Free Rad. Res. Comms., Vol. 19, No. 4, pp. 267-278
Reprints available directly from the publisher
Photocopying permitted by license only

ANALYSIS OF LIPOPROTEIN DIENE FORMATION IN HUMAN SERUM EXPOSED TO COPPER

JAN REGNSTRÖM,* KERSTIN STRÖM,† PETER MOLDEUS,† and JAN NILSSON*

Department of Medicine and King Gustaf V Research Institute and Department of Toxicology,† Karolinska Hospital, Karolinska Institute, Stockholm, Sweden*

(Received June 22nd, 1993; in revised form July 16th, 1993)

The susceptibility of low density lipoprotein (LDL) to oxidative modification can be determined by analyzing the lag phase for initiation of diene formation in isolated LDL exposed to Cu^{2+}. However, the applicability of this assay for clinical studies is limited by the requirement of a preparative ultracentrifugation of LDL and that the influence of water soluble antioxidants and other lipoproteins is not accounted for. The present paper describes a modification of this assay allowing determination of lag phase for lipoprotein diene formation in serum. The formation of dienes in serum exposed to Cu^{2+} begins following the consumption of serum α-tocopherol, correlates to the formation of thiobarbituric acid reactive substances ($r = 0.987$, $n = 8$), is inhibited by the addition of ascorbic acid and is absent in lipoprotein-deficient serum. It is also accompanied by an increased mobility of serum lipoproteins on agarose gel electrophoresis and with an ability of serum to displace isolated copper-oxidized LDL from binding sites mediating degradation in mouse peritoneal macrophages. The coefficient of variance of the analysis is below 3%. It is concluded that this technique allows analysis of lipoprotein oxidation susceptibility in serum samples and may prove to be useful in clinical analysis of the lipoprotein oxidation susceptibility.

KEY WORDS: antioxidant, diene conjugation, lipid peroxidation, low density lipoprotein, serum.

Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; TBAR, thiobarbituric acid reactivity.

INTRODUCTION

Lipid peroxidation is initiated by a free radical attack on fatty acid double bonds. Conjugated dienes are formed which absorb ultraviolet light in the wavelenght range 230–235 nm. Many of these are then further converted into lipid hydroperoxides and other reactive molecules such as aldehydes.[1] The growing body of evidence implicating lipid peroxidation as a pathogenic factor in several types of human diseases has made it important to develop methods allowing quantification of lipid oxidation. However, the short half-life, extreme reactivity and low basal concentrations of lipid peroxides has made this a difficult task. One approach has been to analyze oxidation end products, such as aldehydes by their thiobarbituric acid reactivity (TBAR), but the applicability of this method has been questioned since oxidation of lipids is believed to take place in tissues rather than in the circulation. Another approach has been to analyze oxidation susceptibility by determining the lag phase for diene

All correspondence should be addressed to: Dr Jan Regnström, King Gustaf V Research Institute, Box 60004, S-104 01 Stockholm, Sweden

formation in low density lipoprotein (LDL) exposed to Cu^{2+} *in vitro*. This method was originally developed by Esterbauer and coworkers who showed that dienes form as LDL become antioxidant depleted[2]. However, this method has some weaknesses. It does not account for the water soluble antioxidants in serum and requires isolation of LDL by preparative ultracentrifugation. To overcome these problems we have investigated the possibility of monitoring lipoprotein diene formation directly in serum.

MATERIALS AND METHODS

Materials

Medium Ham's F-10, fetal calf serum (FCS), penicillin (10,000 U/ml) and streptomycin (10 mg/ml) were purchased from Gibco (Glasgow, Scotland); disposable tissue culture dishes from Costar (Cambridge, MA); filters from Millipore (Bedford, MA). Female NMRI mice (average weight 25 g) were obtained from Alab (Sollentuna, Sweden). Sodium [^{125}I] iodide, carrier free, was purchased from Amersham (U.K.). All other reagents were analytical grade obtained from Merck or Sigma.

Determination of the Susceptibility of Serum and LDL to Oxidation

Venous blood taken after a 12 h fast was drawn into vacutainer tubes, left for 30 min in darkness at room temperature and then centrifuged at 1400 g for 20 min in darkness at room temperature. When not used immediately after preparation, serum was stored under nitrogen at 4°C in darkness. Heparin plasma was obtained by collecting venous blood into 5 ml vacutainer tubes containing 143 U.S.P. units of sodium heparin and 20 mg of sodium fluoride. Citrate plasma was prepared by collecting venous blood into 4.5 ml vacutainer tubes containing 0.5 ml of 0.129 M buffered sodium citrate. The plasma was centrifuged immediately after collection and stored as described above.

Serum and plasma were diluted to a final concentration of 0.67% (v/v) in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl prewarmed to 30°C and oxidation initiated by the addition of a freshly prepared aqueous $CuSO_4$ solution with a final concentration of 50 μM. The formation of dienes was followed by monitoring the change in the 234 nm absorbance at 30°C on a Beckman DU 60 spectrophotometer equipped with a six-position automatic sample changer. The initial absorbance at 234 nm was regarded as baseline and the change in absorbance was recorded every 5 min for 4 h.

Lipoprotein-deficient serum was prepared by standard procedure at d. = 1.21 kg/l and dialyzed against 0.9% NaCl.[3] Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL and high density lipoprotein (HDL) + d. > 1.21 kg/l fractions were prepared by ultracentrifugation in a density gradient[4] without the presence of antioxidants or ethylenediaminetetraacetic acid (EDTA). In short, serum was adjusted to a density of 1.10 kg/l by addition of NaCl. A density gradient was formed by first adding 4 ml of 1.10 kg/l density serum, then 3 ml bands of 1.065, 1.020 and 1.006 kg/l NaCl solutions were layered carefully on top in cellulose nitrate tubes (Ultraclear tubes, Beckman) and centrifuged (Beckman SW 40 Ti) at 202.000 g at 1°C for 16 h. The VLDL fraction was isolated from the top 0.5 ml, IDL from the next 2.5 ml, LDL from the next 4 ml and HDL + d. > 1.21 kg/l fraction from the remaining 6 ml of the tube.

Determination of the susceptibility of LDL to *in vitro* oxidation was performed essentially according to Esterbauer *et al.*[2] and described in detail earlier.[5] Total protein was determined using the Lowry technique.[6]

To study the effect of oxidation on the electrophoretic mobility of serum lipoproteins, serum was diluted to a final concentration of 2% in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl and incubated with 50 μM $CuSO_4$ at 30°C for up to 4 h with or without the addition of 20 μM butylated hydroxytoluene (BHT). The incubation was terminated by addition of 20 μM BHT and the samples were put on ice in darkness. Serum lipoproteins were isolated and concentrated by preparative ultracentrifugation at d. = 1.006 kg/l, recovering the VLDL in the top fraction and LDL and HDL in the bottom fraction.[7] From a volume of 15 ml diluted serum the VLDL fraction was recovered in 2.5 ml and the LDL + HDL bottom fraction in 2.0 ml. Electrophoresis in agarose gel was performed according to Noble.[8] The electrophoretic mobility of the lipoprotein density peaks from the application well was determined by densitometric scanning (Ultrascan XL, LKB, Bromma, Sweden) and expressed as the relative mobility (Rf) of the α-lipoprotein band (HDL) of the control serum applied to each gel.

The extent of lipid peroxidation in serum diluted to a final concentration of 0.67% and exposed to copper was also measured in terms of thiobarbituric acid reactive substances and expressed as malondialdehyde equivalents, as described by Yagi.[9]

Determination of α-Tocopherol

The analysis of α-tocopherol was performed essentially as described previously.[10,11] Briefly, 200 μl of ethanol was added to 50 μl of sample, vortexed for 5 sec and then mixed with 200 μl of hexane. Following phase separation 100 μl of the upper hexane layer was transported to a small glass container and evaporated to dryness under nitrogen. The dried material was dissolved in 50 μl methanol and 10 μl was used for analysis. α-Tocopherol was separated and analyzed by HPLC with electrochemical detection.[10] The column used was a CT-sil (250 × 4.6 mm) packed with ODS (C_{18}) 5 μm and the mobile phase was ethanol/methanol/isopropanol 66.5/30.0/3.5 (v/v) with 30 mM lithium perchlorate. The flow rate was 1 ml/min. The electrochemical detector was from Antec Instrument, Leiden, The Netherlands working in the oxidative mode (+0.7V) versus a Ag/Ag Cl_2 reference electrode.

Preparation of Radiolabelled LDL

Iodination of LDL was performed essentially as described by McFarlane[12]. The ^{125}I-LDL was dialyzed extensively against 0.15 M NaCl/1 mM EDTA, pH 7.4 overnight, filtered through a 0.45 μm filter and stored at 4°C. The specific activity of the preparations ranged from 400–600 cpm/ng protein. More than 95% of the activity was trichloroacetic-acid precipitable. 200 μg of ^{125}I-LDL was dialyzed against 0.15 M NaCl without EDTA and then incubated in 1 ml of medium Ham's F-10 with the addition of 50 μg/ml penicillin, 50 U/ml streptomycin and 5.0 μM $CuSO_4$ for 18 h at 37°C. The oxidatively modified ^{125}I-LDL showed increased electrophoretic mobility on agarose gel and contained 10–30 times more thiobarbituric acid reactive material than native ^{125}I-LDL.

Degradation of ^{125}I-LDL in Macrophages

Resident peritoneal macrophages were harvested from the peritoneal cavity of female NMRI mice[13] and seeded out in 12 multiwell plates in Ham's F-10 medium supplemented with 10% fetal calf serum (FCS), 50 μg/ml penicillin and 50 U/ml streptomycin. After 24 h the cultures were washed once with Ham's F-10 medium and incubated in 1.0 ml Ham's F-10 with the addition of 10 μg/ml of ^{125}I-LDL or ^{125}I-oxidatively modified LDL for 5 h at 37°C. The degradation process was arrested by cooling the cells to 4°C. The medium was removed and mixed with 100 μl of bovine serum albumin (10 mg/ml) and 200 μl of 50% trichloroacetic acid. The precipitated protein was removed by centrifugation for 10 min at 2000 g. 0.5 ml of 5% $AgNO_3$ was then added to 1 ml of the supenatant to precipitate free iodide. After centrifugation at 2000 g for 10 min, the trichloroacetic acid-soluble ^{125}I in the supenatant was determined in an LKB gamma counter. The cells were lyzed through incubation with 0.25 M NAOH and the protein content determined according to Lowry.

In order to study the effect of oxidation of serum on the uptake of oxidized LDL through the scavenger receptor, serum was diluted in medium Ham's F-10 supplemented with 50 μg/ml penicillin and 50 U/ml streptomycin to a final concentration of 4 % and incubated for 14 h at 37°C with the addition of 5 μM $CuSO_4$. The incubation was ended by the addition of 0.05% EDTA. As control, serum was prepared as described above with the addition of 0.05% EDTA immediately before the degradation experiment. The LDL band in oxidatively modified serum, but not in control serum, showed increased electrophoretic mobility on agarose gel. 10 μg/ml of ^{125}I-oxidatively modified LDL was incubated in 950 μl oxidatively modified or control serum as described above.

Statistical Analysis

Conventional methods were used for calculation of means, standard deviations and Pearson correlation coefficient. Differences in continuous variables between two groups were tested by Student's paired t-test.

RESULTS

Different serum and Cu^{2+} concentrations were tested to identify optimal conditions for analysis of diene formation in serum. Dilution of serum was necessary when using the assay since serum concentrations above approximately 3% had an absorbance at 234 nm outside the range of the spectrophotometer. At Cu^{2+} concentrations of 10 μM and below diene formation was not consistent, whereas at concentrations above 100 μM the serum became opalescent. Thus, a concentration of 50 μM was chosen. The variation in absorbance at 234 nm was found to increase with increasing serum concentrations, but was almost negligible at concentrations below 1%. In experiments using a constant Cu^{2+} concentration of 50 μM and a serum concentrations ranging from 0.25–1%, the peak of diene formation increased in relation to the serum concentration. A serum concentration of 0.67% was found to provide the most reliable and reproducible results. Figure 1 shows the spectral changes occurring in serum following addition of Cu^{2+} with an absorbance maximum evolving at 234 nm. Exposure of lipoprotein-deficient serum to Cu^{2+} did not result in an increased absorbance at 234 nm. The increase in absorbance at 234 nm in serum

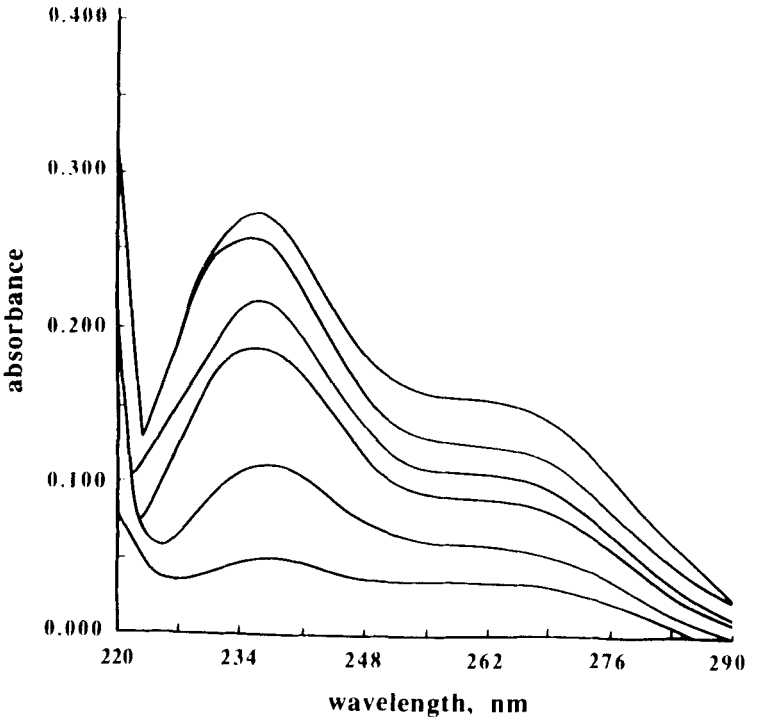

FIGURE 1 Change in the UV spectrum during oxidation of serum. Serum was diluted in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl to a final concentration of 0.67% and exposed to 50 μM $CuSO_4$ to initiate oxidation. The initial absorbance after the addition of $CuSO_4$ was regarded as reference and subtracted. The UV spectrum was measured at 47, 62, 82, 95, 118 and 180 min (from bottom to top).

exposed to Cu^{2+} was closely related to the formation of TBAR ($r = 0.987$, $n = 8$, Figure 2). No increase in the TBAR was observed in lipoprotein-deficient serum or serum supplemented with 20 μM BHT when exposed to CU^{2+}. To determine the oxidation susceptibility of serum lipoproteins following the addition of Cu^{2+}, the initial absorbance at 234 nm was set to zero and the absorbance was recorded every 5 min during 4 h (Figure 3). As has been described for LDL[2] the kinetics of the diene formation could be divided into three phases, a lag phase, a propagation phase and a decomposition phase. During the lag phase serum α-tocopherol was consumed in a time-dependent manner (Figure 4). Supplementation of serum with ascorbic acid was shown to prolong the lag phase in a concentration dependent manner (Figure 5). Since the transition from lag phase to propagation phase was continuous, lag phase was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the baseline and expressed in min. The lag phase was not affected by dilution in the concentration range 0.25–1%. To study the variance of the lag phase determination, six separate analyses of the lag phase were performed on one serum sample. The mean (SD) value was 138.7 (3.7) min and the coefficient of variance 2.7%. Repeated analysis at 30 min intervals during the first 2 h following the preparation of a serum sample demonstrated that the lag phase for diene formation was stable during this period (coefficient of variance 2.1%). Comparison of

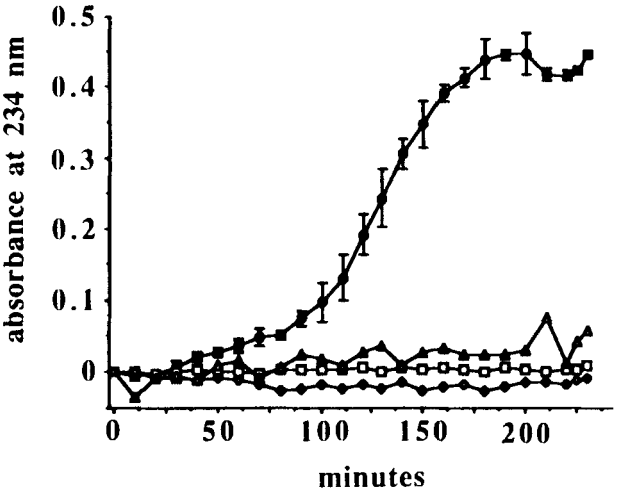

FIGURE 2 Relation between the amount of malondialdehyde (MDA) equivalents formed and increase in absorbance at 234 nm in one serum sample during $CuSO_4$-induced oxidation. Experimental conditions were the same as described in Figure 1. The initial absorbance at 234 nm was set to zero.

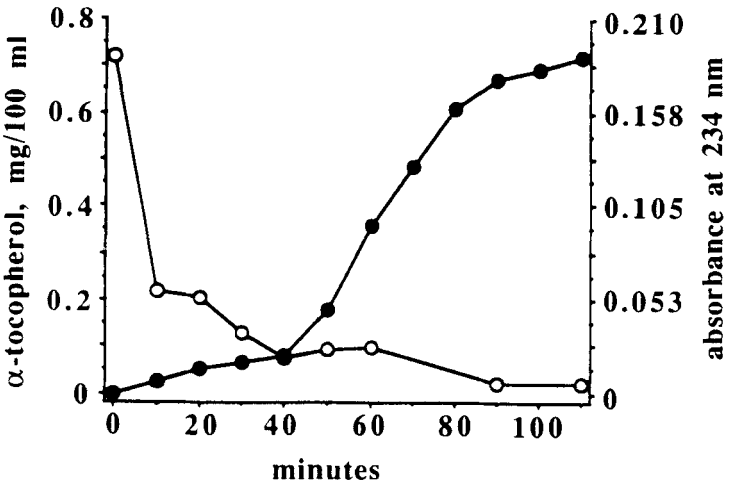

FIGURE 3 The absorbance at 234 nm during oxidation of serum. Serum was diluted in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl to a final concentration of 0.67% and exposed to 50 μM $CuSO_4$ in triplicate samples (●). The diluted serum preparation was supplemented with 20μM BHT (♦) or 0.05% EDTA (▲) prior to the addition of 50 μM $CuSO_4$. Lipoprotein-deficient serum was diluted as described above and supplemented with 50 μM $CuSO_4$ (□). The initial absorbance was set to zero. Bars indicate the SD.

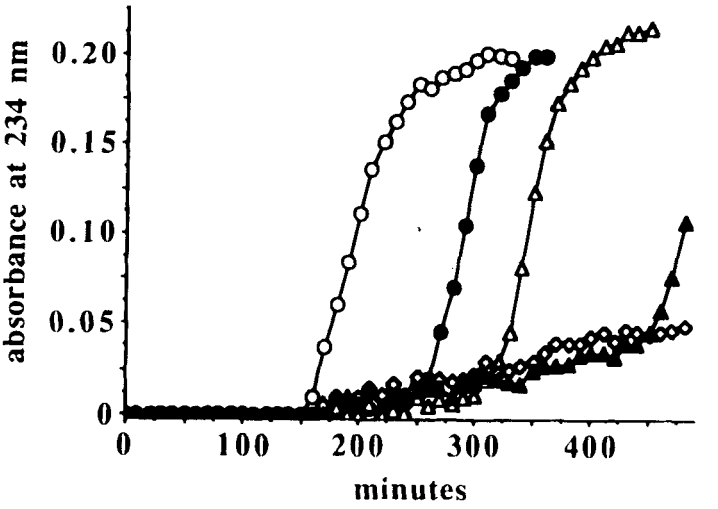

FIGURE 4 Time-course of the consumption of α-tocopherol (○) and the increase in absorbance at 234 nm (●) during serum oxidation. Experimental conditions were the same as described in Figure 1. The initial absorbance was set to zero.

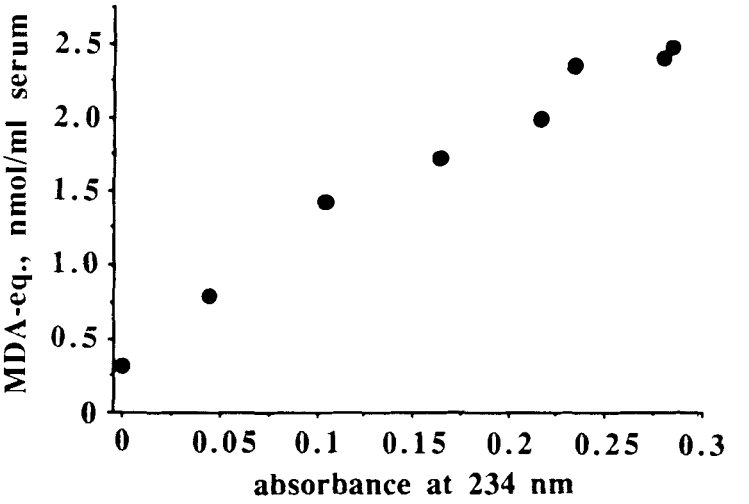

FIGURE 5 The absorbance curve at 234 nm during serum oxidation with the addition of increasing concentrations of ascorbic acid (○ = 0 μM, ● = 5 μM, △ = 10, μM, ▲ = 20 μM, ◇ = 30 μM). Experimental conditions were the same as described in Figure 1 and the ascorbic acid was added to the diluted serum. The initial absorbance was set to zero.

analysis of serum lag phase performed immediately following blood sampling and after storing the serum samples for 4 days at 4°C at darkness under nitrogen demonstrated a high reproducibility ($r = 0.996$, $p < 0.0001$, $n = 6$) and the mean lag phase did not change significantly (124.2 ± 35.2 versus 125.5 ± 36.4 min, $p = 0.415$). To study the effect of freezing on the serum lag phase, analysis was performed immediately following preparation and after one week of storage at −80°C.

There was a correlation between the two determinations ($r = 0.914$, $p < 0.011$, $n = 6$), but freezing was found to result in a significantly prolonged lag phase (59.7 ± 9.2 versus $118.36.3 \pm 36.3$ min, $p = 0.0002$).

The lag phase analyses were performed in serum rather than in plasma in order to avoid a possible influence of substances such as EDTA, heparin and citrate which are required for plasma preparation. However, analysis of lag phase in serum, heparin plasma and citrate plasma obtained from 8 healthy controls revealed a significant relation between the lag phase of serum and heparin plasma ($r = 0.976$, $p = 0.0001$), whereas there was no relation between serum and citrate plasma or between citrate and heparin plasma. Heparin plasma was found to have a significantly longer lag phase compared with serum (111.6 ± 21.2 versus 100.0 ± 23.6 min, $p = 0.0005$), whereas citrate plasma had a significantly shorter lag phase compared with serum (42.9 ± 13.4 min, $p = 0.004$)

VLDL, LDL and HDL isolated from oxidized serum by preparative ultracentrifugation at d. = 1.006 kg/l showed an increase in electrophoretic mobility on agarose gel. The increase in mobility of the pre-β-lipoprotein (VLDL) and β-lipoprotein (LDL) bands was inhibited by the addition of the antioxidant BHT to the serum before the initiation of oxidation, whereas the increase in mobility of the α-lipoprotein (HDL) band was unaffected by the addition of BHT (Figure 6). The relative electrophoretic mobility (Rf) of the lipoprotein density peak at 0, 1, 2, 3, 4 h and at 4 h with the addition of BHT was for pre-β-lipoprotein (VLDL); 0.60, 0.59, 0.59, 0.65, 0.75 and 0.60, β-lipoprotein (LDL); 0.24, 0.26, 0.27, 0.67, 0.80 and 0.28 and α-lipoprotein (HDL); 0.93, 0.95, 0.97, 1.01, 1.02 and 1.02.

In competition degradation experiments, Cu^{2+} oxidized serum was found to effectively compete the degradation of oxidized ^{125}I-LDL, whereas control serum lacked effect in this respect (Table 1).

To study the significance of different lipoprotein fractions on the susceptibility of serum to oxidation, VLDL, IDL, LDL and HDL + d. > 1.21 kg/l fractions were prepared by ultracentrifugation in a density gradient without the presence of antioxidants or EDTA. Serum was reconstituted without prior dialysis by adding the lipoprotein fractions to the HDL + d. > 1.21 fraction, (all diluted to a final concentration of 2% in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl prewarmed to 30°C) and oxidation initiated by the addition of a freshly prepared aqueous $CuSO_4$ solution with a final concentration of 50 μM. Table 2 shows the lag phase for formation of dienes of control serum, reconstituted serum and different combinations of the HDL + d. > 1.21 fraction and the other lipoprotein fractions. The fully reconstituted serum had a shorter lag phase than the control serum indicating that the gradient ultracentifugation without the presence of antioxidants and EDTA or the presence of NaCl affected the susceptibility to oxidation. Only the addition of the LDL fraction to the HDL + d. > 1.21 kg/l fraction with or without the presence of the VLDL and IDL fractions resulted in a shorter lag phase, indicating an important role of LDL in determining the lag phase of serum.

In order to study the relation between serum and LDL susceptibility to oxidation, serum and LDL were prepared from 6 healthy individuals. Determination of lag phase for diene formation in serum was performed immediately after preparation, and lag phase of LDL was determined two days later following isolation and dialysis. No correlation was found between serum and LDL susceptibility to oxidation ($r = 0.08$, $p = 0.88$).

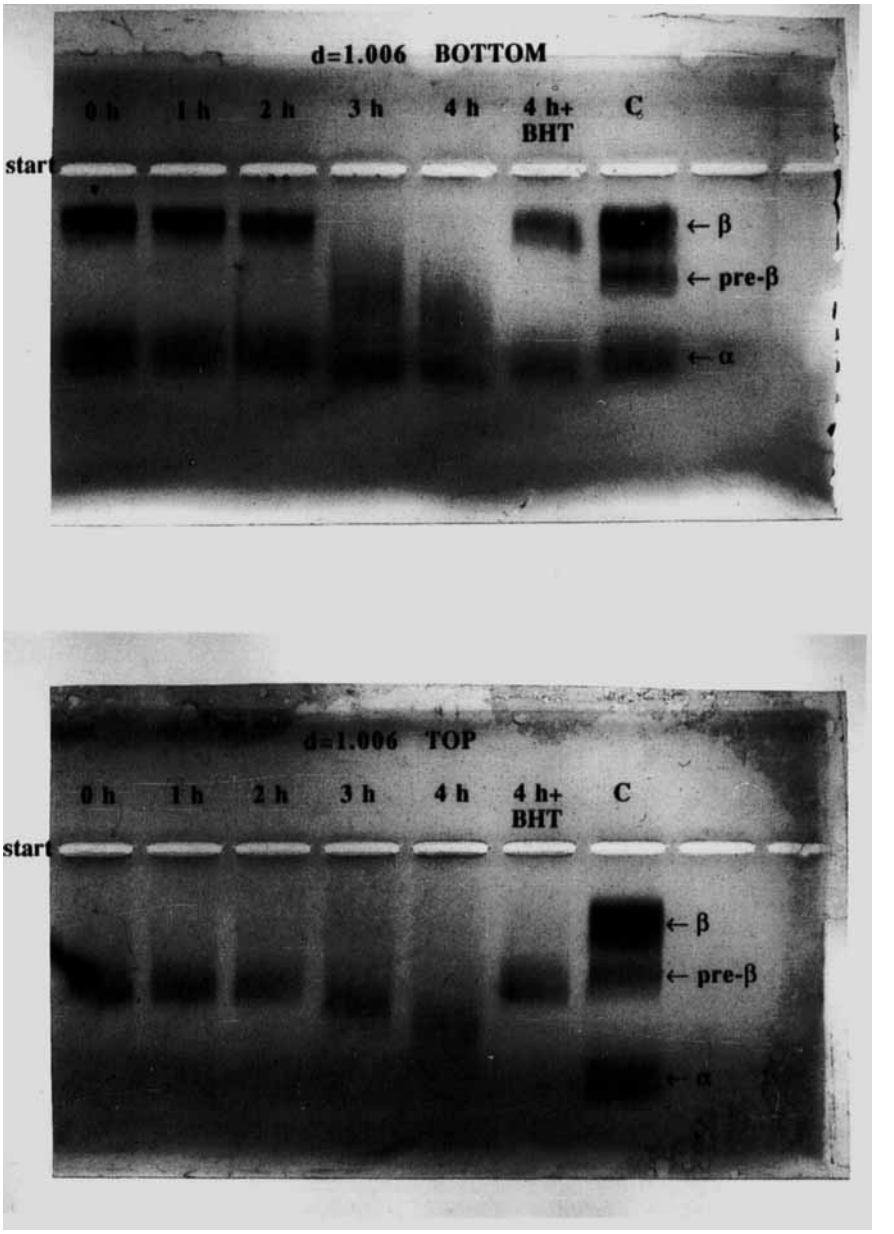

FIGURE 6 Agarose gel lipoprotein electrophoresis of the top (VLDL) and bottom (LDL + HDL) fractions obtained after ultracentrifugation of serum at d. = 1.006 kg/l. Serum was diluted in 0.02 M phosphate buffer pH 7.4/0.16 M NaCl to a final concentration of 2% and incubated with 50 μM $CuSO_4$ at 30°C for up to 4 h with or without the addition of 20 μM BHT. The incubation was terminated by addition of 20 μM BHT. *C* denotes a fresh, unincubated control serum where the β-lipoprotein (LDL), pre-β-lipoprotein (VLDL) and α-lipoprotein (HDL) bands have been indicated by arrows. 20 μl of sample was applied to each well.

TABLE 1
Effect of oxidized and control serum on macrophage degradation of oxidized LDL

	native LDL	oxidized LDL	oxidized LDL + oxidized serum	oxidized LDL + control serum
Macrophage degradation (ng/mg cell protein)	190.5 ± 24.8	1173.3 ± 14.8	332.1 ± 25.5	1140.2 ± 14.4

Cultured resident mouse peritoneal macrophages were incubated with ^{125}I-labelled native and oxidized LDL (10 μg/ml). Oxidized LDL was also incubated with the addition of 950 μl oxidized or control serum diluted to 4% in medium Ham's F-10. Degradation was determined by analyzing the radioactive, non-protein degradation products in the culture medium after 5 h at 37°C. Each value is a mean ± SD of triplicate incubations from one experiment.

TABLE 2
Lag phase for formation of dienes in control and different combinations of reconstituted serum

	lag phase (min)
control serum	119.0
d. > 1.21 kg/l + HDL	116.3
d. > 1.21 kg/l + HDL + VLDL	129.2
d. > 1.21 kg/l + HDL + VLDL + IDL	118.1
d. > 1.21 kg/l + HDL + VLDL + IDL + LDL	77.8
d. > 1.21 kg/l + HDL + LDL	77.3

VLDL, IDL, LDL, and d. > 1.21 kg/l + HDL fractions were prepared by ultracentrifugation in a density gradient without the addition of antioxidants or EDTA. Serum was reconstituted without prior dialysis by adding the lipoprotein fractions to the HDL + d. > 1.21 kg/l fraction to a final concentration of 2% and oxidation was initiated by the addition of $CuSO_4$ with a final concentration of 50 μM. Lag phase determination on control serum was performed immediately after preparation.

DISCUSSION

Several methods, among them analysis of TBAR and cholesterol oxides, have been applied in order to detect the presence of lipid oxidation products in plasma and LDL. Because of the low basal values of lipid peroxides in plasma, sample purification has been required. Using these techniques increased plasma and LDL lipid peroxidation has been demonstrated in patients with cardiovascular disease,[14,15] diabetes mellitus[16] and hypertension.[17]

Another approach recently applied by several groups is to analyze oxidation susceptibility by determining lag phase for diene formation in LDL exposed to Cu^{2+} [2] or the formation of oxidation products in plasma after exposure to Cu^{2+} or hydrogen peroxide.[18] The disadvantages of the former technique include the requirement for isolation of LDL by preparative ultracentrifugation and the failure to account for the pro- and antioxidant factors in plasma such as water soluble antioxidants and other lipoproteins, whereas the latter requires multiple analyses. The method described in the present paper allows the direct determination of the lipoprotein susceptibility to oxidation by continuous monitoring of diene formation in serum exposed to Cu^{2+}. The increase in absorbance at 234 nm in serum after exposure to Cu^{2+} was found to be dependent on the presence of lipoproteins since no increase ocurred in lipoprotein-deficient serum. However an influence of secondary oxidation of other substances in serum can not be excluded. The increase in

absorbance at 234 nm occurred after the consumption of the main part of α-tocopherol and was strongly related to the serum level of TBARS. The present method has several advantages. It only requires dilution of freshly prepared serum and the addition of Cu^{2+} before spectrophotometric analysis, it requires very small sample volumes (13 μl) and allows a precise determination of the individual susceptibility to oxidation with a small intra- and inter-assay variation. It is however possible that lipoprotein lipid peroxidation occurs mainly in the subendothelial space and that measurement of the oxidation susceptibility of serum is of little physiological relevance. Furthermore, it cannot be excluded that the relative antioxidant capacities are distorted due to dilution of serum. Clearly, the value and applicability of this assay requires examination in clinical trials.

Although lag phase determinations of different combinations of reconstituted serum indicated that the LDL fraction increased the susceptibility of serum to oxidation, no relation was found between the oxidation susceptibility of serum and LDL isolated from this serum in six healthy controls. This difference may be due to the influence of water soluble antioxidants such as ascorbate, urate, protein sulfhydryl groups, albumin, albumin-bound bilirubin and to the plasma proteins transferrin and ceruloplasmin which also are considered as antioxidants due to their ability to sequester transition metals[19] or to the presence of other lipoproteins.

Lipid peroxidation has been suggested as a pathogenic factor in several human diseases, including atherosclerosis. Oxidation of LDL initiates a series of events leading to enhanced uptake by the scavenger receptor in macrophages and subsequent foam cell formation.[20] The role of lipid oxidation for development of atherosclerosis has been demonstrated in animal studies,[21,22] and by using immunological and biochemical techniques the presence of oxidatively modified LDL has been identified in atherosclerotic lesions.[23] Several recent clinical studies have indicated that LDL oxidation plays an important role in coronary heart and peripheral artery disease. Low levels of the antioxidant α-tocopherol (vitamin E) have been found in patients suffering from angina pectoris,[24] and an increased susceptibility of LDL to oxidative modification is associated with a proneness to developing premature coronary atherosclerosis.[5]

The assay described here represents a simple and reproducible method to estimate serum lipoprotein oxidation susceptibility which should be possible to apply in large clinical studies.

Acknowledgements

We thank K. Carlson and K. Eriksson for assistance provided during this study. This study was supported by grants from the Swedish Medical Research Council (204, 7140, 8311 and 8691), the Swedish Heart-Lung Foundation, the Knut and Alice Wallenberg Foundation, King Gustaf V 80th Birthday Fund and Professor Nanna Svartz Fund. Dr Nilsson is a Career Investigator of the Swedish Medical Research Council.

References

1. B. Halliwell (1989) Current status review: free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *British Journal of Experimental Pathology*, **70**, 737–757.
2. H. Esterbauer, G. Striegl, H. Puhl and M. Rothender (1989) Continous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Research Communications*, **6**, 67–75.
3. B.L. Knight and A.K. Soutar (1982) Degradation by cultured fibroblasts and macrophages of unmodified and 1,2-cyclohexanedione-modified low-density lipoprotein from normal and homozygous familial hypercholesterolaemic subjects. *Biochemical Journal*, **202**, 145–152.

4. T.G. Redgrave, D.C.K. Roberts and C.E. West (1975) Separation of plasma lipoproteins by density gradient ultracentrifugation. *Analytical Biochemistry*, **65**, 42–49.
5. J. Regnström, J. Nilsson, P. Tornvall, C. Landou and A. Hamsten (1992) Susceptibility to low density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet*, **339**, 1183–1186.
6. O. Lowry, N. Rosenbrough, A. Farr and R. Randall (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
7. K. Carlson (1973) Lipoprotein fractionation. *Journal of Clinical Pathology*, **5**, 32–37.
8. R.P. Noble (1968) Electrophoretic separation of plasma lipoproteins in agarose gel. *Journal of Lipid Research*, **9**, 693–700.
9. K. Yagi (1976) Short communications. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochemical Medicine*, **15**, 212–216.
10. J.K. Lang, K. Gohil and L. Packer (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Analytical Biochemistry*, **157**, 106–116.
11. P.P. Chou, P.K. Jaynes and J.L. Balley (1985) Determination of vitamin E in microsamples of serum by liquid chromatography with electrochemical detection. *Clinical Chemistry*, **31**, 880–882.
12. A.S. McFarlane (1956) Labelling of plasma proteins with radioactive iodine. *Biochemical Journal*, **62**, 135–143.
13. J. Thyberg (1980) Internalization of cationized ferritin into the Golgi complex of cultured mouse peritoneal macrophages. Effects of colchicine and cytochalasin B. *European Journal of Cell Biology*, **23**, 95–103.
14. M.D. Stringer, P.G. Görög, A. Freeman and V.V. Kakkar (1989) Lipid peroxides and atherosclerosis. *British Medical Journal*, **298**, 281–284.
15. K. Liu, T.E. Cuddy and G.N. Pierce (1992) Oxidative status of lipoproteins in coronary disease patients. *American Heart Journal*, **123**, 285–290.
16. Y. Sato, N. Hotta, N. Sakamoto, S. Matsuoka, N. Ohishi and K. Yagi (1979) Lipid peroxide level in plasma of diabetic patients. *Biochemical Medicine*, **21**, 104–107.
17. M. Uysal, H. Buler, D. Sener and H. Oz (1986) Lipid peroxidation in patients with essential hypertension International Journal of Clinical Pharmacological Research, **24**, 474–476.
18. M.A.Q. Arshad, S. Bhadra, R.M. Cohen and T.R. Subbiah (1991) Plasma lipoprotein peroxidation potential: a test to evaluate individual susceptibility to peroxidation. *Clinical Chemistry*, **37**, 1756–1758.
19. B. Frei, R. Stocker and B.N. Ames (1988) Antioxidant detences and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 9748–9752.
20. D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witztum (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*, **320**, 915–925.
21. T.E. Carew, D.C. Schwenke and D. Steinberg (1987) Antiatherogenic effects of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic (WHHL) rabbit. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 7725–7729.
22. T. Kita, Y. Nagano, M. Yokode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida and C. Kawai (1987) Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 5928–5931.
23. S. Ylä-Herttuala, W. Palinsky, M.E. Rosenfeld, S. Parthasarathy, T.E. Carew, S. Butler and J.L. Wiztum (1989) Evidence for presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *Journal of Clinical Investigation*, **84**, 1086–1095.
24. R.A. Riemersma, D.A. Wood, C.C.A. Macintyre, R.A. Elton, K.F. Gey and M.F. Oliver (1991) Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. *Lancet*, **337**, 1–5.

Accepted by Professor B. Halliwell