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Factors Affecting Events During Oxidation of Low Density Lipoprotein: Correlation of Multiple Parameters of Oxidation

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The present study shows that copper oxidation of LDL is a tightly-ordered process which can be finely controlled by appropriate selection of duration of oxidation and of concentrations of LDL and copper. Oxidation of LDL (0.1–2.0 mg LDL protein/ml) was carried out by copper catalysis (in the ratio of 2.5 μM Cu^{2+} to 0.1 mg LDL protein/ml) in phosphate-buffered saline, and was monitored by agarose gel electro-phoresis, gas chromatography (GC), anion exchange fast protein liquid chromatography (FPLC), fluorescence spectroscopy and dynamic light scattering. Analysis of the data showed strong cross correlations between many of the parameters of oxidation. Oxidation was more rapid for lower concentrations than for higher concentrations of LDL, despite the same ratio of copper to LDL being employed. Chemical kinetics analysis of the GC data suggested that 7β-hydroxycholesterol formation occurred as a first order (or pseudo first order) consecutive reaction to the oxidation of linoleate. The first order rate constants for decomposition of linoleate and production of 7β-hydroxycholesterol correlated closely with the theoretically-calculated times between collision of LDL particles. LDL particle diameter, measured by dynamic light scattering, increased by ca. 50% over 24 h oxidation, suggesting unfolding of apo B-100. Prolonged oxidation of LDL at low concentration suggested that the radical chain reaction was able to propagate, albeit slowly, on cholesterol after all the polyunsaturated fatty acid was consumed. For higher concentrations of LDL, prolonged oxidation resulted in partial aggregation. These findings are applicable to preparing oxidised LDL with different degrees of oxidation, under controlled conditions, for studying its biological properties.

Keywords: Low density lipoprotein (human), oxidation, 7β-hydroxycholesterol, polyunsaturated fatty acids, particle diameter, negative charge

INTRODUCTION

There is now much evidence for the oxidation of low-density lipoprotein (LDL) during the pathogenesis of atherosclerosis.[1] Part of this evidence is the detection of modified, probably oxidised, LDL in human and animal lesions using immunohisto-

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chemistry.[2–6] Moreover lipid oxidation products (oxysterols and hydroxyoctadecadienoic acids) have been detected in human lesions,[7–10] and in LDL oxidised in vitro by macrophages or copper.[11–13] LDL isolated from arteries also shows signs of oxidation, in terms of increased electrophoretic mobility relative to plasma LDL,[14,15] increased tendency to aggregate and increased fluorescence[16] and diminished ratio of linoleate (18:2) to oleate (18:1),[14] a feature also observed for lipids extracted from foam cell-rich lesions.[10,17] There is tentative evidence that oxysterols are present in arterial LDL itself.[18]

LDL can be readily oxidised in vitro. The method most commonly used is Cu^{2+} catalysed oxidation of LDL in the absence of cells.[19] Macrophage-mediated modification of LDL produces a form closely resembling copper-oxidised LDL,[13,20,21] and this is also true of the other cell-types found in lesions.[22–24] However chemical evidence suggests that areas rich in macrophage foam cells are responsible for the most oxidative activity.[10,25]

Oxidised LDL possesses a variety of biological properties, including increased uptake by macrophages,[22] chemotactic attraction of monocytes[26,27] and toxicity to various cell-types, including macrophages, endothelial cells and smooth muscle cells.[28–31] These properties may be relevant to lesion progression. However, a defined oxidised LDL does not exist, in the strict chemical sense.[19,32] There are many variables in the oxidation conditions, even when consideration is limited to copper-oxidised LDL. These include copper concentration, time of oxidation, temperature, nature of medium (e.g. phosphate-buffered saline (PBS) or Ham's F-10) and an aspect which is frequently neg-lected, the concentration of the LDL itself. Each laboratory appears to use different combinations of conditions, and the extent of oxidation is often unclear, especially as different laboratories use different methods, or variants of methods, of measuring oxidation.[32] The present study was therefore undertaken to ascertain what interrelationships, if any, exist amongst various parameters of LDL oxidation, and to explore the effect of LDL concentration on its subsequent oxidation.

MATERIALS AND METHODS

LDL Preparation and Oxidation

Each LDL preparation was obtained from the pooled plasma of normal individuals (at least 3 donors per preparation) as described previously.[31] It was stored in Tris/EDTA buffer, pH 7.4, containing 1 mM EDTA at 4°C, for no longer than 4 weeks. Immediately prior to each experiment, the LDL was dialysed for 24 h against phosphate-buffered saline (PBS; no Ca^{2+} or Mg^{2+}) (3 times 1000 ml) with no EDTA. The concentration of the LDL solution was then measured by the method of Lowry, and the LDL diluted as desired (0.1 mg LDL protein/ml—2.0 mg LDL protein/ml) using PBS (no EDTA) and appropriate aliquots of copper added (from a stock solution of 5 mM copper (II) sulphate). The ratio of copper to LDL employed was constant, at 2.5 μM copper to 0.1 mg LDL protein/ml, unless stated otherwise. LDL oxidation was performed at 37°C for the required times (up to 168 h), and the oxidation terminated by addition of EDTA (1 mM final concentration) and butylated hydroxy-toluene (BHT) (20 μM final concentration, from a stock solution in methanol). Samples were stored at 4°C prior to gel electrophoresis, fast protein liquid chromatography, and fluorescence measurements, and at –20°C prior to extraction of lipids and oxidised lipids for gas chromato-graphy. All experiments were repeated at least twice and data from a representative experiment reported in each case.

Analysis of Oxidised LDL

(a) Agarose Gel Electrophoresis. This was carried out using a Beckman Lipogel system (Beckman Instruments Inc., Brea, CA, USA) using the manufacturer's standard procedure. Essentially,

3–5 µl of each sample was applied to the Beckman Lipogel, which was run in barbital buffer at pH 8.6 at 100 V for 30 minutes, then fixed in ethanol/deionised water/acetic acid 540/270/90 (v/v/v), dried, and stained with Beckman Lipostain (Sudan Black B).

For the samples in the fluorescence study and for those in the FPLC study, agarose gel electrophoresis was carried out using a Corning system, on Universal Gel 8 agarose gels (Ciba-Corning, Palo Alto, CA, USA), run in barbital buffer at pH 8.6 at 90 V for 40 minutes, fixed in 10% trichloroacetic acid, dried, and stained with Coomassie Blue.

(b) Gas Chromatography. Lipids and oxidised lipids were extracted, processed, and analysed by gas chromatography (GC) as described previously, using a DB-1 capillary column.[13] Essentially, the procedure involved addition of internal standards, Bligh and Dyer extraction, reduction by sodium borohydride, saponification, and derivatisation to methyl esters and trimethylsilyl ethers, followed by GC analysis. Care was taken to minimise exposure of samples to air, and the reduction, saponification, and derivatisation steps were all under nitrogen or argon. Storage of processed samples (prior to GC) was at –20°C under nitrogen or argon.

(c) Fast Protein Liquid Chromatography. Anion exchange chromatography was carried out using a Mono Q column (Pharmacia, Uppsala, Sweden) on a Pharmacia fast protein liquid chromatography (FPLC) system (LCC-500 programmer plus two P-500 pumps) according to the method of Vedie et al..[33] Buffer A was 0.01 M Tris-HCl, pH 7.4, containing 1 mM EDTA; buffer B was 1 M NaCl in buffer A. Samples of oxidised LDL were dialysed overnight against buffer A, and then diluted to 0.1 mg LDL protein/ml using buffer A. Samples were injected via a 0.5 ml loop. Essentially, the chromatography involved programmed mixing of buffers A and B to generate a stepwise gradient of increasing concentration of sodium chloride, as follows. From 0 to 10 mins, linear gradient of 0–0.1 M NaCl; 10–15 mins, 0.2 M NaCl; 16–20 mins, 0.3 M NaCl; 21–25 mins, 0.4 M NaCl; 26–30 mins, 0.5 M NaCl; 31–35 mins, 0.6 M NaCl; 36–40 mins, 1 M NaCl, 41–45 mins, 0 M NaCl. The flow rate was 1 ml/min. Detection was at 280 nm.

(d) Fluorescence Spectroscopy. This was carried out on a Perkin-Elmer LS-5B luminescence spectrometer (Perkin-Elmer Corporation, Norwalk, CT, USA) operated in the fluorescence mode, using an excitation wavelength of 360 nm and scanning the emission spectrum between 380 nm and 490 nm. The emission maximum of oxidised LDL was at 420 nm. All fluorescence intensity results presented for this study are standardised for LDL protein, i.e. the raw fluorescence intensity units reading obtained by spectrofluorimetry is divided by the LDL protein concentration in mg/ml, in each case.

(e) Dynamic Light Scattering. The dynamic light scattering technique measures the translational diffusion coefficient of the particle which can be related to the particle size (diameter) according to the Stokes-Einstein equation, $R_h = k_BT/6\pi\eta D$ (R_h = hydrodynamic radius, k_B = Boltzmann constant, T = temp, η = viscosity of the solvent, D = diffusion coefficient). Measurements were performed using a Malvern 4700 photon correlation spectroscopy (PCS) system (Malvern Instruments) equipped with a 25 mW HeNe laser emitting vertically polarised light at a wavelength of 633 nm. All measurements were carried out at 37.0 ± 0.1°C. By use of this PCS apparatus the angular dependence of the scattering could be measured over the range 30–150°. In all light scattering experiments cylindrical quartz cells of 10 mm diameter were used. The cells were soaked in nitric acid, rinsed with distilled water and finally rinsed with freshly distilled acetone before use. For the time-course measurements, the native LDL and copper solutions were filtered through a 0.22 µm Millipore filter directly into the light scattering cells, and the oxidation was allowed to proceed in situ within the PCS appara-

tus. For the 24 h single timepoint oxidations, the LDL was oxidised with copper in a glass vial at 37°C and the oxidation terminated by addition of 1 mM EDTA and 20 μM BHT. This oxidised LDL was then filtered through a 0.22 μm Millipore filter directly into the light scattering cells. The purpose of the filtration was to remove dust, which, if present, would interfere with the light scattering measurements and is standard practice for PCS.

RESULTS

(a) Agarose Gel Electrophoresis. Oxidation proceeded rapidly for the dilute LDL incubations and more slowly for the higher LDL concentrations (Fig. 1), despite the ratio of copper to LDL being the same in each case. By 24 h the 0.1 mg/ml LDL had reached an REM of 5.6, whereas the 2.0 mg/ml LDL had only achieved an REM of 2.8. By 120 h the 2.0 mg/ml LDL had attained an REM of 5.5.

There appeared to be some degree of aggregation of the LDL oxidised at the higher concentrations. From 48 h onwards the 2.0 mg/ml LDL showed stained material extending backwards from the main band to the origin, with some stained material remaining in the starting wells for the 72 h and 120 h oxidised LDLs. Backward streaking was also apparent for the 1.0 mg/ml LDLs from 48 h onwards, although this was not as pronounced as for the 2.0 mg/ml LDL and the streaking did not extend as far back as the origin. A slight degree of backward streaking was seen for the 0.5 mg/ml LDL from 48 h onwards. The backward streaking was not due to overloading of gels, as the samples at the later timepoints which displayed streaking were run at the same loading as those of the earlier timepoints which did not display streaking.

In an attempt to examine the limit of the increase in electrophoretic mobility of oxidised LDL, a separate batch of LDL was oxidised at 0.2 mg LDL protein/ml using 5 μM copper, for up to 168 h. The REM at 24 h was 6.8, and from 120–168 h appeared to have reached a plateau at an REM of 8.3. Analysis of the lipids by GC

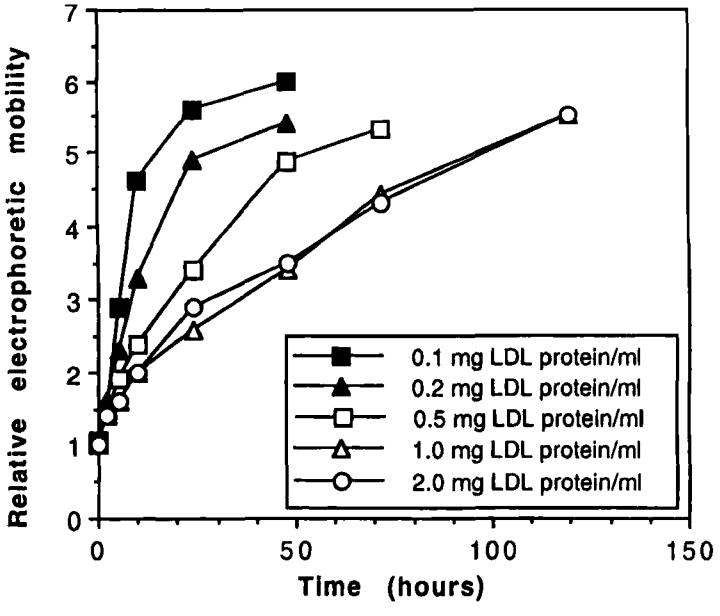

FIGURE 1 Relative electrophoretic mobility (i.e. distance migrated by sample divided by distance migrated by native LDL) on agarose gel electrophoresis (Beckman LipoGel) versus time (h) for LDL oxidised at 0.1, 0.2, 0.5, 1.0 and 2.0 mg LDL protein/ml by copper (II) sulphate at the ratio of 2.5 μM Cu^{2+} to 0.1 mg LDL protein/ml, in PBS at 37°C.

(see below) showed that only 3% of the original linoleate remained at 24 h, and only 1.6% by 168 h. This suggests that the increase in REM seen after 24 h was largely due to changes to the protein other than simple capping of lysines by aldehydes derived from oxidation of polyunsaturates. Alternatively some of the less readily accessible lysine residues might be slow to react with aldehydes, so that their capping might lag behind aldehyde production.

(b) Gas Chromatography. LDL oxidation resulted in lipid oxidation, as judged by the diminution of linoleate, arachidonate and cholesterol, and the production of 7β-hydroxycholesterol. As by agarose gel electrophoresis, the oxidation of LDL measured by GC appeared faster for the more dilute LDLs and slower for the more concentrated LDLs, despite the ratio of copper to LDL being the same in each case. This is exemplified by the results for 7β-hydroxycholesterol production (Fig. 2). The fall in linoleate correlated closely with the increase in relative electrophoretic mobility (Fig. 3a) and with the increase in 7β-hydroxycholesterol (Fig. 3b). The negative exponential form of the curve in Figure 3b indicates that the initial steep falls in linoleate are accompanied by modest increases in 7β-hydroxycholesterol; as linoleate falls to its lowest levels, 7β-hydroxycholesterol increases more dramatically. This suggests that 7β-hydroxycholesterol formation might be secondary to oxidation of linoleate. This interpretation is reinforced by analysis of the data using standard chemical kinetics calculations, as follows.

The diminution of linoleate appeared to be first order, or pseudo first order, as graphs of $\ln c_0/c_t$ vs time (where c_0 is the concentration of linoleate in moles litre $^{-1}$ at time 0 and c_t is the concentration of linoleate at time t seconds or hours) were straight lines, i.e. $\ln c_0/c_t = kt$. The gradient of such a line is the rate constant k (units s^{-1} or h^{-1}). For each of the LDL concentrations tested (using the standard ratio of copper to LDL of 2.5 μM copper to 0.1 mg

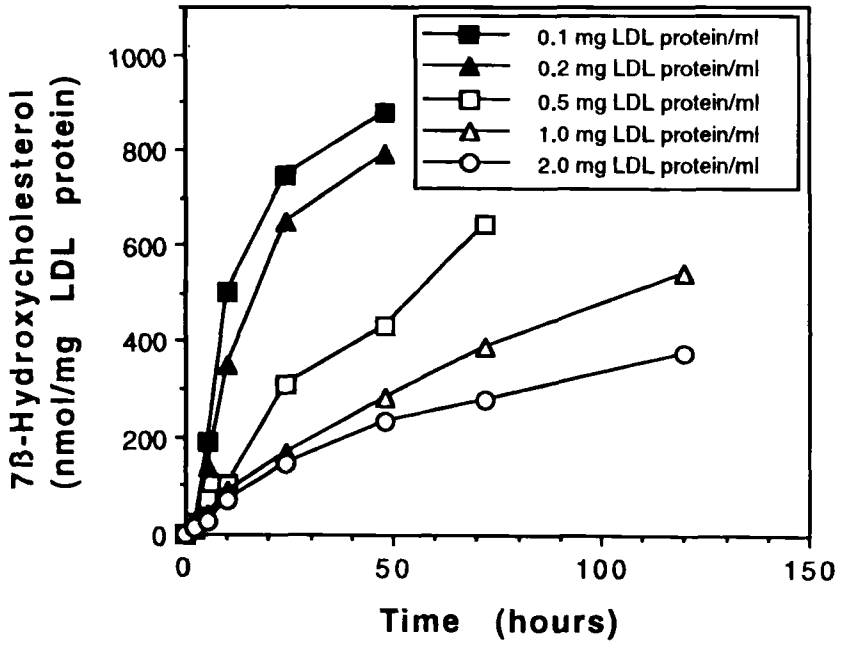

FIGURE 2 Levels of 7β-hydroxycholesterol (nmol/mg LDL protein) versus time (h) for LDL oxidised at 0.1, 0.2, 0.5, 1.0 and 2.0 mg LDL protein/ml by copper (II) sulphate at the ratio of 2.5 μM Cu^{2+} to 0.1 mg LDL protein/ml, in PBS at 37°C. The data are from the same experiment and same batch of LDL as for Figure 1.

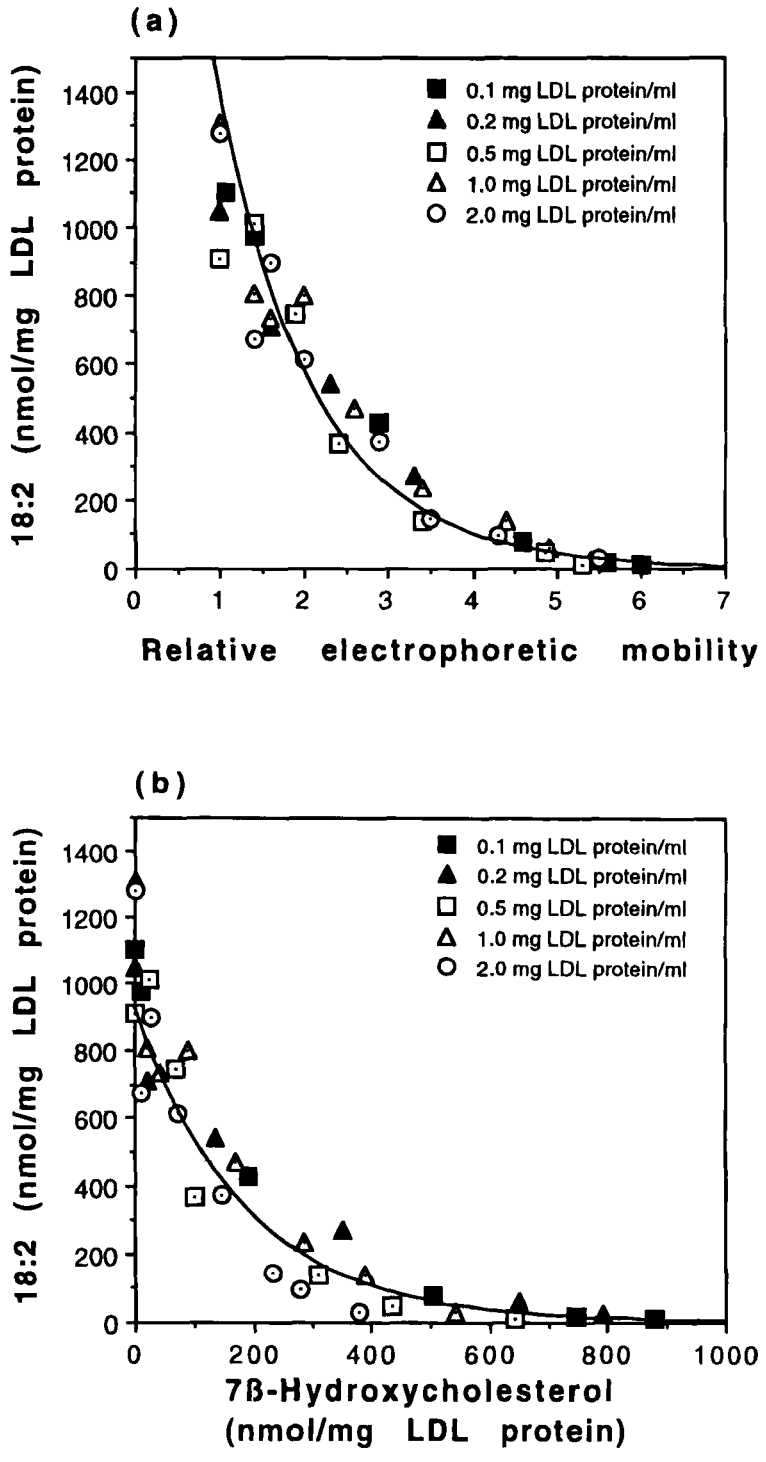

FIGURE 3 Levels of linoleic acid (18:2) (nmol/mg LDL protein) versus (**a**) relative electrophoretic mobility and (**b**) levels of 7β-hydroxycholesterol (nmol/mg LDL protein) for LDL oxidised at 0.1, 0.2, 0.5, 1.0 and 2.0 mg LDL protein/ml by copper (II) sulphate at the ratio of 2.5 μM Cu^{2+} to 0.1 mg LDL protein/ml, in PBS at 37°C. The data are from the same experiment and same batch of LDL as for Figure 1. Curve fitting was performed with Cricket Graph software. The fitted curves were (a) $y = 936 \times 10^{-0.380x}$, with $r^2 = 0.952$, and (b) $y = 260 \times 10^{-0.00586x}$, with $r^2 = 0.909$.

LDL protein/ml) a different rate constant was obtained, the more dilute LDLs giving larger rate constants than the more concentrated ones. The production of 7β-hydroxycholesterol appeared to fit the criteria for a first order (or pseudo first order) consecutive reaction to the oxidation of linoleate, as graphs of $\ln c_0/(c_0 - z)$ vs time (where c_0 is as above, and z is the concentration of 7β-hydroxycholesterol in moles litre^{-1} at time t seconds or hours) are straight lines, i.e., $\ln c_0/(c_0 - z) = k't$, where k' is the rate constant for the consecutive reaction (Fig. 4). As can be seen, the gradient k' was larger for the more dilute LDLs than for the more concentrated ones. It should be noted that for the two most dilute LDL concentrations (0.1 and 0.2 mg LDL protein/ml), first order kinetics held for the first 10 h and 24 h respectively, but not at the later timepoints, due to slowing down of the reaction because of extensive depletion of linoleate at these later timepoints. This phenomenon is a well-recognised feature of reaction kinetics in general which occurs when the substrate is nearly exhausted.

The production of 7β-hydroxycholesterol thus shows a strong quantitative association with the oxidation of linoleate. In order to investigate whether 7β-hydroxycholesterol production could carry on after supplies of linoleate were exhausted by the oxidation, we carried out prolonged oxidation of a separate batch of LDL at 0.2 mg LDL protein/ml using 5 μM copper (see also Results section (a) above). By 24 h linoleate had fallen to 3% of its initial level, whilst 7β-hydroxycholesterol had risen from zero to 638 nmol/mg LDL protein. At 72 h, linoleate was still present at 3% of its initial level, and the level of 7β-hydroxycholesterol was 881 nmol/mg LDL protein. By 168 h linoleate had fallen to 1.6% of its initial level, and 7β-hydroxycholesterol had risen to 939 nmol/mg LDL protein. Thus it appeared that although

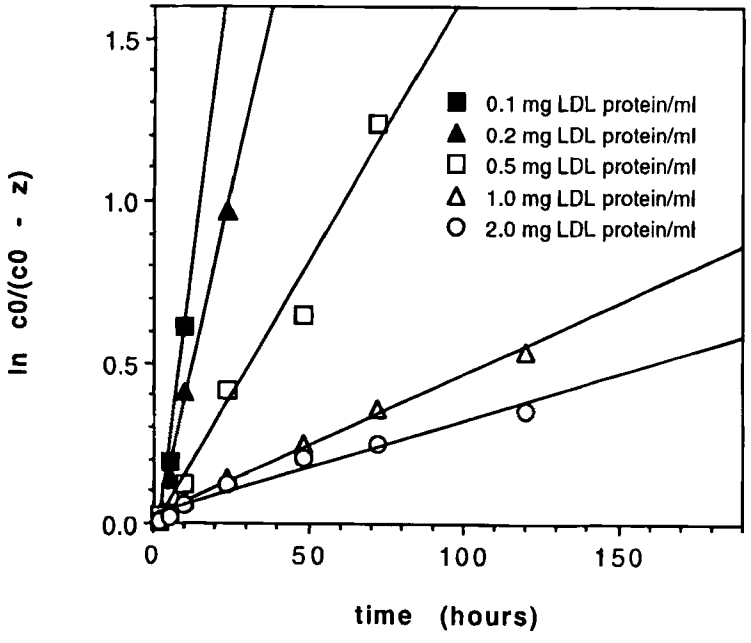

FIGURE 4 Plots of $\ln c_0/(c_0 - z)$ versus time (h), where c_0 is the concentration of linoleate (in moles litre^{-1}) at time zero, and z is the concentration of 7β-hydroxycholesterol (in moles litre^{-1}) at time t hours. The data are from the same experiment and same batch of LDL as for Figure 1. Line fitting (least squares linear regression) was performed with Cricket Graph software. The gradient of each line is equal to k', the rate constant (first order or pseudo first order) for the production of 7β-hydroxycholesterol as a consecutive reaction to the decomposition of linoleate. Values of k' so obtained for 0.1, 0.2, 0.5, 1.0, and 2.0 mg LDL protein/ml were, respectively, 7.59×10^{-2}, 4.32×10^{-2}, 1.65×10^{-2}, 4.43×10^{-3}, and 2.88×10^{-3} h^{-1}. Values of r^2 were, respectively, 0.993, 0.997, 0.980, 0.995 and 0.963.

linoleate was virtually all consumed, 7β-hydroxycholesterol continues to rise, though not as steeply as earlier on, suggesting that the radical chain reaction was able to propagate on cholesterol. By 24 h cholesterol had fallen to 53% of its initial level, and by 168 h to 34%. This suggests that further, slow oxidation of cholesterol might have occurred if the oxidation time had been prolonged even more.

(c) Fast Protein Liquid Chromatography. Anion exchange FPLC using a stepwise elution gradient of NaCl showed that LDL oxidised at 2 mg/ml did not become as negatively charged as LDL oxidised at 0.2 mg/ml, despite the ratio of copper to LDL being the same in each case. After 48 h oxidation, the main peak of the former had a retention time of 17 min whereas that of the latter was 27 min (Fig. 5). Agarose gel electrophoresis and GC analysis supported these findings.

Because the FPLC elution gradient was stepwise, rather than a continuous gradient, more than one peak was seen in some cases for the oxidised LDLs, which were thus split into subfractions each with a narrowly defined range of negative charge. When an oxidised LDL sample which showed two FPLC peaks under the original stepwise gradient was rerun on a continuous gradient, the two peaks merged to form a single broadened peak with a slight shoulder (data not shown). This indicates that oxidised LDL subfractions are not truly discrete and that a range of particles with a spread of degrees of oxidation is being produced.

The retention time of the main peak on FPLC correlated linearly with relative electrophoretic mobility for LDL oxidised at 0.2 mg/ml (r^2 = 0.849). However, no such linear relationship was apparent for LDL oxidised at 2 mg/ml, because the increase in ionic strength of each gradient step of the FPLC buffer was as large as the full extent of the increase in electronegativity of the LDL protein for this comparatively weakly oxidised LDL, the retention time of the main peak only lengthening by the duration of one gradient step over the 48 h of oxidation.

GC analysis of oxidised LDL pre-FPLC and of fractions recovered post-FPLC were consistent with other LDL oxidations in this study, i.e. dilute LDL being much more rapidly oxidised than concentrated. Increased retention time on FPLC was associated with increased degree of oxidation as measured by GC (loss of polyunsaturates and production of 7β-hydroxycholesterol).

(d) Fluorescence Spectroscopy. This was done at various LDL concentrations (0.5–4.0 mg LDL protein/ml), copper concentrations (2.5, 5, 10 and 15 μM) and oxidation times (0–24 h). The results showed that increasing the copper concentration speeded the oxidation, as did decreasing the LDL concentration, as judged by the increases in fluorescence intensity. Representative spectra are shown in Figure 6. It can be seen that fluorescence intensity (standardised for protein content) was more pronounced for LDL oxidised at lower concentrations than at higher concentrations, for a given ratio of copper to LDL. Fluorescence intensity correlated linearly with relative mobility on agarose gel electrophoresis, tested for LDL oxidised at 0.2 mg LDL protein/ml using 5 μM copper (r^2 = 0.997) (data not shown). Results from GC showed levels of 7β-hydroxycholesterol correlated linearly with fluorescence intensity (r^2 = 0.978) (Fig. 7a). The graph of fluorescence intensity vs 18:2 level was a negatively sloped exponential (r^2 = 0.882) (Fig. 7b).

(e) Dynamic Light Scattering. 24 h oxidation of LDL at 0.1–1.0 mg LDL protein/ml, using the standard ratio of copper to LDL of 2.5 μM copper to 0.1 mg LDL protein, produced an approximately 50% increase in LDL particle diameter in each case. The particles of oxidised LDL were judged to be spherical, by virtue of the fact that changing the angle of measurement of dynamic light scattering produced no apparent change in diameter.

Detailed time course measurements of particle diameter, for 0.2 mg LDL protein/ml and 5 μM copper showed a sigmoid-shaped curve, with a "lag phase" of about 7 h, followed by a steep increase in particle size up to 12 h and a less steep

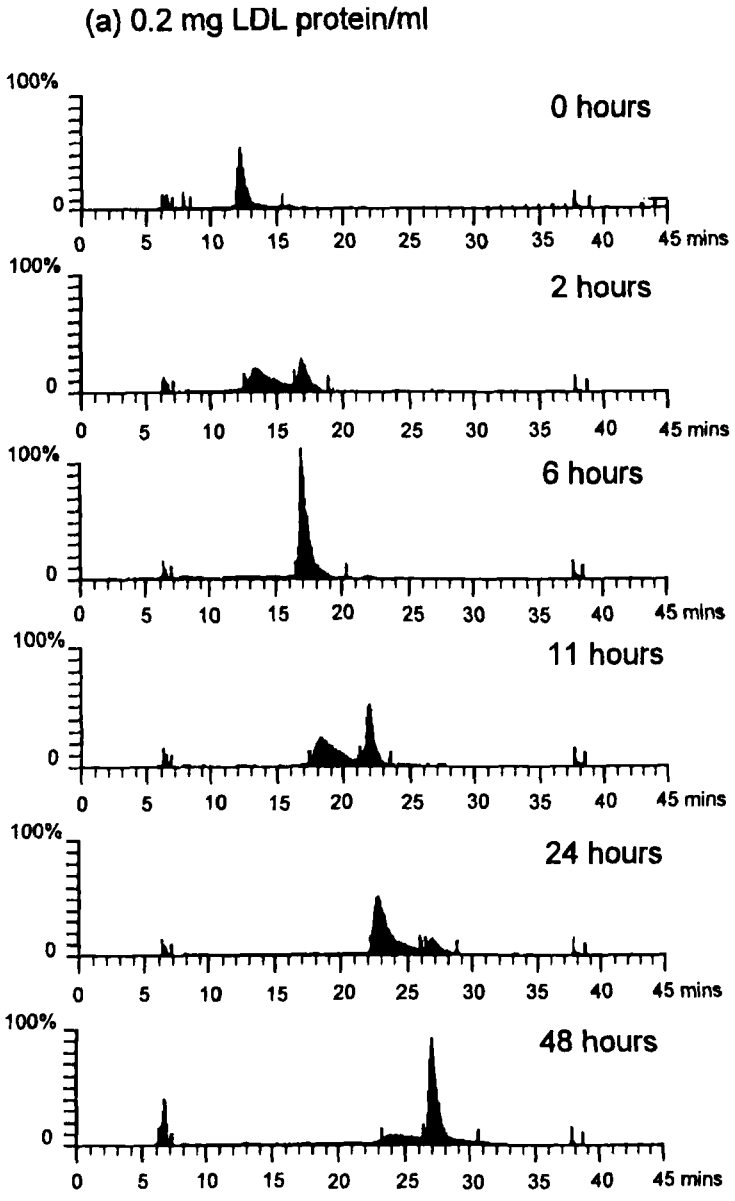

FIGURE 5 Anion exchange FPLC traces for LDL oxidised for 0, 2, 6, 11, 24 and 48 hours at (**a**) 0.2 mg LDL protein/ml using 5 μM Cu^{2+} and (**b**) 2.0 mg LDL protein/ml using 50 μM Cu^{2+}. These data were obtained using a different batch of LDL to that in Figure 1. Further details of FPLC conditions are given in the Materials and Methods section.

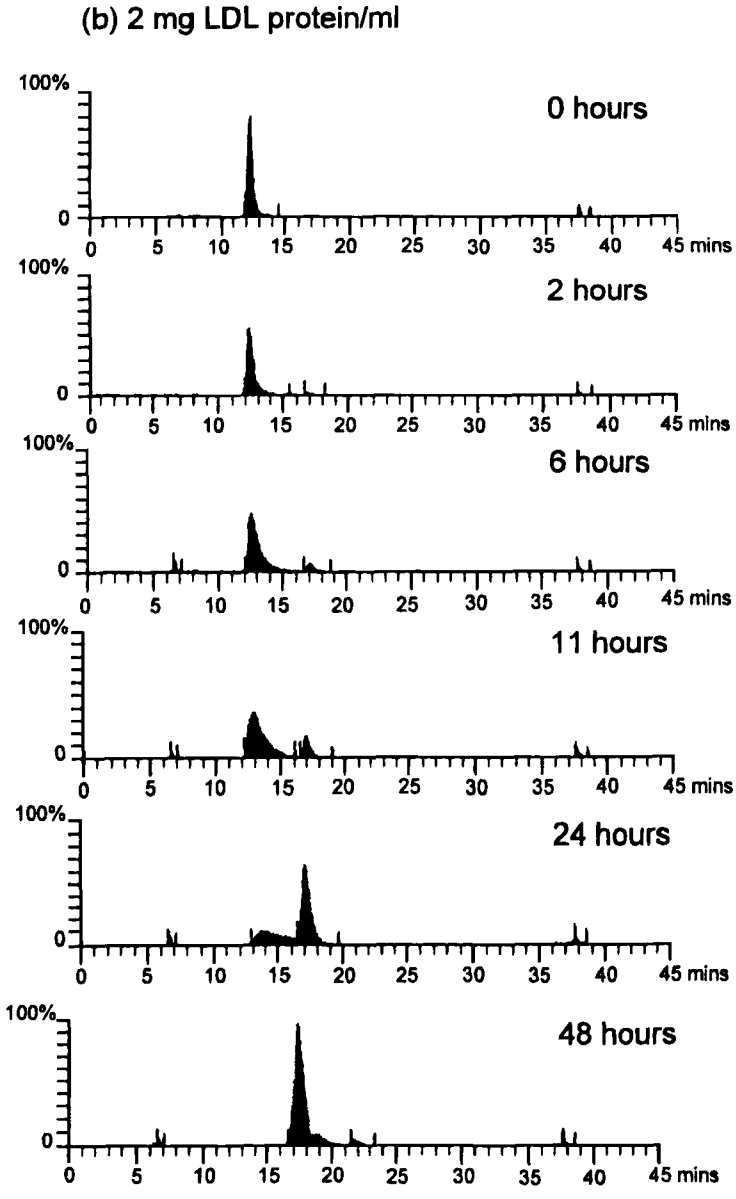

FIGURE 5b *Continued.*

increase up to 24 h (Fig. 8). The curve did not appear to plateau at 24 h, implying that prolonging the incubation would have resulted in larger particle diameter.

The PCS light scattering technique as utilised would not have detected small, detached protein fragments, but would have been capable of detecting aggregates of LDL particles (e.g., dimers, trimers, etc.) if present. No evidence was found for aggregation in the samples measured by light scattering, judged by the spherical nature of the oxidised LDL particles and the observation that the distribution of particle size showed little broadening as a result of the oxidation. Aggregates of LDL particles are heterogeneous in size and shape, as demonstrated by electron

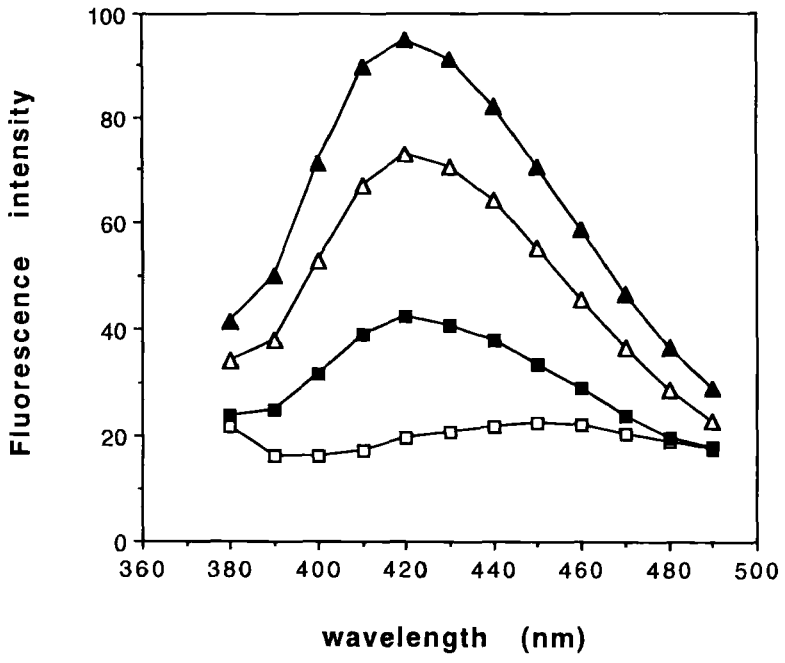

FIGURE 6 Fluorescence spectra (using excitation at 360 nm), for LDL oxidised for 6 h using the following concentrations of LDL and Cu^{2+}: 4 mg LDL protein/ml, 10 μM Cu^{2+} (open squares); 2 mg LDL protein/ml, 5 μM Cu^{2+} (solid squares); 1 mg LDL protein/ml, 10 μM Cu^{2+} (open triangles); 0.5 mg LDL protein/ml; 5 μM Cu^{2+} (solid triangles). The fluorescence intensity values have been standardised for LDL protein content by dividing the raw fluorescence intensity units obtained on the spectrometer by the LDL protein concentration of the sample (mg LDL protein/ml). These data are from a different batch of LDL to that in Figure 1.

microscopy,[16,34–36] and so exhibit a very broad particle size distribution. In the present study, results of agarose gel electrophoresis, on a different batch of LDL, indicated that aggregation occurred for LDL oxidised at high concentration (1 or 2 mg LDL protein/ml) for prolonged durations (48 h or more); see (a) above.

DISCUSSION

The results show that the rate of LDL oxidation depends strongly on the concentration of LDL. In the majority of experiments the ratio of copper to LDL protein was 2.5 μM Cu^{2+} to 0.1 mg LDL protein/ml, i.e. 12.5 moles of copper per mole of apo B-100 (assuming a molecular weight of 500,000 Daltons for apo B-100). Binding of copper to the apo B-100 is believed to be important for LDL oxidation, possibly involving tryptophan and/or histidine residues.[19,37] The number of binding sites per apo B-100 molecule is not known with certainty, although there is evidence that there are at least two per molecule.[19] The number of binding sites detected appears to depend on the method of measurement, and other estimates put the number of copper-binding sites per LDL particle at 6–8[38] or at least 100.[39] This would suggest that at the copper to protein ratio we have chosen there are probably several bound copper ions on each LDL particle, although we may or may not have saturated all the binding sites.

The observation that under these conditions the oxidation proceeds faster the more dilute the LDL might be explained as follows. It is plausible that oxidation commences in the phospholipid shell of the particle, close to a copper binding site. Some of the lipid peroxyl radicals so produced might be scavenged by α-tocopherol which is also believed to be located predominantly in the

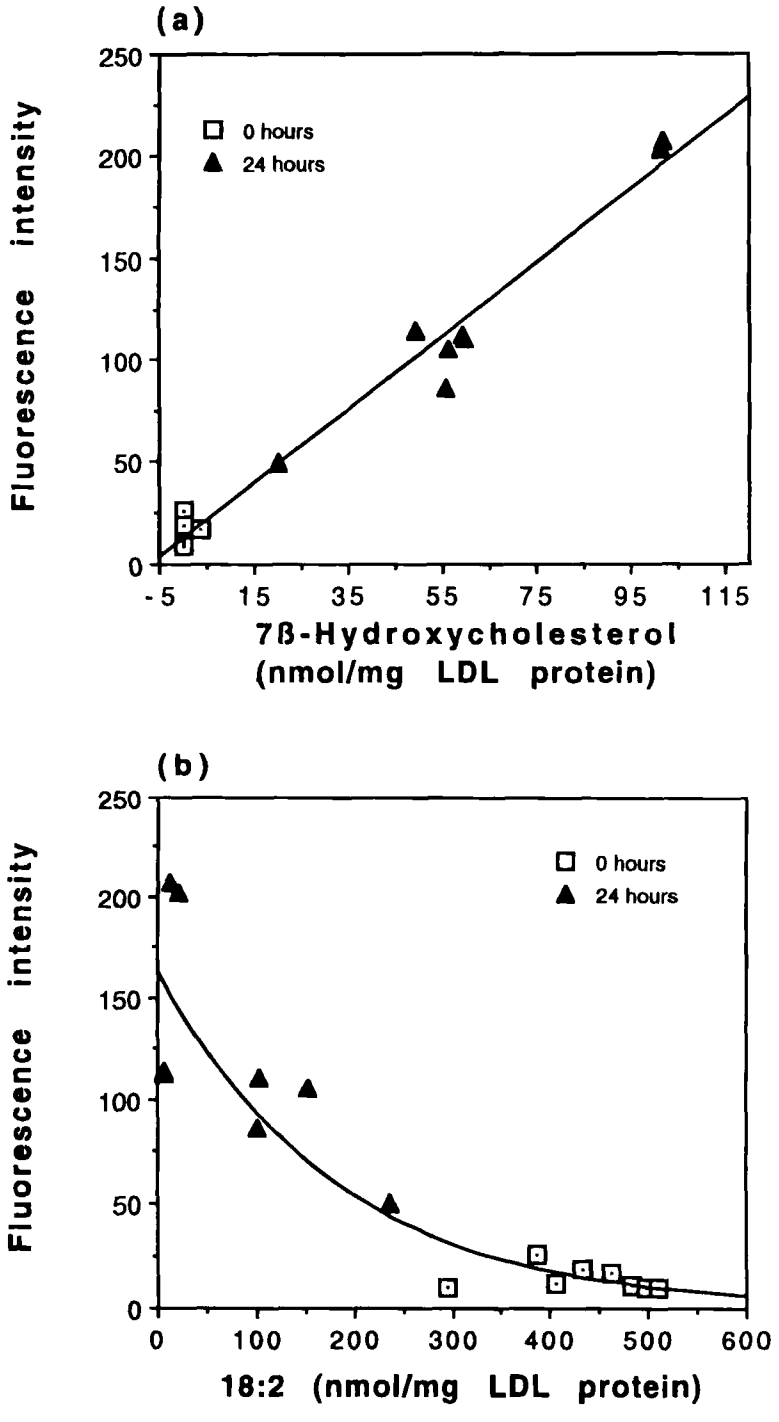

FIGURE 7 Fluorescence intensity (at 420 nm emission, using excitation at 360 nm) versus (**a**) 7β-hydroxycholesterol levels (nmol/mg LDL protein) and (**b**) linoleate (18:2) levels (nmol/mg LDL protein) for LDL oxidised at 0.5, 1.0, 2.0 and 3.0 mg LDL protein/ml using 5 and 10 μM Cu^{2+}. These fluorescence intensity data have been standardised for LDL protein content by dividing the raw fluorescence intensity units obtained on the spectrometer by the LDL protein concentration of the sample (mg LDL protein/ml). These data are from a different batch of LDL to that in Figure 1.

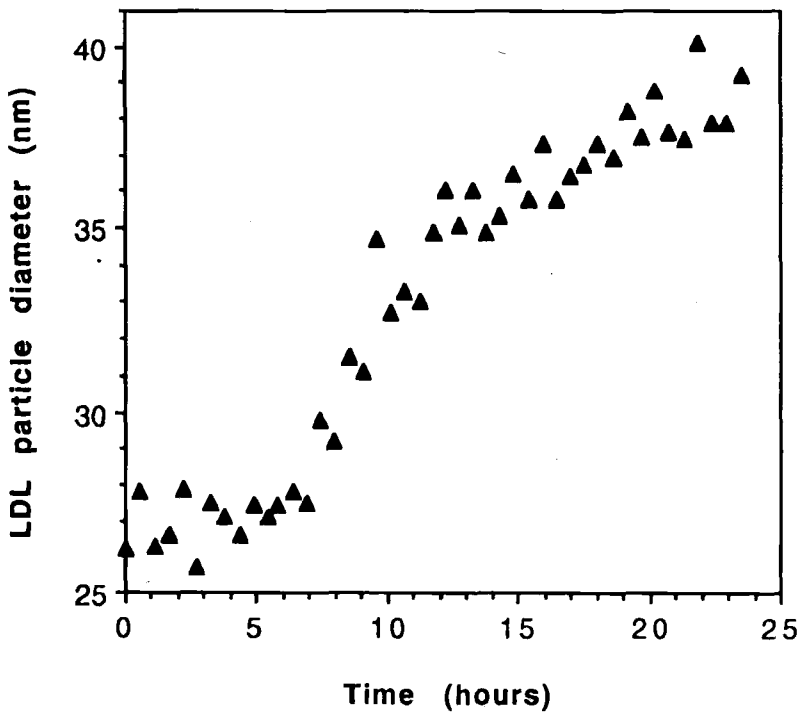

FIGURE 8 Particle diameter (nm) versus time (h) for LDL oxidised at 0.2 mg LDL protein/ml using 5 μM Cu^{2+} from 0–24 h. Dynamic light scattering measurements were made on the same sample at 30 minute intervals. These data are from a different batch of LDL to that in Figure 1. For further technical details of dynamic light scattering, see the Materials and Methods section.

shell.[40] The free radical chain reaction then can spread into the core of the particle which is largely composed of cholesterol esters and triglycerides. Noguchi and colleagues reported that oxidation proceeded ca. five times faster in the cholesterol esters of the core than in the phosphatidylcholines of the shell.[40] These workers suggested that the presence of α-tocopherol in the phospholipid shell delays LDL oxidation by scavenging radicals generated in the outer phospholipid layer, thereby preventing radicals from transmitting from the shell to the more vulnerable core.[40] LDL particles are in a constant random state of motion, making collisions with other LDL particles many times a second, and the more concentrated the LDL, the more frequently two LDL particles collide, and remain in association for a fraction of a second, before moving apart. Thus a free radical reaction started in the shell of one particle might propagate into the shell of another, hitherto unoxidised particle, with the possibility of being scavenged by α-tocopherol. Also exchange of some of the shell phospholipids and tocopherol can occur between colliding particles. It may be thus that encounters between LDL particles slow the oxidation by effectively increasing the chances of radical scavenging. At low concentrations of LDL encounters occur less frequently, and so the likelihood may increase of the oxidation proceeding from the shell into the core of the same particle rather than into the shell of another particle.

The time (t′) in seconds between collisions can be estimated from the following equation:

$$t' = (8 \pi \eta R / k_B T) \cdot [1000 c N_A / M]^{-2/3}$$

where η is the viscosity of the solvent in Newton seconds metre^{-2}, R is the radius of the particle in metres, k_B is Boltzmann's constant, T is the tem-

perature in Kelvin, c is the concentration in g l^{-1}, N_A is Avogadro's number, and M is the molecular weight in g mol^{-1}.

It was observed that the experimentally determined rate constants k and k′ for the first order and consecutive reactions, for decomposition of linoleate and production of 7β-hydroxycholesterol respectively, followed a similar trend to the theoretical times between collisions, i.e. at low concentration of LDL there are fewer collisions per unit time and faster reactions, i.e. larger values of k and k′, which would support this theory (Fig. 9a). Moreover the values of k and k′ correlated closely with the theoretical collision times, k vs collision time giving a positively sloped exponential with an r^2 of 0.979, and k′ vs collision time giving a positively sloped straight line with an r^2 of 0.996 (least squares linear regression) (Fig. 9b).

Half-times ($t_{1/2}$) for α-tocopherol exchange have been estimated by Massay at 20–70 mins,[41] for transfer between various donor and acceptor lipoproteins and model lipoproteins. The α-tocopherol transfers followed first order kinetics. This would give the rate constant k for α-tocopherol transfer (N.B. for first order kinetics, $k = \ln 2/t_{1/2}$) as 0.59–2.1 h^{-1}. In Massay's study, the donor lipoprotein was always a different species to the acceptor lipoprotein, and no data are presented for α-tocopherol transfer between LDL particles, as presumably this would be much harder to measure. However if we assume that it is of a similar order of magnitude to the above, then we can compare this with the rate constants for the LDL oxidation. The first order rate constant (k) for decomposition of linoleate is 0.028–0.32 h^{-1} in our experiments and the first order rate constant for production of 7β-hydroxycholesterol as a consecutive reaction (k′) is 0.0029–0.076 h^{-1}. These values would appear to be consistent with our theory that increasing the concentration of LDL slows oxidation by increasing the rates of encounters of lipid radicals with α-tocopherol, since if α-tocopherol transfer were significantly slower than the oxidation (which does not appear to be the case), α-tocopherol would stand little chance of interfering with the oxidation process. We do not know of any published data quantifying the rate of transfer of oxidation from the shell to the core of the LDL particle, although, as mentioned above, the rate of peroxidation of phospholipid in the shell of LDL is ca. one-fifth of that of the rate of peroxidation of cholesterol ester in the LDL core.[40]

The increase in diameter of the LDL particle by 50% over 24 h, whilst remaining spherical, over 24 h, as a result of oxidation, suggests possible unfolding of apo B-100. In native LDL, apo B-100 takes the form of a "belt" which snakes around the LDL particle, doubling back on itself to form a "bow" at one end.[42] There is thus, in principle, ample scope for unkinking it as a result of oxidative modification of the protein, e.g. cleavage of disulphide bridges, and disruption of hydrogen bonding as a result of capping of amino groups of lysine residues by aldehydes. These and other oxidative changes in the protein could thereby slacken the "belt" and effectively increase the circumference. Oxidation of LDL can result in a variable degree of fragmentation of apo B-100 depending on conditions,[43,44] and possibly at least some of the fragments may stay associated with the phospholipid shell of the particle, which would thus not be surrounded by an entire "belt" but would bear fragments of it. The phospholipid shell, and cholesterol ester core, may thus expand as the result of a slackened or broken "belt".

The concentrations of LDL in normal intima appear to be approximately double those in the plasma of the same individuals.[45,46] Thus a mean level of LDL in normal intima is around 2 mg LDL protein/ml. The LDL concentrations within lesions might be higher than in normal intima, as judged by the elevated lipid contents.[10] For example, the mean cholesterol level of fatty streaks is over eight times higher than that of normal intima.[10] However, it may not always be the case that the LDL concentration in lesions is higher than in normal intima. In human fatty streaks the LDL concentration has been reported to be lower, whereas in proliferative lesions it is higher than in normal intimas.[46] In the lesion with the highest

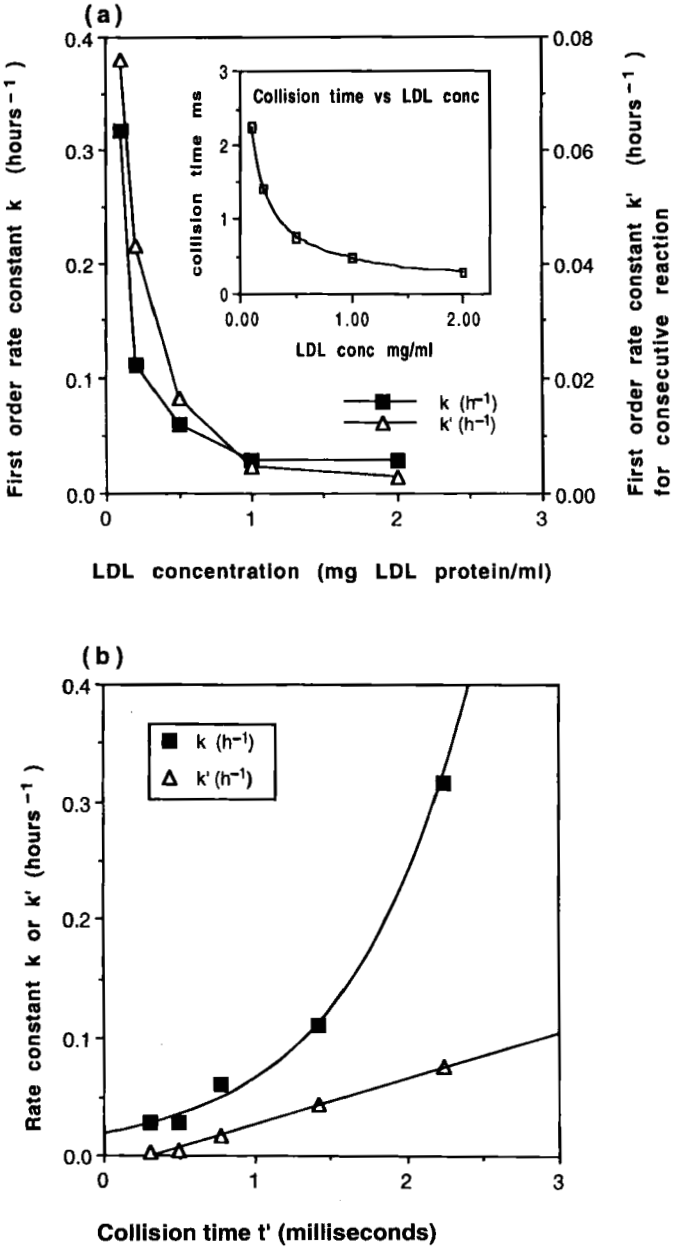

FIGURE 9 (**a**) Main graph: First order rate constant k (h^{-1}) for the decomposition of linoleate, and first order rate constant k′ (h^{-1}) for production of 7 β-hydroxycholesterol as a consecutive reaction to the decomposition of linoleate, plotted versus LDL concentration (mg LDL protein/ml). Values of k′ were obtained from Figure 4. Data are from the same experiment and same batch of LDL as for Figure 1. Inset: time between collisions (milliseconds) versus LDL concentration (mg LDL protein/ml). Time between collisions (t′) was calculated as explained in the Discussion section. (**b**) First order rate constant k (h^{-1}) for the decomposition of linoleate, and first order rate constant k′ (h^{-1}) for production of 7β-hydroxycholesterol as a consecutive reaction to the decomposition of linoleate, plotted versus time between collisions t′ (milliseconds). Values of k, k′, and collision time (t′) are taken from Figure 9a. Curve fitting was performed using Cricket Graph software. Curve fit equations were $k = 0.0184 \times 10^{0.555t'}$ and $k' = -0.0122 + 0.0390\ t'$. Values of r^2 were 0.979 and 0.996 respectively.

concentration of those examined, LDL was more than ten times the plasma concentration.[46] The lipid compositions of arterial LDL and of atherosclerotic lesions suggest a less heavily oxidised form of LDL compared with LDL oxidised in vitro by macrophages or copper.[10,13,14] LDL concentrations used for in vitro oxidations are frequently around 100 μg LDL protein/ml and copper concentrations are a few micromolar.[19] This gives, after 24 h of oxidation, a markedly oxidised form of LDL.[19] However we have observed that when a much higher concentration of LDL is used (e.g. 1 or 2 mg LDL protein/ml) for preparing copper-oxidised LDL, the oxidation at 24 h appears less pronounced, despite the ratio of copper to LDL being unchanged. This might have implications for oxidation within the lesion, where LDL concentrations, as mentioned above, may be high. Thus, in vitro oxidation of LDL at several mg/ml might be more relevant to the lesion than the more usual dilute LDL oxidations. Concentrations of "catalytic" copper in lesions vary widely, reportedly ranging from 0.2–28.6 μM.[47] However the complex nature of the lesion suggests that other factors besides simple concentrations of LDL and copper are undoubtedly also involved, e.g. pH, presence of "catalytic" iron, activity of cells, trapping of LDL by proteoglycan or other macromolecules, etc..

A marked feature of the results of the present study are the strong correlations between most of the parameters of oxidation. This suggests that the oxidative changes occuring in the LDL particle all occur in a tightly co-ordinated way. It is thus likely that the driving force behind the transformations is the peroxidation of the polyunsaturated fatty acid chains. This is in keeping with the results of Reaven and colleagues, who found that LDL rich in oleate (a monounsaturate) was less susceptible to oxidation than LDL rich in linoleate (a polyunsaturate).[48] Peroxidation of polyunsaturates gives rise to fatty acid pentadienyl, peroxyl and alkoxyl radicals which may then attack cholesterol, producing 7-oxysterols. Fatty acid hydroperoxides break down to give aldehydes which react with protein, capping the lysyl residues and thus giving a net increase in negative charge. Oxidation of the lipoprotein also gives increased fluorescence, although the exact nature of the fluorophore is unknown. Unfolding, fragmentation and aggregation of apo B-100 occurs as the protein becomes oxidised. Overall, the oxidation of LDL appears to be a well-defined, ordered process rather than a loose collection of changes.

The close correlation of the various parameters measured in this study means that the state of oxidation can be assessed reasonably accurately using any of the parameters. For example, knowing the relative electrophoretic mobility we can calculate how much 7β-hydroxycholesterol is present to a good approximation. The results of this study show that copper oxidation of LDL can be controlled by appropriate selection of duration of oxidation and of concentrations of LDL and copper. These results are thus applicable to preparing oxidised LDL under controlled conditions for studying its biological properties.

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