note on methodology

Effects of storage on the distribution of high density lipoprotein subfractions in human sera

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Summary The current growing interest in the separation of the high density lipoprotein (HDL) subfractions suggested a comparative analysis of the HDL_2 and HDL_3 distribution in fresh and stored serum samples. Human sera were processed by rate zonal ultracentrifugation in a swinging bucket rotor, immediately after collection and after a 7-day storage at 4°C, both in the presence and in the absence of 5.1 M sodium bromide. Samples stored in absence of salt show a marked decrease of the HDL_3 mass, a reduction of its flotation rate, and significant changes in the lipid composition. The HDL_2 concentration and composition are not altered by storage. The reported findings indicate that significant HDL_3 modifications may occur in serum samples stored at 4°C; these changes can be prevented by the addition of high concentrations of salt before storage.—**Franceschini, G., C. Tosi, Y. Moreno, and C. R. Sirtori.** Effects of storage on the distribution of high density lipoprotein subfractions in human sera. *J. Lipid Res.* 1985. **26:** 1368–1373.

Supplementary key words HDL_2 • HDL_3 • HDL interconversion • lipoprotein metabolism • LCAT

A dramatic rise in interest for the high density lipoprotein (HDL) subfraction distribution has occurred in the past several years, because of the emerging evidence for a differential role of the two major HDL subclasses, HDL_2 and HDL_3, in the pathophysiology of lipoprotein metabolism (1–3). Earlier studies had suggested that HDL_2 might exert a protective effect against atherosclerosis (4, 5); on the other hand, the dense HDL_3 particles can promote the efflux of cholesterol from cells, as well as enhance the low density lipoprotein (LDL) receptor activity (6, 7).

The two HDL subfractions are metabolically interrelated, HDL_2-like particles being produced during lipolysis (8) and during incubation of serum at 37°C (9). Multiple enzyme activities, i.e., lecithin:cholesterol acyltransferase (LCAT), hepatic and lipoprotein lipases, and different lipid transfer proteins are involved in the in vivo and in vitro interconversion (10, 11).

Several problems are encountered when concentration and composition of HDL_2 and HDL_3 have to be determined in serum. A simple double-precipitation method has recently been proposed (12), but an incomplete separation of the subfractions has been reported (13). Analytical ultracentrifugation, time-consuming and requiring sophisticated equipment, produces an estimate of the HDL subfraction distribution and concentration in serum (14), but does not allow one to accurately determine composition. Gradient polyacrylamide gel electrophoresis (PAGE) gives the best separation of the HDL subpopulations (15), and is very useful in the evaluation of HDL particle interconversion (16), but provides only semi-quantitative results.

Rate zonal ultracentrifugation (17) is the most useful method to separate HDL_2 and HDL_3, as well as to evaluate their concentration and composition. The recently introduced procedure for rate zonal ultracentrifugation in swinging bucket rotors (18) can overcome the major drawback of the classical zonal ultracentrifugation, i.e., the analysis of only one sample at a time. This procedure was recently used to characterize the effects of fat ingestion (19), and by us for the separation and compositional studies on the HDL subclasses in subjects with the apolipoprotein AI_{Milano} variant (G. Franceschini, C. R. Sirtori, and A. V. Nichols, unpublished observations). In this latter study, the necessity for analyzing multiple samples taken at the same time from more than 50 subjects raised the problem of sample storage before ultracentrifugation. In the present study we report how the simple storage of EDTA-containing serum at 4°C for 1 week, can modify the HDL subfraction distribution and composition, and how these modifications may be prevented by the addition of high concentrations of salt.

MATERIALS AND METHODS

Subjects

Six male volunteers, aged 22–50 years, were selected from the members of our department and medical students. Their serum cholesterol and triglyceride (TG) levels ranged between 163 and 228 mg/dl and 50 and 125 mg/dl, respectively. Six type IV hypertriglyceridemic (HTG) patients were selected from those attending our Lipid Research Clinic. Serum cholesterol and TG levels in

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; HDL_2, HDL subfraction 2; HDL_3, HDL subfraction 3; LCAT, lecithin:cholesterol acyltransferase; PAGE, polyacrylamide gel electrophoresis; TG, triglycerides; TC, total cholesterol; FC, free cholesterol; CE, cholesteryl esters; PL, phospholipids.

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these patients ranged, respectively, between 180 and 239 mg/dl and 252 and 1,039 mg/dl.

Blood was taken after an overnight fast and allowed to clot for 3 hr at 0°C; sera were obtained by low speed centrifugation. EDTA was immediately added to two 1.0-ml serum samples from each subject (1 mM final) and solid NaBr was added to raise the non-protein density to 1.40 g/ml; one of these samples was processed within the same day, the other was stored at 4°C for 7 days. EDTA was added immediately to a third serum sample which was analyzed after 7 days storage at 4°C without NaBr.

Preparation of the HDL subfractions

The HDL subfractions were isolated by a modification of the procedure described by Groot et al. (18). Aliquots (0.5 ml) of the d 1.40 g/ml serum solutions were placed at the bottom of SW41 polyallomer tubes (Beckman, Palo Alto, CA) and then overlayered with NaBr-NaCl (0.15 M)-EDTA (1 mM) solutions (pH 7.4) of d 1.25 (4.5 ml), d 1.19 (5.5 ml), and d 1.006 g/ml (0.8 ml). The tubes were run at 15°C for 23 hr at 40,000 rpm. At the end of the run the tubes were punctured at 16 mm from the bottom with a G22 butterfly needle and connected to a Pharmacia (Uppsala, Sweden) P1 pump. The gradients were pumped at a flow rate of 1.0 ml/min and the effluent was passed through a Pharmacia UV1 monitor equipped with a 280-nm filter. Two fractions, corresponding to HDL_3 and HDL_2, were collected with a Pharmacia FRAC 100 fraction collector by pooling all material between the minimal optical densities recorded at 280 nm. The elution volumes of the HDL_3 and HDL_2 peaks and the total volumes of the two collected fractions were automatically monitored by the FRAC 100. Coefficients of variation for the same sample, recorded for ten runs performed on 2 different days, were 1.81% for the elution volumes and 1.73% for the fraction volumes. For the determination of the density profile along the gradients, fractions of 0.3 ml were collected from a "blank" gradient and the density of each fraction was measured at 15°C with an Abbe refractometer.

Chemical analyses

Total and free cholesterol (TC, FC), TG, and phospholipids (PL) were determined by enzymatic procedures (20-22). The cholesteryl ester (CE) mass was calculated as (TC - FC) × 1.68. The protein content of the HDL_2 and HDL_3 subfractions was determined by measuring the absorbances at 280 nm; the extinction coefficients determined in our laboratory for each subfraction were: 0.693 mg protein × A_{280} for HDL_2 and 0.782 mg protein × A_{280} for HDL_3. The apoprotein composition of delipidated fractions was analyzed by SDS-PAGE and apoproteins were quantitated by densitometry (23).

Statistical methods

Results are expressed as mean ± SD. Values from different groups were compared by a two-tailed paired Student's *t* test.

RESULTS

The effect of storage of serum samples at 4°C for 7 days, both in the presence (+NaBr) and in the absence (-NaBr) of 5.1 M sodium bromide, on the distribution of HDL subfractions, was analyzed by rate zonal ultracentrifugation in a swinging bucket rotor. Basal concentrations of HDL_2 varied substantially in the subjects, being markedly reduced in HTG patients, whereas the HDL_3 levels were relatively constant (**Table 1**). The UV elution profiles obtained from two subjects,

TABLE 1. Concentration of HDL_2 and HDL_3 constituents in serum before (basal) and after 7 days storage at 4°C, both in the presence (+NaBr) and absence (-NaBr) of 5.1 M NaBr

	Mass	FC	CE	PL	TG	Protein
			mg/dl			
HDL_2						
Basal	45.6 ± 35.7[a]	2.2 ± 1.3	9.2 ± 7.1	12.7 ± 8.8	2.0 ± 1.3	19.7 ± 11.6
+NaBr	43.9 ± 36.4	2.1 ± 1.2	9.5 ± 7.7	12.3 ± 9.8	1.9 ± 1.1	18.2 ± 10.3
-NaBr	43.2 ± 32.7	2.0 ± 1.2	8.8 ± 6.8	12.3 ± 8.0	1.9 ± 0.8	18.2 ± 9.7
HDL_3						
Basal	234.2 ± 28.1	5.4 ± 1.7	40.9 ± 10.7	59.0 ± 6.8	4.8 ± 1.8	124.0 ± 13.8
+NaBr	221.3 ± 25.6	5.2 ± 1.7	40.2 ± 10.5	57.9 ± 6.0	4.7 ± 1.8	113.3 ± 11.3[b]
-NaBr	201.8 ± 22.5[c]	3.5 ± 1.2[c]	38.6 ± 9.0[b]	48.4 ± 4.6[d]	5.6 ± 1.9	105.6 ± 10.8[c]

[a] Mean ± SD of 11 samples.
[b] $P < 0.05$, paired *t*-test versus Basal.
[c] $P < 0.001$.
[d] $P < 0.005$.

a normolipidemic with a relatively high serum content of HDL_2 and a hypertriglyceridemic with a low HDL_2 level, are shown in **Fig. 1.** HDL_3 from HTG patients constantly show lower elution volumes, compared to normolipidemic subjects (**Table 2**). This is probably due to an increased content of the denser HDL_{3D} subfraction (17). In all examined subjects, the absorption profiles of samples stored in the presence of NaBr were superimposable on the basal tracings. By contrast, significant variations were recorded for samples stored in the absence of salt. The absorption at 280 nm in the HDL_3 region was slightly decreased, compared to basal values, and the absorption maxima shifted to lower elution volumes (Table 2). The change in the elution profile of stored HDL_3 was particularly dramatic in the HTG patient with the highest TG level (1,039 mg/dl), in whom no separation could be achieved between HDL_3 and serum proteins (**Fig. 2**). No significant modifications were recorded in the HDL_2 region.

Changes in the HDL_3 elution profile are associated with altered concentration and composition, both in normolipidemic and HTG subjects (Table 1 and **Table 3**). Total lipoprotein mass decreased, in fact, by 15% in samples stored in the absence of salt, the reduction being evident for all the HDL_3 constituents, except for TG. A slight decrease (<10%) of the serum HDL_3 protein concentration was also noted in samples stored in the presence of NaBr (Table 1). A slight, nonsignificant reduction of the apoA-I/apoA-II ratio was recorded in HDL_3, isolated from sera stored both in the presence and absence of NaBr (basal, 3.99 ± 0.47; − NaBr, 3.77 ± 0.26; + NaBr, 3.81 ± 0.26). The lipid composition of HDL_3 was strongly modified during storage in the absence of salt; the TG and CE contents increased and the FC percentage decreased (Table 3), thus resulting in a highly significant reduction of the FC/CE ratio (basal, 0.133 ± 0.035; −NaBr, 0.095 ± 0.026, $P < 0.001$). The serum concentration and composition of the HDL_2 subfraction were not modified during storage. As a consequence, the HDL_2/HDL_3 mass ratio rose significantly in samples stored for 7 days in the absence of NaBr (basal, 0.192 ± 0.143; −NaBr, 0.215 ± 0.160, $P < 0.005$).

DISCUSSION

The HDL density fraction of human serum contains particles heterogeneous in size and lipid and apoprotein composition (24). A bimodal distribution can be obtained by analytical (14) and rate-zonal (17) ultracentrifugation, both producing two major subfractions, identified as

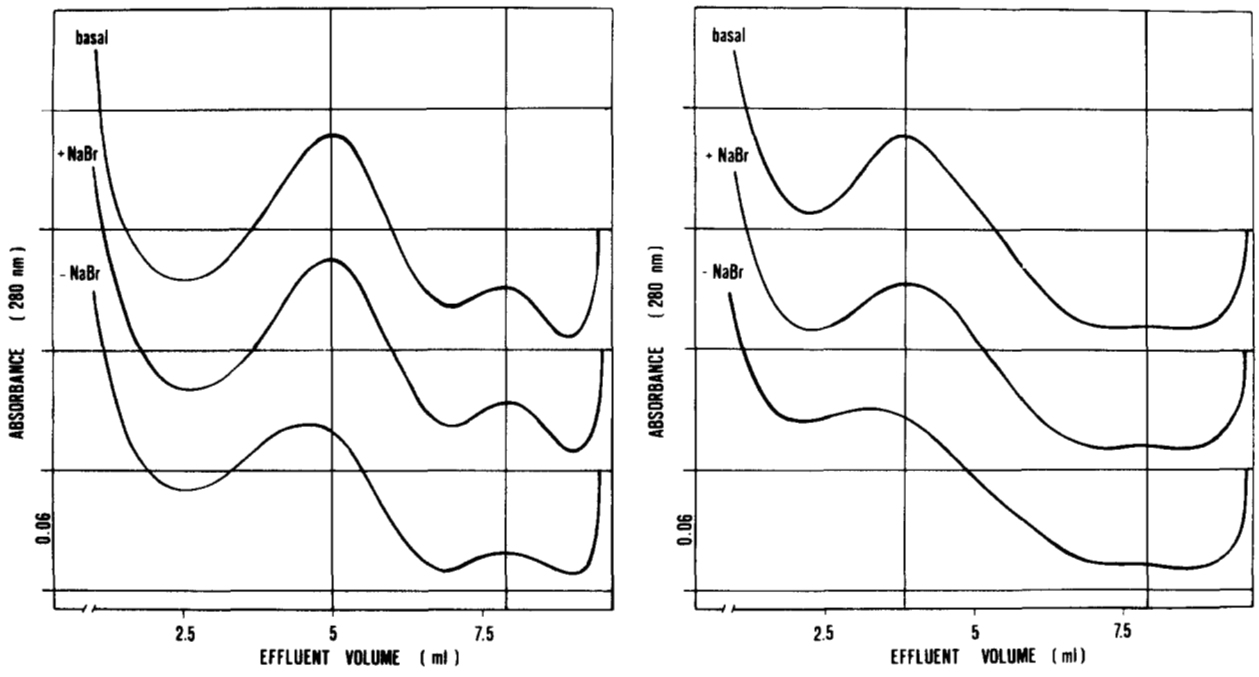

Fig. 1. HDL subfraction profiles after rate-zonal ultracentrifugation of sera from a normolipidemic volunteer (left) and from a hypertriglyceridemic patient (right) processed immediately after sampling (basal) and after 7 days storage at 4°C, both in the presence (+NaBr) and absence (−NaBr) of 5.1 M NaBr. Details of the ultracentrifugal and recording modalities are reported in Materials and Methods. In the absence of salt, the HDL_3 subfraction (left peaks) is shifted to lower elution volumes, with marked changes in the density profile.

TABLE 2. Elution volumes and densities of HDL_2 and HDL_3 before (basal) and after 7 days storage at 4°C, both in the presence (+ NaBr) and absence (− NaBr) of 5.1 M NaBr

	Elution Volume		Density	
	NTG[a]	HTG[b]	NTG	HTG
	ml		*g/ml*	
HDL_2				
Basal	8.16 ± 0.35	8.17 ± 0.37	1.153 ± 0.006	1.153 ± 0.007
+ NaBr	7.99 ± 0.24	8.00 ± 0.41	1.156 ± 0.004	1.156 ± 0.008
− NaBr	7.97 ± 0.29	7.95 ± 0.51	1.157 ± 0.005	1.157 ± 0.010
HDL_3				
Basal	5.06 ± 0.27	3.71 ± 0.10	1.203 ± 0.004	1.225 ± 0.003
+ NaBr	4.85 ± 0.32	3.72 ± 0.16	1.206 ± 0.005	1.224 ± 0.003
− NaBr	4.31 ± 0.38[c]	3.28 ± 0.13[c]	1.215 ± 0.006[c]	1.232 ± 0.003[c]

[a] Mean ± SD of six samples from normotriglyceridemic (NTG) subjects.
[b] Mean ± SD of five samples from hypertriglyceridemic (HTG) patients.
[c] $P < 0.01$, paired *t*-test versus Basal.

HDL_2 and HDL_3 according to their flotation characteristics. HDL_2 and HDL_3 differ in their lipid and apoprotein composition, HDL_3 being lipid-depleted and apoA-II-enriched (24).

The growing interest in the determination of the HDL subfraction distribution is hampered by the unavailability of simple and reliable methods of separation and quantitation. The recently proposed rate-zonal ultracentrifugal procedure in swinging bucket rotors (18) improves the classical method introduced by Patsch et al. (17), allowing the simultaneous separation of six samples at a time. However, a short storage period is still necessary if multiple samples have to be analyzed. In the present report, the effect of storage on the distribution and composition of HDL subfractions separated by this method is investigated. The HDL_2 concentration and composition do not change during prolonged storage at 4°C, but the HDL_3 subfraction is markedly modified. Total lipoprotein mass is reduced, flotation rate is decreased, and the lipid composition is significantly altered. These variations are prevented by the simple addition of solid NaBr to the serum sample before storage.

The changes in the lipid composition of HDL_3 (Table 3), i.e., TG and CE enrichment and FC depletion, suggest that some transfer of lipids to other lipoprotein fractions may occur. In particular, the reduced FC/CE ratio indi-

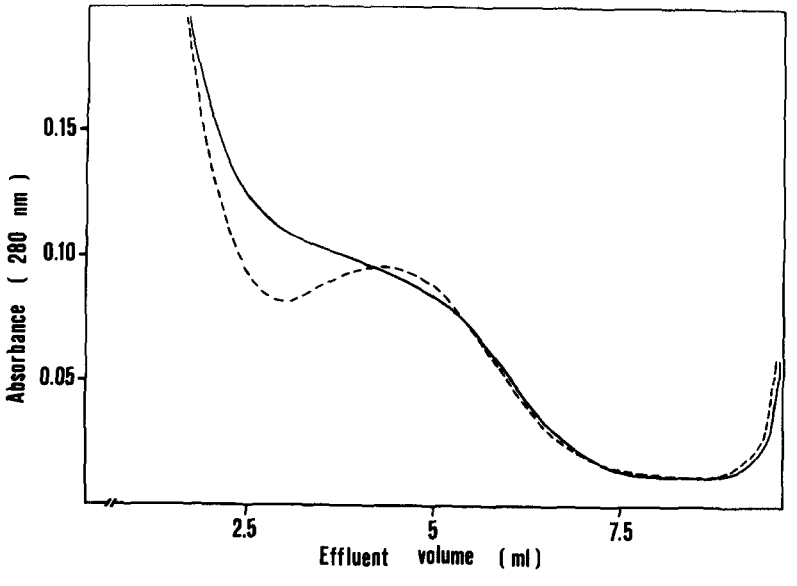

Fig. 2. Elution profiles of HDL subfractions from a severely hypertriglyceridemic patient (TG = 1,039 mg/dl), processed after 7 days storage of the serum at 4°C in the presence (----------) and absence (————) of 5.1 M NaBr. No separation between HDL_3 and serum proteins could be achieved in the sample stored in the absence of salt.

TABLE 3. Chemical composition of HDL_2 and HDL_3 before (basal) and after 7 days storage at 4°C, both in the presence (+ NaBr) and absence (− NaBr) of 5.1 M NaBr

	FC	CE	PL	TG	Protein
			% of total weight		
HDL_2					
Basal	4.1 ± 1.2[a]	18.5 ± 5.1	26.2 ± 3.2	6.1 ± 3.5	45.3 ± 4.5
+ NaBr	3.9 ± 1.2	18.7 ± 4.7	27.0 ± 4.3	5.8 ± 3.1	44.8 ± 5.5
− NaBr	3.8 ± 1.3	18.0 ± 5.0	27.2 ± 4.0	6.1 ± 3.1	44.8 ± 4.5
HDL_3					
Basal	2.3 ± 0.6	17.3 ± 2.5	25.2 ± 1.4	2.1 ± 0.9	53.0 ± 2.3
+ NaBr	2.3 ± 0.6	17.9 ± 2.6	26.2 ± 1.3	2.2 ± 0.9	51.3 ± 1.6
− NaBr	1.7 ± 0.6[b]	19.0 ± 2.4[c]	24.0 ± 1.2	2.9 ± 1.0[c]	52.4 ± 1.7

[a] Mean ± SD of 11 samples.
[b] $P < 0.001$, paired *t*-test versus Basal.
[c] $P < 0.01$, paired *t*-test versus Basal.

cates a possible involvement of LCAT in these modifications. LCAT activity is also known to be inhibited by elevated salt concentrations (25). Furthermore, the TG enrichment suggests that a net transfer of these lipids from other lipoprotein classes, catalyzed by a lipid transfer protein (26), may be operative not only at 37°C (27), but also at lower temperatures. That TG-rich lipoproteins may be involved in these modifications is indicated by the dramatic variation of the HDL subfraction distribution in the serum of the patient with the most severe hypertriglyceridemia.

Earlier studies, dealing with the determination of total HDL cholesterol, indicated reduced levels after prolonged storage at 4°C (28). This reduction, according to our data, is probably related to a significant loss of HDL_3-associated cholesterol. As the decrease in the total HDL cholesterol has been observed after precipitation of the apoB-containing lipoproteins, by measuring cholesterol in the supernatant, the lost HDL_3 cholesterol probably associates to apoB-containing particles.

A slight decrease of the A-I/A-II ratio has been detected in stored HDL_3. Apoprotein levels in sera, known to be stable for at least 46 days at 4°C (29), were not determined. The loss of apoA-I from HDL_3 is probably due to the storage conditions (30) and may account for the minimal decrease of the HDL_3 protein content. A special comment relates to serum or plasma samples, stored at 4°C, used in metabolic and biochemical in vivo and in vitro studies. The reported findings suggest limitations to their validity, and alert one to the fact that stored lipoproteins may not be comparable to their native counterparts.

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