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Advantages and limitations of density gradient ultracentrifugation in the fractionation of human serum lipoproteins: role of salts and sucrose

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Abstract Two density gradient ultracentrifugation methods, Redgrave et al. (1975. *Anal. Biochem.* **65:** 42–49) and Nilsson et al. (1981. *Anal. Biochem.* **110:** 342–348), currently used for the separation and analysis of plasma lipoproteins were compared with respect to their resolving power and capacity to obtain pure products as a function of time of ultracentrifugation using the same rotor (Beckman SW-40), speed (150,000 *g*), and temperature (14°C). The effects of sucrose and salts were also investigated. The Redgrave gradient insured the separation of the major classes of plasma lipoproteins after 24 hr of centrifugation; however, equilibrium conditions were only reached after 48 hr, at which time the lipoproteins were contaminated by albumin. When the effluents from each rotor tube were continuously monitored at 280 nm, each lipoprotein band gave values that were higher than those from mass analyses. This was due to a light scattering effect, the extent of which was dependent on the concentration of lipoproteins and salts. Sucrose prevented the scattering effect and was found to bind irreversibly to the apolipoproteins. In contrast, after 66 hr centrifugation, the lipoproteins obtained from the Nilsson gradient exhibited a close correspondence between protein mass and absorbance values at 280 nm, had no scattering effect, and were uncontaminated by albumin. The difference in spectroscopic behavior between the Redgrave and the Nilsson procedures was attributed to three factors: *1*) the presence of sucrose in the latter gradient and incorporation of this sugar into lipoproteins as assessed by mass and radioactivity measurements; *2*), the salt density to which the serum samples were exposed to at the beginning of the ultracentrifugation; and *3*) the final lipoprotein concentration. The results of these studies indicate that the banding and spectroscopic properties of the plasma lipoproteins can be influenced by the ultracentrifugal conditions of isolation due to interactions with medium components. An important message of this work is that sucrose is not an inert medium component and its uptake by lipoproteins during the ultracentrifugal procedures of isolation must be taken into account.—**Edelstein, C., D. Pfaffinger, and A. M. Scanu.** Advantages and limitations of density gradient ultracentrifugation in the fractionation of human serum lipoproteins: role of salts and sucrose. *J. Lipid Res.* 1984. **25:** 630–637.

Supplementary key words high salts and lipoproteins • sucrose and lipoproteins

Density gradient ultracentrifugation (DGU) is among the techniques used to study plasma lipoproteins both from the analytical and preparative standpoints. The advantages of this technique are that gradients of different density range and steepness can be generated and in the isopycnic mode, information can be gained on the actual hydrated density of the lipoprotein species under study. We have previously described a DGU technique which permits good resolution of the main classes and subclasses of plasma lipoproteins as assessed by the continuous graphic display of the absorbance at 280 nm of the eluted fractions (1). One disadvantage of the original procedure was the 66-hr requirement for equilibrium conditions to be reached. In contrast, the DGU method by Redgrave, Roberts, and West (2) was described to require only 24 hr to separate the main classes of plasma lipoproteins; however, whether equilibrium conditions were reached was not established. In order to gain a better insight into the actual capabilities of DGU in lipoprotein studies, we have compared the Redgrave and Nilsson procedures in order to determine: *1*) relative efficiency of the two methods in terms of their resolving power as a function of time; *2*) conditions for attainment of equilibrium; *3*) properties of the resulting products. The observations derived from these studies have permitted us to recognize advantages and limitations of the two methods and identify problems in lipoprotein isolation which may arise from medium composition and gradient design.

MATERIALS AND METHODS

Human sera

Blood was collected from the cubital vein of two healthy normolipidemic female subjects (Subject 1, plasma cho-

Abbreviations: DGU, density gradient ultracentrifugation; VLDL, very low density lipoprotein, density 1.006 g/ml; LDL, low density lipoprotein, density 1.006–1.063 g/ml; HDL_2, high density lipoprotein, density 1.063–1.121 g/ml; HDL_3, high density lipoprotein, density 1.121–1.21 g/ml; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate.

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lesterol 197 mg/dl, triglyceride 53 mg/dl and Subject 2, cholesterol 155 mg/dl, triglyceride 97 mg/dl) after an overnight fast and placed in a solution containing EDTA, final concentration 1.5 g/L; sodium azide, 0.1 g/L; and aprotinin, 10,000 U/L. The plasma was separated by centrifugation at 4°C (4,500 *g*, 10 min) and was either used immediately or stored at 4°C for no longer than 1 day.

Density gradient ultracentrifugation

Two kinds of density gradients were constructed following published procedures (1, 2). A schematic representation of the gradients is shown in **Fig. 1.** Typically, 1 ml of plasma was applied per gradient. Centrifugations of both gradients were conducted at 14°C with a swinging bucket rotor (SW-40, Beckman Instruments, Co., Palo Alto, CA) in a Beckman L8-70 ultracentrifuge at 39,000 rpm. The duration of the ultracentrifugation run was 24, 48, or 66 hr. Fractions of 0.4 ml were collected with a density gradient fractionator (Model 640, Instrumentation Specialties, Co., Lincoln, NE) and were continuously monitored at 280 nm on an ISCO Model UA-5 absorbance monitor and recorder as previously described (1).

Analysis of fractions

The density of the fractions eluted after centrifugation was determined in a digital precision magnetic oscillator (Model DMA 02C, Mettler-Paar, Hightstown, NJ) at 14°C. Each pooled lipoprotein fraction was dialyzed overnight at 4°C against 0.15 M NaCl solution containing 1 mM EDTA, pH 7.0, and analyzed by polyacrylamide gel electrophoresis in 10% acrylamide, 0.1% SDS (3). Protein analyses were carried out according to Markwell, et al. (4).

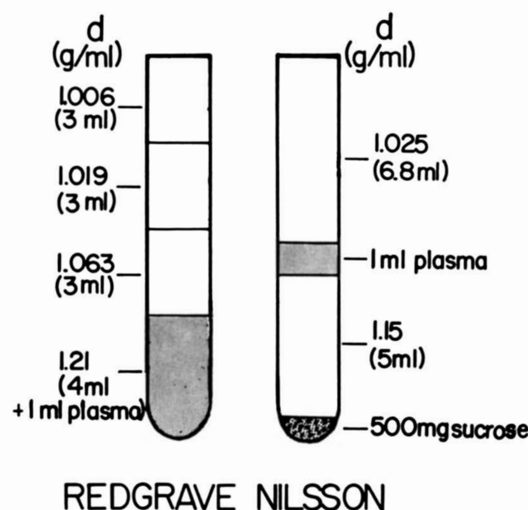

Fig. 1. Schematic representation of the constitutents in the Redgrave (left) and Nilsson (right) gradients. The solutions were layered consecutively into 14 × 95 mm ultraclear Beckman centrifuge tubes (capacity, 13.2 ml). The density of the solutions was measured at 14°C in a magnetic oscillator as described in Materials and Methods.

Studies on the effects of salt and sucrose

Redgrave system. This gradient is composed of NaCl and NaBr and does not contain sucrose; the plasma sample is brought to d 1.21 g/ml and positioned at the bottom of the gradient (2). To study the effect of sucrose on lipoprotein scattering in this system, we initially incubated the plasma for 2 hr at room temperature with sucrose (20 mg of sucrose/ml of plasma). We then dialyzed it against 0.15 M NaCl, 1 mM EDTA, pH 7.0, and brought it to d 1.21 g/ml with solid NaBr to permit its positioning at the bottom of the gradient as originally described (2). To study the effect of salt, the plasma at d 1.006 g/ml was positioned at the top of the gradient.

Nilsson system. As described in the original method (1), this gradient is composed of NaCl and sucrose and the plasma is layered in the d 1.006 g/ml position (see Fig. 1). To study the effect of salt, the plasma was brought to d 1.15 g/ml with NaCl and placed in this density zone; sucrose was replaced by a d 1.21 g/ml solution (NaCl and NaBr). To study the effect of sucrose, this sugar was omitted from the gradient and replaced by d 1.21 g/ml solution (NaCl and NaBr). To study the combined effect of salt and sucrose, the plasma was brought to d 1.15 g/ml with NaCl and placed in the d 1.15 g/ml zone of the gradient which contained 500 mg of sucrose.

Measurement of light-scattering contributions

The absorbance contribution due to light-scattering was determined in a UV-Vis double-beam spectrophotometer (DMS-90, Varian Assoc., Palo Alto, CA), following the procedure of Leach and Scheraga (5). Briefly, absorbance recordings of each of the lipoprotein samples were obtained in the visible region (600–325 nm) where absorption due to the chromophore (tyrosine and/or tryptophan) of the protein is not a contributing factor. A log-log plot of the experimental data was then constructed. Extrapolation of the straight line portion into the 280 nm region gave the absorption due to scattering. This value was then subtracted from the original absorbance at 280 nm to give the corrected absorbance.

Delipidation

Lipoproteins were delipidated with mixtures of ethanol and diethyl ether at −20°C as previously described (6).

Reagents

All of the general chemicals were of reagent grade purity. Apoprotein standards, apolipoprotein A-I, A-II, and C peptides were prepared as previously described (7). Lipoprotein fractions, LDL, HDL_2, and HDL_3 were

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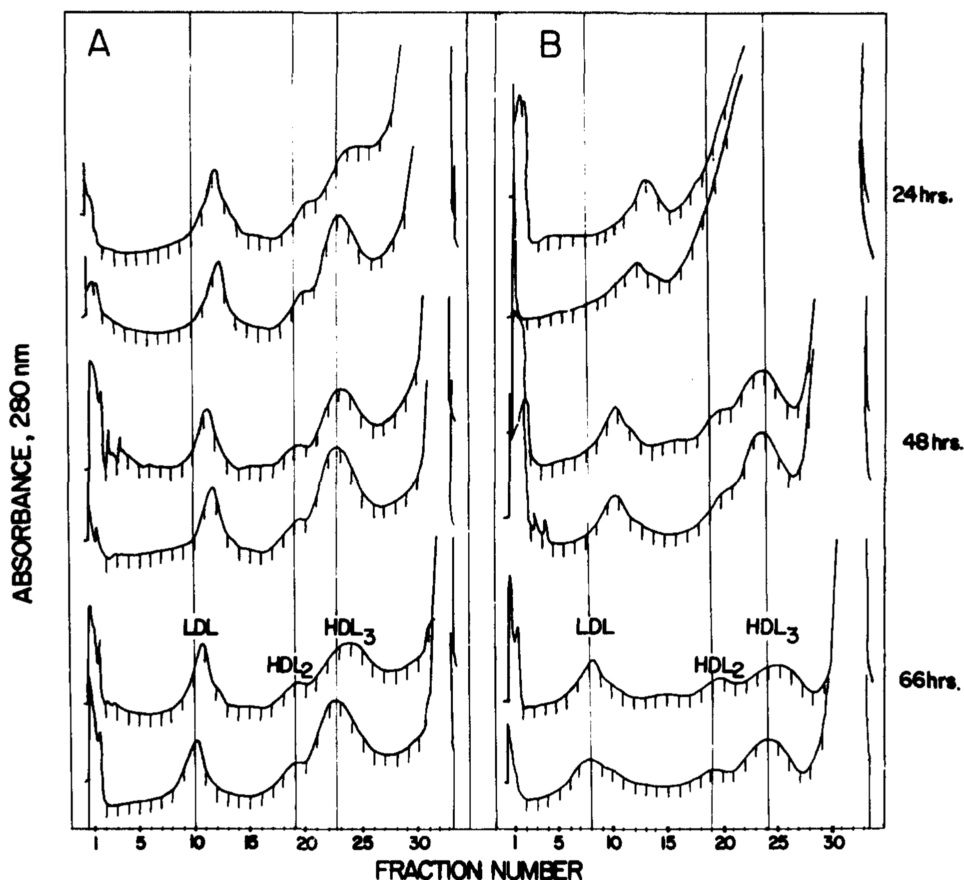

Fig. 2. Absorbance profiles of ultracentrifuged plasma obtained using the Redgrave (A) and the Nilsson (B) density gradients. Plasma was obtained from two normolipidemic female subjects, Subject 1, top, and Subject 2, bottom, and centrifuged for 24, 48, and 66 hr. Formation of density gradients and collection of fractions were as described in Fig. 1 and in Materials and Methods. Each fraction is designated by a tick mark and corresponds to 0.4 ml. Identification of lipoprotein classes is based on electrophoretic mobilities in 1% agarose gels, apoprotein banding patterns in 0.1% SDS-PAGE and compositional analysis. The three vertical lines drawn through the profiles in each panel represent the position of the lipoprotein maxima when equilibrium has been reached.

prepared by conventional ultracentrifugal techniques described elsewhere (8). [U-^{14}C]Sucrose, specific activity 1.53 mCi/mg, was obtained from Amersham Corp., Arlington Heights, IL. The purity of labeled and unlabeled sucrose was checked by paper chromatography in a solvent system consisting of n-butanol–ethanol–water 104:66:30 (v/v/v) and detected with potassium periodocuprate according to Amersham Corp. The recovery of [U-^{14}C]-sucrose was greater than 90% and its mobility was identical to that of the unlabeled material.

RESULTS

Plasma samples from the two female subjects were separated under the density gradient conditions of Redgrave and Nilsson for 24, 48, and 66 hr. After 24 hr, the Nilsson gradient was unable to resolve the three main lipoprotein classes, VLDL, LDL, HDL_2, and HDL_3 (**Fig. 2B**); in turn, the Redgrave gradient was relatively more successful (Fig. 2A) although the lipoprotein fractions obtained were all significantly contaminated by albumin even after 66 hr of centrifugation as assessed by SDS-polyacrylamide gel electrophoresis (**Fig. 3**). The salt gradients formed by the two methods were curvilinear but differed in that comparable density distribution was obtained after 48-hr and 66-hr centrifugations by the Nilsson method (**Fig. 4A**). In contrast, the Redgrave method generated density distributions that were comparable at 24 and 48 hr but deviated considerably at 66 hr (Fig. 4B). In terms of lipoprotein densities, the LDL fraction obtained after 48 hr centrifugation was comparable in both procedures, and the HDL_2 and HDL_3 in the Redgrave 48-hr gradient were comparable to the Nilsson 48- and 66-hr gradients

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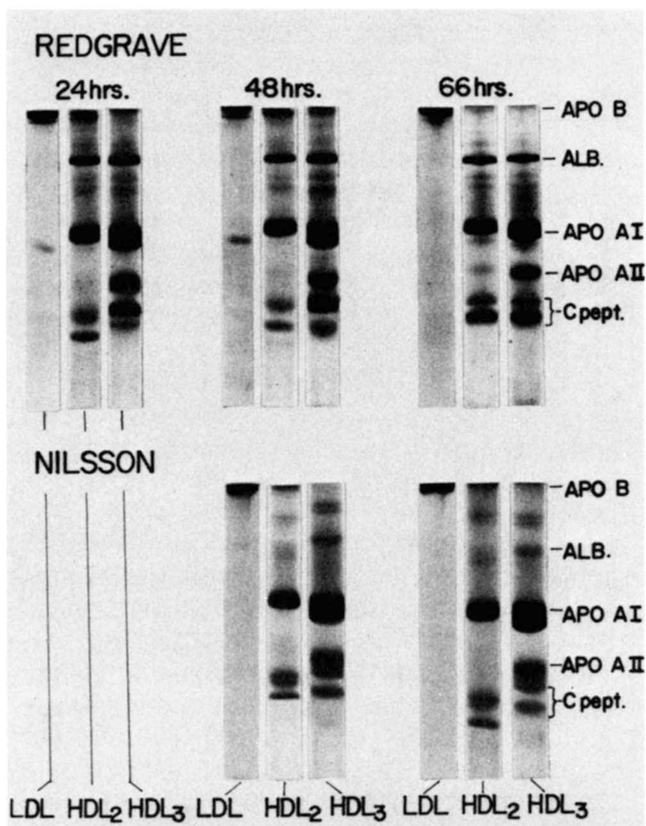

Fig. 3. SDS-PAGE of apoproteins obtained from the ultracentrifugal fractions of the Redgrave and Nilsson gradients. The collected fractions were dialyzed against 0.15 M NaCl, 1 mM EDTA, pH 7.0, and incubated in 1% SDS at 37°C for 30 min. The samples were applied to 10% polyacrylamide gels in 0.1% SDS and stained with Coomassie Blue. The bands were identified by running apoprotein standards in parallel gels.

(**Table 1**). As assessed by their absorbance at 280 nm, at all times of centrifugation, the lipoprotein peaks, LDL in particular, in Fig. 2A (Redgrave) appeared higher and sharper than in Fig. 2B (Nilsson). Manual absorption readings at 280 nm of pooled lipoprotein fractions obtained from the Redgrave gradient were significantly higher than those from the Nilsson gradient. On the other hand, protein concentrations of these pooled fractions were comparable (**Table 2**). Thus, the Redgrave gradient appeared to contribute an additional optical component to the absorbance; this was especially true for LDL. To further explore this phenomenon, the lipoprotein fractions were monitored between 600 and 325 nm. Following the procedures of Leach and Scheraga (5), the absorption readings between 600 and 325 nm of each peak fraction were plotted on a log-log scale and the straight line portion of the curve was extrapolated to 280 nm (**Fig. 5**). The intercept at 280 nm was considered as representing the scattering contribution which was then subtracted from the total absorbance at 280 nm. Table 2 shows the corrected absorptions of the pooled fractions for each lipoprotein class in the two gradients. At 48 hr centrifugation, compared to the Nilsson gradient (Fig. 5A), the Redgrave gradient (Fig. 5B) required larger scattering corrections for all of the lipoproteins. This was also the case for the 24- and 66-hr gradients. However, determination of protein concentrations across the absorption

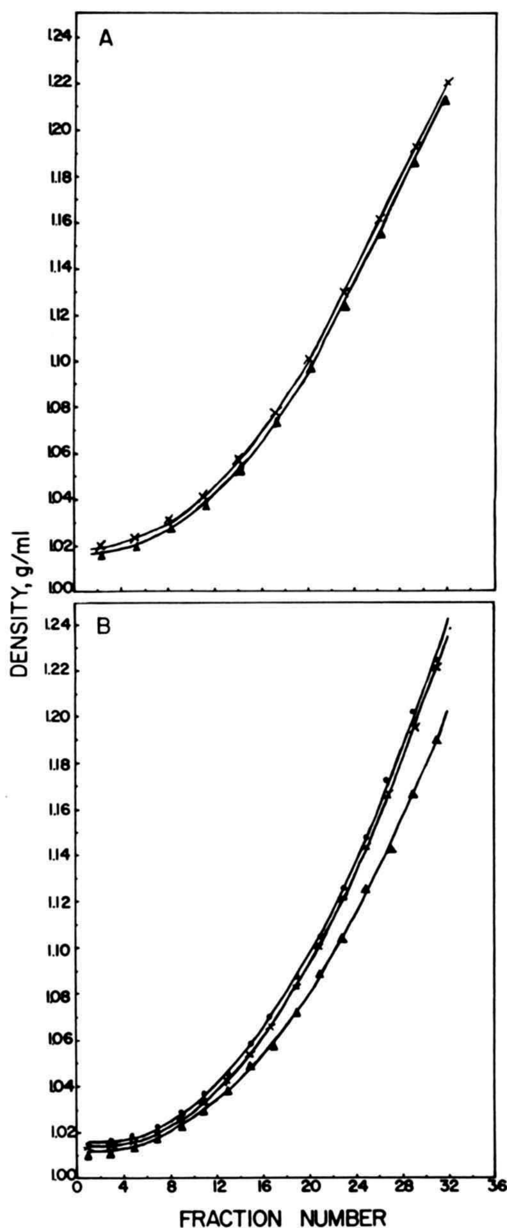

Fig. 4. Profiles of the density versus fraction number of the Nilsson (A) and Redgrave (B) gradients. The density of each fraction was measured in a magnetic oscillator at 14°C. (●) 24 hr; (×) 48 hr; (▲) 66 hr. The solid curves represent the best fit to the least squares analysis of the data points.

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TABLE 1. Peak density of lipoproteins after 24, 48, and 66 hr centrifugation

	Redgrave			Nilsson		
	Centrifugation Time (hr)					
	24	48	66	24	48	66
	d (g/ml)					
LDL	1.041	1.036	1.030	nd	1.037	1.027
HDL_2	1.097	1.085	1.077	nd	1.090	1.086
HDL_3	1.127	1.123	1.108	nd	1.124	1.128

nd, Not determined.

profile showed that the fractions in the peak tube (Fig. 2A, tubes 11, 19, 23 and Fig. 2B, tubes 10, 19, 23) of each of the lipoprotein bands of the Redgrave gradient contained more mass than in the Nilsson gradient suggesting that the light scattering was dependent, at least in part, on lipoprotein concentration. This was confirmed by measuring the scattering contribution of solutions of LDL varying in protein concentrations between 1 and 10 mg of protein/ml. The scattering increased linearly as a function of protein concentration and was unaffected after 24 hr standing at room temperature (data not shown).

From these results, we considered two additional factors which might have contributed to the scattering. The first was the presence of sucrose in the Nilsson system and its absence in the Redgrave gradient and the second, the placement of the serum sample in low salt in the Nilsson gradient and in a high salt medium in the Redgrave gradient. To verify the effect of sucrose, [U-^{14}C]sucrose was mixed with unlabeled sugar and added to the Nilsson gradient according to the scheme in Fig. 1. After ultracentrifugation, the fractions were monitored both for absorbance at 280 nm and radioactivity. As shown in **Fig. 6,** a peak of radioactivity co-eluted with LDL as well as HDL_2, HDL_3, and the bottom fraction. Exhaustive dialysis against 0.15 M NaCl, 1 mM EDTA, pH 7.0, did not change the amount of radioactivity in each lipoprotein fraction. Subsequently, LDL (d 1.006–1.063 g/ml) prepared by conventional preparative ultracentrifugation was incubated with [U-^{14}C]sucrose (sp act 510 dpm/mg sucrose) in a weight ratio of 1 to 20 mg of sucrose per mg of LDL protein for 2 hr at room temperature and the scattering was measured. The scattering, when extrapolated to 280 nm, decreased as the sucrose concentration increased (**Fig. 7**). Moreover, upon dialysis, the scattering remained unchanged. Delipidation of the lipoprotein with chloroform–methanol 2:1 (v/v) resulted in the recovery of 99% of the radioactivity with the lipid free protein. In addition, when serum was incubated with [U-^{14}C]-sucrose (20 mg/ml serum), dialyzed extensively (eight changes of 4 liters each) against 0.15 M NaCL, 1 mM EDTA, pH 7.0, and then fractionated using the Redgrave method, the radiolabeled sucrose was found to band with LDL, HDL_2, and HDL_3. Moreover, the scattering effect in each of these lipoprotein classes decreased markedly (**Table 3,** Redgrave system, columns 2 and 3). In a parallel experiment, sucrose, which is normally present in the Nilsson gradient, was excluded from it and substituted with 1 ml of d 1.21 g/ml solution. When the plasma was fractionated in this gradient system, the lipoproteins resolved as well as in the presence of sucrose and the scattering was comparable (Table 3, Nilsson system, columns 3 and 4).

To study the effect of salt concentration, the positioning of the plasma sample was modified in both the Redgrave and Nilsson systems; in the Redgrave system, the plasma

TABLE 2. Absorption and protein concentration of lipoproteins after 48 hr centrifugation

	Redgrave[a]			Nilsson[b]		
	Abs 280 nm	Abs 280 nm (corr)[c]	Lowry Protein mg/ml	Abs 280 nm	Abs 280 nm (corr)[c]	Lowry Protein mg/ml
LDL	1.145	0.695	0.718	0.584	0.557	0.701
HDL_2	0.531	0.402	0.512	0.420	0.407	0.525
HDL_3	0.753	0.665	0.680	0.561	0.547	0.670

[a] Combined fractions were #11–12 for LDL; #18–19 for HDL_2 and #22–23 for HDL_3.
[b] Combined fractions were #10–11 for LDL; #18–19 for HDL_2 and #23–24 for HDL_3.
[c] Corr, absorbance which has been corrected for light scattering, see Materials and Methods.

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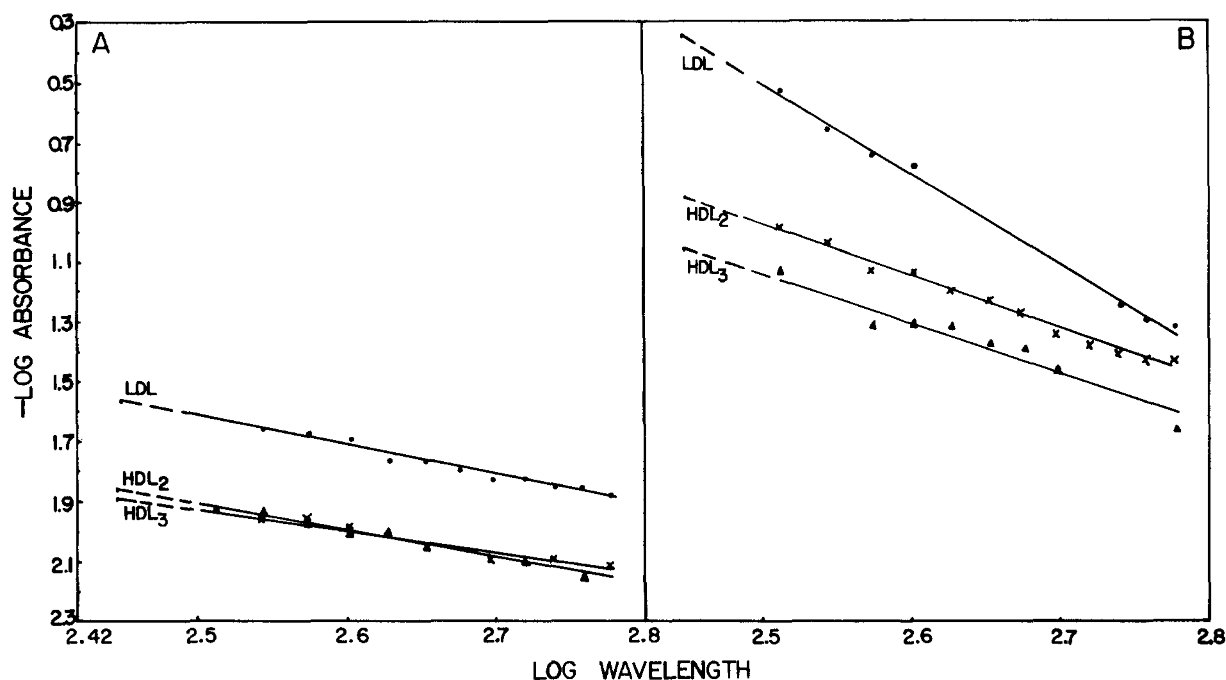

Fig. 5. Double logarithmic plot of absorbance versus wavelength. The absorptions of combined lipoprotein fractions obtained with the Nilsson or Redgrave method (see Materials and Methods) were measured as a function of wavelength and plotted on a log-log scale. The extrapolation to 280 nm, dashed line, gave the scattering contribution. A, Nilsson method, ●——● LDL, fractions 10–11; ×——× HDL_2, fractions 18–19; ▲——▲ HDL_3, fractions 23–24. B, Redgrave method, ●——● LDL, fractions 11–12; ×——× HDL_2, fractions 18–19; ▲——▲ HDL_3, fractions 22–23. The solid lines represent the statistical best fit to the data.

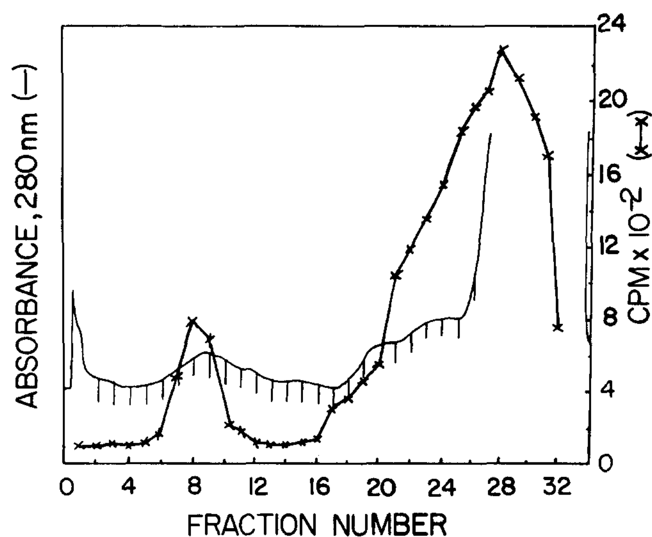

Fig. 6. Absorbance and radioactivity profiles of serum fractionated with the Nilsson gradient. Sucrose (500 mg) was mixed with [U-^{14}C]sucrose and placed at the bottom of the gradient as described in Fig. 1 and 1 ml of serum was added into the d 1.006 g/ml portion of the tube. After centrifugation for 48 hr, the lipoproteins were continuously monitored for their absorbance at 280 nm. Each fraction number designated by a tick mark corresponds to 0.4 ml. For radioactivity measurements, 0.1-ml aliquots were mixed with 10 ml of scintillation fluid and counted.

sample was placed in the top d 1.006 g/ml portion of the gradient whereas in the Nilsson method, the plasma was placed in the d 1.15 g/ml portion (Fig. 1 and Table 3 Redgrave column 4, Nilsson column 2). Moreover, in the Nilsson system, to rule out a potential additional effect by sucrose, this sugar was omitted from the gradient and replaced by d 1.21 g/ml solution (Table 3, Nilsson column

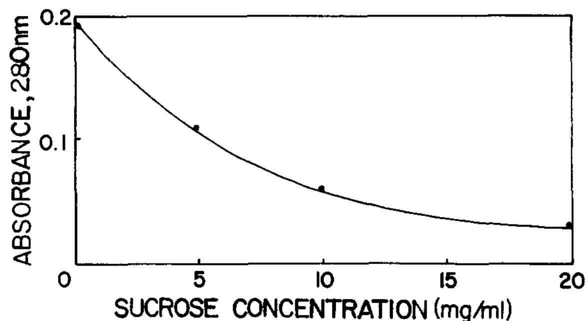

Fig. 7. Effect of sucrose concentration on the scattering absorbance of LDL. Sucrose was incubated in a weight ratio of 1 to 20 mg per mg of LDL protein for 2 hr at room temperature. The absorption from 600 to 325 nm was measured and the scattering absorbance was obtained by extrapolation of the straight line portion of the curve to 280 nm.

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TABLE 3. Factors contributing to the scattering of plasma lipoproteins in the Nilsson and Redgrave systems

Lipoprotein Class	Redgrave			Nilsson			
	Plasma[a] (d 1.21 g/ml) + Sucrose	Plasma[b] (d 1.21 g/ml)	Plasma[c] (d 1.006 g/ml)	Plasma[d] (d 1.15 g/ml) + Sucrose	Plasma[e] (d 1.15 g/ml)	Plasma[f] (d 1.006 g/ml)	Plasma[g] (d 1.006 g/ml) + Sucrose
LDL	0.217 ± 0.040	0.499 ± 0.082	0.147 ± 0.034	0.114 ± 0.017	0.105 ± 0.024	0.060 ± 0.018	0.042 ± 0.012
HDL_2	0.062 ± 0.016	0.144 ± 0.032	0.063 ± 0.015	0.050 ± 0.011	0.042 ± 0.012	0.031 ± 0.010	0.030 ± 0.010
HDL_3	0.057 ± 0.013	0.131 ± 0.040	0.062 ± 0.015	0.044 ± 0.011	0.042 ± 0.012	0.032 ± 0.010	0.034 ± 0.010

All values represent the scattering correction calculated from four separate experiments as described in the legend to Fig. 5. Values ± SD.
[a] Plasma initially incubated with sucrose, dialyzed against 0.15 M NaCl, 1 mM EDTA, pH 7.0, then brought to d 1.21 g/ml and applied to gradient as in Fig. 1.
[b] Plasma brought to d 1.21 g/ml as in original Redgrave study (Fig. 1).
[c] Plasma placed in d 1.006 g/ml portion of gradient.
[d] Plasma brought to d 1.15 g/ml, then placed on gradient containing sucrose as in Fig. 1.
[e] Plasma brought to d 1.15 g/ml, gradient prepared without sucrose and replaced by d 1.21 g/ml (Fig. 1).
[f] Plasma placed in d 1.006 g/ml portion of gradient as in Fig. 1, but sucrose is replaced by d 1.21 g/ml solution.
[g] Plasma placed in d 1.006 g/ml portion of gradient and sucrose added as in Fig. 1.

3). These modifications did not alter the resolution of the lipoprotein profiles. The results summarized in Table 3 reveal two main factors. First, when the plasma is initially exposed to a high salt solution and then separated by either Nilsson procedure, i.e., with or without sucrose (Table 3, Nilsson columns 1 and 2), the lipoprotein bands exhibit an increased scatter over a plasma exposed to low salt (compare Table 3, Nilsson columns 1, 2 with columns 3, 4). This is also true for the Redgrave system, except that the scattering is higher than in the Nilsson system. Second, the above increase in scattering due to high salt can be partially but not totally prevented by the presence of sucrose in the Redgrave system, an effect not encountered in the Nilsson procedure.

DISCUSSION

The results of the current studies confirm that DGU is a valuable technique for separating the main classes of human plasma lipoproteins, and also show that attention must be paid to gradient constituents, geometry, lipoprotein concentration, and time of ultracentrifugation. These points emerged from the study of two previously reported gradient procedures, each of which appeared to offer points of advantage and disadvantage as summarized in **Table 4.** The Redgrave method, as originally described, did have the capacity to separate the main classes of plasma lipoproteins in 24 hr, however, in this time interval, equilibrium conditions were not reached. For the HDL class, equilibrium was attained after 48 hr and for the LDL class after 66 hr. Similar results were obtained with the Nilsson procedure. In addition, in the Redgrave system, a major albumin contaminant was present in the HDL subclasses at all times of centrifugation. This contamination was minimal or absent in the Nilsson system.

TABLE 4.

	Redgrave	Nilsson
Gross separation of lipoproteins after 24-hr run	Present	Absent
Lipoprotein resolution at 48 hr and 66 hr	Comparable	Comparable
Contamination by albumin	Present	Absent
Light scattering effects	Present	Absent

A peculiarity of the Redgrave gradient system was the presence after ultracentrifugation of a light scattering or turbidity effect which contributed significantly to the total absorbance of the lipoprotein fractions. This effect was proportional to lipoprotein concentration and was particularly evident with the LDL class and comparatively less with HDL_2 and HDL_3. This phenomenon may reflect a lipoprotein-packing anomaly, particle aggregation, or shape change contributed by a combination of high salt, lipoprotein concentration, and gravitational force. This effect was prevented by sucrose, which was irreversibly incorporated into the lipoprotein particles thus probably favoring their stabilization. The property of sucrose as a stabilizing agent for lipoproteins in serum (9) and proteins in general (10, 11) has been documented and also shown to favor their preferential hydration. The irreversibility of the sucrose incorporation into the lipoprotein, as indicated by its resistance to dialysis, suggests that sucrose bound covalently to the lipoprotein particles and, as our studies indicated, to the protein moiety. The precise mode of covalent attachment was not established but, since sucrose is a non-reducing sugar, interaction between the primary amino groups of the apoprotein may be ruled out (12). Sucrose may have also affected the interactions of salts and water with the lipoprotein particles. The Redgrave gradient contained both KBr and NaCl whereas

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the Nilsson system contained only NaCl. Previous studies have shown that species such as NaCl cause preferential hydration of proteins and a stabilizing effect on them (13–16). On the other hand, the effect on proteins of KBr and particularly of the Br^- anion has not been studied as extensively. Based on previous reports (17), our present observations could be interpreted to mean that this anion may favor destabilization of the lipoproteins by binding to the protein moiety leading to an increase in net charge, particle to particle repulsion, and also a salting-in effect. The postulated increase in net charge may explain the smaller effect exhibited by sucrose alone in preventing lipoprotein scattering (Table 3, Redgrave system column 2). Regardless of the mechanism of action, however, the notion that sucrose can bind irreversibly to the lipoprotein particles puts limitations on its use and should alert us to the fact that lipoproteins exposed to sucrose and high salts may not be comparable to their native counterparts. It remains for future studies to establish the chemical nature of the interaction between sucrose and lipoproteins.

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