Lipoprotein fractionation in deuterium oxide gradients: a procedure for evaluation of antioxidant binding and susceptibility to oxidation

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Abstract Oxidative modifications of lipoproteins appear to contribute to their atherogenecity. Very low and low density lipoproteins (VLDL and LDL) are protected against these modifications by antioxidants that can be incorporated in vivo or in vitro into the particles. We describe here ultracentrifugal procedures for isolation of VLDL and LDL that do not require subsequent dialysis or buffer equilibration. Lipoproteins were isolated in buffers with physiological ionic composition prepared in D_2O (deuterium oxide). This allowed measurements of the content of antioxidants and of the susceptibility to oxidation of the isolated LDL without further manipulations. Conventional ultracentrifugal methods use high salt concentrations and require additional steps to eliminate them. This introduces uncertainties in the evaluation of antioxidant binding and on measurements of their effect on VLDL and LDL oxidation. With the method described, the composition of the isolated VLDL and LDL was indistinguishable from that of fractions isolated with KBr gradients. Also, the content of α-tocopherol was similar. LDL isolated with KBr solutions appeared to bind 20–45% more of the probucol present in serum than LDL isolated in isotonic solutions prepared with D_2O. This was the case with probucol incorporated into plasma or serum in vivo or in vitro. Five out of seven LDL isolated with the D_2O procedure from different human sera appeared more resistant to Cu^{2+}-catalyzed oxidation than those obtained with KBr gradients from the same serum. In addition to the gradient procedure, we also describe a preparative version of the method that can be used with multiple samples.—**Hallberg, C., M. Hådén, M. Bergström, G. Hanson, K. Pettersson, C. Westerlund, G. Bondjers, A-M. Östlund-Lindqvist, and G. Camejo.** Lipoprotein fractionation in deuterium oxide gradients: a procedure for evaluation of antioxidant binding and susceptibility to oxidation. *J. Lipid Res.* 1994. **35:** 1–9.

Supplementary key words serum lipoproteins • ultracentrifugation in deuterium oxide • probucol binding to lipoproteins

Differential or density gradient ultracentrifugations in solutions of NaBr or KBr are the most used procedures for preparative and analytical separation of plasma lipoproteins (1, 2). These procedures, based on the fundamental work of De Lalla and Gofman (3) and Havel, Eder, and Bragdon (4) are flexible, provide good resolution and yield, when properly used, homogeneous lipoprotein classes suitable for most studies. The main drawback of these methods is the exposure of low density lipoproteins (LDL) and high density lipoproteins (HDL) for long periods to high concentrations of salts. This has been shown to introduce redistribution of apolipoproteins, compared with procedures that maintain the lipoproteins at isotonic ionic conditions, such as gel filtration and immunoadsorption (5, 6). High salt concentrations may obviously displace substances that are associated with lipoproteins by ionic forces. However, the forces between nonpolar molecular surfaces and those controlling the association between apolar substances and lipoproteins are also affected by electrolytes in aqueous solutions (7, 8).

Presently, there is interest in modifications introduced in LDL by free radical-mediated processes because of their potential contribution to atherogenesis (9). One factor that modulates the effect of free radicals on lipoproteins is the presence of lipophilic antioxidants associated with the particles (10). These substances can be bound to the lipoproteins in plasma both in vivo or in vitro. The synthetic free radical scavenger probucol and the naturally occurring α-tocopherol, carotenes, and ubiquinones are associated with nonpolar regions of LDL (10, 11). These antioxidants effectively protect LDL in vitro and ex

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HEPES, N-[hydroxyethyl]piperazine-N'-[ethanesulfonic acid]; HPLC, high performance liquid chromatography.

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vivo against oxidation and some of them reduce the progress of atherosclerosis in animal models (11–14). To study the mechanisms behind the protective effect of antioxidants, it is important to evaluate their in vivo and in vitro association with lipoproteins. To our knowledge, most of these studies have been carried out with lipoproteins isolated by ultracentrifugation in the presence of high salt concentrations (10, 11, 14, 15). High salt concentrations, and the subsequent steps required for desalting, may alter the association of nonpolar and polar substances with lipoproteins. Therefore, we developed a procedure in which VLDL and LDL were obtained with D_2O (deuterium oxide) gradients, isotonic in NaCl, without exposure to high salt concentrations. The D_2O isotonic gradient allowed fast (24 h) preparation of homogeneous VLDL and LDL fractions with compositions that were indistinguishable from those of fractions obtained with KBr gradients. The lipoproteins could be used immediately for analysis of susceptibility to oxidation by cells or transition metals without the need for dialysis or desalting. With this method we evaluated the effect of centrifugation at high salt concentrations on the association of probucol with LDL. We also compared the susceptibility of LDL to oxidation separated with KBr and deuterium oxide solutions. The results suggest that the extent of association of exogenous antioxidants, such as probucol, with LDL could be influenced by centrifugation in salt gradients.

MATERIALS AND METHODS

Chemicals

Probucol, D_2O, D-α-tocopherol and HEPES buffer (1 M solution) (H-0877) were purchased from Sigma (St. Louis, MO). Polyvinyl alcohol (mol wt 72,000), NaCl, KBr, $CaCl_2$, and $MgCl_2$ were analytical grade and were obtained from Merck (Darmstadt, Germany). Solvents used for lipid extraction and high performance liquid chromatography were from Rathburn, Walkerburn, Scotland. The internal standard used for probucol determination, 2-pentanone bis (3,5-di-tert) mercaptole, was a generous gift from Dr. Thomas Carew, University of California, San Diego.

Serum samples

Human lipoproteins were prepared from fresh serum samples obtained from apparently healthy subjects. No preservatives were added. Serum lipoproteins were also isolated from WHHL receptor-defective rabbits that had received a diet containing 1% (w/w) probucol for 3 months. The serum samples from rabbits were frozen at −80°C for 4 months. The frozen serum samples were thawed overnight at 2–4°C. After homogenization by inversion of the tubes, the samples were used directly for fractionation. In experiments in which lipoprotein composition, susceptibility to oxidation, and probucol content were compared, lipoprotein fractionation and characterization were done in parallel. This was required to insure that the time for fractionation and storage (never more than 24 h) of the lipoproteins was similar in the samples that were compared.

Analytical procedures

Total cholesterol, free cholesterol, and total phospholipids were evaluated using commercially available procedures (Boehringer Mannheim, Germany) adapted to microtiter plate versions. ApoB was measured by single radial immunodiffusion with the NOR-Partigen kit from Behring (Behringwerke AG, Marburg, Germany). Protein was measured with the Bio-Rad protein assay procedure (Bio-Rad, Richmond, CA) with the use of γ-globulin as a standard. This method gave a better agreement with gravimetric measurements of LDL-protein than Lowry's method using albumin as standard. Individual cholesteryl esters were quantitatively measured using high performance liquid chromatography (HPLC) (16). Probucol and α-tocopherol in LDL and serum were also evaluated by HPLC. Serum or LDL (100 μl) were precipitated with 250 μl ethanol containing the internal standard 2-pentanone bis (3,5-di-tert) mercaptole. The samples were extracted with hexane (2 ml), and after centrifugation, the organic phase was transferred to new tubes and 20 μl octanol was added. The hexane was evaporated under nitrogen and the octanol phase was mixed with 150 μl of mobile phase. The mobile phase was made of methanol–acetonitrile–water 45:45:10 (v/v). Chromatography was performed on a Nucleosil 100-5C_{18} column (150 × 4.6 mm) (Macherey-Nagel, Germany). Probucol and the internal standard were detected at 242 nm. The limit of detection was 0.3 μmol/l. Alpha-tocopherol was also evaluated by HPLC using the same type of extraction procedure and column as with probucol. The internal standard was α-tocopherol acetate. The mobile phase was methanol–acetonitrile–water 48:48:4 (v/v). The detector was set at 291 nm.

Electrophoresis of isolated lipoproteins was performed on agarose (Sea-Kem, LE, FMC BioProducts, Rockland, ME), 0.7% (w/v). The gel was prepared in Tris-borate-EDTA buffer, pH 8.4 (Sigma, TEB-9) and spread on Gelbond films (FMC) to approximately 2.0 mm thickness. The samples containing 10% (v/v) glycerol were applied and electrophoresis was run in a "submarine" apparatus for 80 min at 60 V (constant) with the electrode buffer the same as used for the gel. The fixed and dried gels were stained with Coomassie brilliant blue.

Fractionation of lipoproteins in D_2O gradients

Three solutions were used for gradient preparation (**Table 1**): solution A, 10 mM HEPES buffer, pH 7.2,

TABLE 1. Solutions used for isolation of lipoproteins by density gradient and differential centrifugation in isotonic conditions

Solutions	Density, g/ml, 20°C	Mixture	Respective Volumes to Mix
A	1.116	None[a]	None
B	1.006	None[b]	None
C	1.072	Sol A + Sol B	3.0 + 2.0
D	1.019	Sol B + Sol A	3.0 + 0.4
E	1.062	Sol D + Sol A	2.0 + 1.6

[a]Solution A contained 10 mM HEPES buffer, pH 7.2, and 140 mM NaCl prepared in D_2O.
[b]Solution B contained 10 mM HEPES buffer, pH 7.2, and 140 mM NaCl prepared in H_2O.

140 mM NaCl prepared in D_2O, density 1.116 g/ml; solution B, 10 mM HEPES buffer, pH 7.2, 140 mM NaCl prepared in H_2O, density 1.006 g/ml; solution C, density 1.072 g/ml made by mixing 3 parts of solution A with 2 parts of solution B, density 1.072 g/ml. The HEPES buffer used was purchased as a 1 M dilution to simplify the preparation of the gradient solutions. These solutions contained no antioxidant or enzyme inhibitors. The gradients were formed in Ultra-Clear tubes that were made hydrophilic by pre-treatment with polyvinyl alcohol, a non-water-soluble polymer (17). Two ml serum was mixed with 3 ml solution A. This produced a solution with a final background density of 1.072 g/ml. The resulting 5 ml was placed at the bottom of the tube and an exponential gradient (1.006–1.072 g/ml) was layered on top with the use of a simple disposable syringe device (18). The lower chamber of the syringe was filled with 3 ml of solution C and the upper chamber with 5 ml of solution B. A multihead peristaltic pump and a magnetic stirrer were used to prepare six gradients simultaneously. The tubes were centrifuged in a SW-40 Beckman rotor (Beckman, Palo Alto, CA) or RP5 40T LKB (LKB, Bromma, Sweden) at 35,000 rpm (184,000 g_{av}) for 24 h at 20°C. After centrifugation, 1.2-ml fractions were collected by bottom displacement with the use of an ISCO UV-A5 gradient fractionator (ISCO, Lincoln, NE). The density of the solutions and of the fractions obtained from the gradients was monitored gravimetrically. HEPES buffer was selected for the preparation of the solutions because it does not interfere with Cu^{2+} oxidation of LDL and is one of the most used buffers in cell culture systems.

Fractionation of lipoproteins by differential centrifugation on D_2O solutions

Sequential isolation of VLDL, LDL, and HDL plus serum proteins was carried out in a fixed-angle rotor with capacity for 44 samples (Beckman 50.4) and the use of polycarbonate tubes with no caps. Three ml of serum (plasma can be also used) was mixed with 0.4 ml of solution A (Table 1, d 1.116 g/ml) to a final density of 1.019 g/ml. The tubes were centrifuged at 40,000 rpm (111,000 g_{av}, inner row, 132,000 g_{av} in the outer row) for 20 h at 20°C. The VLDL was collected by aspiration of 1.4 ml from the top of each tube. The VLDL fraction could be recentrifuged by mixing with solution D (d 1.019 g/ml) made of 3 volumes of solution B (Table 1, d 1.006 g/ml) plus 0.4 volume of solution A (d 1.116 g/ml). The infranatant containing LDL, HDL, and serum or plasma proteins was adjusted to 3.4 ml with solution D and recentrifuged under the same conditions. After discarding the residual VLDL in the upper 1.4 ml, the volumes of each tube were adjusted to 2.0 ml with solution D. This was conveniently done with a balance knowing that the density of the liquid was 1.019 g/ml. The resulting 2 ml was mixed with 1.6 ml of solution A (d 1.116 g/ml) to a final density of 1.062 g/ml. The tubes were centrifuged as before and the LDL fraction was recovered from the meniscus. This fraction was homogeneous when examined by gel electrophoresis. However, it could be recentrifuged by mixing with solution E (d 1.062 g/ml) made of 2 volumes of solution D and 1.6 volumes of solution A. The purified LDL fraction was recovered from the top of the tubes by aspiration. This procedure can be easily adapted to other rotors and tubes that provide more than 110,000 g_{av}.

Density gradient centrifugation in KBr gradients

Serum lipoproteins were also fractionated by the single spin, density gradient procedure of Redgrave, Roberts, and West (19). Solutions of KBr were used to prepare the initial discontinuous gradient. After centrifugation, fractions were collected as described for the deuterium oxide gradients. Fractions were analyzed for probucol content after extraction with hexane (see Analytical Procedures) and total cholesterol. In these procedures no interference of KBr could be detected. For other analyses and evaluation of susceptibility to oxidation, the fractions containing LDL were pooled. The fractions containing LDL were desalted by equilibration with solution B (Table 1) in PD-100 gel chromatography columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Because the aim of the work was to compare the LDL obtained with this gradient and the one obtained with D_2O gradient, here also no chelator or other substances were included in the solutions.

In vitro binding of probucol to serum lipoproteins

Probucol (1 mM) was dissolved in ethanol. The appropriate amounts of this solution were added to serum samples to reach final concentrations of 10 and 20 μM. The samples were allowed to stand protected from light at 20 ± 2°C for 1 h before centrifugation. The serum of the WHHL rabbits treated with probucol was frozen at −80°C for 4 months before fractionation and analysis.

Evaluation of susceptibility to oxidation

A microtiter version of the thiobarbituric acid reaction (TBARS) was used to measure the Cu^{2+}-catalyzed oxidation of LDL. In this procedure, oxidation and TBARS evaluation were carried out in the same plate as described (20). Oxidation was induced by addition of 10 μM $CuSO_4$ (final concentration) to solutions of the lipoproteins (200 μg protein/ml) in 10 mM HEPES buffer containing 140 mM NaCl. This procedure, when used with purified LDL, showed an excellent correlation with the rate of increase in absorption at 232 nm associated with diene formation. Formation of peroxides was measured in a microtiter version of the method of El-Saadani et al. (21) that was also found to correlate well with diene formation.

Measurement of copper in KBr and deuterium oxide solutions

Copper was measured in the solutions of deuterium oxide and KBr used for ultracentrifugal fractionation of lipoproteins by atomic absorption spectrophotometry (22).

RESULTS

Lipoprotein distribution after centrifugation in D_2O or KBr gradients

Fig. 1 compares the fractionation of a human serum with normal levels of triglycerides (1.1 mmol/l) and cholesterol (4.8 mmol/l) in density gradients made with deuterium oxide or KBr solutions. The average cholesterol profile of 20 × 0.6 ml fractions obtained from five samples of the same serum that were run in the same rotor is presented. Also the density profiles measured at the end of the run are shown. The D_2O gradient after 24 h centrifugation became a shallow and almost linear gradient which provided a good separation of VLDL from LDL, and of both from HDL and other serum proteins. The shallow gradient displays the density heterogeneity of LDL. The KBr gradient remained, on the other hand, an exponential gradient with LDL and HDL close to each other but separated from other plasma proteins as described in the original procedure (19). This was the case also for newer versions of single-spin salt gradients (2). The somewhat higher standard deviations in the cholesterol distribution profiles of the KBr gradient compared with the D_2O gradient samples are probably a reflection of the steeper gradient. This magnifies the errors between tubes during gradient formation and fraction collection.

Properties of LDL isolated with D_2O or KBr gradients

The percentage composition of the d 1.020–1.063 g/ml fraction (LDL) isolated from aliquots of the same serum sample with the use of deuterium oxide and KBr gradients is presented in **Fig. 2 (A)**. The data obtained show that in terms of composition the fractions obtained by the

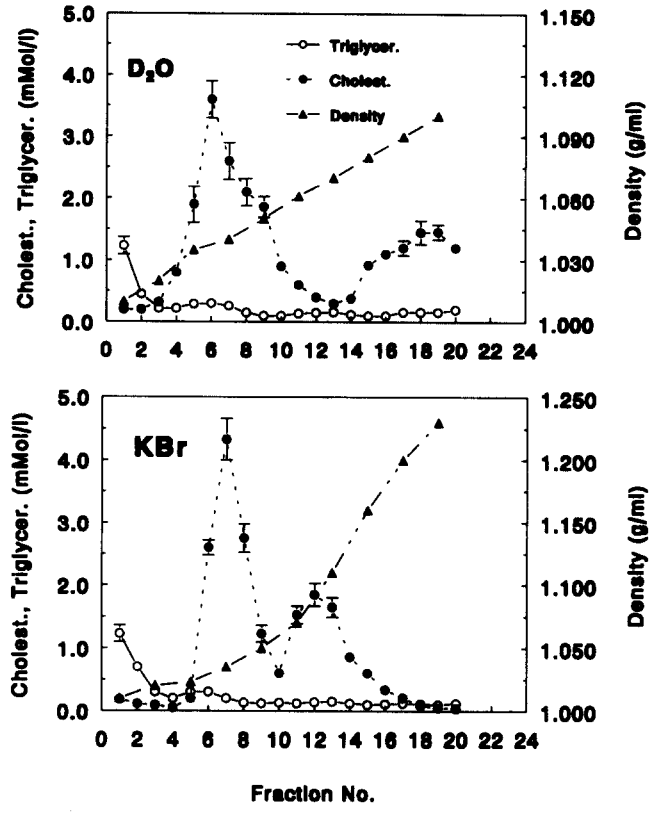

Fig. 1. Density gradient ultracentrifugation of human serum. Cholesterol distribution (filled circles), triglycerides (empty circles), and density profiles (filled triangles) of deuterium oxide (D_2O) and KBr gradients. Each experiment consisted of six tubes containing samples of the same human serum. Cholesterol and triglycerides were measured in 0.6-ml fractions collected after centrifugation. The density profiles were measured in 1.2-ml fractions obtained from gradients run with "mock serum." The values are the mean of determinations in five individual tubes and the bars at each point are the standard deviations of the means.

two gradients were indistinguishable. Fig. 2 (B) presents the results of analysis of the cholesteryl ester composition of the LDL fraction obtained from the same serum prepared with the two types of gradient. Here also no differences could be observed. The fraction less than d 1.019 g/ml (VLDL) was also analyzed, and again no differences could be detected between the VLDL isolated with the D_2O and KBr gradients (data not shown). The small standard deviations suggested that the procedures yielded LDL fractions with very similar composition. One important aspect of a lipoprotein fractionation procedure is the recovery. This determines whether the method could be used for a quantitative purpose. We used immunological evaluation of apoB in the unfractionated serum and of the LDL fractions isolated from the gradients to evaluate recovery. The amount of LDL-apoB present in the region d 1.020–1.063 g/ml of the deuterium oxide and the KBr gradients was measured by radial immunodiffusion for six

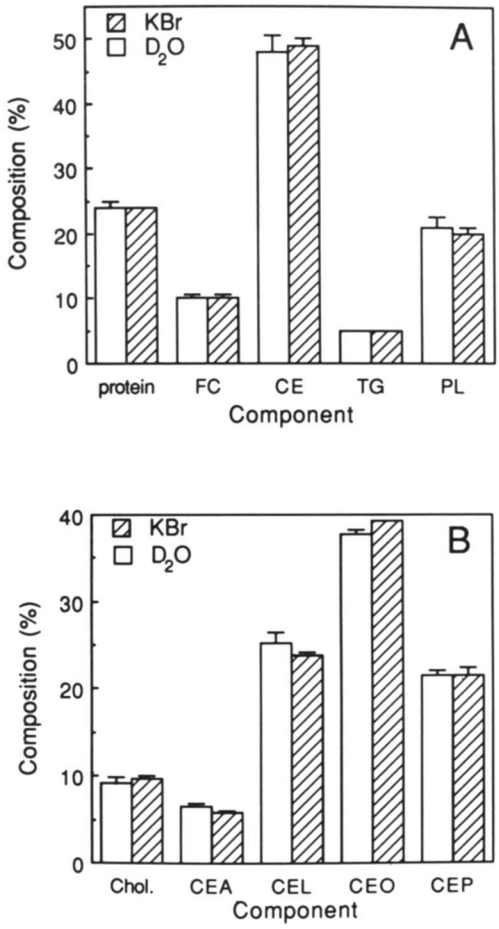

Fig. 2. Panel A, percentage composition of LDL prepared in D_2O and KBr gradients. Panel B, percentage composition of cholesteryl esters of LDL isolated with D_2O and KBr gradients. The abbreviations represent (A): FC, free cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids; (B): Chol, free cholesterol; CEA, cholesteryl arachidonate; CEL, cholesteryl linoleate; CEO, cholesteryl oleate; CEP, cholesteryl palmitate. Each experiment consisted of six individual tubes in which the same human serum was run with each gradient. The values are the mean of duplicate determinations in LDL separated in three individual tubes. The segments on top of the bars represent the positive standard deviation.

samples of the same human serum. The two procedures gave essentially the same recovery, 0.61 ± 0.05 (D_2O) and 0.62 ± 0.03 mg/ml of serum (KBr). In terms of the total apoB present in the sera analyzed, this represented a recovery better than 85%. The rest of the apoB probably was associated with the VLDL fraction. Total cholesterol recovery in the added fractions of the D_2O gradient was better than 90% if no leaks occurred during fraction collection.

"Submarine" agarose gel electrophoresis of the fractions obtained with the two types of gradients showed no other proteins or lipoproteins either in the fraction with density less than 1.019 g/ml or in the fraction of d 1.020–1.063 g/ml. Also, in VLDL and LDL fractions purified by the differential version of the deuterium oxide centrifugation procedure, the agarose electrophoresis analyses showed no cross-contamination or the presence of other serum proteins. **Fig. 3** shows the results of a gel containing LDL fractions from the same serum obtained with the D_2O and KBr gradients. These results, with those from the composition analysis, suggest that the deuterium oxide gradient and differential centrifugation versions yielded VLDL and LDL of similar homogeneity as that achieved with KBr gradients.

Binding of probucol to lipoproteins isolated with D_2O or KBr gradients

Probucol administered orally to animals and humans appears in plasma mainly associated with LDL and VLDL (10, 11, 15). When added directly to human plasma at saturating or nonsaturating conditions, probucol partitions preferentially toward LDL. However, the relative proportion bound by LDL and VLDL seems to depend on the mass of neutral lipids present in these lipoproteins. The affinity of probucol for HDL is much lower, probably because of its low content of cholesteryl esters and triglycerides (23). The affinity of probucol for other plasma proteins or erythrocyte membranes is low (23). However, as mentioned before, the cited work was conducted with lipoproteins isolated with centrifugation in high salt concentrations. We compared the distribution of probucol in different sera that were fractionated with the deuterium oxide gradient or the KBr gradient. **Fig. 4** shows the distribution of probucol as percentage of total serum probucol in human serum fractionated by the two procedures (cholesterol 6.1 mM, triglycerides 0.9 mM). Probucol was added in vitro to these serum aliquots as an ethanolic solution and to final concentrations of 10 μM (A) and 20 μM (B). Evidently, in the deuterium oxide gradients,

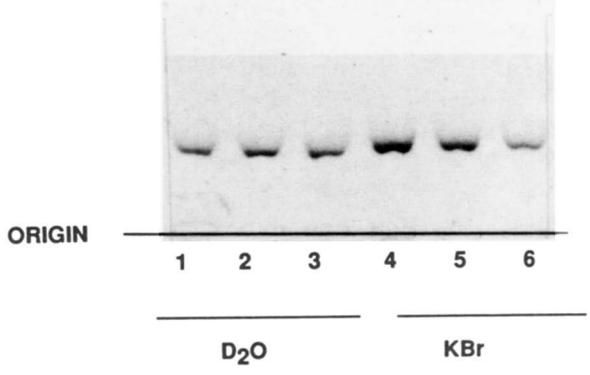

Fig. 3. Agarose electrophoresis of LDL fractions isolated with D_2O (samples 1–3) and KBr gradients (samples 4–6). The samples were run in a "submarine chamber" and were stained with Coomassie brilliant blue.

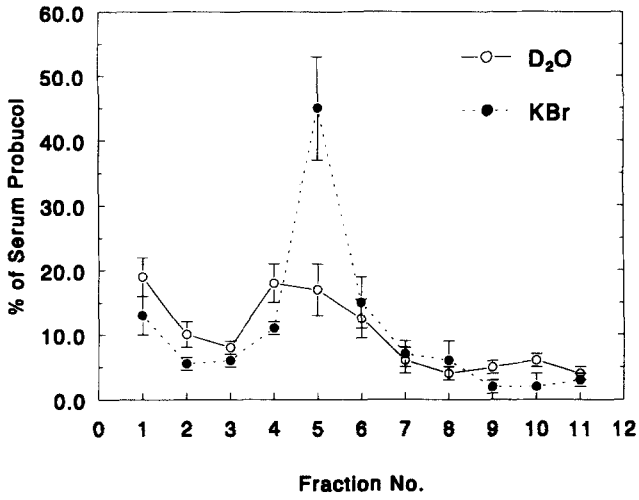

Fig. 4. Distribution of probucol in human serum lipoproteins fractionated with D_2O (solid lines) and KBr gradients (dashed lines). The serum aliquots were incubated with 10 μM probucol. The content of probucol was measured in each 1.2-ml fraction from the gradients by HPLC as described in Materials and Methods. Each experiment consisted of six tubes containing the same human serum. The values are the means of duplicate analysis obtained from three individual tubes from each gradient type. The bars at each point represent the standard deviations of the probucol determinations.

the amounts of probucol associated with LDL were lower than in the KBr gradients. **Table 2** shows the results obtained with two different sera with 10 μM probucol and one serum with 10 and 20 μM. The results are expressed as percentage of total serum probucol present in the VLDL (fractions 1 and 2 of the gradients) and LDL (fractions 3 to 7 of the gradients). The data suggested that the LDL obtained with KBr solutions contained 20–45% more of the serum probucol than a similar fraction obtained with the deuterium oxide gradients. Probucol appears not to bind symmetrically along the LDL-cholesterol profiles shown in Fig. 4. We do not have an explanation for this observation. An evaluation of the probucol content per LDL particle of different LDL subclasses will be required to explain this finding.

One important question not addressed by the above experiments is the possibility that the probucol incorporated in vivo into VLDL and LDL may be affected differently by the presence of high salts in the isolation procedure. We explored this possibility with the use of plasma obtained from WHHL rabbits that had been fed 1% (w/w) probucol for 3 months. The results presented in **Fig. 5** show that here also the LDL fraction obtained with the KBr gradients contained more of the serum probucol than the corresponding fractions prepared with D_2O gradients. Table 2 presents the quantitative probucol distribution in VLDL and LDL from WHHL rabbits isolated with the two types of gradient. It is possible that storage at −80°C modified the partition of probucol between the different lipoproteins. However, the differences observed in probucol distribution in the two gradients in the experiments presented in Fig. 5 and Table 2 were probably not related to the storage at −80°C because the sera were frozen for the same period.

Susceptibility to oxidation of LDL isolated in D_2O or KBr gradients

Because the LDL fractions can be used directly for measurements of sensitivity to oxidation, we compared the kinetics of Cu^{2+}-catalyzed oxidation of LDL from the same sera prepared with D_2O and KBr gradients. The fractions from the KBr gradients were rapidly equilibrated in PD-10 columns (Pharmacia, Uppsala, Sweden)

TABLE 2. Distribution of probucol in VLDL and LDL of human and WHHL rabbit sera fractionated with the deuterium oxide and KBr gradients

			% Probucol in VLDL[b]		% Probucol in LDL[b]		
Serum	Chol[a]	TG[a]	D_2O	KBr	D_2O	KBr	P^c
1.10 μM Probucol	4.6	0.8	13.3 ± 1.4	10.7 ± 2.2	39.8 ± 0.4	81.6 ± 5.2	3 vs 4 < 0.002
2.10 μM Probucol	6.1	0.9	11.3 ± 0.9	7.6 ± 1.5	62.7 ± 10.4	81.0 ± 10.2	3 vs 4 < 0.05
2.20 μM Probucol	6.1	0.9	28.2 ± 4.9	17.5 ± 2.3	58.6 ± 7.8	81.1 ± 6.5	1 vs 2 < 0.02
							3 vs 4 < 0.005
3. WHHL[d]	11.8	1.3	5.9 ± 1.0	6.9 ± 1.6	24.6 ± 3.4	43.3 ± 6.8	3 vs 4 < 0.01

[a]Total serum cholesterol (Chol) and triglycerides (TG) (mM).
[b]Percentage of total serum probucol in the lipoprotein fractions. For VLDL, fractions 1 and 2 (d < 1.019 g/ml) from the gradients were pooled. For LDL, fractions 3 to 7 were pooled (d 1.020–1.063 g/ml). Total serum probucol was measured in aliquots of the incubated serum. Each experiment included six samples from the same serum that were fractionated simultaneously using the two types of gradient. Each gradient was collected in 10 to 11 fractions of 1.2 ml that were subsequently analyzed individually for their probucol content. The values represent the mean probucol content of three individual LDL fractions analyzed in duplicate ± SD.
[c]Statistical significance of the differences between the indicated fractions.
[d]A pool of sera from four animals that were treated for 3 months with probucol was used. The final concentration of the drug was 85 μM in the pool used.

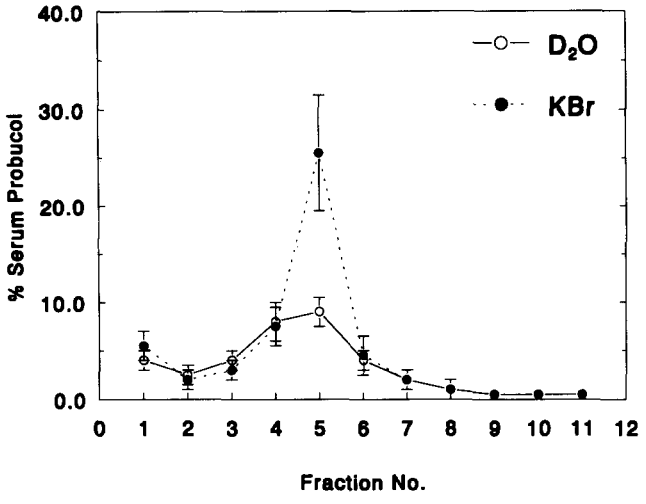

Fig. 5. Distribution of probucol in a pool of sera from four WHHL rabbits that were treated for 3 months with 1% (w/w) of the drug. The same serum was fractionated in D_2O (solid line) and KBr (dashed line) gradients. Analysis and conditions were the same as those described in Fig. 4.

with the buffer present in the D_2O gradient but prepared in Milli-Q water (Millipore Corp. Marlborough, MA). The LDL fractions obtained with the two gradients from seven different sera were oxidized and the kinetics of formation of TBARS and lipid hydroperoxides were evaluated in a single microtiter plate version of the procedure (20). The results indicated that in four out of seven sera the LDL obtained in deuterium oxide gradient was more resistant to oxidation than a similar fraction prepared from the same serum with a KBr gradient. In **Fig. 6** and **Fig. 7** are presented the results of kinetics of TBARS and hydroperoxide formation for three individual sera: two showing the differences in kinetics of oxidation when isolated in the two gradients (serum 1 and serum 3), one in which no difference was observed (serum 2). These fractions were isolated in parallel in the same rotor with the two gradients and were stored at similar concentration (2–3 mg protein/ml) for similar periods, less than 24 h. LDL fractions obtained by the two gradients from each serum had indistinguishable fatty acid compositions (Fig. 2). Also the α-tocopherol content was similar in LDL isolated with KBr and equilibrated in PD-10 columns (3 samples, 9.9 ± 2.6 nmol/mg protein) and LDL from the same human serum isolated with D_2O (three samples, 11.7 ± 2.7 nmol/mg protein) or for three LDL samples from three different human sera run in parallel in the two procedures (KBr, 9.3 ± 2.4 nmol/mg protein; D_2O, 10.3 ± 2.6 nmol/mg protein). Therefore, the differences may be caused by dissimilar content of other antioxidants (not measured) or to subtle alteration of lipoprotein structure induced by the separation under high KBr concentrations or the subsequent equilibration step. A third possibility was suggested by the copper analyses of the KBr solutions used for LDL isolation. They indicate that the lipoproteins were exposed to a maximum of 6–7 μM copper when KBr (pro analysis) was used for the isolation. On the other hand, in the D_2O method, serum or LDL were exposed to a maximum of 1.5 μM copper. LDL has a high affinity for copper (10, 24). The bound metal may add to the copper in the oxidation experiments and contribute to the higher oxidation rate observed or may initiate lipid peroxidation during isolation. That this may be the case is supported by results in which the spontaneous accumulation of hydroperoxides (no Cu^{2+} added) was followed in LDL obtained with the two gradients from the same serum. **Fig. 8** presents these results showing that in these LDL fractions the spontaneous accumulation of lipid

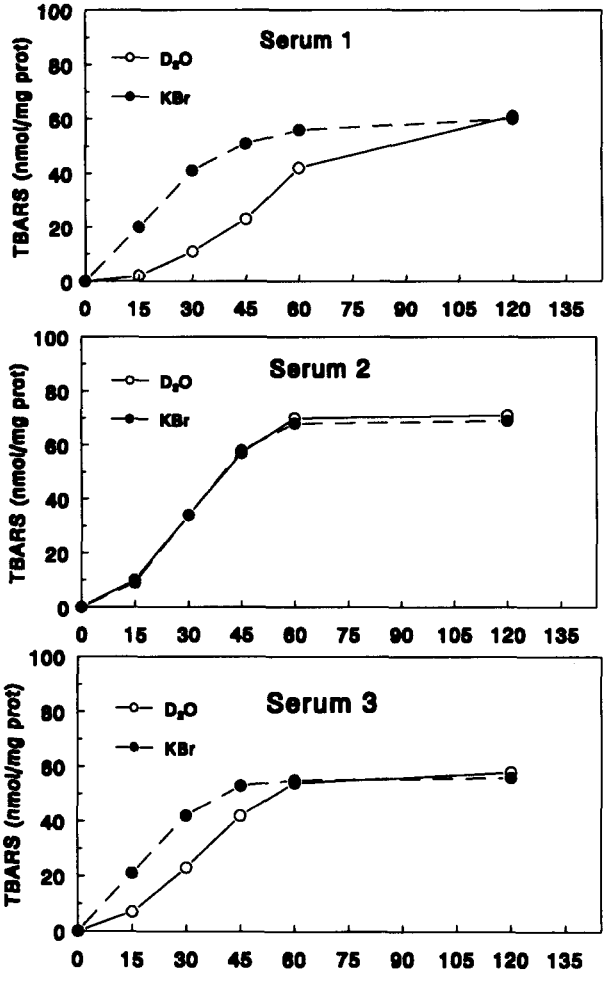

Fig. 6. Kinetic of Cu^{2+}-catalyzed oxidation of human LDL obtained with D_2O (solid lines) and KBr (dashed lines) gradients from three different sera. Oxidation was followed by measurements of TBARS. The points are the means of duplicate determinations conducted in the microtiter plate version of the procedures (see ref. 20).

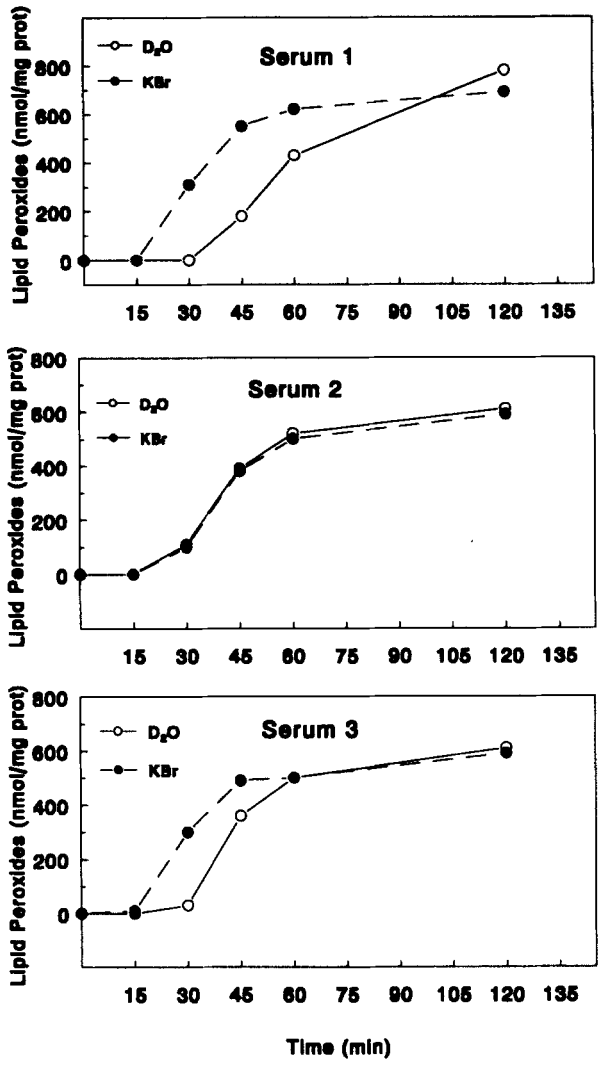

Fig. 7. Kinetic of Cu^{2+}-catalyzed oxidation of human LDL obtained with D_2O (solid lines) and KBr (dashed lines) gradients from three different sera (the same preparations as in Fig. 6). Oxidation was followed by measurements of lipid hydroperoxides. The points are the means of duplicate determinations conducted in the microtiter plate version of the procedures (see ref. 20).

hydroperoxides occurs at a higher rate. A fourth possibility is that D_2O inhibits the spontaneous or transition metal-mediated oxidation of LDL. The lack of effect of additions of D_2O solutions to LDL isolated with KBr solutions does not support this possibility.

DISCUSSION

Deuterium oxide was used in several fundamental studies on characterization of lipoproteins with the analytical ultracentrifuge (4). D_2O is not widely used presently for preparative lipoprotein fractionation because of its price and because it is not possible to use it to separate HDL from the rest of serum or plasma proteins. However, in experiments in which only VLDL and LDL are required, density gradient or differential centrifugation in isotonic solutions of deuterium oxide appear as convenient alternatives. The price difference with the high salt methods becomes insignificant because of the time saved in the deuterium oxide procedure as no dialysis or equilibration is needed. However, the most important consideration in favor of the use of deuterium oxide solutions is that they yield homogeneous VLDL and LDL that have not been in contact with nonphysiological concentrations of salts. This is a condition that may introduce modifications in lipoprotein structure and composition (5, 6). This aspect is critically relevant if the intention of the experiment is to analyze association of substances in plasma or serum with lipoproteins. Ionic associations between charged groups at the surface of lipoproteins and polar compounds are strongly diminished by the presence of high salts. On the other hand, depending on the dipole moment of the compounds and the structure of the surface monolayer and nonpolar core of lipoproteins, the association with nonpolar substances may be favored by high salts (7, 8). Probucol, a hydrophobic substance, has been postulated to be associated with the nonpolar core of VLDL and LDL. The amount of probucol bound to these lipoproteins in serum is related to the mass of triglycerides and cholesteryl esters, respectively (8, 10, 11, 23). The results obtained with probucol in our experiments suggest that the affinity for LDL could be increased when the serum is mixed with high KBr concentrations. We believe that the distribution obtained with the use of isotonic solutions of deuterium oxide would better reflect that

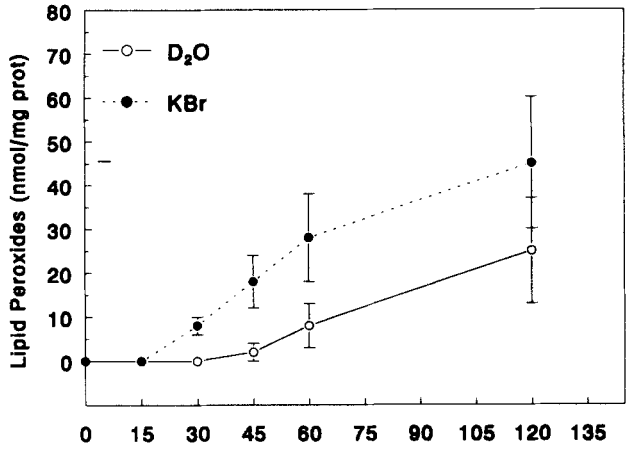

Fig. 8. Kinetics of spontaneous formation of lipid hydroperoxides in LDL incubated at 37°C. The solid line represents the results from LDL prepared with the D_2O gradient, the broken one those from LDL from the same serum prepared with KBr gradients. The values are means obtained from three tubes of each gradient type and the bars are the standard deviations.

present in vivo. The results also indicate that the in vivo effects of probucol related to its binding to LDL and antioxidant protection conferred to the lipoprotein depend on fewer molecules per particle than previously suggested (9–11). The following interesting situation may arise upon isolation of LDL from animals and subjects treated with probucol with the use of KBr. These isolated particles may contain more probucol than in serum or plasma and this could make them more resistant to oxidation. But, at the same time, KBr fractionation appears to potentiate oxidation. The net effect of these actions will be difficult to predict because of the potential variations in the native LDL of its affinity for probucol and susceptibility to oxidation.

An additional convenience in the use of D_2O for isolation of LDL may be the preparation of particles that retain their native complement of polar and nonpolar antioxidants and prooxidants that may be lost during dialysis or equilibration. The use of such preparations in studies of LDL susceptibility to oxidation by cells and transition metals could provide a material closer to the native one than the one provided by centrifugation in high salt solutions.

We are grateful to Hans Jönsson for his help on the measurements of probucol and to Lars Svensson for the measurements of copper content. The part of this work conducted at the Wallenberg Laboratory was partially supported by grant 4531 of the Swedish Medical Research Council and Grants from The Swedish Heart and Lung Foundation.

Manuscript received 8 December 1992, in revised form 15 June 1993, and in re-revised form 16 August 1993.

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