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A Simple and Rapid Purification Procedure Minimizes Spontaneous Oxidative Modifications of Low Density Lipoprotein and Lipoprotein (a)

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Usual purification procedures of LDL and Lp(a) require numerous, extensive and prolonged sample handlings: this greatly increases the possibility of spontaneous oxidation. We have developed a method which, making use of two short-run ultracentrifugations in vertical rotors alternated by two rapid column-chromatography steps (SRUC), significantly shortens the preparation time to 3.5 h (LDL) and does not demand additional instrumentation or particular accuracy. Purification of Lp(a) requires a further wheat germ agglutinin chromatographic step, which can be accomplished within 30 min. More importantly, the method significantly reduces spontaneous oxidation as compared with classical isolation procedures. LDL isolated by the standard sequential method exhibits more extensive apolipoprotein B_{100} degradation, lipid peroxidation, and endogenous antioxidant (vitamin E) loss than the same lipoproteins obtained by means of the SRUC. This procedure may have be particularly valuable in experiments evaluating the effects of oxygen radical-induced modifications, especially *in vitro*.

Key words: atherosclerosis, LDL, Lp(a), oxidation, ultracentrifugation.

A large body of studies both *in vivo* and *in vitro* (*1-3*) has established that oxidative modifications of low density lipoproteins (LDL) play a pivotal role in the pathogenesis of atherothrombosis. It has been hypothesized that modification or oxidation of LDL is a prerequisite for macrophage uptake, while the accumulation of lipids within these cells leads to foam-cell formation and subsequent appearance of the fatty streak lesion (*1*). More recent evidence seems to suggest a similar role for the lipoprotein (a) [Lp(a)], a particle which differs from LDL in having one additional protein [apoprotein (a)], to which apolipoprotein B_{100} (apo-B_{100}) is bound through an S-S bridge. Lp(a) has been proposed as a leading inherited risk factor for a variety of vascular diseases (*4-6*), and this protein is preferentially deposited in atherosclerotic plaques compared to LDL (*7*): once deposited at the injury site the LDL-like portion of the Lp(a) may undergo oxidative modification (*8*), becoming liable to internalization by a scavenger receptor-like mechanism (*9*). If this is the case, protection against oxidative damage should decrease the uptake/degradation by macrophages. In fact it has been observed that addition of antioxidants *in vitro* (*8*) or *in vivo* (*10*) protects Lp(a) against oxidative modification. Thus, in oxidative studies, it is extremely important to control strictly the lipoprotein preparation to prevent, or at least minimize, oxidation. Although a number of strategies have been used to impede the occurrence of such modifications, the methods [*i.e.*, the original procedure of Havel *et al.* (*11*) and its modifications] used to isolate and characterize these lipoproteins have remained essentially unchanged up to now. These techniques require several manipulations including prolonged ultracentrifugation and dialysis steps. Now we have developed a rapid ultracentrifuge/chromatography-based procedure which yields purified LDL, within a few hours, directly in the required solvent.

The method, outlined below, presents some real advantages over established methods such as the classical technique of Havel *et al.* (*11*). The latter procedure takes up to 4 days and involves long dialysis steps between the ultracentrifugations. The long time and the numerous manipulations to which samples are subjected, render LDL and Lp(a) highly susceptible to oxidation (*12*). In contrast, the present non-equilibrium density gradient-based isolation procedure, exploiting previous observations (*13*), consists of a smaller number of steps and can be accomplished within 3.5 h. It required, further purification of Lp(a) can be achieved by an additional rapid wheat germ agglutinin (WGA) column chromatography step.

MATHERIALS AND METHODS

Plasma Samples—Plasma samples were obtained from young healthy normolipidemic male volunteers (between 18 and 35 years of age) after an overnight fast and collected

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in tubes containing EDTA (1 mg/ml) as an anticoagulant, antioxidant (it chelates oxidizing heavy-metal ions such as Cu^{2+}), and antibacterial agent (being a phospholipase C inhibitor) (*14*). Trasylol was added (final concentration of 10 U/ ml of plasma) (*14*) to prevent apolipoprotein B_{100} degradation by plasma kallikrein. Phenylmethylsulfonyl fluoride and sodium azide were not used because the first causes considerable hemolysis in whole blood, and the second has been shown to promote the oxidation of lipids and cleavage of polypeptide chains (*14*). All volunteer donors gave informed consent for the investigation, which was approved by the Human Study Committee of the "Federico II" School of Medicine. At the time of recruitment their diet was isocaloric [contained 23% of energy as fat (3% poly- and 20% mono-unsaturated fatty acids as olive oil), 18% of energy as protein and 59% of energy as carbohydrate (75% as complex carbohydrate)]. The average amounts of cholesterol and fiber were 220 mg/day and 58 g/day, respectively. No drugs or vitamins were included in the diet.

Lipoproteins—LDL obtained by the classical sequential and the SRUC procedures were analyzed at the same time. For this purpose the initiation of the LDL-isolation with the classical procedure (*11*) from a number of plasma samples was timed appropriately.

LDL Isolation by Short-Run Ultracentrifugations (SRUC) in a Vertical Rotor—Ten milliliters of freshly collected plasma was centrifuged for 20 min at 20,000 rpm at 20°C to remove floating chylomicrons. In our experience this step could be omitted, except when the sample was noticeably turbid. In normal conditions the LDL separation is not affected by the presence of small amounts of non removed chylomicrons. Otherwise, floating chylomicrons were aspirated with a Pasteur pipette, discarded, and replaced with an identical volume of 0.191 M NaCl and 1 mM EDTA ($d=1.006$ g/ml). Before filling the ultracentrifugation tubes (39 ml, Beckman #342417), solid anhydrous KBr (350 mg/ml) was added slowly. Particular attention was paid during this step to avoid protein denaturation and local KBr hyper-concentration. This solution was then carefully poured into a Quick-seal polyallomer tube. The estimated density at 20°C of this solution was 1.225 mg/ml (*15*). Finally, the remainder of the tube was filled with isotonic saline ($d=1.006$ mg/ml) containing 1 mM EDTA, which was carefully layered on top of the sample. Although anhydrous (oven-dried) KBr was used in several experiments, we found that small changes in its concentration (say between 340 to 380 mg/ml), did not negatively affect the LDL purification. The sample was centrifuged in a Beckman L65 ultracentrifuge (Beckman Instruments, Palo Alto, CA) in a VTi 50 vertical rotor at 45,500 rpm for 2.0 h at 10°C. At the end of the centrifugation the gradient was collected with the aid of a fraction collector by piercing the centrifuge tube at the bottom with a syringe needle to which a peristaltic pump was attached. About 80 fractions of 450 μl were collected and analyzed for proteins and cholesterol (see "*Other Methods*" section). The LDL lipoprotein banded near the middle of the tube and could be easily identified as a yellow disk. In subsequent separations the LDL was directly aspirated through the wall by means of a 5 ml syringe. Usually, 2 ml of LDL protein solution (or less) was withdrawn with a protein concentration in the range of 1.8-2.5 mg/ml. Then ~2 ml of LDL-enriched solution was directly poured on the top of a Sephadex G 25 prepacked column (PD10) equilibrated with EDTA (1 mM) and KBr (1.225 mg/ml). The same solution was used to elute the column. As advised by the column manufacturer (Pharmacia), the first 3 ml of the eluate was discarded, and the second 3 ml was directly collected into Quick-seal polyallomer tubes (5.1 ml, Beckman #342883). Again, the tube was filled with isotonic saline ($d=1.006$ mg/ml) containing EDTA (1 mM, final concentration), then centrifuged for 40 min at 68,000 rpm at 10°C in a Beckman VTi 80 vertical rotor. Once again the LDL-containing band near the middle of the tube was aspirated by piercing the wall with a 2 ml syringe and directly applied to a Sephacryl S 300 column (5×0.9 cm) equilibrated with the required buffer. For example, Tris-HCl or PBS containing buffers were used as required for electrophoresis, lipid or protein determinations, chemical analysis, addition to cells in culture, *etc.* This final step contributes to the removal of possible extraneous proteins (*i.e.*, serum albumin and immunoglobulins) which may have been co-purified with the LDL/Lp(a). The purified LDL were finally sterilized by filtration (Nalgene disposable syringe filter, 0.45 μm) and kept at 4°C under nitrogen until used.

Lipoprotein (a) Purification—A new method to isolate Lp(a) from human plasma has recently been described (*16*). Lp(a) contains large amounts of *O*-linked sugars, namely *N*-acetyl-D-neuramic acid (NANA) and *N*-acetyl-D-glucosamine (GlcNAc), which account for 25-40% of the total Lpa mass (*17, 18*). This makes Lp(a) one of the most heavily glycosylated proteins in human plasma, and a good ligand for WGA. In our study we have exploited this property by applying the LDL-enriched Lp(a) fractions to a WGA column. In brief, plasma from selected donors, whose Lp(a) levels were between 30 and 80 mg/dl (ELISA, Immuno, Vienna) was treated as described for the normal preparation of LDL. At the time of the final sample syringe aspiration, a larger volume of fluid, spanning from 2 mm above to 2 mm below the β-carotene of the LDL band, was collected. Pools of this material (about 1.2 ml/tube; total 4-5 ml) were immediately applied to a 10 ml disposable plastic column (Pharmacia) filled with 2.5 ml of packed 4% beaded agarose to which WGA was cross-linked (Sigma-Aldrich) (7 mg/ml). The column had been pre-washed with 25 ml of buffer A (1 M NaCl), and equilibrated with 50 ml of buffer B (1 mM PBS, 0.15 M NaCl, 1 mM EDTA, pH 7.4). The gravity-obtained flow-through fraction was recycled three additional times. The column was then washed with 50 ml of buffer B and the lectin-bound material was stripped with 7.5 ml of buffer C (buffer B containing 0.1 M *N*-acetyl-D-galactosamine). Eluates (500 μl) were quickly tested for protein content by mixing a few microliters of each fraction with appropriate amounts of biuret reagent. Typically a biuret-positive material was detected in fractions 3, 4, and 5. These fractions were then pooled and used as purified Lp(a). The purity of this material was finally checked by Western blot analysis using the anti-human apo (a) monoclonal antibody (MIA_2, Boehringer-Mannheim, Mannheim, Germany), as described in detail by Lackner *et al.* (*19*). The nitro-cellulose membrane was from Amersham Italia srl, Milan, Italy. The column was restored by washing with 10 ml of buffer D (1 M NaCl, 0.1 M *N*-acetyl-D-galactosamine, and then with 25 ml of buffer A).

Lipid Analysis—Lipids of both LDL and Lp(a) fractions were extracted according to Folch's method (*20*). The lipid mixture in chloroform was applied to an amino-propyl bonded phase column under vacuum pressure. Recoveries and homogeneity of polar and neutral lipid classes, isolated according to Kaluzny's method (*21*) with the modifications we have recently introduced (*22, 23*), were determined according to Bittman and Wood (*24*), and were greater than 95% for all classes. The subsequent analysis of fatty acids was done by using about one half of each fraction (cholesterol esters, phospholipids, and triglycerides). Samples were placed in a derivatization tube together with 100 μg of heptadecanoic acid (internal standard), dried under a stream of nitrogen, and hydrolyzed for 30 min at 80°C with 0.3 ml of KOH (8.9 M) in 2 ml of methanol. After cooling, 2 ml of water and 0.5 ml of HCl (6 N) were added to the reaction tube and the free fatty acids were extracted three times with 2 ml of hexane. The extracts were washed with saline and dried under a stream of nitrogen. Fatty acids were methylated with diazomethane at room temperature for 10 min, dried a second time and finally re-dissolved in 1 ml of hexane. Then 50 μl of this solution was injected into a gas chromatograph/mass spectrometer (GC 5890, MSD 5970, Hewlett Packard, Palo Alto, CA, USA). A detailed description of the whole procedure, including the identification and quantitative evaluation of methylated fatty acids by mass spectrometry, has been reported in a previous paper from this laboratory (*23*). Similarly, vitamin E was extracted with hexane from ethanol-precipitated lipoproteins, evaporated, and redissolved in methanol. Its HPLC separation and quantitative determination are described in the paper mentioned above (*23*); a 3 μm particle size Supelcosil LC8DB analytical column (150×4.5 mm), was used.

Evaluation of Oxidation Products of LDL and Lp(a)—LDL obtained by the classical technique and by the SRUC procedure were analyzed at the same time.

a) Apoproteins: Apolipoprotein B_{100} (apo B_{100}) and apolipoprotein (a) [apo(a)] were quantitatively delipidated in 2.5% SDS plus dithiothreitol by heating in a boiling bath for 45 s. Isolated apolipoproteins were electrophoresed overnight on 5-16% sodium dodecyl sulfate polyacrylamide discontinuous-gradient gels at 5 mA/gel. Both purity and/or extent of degradation of apo B_{100} and apo (a) were quantitatively estimated by densitometric scanning (LKB, Pharmacia Produkter AB, Sweden) of the Coomassie Blue stained gels.

b) Lipids: Lipid peroxidation of lipoproteins was evaluated from the amount of both lipid peroxides (LPO kit, Kamiya Biomedical, Thousand Oaks, CA, USA) and malonyldialdehyde (MDA) produced, as previously described in detail (*23, 25-27*). The latter compound, a widely used marker of lipid peroxidation, represents one of the main end-products of peroxidation of unsaturated fatty acids. It was assayed by means of the thiobarbituric acid assay. Peroxidative chain-reactions were prevented by adding to samples the chain-breaker butylated hydroxytoluene (0.16%), the iron chelator deferoxamine (100 μM final concentration), and ethanol (8%) (*23, 25-27*). This mixture prevents artifacts due to variations in sample lipid content and/or antioxidant concentration and possible heavy metal ion contamination of reagents.

Other Methods—Total cholesterol, unesterified (free) cholesterol, phospholipids, and triglycerides were detected by commercially available enzymatic methods (nos. 237574, 310328, and 691844, Boehringer-Mannheim, and no. 6639, Sera Pak, Italy, respectively). Protein content of LDL was measured by the Lowry method (*28*). Results are expressed as the mean±SD of 12 different preparations from different donors. Statistical evaluation of the data was performed by the Wilcoxon signed-rank test for paired observations. For unpaired data the Wilcoxon rank-sum test was used. Correlation coefficients were determined according to Spearman's rank correlation method. A two-tailed probability value of $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

LDL and Lp(a) are unstable molecules: this property is intrinsic and is probably related to their physiological function. Both macromolecules contain lipids and protein: the lipids are extremely susceptible to peroxidative chain-reactions, and the polypeptide chains may be directly broken or may give rise to covalent adducts with aldehydes and/or other side products derived from oxidation of lipid moieties. *In vivo*, these modifications may take place as a consequence of physiological and/or pathophysiological injury, and *in vitro*, spontaneous oxidation occurs during purification, handling, and storage. However it is still not clear to what extent the oxidation process can be controlled. Although all this is well known, the isolation of LDL and Lp(a) is normally achieved by using a procedure consisting of prolonged ultracentrifugation [which may influence the protein composition of lipoproteins (*13*)] alternated with extensive dialysis steps. The need for faster preparative procedures, then, should be considered a major goal, particularly for studies (either *in vivo* or *in vitro*), which aim to correlate oxidation of molecules such as LDL and Lp(a) with diseases as atherosclerosis and/or thrombosis.

In this report we have compared LDL and Lp(a) purified by SRUC, with the same molecules obtained by the method of Havel *et al.* (*11*). We report the results of chemical analysis, including protein distribution (a), relative amounts of single classes of lipids (b_1), fatty acid distribution (b_2), and total amount of the naturally occurring antioxidant vitamin E (c) (Table I), major lipid peroxidation products (MDA, LPO, Table II), and the relative electrophoretic profiles (purity, number and amounts of additional species) (Table III).

The chemical compositions of lipoproteins prepared by the SRUC and classical methods showed no meaningful differences. This was also the case for total lipid and fatty acid residues distribution (Table I, sectors a, b_1, and b_2). In contrast, after isolation by SRUC coupled with a Sephacryl S-300 column, mean levels of vitamin E were similar in LDL and Lp(a), while vitamin E in LDL-classic was reduced by a factor of ~2 (Table I, sector c) with respect to LDL-SRUC.

The quantitative estimates (reported in Table II) clearly reveal that, under our experimental conditions, typical indices of spontaneous lipid peroxidation in lipoproteins are much lower (4-5-fold) in molecules [either LDL or Lp(a)] prepared according to our procedure than in molecules prepared according to the classical methods. This phenomenon is probably related to the lower rate of peroxidative

chain-reactions (*26*) in LDL isolated with SRUC.

It is well known that the electrophoretic profile of purified LDL is remarkably variable, especially in regard to the number and relative amounts of faster-migrating bands (*29*). This holds true especially when LDL samples are obtained from differents blood donors and/or using different preparation protocols. For this reason, we have conducted 8 different comparative LDL preparations and examined the purified products by means of gel electrophoresis. A typical example is reported in Fig. 1, upper panel, in which are depicted the molecular weight standards (high), the LDL-classic and the LDL-SRUC. It appears that, besides albumin, a major contaminant, four lower-molecular-weight species are present in this LDL-classic preparation. The averaged amounts of each band were obtained by densitometry of the Coomassie Blue-stained gels and expressed as percent of the apo B_{100} (Table III). The same species, marked with small arrows in Fig. 1, are indicated in Table III as I, II, III, and IV (from the top to the bottom). Very likely, in accord with several studies (reviewed in Refs. *3* and *29*), these bands represent early degradation products of apo B_{100} and not proteins such as apo B_{48} or apo A or apo E or apo C. This was supported by comparing the migration of the observed fragments with that of the aforementioned apoproteins. However, while the understanding of the origin of these bands is outside the scope of this work, it is noteworthy that the same species are virtually absent in the electrophoretic pattern of LDL-SRUC (indeed in Fig. 1 they are undetectable). The lower panel of Fig. 1, represents the relative electrophoretic mobility on agarose gel of isolated LDL-SRUC and Lp(a) bands: no significant difference can be detected. This latter observation demonstrates that the additional purification step with the wheat-germ column did not modify Lp(a) net charge. It is well known that an increase in electrophoretic mobility is associated with the loss of lipoprotein positive charge due to chemical modifications of its lysyl and arginyl residues (*1, 2*).

Figure 2 (upper panel) shows a typical SDS-PAGE of isolated LDL (A) and Lp(a) (B) obtained by the SRUC procedure. In both cases the major protein stained is apo B_{100}. The lower panel (D) depicts Lp(a) and its isoforms after WGA purification, electrophoresis on a 5% gel, blotting on nitrocellulose filter, incubation with the MIA_2 antibody and final staining (by chemiluminescence). The C

TABLE I. **Chemical composition of LDL and Lp(a).**

	LDL-classic	LDL-SRUC	Lp(a)-SRUC-WGA
(a) Protein (% of total mass)	26.3±3.6	25.3±2.8	34.7±1.8[a]
(b_1) Cholesterol (% of total mass)	5.5±2.1	5.9±1.8	5.2±1.5
Cholesteryl esters (% of total mass)	37.8±4.1	39.5±3.5	32.9±3.0
Phospholipids (% of total mass)	21.0±3.3	20.5±2.1	18.8±2.2
Triglycerides (% of total mass)	8.2±1.8	7.8±1.5	7.9±1.5
(b_2) Fatty acid residue (%)			
Palmitoleic (16.1)	1.9±1.2	2.0±1.8	2.4±0.9
Palmitic (16.0)	18.8±3.9	18.0±5.4	18.4±2.9
Linoleic (18.2)	29.2±7.6	32.4±5.1	29.5±6.6
Oleic (18.1)	34.5±7.5	33.3±8.3	35.8±7.2
Stearic (18.0)	9.9±0.6	9.1±1.0	9.4±1.2
Arachidonic (20.4)	7.5±0.4	7.1±0.9	6.9±1.3
(c) Vitamin E (nmol/mg LDL)	1.8±0.3	3.5±0.3[a]	3.1±0.4[a]

SRUC, short-run ultracentrifugations in vertical rotors alternated with two rapid column-chromatography steps; WGA, wheat germ agglutinin column. Data are presented as means±standard deviation. [a]$p<0.05$ *vs.* LDL-classic.

TABLE II. **Indices of spontaneous lipid peroxidation in different lipoprotein samples.**

	LDL-classic	LDL-SRUC	Lp(a)-SRUC-WGA
MDA (nmol/mg protein)	2.4±0.3[a]	0.5±0.1	0.7±0.1[b]
LPO (nmol/mg protein)	18.0±4[a]	<5	<5

SRUC, short-run ultracentrifugations in vertical rotors alternated by two rapid column-chromatography steps; WGA, wheat germ agglutinin column. Data are presented as means±standard deviation. [a]$p<0.05$ *vs.* LDL-SRUC and [b]$p=0.69$ (ns) *vs.* LDL-SRUC.

TABLE III. **Electrophoretic profiles, and relative amounts of stainable species.**

Electrophoretic band	LDL-classic	LDL-SRUC
Apo B_{100}	100	100
I	2.50%[a]	1.51%
II	1.70%[a]	0.61%
III	4.90%[a]	2.01%
IV	7.01%[a]	Undetectable
Total	16.11[a]	4.13

SRUC, short-run ultracentrifugations in vertical rotors alternated with two rapid column-chromatography steps; WGA, wheat germ agglutinin column; Apo-B_{100}, apolipoprotein B_{100}. Mean of 8 different experiments. [a]$p<0.05$ *vs.* LDL isolated by SRUC procedure.

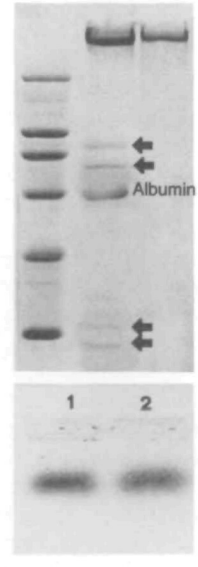

Fig. 1. **(Upper Panel): Electrophoretic profile under denaturing (SDS) and reducing (β-mercaptoethanol) conditions of LDL prepared by the procedure of Havel *et al.* (*11*) compared with that obtained by the SRUC method.** From left: molecular weight standards [myosin, $M_r=200{,}000$; β-galactosidase, $M_r=116{,}000$; phosphorylase *b*, $M_r=97{,}000$; albumin, $M_r=66{,}000$; ovalbumin, $M_r=45{,}000$; soybean trypsin inhibitor (21,500); LDL-SRUC (~50 mg); and LDL-Classic (~35 μg)]. The arrows indicate bands I, II, III, and IV of degraded apo B_{100} (from top to bottom). **(Lower Panel): Agarose gel electrophoresis pattern of isolated LDL and Lp(a) bands.** No significant difference in the respective electrophoretic mobilities is observed.

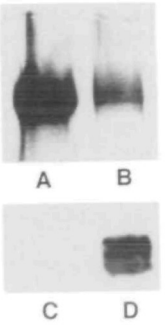

Fig. 2. (Upper panel): LDL (A) and Lp(a) (B) prepared by the SRUC procedure. (Lower panel): Immunostaining of Lp(a) isoforms. After SRUC, Lp(a) isoforms were finally purified through a WGA column, electrophoresed on a 5% polyacrylamide gel in SDS, blotted onto nitrocellulose, treated with anti-Lp(a) M1A, monoclonal anti-Lp(a) and stained by chemiluminescence (D). The empty channel (C) was loaded with the flow-through fraction of the WGA column.

channel (unstained) was loaded with the flow-through material: the total absence of immunostainable material (flow-through) indicates that apo(a) was quantitatively retained by the WGA.

In the light of the sensitivity of LDL and Lp(a) to spontaneous oxidation during handling, it is important to control strictly the experimental conditions of purification before performing measurements of lipid and protein oxidation products. Optimizing these circumstances is a prerequisite for obtaining valuable and reproducible information. Both lipoproteins isolated by our method appeared to have the same density and purity as those isolated by the conventional sequential-isolation method. However, the assays showed some differences between the two preparations. The uses of vertical rotors significantly reduces the total purification time, while the use of PD10 and Sephacryl S 300 columns avoids the need for prolonged dialysis. This is important, because considerable oxidation can occur during the lengthy dialysis step (*12*). The procedure allows experiments to be conducted on the same day as lipoprotein preparation. It appears that LDL obtained by the sequential method exhibits more extensive apo B_{100} degradation and lipid peroxidation than the molecules obtained by means of SRUC: the latter method should consequently be preferred over the conventional ones, especially when oxygen radical-induced modifications are under investigation. Even the endogenous LDL and Lp(a) antioxidant (*i.e.*, vitamin E) appears to be better preserved. Presumably, the limited manipulation of the sample, the storage at −20 to 4°C (*30*) and, above all, the elimination of the prolonged dialysis steps required by the classical purification procedure prevent the detachment of this molecule from the lipoproteins, to which it is bound by means of weak forces.

An additional practical point is that changes in the KBr concentration from 340 to 380 mg/ml do not significantly affect the quality of the product: the method is tolerant of some inaccuracy in preparing samples for the ultracentrifugation (amount of KBr to be added to serum) or small errors in KBr weight due to partial hydration. If necessary, oxidizing heavy metal ions possibly present in plasma can be eliminated before the initiation of the purification: the collected plasma can be gently brought into contact for a few minutes with 50-70 mg/ml of Chelex-100 resin, which, in turn, can be easily removed by low-speed centrifugation at 450×*g* for 2.5 min at 4°C.

It appears that the LDL recovery by the SRUC method is 50 to 70% based on the LDL content of the donors, *i.e.*, of the same order of magnitude as other established methods (*3*). The Lp(a) recovery is very high (≥90%). An indirect proof of the high affinity and capacity of the system comes from the immunoblotting analysis of the fractions collected from the column (Fig. 2): all the immunoreactive material was detected in the eluate and none was present in the flow-through and wash fractions. Unfortunately, there is no reference to recovery rate in the original report which describes the WGA-purification of Lp(a) from the whole plasma (*16*). However, a similar recovery can be assumed considering that Lp(a) was quantitatively retained by the gel matrix and could not be detected either in the flow-through or in wash fractions. Finally, it must be emphasized that the method presented here allows the preparation of undegraded and unoxidized lipoprotein species in less than a working day. Consequently, the problem of the yield is not major, since the amount of lipoprotein necessary for an experiment is usually in the order of a few milligrams or even less. Fresh material may be obtained as required on the day of an experiment.

In conclusion, this study provides a rapid and simple method to obtain LDL and Lp(a) with minimal oxidation, a crucial point for studies on the role of oxygen radical-induced modifications of these lipoproteins in atherothrombosis. It is clear that this method, conveniently adapted, may also be used to prepare other components of the lipoprotein family. Experiments in this direction are in progress in our laboratory.

REFERENCES

1. Witztum, J.L. and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88**, 1785-1792
2. Berliner, J.E. and Heinecke, J.W. (1996) The role of oxidized lipoproteins in atherosclerosis. *Free Rad. Biol. Med.* **20**, 707-727
3. Young, S.G. and Parthasarathy, S. (1994) Why are lipoproteins atherogenic? *Western J. Med.* **160**, 153-161
4. Utermann, G., Scriver, C.R., Beaudet, A.C., Sly, W.S., and Valle, D. (eds.) (1995) Lipoprotein (a) in *The Metabolic Basis of Inherited Diseases*, pp. 1887-1912, McGraw Hill, New York, NY
5. Liu, C.A. and Lawn, R.M. (1994) Vascular interactions of lipoprotein(a). *Curr. Opin. Lipidol.* **5**, 269-273
6. Mancini, F.P., Newland, D.L., Mooser, B., Murata, J., Marcovina, S., Young, S.G., Hammer, R.E., Sanan, D.A., and Hobbs, H.H. (1995) Relative contributions of apolipoprotein(a) and apolipoprotein-B to the development of fatty lesions in the proximal aorta of mice. *Arterioscler. Thromb. Vasc. Biol.* **15**, 1911-1916
7. Pepin, J.M., O'Neil, J.E., and Hoff, H.F. (1991) Quantification of Apo (a) and B in atherosclerotic lesions. *J. Lipid Res.* **32**, 317-327
8. Naruszewicz, M., Selinger, E., and Davignon, J. (1992) Oxidative modification of lipoprotein (a) and the effect of β-carotene. *Metabolism* **41**, 1215-1224
9. Haberland, M.E., Fless, G.M., Scanu, A.M., and Fogelman, A.M. (1992) Malondialdehyde modification of Lp(a) produces avid uptake by human monocyte-macrophages. *J. Biol. Chem.* **267**, 4143-4151
10. Naruszewicz, M., Selinger, E., Dufour, R., and Davignon, J. (1992) Probucol protects Lp(a) against oxidative modification. *Metabolism* **41**, 1225-1228
11. Havel, R.K., Eder, H.A., and Bragdon, J.H. (1955) The distribu-

tion and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**, 1345-1353
12. Scheek, L.M., Wiseman, S.A., Tijburg, L.B.M., and van Tol, A. (1995) Dialysis of isolated low density lipoprotein induces a loss of lipophilic antioxidants and increases the susceptibility to oxidation in vitro. *Atherosclerosis* **117**, 139-144
13. Chung, B.H., Wilkinson, T., Geer, J.C., and Segrest, J.P. (1980) Preparative and quantitative isolation of plasma lipoproteins: rapid single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21**, 284-291
14. Edelstein, C. and Scanu, A.M. (1986) Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol.* **128**, 151-155
15. Weast, R.C. (ed.) (1970-1971) *CRC Handbook of Chemistry and Physics,* 51st ed., Table 34, D-197
16. Seman, L.J., Jenner, J.L., McNamara, J.R., and Schaefer, E.J. (1994) Quantification of lipoprotein (a) in plasma by assaying cholesterol in lectin bound plasma fraction. *Clin. Chem.* **40**, 400-403
17. Seman, L.J. and Breckenridge, W.C. (1986) Isolation and partial characterization of human apolipoprotein (a) from lipoprotein (a). *Biochem. Cell Biol.* **64**, 999-1009
18. Fless, G.M., ZumMallen, M.E., and Scanu, A.M. (1986) Physicochemical properties of apolipoprotein (a) derived from the dissociation of human plasma lipoprotein (a). *J. Biol. Chem.* **261**, 8712-8718
19. Lackner, C., Boerwinkle, E., Leffert, C.C., Rahmig, T., and Hobbs, H. (1991) Molecular basis of apolipoprotein (a) isoform size heterogeneity as revealed by pulse-field gel electrophoresis. *J. Clin. Invest.* **87**, 2153-2161
20. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**, 497-505
21. Kaluzny, M.A., Duncan, L.A., Merritt, M.V., and Epps, D.E. (1985) Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26**, 135-142
22. Nardone, G., D'Armiento, F.P., Corso, G., Coscione, P., Esposito, M., and Boudillon, G. (1994) Lipids of human gastric mucosa: Effect of *Helicobacter pylori* infection and non-alcoholic cirrhosis. *Gastroenterology* **107**, 362-368
23. Napoli, C., Postiglione, A., Triggiani, M., Corso, G., Palumbo, G., Carbone, V., Ruocco, A., Ambrosio, G., Montefusco, S., Malorni, A., Condorelli, M., and Chiariello, M. (1995) Oxidative structural modifications of low density lipoprotein in homozygous familial hypercholesterolemia. *Atherosclerosis* **118**, 259-273
24. Bittman, J. and Wood, D.L. (1982) An improved copper reagent for quantitative densitometric thin-layer chromatography of lipids. *J. Liquid Chromatogr.* **5**, 1155-1160
25. Ambrosio, G., Napoli, C., Oriente, A., Palumbo, G., Chiariello, P., Marone, G., Condorelli, M., Chiariello, M., and Triggiani, M. (1994) Oxygen radical inhibit human plasma acetylhydrolase, the enzyme that catabolizes platelet activating factor. *J. Clin. Invest.* **93**, 2408-2416
26. Napoli, C., Ambrosio, G., Palumbo, G., Elia, P.P., and Chiariello, M. (1991) Atherogenic oxidative modification of human lipoproteins may involve peroxidative chain reactions following initial oxygen free radicals attack. *J. Mol. Cell. Cardiol.* **23** (Suppl. V), 115A-118B
27. Napoli, C., Chiariello, M., Palumbo, G., and Ambrosio, G. (1996) Calcium channel blockers inhibit human low density lipoprotein oxidation by oxygen radicals. *Cardiovasc. Drugs Ther.* **10**, 417-424
28. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
29. Fong, G.L., Parthasarathy S., Witztum J.L., and Steinberg D. (1987) Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J. Lipid Res.* **28**, 1466-1477
30. Evans, K., Mitcheson, J., and Laker, M.F. (1995) Effect of storage at 4 degrees C and −20 degrees C on lipid, lipoprotein, and apolipoprotein concentrations. *Clin. Chem.* **41**, 392-396