Preparation of biologically active analogs of serum low density lipoprotein

Bo Lundberg[1] **and Liisa Suominen**

Department of Biochemistry and Pharmacy, Åbo Akademi, SF-20500 Åbo 50, Finland

Abstract A method for the preparation of stable and water-soluble analogs of low density lipoprotein (LDL) is presented. The experimental protocols start with the preparation of a cholesteryl ester/phospholipid microemulsion by a combined injection-sonication procedure and delipidation of apoprotein B (apoB) with sodium deoxycholate (NaDOC). The association of lipid microemulsion and NaDOC-solubilized apoB is achieved by incubation and sonication of the components above the melting point of the cholesteryl ester. The reconstituted model LDL (m-LDL) proved to be quite homogeneous both with respect to particle size and composition. Negative-stain electron microscopy shows spherical particles with a mean diameter of 21 nm. The mean density of the reconstituted LDL was 1.07 g/ml as determined by sucrose density gradient centrifugation. The reconstituted LDL retained its β-mobility on agarose gel electrophoresis, and sodium dodecyl sulfate (SDS)-gel electrophoresis showed no degradation of apoB during the reconstitution procedures. Studies of biological activity showed that the m-LDL particles are bound, incorporated, and degraded by human fibroblasts in a way similar to native LDL. The reconstituted m-LDL has potential use for metabolic, physicochemical, and enzymatic studies of lipoproteins.—**Lundberg, B., and L. Suominen.** Preparation of biologically active analogs of serum low density lipoprotein. *J. Lipid Res.* 1984. **25:** 550–558.

Supplementary key words apoB delipidation • lipid microemulsion • sonication • cholesteryl ester • phospholipid

Low density lipoprotein (LDL) has an important role in cholesterol transport and metabolism. LDL consists of an apolar core of neutral lipids (cholesteryl esters, triglycerides) surrounded by a polar shell (phospholipids, cholesterol, protein) (1, 2). LDL delivers cholesterol to peripheral cells via receptor-mediated endocytosis and the cholesterol homeostasis in cells under normal conditions is regulated within narrow boundaries (3). However, atherosclerosis is featured by an excessive uptake of cholesterol into foam cells. Much attention has been attached to the uptake of modified LDL. Chemical modification of apoB has been shown to alter the binding and uptake of LDL (4), but alterations of the composition and physical properties of the lipid part may also affect the uptake of LDL. Lipoproteins from hypercholesterolemic animals exhibit order-disorder transitions above physiological temperatures (5) and these animals are usually more prone to atherosclerosis. Thus there is an obvious need to modify the lipid composition of LDL in a systematic way for physical and metabolic studies.

Krieger et al. (6) have described a reconstitution procedure by which the core cholesteryl esters can be removed from the LDL particle by heptane extraction and replaced with an equal amount of exogeneous cholesteryl ester. Microemulsions of phospholipids and neutral lipids, as protein-free models for LDL, have been prepared by sonication (7–10) and by injection techniques (11). The particle diameter of these microemulsions is in the same order of magnitude as that of native LDL. The method for reconstitution of LDL-like particles presented in this study starts from a phospholipid-cholesteryl ester microemulsion system and detergent-delipidated apoB.

MATERIALS AND METHODS

Isolation of LDL

LDL (d 1.025–1.050 g/ml) was isolated from blood of healthy donors who had fasted overnight. A mixture of preservatives was added to the freshly drawn blood; final concentrations were 500 units/ml penicillin-G, 50 μg/ml streptomycin sulfate, 0.05% EDTA, 0.05% NaN_3, 0.2 mM phenylmethylsulfonyl fluoride, and 0.005% 4-methyl-2,6-*tert*-butylphenol. Isolation of LDL was started at once by the density-gradient ultracentrifugation method of Redgrave, Roberts, and West (12). The LDL obtained was recentrifuged through an overlayering KBr solution of density 1.050 g/ml. The density of the solutions was monitored by measurement of refractive index and checked with a digital densitometer, Model DM 40 (Anton Paar K.G., Graz, Austria). Both centrifugations

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoB, apolipoprotein B; NaDOC, sodium deoxycholate; m-LDL, model low density lipoproteins; SDS, sodium dodecyl sulfate; EPC, egg yolk phosphatidylcholine; CO, cholesteryl oleate; EDTA, ethylenediamine tetraacetic acid; TLC, thin-layer chromatography; LDS, lipoprotein-deficient serum; GLC, gas–liquid chromatography; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

[1] To whom correspondence should be addressed.

were performed in a Beckman SW-60 Ti rotor at 150,000 *g* at 4°C for 24 hr. The purity of the lipoprotein preparations was assessed by agarose gel electrophoresis (13) with both lipid (Oil Red O) and protein (Coomassie Blue K-250) staining and further with analytical gel filtration on a Sepharose CL-4B column (1 × 80 cm). No sign of impurity could be found by these methods. The isolated LDL was dialyzed in the dark at 4°C for 24 hr against 0.15 M NaCl–1.3 mM EDTA (pH 7.0), filtered through a 0.22-μm filter, and stored at 0°C in sterile bottles.

Delipidation of LDL

ApoLDL was prepared by delipidation with the detergent sodium deoxycholate (NaDOC) (E. Merck AG, Darmstadt, G.F.R.). The NaDOC delipidation was essentially by the method developed by Helenius and Simons (14). Briefly, LDL was dialyzed against 50 mM NaCl–50 mM sodium carbonate (pH 10) overnight, and then 12 mg of solid NaDOC was added per mg of LDL. After a 30-min incubation, with gentle stirring at room temperature, the solubilized protein was separated from the lipid and detergent by gel filtration on a Sepharose CL-4B column (1.6 cm × 60 cm). The column was equilibrated and eluted with 50 mM NaCl–50 mM sodium carbonate–10 mM NaDOC (pH 10) at room temperature. Approximately 2-ml fractions were collected and analyzed for protein, phosphorus, cholesterol, and detergent. The protein peaks were concentrated by ultrafiltration with PM-30 membranes (Amicon Co., Lexington, MA). To remove detergent, the concentrated material was passed through a Sephadex G-75 column (1.6 × 30 cm) and eluted with 0.01 M Tris-HCl buffer, pH 9.0 (standard buffer). The protein eluted in the void volume and the bile salt close to the total volume of the column. The amount of detergent present after gel filtration on the Sepharose CL-4B and Sephadex G-75 columns was determined by including ^{14}C-labeled NaDOC in the incubation mixture and preequilibration of the Sepharose column with eluting buffer containing ^{14}C-labeled NaDOC followed by scintillation counting and protein determination of aliquots from column fractions. SDS-polyacrylamide gel electrophoresis on 3% acrylamide gels was used to monitor the purity of the apoB preparations. The buffer system of Weber and Osborn (15) was used. The running buffer contained 0.1% SDS. The gels were stained with 0.1% Coomassie Blue K-250 in methanol–acetic acid–water 25:10:65 (v/v/v).

Lipids and preparation of microemulsion

Egg phosphatidylcholine (EPC) was isolated in this laboratory (16). Cholesteryl oleate (CO) and tritiated CO were synthesized by the acid chloride method from cholesterol (E. Merck AG), [1,2(n)-^{3}H]cholesterol (47 Ci/mmol, Amersham) and oleic acid (Sigma Chemical Co.). Phosphatidyl [N-methyl-^{14}C]choline (60 mCi/mmol) and [carboxyl-^{14}C]sodium deoxycholate (60 mCi/mmol) were purchased from New England Nuclear. The purity of the lipids was assessed by TLC and GLC to be >99% in all cases. Stock solutions containing 10 mg/ml of the lipids were prepared in benzene and stored at −20°C. Before use, aliquots of these were dried at room temperature for a minimum of 2 hr by vacuum desiccation.

The microemulsions were prepared in two steps. First a suspension of 4 mg of ^{14}C-labeled CO in 4 ml of standard buffer was prepared by injection of a solution of the ester in 400 μl of diethyl ether–chloroform 2:1 through the bottom of a thermostatted sonication vessel (**Fig. 1**). For the injection, an "Agla" micrometer syringe was used. At the same time as the injection was performed, sonication was carried out with 30-s intervals using a MSE sonifier equipped with a titanium probe, at setting 6 (100 watts output). The temperature of the buffer was maintained at 52°C and the surface was covered with N_2 gas. The sonication procedure was completed over a period of about 30 min and resulted in an opalescent suspension with a yield of 85 (±10)% CO.

An aliquot of the ^{14}C-labeled CO suspension was transferred at once to a thin-walled glass ampoule in which ^{3}H-labeled EPC had been dried as a thin film. The CO/EPC weight-ratio routinely used was 2:1. The ampoule was then filled with N_2 gas, sealed, and sonicated for 20 min with a bath-type Branson sonifier at 52°C. This procedure resulted in a slightly opalescent microemulsion (absorbance < 0.1 at 450 nm for a 1 mg/ml CO emulsion) which was stable at 4°C for several days.

Reconstitution of LDL-like particles

The preparation of model LDL (m-LDL) was accomplished by addition of the detergent-delipidated apoB to

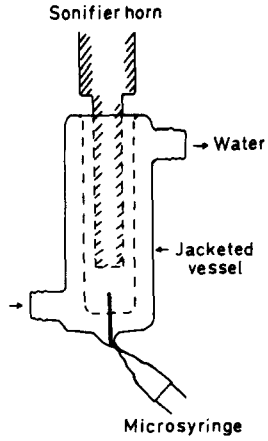

Fig. 1. The apparatus used for preparation of cholesteryl ester suspension. The diethylether–chloroform 2:1 solution containing the lipid was slowly injected through the bottom of the jacketed sonication vessel. Simultaneously, the sonication was performed with the horn dipped about 1 cm into the buffer solution at 52°C.

the CO-EPC microemulsion at a weight ratio of 1:1.2 between EPC and apoB. The mixture was first incubated for 10 min at 52°C and then sonicated in the bath sonifier at the same temperature for 15 min. An optically clear preparation of m-LDL was obtained.

The m-LDL solution was then subjected to density gradient centrifugation using a 0–40% (w/v) linear sucrose gradient in standard buffer. The m-LDL samples (0.5 ml) were layered on top of the gradient and centrifuged at 50,000 rpm for 24 hr at 10°C in a Beckman SW-60 Ti rotor. At the end of the centrifugation the bottoms of the tubes were punctured, and five-drop fractions were collected. The concentrations of ^{14}C-labeled CO, and ^{3}H-labeled EPC were determined by liquid scintillation counting. Protein was determined by a modified Lowry method (17). Sample densities were determined by refractometry and fractions with densities between 1.04 and 1.09 g/ml were pooled, dialyzed against standard buffer, and used for further characterization of m-LDL.

Characterization of microemulsion and reconstituted LDL

Ascending elution gel filtration was performed on a Sepharose CL-4B column (1 × 60 cm) at a flow rate of 5 ml/hr. The samples were <2% of the total volume of the column and fractions of ~1 ml were collected. The column was calibrated for molecular weight with a Pharmacia high molecular weight calibration kit. K_{av} was determined from the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$. The lipid and protein concentrations of eluted fractions were determined and expressed in μg per ml of effluent.

Negative staining electron microscopy was carried out by drying samples (concentration ca. 0.2%) in 2% potassium phosphotungstate, pH 7.4, on Formvar-coated copper grids. The air-dried grids were examined in a Zeiss EM 109 electron microscope, calibrated with a grating replica. The mean value for the diameters was obtained by measurement of 100 particles.

In order to establish the possible presence of EPC vesicles in the final m-LDL preparation, the sonication was performed with 0.1 M 6-carboxyfluorescein in the buffer. EPC vesicles were prepared by sonication in standard buffer containing 0.1 M 6-carboxyfluorescein. Free dye was removed by chromatography on Sephadex G-50. After addition of 0.2% (w/v) Triton X-100, the absorbance at 492 nm was measured (18). The amount of surface-bound dye was measured by the same procedure after mixing 0.1 M 6-carboxyfluorescein with m-LDL and vesicles. The difference between total and surface-bound dye was considered to indicate the vesicle trapped volume.

The electrophoretic integrity and mobility of m-LDL were assessed by agarose gel electrophoresis (13) with both lipid and protein staining. For assessment of apoB degradation after recombination, SDS-polyacrylamide gel electrophoresis with the same procedures as for delipidated apoB was performed.

Cell culture

Human fibroblasts were derived from skin biopsies of normal subjects. Cells were grown in monolayers and used between the 5th and 15th passage. The medium consisted of Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acid solution, 20 mM HEPES buffer, 0.08% (w/v) sodium bicarbonate, 10 μg/ml of gentamycin, and 10% (v/v) fetal calf serum. Cell growth medium and supplements were purchased from Gibco Europe, U.K. Confluent monolayers of cells from stock flasks were dissociated with 0.05% trypsin, 0.02% EDTA solution. About 3×10^5 cells were seeded into 50-mm Petri dishes containing 2 ml of growth medium with 5% (v/v) fetal calf serum. All experiments were performed after the cells had been incubated with 5% lipoprotein-deficient serum (LDS) for 48 hr and while the cells were in the late phase of logarithmic growth.

Binding, incorporation, and degradation of [^{125}I]LDL and [^{125}I]m-LDL

^{125}I-labeled LDL and m-LDL (sp act 200 to 400 cpm/ng of protein) were prepared by the iodine monochloride method as described by Karlin et al. (19). Na^{125}I (16.3 mCi/μg, Amersham) was added at room temperature to 1 mg of LDL in 0.5 M glycine buffer (pH 10) and the reaction was started by addition of 19 nmol of ICl in 0.5 M glycine. After 3 min the reaction was stopped by the addition of 5 μl of sodium metabisulfite (0.2 mg/ml) and fractionated on a Sephadex G-75 column. [^{125}I]LDL and [^{125}I]m-LDL eluted with the void volume and the combined fractions were dialyzed for 24 hr against three changes of 0.15 M NaCl and 0.05% EDTA, pH 7.4. After removing aliquots for protein determination, the lipoproteins were sterilized by filtration.

Determination of binding, incorporation, and degradation of [^{125}I]LDL and [^{125}I]m-LDL were done essentially as described by Brown and Goldstein (20). The cells were incubated at 37°C with 2 ml of growth medium with 5% LDS and [^{125}I]LDL or [^{125}I]m-LDL. After incubation the cells were cooled at 4°C for 30 min. The medium was collected for determination of TCA-soluble material. The cells were washed with ice-cold PBS containing 0.2% albumin and then two times with albumin-free PBS. The cells were then incubated for 1 hr at 4°C with 2.5 ml of a solution containing 5 mg/ml heparin in PBS. An aliquot of the heparin solution was counted and gave values for bound LDL and m-LDL.

The flasks were then washed three times with PBS and

the cells were dissolved in 2 ml of 0.1 M NaOH. One aliquot was counted to determine total lipoprotein internalized into cells (incorporated LDL or m-LDL). Another aliquot was used for protein determination.

To 2 ml of the media, 0.5 ml of 50% TCA was added. After incubation for 30 min at 4°C the precipitate was removed by centrifugation. Ten μl of 40% KI and 40 μl of 30% H_2O_2 were added to 1 ml of the supernatant to remove any free iodine. After 5 min the iodine was extracted from the supernatant with 2 ml of chloroform, and 250 μl of the media was removed for counting (degraded LDL and m-LDL).

Analytical procedures

Cholesterol was assayed by the method of Zlatkis, Zak, and Boyle (21) and phosphate was assayed according to Bartlett (22). The amount of phospholipid was calculated by multiplying the amount of phosphorus by 25. Protein was determined by a modified Lowry method (18). The radioactive lipids were measured by liquid scintillation counting using narrow windows for ^3H and ^{14}C and with Lumagel (Lumac B. V., Holland) as scintillator. ^{14}C cross-over in the ^3H channel was determined using standards and an appropriate amount of buffer. Quantitative TLC was performed with 20 × 20 cm precoated silica gel 60 plates (E. Merck AG). The plates were developed first in chloroform–methanol–acetic acid–water 50:25:8:4 to half the height and then in petroleum ether (40–60°C)–benzene–acetic acid 30:8:1. The densitometric quantification was made after sulfuric acid charring at 180°C for 30 min.

RESULTS

Deplipidation of LDL with NaDOC

The Sepharose CL-4B elution profiles of protein and lipid components of LDL after solubilization with NaDOC are presented in **Fig. 2 A.** A minor and variable part (about 6%) of the protein eluted in the void volume as an opalescent solution (pool I). The protein in this fraction did not penetrate the SDS-polyacrylamide electrophoresis gel and was not used in the subsequent reconstitution experiments. The rest of apoB eluted as a single symmetrical fraction of clear solution (pool II). The protein recovery in pool II was 83 (±6)%. The lipid components of holo-LDL eluted together with NaDOC near V_t of the column. The protein pool II was concentrated by ultrafiltration and subjected to gel filtration on a Sephadex G-75 column which was run with detergent-free standard buffer. The protein eluted in the void volume and the NaDOC eluted near the total volume of the column.

When radioactive NaDOC was included in the delipidation mixture, a value of 3 μg NaDOC per mg protein was obtained after the Sephadex G-75 separation. The final apoB preparation remained optically clear for several weeks at 4°C. SDS-polyacrylamide electrophoresis of the protein showed high molecular weight subunits with an apparent molecular weight, M_r, of approximately 320,000 daltons (Fig. 2 B). The NaDOC-solubilized apoB was also subjected to analytical gel filtration on a Sepharose CL-4B column. A K_{av} value of 0.37 (±0.02) was obtained which gave an apparent molecular weight of 520,000 (±30,000) daltons.

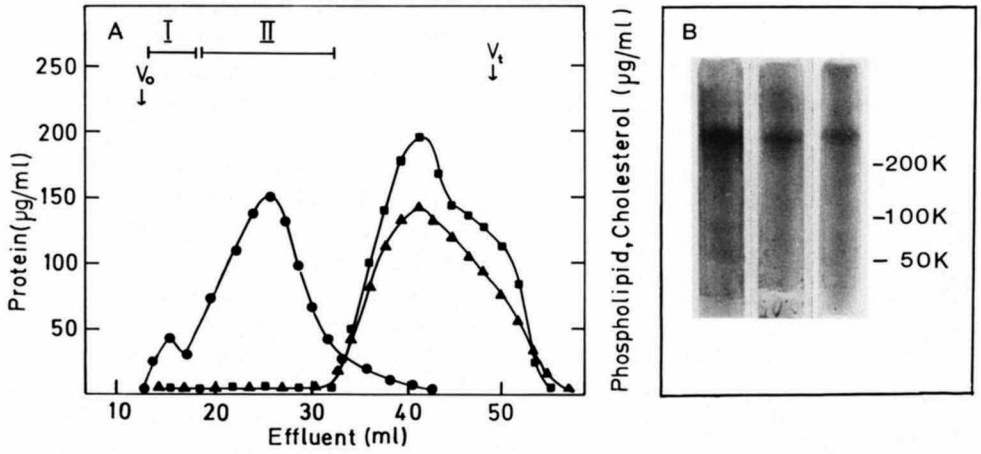

Fig. 2. (A) Sepharose CL-4B column chromatography of LDL incubated with NaDOC. A sample of 0.5 ml containing 2 mg of protein and 120 mg of NaDOC in 50 mM NaCl–50 mM sodium carbonate, pH 10, was applied to the column (1.6 × 60 cm). The elution was performed with the same buffer with 10 mM NaDOC added. Arrows mark the void (V_0) and total (V_t) volumes of the column. (●) Protein; (▲) phospholipid; (■) total cholesterol. NaDOC not shown. (B) SDS-polyacrylamide gel (3%) electrophoresis of apoB at different stages of the reconstitution procedure. From left to right: LDL; NaDOC-solubilized apoB (protein fraction II in Fig. 2 A); reconstituted m-LDL.

Quantitative TLC of chloroform–methanol 2:1 (v/v) extracts from lyophilized apoB showed that the protein was virtually free of lipids in agreement with other similar studies (14, 23).

Cholesteryl ester-phosphatidylcholine microemulsion

The CO suspension prepared by the combined injection-sonication method was opalescent and had a mean particle diameter of about 100 nm as judged by electron microscopy. The subsequent sonication of the CO suspension, together with EPC at an initial ratio of 2 to 1, gave a microemulsion with a mean particle diameter of approximately 30 nm.

The gel filtration elution profile of the CO-EPC microemulsion is shown in **Fig. 3 A.** There was a peak with high CO to EPC ratios close to the void volume (V_0) and a second peak with fairly constant CO to EPC ratios. The major part of this peak had CO/EPC ratios about 1.5.

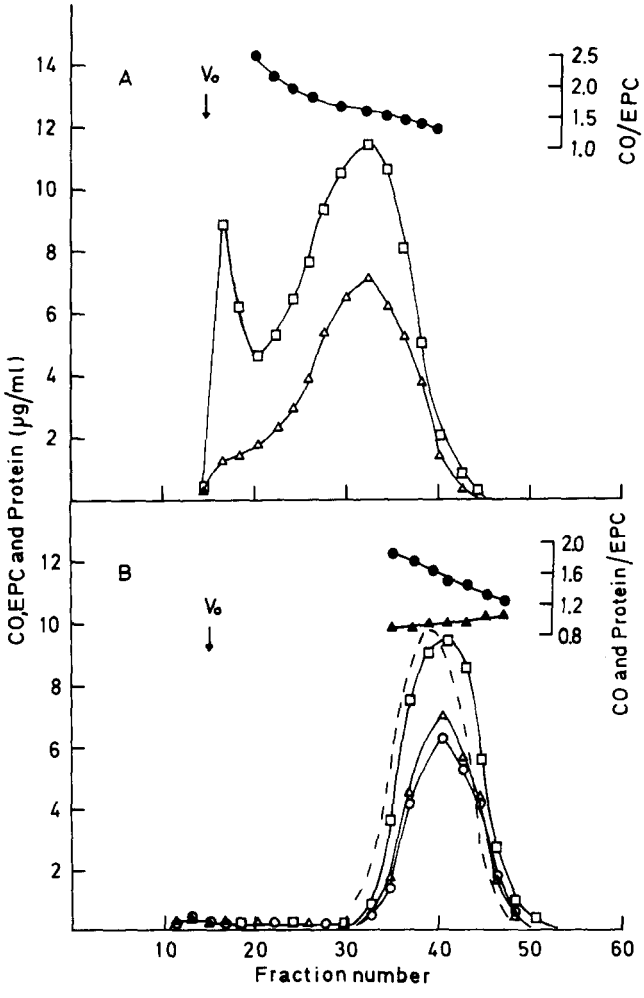

Fig. 3. Sepharose CL-4B gel filtration of (A) CO/EPC microemulsion (initial weight ratio 2:1) and (B) reconstituted m-LDL after separation by sucrose gradient centrifugation. (○) Protein; (△) EPC; (□) CO. Inserts: (●) CO/EPC weight ratio; (▲) protein/EPC weight ratio. The dotted line in (B) represents native LDL.

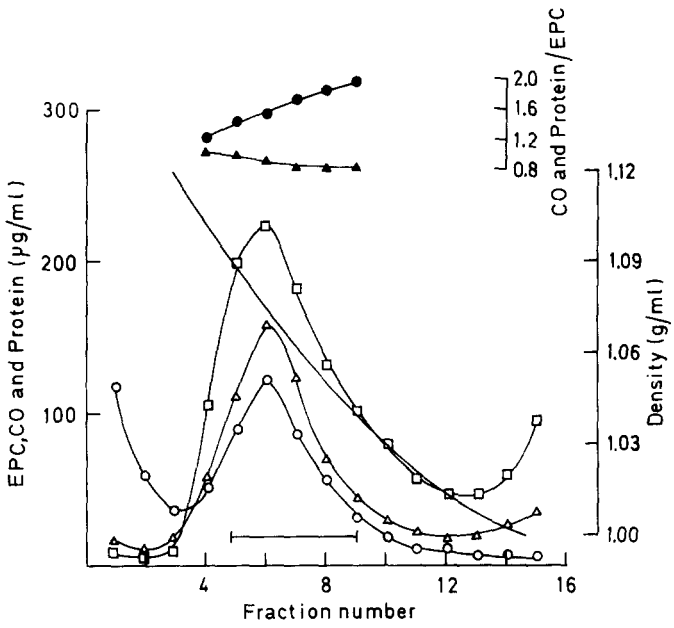

Fig. 4. Separation of reconstituted m-LDL by centrifugation in a 0–40% linear sucrose gradient. The bottom is to the left. Fractions were collected after centrifugation (50,000 *g*, 24 hr) and measured for EPC (△), CO (□), and protein (○). The density was assessed by refractive index measurements. The insert shows the weight ratios CO/EPC (●), and protein/EPC (▲).

Reconstituted LDL

The isolation of lipid-protein complexes, after addition of apoB to the CO-EPC microemulsion, by sucrose gradient centrifugation is illustrated in **Fig. 4.** The m-LDL particles were concentrated to a peak with maximum at d ~ 1.07 g/ml. The weight ratios between the components are quite constant in the major part of the peak with values around 1.5 for CO/EPC and 0.8 for protein/EPC. The fractions with densities between 1.04 and 1.09 g/ml were pooled for further characterization. The recovery of the original CO in the final m-LDL was 60 (±10)%.

When subjected to analytical gel filtration on a Sepharose CL-2B column, the m-LDL preparation isolated by gradient centrifugation appeared as a symmetrical peak at K_{av} = 0.50 (Fig. 3 B). The ratios between the components were fairly constant throughout the peak. For comparison the elution profile of native LDL with a K_{av} of 0.47 is shown. The K_{av} value of m-LDL gave an apparent molecular weight of approximately 2.2×10^6 daltons. Negative staining electron microscopy of the m-LDL particles revealed spherical structures with the diameters varying between 16 and 29 nm (**Fig. 5**). Quantitative analysis of the particle diameter (**Fig. 6**) gave an average value of 21 nm.

Examined by electron microscopy, the m-LDL preparation showed no vesicle contamination. This observation

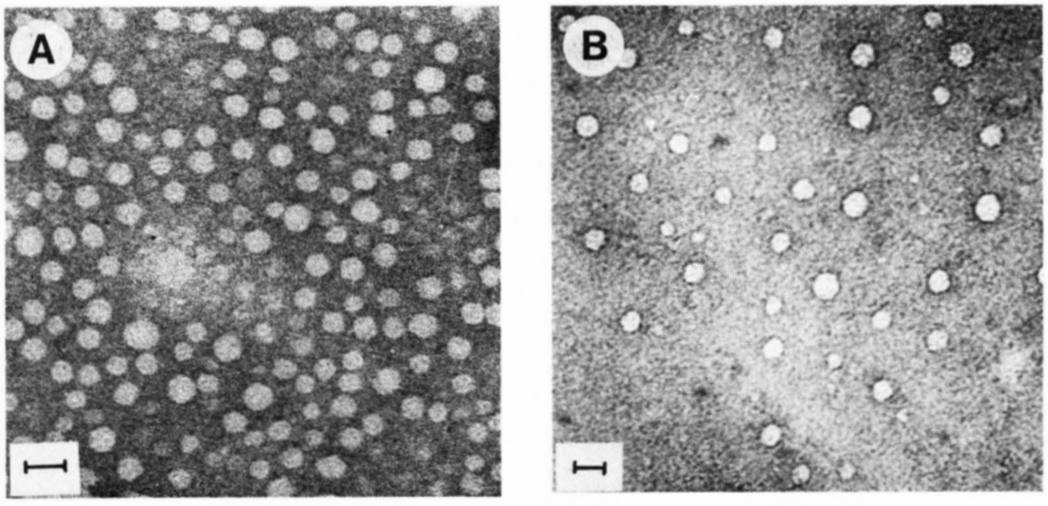

Fig. 5. Electron microscopic photographs of (A) native LDL and (B) reconstituted m-LDL. The particles were negatively stained with phosphotungstate. The bar equals 40 nm.

was confirmed by the experiment with 6-carboxyfluorescein. The dye trapped inside vesicular structures gave no measurable absorption at 492 nm. The smallest amount of EPC vesicles that could be detected by measuring the absorption of 6-carboxyfluorescein trapped inside vesicles was found to be 0.5 μg of EPC/ml. From this value it can be calculated that in the m-LDL preparation with 0.5 mg EPC/ml less than 0.1% of the EPC was in the form of vesicular structures. Agarose gel electrophoresis of m-LDL in parallel with native lipoproteins gave one band with β-mobility (**Fig. 7**). Staining with both lipid and protein stains showed that the components of m-LDL coincide completely. The possible degradation of apoB during the preparation of m-LDL was tested by SDS-polyacrylamide electrophoresis (Fig. 2 B).

The degradation of CO and EPC during sonication was tested by TLC on silica gel plates. The radiochemical purity of the ^{3}H-labeled CO was 99.8 and 99.1% before and after sonication, respectively, as calculated from the appearance of free [^{3}H]cholesterol. No measurable degradation of EPC was found (<0.05%).

Cell culture experiments

As a test of the preservation of biological activity in m-LDL, the ability of native LDL to compete with [^{125}I]m-LDL for binding, incorporation, and degradation was studied in cultured human fibroblasts. The data in **Fig. 8** show that native LDL competes with m-LDL, which

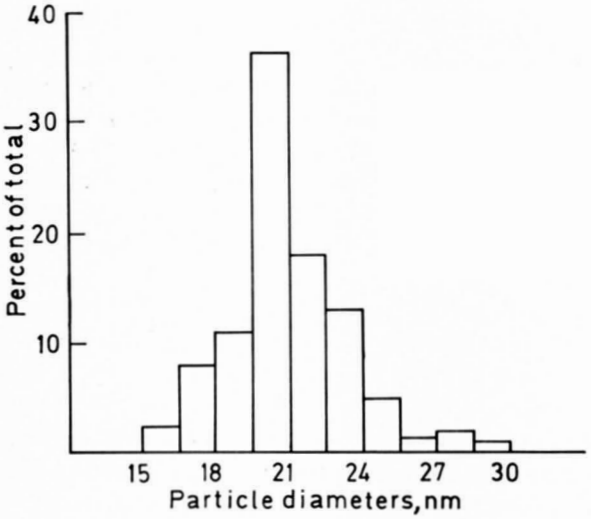

Fig. 6. Range of diameters of negatively stained reconstituted m-LDL. One hundred particles were selected at random and measured.

Fig. 7. Agarose gel electrophoresis of native LDL (A) and reconstituted m-LDL (B). The gel was stained with Oil Red O.

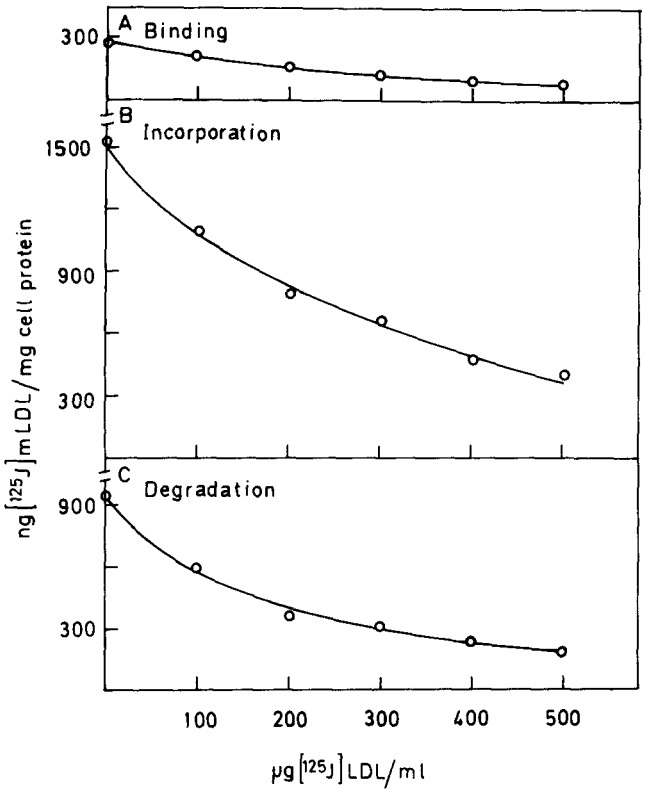

Fig. 8. Ability of native LDL to compete with reconstituted [^{125}I]m-LDL for binding, incorporation, and degradation in human fibroblasts at 37°C. Cells were incubated with 5% lipoprotein-deficient serum for 24 hr before the addition of 50 μg of [^{125}I]m-LDL per ml of medium and the indicated concentration of native LDL. After 5 hr incubation, the amounts of [^{125}I]m-LDL associated with the cell surface (A), internalized (B), and degraded (C) were determined as described under Materials and Methods.

demonstrates that m-LDL is recognized and taken up by the LDL receptor pathway (3).

To compare the metabolism of [^{125}I]LDL and [^{125}I]m-LDL, each lipoprotein preparation was incubated with fibroblasts and the binding, uptake and degradation as a function of time (**Fig. 9 A**) were measured. In the case of [^{125}I]LDL, the binding and cellular content of radioactivity reached a steady state plateau by 2 hr. Nearly all of the [^{125}I]LDL that was hydrolyzed by the cells appeared in the medium as TCA-soluble radioactivity and the rate of appearance was time-dependent. The metabolism of [^{125}I]m-LDL was similar to that of the [^{125}I]LDL. Thus, the binding and cellular content of unhydrolyzed [^{125}I]m-LDL reached a plateau within 2.5 hr at nearly the same level as the native LDL. The degradation of [^{125}I]m-LDL was slightly slower than that for [^{125}I]LDL. Lineweaver-Burk plots of the incorporation values gave maximum velocities of 1282 ng of LDL and 1163 ng of m-LDL per mg of cell protein. The corresponding lipoprotein concentrations at half-maximum velocity were 29.2 μg and 34.8 μg per ml, respectively.

The uptake and hydrolysis of [^{125}I]LDL and [^{125}I]m-LDL as a function of the concentration of lipoprotein is compared in Fig. 9 B. The similarity in shapes of the saturation curves for both preparations indicates that the uptake of [^{125}I]m-LDL, like that of [^{125}I]LDL, is receptor-mediated.

DISCUSSION

In the current study we describe a procedure by which biologically active LDL-like particles can be prepared. The present experimental protocols are continuations of those given for the preparation of protein-free models of LDL and VLDL (7, 9). The main objective was to develop a reconstitution method by which the lipid components of LDL could be varied in a systematic way. The procedure described by Krieger et al. (6) gives a biologically active particle with proper composition, but it does not offer the possibility of replacing the endogenous phospholipids. Important drawbacks also are the low recovery of labeled cholesteryl esters (about 10%) and the altered physical properties probably due to retention of heptane (24).

A prerequisite for a reconstitution method is the production of completely delipidated and water-soluble apoB. Detergent solubilization was regarded as the potentially most promising method and NaDOC was chosen because it is a mild ionic surfactant which is, moreover, relatively easily removed by dialysis. By using the protocols of Helenius and Simons (14) with small modifications, it was possible to obtain a delipidated and soluble apoB with only three molecules of NaDOC per native mass of the protein.

The gel filtration value of approximately 520,000 daltons for the molecular weight of NaDOC-solubilized apoB indicated that it remains associated in the dimeric state after delipidation. This conclusion is supported by several other reports (25, 26). The amount of protein in the final reconstituted m-LDL is 24% and because the molecular weight of m-LDL was found to be about 2.2×10^6 daltons the total mass of apoB in the particle is ca. 530,000 daltons. It seems quite likely that the NaDOC-solubilized apoB associates with microemulsion particles in its dimeric state.

Regarding the lipid microemulsion, the aim was to design a method that reduces the sonication time as much as possible in order to reduce the risk for degradation and autoxidation. The procedure presented in this study includes sonication in two steps and a combination of sonication with the injection technique. Ginsburg, Small, and Atkinson (10) obtained a quite good product after a very long sonication time (300 min). The microemulsion obtained by much less sonication in this study is not equally

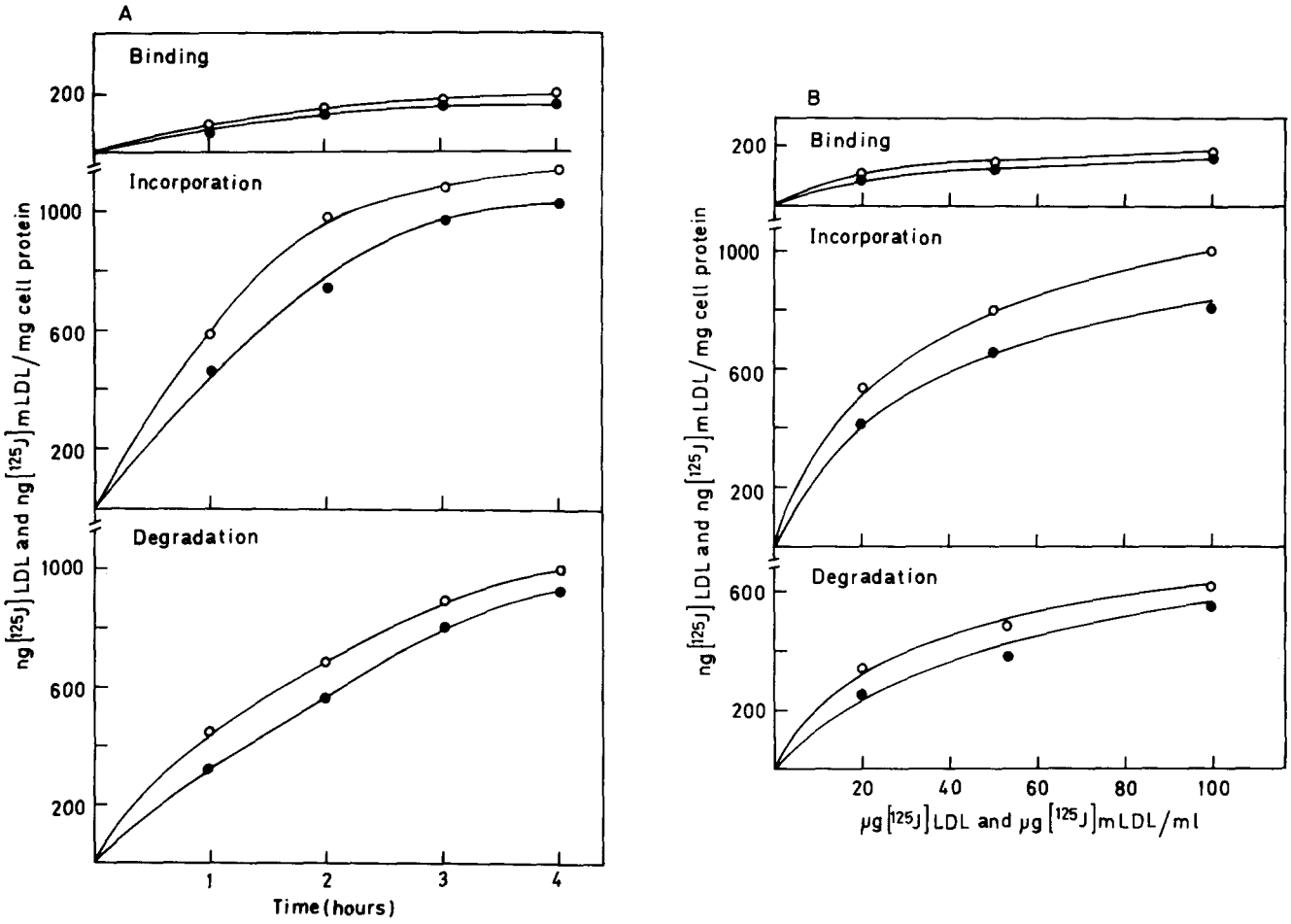

Fig. 9. Time course (A) and saturation kinetics (B) for binding, incorporation, and degradation of native human [^{125}I]LDL (○), and reconstituted [^{125}I]m-LDL (●) in human skin fibroblasts at 37°C. Each monolayer of normal cells received 2 ml of medium containing 5% lipoprotein-deficient serum and [^{125}I]LDL (27,500 cpm/μg of protein) or [^{125}I]m-LDL (32,400 cpm/μg of protein). The amount of bound (heparin-releasable), incorporated (heparin-resistant), and degraded (acid-soluble) lipoprotein was determined as described under Materials and Methods.

homogeneous but the proceeding incubation and sonication with apoB gave a satisfactory final product.

The temperature control during sonications is critical. In order to obtain a proper microemulsion and an effective association between the microemulsion and apoB, the temperature must be above the melting point of the cholesteryl ester. Special attention should also be given to the sonication effect, which must be optimized. Once a proper microemulsion has been obtained it can be stored for several days at 4°C.

The apparent molecular weight of LDL-apoB, NaDOC-solubilized apoB, and m-LDL-apoB was the same (~320,000) as determined by SDS-polyacrylamide electrophoresis on 3% gels, and was in agreement with other investigations (23, 27). The fact that the electrophoretic mobility of m-LDL and m-LDL-apoB on agarose and polyacrylamide, respectively, was the same as that of the native species indicates that the protein retains its secondary structure. This is in agreement with a recent study using NaDOC (23) and other studies using other detergents (26, 27).

The incubation and mild sonication of the lipid microemulsion, together with the delipidated apoB, caused a considerable reduction in particle diameter from 30 nm to 21 nm, but the CO/EPC ratio remains almost unchanged. This suggests that apoB is located mainly in the interfacial region and takes part in the emulsification of the cholesteryl ester.

Reconstitution approaches similar to that described here have recently been presented by Via et al. (11) and Walsh et al. (28) in preliminary studies showing that apoE and apoB can associate with microemulsions rich in cholesteryl esters. A different line of action has also been used (23, 29) demonstrating that detergent-solubilized apoB can form complexes with phospholipids in vesicular structures. These results may indicate the importance of the amphiphilic milieu offered by the phospholipid layer around the microemulsion particle. However, it is still

too early to speculate about the contribution of the interaction between hydrophobic parts of apoB and the neutral lipid core to the association process.

The test for biological activity of the m-LDL with human fibroblasts revealed that the reconstituted LDL was bound to the receptor with the same affinity as the native LDL. It was also shown that the particles were taken up and hydrolyzed like native LDL. This fact renders them suitable for metabolic studies. We also think that reconstituted m-LDL particles provide a useful system for further studies on the molecular organization and lipid-lipid and lipid-protein interactions.

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