methods

Rapid isolation of VLDL subfractions: assessment of composition and susceptibility to copper-mediated oxidation

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Abstract VLDLs, synthesized and released by the liver, are a heterogeneous group of particles of varying composition and metabolic fates. A method is described for the rapid isolation of VLDL into four subfractions (A–D) and assessment of their susceptibility to oxidation. The total isolation procedure required less than 3.5 h, and was achieved by gradient ultracentrifugation. Each subfraction was assessed for triglyceride, cholesterol, and apolipoprotein B (apoB) composition and for the presence of contaminants such as albumin and urate. The oxidation potential, in the presence of copper ions, of each subfraction was also assessed. This rapid procedure produced VLDL fractions analogous to those produced by a previously reported but more prolonged isolation method. Comparison of the two procedures demonstrated that lipid and apoB were similar, while the rapid procedure produced subfractions void of albumin and urate contamination and lower in preformed hydroperoxides. Compositional changes were found between the subfractions: as the subfractions became smaller and more dense (A→D), there was a decrease in the ratio of triglyceride to apoB and an increase in the ratio of cholesterol to apoB, also arachidonic acid was increased in subfraction D compared with subfractions A, B, and C. The smaller subfractions were more susceptible to oxidation, a trend similar to that reported previously for the oxidation of LDL subfractions.—McEneny, J., C. McMaster, E. R. Trimble, I. S. Young. **Rapid isolation of VLDL subfractions: Assessment of composition and susceptibility to copper-mediated oxidation. *J. Lipid Res.* 2002.** 43: **824–831.**

Supplementary key words ultracentrifugation • fatty acid • lipid composition

VLDL secreted by the liver is a heterogeneous mixture of particles (1) which may be classified either on the basis of density (0.94–1.006 kg/l), diameter (20–75 nm), or flotation [Svendberg floatation rate (Sf) 20–400]. The major lipid component is triglyceride, making up approximately 60% of the particle.

Although the association of triglyceride-rich particles such as VLDL with the development of cardiovascular disease remains under debate (2), there is increasing support for the importance of hypertriglyceridaemia as an independent risk factor for cardiovascular disease (CVD) (3). VLDL particles are released from the liver in a range of sizes and compositions. When the liver is supplied with increased substrate (non-esterified fatty acids or chylomicron remnants) there is a tendency to synthesize and release large triglyceride rich VLDL (4), the remodelling of which leads to small dense LDL (5, 6). These particles have a strong association with the development of cardiovascular disease (7–9), as small dense LDL has a decreased affinity for its native receptor and subsequently an increased residence time within the circulation and extra vascular fluid. As a consequence of this, together with the fact that small dense LDL particles are more susceptible to oxidative modification, there is a greater probability that such LDL particles will become modified. Modified LDL taken up by scavenger receptors leads to an accumulation of lipid within the macrophage and the production of characteristic foam cells (10, 11). Large triglyceride-rich VLDL particles not remodelled to LDL are themselves pro-atherogenic and can be removed by macrophage receptors without the need for oxidative modification (12), leading to the formation of foam cells and ultimately the development of atherosclerosis.

Several methods requiring long preparation times have previously been developed which enable VLDL to be subfractionated (13–15). However, the lengthy time required has proved prohibitive for routine analysis. We have therefore established a technique that enables the isolation of VLDL subfractions in a much shorter time and also compares favorably with currently published methods.

A method is described that enables isolation of four VLDL subfractions with a total preparation time of just over 3 h. This rapid method produces sufficient quantities of each subfraction for the determination of composition and assessment of susceptibility to copper mediated oxidation. The application of this method to patient groups with increased incidence of premature atherosclerosis

Abbreviations: FOX 2, ferrous oxidation of xylenol orange version 2.

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and hypertriglyceridaemia enables measurement of the lipid profile of each subfraction and their potential for oxidation to be rapidly assessed.

MATERIALS AND METHODS

Materials

All materials were obtained from the Sigma Chemical Company, Poole, Dorset, UK, unless otherwise stated.

Methods

Plasma separation. Fasting peripheral venous blood was collected into heparinised tubes (Li Heparin 500 U/10 ml) on ice and centrifuged (Jouan CR 412) at 950 × *g* for 10 min at 4°C. Plasma was removed within 30 min from venipuncture and stored frozen at −70°C in 2 ml aliquots until utilization.

VLDL isolation. To facilitate the subfractionation of VLDL, total VLDL was first isolated by rapid ultracentrifugation by the method of McEneny et al. (16). In brief, 1.8 ml heparinised plasma was added to a 3 ml ultracentrifuge tube (Polyallomer Bell-top; Beckman) and was gently overlaid with normal saline, 1.2 ml (d = 1.006 Kg/l; 0.196 mol). Ultracentrifugation was performed in a Beckman Table Top Ultracentrifuge (TL-100) using a Beckman fixed angle rotor (TL-100.3) and the following parameters: 541,000 × *g* (100,000 rpm) for 60 min at 4°C. On completion of ultracentrifugation, due to flotation, the VLDL was located at the top of the ultracentrifuge tube. Its extraction was performed by aspiration after tube slicing; this extract equates to "whole" or "crude" VLDL. VLDL subfractions were isolated from blood tubes containing the anticoagulant lithium heparin in preference to EDTA. This latter anticoagulant influences the subfractions' susceptibility to oxidation by chelating the metal ions used to promote oxidation (see later).

Prior to subfractionation, the purity of this isolated VLDL was assessed by lipoelectrophoresis using a Beckman Paragon Lipoprotein (Lipo) Electrophoresis Kit (Beckman, UK) following the manufacturer's instructions.

VLDL subfractionation. Isolation of VLDL into subfractions was achieved by gradient ultracentrifugation. The rapid method described here is an adaptation of the method of Saheki et al. (15), using a different rotor, (Beckman TL-100.3 V's Beckman TLS-55). Run parameters for the TL100.3 rotor were calculated using technical information sheets and the clearing factor (*k*) supplied by the manufacturer for this purpose. In contrast to the 18 h required for the original Saheki et al. method, total preparation time for the collection of the four subfractions from VLDL was 3 h 26 min (allowing for a total of 60 min deceleration time), the sum of four ultracentrifugation runs. The rapid method was as follows: to a Beckman open top ultracentrifugation tube (Polycarbonate, 13 × 51 mm) was added 0.1225 g KBr, followed by 0.875 ml crude VLDL; these were gently mixed and resulted in adjustment of the VLDL density to 1.1 g/ml. To this density adjusted VLDL the following three solutions of decreasing density were gently overlaid in the following proportions: 0.688 ml, d = 1.065 g/ml; 0.688 ml, d = 1.020 g/ml; 0.75 ml, d = 1.006 g/ml.

Ultracentrifugation was again performed in a Beckman Table Top Ultracentrifuge (TL100) utilizing a Beckman fixed angle rotor (TL100.3) with an acceleration setting of five and deceleration setting of zero. Temperature was maintained at 4°C for all four runs. *1*) Subfraction A was obtained after ultracentrifugation at 86,000 × *g* (40,000 rpm) for 16 min (plus 8 min deceleration time). This fraction was removed from the top section of the ultracentrifuge tube by careful aspiration in a volume of 0.75 ml. After the removal of fraction A, the volume of the ultracentrifuge tube was replenished with 0.75 ml saline (d = 1.006 kg/l). The replenishment of the volume removed with saline was performed after each ultracentrifugation step; *2*) Subfraction B was isolated after ultracentrifugation at 346,200 × *g* (80,000 rpm) for 12 min (plus 16 min deceleration time), in a volume of 0.75 ml; *3*) Subfraction C was isolated after ultracentrifugation at 346,2000 × *g* (80,000 rpm) for 13 min (plus 16 min deceleration time), in a volume of 0.75 ml; and *4*) Subfraction D was isolated after ultracentrifugation at 541,000 × *g* (100,000 rpm) for 1 h 45 min (plus 20 min deceleration time), in a volume of 0.75 ml. (In preliminary experiments, infranatant E was removed and tested to verify complete harvesting of VLDL.)

All four subfractions together with infranatant E were subjected to lipoprotein electrophoresis using a Beckman Paragon Lipoprotien (Lipo) Electrophoresis Kit (Beckman, UK) following the manufacturer's instructions. On completion of each ultracentrifugation step, the removed subfraction was stored on ice until all four subfractions had been obtained.

To validate this rapid method the results obtained were compared directly with the prolonged method of Saheki et al. (15), with application for use in the Beckman TL 100 Ultracentrifuge containing a Beckman TL100.3 rotor. Ratios and quantities of solutions were as described in the rapid method with the following ultracentrifugation parameters: *1*) Subfraction A obtained after ultracentrifugation at 48,700 × *g* (30,000 rpm) for 29 min (plus 6 min deceleration time); *2*) Subfraction B obtained after ultracentrifugation at 135,200 × *g* (50,000 rpm) for 30 min (plus 10 min deceleration time); *3*) Subfraction C obtained after ultracentrifugation at 135,200 × *g* (50,000 rpm) for 34 min (plus 10 min deceleration time); and *4*) Subfraction D obtained after ultracentrifugation at 55,400 × *g* (32,000 rpm) for 15.75 h (plus 7 min deceleration time). Total preparation time for VLDL subfractions isolated by the above method was 17 h 53 min.

The infranatant E was again collected to ensure complete isolation of VLDL and all subfractions were subjected to lipoelectrophoresis.

VLDL composition

Validation of this rapid method, and comparison to the prolonged procedure (modified for the TL 100 ultracentrifugation) was assessed using the following methods:

Protein determination. For both procedures each of the four subfractions of VLDL, together with the infranatant E, were analyzed for total protein concentration using a commercial kit based on the Coomassie Blue reaction with proteins (Biorad; 500-006), following the manufacturer's instructions. This methodology enabled a direct comparison of protein concentration between each of the isolation procedures.

Cholesterol and triglyceride

RECOVERY. Total cholesterol and triglyceride within the VLDL subfractions and the infranatant E were measured using enzymatic assays (Boehringer Mannheim) on a Cobas Bio Analyser. The sum of triglyceride and cholesterol from subfractions A–E was compared with the original triglyceride and cholesterol concentrations in the initial crude VLDL.

RATIO TO APOLIPOPROTEIN B. Triglyceride and cholesterol were standardised for apolipoprotein B (apoB) content and expressed as μmol triglyceride/mg apoB or μmol cholesterol/mg apoB.

FATTY ACID DETERMINATION. The distribution of fatty acid, expressed as percent of saturated fatty acid, monounsaturated fatty acid, and PUFA in crude VLDL and subfractions A–D was determined by gas chromatography using a Hewlett Packard GC system as described in McEneny et al. (17).

APOB DETERMINATION. The concentration of apoB in each subfraction was determined by single radial immunodiffusion as

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described in McEneny et al. (16), a modification of the method of Mancini et al. (18), and Becker (19).

ALBUMIN DETERMINATION. Albumin is a known antioxidant whose presence can alter the oxidation profile of lipoproteins (20). Albumin concentration was assessed by single radial immunodiffusion as described in McEneny et al. (16).

PERFORMED HYDROPEROXIDES. Preformed hydroperoxides were measured in crude VLDL and each of the VLDL subfractions by the ferrous oxidation of xylenol orange version 2 (FOX 2) method of Wolff (21) and Naurooz-Zadeh et al. (22) and as described in McEneny et al. (16).

URATE DETECTION. Previous experiments had demonstrated that crude VLDL isolated by a single spin was contaminated with urate [57 μmol/l (range 45–65)] (23). As urate can greatly alter the susceptibility of lipoproteins to oxidation (16, 24, 25), VLDL subfractions were assessed for contamination with urate carried over from the crude VLDL. Urate was measured by HPLC with electrochemical detection using a modification of the method of Chevion et al. (26).

Susceptibility of VLDL subfractions to oxidation

Optimum copper concentration. In the oxidation of LDL a relationship exists between lag time and copper concentration; with increasing copper concentrations between 0.2 μmol/l and 5 μmol/l, there is a resultant decrease in lag time. However this trend does not continue when copper concentrations exceed 5 μmol/l, where further increases in copper have no effect on lag time (27, 28). We have found that VLDL becomes more susceptible to oxidation in the presence of copper ions within the range 0.1 to 17.5 μmol/l, showing a characteristic decrease in lag time with increasing copper concentration. However, increasing the copper concentration above 17.5 μmol/l causes no further decrease in lag time (23). This relationship between copper ions and lag time was tested on the VLDL subfractions. Copper concentrations in the range 2.5 to 15 μmol/l were employed with the VLDL sample being standardised to 10 μg/ml protein.

Routine oxidation procedure. The routine oxidation of the VLDL subfractions was as follows: after standardisation of each of the subfractions of VLDL to 10 μg/ml protein with PBS, oxidation was initiated by the addition of 10 μmol/l copper II chloride (final concentration). The oxidation process was carried out in a thermostatically controlled spectrophotometer at 37°C (Hitachi U2000), containing an automatic six cell positioner. The production of conjugated dienes was followed by change in absorbance at 234 nm. The lag time was calculated as the intercept of the initial and rapid phases of the reaction using a specially written macro (29).

Storage of VLDL subfractions. Both the rapid and prolonged ultracentrifugation procedures required that the isolated VLDL subfractions be stored on ice until the completion of the four ultracentrifugation runs (rapid ultracentrifugation subfraction A 3 h 2 min, subfraction B 2 h 34 min, subfraction C 2 h 5 min, prolonged ultracentrifugation subfraction A 17 h 16 min, subfraction B 16 h 36 min, subfraction C 15 h 52 min). To assess if this storage affected the susceptibility to oxidation the following experiments were performed.

As subfraction A was collected, an aliquot was oxidized immediately and another stored on ice. This procedure of oxidising one aliquot immediately and a second after storage was followed for the other subfractions until completion of the four runs. All samples were stored for the times described above.

Reference values for VLDL subfraction oxidation

Heparinised plasma was obtained from healthy laboratory staff with no known history of hyperlipidaemia. Controls were excluded if they were taking antioxidant supplements or if they smoked.

Statistical analysis

Calculations were performed using the statistics software package SPSS for Windows. Paired samples were analyzed using the Mann Whitney U Test and results are given as median (range) unless otherwise stated.

RESULTS

VLDL subfractionation

Electrophoresis pattern of isolated VLDL and its subfractions Typical electrophoresis results for VLDL subfractions isolated by rapid ultracentrifugation are shown in **Fig. 1**, demonstrating that the crude VLDL was not contaminated with either LDL or HDL and showed typical pre-β mobility. The electrophoresis patterns for subfractions A–D along with the infranatant E are also shown in Fig. 1. Infranatant E did not contain any pre-β lipid indicating complete harvesting of the VLDL particles. The electrophoresis pattern for VLDL subfractions isolated by prolonged ultracentrifugation displayed a similar pattern (results not shown).

VLDL composition

Protein concentration. Isolation of the subfractions by either rapid or prolonged ultracentrifugation produced very similar protein profiles (**Table 1**). Protein found in infranatant E was the result of albumin contamination.

Lipid parameters

Cholesterol and triglyceride

RECOVERY. When isolated by either prolonged or rapid ultracentrifugation, VLDL subfractions A–D, together with infranatant E, had very similar triglyceride and cholesterol profiles showing no difference between the two isolation procedures (**Table 2**). The sums of the percent triglyceride and cholesterol for subfractions A–D were compared with crude VLDL and showed very similar recoveries (Table 2). Results indicate that infranatant E contained cholesterol and triglyceride; however, no statistically significant difference was found between the ultracentrifugation techniques. Lipoelectrophoresis did not detect the presence of a characteristic pre-β band.

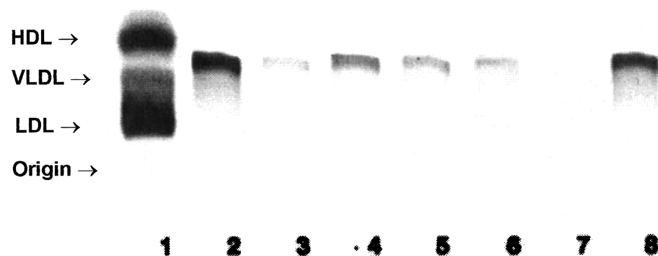

Fig. 1. Electrophoresis patterns of crude VLDL and subfractions A–D together with infranatant E. Well 1, plasma; wells 2 and 8, crude VLDL; well 3, subfraction A; well 4, subfraction B; well 5, subfraction C; well 6, subfraction D; well 7, infranatant E. Gels stained with Sudan Black to identify lipid.

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TABLE 1. Protein, apoB, and albumin content of VLDL subfractions A–D and infranatant E isolated by either rapid or prolonged ultracentrifugation, n = 5

Subfraction	Rapid U/C			Prolonged U/C		
	Total Protein	ApoB	Albumin	Total Protein	ApoB	Albumin
	mg/l	*mg/l*	*mg/l*	*mg/l*	*mg/l*	*mg/l*
A	17 (7–37)	15 (10–20)	0	14 (6–34)	14 (9–23)	0
B	110 (105–156)	55 (41–67)	0	94 (80–140)	51 (41–63)	0
C	80 (70–90)	41 (37–55)	0	78 (58–99)	40 (35–49)	0
D	31 (22–35)	36 (35–49)	0	32 (24–45)	37 (33–41)	15 (11–19)
E	212 (121–270)	14 (7–23)	190 (118–218)	198 (94–280)	19 (7–31)	142 (121–177)
Total A–E	450 (325–588)	161 (137–214)	190 (118–218)	416 (262–598)	161 (125–207)	157 (132–196)
Crude VLDL	462 (411–519)	153 (134–165)	166 (105–182)	387 (302–519)	155 (135–165)	132 (105–155)
% Recovery	97 (80–113)	105 (85–130)	114 (71–130)	107 (86–115)	104 (81–133)	119 (100–148)

Results are given as median (range). Paired analysis of the subfractions isolated by either rapid or prolonged ultracentrifugation showed no significant difference in protein and apoB content, subfraction D was contaminated with albumin when isolated by prolonged ultracentrifugation.

Ratio to apoB. Results shown in **Table 3** demonstrate that for both ultracentrifugation procedures the ratio of triglyceride to apoB significantly decreased as the subfractions increased in density (rapid ultracentrifugation; subfractions A vs. D, $P = 0.009$: prolonged ultracentrifugation; subfractions A vs. D, $P = 0.009$). For both ultracentrifugation procedures the ratio of cholesterol to apoB increased when subfractions B and C were compared with subfraction A; this trend was less pronounced with subfraction D. The decrease in the ratio of cholesterol to apoB in subfraction D may be the result of loss of apoB into infranatant E, with this loss being more pronounced during the prolonged ultracentrifugation procedure (Table 1).

Fatty acid determination. Results, expressed as percent of total chromatogram, show no difference in the total saturated, monounsaturated, and polyunsaturated fatty acids between subfractions A, B, C, and D (**Table 4**). Examination of individual fatty acids showed no difference in the distribution of oleic or linoleic acid as the subfractions increased in density; however, arachidonic acid statistically increased with increasing density (**Table 5**).

TABLE 2. Triglyceride and cholesterol content of VLDL subfractions A–D and infranatant E expressed as percent of total triglyceride or total cholesterol content in A–E, n = 5

Subfraction	Rapid U/C		Prolonged U/C	
	Triglyceride	Cholesterol	Triglyceride	Cholesterol
	%	%	%	%
A	12 (11–14)	7 (2–9)	11 (6–15)	7 (4–9)
B	46 (47–50)	40 (34–46)	43 (38–45)	34 (32–38)
C	27 (22–29)	28 (23–29)	28 (26–32)	30 (27–33)
D	11 (10–13)	19 (16–20)	12 (9–17)	21 (17–25)
E	4 (4–5)	6 (4–8)	7 (5–11)	8 (4–14)
% Recovery of Crude VLDL	92 (82–95)	89 (78–99)	93 (89–105)	88 (78–96)

Results given as median (range). Paired analysis of the 4 subfractions (A–D) and infranatant E isolated by both the rapid and prolonged ultracentrifugation procedures showed no difference in lipid content.
P = ns.

ApoB determination. Results expressed as mg/l and given as mean (range) are shown in Table 1. ApoB levels were very similar in the subfractions isolated by the two procedures. However in each procedure some apoB was found in infranatant E indicating that apoB isolation was not complete. From this result, together with the appearance of triglyceride and cholesterol in infranatant E (results above) and the absence of a pre-β band on electrophoresis, it was concluded that some fragmentation of the particles had occurred and that fragmentation was greater in the prolonged isolation procedure.

Albumin determination. Albumin results, also shown in Table 1 and given as mean (range), indicate that no subfraction from the rapid procedure was contaminated with albumin. However, subfraction D from the prolonged procedure was contaminated with albumin (mean: 15 mg/l total protein; range: 11–19).

Urate detection. The detection limit of HPLC analysis of urate was <0.5 μmol/l. Results showed that urate in subfractions A–D was below the level of assay detection (all results <0.5 μmol/l). We have demonstrate previously that urate below this concentration has no influence during copper mediated oxidation of VLDL (23). Subsequently these subfractions did not require size exclusion chromatography for the removal of urate.

Susceptibility of VLDL subfractions to oxidation

Optimum copper concentration. There is a characteristic fall in lag time with increasing copper concentration, up to an optimum concentration, after which addition of copper has no further effect (**Fig. 2**). This indicates that the oxidation of VLDL subfractions follow a similar trend to that previously shown for LDL (27, 28) and VLDL (23). For all future experiments a copper concentration of 10 μmol/l was used (a concentration found on the plateau phase of this profile).

Routine procedure. Comparison of lag time results is given in **Fig. 3**. This indicates that subfraction A was more resistant to copper mediated oxidation when compared with the other three subfractions ($P < 0.05$). The results also demonstrated that subfractions B and C were more re-

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TABLE 3. Triglyceride and cholesterol content within each VLDL subfraction standardized for apoB content, n = 5

Subfraction	Rapid U/C		Prolonged U/C	
	μmol trig/mg apoB	*μmol chol/mg apoB*	*μmol trig/mg apoB*	*μmol chol/mg apoB*
A	19.29 (12.94–25.00)	2.50 (2.00–4.29)	18.46 (10.77–24.44)	3.04 (1.54–4.29)
B	16.57 (12.09–17.14)	4.15* (3.88–4.67)	15.37 (12.61–20.44)	4.29* (3.56–4.66)
C	12.89* (10.67–13.9)	4.86* (3.66–6.00)	11.08* (9.51–15.41)	4.59* (3.41–5.68)
D	5.43* (4.69–7.30)	3.78 (2.97–5.43)	5.41* (4.32–6.29)	3.51 (2.7–4.29)

Results given as median (range). Analysis of subfractions A–D isolated by either rapid or prolonged ultracentrifugation demonstrated a statistically significant stepwise depletion of triglyceride when standardized for apoB content and enrichment of cholesterol as the subfractions decreased in size and increased in density.

* $P < 0.05$ comparison of subfraction A with subfractions B, C, and D. No difference was found between the two procedures.

sistant to copper mediated oxidation when compared with subfraction D ($P < 0.05$). However, there was no significant difference in lag time between subfractions B and C.

Storage of VLDL subfractions. The changes in lag time, after storage on ice, are shown in **Table 6**. The results indicate that storage of the subfractions for completion of the rapid isolation procedure did not result in a more oxidatively susceptible particle. However, using the prolonged isolation procedure a more readily oxidized subfraction was harvested. Preformed peroxides, measured by the FOX 2 assay (prior to oxidation), were shown to be higher in the subfractions isolated by prolonged ultracentrifugation (**Table 7**). Copper ions are potent promoters of free radical reactions; they can participate in lipid peroxidation in two ways. First, they can initiate chain reactions with PUFAs; however, as this reaction is thermodynamically unfavorable it is unlikely to occur in vivo. Secondly, and more importantly, copper ions decompose peroxides to peroxyl and alkoxyl radicals when added to systems already containing trace amounts of preformed lipid peroxides. The peroxyl radical so formed can participate in hydrogen abstraction from neighboring PUFA side chains and thus perpetuate the chain reaction. Therefore, the higher levels of lipid peroxides found within the VLDL from prolonged ultracentrifugation could enhance the oxidation process resulting in a more easily oxidized particle, a finding consistent with our oxidation results. The FOX 2 results were negatively correlated with lag time results, demonstrating that as the level of preformed peroxides increased so the subfractions' susceptibility to copper mediated oxidation increased, resulting in a decrease in lag time, $r = -0.423$, $P = 0.044$.

DISCUSSION

An ultracentrifugation method has been described which enables VLDL to be separated into four subfractions in a substantially shorter isolation time (less than 3.5 h) than previously reported methods (13, 14, 15). A study performed by Leonhardt et al. (30) demonstrated that a short run-time for the separation of lipoproteins was desirable over prolonged ultracentrifugation. They found that very fast ultracentrifugation resulted in moderate changes in the composition of the lipoprotein particle and provided lipoproteins void from albumin contamination, a finding that is consistent with our results. We also demonstrated that prolonged ultracentrifugation produced lipoprotein particles with increased susceptibility to copper mediated oxidation and which also contained higher levels of preformed peroxides. The speed and ease of practice of this procedure are important considerations when large sample numbers require analysis; the method allows assessment of the susceptibility of VLDL subfractions to oxidation. According to the results described by Redgrave and Carlson (14) the VLDL subfractions isolated using

TABLE 4. Fatty acid content (percent of total chromatogram) of crude VLDL and VLDL subfractions (A–D) isolated by rapid ultracentrifugation, n = 5

	Fatty Acid		
Subfraction	Saturated	Monounsaturated	Polyunsaturated
	%	%	%
A	37.7 (36.8–39.2)	39.4 (37.4–41.1)	22.9 (19.6–25.6)
B	36.7 (34.7–40.4)	37.5 (34.5–40.4)	25.7 (22.1–30.3)
C	36.8(34.7–38.3)	36.8 (34.0–39.3)	26.3 (25.2–27.8)
D	35.9 (34.1–37.0)	36.2 (34.3–38.4)	27.8 (27.4–28.6)
Crude VLDL	38.6 (35.9–40.7)	36.4 (34.8–39.2)	24.9(24.2–25.1)
% Recovery	95 (90–99)	103 (101–106)	104 (97–116)

Results given as median (range). Percent saturated and monounsaturated fatty acid showed a tendency to decrease as the subfraction increased in density, while percent polyunsaturated fatty acid showed a tendency to increase with increasing density.

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TABLE 5. Oleic, linoleic, and arachidonic acid content (percent of total chromatogram) of crude VLDL and VLDL subfractions (A–D) isolated by rapid ultracentrifugation, n = 5

Subfraction	Fatty Acid		
	Oleic Acid	Linoleic Acid	Arachidonic Acid
	%	%	%
A	35.2 (33.4–37.5)	18.0 (16.7–20.3)	1.6 (1.3–2.2)
B	32.6 (30.1–36.0)	19.5 (17.4–22.3)	1.9 (1.6–2.2)
C	32.5 (30.7–35.0)	19.6 (17.8–21.8)	2.1 (1.8–2.4)
D	31.8 (30.4–34.6)	20.6 (19.8–22.3)	2.6* (2.3–2.9)
Crude VLDL	31.5 (27.7–33.8)	18.5 (17.1–19.6)	2.0 (1.8–2.1)

Results given as median (range). Percent oleic and linoleic acid remained unaltered between the subfractions, however as the subfractions increased in density, arachidonic acid content increased.
* $P < 0.05$.

this rapid ultracentrifugation technique will have the following diameters and flotation rates (Sf): Subfraction A >75 nm, Sf >400; Subfraction B 50–75 nm, Sf 175–400; Subfraction C 37–50 nm, Sf 100–175; Subfraction D 20–37 nm, Sf 20–100. Subfraction A being the largest and most buoyant subfraction and subfraction D being the smallest and most dense subfraction.

When compared directly to the prolonged procedure of Saheki et al. (15), the VLDL subfractions isolated by this rapid method had very similar lipid, protein, and apoB contents along with lower preformed hydroperoxide levels and an increased resistance to copper mediated oxidation.

This study has shown for the first time that as VLDL subfractions decrease in size and increase in density (A→D), there was a significant trend for the particles to become more susceptible to copper mediated oxidation. A similar trend has been reported previously for LDL subfractions (10, 11).

Patients with hypertriglyceridaemia produce a greater percent of the larger VLDL subfractions. A preponderance of these large subfractions may have several detrimental effects, leading to the development of atherosclerosis. *1*) They have the potential to deposit large amounts of cholesterol under conditions of oxidative stress. Large VLDL particles have the capacity to carry up to five times more cholesterol per molecule when compared with LDL (31); *2*) Unlike LDL, which requires modification before its removal by scavenger receptors (32), large VLDL subfractions can be taken up by macrophage receptors without their prior modification. These receptors, although distinct from the acetyl LDL receptor, are not subject to down-regulation, thus leading to cholesterol accumulation and characteristic foam cells production (12, 33, 34); and *3*) Compared with their smaller counterparts, large VLDL subfractions have an altered metabolism. They have a prolonged residence time within the circulation; subsequently they are exposed to the action of the enzyme CETP for extended periods. This enzyme influences the

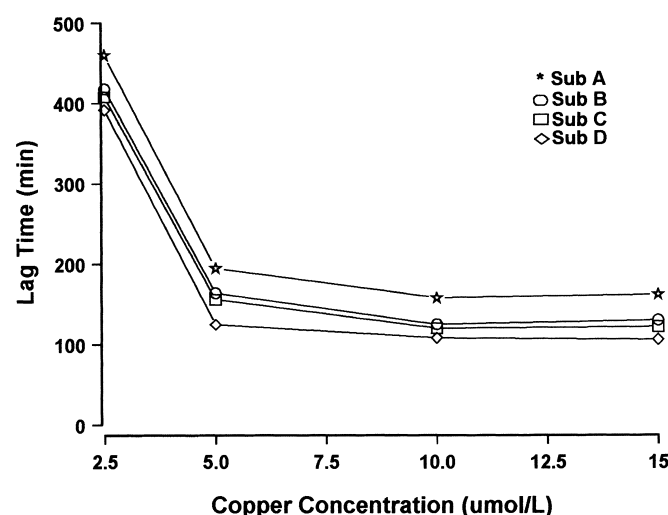

Fig. 2. Effect of copper chloride concentration on VLDL lag time (μmol/l), after each subfraction was standardised to 10 μg/ml protein, n = 3.

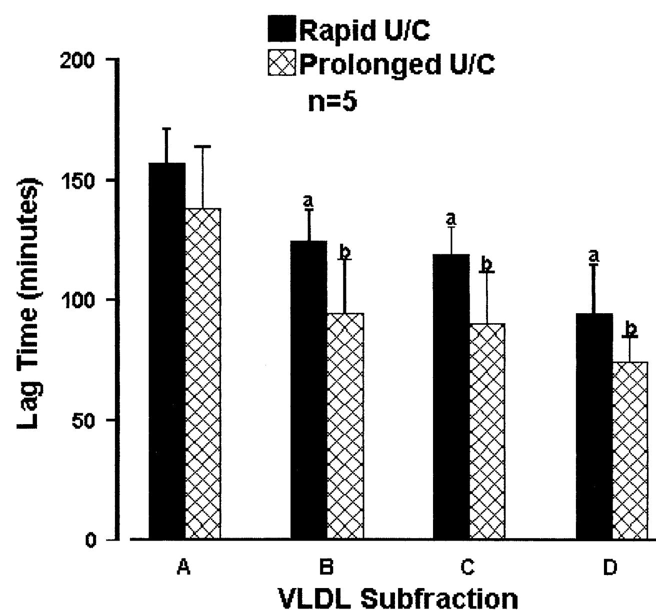

Fig. 3. Lag time results of VLDL subfractions (A–D) oxidized in the presence of 10 μmol/l copper chloride, after protein standardised to 10 μg/ml. a = $P < 0.05$; subfraction A compared with subfractions B, C, and D from rapid ultracentrifugation. b = $P < 0.05$; subfraction A compared with subfractions B, C, and D from prolonged ultracentrifugation.

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TABLE 6. Change in lag time when subfractions A–C are stored for the duration of both the rapid and prolonged ultracentrifugation procedures, n = 3

Subfraction	Rapid Ultracentrifugation Change in Lag Time	Prolonged Ultracentrifugation Change in Lag Time
	min	*min*
A	−3* (−2 to −4)	−34 (−22 to −130)
B	0* (−6 to +4)	−94 (−16 to −124)
C	+2* (−2 to +8)	−110 (−28 to −120)
D	No storage	No storage

Results are given as median (range). Prolonged ultracentrifugation produced subfractions which were more susceptible to oxidation. Subfractions were either oxidized immediately on removal from the ultracentrifuge or stored on ice under air for the duration of the remaining ultracentrifugation steps. Storage times for rapid ultracentrifugation were subfraction A 3 h 2 min; subfraction B 2 h 34 min; and subfraction C 2 h 5 min. Storage times for prolonged ultracentrifugation were subfraction A 17 h 16 min; subfraction B 16 h 36 min; and subfraction C 15 h 52 min. Statistical analysis demonstrated that each subfraction from the prolonged ultracentrifugation procedure was more readily oxidized compared to the same subfraction from the rapid ultracentrifugation procedure.

* $P < 0.05$.

remodelling of other lipoproteins leading to an increased transfer of triglyceride from VLDL to HDL and LDL in exchange for cholesterol. The triglyceride enrichment of HDL and LDL produces particles that are ready substrates for the action of hepatic lipase, the consequence of which may lead to the production of small dense atherogenic LDL (5, 6) together with cholesterol deplete HDL, the latter not participating to the same extent in reverse cholesterol transport (35). Thus, triglyceride enrichment of plasma is a consequence of large triglyceride-rich VLDL subfractions, ultimately resulting in the production of small dense LDL particles. These particles in turn have a close association with the development of premature atherosclerosis and cholesterol deplete HDL, which has reduced anti-atherogenic properties.

Small VLDL subfractions have also been linked with the development of atherosclerosis and studies have demonstrated their ability to induce macrophage cholesteryl ester formation, even in normolipoproteinaemic subjects (36). We have demonstrated that as with small dense LDL, small dense VLDL is more susceptible to oxidative modification. As small VLDL is the major precursor of LDL, abnormalities such as increased oxidation within this parent molecule may ultimately result in enhanced seeding of LDL with preformed hydroperoxides, producing an oxidatively compromised particle. Therefore, analysis of the oxidation potential of VLDL subfractions enables both the assessment of their ability to oxidize and their potential to compromise LDL by donation of preformed hydroperoxides.

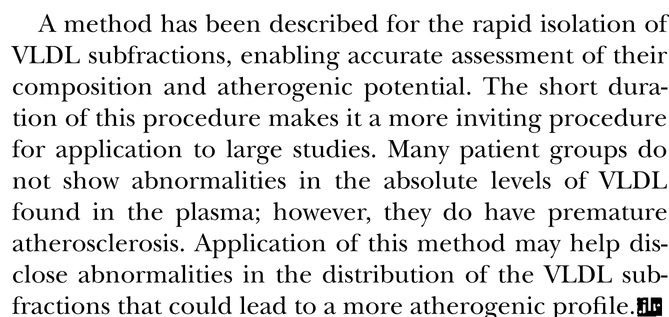
TABLE 7. Preformed hydroperoxides in subfractions A–D isolated by both rapid and prolonged ultracentrifugation and measured by the FOX 2 method, n = 5

Subfraction	Rapid U/C	Prolonged U/C
	nmol/mg protein	*nmol/mg protein*
A	6.2* (4.6–8.5)	38.2 (13.6–56.8)
B	8.1* (6.5–15.9)	44.4 (22.8–76.7)
C	8.1* (4.6–11.3)	35.9 (30.4–39.9)
D	6.0* (5.9–6.9)	16.6 (9.9–44.5)

Results given as median (range). The prolonged ultracentrifugation method produced subfractions with higher levels of preformed peroxides, indicating that prolonged ultracentrifugation was detrimental to the particles.

* $P < 0.05$ prolonged versus rapid ultracentrifugation.

CONCLUSION



A method has been described for the rapid isolation of VLDL subfractions, enabling accurate assessment of their composition and atherogenic potential. The short duration of this procedure makes it a more inviting procedure for application to large studies. Many patient groups do not show abnormalities in the absolute levels of VLDL found in the plasma; however, they do have premature atherosclerosis. Application of this method may help disclose abnormalities in the distribution of the VLDL subfractions that could lead to a more atherogenic profile.

Manuscript received 27 August 2001 and in revised form 15 January 2002.

REFERENCES

1. Packard, C. J., A. Munro, A. R. Lorimer, A. M. Gotto, and J. Shepard. 1984. Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects. *J. Clin. Invest.* **74:** 2178–2192.
2. Austin, M. A. 1999. Epidemiology of hypetriglyceridemia and cardiovascular disease. *Am. J. Cardiol.* **83:** 13F–16F.
3. Austin, M. A., B. McKnight, K. L. Edwards, C. M. Bradley, M. J. McNeeley, B. M. Psaty, J. D. Brunzell, and A. C. Motulsky. 2000. Cardiovascular disease mortality in familial forms of hypertriglyceridaemia: A 20-year prospective study. *Circulation.* **101:** 2777–2782.
4. Bjorkegren, J. C., J. Packard, A. Hamsten, D. Bedford, M. Caslake, L. Foster, J. Shepard, P. Stewart, and F. Karpe. 1996. Accumulation of large very low density lipoprotein in plasma during intravenous infusion of a chylomicron-like emulsion reflects competition for a common lipolytic pathway. *J. Lipid Res.* **37:** 76–86.
5. Krauss, R. M. 1987. Relationship of intermediate and low-density lipoprotein subspecies to risk of coronary artery disease. *Am. Heart J.* **113:** 578–582.
6. Griffin, B. A., D. J. Freeman, G. W. Tait, J. Thomson, M. J. Caslake, C. J. Packard, and J. Shepard. 1994. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. *Atherosclerosis.* **106:** 241–253.
7. Austin, M. A., J. L. Breslow, C. H. Hennekens, J. E. Buring, W. C. Willett, and R. M. Krauss. 1988. Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA.* **260:** 1917–1921.
8. Tornvall, P., F. Karpe, L. A. Carlson, and A. Hamsten. 1991. Relationships of low density lipoprotein subfractions to angiographically defined coronary artery disease in young survivors of myocardial infarction. *Atherosclerosis.* **90:** 67–80.
9. Austin, M. A. 1992. Genetic epidemology of low-density lipoprotein subclass phenotypes. *Anal. Med.* **24:** 477–481.
10. DeGaff, J., L. M. Hak-Lemmers, M. P. C. Hectors, P. N. M. Decmaker, J. C. M. Hendriks, and A. F. H. Stalenhoef. 1991. Enhanced susceptbility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arteriosclerosis.* **11:** 298–306.
11. Dejager, S, E. Bruckert, M. J. Champman. 1993. Dense low density lipoprotein subspecies with diminished oxidative resistance predominate in combined hyperlipidemia. *J. Lipid. Res.* **34:** 295–308.
12. Bradley, W. A., and S. H. Gianturco. 1994. Triglyceride-rich lipo-

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proteins and atherosclerosis: pathophysiological considerations. *J. Intern. Med.* **236 (Suppl. 736):** 33–39.
13. Lindgren, F. T., L. C. Jensen, and F. T. Hatch. 1972. The isolation and quantitative analysis of serum lipoproteins. *In* Blood Lipids and Lipoprotein: quantitation, composition and metabolism. Chapter 5. G. J. Nelson, editor. Wiley-Interscience, New York. 181–274.
14. Redgrave, T. G., and L. A. Carlson. 1979. Changes in plasma very low density lipoprotein content, composition, and size after a fatty meal in normo- and hypertriglyceridemic man. *J. Lipid Res.* **20:** 217–229.
15. Saheki, S., Y. Hitsumoto, S. Bando, M. Murase, N. Takeuchi, and K. Uchida. 1995. Compositions of very low density lipoprotein subfractions from patients with polydisperse low density lipoproteins. *Clin. Chim. Acta.* **238:** 43–57.
16. McEneny, J., C. M. Loughrey, P. T. McNamee, E. R. Trimble, and I. S. Young. 1997. Susceptibility of VLDL to oxidation in patients on regular haemodialysis. *Atherosclerosis.* **129:** 215–220.
17. McEneny, J., M. J. O'Kane, K. W. Moles, C. McMaster, D. McMaster, C. Mercer, E. R. Trimble, and I. S. Young. 2000. Very low density lipoprotein subfractions in Type II diabetes mellitus: Alterations in composition and susceptibility to oxidation. *Diabetologia.* **43:** 485–493.
18. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2:** 235–254.
19. Becker, W. 1969. Determination of antisera titres using the single radial Immunodiffusion method. *Immunochem.* **6:** 539–546.
20. Halliwell, B. 1988. Albumin-an important extracellular antioxidant. *Biochem. Pharmacol.* **37:** 569–571.
21. Wolff, S. P. 1994. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* **233:** 182–189.
22. Nauroozi-Zadeh, J., J. Tajaddini-Sarmadi, and S. P. Wolff. 1994. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal. Biochem.* **220:** 403–409.
23. McEneny, J., E. R. Trimble, and I. S. Young. 1998. A simple method to assess copper-mediated oxidation of VLDL isolated by rapid ultracentrifugation. *Anal. Clin. Biochem.* **35:** 504–514.
24. Frei, B., R. Stocker, and B. N. Ames. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. USA.* **85:** 9748–9752.
25. Ma, Y. S., W. L. Stone, and I. O. Leclair. 1994. The effects of vitamin C and urate on the oxidation kinetics of human low density lipoprotein. *Proc. Soc. Exp. Biol.* **206:** 53–59.
26. Chevion, S., E. M. Berry, N. Kitrossky, and R. Kohen. 1997. Evaluation of plasma low molecular weight antioxidant capacity by cyclic voltammetry. *Free Radic. Biol. Med.* **22:** 411–421.
27. Heinecke, J. W., H. Rosen, and A. Chait. 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
28. Ramos, P., S. P. Gieseg, B. Schuster, and H. Esterbauer. 1995. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. *J. Lipid Res.* **36:** 2113–2128.
29. McDowell, I. F. W., J. McEneny, and E. R. Trimble. 1995. A rapid method for measurement of the susceptibility to oxidation of low-density lipoprotein. *Anal. Clin. Biochem.* **32:** 167–174.
30. Leonhardt, W., J. Pietzsch, and S. Nitzsche. 1994. Very-fast ultracentrifugation of human plasma lipoproteins: influence of the centrifugal field on lipoprotein composition. *Clin. Chim. Acta.* **224:** 21–32.
31. Bradley, W. A., and S. H. Gianturco. 1994. Triglyceride-rich lipoproteins and atherosclerosis: pathophysiological considerations. *J. Int. Med.* **236:** 33–39.
32. Goldstein, J. L., Y. K. Ho, S. K. Basu, M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
33. Gianturco, S. H., W. A. Bradley, A. M. Gotto, J. D. Morrisett, and D. L. Peavy. 1982. Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. *J. Clin. Invest.* **70:** 168–178.
34. Gianturco, S. H., S. A. Brown, D. P. Via, and W. A. Bradley. 1986. The beta-VLDL receptor pathway of murine P388D1 macrophages. *J. Lipid Res.* **27:** 412–420.
35. Golay, A., L. Zech, M-Z. Shi, Y-A. M. Chiou, G. M. Reaven, and Y-D. I. Chen. 1987. High density lipoprotein (HDL) metabolism in non-insulin-dependent diabetes mellitus: Measurement of HDL turnover using tritiated HDL. *J. Clin. Endocrinol. Metab.* **65:** 512–518.
36. Sacks, F. M., J. L. Breslow. 1987. Very low density lipoproteins stimulate cholesteryl ester formation in U937 macrophages. Heterogeneity and biologic variation among normal humans. *Atherosclerosis.* **7:** 35–46.

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