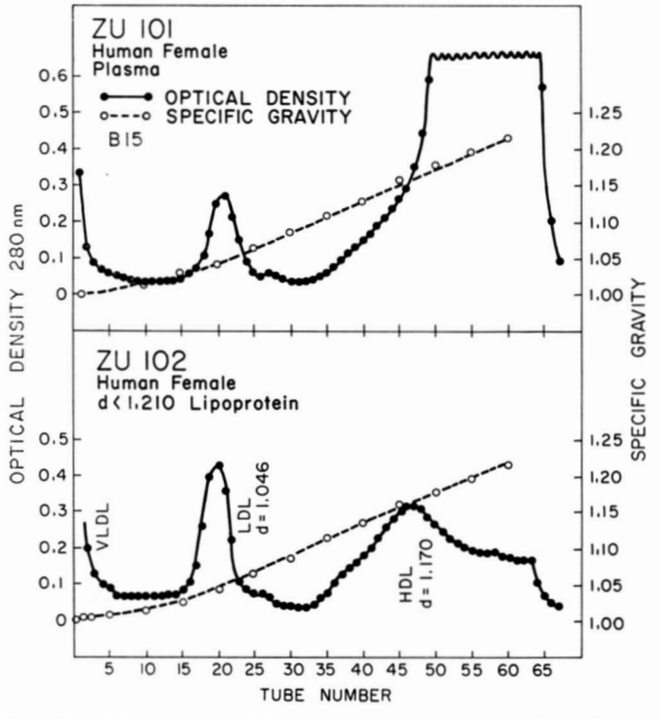

FIG. 11. Plasma or total lipoproteins with d < 1.210 from plasma of human females was centrifuged in the B15 rotor. 25 ml of pooled plasma from four adult females was used (ZU 101). The d < 1.210 lipoprotein (ZU 102) sample was isolated from 48 ml of whole plasma. See Fig. 8 for details of the ultracentrifugation.

electrophoretic mobility of the VLDL in this manuscript, since the VLDL, as reported here, was defined as consisting of both chylomicrons and endogenous VLDL; furthermore, the VLDL and chylomicrons cannot be separated electrophoretically from each other on Whatman 3MM filter paper (3). This paper was used to describe the mobility of the LDL and HDL isolated by zonal ultracentrifugation from human or animal plasma.

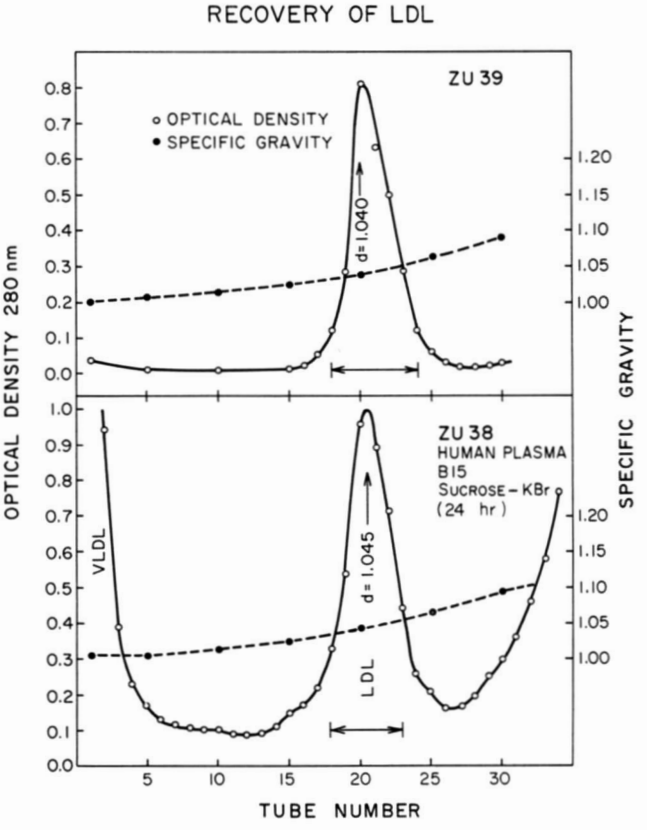

FIG. 12. The gradient material was sucrose–KBr (2:1, w/w) in the density range of 1.00–1.20 (see Fig. 6). The sample (ZU 38) was 100 ml of human plasma, obtained from outdated blood-bank blood. The specific gravity of the sample was not adjusted before injection into the system. In both ZU 38 and ZU 39, the rotor was allowed to build its own gradient. An overlay of 790 ml of distilled water was pumped into the rotor. This was followed, in order, by 766 ml of the d 1.20 sucrose–KBr solution, the sample, and sufficient high density solution to fill the rotor. Centrifugations were carried out for 24 hr at 27,000 rpm in the B15 rotor. At the termination of experiment ZU38, tubes 18–23 inclusive (volume 150 ml) of the LDL peak were combined, and 100 ml of the total, without any further adjustments of specific gravity, were used as the sample for ZU 39.

Several experiments were carried out to determine whether the LDL isolated from plasma by zonal ultracentrifugation could be recentrifuged and recovered from the same location in the gradient. The results of these experiments may be seen in Figs. 12 and 13. It is clear that the LDL, when recentrifuged under identical conditions, reappeared at the same position and density in the gradient as did the original sample of plasma. Furthermore, the recovery of protein, as measured by absorption at 280 nm was approximately 100%. As an additional estimate of recovery, the content of cholesterol and phospholipid in the LDL was measured. The recovery of lipid was complete when the LDL was recentrifuged in either the KBr–NaCl or sucrose–KBr gradients (Table 2).

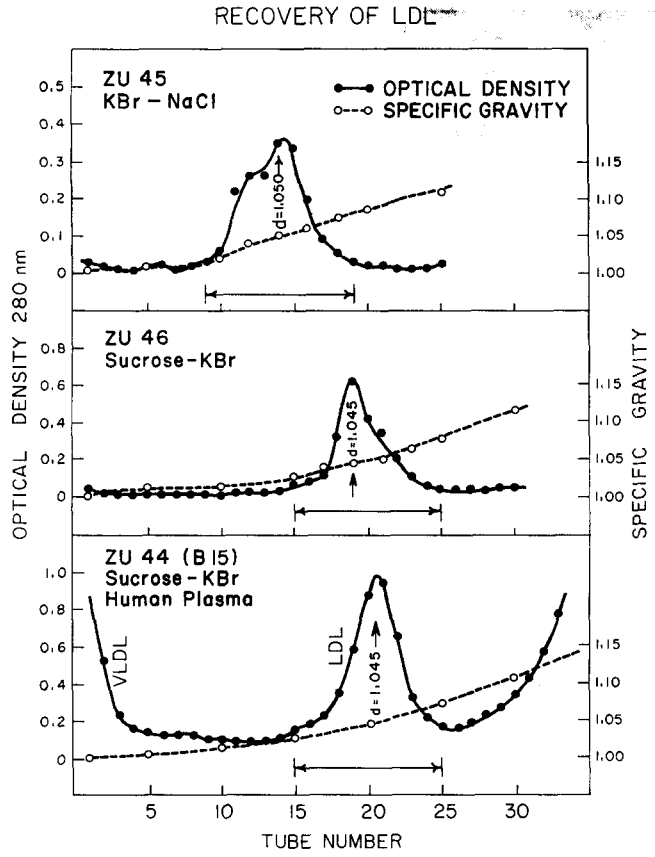

FIG. 13. The gradient material was sucrose–KBr (2:1, w/w) in the density range 1.00–1.28 (ZU 44 and ZU 46) or NaCl–KBr in the density range 1.00–1.30 (ZU 45). The sample (ZU 44) was 100 ml of human plasma obtained from outdated blood-bank blood. The specific gravity of the sample was not adjusted before injection into the system. An overlay of 845 ml distilled water was pumped into the rotor. This was followed, in order, by 710 ml of concentrated gradient solution (sucrose–KBr in ZU 46, and NaCl–KBr in ZU 45), the sample, and sufficient high density solution to fill the rotor. Centrifugations were carried out for 24 hr at 27,000 rpm in the B15 rotor. At the termination of experiment ZU44, tubes 15–25 inclusive (volume 278 ml) of the LDL peak were combined, and 100-ml aliquots of the total were used as the samples for ZU45 and ZU46 without any further adjustments of specific gravity.

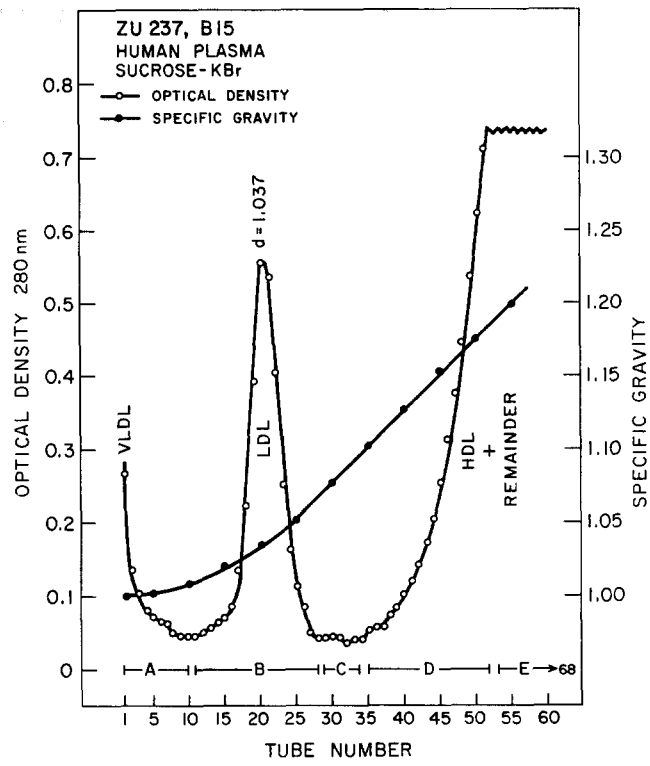

FIG. 14. The gradient material was sucrose–KBr, as described in the legend for Fig. 8. All solutions were 350 μM with respect to EDTA. The sample was 50 ml of fresh pooled human plasma (adult males). The plasma was adjusted to a specific gravity of 1.28. Centrifugation was carried out for 24 hr at 27,000 rpm in the B15 rotor. Fractions A–E were analyzed for cholesterol and phospholipid (Table 1).

The LDL isolated by zonal ultracentrifugation was compared to that fraction with d 1.006–1.063 isolated by sequential methods with an angle-head rotor. The fraction with d 1.006–1.063 (LDL) was isolated as described by De Lalla and Gofman (24), after initial removal of the lipoproteins with d < 1.006 by centrifugation of the plasma in a Spinco No. 40 rotor at 40,000 rpm for 18 hr. The LDL isolated by zonal ultracentrifugation (fraction B, ZU 237, Fig. 14) was dialyzed and concentrated using the Diaflo apparatus and an XM-50 membrane filter. Both samples of LDL were dialyzed exhaustively against a solution of NaCl (d 1.063) which was 350 μM with respect to EDTA. The volume of each sample was adjusted with the NaCl solution to contain 3.5 mg of protein per ml; this amount of LDL protein was present in 3.7 ml of the original sample of human plasma. The samples of LDL were then compared by the technique of double immunodiffusion (22) and by centrifugation in the Spinco Model E analytical ultracentrifuge (24). The mean flotation constant ($S_{f,1.063}$) for either preparation of LDL was 6.0 (Fig. 15). Both preparations of LDL reacted with rabbit antihuman β-lipoprotein and formed bands of identity with each other and with whole plasma (Fig. 16). The greater intensity of the bands obtained with the samples of LDL in comparison to those obtained with plasma reflects probably the greater concentration of LDL protein in the isolated samples (the preparations of LDL contained 3.7 times the concentration of LDL in the original sample of plasma).

Analyses for cholesterol and phospholipid in the fractions designated A–E (Fig. 14) are presented in Table 1.

A variety of animals have been used experimentally for the investigation of blood lipids and lipoproteins. It would be of value, therefore, if one could isolate and characterize the plasma lipoproteins from different species of animals in a continuous density gradient in the zonal rotors and compare the patterns obtained with that of the human.

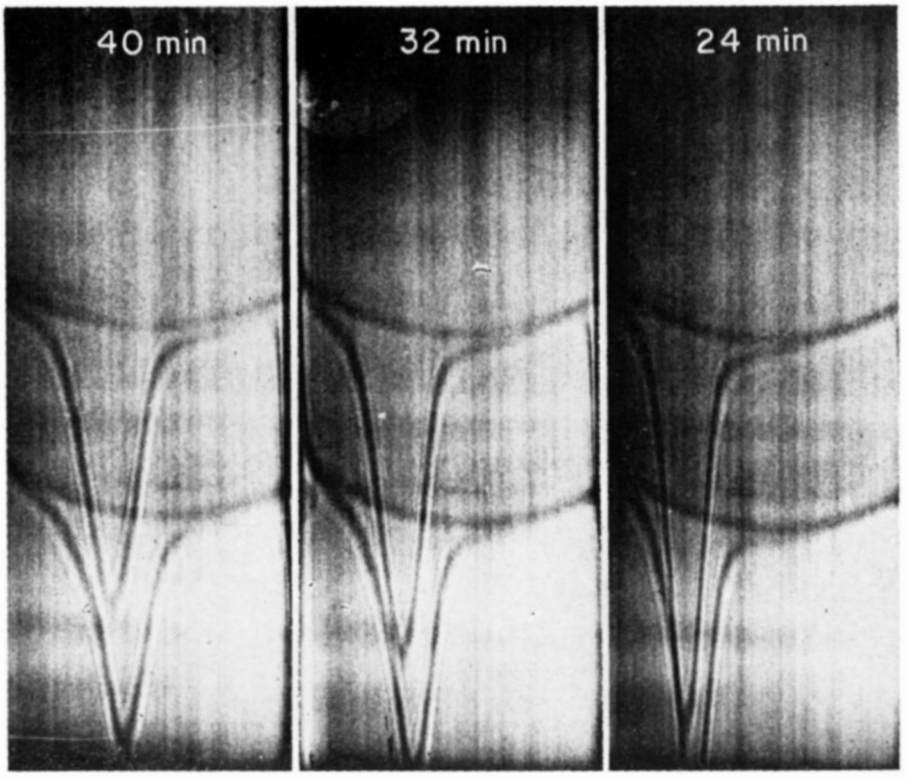

FIG. 15. Analytical ultracentrifugation of LDL. Centrifugation was carried out in the AN-D rotor at 52,640 rpm in the Spinco Model E ultracentrifuge. Temperature was maintained at 23.5°C. The lipoprotein was in a NaCl solution (d 1.063) which was 350 μM with respect to EDTA. The wedge window cell (upper pattern in each photograph) contained 3.5 mg of protein per ml of the d 1.006–1.063 lipoprotein isolated with the Spinco No. 40 rotor (24); the regular double-sector cell contained 3.5 mg of protein per ml of the LDL isolated by zonal ultracentrifugation (Fig. 14, fraction B). Photographs were taken at speed and at 8-min intervals thereafter. Estimates of the flotation constant ($S_{f,\ 1.063}$) were calculated from measurements of the distance of peak migration during the period 24–64 min. The photographs presented in this figure were taken at 24, 32, and 40 min, respectively. Direction of flotation is from left to right.

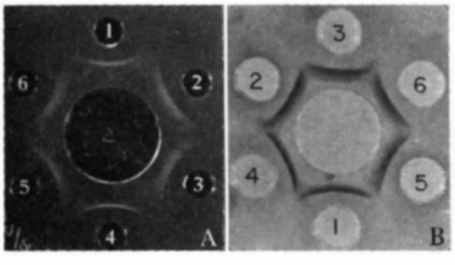

FIG. 16. Double immunodiffusion of LDL. The center well contained 50 μl of rabbit antihuman β-lipoprotein antiserum. Wells 1 and 4 contained 10 μl of human plasma; wells 2 and 5 contained 10 μl of LDL isolated by zonal ultracentrifugation (3.5 mg of protein per ml, fraction B, Fig. 14), and wells 3 and 6 contained 10 μl of LDL (d 1.006–1.063) isolated by sequential procedures (3.5 mg of protein per ml). Panel *A* was photographed directly in a dark field. Panel *B* was photographed after staining with Oil Red O.

Plasma from a normal female dog was centrifuged in the B15 rotor (Fig. 17). Prominent peaks were seen at d 1.040 (LDL) and d 1.188 (HDL). A sample of total lipoproteins with d < 1.210 from the plasma of the same dog was centrifuged under identical conditions; a similar pattern was obtained except that the mean density of the HDL in the absence of the remaining plasma proteins was 1.150. The distribution of cholesterol and phospholipid in the lipoproteins followed the absorption at 280 nm. The electrophoresis of fractions from ZU 116 (Fig. 17) are shown in Figs. 18 and 19. The peak at d 1.04 contained two components; one migrated as a β- and the other as an α_2-globulin. The fraction at d 1.08 also migrated to the α_2 position. The major peak (d 1.188) contained a single band that migrated as an α_1-globulin. When the two peaks were combined and then separated by electrophoresis, three separate components could be identified. On electrophoresis of fractions from the d < 1.210 plasma lipoproteins, results similar to those obtained with whole plasma were found. The existence of an α_2-globulin in the LDL was further verified as follows: aliquots of the d < 1.210 plasma lipoproteins were separated into lipoproteins with d < 1.006 (VLDL), d 1.006–1.063 (LDL), and d > 1.063 (HDL) by sequential procedures after adjustment of the density with KBr. Both β- and α_2-glob-

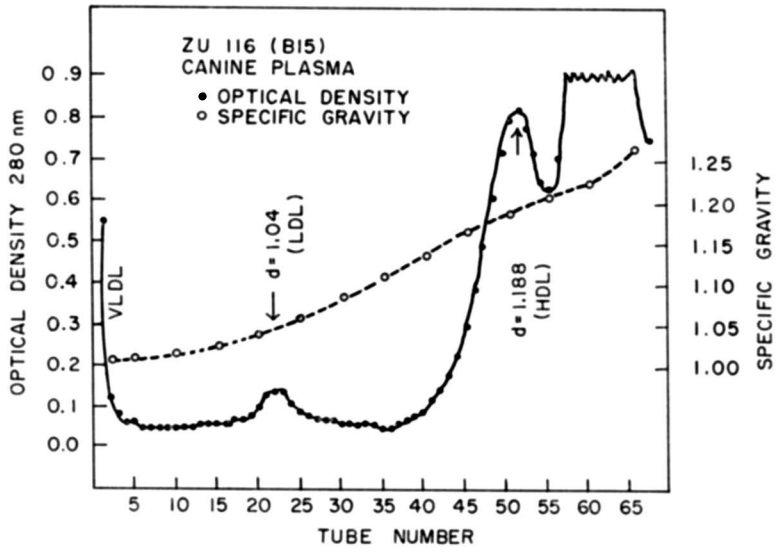

FIG. 17. 50 ml of plasma from a dog were centrifuged in the B15 rotor in a sucrose–KBr gradient for 24 hr. Refer to legend for Fig. 8 for further details.

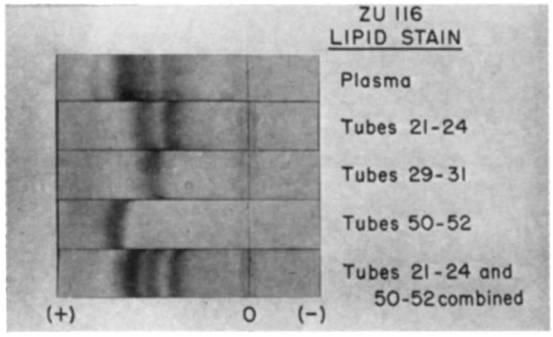

FIG. 18. Paper electrophoresis of canine plasma lipoproteins. Fractions obtained after centrifugation (ZU 116, Fig. 17) were separated by electrophoresis and stained for lipid.

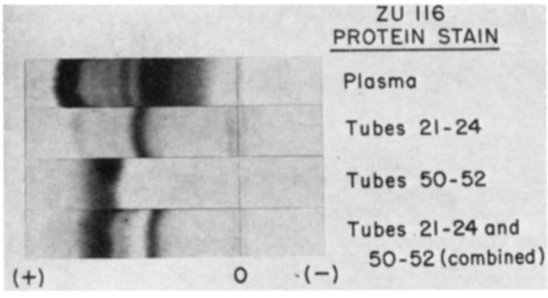

FIG. 19. Paper electrophoresis of canine plasma lipoproteins. Fractions obtained after centrifugation (ZU 116, Fig. 17) were separated by electrophoresis and stained for protein.

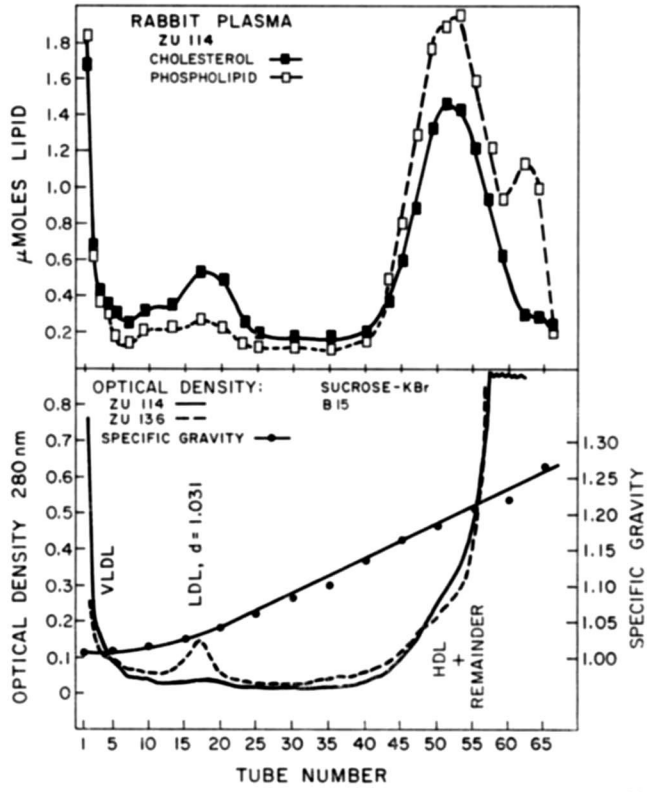

FIG. 20. The gradient material was sucrose–KBr. In ZU 114, 50 ml of pooled plasma from two white rabbits was used. In ZU 136, 30 ml of pooled plasma from three white rabbits was the sample. Refer to legend for Fig. 8 for details.

ulins were found in the d 1.006–1.063 lipoprotein fraction, whereas the HDL contained only an α_1-globulin. Similar results were obtained when plasma from a second dog was centrifuged in the B15 zonal rotor. The VLDL, LDL, and HDL were located at positions in the density gradient similar to those observed in ZU 116 (Fig. 17). On electrophoresis, the LDL again was observed to contain β and α_2 components, whereas the HDL contained an α_1-globulin only.

The distribution of lipoproteins from chicken plasma in the density gradient was observed to possess certain

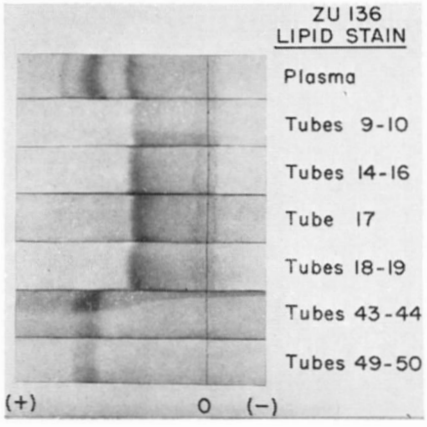

FIG. 21. Paper electrophoresis of rabbit plasma lipoproteins. Fractions obtained after centrifugation (ZU 136, Fig. 20) were separated by electrophoresis and stained for lipid.

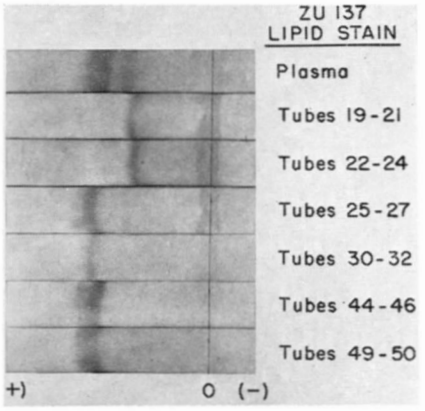

FIG. 23. Paper electrophoresis of rat plasma lipoproteins. Fractions obtained after centrifugation (ZU 137, Fig. 22) were separated by electrophoresis and stained for lipid.

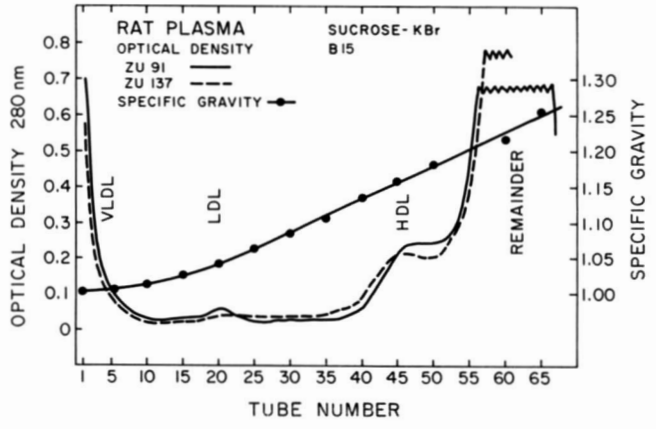

FIG. 22. The gradient material was sucrose–KBr. In ZU 91, 50 ml of pooled rat plasma was used; in ZU 137, 43 ml of pooled rat plasma was introduced into the rotor. Refer to legend for Fig. 8 for details.

characteristics in common with canine plasma. When chicken plasma was centrifuged in the B15 rotor for 24 hr in sucrose–KBr (1.0–1.28), a small peak was observed at d 1.05 (LDL) and a major peak at d 1.185 (HDL). On electrophoresis, the LDL did not migrate with the mobility of a β-globulin but trailed from the origin to the β position. The HDL, however, migrated to the α_1 position as a single sharp component.

The distribution of lipoproteins in the density gradient after zonal ultracentrifugation of plasma from white female rabbits is seen in Fig. 20. The VLDL was readily detectable, as was the LDL (d 1.03). On electrophoresis, fractions from the LDL migrated as a β-globulin, while material in the gradient at d 1.150 (HDL) migrated as an α-lipoprotein (Fig. 21). When the total lipoproteins with d $<$ 1.210 from rabbit plasma were centrifuged, patterns similar to that of whole plasma were obtained, except that the HDL component was now visible with a mean density at 1.160. The distribution of cholesterol and phospholipid in the gradient is seen in Fig. 20.

The distribution of lipoproteins in the density gradient following zonal ultracentrifugation of pooled rat plasma is shown in Fig. 22. The three major lipoprotein fractions, the VLDL, the LDL (d 1.04), and the HDL (d 1.16), were observed. The HDL was separated incompletely from the more dense residual plasma proteins. In general the absorption of the LDL at 280 nm was small in the rat in comparison to the LDL of other species. On electrophoresis, the LDL and HDL migrated as β- and α-globulins, respectively (Fig. 23). It is of interest that the area between the small peak of LDL and the large peak of HDL contained lipoproteins which had the electrophoretic mobility of an α_1-globulin (Fig. 23). When a sample of the total d $<$ 1.210 lipoproteins of rat plasma was centrifuged, patterns similar to that of whole plasma were obtained, except that the HDL (d 1.13) was separated completely from the small amount of higher density plasma proteins which contaminated the sample.

The percentage distribution of phospholipid and cholesterol in the lipoproteins isolated by zonal ultracentrifugation from the plasma of various species is presented in Table 3; these data have been compared with the analytical results of other workers (Table 4). In general, the distribution of cholesterol and phospholipids in the major lipoproteins was similar to that reported by Havel et al. (19) and Hillyard, Entenman, Feinberg, and Chaikoff (26).

DISCUSSION

One of the primary goals of this work was the separation of the major classes of the serum lipoproteins with a single centrifugation. Clearly, both the VLDL and LDL

were separated from the large quantity of the heavier serum proteins as well as from the HDL. The HDL was separated only partially from the more dense plasma proteins in the various gradients using the B14 and B15 titanium zonal rotors. Separation of the VLDL and LDL from serum was achieved without isopycnic equilibrium in as short a time as 1 hr (B14) or 2 hr (B15); furthermore, this separation was achieved using as little as 6 ml (B14) or as much as 150 ml (B15) of serum. Rate-zonal flotation of lipoproteins thus has certain advantages over previous methods and results in a considerable saving of time and labor. One can isolate all the lipoprotein classes with the gradients and rotors that have been employed if the starting sample is the lipoproteins with $d < 1.210$ rather than serum. This would require the initial isolation of the total lipoproteins from serum using angle-head rotors, and, therefore, much of the advantage of the zonal procedure would be lost.

The large peak of the LDL was always present in human plasma and reached its isopycnic position in the gradient (d 1.04) readily. It may be presumed that, because of its relatively low density and high molecular weight, the LDL migrated rapidly to its isopycnic position in the gradient during zonal ultracentrifugation and was thereby separated easily from the other plasma proteins even though the gradients were, as in the case of the sucrose–KBr, quite viscous. The LDL when isolated by zonal ultracentrifugation is not denatured and can be recentrifuged and recovered from the same position in the density gradient. The zonal LDL fraction which had been concentrated by molecular filtration, and the lipoprotein class defined by the density limits 1.006–1.063 but isolated by sequential procedures, have a similar lipid composition, migrate electrophoretically as β-globulins on paper, and are immunologically identical. The $S_{f(1.063)}$ value obtained at 23.5°C for the LDL isolated by either of these methods was 6.0; this figure agrees well with those values measured at 26°C and reported earlier in the literature (25).

The sucrose–KBr gradient was selected for comparison of the plasma lipoproteins from various animal species since the mixture is inexpensive and the gradient is sufficiently stable to allow for the isopycnic separation of the LDL. This gradient is, however, not ideal for the separation of the HDL because of its high viscosity.

When plasma from humans or from rabbits was centrifuged in this gradient, the peak corresponding to the HDL as measured by absorption at 280 nm was not separated from the bulk of the plasma proteins. The distribution of cholesterol and phospholipid, however, was indicative of a distinct peak of lipoprotein in this region. When plasma from dogs or chickens was centrifuged, the peaks of HDL were very much in evidence, although the HDL was separated incompletely from the more dense plasma proteins. The appearance of the HDL under these conditions of centrifugation must reflect the high concentration of the HDL in the plasma of the dog and the chicken. The HDL as measured by absorption at 280 nm in fractions of rat plasma was visualized easily after zonal ultracentrifugation. The separation of the HDL from the more dense plasma proteins in the rat may have resulted from a lower mean density of the HDL of rat plasma in comparison to the other species examined. The lower mean density for the HDL of rat plasma is in agreement with observations made by Camejo (27) and by Lewis, Green, and Page (28) using procedures other than zonal ultracentrifugation.

A clear advantage of the zonal procedure for the isolation of serum lipoproteins is that it is simultaneously preparative and analytical. A second advantage of the zonal procedure is the reduction of time and labor required to isolate the lipoproteins in a state of relative purity in comparison to the usual sequential technique. Patterns of distribution of the serum lipoproteins throughout the continuous density gradient have been obtained for the human and for a number of animal species. From such patterns, we can obtain quantitative data of the amount of protein and lipid in the various peaks, flotation rates, and peak density. It has been observed that various animal species have a dominance of one lipoprotein class over another. This information should be of value in the selection of animal models for study of human disease or for investigations in comparative biochemistry and physiology. These lipoprotein patterns may be altered in man with age, sex, diet, endocrinopathies, and various pathologic states, and these changes should be readily detectable by zonal ultracentrifugation.

One obvious disadvantage of zonal ultracentrifugation is that the lipoproteins are diluted in the gradient during separation, whereas they are concentrated in the upper layers of the solution when they are isolated by the conventional flotation techniques. When lipoproteins are isolated by zonal ultracentrifugation, it may be necessary to concentrate the fractions before they can be analyzed electrophoretically, immunologically, in the analytical ultracentrifuge, or by other procedures. Membrane filters were found to be very effective for this purpose. We suggest that the potential of the procedure of zonal ultracentrifugation for the isolation of the lipoproteins from serum is of sufficient magnitude to outweigh the particular disadvantage of dilution.

We wish to thank Mr. Guinn Dishmon for his excellent technical assistance in this research work, and Dr. W. M. Mitchell for the determination of the S_f of the LDL in the analytical ultracentrifuge.

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