Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation

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Abstract The major lipoprotein density classes (chylomicrons–VLDL, LDL, HDL_2 and HDL_3) were isolated from human plasma in a two-step ultracentrifugal procedure using the Ti-14 zonal rotor. The isolation of the two major high density lipoprotein subclasses (HDL_2 and HDL_3) was achieved in a 24-hr run using a nonlinear NaBr gradient in the density range of 1.00–1.40. The lipoproteins with a density < 1.063 found in the rotor's center were isolated in a second run of 140 min duration using a continuous linear NaBr gradient in the density range of 1.00–1.30. The isolated lipoproteins were analyzed for chemical composition and for electrophoretic mobility; purity of isolated fractions was checked by immunochemistry. The lipoproteins exhibited flotation rates, chemical compositions, and molecular weights similar to those found with the common sequential procedures in angle-head rotors. The amount of lipoprotein lipids in the bottom fraction of the zonal rotor was comparable to that of the angle-head rotor. The described method yields the main lipoprotein density classes free from albumin in a very short running time; compared with the rate-zonal techniques already in use, this method allows the quantitative separation of an additional lipoprotein density class (HDL_2) without increasing the running time. Furthermore, this procedure proved to be suitable for isolation of plasma lipoproteins from subjects with various types and varying degrees of hyperlipoproteinemia.

Supplementary key words nonlinear density gradient · continuous linear density gradient · lipoprotein protein · lipoprotein lipids

The quantitative determination of the different lipoprotein density classes plays an increasingly important role in the clinical laboratory. In particular, a quantification and characterization of the lipoproteins are relevant for the classification and control of primary endogenous hyperlipoproteinemias and for the detection of familial lipoprotein deficiencies. Furthermore, a characteristic pattern of the different lipoprotein density classes or the appearance of an additional lipoprotein in the plasma may occur in certain metabolic situations or in several diseases such as type III hyperlipoproteinemia (1–3), bile duct obstruction (4), dysfunctions of the thyroid gland (5), and malignant tumors (6, 7). In such cases, and probably in many other diseases, a characteristic lipoprotein profile is essential for diagnosis and measurement of the effect of drugs or diet on the metabolism of lipids or lipoproteins.

The development of high-performance titanium zonal rotors (8) has opened up new possibilities for lipoprotein preparation. Wilcox and Heimberg (9, 10), Viikari et al. (11), and Wilcox, Davis, and Heimberg (12) made use of the new titanium zonal rotors for the isolation of lipoproteins and described their advantages and disadvantages in lipoprotein preparation in some excellent reviews. However, separation of lipoproteins in zonal rotors, especially in the clinical laboratory, has not been used very much. In spite of some drawbacks, such as considerable dilution of the lipoprotein fractions, troublesome concentration steps that are occasionally needed, and the development of only a single, relatively small sample per centrifuge rotor, this rate-zonal ultracentrifugal technique offers some advantages over the usual preparative procedures in angle-head rotors for certain purposes. These advantages are a shorter running time, a possibility for immediate quantification of the various lipoprotein density classes by monitoring the protein content without the necessity of using the analytical ultracentrifuge, and, finally, immediate information about the separation quality. Moreover, this procedure opens up the possibility for fractionation and investigation of the rotor contents arbitrarily at any density region. The former technique, originally described by Wilcox, Davis, and Heimberg (12), did not succeed properly in separating the two major HDL density classes. Yet, HDL_2 might play an important role in many pathophysiological states (13). Thus, the purpose of our study was the isolation of the main lipoprotein density classes, particularly the separation of high density lipoproteins.

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Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; VHDL, very high density lipoproteins; C_T, total cholesterol; TG, triglyceride; PL, phospholipid.

The human plasma lipoprotein density classes, isolated by rate-zonal ultracentrifugation, have been examined with regard to their plasma concentrations, distribution, and some physicochemical properties.

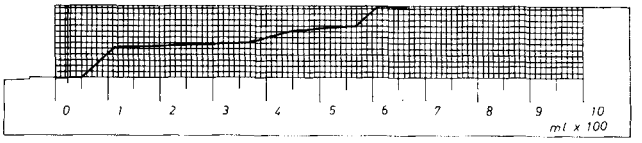

Fig. 1. Contour of the program cam "A," which limits the stroke of the program cam follower and provides in this way the gradient for ultracentrifugal step I. The contour has been drawn on an original program cam-form available from the producer of the pump (Beckman no. 324813).

MATERIALS AND METHODS

Samples and material

Blood was obtained after an overnight fast from 30 clinically healthy volunteers of both sexes, 15–63 yr of age, and was collected in heparin (7 USP units/ml of whole blood) or in disodium EDTA (1 mg/ml of whole blood) or without any anticoagulants. In these subjects, the plasma lipid concentrations and lipoprotein patterns on agarose gel proved to be within the normal limits. Only blood in which no Lp(a) antigen (14) could be detected with specific antibodies was used. Plasma or serum separated from the blood was used immediately for ultracentrifugation. All runs were carried out in a Beckman Spinco model L2-65B ultracentrifuge equipped to accept the Ti-14 zonal rotor. For forming the gradient and filling the rotor, a piston gradient pump, Beckman model 141, was used. The stock solutions for forming the gradient (distilled water, NaBr d 1.30, NaBr d 1.40) were prepared and controlled with respect to their densities with hydrometers at 20°C. At 15°C, all solutions containing 350 μM disodium EDTA were adjusted to pH 7.6 by the addition of 1 M NaOH and then stored; only analytical grade chemicals were used to prepare the solutions (E. Merck, Darmstadt, Germany).

Isolation of lipoprotein density classes

For the preparation of the major lipoprotein density classes (chylomicrons–VLDL, LDL, HDL_2, and HDL_3), we made use of the following two-step procedure.

Step I: Preparation of chylomicrons–VLDL plus LDL, HDL_2, and HDL_3. The gradient was produced in the density range of 1.00–1.40 using distilled water and NaBr solution of d 1.40. In order to form a nonlinear density gradient, the pump was programmed by means of a program cam (cam "A", **Fig. 1**) providing a gradient with three major density grades. At the start of the program area (0–50 ml), the density of the solution pumped into the empty rotor was 1.00. At the end of the program area (665 ml) only the heavy solution (NaBr d 1.40) was pumped into the rotor. The loading of the rotor was performed at 3500 rpm; the gradient was pumped into the rotor from its periphery at a rate of 50 ml/min. 15 ml of plasma or serum was adjusted to a density of d 1.40 by addition of solid NaBr and was diluted with 10 ml of the heavy solution (NaBr d 1.40, pH 7.6). After filling the rotor with the gradient, the plasma–NaBr mixture was injected with a hypodermic syringe from the rotor's periphery and was followed by a 20-ml cushion of the heavy solution in order to ensure that the entire sample was within the rotor and that 45 ml of the overlay was displaced (program area 0–45 ml). The rotor was then accelerated and spun at 41,000 rpm (125,000 *g* at the periphery) and 15°C for various periods of time. At the end of the run, the rotor was decelerated and kept running at 3500 rpm for unloading. The contents were displaced by pumping NaBr solution of density 1.40 into the rotor from the periphery at a rate of 20 ml/min, and 10-ml fractions were collected. The rotor content was monitored by continuous measurement of absorbance at 280 nm in an LKB Uvicord II absorptiometer equipped with a quartz flow-through cell. In addition, the absorbances of individual 10-ml fractions were measured at 280 nm with a Zeiss spectrophotometer PM 4. The densities of the rotor's contents were determined in the following way: 10 ultracentrifugal two-step runs were carried out in the same way as described above, except that the plasma sample was replaced by NaBr solutions of densities 1.40 and 1.30 for steps I and II, respectively. The densities of all collected 10-ml fractions were determined by measuring the refractive indices in an Abbe refractometer at 20°C and finding the corresponding values in the Handbook of Chemistry and Physics (52nd ed., 1971–72, Chemical Rubber Co., Cleveland, Ohio). In the same way, the densities of 10-ml fractions representing the protein-free zones from the runs in which lipoproteins had been isolated from the plasma (i.e, 50–70 ml, 140–160 ml and 350–400 ml in step I and 100–150 ml and 300–665 ml in step II) were determined. The densities of the protein-free zones from those runs were compared with the densities of the corresponding rotor regions from the sample-free reference runs. Since these densities agreed closely, only the density courses of reference runs are indicated in the figures.

The first 50 ml leaving the rotor from its center contained the lipoprotein density classes with density $<$ 1.063; this fraction was collected for recentrifugation in the ultracentrifugal step II.

Step II: Separation of the lipoproteins $d < 1.063$ into chylomicrons–VLDL and LDL. The density of the first 50 ml from the ultracentrifugal step I was adjusted to

1.30 by addition of solid NaBr; a pH of 7.6 was produced by addition of 0.2 M NaOH. A continuous linear density gradient in the range 1.00–1.30 was formed from distilled water and NaBr solution of d 1.30 by means of the gradient pump equipped with an appropriate program cam. At the start of the program area (0–50 ml), the density of the solution pumped into the empty rotor was 1.00. At the end of the program area (665 ml), only the heavy solution (NaBr d 1.30) was pumped into the rotor. The loading of the rotor with gradient and sample was accomplished in the same way as described for step I with a cushion of 10 ml of NaBr solution (d 1.30). Ultracentrifugations were carried out at 42,000 rpm (131,000 *g* at the periphery) and 15°C for various periods of time. Afterwards, the rotor content was displaced from the rotor at 3500 rpm with the heavy solution (d 1.30) and monitored and fractionated as described above.

Isolation of lipoprotein density classes from hypertriglyceridemic sera

In order to prove the method to be suitable for the separation of plasma lipoproteins from patients with hyperlipoproteinemia, serum from different hyperlipemic individuals was used. By separation of plasma lipoproteins from patients with severe hypertriglyceridemia, an overlap of the lipoprotein fraction of density < 1.063 and HDL_2 may occur. In order to avoid this problem, we successfully applied the following modification.

Chylomicrons–VLDL and LDL were removed by precipitation with polyanions according to the method of Burstein, Scholnick, and Morfin (15): 3 ml of 4% sodium phosphotungstate, pH 7.6, and 0.75 ml of 2 M $MgCl_2$ were added to 30 ml of serum, and the precipitate was removed by centrifugation at 6000 *g* for 10 min. 15 ml of the clear supernatant solution was adjusted to a density of 1.40 by the addition of solid NaBr and was diluted with 10 ml of the NaBr solution, d 1.40, pH 7.6. Separation of HDL was carried out as described for step I. Chylomicrons–VLDL and LDL from hypertriglyceridemics were isolated directly from 15 ml of whole serum by using the linear density gradient 1.00–1.30 as described for step II. The specific details are given with Table 4 and Fig. 5.

Chemical analysis

The separated lipoprotein density classes were analyzed for total cholesterol, triglyceride, lipid phosphorus, and protein. The fractions of the rotor contents belonging to one lipoprotein peak were pooled and dialyzed against a 200-fold volume of 0.15 M NaCl, pH 8.0, containing 350 μM disodium EDTA at 4°C. For chemical analyses, 60 min of dialysis proved to be satisfactory; for electrophoretic and immunological studies, lipoprotein fractions were dialyzed exhaustively against several changes of 0.15 M NaCl, pH 8.0. Protein content of the dialyzed fractions was measured by the method of Lowry et al. (16), using bovine serum albumin as a standard. The turbidity caused in some fractions by high lipid concentrations was cleared by extracting the solutions with diethyl ether at room temperature after color development was complete. Aliquots of the lipoprotein fractions were extracted (17) and analyzed for total cholesterol (18), triglyceride (17), and lipid phosphorus (19). A factor of 25 was used to convert values of phosphorus to phospholipids. For exact reading of extinctions in a linear region of the reference curves, the chloroform phases of the lipid extracts were evaporated under nitrogen and redissolved in a smaller volume of chloroform.

Only for lipoprotein electrophoresis, double immunodiffusion, and sedimentation and flotation experiments were the lipoproteins dialyzed against saline for 48 hr and concentrated by dialysis against polyethylene glycol, mol wt $>$ 17,000 (Fluka, A.G., Buchs, Switzerland). In some cases, concentration of lipoproteins displaced from the zonal rotor was carried out by spinning the solutions in a fixed-angle rotor at appropriate densities and *g*-hours in the Beckman L2-65B ultracentrifuge, or in a few other cases by means of ultrafiltration in a Diaflo cell, model 32, using a PM-30 membrane (Amicon Corp., Lexington, Mass.). Because of some possible disadvantages of the two latter procedures (see Discussion), if not stated otherwise all given results with regard to immunochemical and physicochemical properties are based on investigation of lipoproteins concentrated by polyethylene glycol.

Electrophoresis, immunochemical methods, and physicochemical investigation

Lipoprotein electrophoresis was carried out on 0.5% agarose gel in a barbital buffer, pH 8.2, ionic strength 0.045. For staining the lipoprotein bands, Sudan black B in 60% (v/v) ethanol was used (20).

The presence of albumin contamination in the concentrated lipoprotein fractions was checked before and after delipidation (21) by the double immunodiffusion technique and immunoelectrophoresis described by Ouchterlony (22), using anti-human albumin serum (Behringwerke A.G., Marburg/Lahn, Germany). Antiserum to Lp(a) antigen was a gift from H. N. Magnani, Birmingham, England. Sedimentation and flotation experiments (23, 24) were performed in an analytical ultracentrifuge (Beckman model E) equipped with electronic speed control. Flotation coefficients were calculated from the formula:

$$(F_c)_{app} = -\frac{\ln r_2 - \ln r_1}{60\omega^2(t_2 - t_1)}$$

where $(F_c)_{app}$ is the apparent flotation coefficient at concentration c, r_1 and r_2 are the distances of boundaries from the rotor axis at different time intervals (t_1 and t_2),

and ω is the angular velocity. For calculation of molecular weights, the high-speed equilibrium technique of Yphantis (25) was carried out using the Rayleigh interference optic and a six-channel centerpiece. Protein concentrations of the solutions analyzed were between 0.15 and 2.0 mg/ml. Equilibrium runs were performed at 14,000 rpm for 30–48 hr. For calculation of the molecular weight, the equation

$$M_{app} = \frac{2RT}{(1 - \bar{v}\rho)} \cdot \frac{1}{\omega^2} \cdot \frac{d \ln c}{d(r^2)}$$

was used, where $d \ln c/d(r^2)$ was obtained from the slope of the $\ln y$ vs. r^2 plot.

The partial specific volume was determined using a precision density-measuring device (DMA 02/C, A. Paar Instruments, Graz, Austria) allowing an accuracy in density determination of aqueous solutions of $\pm 3 \times 10^{-6}$ g/cm^3. The determination was carried out in 0.15 M NaCl (pH 7.4) at 25.02°C.

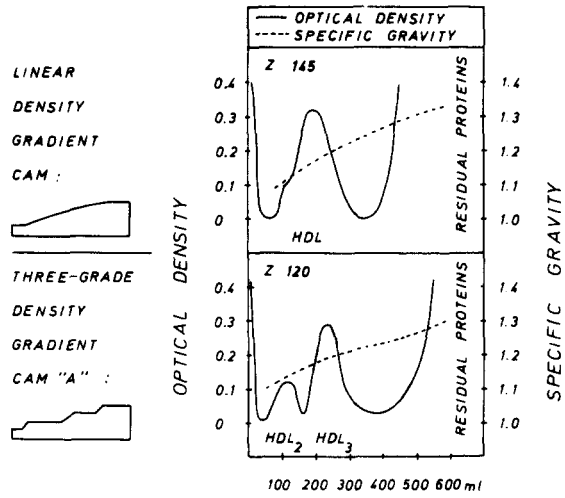

Fig. 2. Effect of the nonlinear density gradient on preparation of HDL_2 and HDL_3 (cam "A") (Z 120) compared with that obtained in a linear density gradient (Z 145) in the same density range (1.00–1.40). 15 ml of heparinized plasma from the same blood sample was centrifuged in each experiment. The centrifugations were carried out for 24 hr, 15°C, 41,000 rpm.

RESULTS

Separation of HDL_2 and HDL_3 (step I)

In order to achieve optimal separation of HDL_2 from HDL_3, a series of experiments was carried out using various density gradients and running times. Because in a continuous linear density gradient the HDL_2 usually appears as a small shoulder on the HDL_3 peak at the light side of the gradient (11, 12), we tried to achieve better resolution of these two density classes in a single run. Considering the fact that in the angle-head rotor HDL_2 floats at a density of 1.125 whereas HDL_3 floats at d 1.210, we tried to achieve a similar result in the zonal rotor.

After testing several nonlinear density gradients of NaBr, a density gradient with three grades was found to be optimal for the separation of HDL_2 from HDL_3, even though the form of the gradient shifted during the run as a result of the high diffusion constant of NaBr.

Finally, the density gradient provided by the program cam "A" (Fig. 1) proved to be the most appropriate one. In order to get information on the actual form of the gradient in the rotor during centrifugation, we examined the density course at various points of time. The total volume of the gradient in the Ti-14 rotor (665 ml) was collected in 10-ml fractions, which were examined with respect to their specific gravity: *a,* straight out of the pump; *b,* pumped into the rotor at 3500 rpm and promptly displaced with NaBr solution d 1.40; and *c,* introduced into the rotor at 3500 rpm and run at 41,000 rpm for 5, 14, 24, and 30 hr, respectively.

These studies showed that the gradient at density 1.30–1.40 in the peripheral 100 ml persists over a period of roughly 24 hr (d 1.28–1.35). This steepness of the gradient keeps back the residual plasma proteins at the rotor's very periphery and in this way extends the path length for floating lipoproteins. **Fig. 2** shows an HDL preparation in a linear density gradient compared with the three-grade density gradient provided by program cam "A" (Fig. 1).

The two other density grades (1.00–1.17 and 1.18–1.30) become flattened in a relatively short time, but, in fact, they are essential for a good result in the separation of HDL_2 from HDL_3.

After finding the gradient created by program cam "A" to be the most effective one for separation of HDL_2 from HDL_3, various running times were tested, and 24 hr was found to be optimal.

Using this procedure the lipoproteins of density $<$ 1.063 were found in the rotor's center clearly separated from HDL_2 and HDL_3. In **Fig. 3** a representative pattern of separation of HDL_2 from HDL_3 and the residual plasma proteins is shown (step I).

Separation of chylomicrons–VLDL and LDL (step II)

The separation of the lipoprotein mixture of density $<$ 1.063, found after step I in the rotor's center, into chylomicrons–VLDL and LDL was achieved in step II. For this purpose, another series of experiments with various gradients was carried out.

Since the flotation rates of the lipoprotein classes of d $<$ 1.063 differ from each other to a much higher degree than

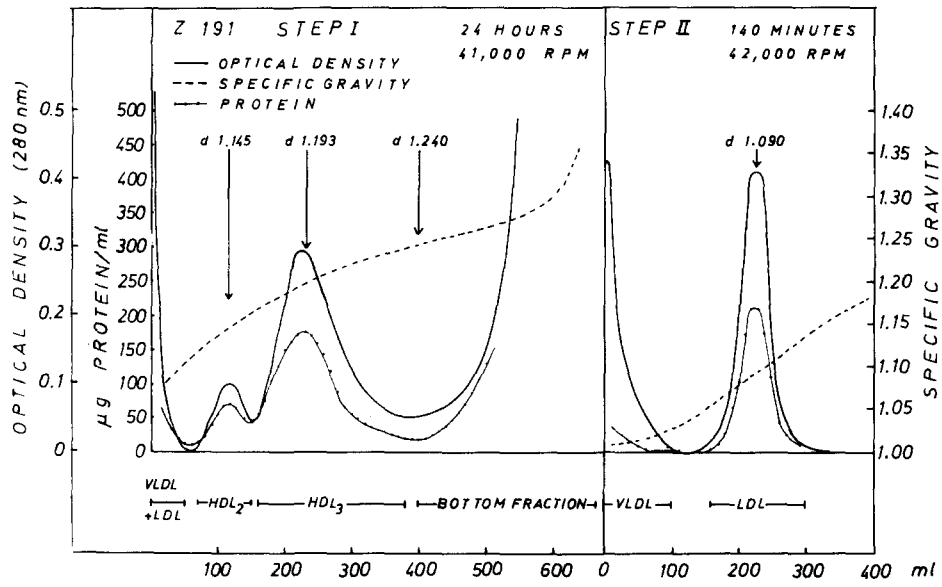

Fig. 3. Representative pattern of lipoprotein isolation in the described two-step procedure. For ultracentrifugal step II, the first 50 ml (VLDL + LDL) leaving the rotor after step I was used.

those of HDL_2 and HDL_3, a continuous linear density gradient proved to be satisfactory. It was our aim to separate VLDL and LDL in sharp zones near the rotor's center to keep the dilution volume small; on the other hand, a certain distance between VLDL and LDL is desirable because of the occasional appearance in high concentrations of an additional lipoprotein fraction, as found in type III hyperlipoproteinemia between the two regular lipoprotein density classes (26). For this purpose a continuous linear density gradient in the range d 1.00–1.30 proved to be very effective. The most satisfactory running time was 140 min at 42,000 rpm and 15°C. Under these conditions a clear separation of VLDL and LDL into sharp zones and at a suitably long distance was achieved (Fig. 3). An increase in the running time up to a maximum of 420 min resulted in a decrease of the distance between VLDL and LDL. **Table 1** shows the plasma concentrations and chemical compositions of the main plasma lipoprotein density classes isolated by means of the suggested two-step procedure.

Disodium EDTA-treated plasma, heparinized plasma, and serum, as well as a mixture of aliquots of the above from one blood sample, were used as sources for lipoprotein isolation in separate runs. The results of lipoprotein isolation with respect to concentration, chemical composition, and electrophoretic mobility were identical, as proved in samples from normolipemic and hypertriglyceridemic subjects.

In order to prove that the suggested method gives reproducible results, we performed the following series of experiments. Four healthy volunteers were kept on a standard isocaloric diet (40% fat, 40% carbohydrate, and

TABLE 1. Plasma concentrations and chemical compositions of lipoproteins isolated in the zonal rotor[a]

Lipoprotein Density Class[b]	Plasma Concentration[c]	Percentage Composition				C_T/PL[d]
		Cholesterol (Total)	Triglyceride	Phospholipid	Protein	
VLDL	70 ± 40	14.3 ± 2.8	53.7 ± 7.0	19.9 ± 3.7	12.0 ± 4.5	0.72
LDL	298 ± 58	40.9 ± 3.4	7.8 ± 1.7	25.5 ± 3.0	25.8 ± 3.8	1.60
HDL_2	105 ± 37	23.3 ± 3.0	6.6 ± 2.9	29.9 ± 4.7	40.1 ± 5.1	0.78
HDL_3	267 ± 41	16.1 ± 2.5	3.9 ± 2.1	27.4 ± 3.3	52.5 ± 4.5	0.59

In 19 runs, the recovery of total cholesterol in the lipoproteins without the bottom fraction was 92.1 ± 8.2% (SD).

[a] The given data represent the mean values ± SD of lipoprotein preparations from the blood of 30 healthy volunteers of both sexes (18 males, 12 females), 15–63 yr of age (mean 29 yr), after a 14-hr fast.

[b] The lipoprotein fractions are those defined representatively in Fig. 3.

[c] Plasma concentrations in mg of lipoprotein/100 ml of plasma were calculated from the sum of the amounts of protein, total cholesterol (for weight calculation, the ratios of unesterified cholesterol to cholesterol ester [linoleate] in different lipoprotein density classes from Ref. 32 were used), triglyceride, and phospholipid.

[d] Ratio of mg of total cholesterol to mg of phospholipid.

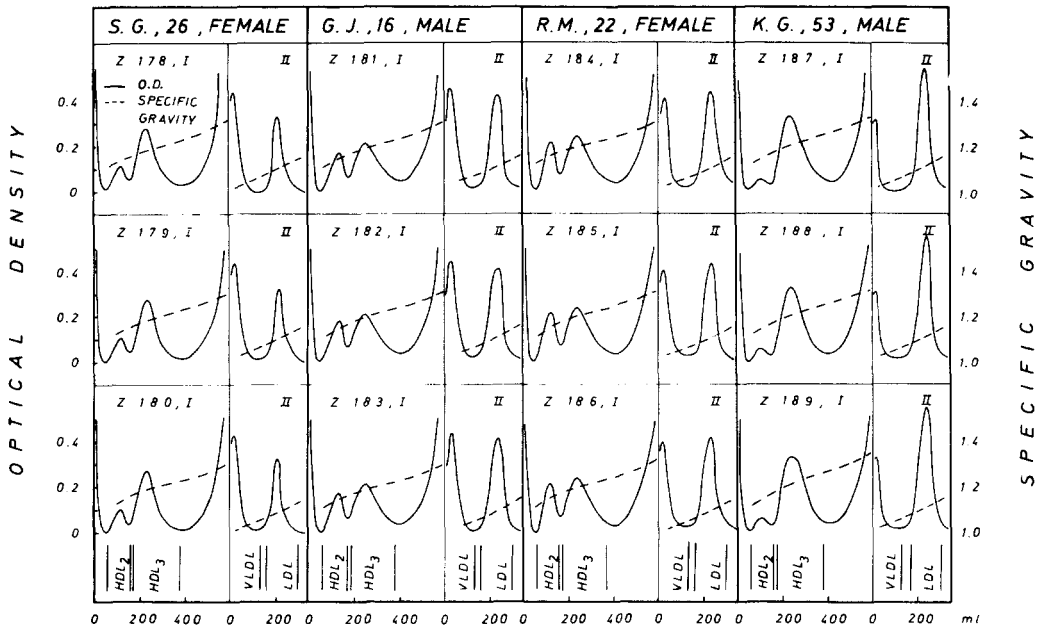

Fig. 4. Separation of lipoproteins from the plasma of four volunteers, carried out on 3 consecutive days after an overnight fast. For ultracentrifugation step I (*I*) the NaBr density gradient in the range 1.00–1.40 provided by program cam "A" was employed. For step II (*II*), a continuous linear NaBr density gradient in the range 1.00–1.30 was used.

20% protein). Plasma, freshly obtained on 3 consecutive days after an overnight fast, was centrifuged immediately in the zonal rotor. The separation of lipoproteins from these experiments is shown in **Fig. 4**; the corresponding lipoprotein concentrations are given in **Table 2**.

Estimation of the "bottom fraction" at step I

In the "bottom fraction," i.e., in the most peripheral 265 ml of the rotor, d > 1.240, 1.7–2.8% of the total plasma cholesterol and 4.2–11.9% of the plasma phospholipids were found. These findings correspond well to the data reported by Havel, Eder, and Bragdon (27) for the d 1.21 infranate. The ratio mg C_T/mg PL in the bottom fraction was 0.18–0.36 (range). In three single runs the 265-ml "bottom fraction" was subfractionated into 50-ml fractions, and total cholesterol as well as phospholipids were determined for calculation of the ratio mg C_T/mg PL. From the center to the periphery, the ratio tended to decrease (**Table 3**). The ratio mg C_T/mg PL reported by Havel et al. (27) for the d 1.21 infranate (< 0.20) and the ratio mg C_T/mg PL reported by Alaupovic et al. (28) for the VHDL (0.14) correspond to those found after Z 195, Z 196, and Z 230 in the most peripheral 65 ml of the zonal rotor.

Recentrifugation of HDL

In order to test for a possible breakdown of HDL during ultracentrifugation and concentration by polyethylene glycol, the following experiment was performed. HDL_2 and HDL_3 were isolated from 15 ml of plasma by step I. After chemical analysis, HDL_2 and HDL_3 were mixed (300 ml) and concentrated to 30 ml. The density of the fraction was adjusted to d 1.40 (NaBr), and separation of HDL_2 and HDL_3 was performed according to step I once again. A lipoprotein pattern was found with a ratio of HDL_2 to HDL_3 similar to the first preparation: the recovery for HDL_2 was 81% and for HDL_3 was 85%. No difference in chemical composition could be noticed in li-

TABLE 2. Lipoprotein concentrations,[a] from four normolipemic volunteers,[b] after zonal centrifugation carried out three times on 3 successive days

Initials, Age, and Sex	Run	VLDL[c]	LDL	HDL_2	HDL_3
S. G., 26, female	Z 178 I/II	114	180	76	289
	Z 179 I/II	78	191	78	282
	Z 180 I/II	71	197	85	294
G. J., 16, male	Z 181 I/II	149	324	142	261
	Z 182 I/II	133	340	128	270
	Z 183 I/II	119	334	133	278
R. M., 22, female	Z 184 I/II	101	379	237	254
	Z 185 I/II	78	385	230	248
	Z 186 I/II	63	361	233	262
K. G., 53, male	Z 187 I/II	34	448	62	308
	Z 188 I/II	30	464	77	317
	Z 189 I/II	42	462	68	304

[a] mg of lipoprotein/100 ml of plasma.
[b] Volunteers were kept on a standard isocaloric diet (40% fat, 40% carbohydrate, and 20% protein). Blood was drawn after an overnight fast.
[c] Lipoproteins are those defined in Fig. 4.

TABLE 3. Distribution of total cholesterol and phospholipids in five subfractions of the "bottom fraction"[a]

	Subfraction[b]														
	400–450			450–500			500–550			550–600			600–665		
	C_T[c]	PL[d]	Ratio[e]	C_T	PL	Ratio	C_T	PL	Ratio	C_T	PL	Ratio	C_T	PL	Ratio
Z 195	10	18	0.56	23	48	0.48	58	132	0.44	86	344	0.25	98	795	0.12
Z 196	5	14	0.35	26	67	0.39	112	386	0.29	134	670	0.20	195	1080	0.18
Z 230	0	0		30	45	0.67	110	180	0.61	120	330	0.36	180	1300	0.14

[a] The most peripheral 265 ml in the zonal rotor (d > 1.240), defined representatively in Fig. 3.
[b] ml in the Ti-14 rotor.
[c] μg C_T (total cholesterol) in one subfraction.
[d] μg PL (phospholipid) in one subfraction.
[e] Ratio μg C_T/μg PL.

poprotein fractions before or after recentrifugation. Also, lipoprotein electrophoresis showed no indication of an alteration of the lipoprotein fractions. In the bottom fraction of the second run, less than 3% of the original HDL protein and less than 1% of the original HDL lipids were found.

Modification in lipoprotein isolation in hypertriglyceridemia

In order to prove the method to be suitable in hyperlipoproteinemia, we centrifuged plasma from different hyperlipemic individuals. In hyperbetalipoproteinemia, no modification of running conditions was necessary. A clear separation of the various main lipoprotein density classes was achieved from plasma with a cholesterol content as high as 720 mg/100 ml of plasma.

In hypertriglyceridemia with more than 350 mg TG/100 ml of plasma, an overlap of the lipoprotein fraction d < 1.063 with HDL_2 could occur because of the high amount of VLDL. In order to avoid the merging of HDL_2 with less dense lipoproteins, the lipoproteins d < 1.063 were removed by precipitation with sodium phosphotungstate and $MgCl_2$, as described in Materials and Methods. This modification has been compared with the cam "A" procedure without precipitation in serum with normal lipid concentration and has proved to be equally suitable (**Table 4**). In hyperlipoproteinemic sera it was applied successfully (**Fig. 5**).

TABLE 4. Lipoprotein concentration,[a] from a normolipemic blood donor,[b] found with the suggested zonal ultracentrifugal procedure and with the modification necessary in case of hypertriglyceridemia

Run	Method	VLDL	LDL	HDL_2	HDL_3
Z 171 I/II	Cam "A"	93	340	138	271
Z 173 I/II	Precipitation + cam "A"	97	348	131	264

[a] Lipoprotein concentration in mg of lipoprotein/100 ml of plasma has been calculated as for Table 1.
[b] Blood was obtained on 2 consecutive days after an overnight fast from one female subject, aged 32 yr, kept on a standard isocaloric diet (40% fat, 40% carbohydrate, 20% protein). Serum was separated from the freshly obtained blood and used for ultracentrifugation immediately.

Physicochemical investigation of isolated lipoproteins

Lipoproteins isolated by rate-zonal ultracentrifugation were concentrated and investigated by agarose gel electrophoresis (**Fig. 6**). VLDL from fasting normals showed a migration in the pre-β position and LDL in the β position. HDL_2 exhibited a somewhat faster migration rate than HDL_3.

Concentrated lipoprotein fractions were investigated for the presence of albumin by immunodiffusion and immunoelectrophoresis. Lipoprotein concentrations were in the range of 5–20 mg/ml. No positive reaction was found with any density class. By investigating total delipidized VLDL, LDL, and HDL_2 for the presence of albumin, no positive reaction could be observed using various antigen/antibody concentrations. By this method an albumin contamination of at least 1% of the total apoprotein would have been detected. From 10 delipidized HDL_3 preparations tested, 3 formed a weak precipitin line with anti-albumin serum.

In flotation experiments, LDL, HDL_2, and HDL_3 showed a single peak in the analytical ultracentrifuge.

TABLE 5. Values of some physicochemical characteristics of lipoproteins[a] isolated in several runs with the suggested two-step procedure

Lipoprotein Density Class	$S_{f,1063}$	$F_{1.21}$	$\bar{v}$[b]	Mol wt[c]
LDL	6–8		n.d.[d]	n.d.
HDL_2		6.5–8.7	0.903	345,000
HDL_3		3–5	0.871	187,000

For details see Materials and methods.
[a] Lipoproteins are those defined representatively in Fig. 3.
[b] Partial specific volume, cm^3/g.
[c] Molecular weight determined by equilibrium runs.
[d] Not determined.

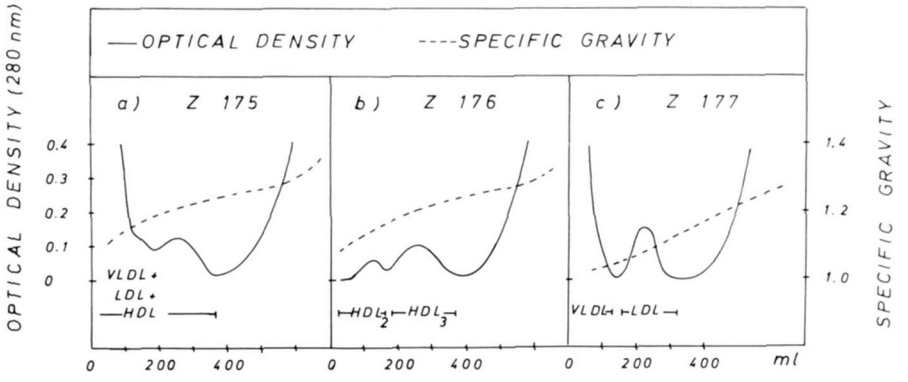

Fig. 5. Lipoprotein isolation in marked hypertriglyceridemia (915 mg of TG/100 ml of plasma). *a*) Merging of HDL with lipoproteins of lower density (cam "A" without precipitation); *b*) isolation of HDL_2 and HDL_3 from the clear supernate obtained after precipitation of the lipoproteins d < 1.063 with polyanions from serum (precipitation + cam "A"); *c*) isolation of chylomicrons–VLDL and LDL directly from 15 ml of whole serum by using the linear density gradient 1.00–1.30 as described for step II. The sera were separated from one blood sample.

Fig. 7 shows the patterns of HDL_2 and HDL_3 at d 1.21 in the analytical ultracentrifuge. Whether concentrated by means of polyethylene glycol or by spinning the solutions in the angle-head rotor, the same behavior of LDL, HDL_2, and HDL_3 in the analytical ultracentrifuge was observed. Values of some physicochemical characteristics of lipoproteins isolated by rate-zonal ultracentrifugation are shown in **Table 5**.

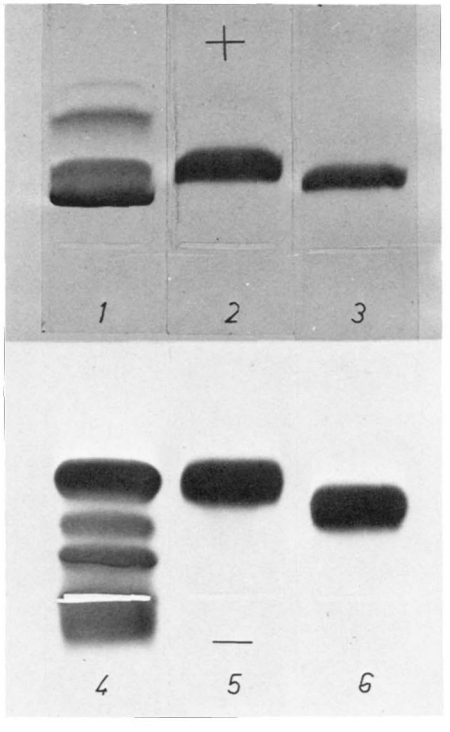

Fig. 6. Agarose gel electrophoresis of lipoprotein density fractions isolated by rate zonal ultracentrifugation of normal plasma. *1* and *4*, samples of normal plasma; *2*, VLDL, *3*, LDL; *5*, HDL_2; *6*, HDL_3. Slide *1* was stained with Sudan black B and slides *2–6* were stained with amido black 10B. Samples *1–3* and *4–6*, respectively, were run simultaneously.

DISCUSSION

The aim of this study was the isolation of all main lipoprotein density classes free from other serum contaminants in a short running time. For this purpose we made use of the rate-zonal ultracentrifugal technique. Using a nonlinear density gradient in the range d 1.00–1.40, achieved by a new designated program cam in the Beckman gradient pump model 141, we succeeded in separating the two major HDL subclasses from each other and the residual plasma proteins in a 24-hr run (step I). The lipoproteins of density < 1.063, merged after step I in the rotor's center, were separated into VLDL and LDL in the ultracentrifugal step II. In a continuous linear density gradient in the range d 1.00–1.30, a clear separation of the lipoproteins of d < 1.063 was achieved. VLDL and LDL float in this gradient in sharp zones comparable to those obtained in a steeper gradient (e.g., 1.00–1.40). Furthermore, between these two regular density classes a satisfactory distance remains in order to accommodate oc-

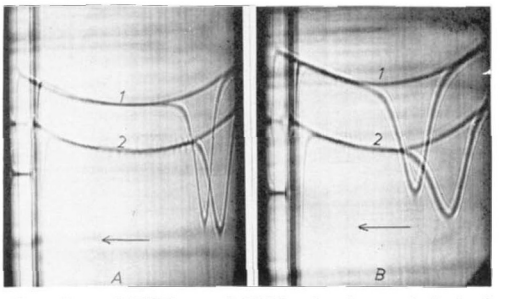

Fig. 7. Flotation of HDL_2 and HDL_3 in the analytical ultracentrifuge. Samples displaced from the Ti-14 rotor were concentrated by spinning in a type 65 fixed-angle rotor at d 1.21 for 24 hr at 140,000 *g*. Floating lipoproteins were dialyzed against NaBr solution of d 1.210 and investigated by means of the analytical ultracentrifuge at 48,000 rpm. *A*, after 25 min; *B*, after 90 min. *1*, HDL_2; *2*, HDL_3. The arrows show direction of flotation.

casionally appearing lipoproteins with flotation rates between those of VLDL and LDL (26).

The course of the three-grade density gradient used in step I made it necessary to isolate the HDL_2 and HDL_3 first and to separate VLDL and LDL from the lipoprotein mixture $d < 1.063$ in a second step. This sequence differs from the method of Wilcox et al. (12), who isolated the VLDL and LDL first and reaped these two density classes by incomplete emptying of the rotor; afterwards, the light side of the gradient was replaced from the rotor's center, and HDL was isolated in the second step using an identical gradient. In our experience, the recentrifugation of the lipoproteins $d < 1.063$ in the ultracentrifugal step II yields comparable results. Furthermore, we see a major advantage in this sequence of preparation: the VLDL, prepared in step II, never showed a contamination with albumin, whereas that of Wilcox et al. (12) did. Perhaps our results were due to the long running time in step I and/or by the recentrifugal effect of step II in the described procedure.

Principally, the isolation of the main density classes of the whole plasma lipoprotein spectrum in two separate runs offers the ability to use individual appropriate gradient density ranges for individual lipoprotein density classes (1.00–1.40 for HDL_2 and HDL_3; 1.00–1.30 for lipoproteins of $d < 1.063$) as discussed above for step II.

The technique of recentrifugation can also be applied in rate-zonal ultracentrifugation equally as in angle-head rotors, if a special problem has to be solved.

In lipoprotein preparation from plasma of hyperlipemic individuals, the described method proved to be suitable; in the case of hyperbetalipoproteinemia, the two-step procedure can be applied in an unmodified form. In marked hypertriglyceridemia, a precipitation was necessary because of the merging of lipoproteins $d < 1.063$ and HDL. It should be mentioned, however, that for the precipitation of lipoproteins $d < 1.063$ with polyanions, only serum and not plasma must be used; otherwise, considerably smaller HDL peaks are found. As proved in several experiments, serum was just as suitable as plasma for lipoprotein preparation in the zonal rotor if a precipitation procedure is not necessary.

The concentration procedure of the lipoprotein fractions in our experiments was necessary only in order to investigate their purity and physicochemical properties. In our hands the concentration by polyethylene glycol gave the most satisfactory results because no precipitation of lipoproteins occurred. Ultrafiltration in a Diaflo cell may result in precipitation and loss of lipoprotein on the membrane. Using the angle-head rotor for concentration, an additional fractionation of isolated fractions cannot be excluded. If this were the case, any analysis of lipoproteins concentrated in the angle-head rotor would minimize any differences to be obtained by lipoprotein preparation in zonal rotors or angle-head rotors.

In order to determine to what degree the described centrifugation technique on the one hand and concentration by polyethylene glycol on the other may cause a possible loss or alteration of lipoprotein fractions, the experiment of recentrifugation of HDL was performed. The identical lipoprotein pattern, HDL_2/HDL_3 ratio, chemical composition, and electrophoretic behavior indicate that no detectable alteration of lipoproteins occurred. Over 80% of HDL_2 and HDL_3 was recovered. The several experimental steps between the two runs most probably account for the observed loss, because less than 3% of the original HDL protein and less than 1% of the original HDL lipids were found in the bottom fraction of the second run. This result indicates that the lipids found in the bottom fraction of HDL separation from serum belong to VHDL that were most probably already present in the inserted serum sample. Furthermore, it can be assumed that no significant breakdown of HDL occurs by increasing the density of samples by NaBr to d 1.40, as carried out in our experiments. The lipoproteins isolated by the described method exhibited a chemical composition similar to those isolated by common sequential methods in angle-head rotors (13, 29, 30).

The ratios of mg C_T to mg PL for the VLDL and LDL (0.72 and 1.60, respectively) agree with those reported by Havel et al. (27) for lipoproteins isolated by the usual sequential procedures and with those found by Wilcox et al. (12) in lipoproteins prepared in the zonal rotor using a continuous linear density gradient. The mg C_T/mg PL ratio in total HDL (0.62) reported by Alaupovic et al. (28) lies between the ratios of HDL_2 and HDL_3 isolated by the described procedure (0.78 and 0.59, respectively). The plasma lipoprotein concentrations (VLDL, LDL, HDL_2) revealed by our method are in agreement with those reviewed by Barclay (13), with the exception of HDL_3. With respect to HDL_3, our data correspond well to those of Hatch and Lees (31).

The flotation rates ($S_{f,\ 1.063}$) of our LDL preparations ranged between 6 and 8. The HDL_2 and HDL_3 flotation rates ($F_{1.21}$) and molecular weights (Table 5) correspond quite well to those reviewed by Skipski (32).

The positions of the separated lipoprotein zones (LDL, HDL_2, and HDL_3) are very dependent on running time. It is not necessary, however, that the various lipoprotein density classes reach isopycnic equilibrium to achieve a clear-cut separation. The lipoprotein isolation in the NaBr gradients of the density ranges used depends rather on the flotation rates of the different lipoprotein density classes, as shown and discussed for similar gradients by other investigators (10, 12).

It is to be expected that by adapting the formation of

density gradients to Ti-15 rotors a separation of the plasma lipoprotein density classes should be obtained in the same way as in the Ti-14 rotor. In Ti-15 rotors, it should be possible to separate approximately 40–50 ml of plasma.

Because of the fact that by using a rate-zonal ultracentrifugation technique a considerable dilution of different lipoprotein density classes is obtained, only one sample at a time can be fractionated and a maximum of 20 ml of serum can be applied if all density classes are to be separated. The usefulness of this method is limited to particular problems. In spite of these drawbacks, the described method seems to be very useful for the solution of certain problems because of the following facts. (*1*) The major lipoprotein density classes can be isolated quantitatively free from albumin within 24 + 2 hr; (*2*) changes of quantitative distribution of different lipoprotein density classes can be directly visualized from the preparation pattern if monitored at 280 nm; (*3*) lipoprotein patterns differing from normal can be easily detected; and (*4*) fractionation of the rotor content corresponding to the elution pattern can be carried out arbitrarily; in this way lipoprotein fractions can be separated from each other at regions of minimum concentrations.

On the basis of the above benefits, we regard the procedure for the isolation of human plasma lipoproteins as a very promising technique, especially for the study of lipoprotein metabolism in normal and pathological states and for the diagnosis of disorders of lipid metabolism.

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