note on methodology

Rapid preparative isolation of concentrated low density lipoproteins and of lipoprotein-deficient serum using vertical rotor gradient ultracentrifugation

Yves Poumay[1] and Marie-France Ronveaux-Dupal[2]

Unité de Cytologie, Facultés Universitaires Notre-Dame de la Paix, 61 rue de Bruxelles, 5000 Namur, Belgium

Summary In order to study cellular metabolism of low density lipoproteins (LDL), ultracentrifugal methods have been used to isolate the lipoproteins. The use of vertical rotor ultracentrifugation very quickly produces small quantities of diluted lipoproteins per gradient, as well as small volumes of lipoprotein-deficient serum. We present modifications to this method in order to prepare routinely more concentrated LDL and a sufficient volume of lipoprotein-deficient serum in a relatively short time with minimal cost and handling. —**Poumay, Y., and M-F. Ronveaux-Dupal.** Rapid preparative isolation of concentrated low density lipoproteins and of lipoprotein-deficient serum using vertical rotor gradient ultracentrifugation. *J. Lipid Res.* 1985. **26:** 1476–1480.

Supplementary key words VLDL • LDL • HDL

Since the first in vitro studies on binding and degradation of low density lipoproteins (LDL) by cultured cells (1), many authors have isolated lipoproteins in order to study their metabolism in different tissues. For analytical or preparative purposes, serum lipoproteins have usually been isolated by ultracentrifugation. In addition to the standard sequential flotation method (2), density gradient ultracentrifugation procedures have been developed for the separation of lipoprotein fractions by a single spin (3–5). However, with these latter methods, the volume of plasma is limited to a maximum of about 24 ml per spin and a centrifugation time of at least 24 or 48 hr is required in order to obtain a sufficient separation of lipoproteins for preparative purposes (6). In order to circumvent these limitations, Chung et al. (7) developed a rapid, single spin, ultracentrifugal method suitable for preparative and quantitative isolation, employing a single discontinuous density gradient in a vertical rotor. In spite of the rapidity of the separation, there is only a small quantity of LDL isolated per tube, and the preparation is very dilute and needs to be concentrated.

We present here some modifications to this method in order to prepare large volumes of concentrated LDL. Furthermore, our preparation produces a lipoprotein-deficient serum (LPDS) useful for incubation with cultured cells.

MATERIALS AND METHODS

Freshly collected serum (80 ml) from about 20 healthy volunteers was obtained from the local Red Cross Center. The serum was pooled and used immediately for lipoprotein isolation. The serum was adjusted to a density of 1.25 g/ml with solid KBr and transferred into two 40-ml centrifugation tubes (Beckman, Quick Seal, 25 × 89 mm). Centrifugation was performed in a Beckman vertical VTi 50 rotor for 16 hr at 10°C and 50,000 rpm in a Beckman L5-65 ultracentrifuge equipped with a slow acceleration accessory. The brake was cut off at the end of the run at 2,000 rpm. The top of each tube was then gently sliced with a scalpel and the upper yellow-orange, slightly opalescent layer was cautiously removed using a narrow Pasteur pipette. This layer, corresponding to lipoproteins, was separated by a colorless zone from the rest of serum proteins. Lipoproteins from the two tubes were pooled (10 ml) and the density of this solution was adjusted to 1.30 g/ml with solid KBr as described (7). Thirty ml of 0.9% NaCl was added to a Quick Seal centrifugation tube and the lipoprotein solution was layered under the saline with a syringe and narrow plastic tubing to fill the tube, which was then sealed. Centrifugation was performed as described above for 180 min. At the end of the run, VLDL, LDL, and HDL appeared well separated and the gradient was fractionated using Pasteur pipettes (5). In our first series, a constant volume of 1 ml was removed in order to study the gradient. For preparative purposes, fractions were removed on the basis of the different lipoprotein bands. Fractions of gradient were analysed for density, protein, and total cholesterol content (8, 9). Distribution and size of lipoproteins was determined by negative staining electron microscopy using a 2% potassium phosphotungstate solution, pH 6.3 (10). Electrophoretic migration of intact lipoprotein fractions was studied in 0.8% agarose

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein-deficient serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[1]Y. P. is a Fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (I.R.S.I.A.).

[2]To whom correspondence should be sent.

gels, pH 8.6, according to the method of Noble (11). SDS-polyacrylamide gel electrophoresis was performed in order to ascertain the purity of lipoprotein fractions (12). The lipoprotein-deficient serum was collected after the first centrifugation by piercing the bottom of tubes with a hypodermic needle.

RESULTS AND DISCUSSION

Following the procedure described here, we obtained about 50 ml of LPDS and more than 50 mg of pure LDL protein contained in 8 ml in less than 24 hr. The first centrifugation step gave a well separated, easily removable lipoprotein band distinct from the rest of the serum proteins. However, chylomicrons and VLDL tend to stick onto the inner tube wall during centrifugation in a vertical rotor (7). If the lipoprotein band was quickly removed from the two tubes after the first run, it was possible to considerably reduce the amount of chylomicrons and VLDL in the lipoprotein band. As a consequence of their adsorption onto the inner wall, their reorientation in the tube at the end of the run was indeed much slower than that of the gradient itself. Therefore, only a small part of these lipoproteins was present in the yellow-orange lipoprotein band. Furthermore, this presence of chylomicrons and VLDL represented an important problem for the isolation of the LPDS. After the immediate removal of the lipoprotein band, we observed that storing the LPDS tubes in a cold room at 4°C for 1 hr resulted in the appearance of a white opalescent upper band. This probably represented most of the triglyceride-rich lipoproteins. Using such conditions of storage, we were able to obtain in a reproducible way about 50 ml of LPDS containing less than 4 mg/dl of cholesterol, which is in accordance with the advised limit (13).

The basic aim of our modification was to initially concentrate lipoproteins while isolating them from the rest of the serum. Thereafter, one single gradient would efficiently separate LDL from other lipoproteins. Indeed, after the second centrifugation, we could recognize characteristic bands on the gradient corresponding to the different classes of lipoproteins. Protein and cholesterol distribution on the gradient indicated effective separation of VLDL, LDL, and HDL. There were only a few non-lipoprotein contaminants recovered in the bottom of the gradient, which could represent plasma proteins usually adsorbed on lipoproteins (**Fig. 1**).

We have characterized the isolated bands of the gradient by checking their homogeneity using negative staining electron microscopy, their mobility on agarose gels, and their main apoprotein composition by SDS-PAGE. As

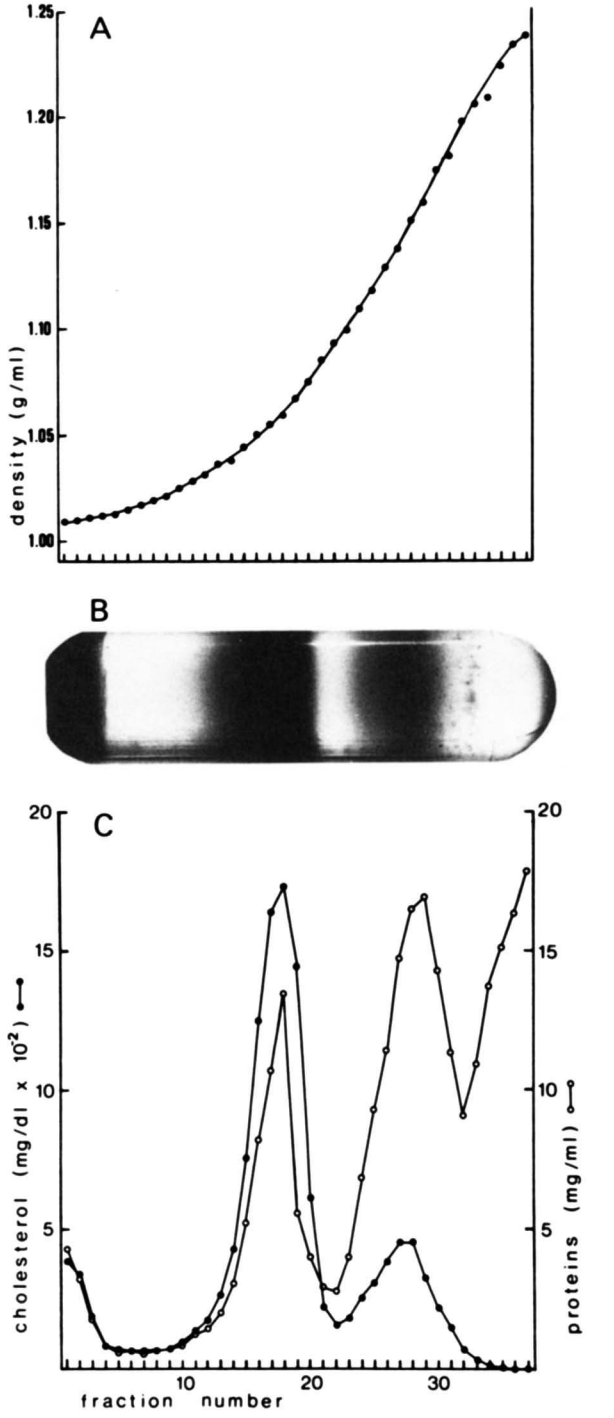

Fig. 1. A. Density distribution in the fractions of a discontinuous gradient of d 1.006 g/ml NaCl solution and d 1.30 g/ml lipoproteins and KBr solution after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 rpm for 180 min. B. Tube with the gradient after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 rpm for 180 min. Separation of serum lipoproteins: from left to right, bands correspond to VLDL, LDL, HDL. C. Distribution of total cholesterol (● — ●) and proteins (○ — ○) in the fractions of the gradient after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 rpm for 180 min.

observed by negative staining electron microscopy, lipoprotein populations were homogeneous, especially the LDL fraction (**Fig. 2**). After Sudan Black B staining, agarose electrophoresis showed one single band of characteristic migration for the VLDL, LDL, and HDL fractions, as well as an absence of chylomicrons in the VLDL fraction. However, staining with a protein-specific dye (Coomassie Blue R 250) revealed the presence of other serum proteins in the HDL fraction (**Fig. 3**). In SDS-PAGE no cross-contamination by apoproteins specific for other lipoprotein classes was observed. However, a small quantity of serum proteins was seen in the HDL fraction. Among these, albumin was detected as a faint band. In the LDL fraction, a very faint band corresponding to a protein of a molecular weight around 31,000 was observed. This could correspond to the apoE (**Fig. 4**). These data confirmed those of Chung et al. (7) on the purification of HDL from serum proteins. Indeed, our first centrifugation served as a first wash, eliminating most of the non-lipoprotein serum proteins. Then, the single gradient step achieved the removal of these proteins in the VLDL and LDL fractions but not completely in the HDL fraction.

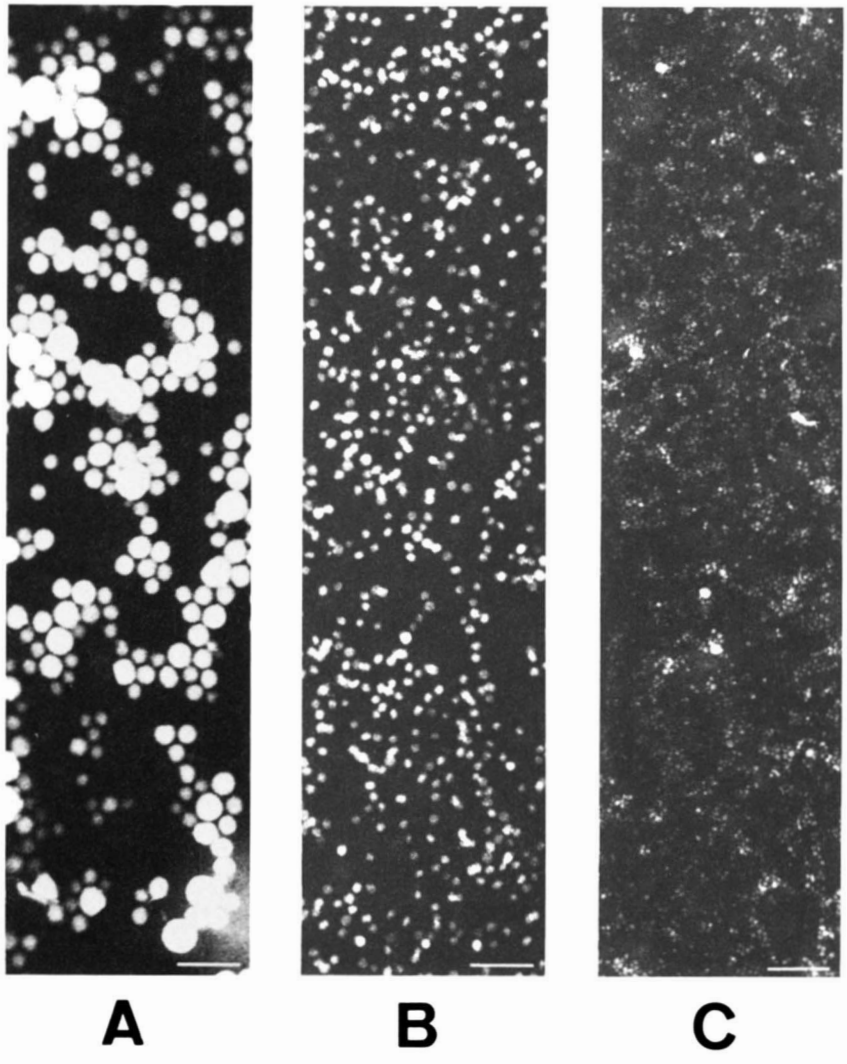

Fig. 2. Electron micrographs of negatively stained VLDL fraction (A), LDL fraction (B), and HDL fraction (C) of the gradient after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 rpm for 180 min. Each bar represents 1,000 Å.

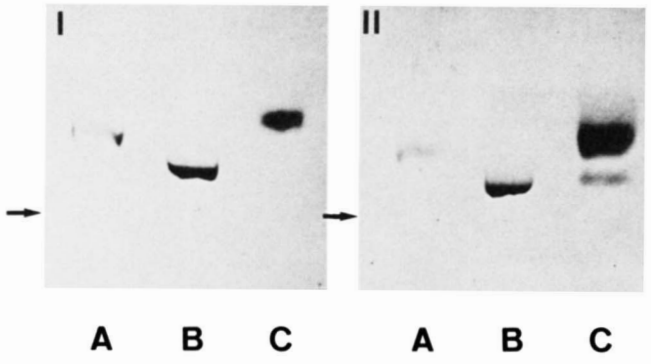

Fig. 3. Electrophoretic mobilities in agarose gel of VLDL fraction (A), LDL fraction (B), and HDL fraction (C) of the gradient after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 rpm for 180 min. The arrows indicate the origin. I. Staining with Sudan Black B. II. Staining with Coomassie Blue R 250.

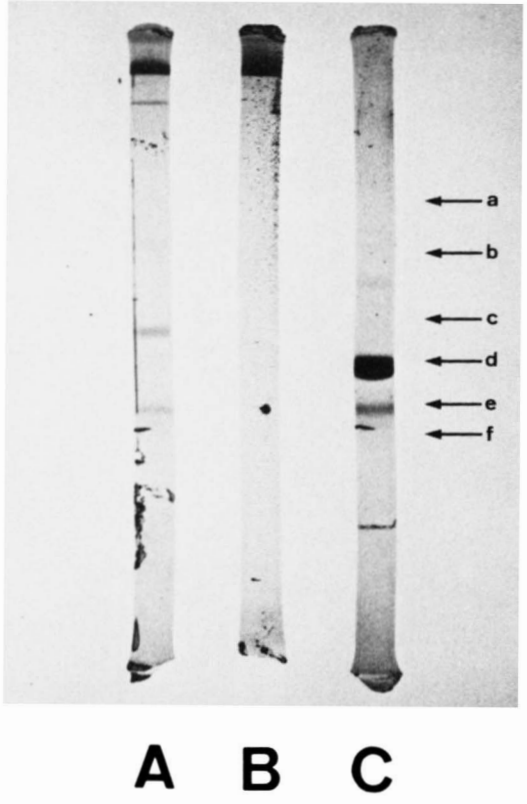

Fig. 4. SDS-PAGE on VLDL fraction (A), LDL fraction (B), and HDL fraction (C) of the gradient after ultracentrifugation in a Beckman VTi 50 vertical rotor at 50,000 RPM for 180 min; 6% acrylamide gels. The arrows indicate molecular weight markers: a, phosphorylase b (94,000); b, albumin (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, trypsin inhibitor (20,100); f, α-lactalbumin (14,400).

Thus our results indicate that the isolated VLDL and LDL are not contaminated by other lipoproteins or serum proteins except for the very small quantity of apoE in the LDL fraction.

We were mainly interested in routine, easy, and rapid LDL isolation. We demonstrate here that the Beckman vertical rotor centrifugation facilities are also suitable for the technique described for Sorvall vertical rotors (7). We confirm that Quick Seal tubes are adequate for lipoprotein isolation (14). The advantages of this technique over those using a swinging bucket rotor and sequential flotation ultracentrifugation seem evident. As we use only three ultracentrifuge tubes, we reduce the cost of the centrifugation while producing comparable quantities of isolated LDL. Furthermore, we directly recover LPDS. Finally, the handling of one single gradient makes isolation easier and shorter and centrifugation time in the ultracentrifuge is considerably reduced. Our LDL has been used for radioiodination, conjugated with colloidal gold, and biological activity has been tested on cultured cells with success (data not shown).

Manuscript received 1 May 1985.

REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
2. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
3. Redgrave, T. G., D. C. K. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
4. Nilsson, J., V. Mannickarottu, C. Edelstein, and A. M. Scanu. 1981. An improved detection system applied to the study of serum lipoproteins after single-step density gradient ultracentrifugation. *Anal. Biochem.* **110:** 342–348.
5. Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Laplaud. 1981. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* **22:** 339–358.
6. Edelstein, C., D. Pfaffinger, and A. M. Scanu. 1984. Advantages and limitations of density gradient ultracentrifugation in the fractionation of human serum lipoproteins: role of salts and sucrose. *J. Lipid Res.* **25:** 630–637.
7. Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.

8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
9. Allain, C. A., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
10. Hamilton, R. L., R. J. Havel, J. P. Kane, A. E. Blaurock, and T. Sata. 1971. Cholestasis : lamellar structure of the abnormal human serum lipoprotein. *Science.* **172:** 475–478.
11. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
12. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
13. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
14. Cole, T. G., and D. W. Gibson. 1984. Evaluation of heat-sealed ultracentrifuge tubes in the isolation of plasma lipoproteins. *J. Lipid Res.* **25:** 312–314.