

Continuous Monitoring of Intermediates and Final Products of Oxidation of Low Density Lipoprotein by Means of UV-Spectroscopy

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The most widely used routine technique for determination of LDL 'oxidizability' is the continuous monitoring of the absorption at 234 nm in the UV spectrum of LDL, following the addition of an oxidation promotor such as copper ions. This absorption is commonly attributed to the conjugated dienes formed upon oxidation as the major intermediate, namely the hydroperoxides of polyunsaturated fatty acids, mostly linoleate hydroperoxides. These, however, are not the only products of oxidation that absorb light at 234 nm. Other products, particularly 7-ketocholesterol, also absorb light at the same wavelength. Furthermore, enals and dienals also absorb in the wavelength range of 210–300 nm. The aim of the present work was to develop a simple spectroscopic method for more detailed investigation of the kinetics of lipoprotein oxidation. The method is based on continuous measurement of the UV spectrum in the wavelength range of 210–300 nm and subsequent decomposition of the spectra into four absorption bands due to hydroperoxides, 7-ketocholesterol, dienals and enals. The sixth derivatives of the spectra, recorded during the first seven hours of copper-induced oxidation of LDL were used to monitor the growth and subsequent decay of the hydroperoxides. The resultant time course, in conjunction with difference spectra

obtained after the concentration of these intermediates decay to zero, enabled us to determine the spectra of the other oxidation products and, by that, to evaluate their time dependencies. Based on these results, we present a series of four simple equations that can be used to evaluate the concentrations of the individual products of LDL oxidation from UV absorption measurements of the mixtures at merely four different wavelengths. The resultant time dependencies of the accumulation of four major products of lipid oxidation are consistent with published data obtained through separation and chemical analysis. This simple method can be used for more meaningful routine kinetic measurements of lipids oxidation.

Key words: Low density lipoprotein, LDL, copper-induced oxidation, atherosclerosis

Abbreviations: LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids

INTRODUCTION

The oxidation of blood low density lipoproteins (LDL) gives rise to the formation of many

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products including peroxides, carbonyl and hydroxy compounds and hydrocarbons. Some of these products are toxic and are involved in the formation of atherosclerotic lesions.¹⁻³ All the components of the lipoprotein particle, including fatty acids derivatives, cholesterol and apolipoprotein B, undergo chemical transformations during oxidation. Many of the oxidation products absorb intensely in the UV region. Hydroperoxides of polyunsaturated fatty acids (PUFA) are formed during the initial stages of the reaction as the main intermediate products. The formation and subsequent decomposition of these compounds are believed to determine the whole process and the consequent biological damage.¹⁻⁴

The most widely used technique for the determination of LDL resistance to oxidation is continuous monitoring of 'conjugated diene absorption' at 234 nm.^{1,5} This method provides a simple way to get reproducible quantitative results. Assuming a definite value of extinction coefficient of dienes, the absorption values can be recalculated as 'conjugated diene concentration' in lipoprotein solutions.⁵ Nevertheless, the results are severely distorted by overlapping of absorption bands due to different intermediates and final products.

Alternative methods are available to monitor the oxidation and the temporal order of production of various intermediates and final products of the reaction. These include determination of the various components present in the system following fractionation by chromatographic techniques,^{1,6-8} colorimetric determination of specific products such as malondialdehyde (MDA)¹ or continuous measurement of free radicals by ESR spectroscopy.⁶ Nonetheless, most of these alternative methods cannot provide continuous monitoring of oxidation. Furthermore, most of these methods require either complicated data processing, unavailable standards or derivatisation of samples, which may lead to uncontrolled chemical changes of the primary products, and cannot provide continuous monitoring of oxidation. Moreover, none of these techniques is

sufficiently simple for routine testing in medical investigations.

This work presents results on the determination of LDL oxidation products by analysis of the whole UV spectra in the range of 210–300 nm. UV spectra were recorded during the first seven hours of Cu(II)-induced oxidation and decomposed into spectra of the various components. The individual absorption bands were then assigned to specific species in the reaction mixture. This procedure provides a simple, quick and continuous measurement of the accumulation of the various products in the time course of the oxidation.

MATERIALS AND METHODS

Human plasma was recovered following blood drawing from individuals fasting for 12 hours. The blood was centrifuged in chilled tubes containing Na₂EDTA (0.1% W/V final concentration; pH 7.4). The successive procedures involved in LDL isolation⁹ were started immediately after blood drawing and were all carried out at 4°C. LDL was isolated by sequential flotation of plasma at 1.019–1.050 g/ml KBr density solutions, containing 1 mM Na₂EDTA, pH 7.4 in an LB-70 Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA) using a 70.1 rotor at 40,000 rpm for 20 h. The LDL was recovered by tube slicing and re-isolated at the limiting density under the same conditions, dialyzed in the dark for 24 h against five changes of 100 volumes of 150 mM NaCl, 1 mM Na₂EDTA, pH 7.4.

Oxidation was monitored using a Hewlett-Packard diode-array spectrophotometer (HP 8452A) and^a Kontron double beam spectrophotometer (Uvikon 930). Measurements were carried out at 37°C in 1 cm quartz cuvetts with PBS in the reference cell, following the addition of freshly prepared CuCl₂ solutions to LDL solutions in PBS (pH 7.4). The ranges of LDL and CuCl₂ concentrations studied were 45–180 nM (25–100 µg protein/ml) and 0.25–20 µM, respectively. The data presented in this communication were

observed upon oxidation of 0.091 μM LDL with 5 μM CuCl_2 . For the whole range of copper concentrations studied the **maximal accumulation** of all the reaction products was proportional to the concentration of LDL and independent of CuCl_2 concentration (not shown). At copper concentrations lower than the 'saturation level' (3–5 μM) the rate of accumulation of all the products increased monotonically with CuCl_2 and above this level the rate became independent of copper concentration.

The apparent reproducibility of OD measurements and subsequent calculations was usually better than 2%.

RESULTS AND DISCUSSION

Four groups of products which are known to be formed in the time course of LDL oxidation have intensive absorption bands in the spectral range of 200–300 nm,^{5–8,10–14} as depicted in Table 1. This table, based on the known character of the UV absorption of each of the components, reflects the main pathways and stages of LDL oxidation. Different compounds of similar spectral characteristics, such as 7-ketocholesterol and 7-ketocholesteryl esters of oxidized and non-oxidized fatty acids are listed as one group of compounds. Similarly, hydroperoxidized PUFAs

(including triglycerides, cholesteryl esters, 7-ketocholesteryl esters and phospholipids) all contribute to the spectra as PUFAs hydroperoxides, reflecting the whole process of oxidation of polyunsaturated chains. Previous experiments have shown that the hydroperoxides are intermediates, thus their level first grows and subsequently decays.^{5,6,8} By contrast, the concentrations of enals and dienals grow up to the complete consumption of polyunsaturated fatty acids,^{1,7} and formation of 7-ketocholesterol proceeds until essentially all the LDL cholesterol becomes oxidized.^{1,8}

The results of a typical experiment are presented in Figure 1. The time-dependent changes in the shape clearly indicate that the spectra are superimpositions of the spectra of several components. To determine the minimal number of contributors to the spectra, as measured at all times (for different concentrations of LDL and CuCl_2), optical density matrices were analyzed for quantitative determination of the number of independent components. This was done according to the well established procedure of Marck^{15,16}: after the orthogonalization of optical density matrix, the number of non-zero rows was determined and considered as being equal to the number of the independent components. The result obtained using this procedure was that, given the accuracy of optical density measurements (0.004 OD units),

TABLE 1 Products formed as a result of LDL oxidation

Product group	Chromophore	Absorption max (nm)	Mean extinction coefficient (range) reported, OD/M	Maximal content by HPLC		
				μM^a	mol/mol LDL	expected absorption, OD ^a
PUFAs hydroperoxides ^{6,8,13} and hydroxyPUFAs ^{8,14} and their derivatives	-(CH=CH) ₂ -CH(OOH)-	234	26730	7–14	80–160	0.2–0.4
	-(CH=CH) ₂ -CH(OH)-	234	23000	1–2	15–30	0.03–0.06
2-heptenal, octenal, nonenal, other conjugated enals and their derivatives ^{7,10,12}	-(CH=CH)-CH=O	213–223	17800 (13200–22400)	9 ^b	100	0.2
2,4-nonadienal, decadienal ^{7,10,12}	-(CH=CH) ₂ -CH=O	260–274	29000 (26300–31600)	14 ^b	160	0.4
7-ketocholesterol ^{1,8,10,11} and its derivatives	-C=CH-C=O	235–238	15000 (14000–15500)	7–100	80–1100	0.1–1.5

^a data recalculated for 50 mg protein/1 LDL solution

^b assuming that the composition of the marked products is similar to that of the decomposition products of linoleate hydroperoxide

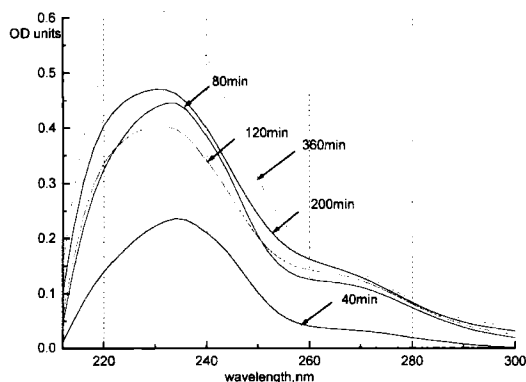


FIGURE 1 Absorption spectra of reaction mixtures of the products of LDL oxidation. Spectra recorded at 5 different time points (as indicated) are presented. The initial absorption spectrum of the mixture (50 mg protein/L, 5 μ M CuCl₂, 37°C) at zero time was subtracted.

at least four different substances must be assumed to simulate the actual spectra obtained during oxidation. This result is consistent with the preliminary considerations (see introduction and Table 1). Based on this considerations, we have assumed that the four groups of products listed in Table 1, are the only major contributors to the spectra.

Since the spectra of these compounds are known, determination of their concentration could have possibly been based on decomposition of the observed spectra. However, this can not be done by any trivial procedure because the UV spectra of conjugated carbonyl compounds are known to depend strongly on the interactions of the chromophoric groups with their surrounding mostly due to polar effects and hydrogen bonding.¹² Published spectra of these products in various solvents can therefore not be used for decomposition since these spectra can be different from the spectra of the same compound in LDL. To obtain the actual UV spectra of the various contributors at different times, we have chosen a strategy based on combination of two approaches, namely analysis of derivative spectra and detailed study of the spectral changes during the late stages of oxidation.

Even derivatives of the spectra were used to find the position of the absorption peaks.^{15,17} Since the peaks due to linoleate hydroperoxide are insensitive to the media,¹² they were identified unambiguously in the derivative spectra. In fact, the sixth derivative of the published spectrum of linoleate hydroperoxide¹³ coincides with the spectra of reaction mixtures in the range of 220–260 nm (Figure 2). This must mean that, in this spectral range, the absorption bands of the other substances are relatively broad and their intensities in the derivative spectra are therefore reduced almost to the noise level. The intensity of the most intensive peak in the derivative spectra, at 244 nm, was therefore used to compute the time dependency of the hydroperoxide content in the mixture. According to the well established fact that both

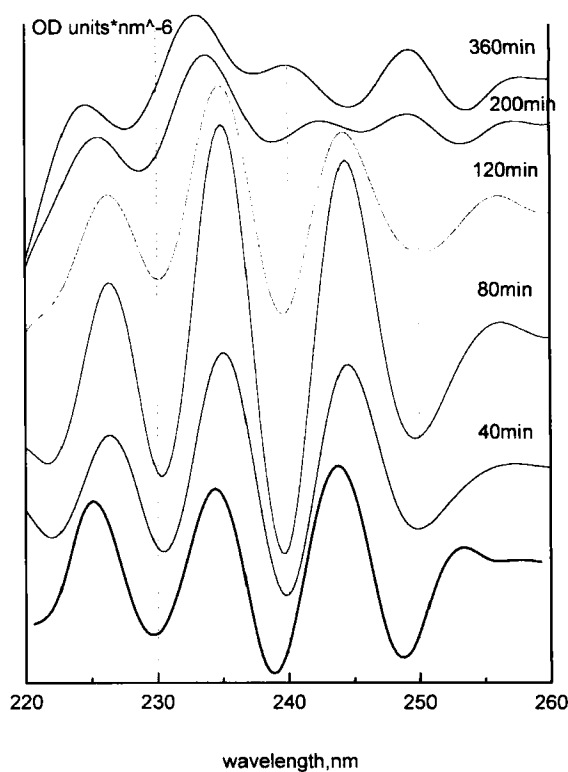


FIGURE 2 Sixth derivatives of the spectra given in Figure 1. The bold (lower) curve depicts the sixth derivative of the published spectrum⁹ of methyl linoleate hydroperoxide. The actual points obtained are presented through each 2 nm and connected by spline curves. No smoothing algorithms were used.

cis-trans and trans-trans isomeric hydroperoxides are formed in equal quantities,¹³ the spectrum of equimolar mixture of the isomers was used as reference for hydroperoxides quantitation. As obvious from Figure 2, peroxides are intermediate products: their concentration first grew and subsequently decreased. In the experiment described in Figure 1, the peroxide level became in fact negligible after 200 min.

At the time when peroxide concentration becomes negligible, the spectrum is of course due to some other components. The continuing increase of absorption following this time (210 min in Figure 3a) shows that the concentration of one or more of these components keeps increasing with time. Difference spectra obtained upon subtraction of the spectrum observed when peroxide

concentration became negligible from the spectra observed at later times are given at Figure 3b. Notably, all these difference spectra converge into one curve by multiplication of the various spectra by scaling factors, as exemplified in Figure 3b. The most straightforward interpretation of this result is that the time-dependence of the spectrum after the peroxide level became negligible resulted from the accumulation of one reaction product.

Based on the following rationale, we conclude that this product was 7-ketocholesterol:

- (i) of the major products of oxidation, besides hydroperoxides, this is the only product with an absorption maximum in this range (Table 1),
- (ii) the concentration of this component continues to grow after the oxidation of PUFA is already completed (as indicated by negligible peroxide content) and
- (iii) the absorption of this component grew to the levels expected for 7-ketocholesterol on the basis of independent chemical analysis (see below).

Based on the difference spectra (e.g. Figure 3b) it can be concluded that 7-ketocholesterol makes only a minor contribution to the spectrum at any wavelength higher than 265 nm. The absorption at 265 nm and higher wavelengths (Figures 1, 3a) is therefore predominantly due to dienals (Table 1). This means that after the peroxide concentration in the system becomes negligible (e.g. after 360 min, as in Figure 4a), the major contributors to the spectrum within the range of 240–300 nm are 7-ketocholesterol and dienals. Using the computed spectrum of 7-ketocholesterol, obtained as described above (Figure 3b), we have computed the various possible contributions of 7-ketocholesterol to the spectra. Of these possibilities only the combination given by the dash line in Figure 4a yields a symmetric absorption band for dienals (dash-and-dot line in Figure 4a). This band could be used to elucidate the time dependence of the content of dienals.

Hence, the analysis of spectra **within the spectral range of 240–300 nm** enabled elucidation of

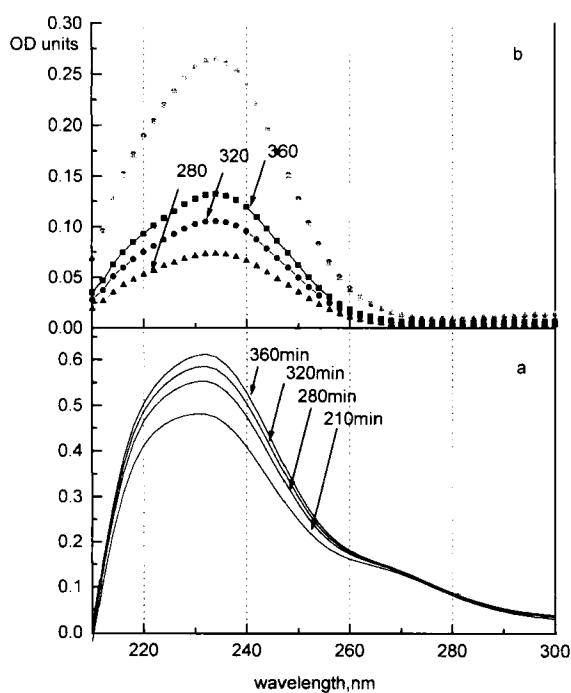


FIGURE 3 Panel a presents the absorption spectra of the reaction mixtures (50 mg protein/L, 5 μ M CuCl₂, 37°C) as measured at the later stages of oxidation. Panel b depicts the difference spectra, obtained by subtraction of spectrum at 210 min from the other spectra. The upper series of data points in panel 3b demonstrates that all the difference spectra converge to one spectrum upon multiplications by scaling factors (2.0 for the difference spectrum calculated for 360 min, 2.5 for 320 min and 3.6 for 280 min).

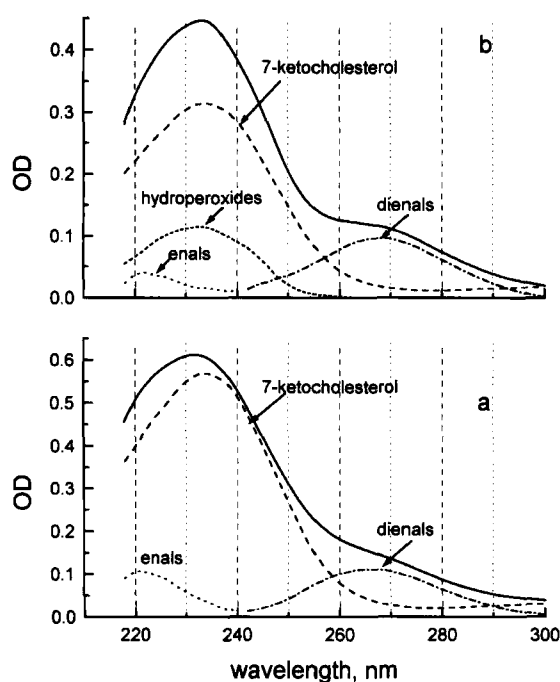


FIGURE 4 Decomposition of the spectra of LDL oxidation products as measured 360 min (panel *a*) and 80 min (panel *b*) after the addition of Cu(II) (5 μ M) to LDL (0.091 μ M). The contributions of hydroperoxides (short dash lines), 7-ketocholesterol (dash lines), dienals (dash-and-dot lines) and enals (dot lines) to the composite spectra (solid line) were evaluated as described in the text.

the contributions of dienals and 7-ketocholesterol as well as that of peroxides to the whole spectra as measured at various times. These contributions were then summed and the computed spectra were subsequently subtracted from the whole spectra **throughout the range of 200–300 nm**. An apparently symmetric absorption band was obtained at 220 nm (dot line in Figure 4b), probably due to enals (Table 1). At earlier times (e.g. after 80 min, as in Figure 4b), the contribution of hydroperoxides was evaluated from derivative spectra (data points in the lower panel of Figure 5), using the value of extinction coefficient given in Table 1. Following subtraction of this contribution from the spectra, we have treated the residual spectra using the same procedure as for Figure 4a. Thus, the time-dependent spectra could be completely decomposed into four spectra, each

resulting from a known contributor. Assuming that the published values of extinction coefficients (ϵ) of each of the contributors at the respective absorption maxima (Table 1) are valid, the spectra evaluated from our computations can be used to determine the extinction coefficients at any wavelength thus the time-dependencies of concentrations. Since the values of ϵ presented by different authors vary by $\approx 10\%$, the inaccuracy in ϵ may be a source of errors independently of the accuracy of the experiment. We have chosen to use the values of ϵ presented in spectroscopic studies which contained the original spectra (Table 1).

Using these values, the spectra obtained at various times can be decomposed into spectra of the four contributing chromophores by multiple linear regression analysis (MLR) of the whole spectral range studied. The use of this procedure has the obvious advantage of using all the available values of absorbancies. However, this procedure has very serious disadvantages due to two major reasons. First, non-characteristic absorbancies within the studied spectral range are also taken into account, which results in accumulation of inaccuracies.^{15,18} Secondly, the use of MLR for decomposition of spectra requires the introduction of sophisticated constraints to the standard procedure.^{15,19} Therefore, rather than using MLR for determination of the concentrations of the four components, with overlapping UV absorption bands, we have employed the commonly used procedure of Vierordt,^{12,15} based on Beer-Lamberts law. Specifically, we have formulated and solved four equations which present the absorbance as measured at each of four chosen analytical wavelengths as the sum of absorbancies of the components at this wavelength (sum of four products of extinction coefficient and respective concentration):

$$OD_{\lambda} = C_{\text{perox}} * \epsilon_{\text{perox}} + C_{\text{dialal}} * \epsilon_{\text{dialal}} + C_{\text{enal}} * \epsilon_{\text{enal}} + C_{7\text{-keto}} * \epsilon_{7\text{-keto}} \quad (1)$$

where OD_{λ} is the optical density at wavelength λ nm (OD units), C values are molar concentrations

of the respective component and the values of ϵ are the extinction coefficients of the respective component at the relevant wavelength.

Solution of four equations of the form of equation 1 was based on the extinction coefficients computed as described above, for actual values of optical densities at four corresponding wavelengths. This results in four empirical equations, which can be used to compute the unknown concentrations of the four major products. The choice of the four analytical wavelengths to be used was based on the following criteria^{12,15}: the wavelength of absorption maxima of the components were preferred (220, 234 and 268 nm, see Table 1 and Figure 4) and one additional wavelength was chosen at which the maximal difference between the normalized extinction coefficients of hydroperoxides and 7-ketocholesterol was maximal (250 nm, see Figure 4). This yielded the following four empirical equations:

$$C_{\text{perox}} = -27.37 \cdot OD_{220} + 76.34 \cdot OD_{234} - 127.5 \cdot OD_{250} + 49.41 \cdot OD_{268} \quad (2)$$

$$C_{7\text{-keto}} = 19.62 \cdot OD_{220} - 54.74 \cdot OD_{234} + 240.2 \cdot OD_{250} - 98.1 \cdot OD_{268} \quad (3)$$

$$C_{\text{enal}} = 68.51 \cdot OD_{220} - 34.36 \cdot OD_{234} - 30.83 \cdot OD_{250} + 11.95 \cdot OD_{268} \quad (4)$$

$$C_{\text{dial}} = -0.567 \cdot OD_{220} + 1.582 \cdot OD_{234} - 7.16 \cdot OD_{250} + 37.26 \cdot OD_{268} \quad (5)$$

Using equations 2–5, the concentration of each of the components can be evaluated at any time from the absorbance at the four chosen wavelengths. This system of equations can be treated easily and rapidly by any PC electronic spreadsheet after importing the original data from the spectrophotometer.

The time courses of accumulation of each of the compounds listed in Table 1 during Cu(II)-induced oxidation of LDL, as computed from equations 2–5, are presented in Figure 5. The time course obtained for hydroperoxides was consistent with that obtained independently from the

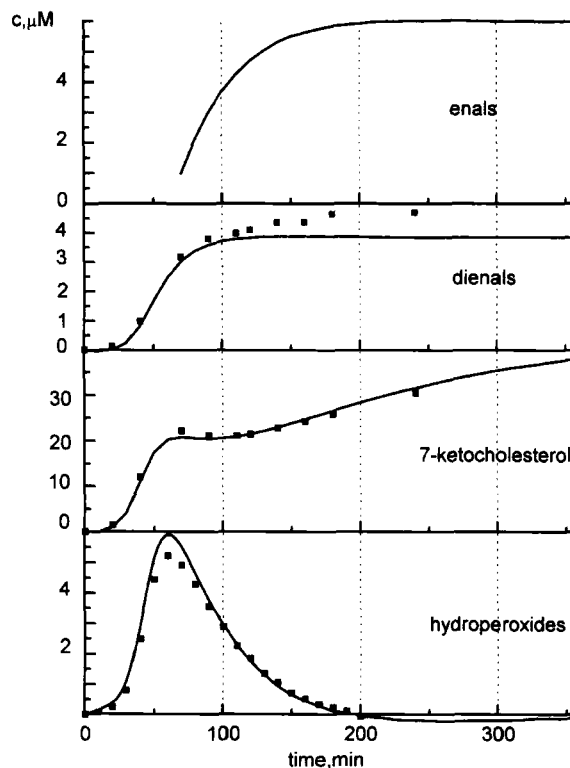


FIGURE 5 Accumulation of the reaction products in the time course of oxidation. The lines demonstrate the accumulation as calculated from the UV-spectra using equations 2–5. For hydroperoxides, the dots depict the hydroperoxide content as estimated from the intensity of the peak at 244 nm in the sixth derivative of the spectra. For 7-ketocholesterol and dienals, the dots represent the concentrations, as determined by decomposition of the residual spectra into Gaussian components (see text for details).

derivative spectra. Moreover, these results were similar, both qualitatively and quantitatively, to the results of previous chromatographic studies.^{1,6–8}

The spectra of **hydroxylinoles** are very similar to those of hydroperoxides, both in terms of band positions and shapes.^{13,14} The same is true for the derivative spectra, although the intensity of the 244 nm peaks in the 6th derivative of the spectrum of hydroxides is approximately 30% lower than in the respective spectrum of hydroperoxide (not shown). The fact that the intensity of the 244 nm peak in the derivative spectra of our reaction mixtures approached a zero level

indicates that formation of hydroxylinoleates in our experiments was very low, in agreement with the HPLC data of Kritharides *et al.*, who found that the maximal level of hydroxylinoleates is 6 times lower than that of hydroperoxides.⁸ We cannot exclude, however, the possibility that minor quantities of hydroxylinoleates were formed in the time course of oxidation and contribute to the absorbance that we attribute to 7-ketocholesterol through the use of equations 2–5.

The general trend of accumulation of **hydroperoxides** was of growth and subsequent destruction and their maximal (transient) level for the sample exemplified in Figures 1–5 was about 6 μM per 0.091 μM LDL i.e. the equivalent of about 66 PUFA peroxides per LDL particle as compared to 80–150 found by HPLC at the time when its level was maximal.^{6,8} Furthermore, the calculated concentrations of **7-ketocholesterol** were also in the range of the concentrations found by direct chromatographic HPLC measurements: when the concentrations of these relatively stable oxidation products reached steady state levels, an LDL particle contains, according to the data in Figure 5, about 400 molecules of **7-ketocholesterol**, 67 enals and 40 dienals, in good agreement with the literature data compiled in Table 1.

To evaluate the correctness and accuracy of our simple 'linear' approach, an attempt was made to determine the concentrations of the components, using the absorption values obtained by decomposition of absorption bands into Gaussian bands due to the absorption of individual components. Assuming, as previously, that after subtraction of peroxide absorption, at any time, the 'residual' spectra in the range 240–300 nm are superimpositions of only two components, namely 7-ketocholesterol and dienals, we decomposed them into two Gaussians with maximal absorptions at 234 and 268 nm, respectively. The results presented on Figure 5 are very similar to those obtained by using equations 2–5.

In evaluating the accuracy of our results, we note that the main sources of inaccuracies in determining the concentrations of the various

products are not experimental. Larger errors can be expected from the assumptions on which our computations were based. The most critical assumption is that the system can always be treated as a mixture of four reaction products. In fact, each group of products presented in Table 1 unites few substances which are slightly different in their spectral characteristics. The values of extinction coefficients are also not free from inaccuracies as mentioned above. Due to these reasons we estimate the accuracy of the whole procedure as $\pm 15\%$ of the maximal products accumulation (with the exception of enals, for which the error may be higher). The estimate for hydroperoxides, 7-ketocholesterol and dienals is based on comparison between the results obtained by using the different independent methods for treatment of experimental data (derivative method, equations 2–5 and decomposition of bands into Gaussian components). It should, however, be noted that the reproducibility of comparative studies is in fact much higher, as mentioned in 'Materials and methods', since the major contributors to errors change only slightly for all the samples.

In conclusion, the time dependencies of the UV spectrum at merely four wavelengths are sufficient to evaluate the kinetics of accumulation of intermediates and products during LDL oxidation. Using this procedure, we find that 7-ketocholesterol actually contributes significantly to the absorbance at 234 nm, which is commonly assigned to conjugated dienes. Production of 7-ketocholesterol begins at early stages of oxidation (in parallel to PUFA's oxidation) and continues at relatively high (and even growing) rate after completion of PUFA's oxidation. The formation of 7-ketocholesterol, as determined by HPLC, was reported to begin in detectable quantities after peroxidation of PUFA chains and leads to the formation of only 80 molecules of 7-ketocholesterol per LDL particle (though data for only few time points were presented).⁸ It must be mentioned, however, that 7-ketocholesterol itself is only one of the products of oxidation of the 'total cholesterol' present in LDL. Since cholesterol and

cholesteryl esters possess approximately the same susceptibility to oxidation,¹ it can be concluded that 7-ketocholesteryl esters must be present in three-fold higher quantities than 7-ketocholesterol itself. This explains the apparent contradiction between our conclusion and the result of the recent HPLC study⁸: 7-ketocholesteryl esters of oxidized and non-oxidized LDL were not identified in the HPLC investigation,⁸ though they are known to be formed¹ (and contribute to the spectra as 7-ketocholesterol).

The time dependence of UV absorbance at 234 nm does not reflect the fast decomposition of conjugated hydroperoxides. Instead, an apparent plateau is observed, which may reflect the production of 7-ketocholesterol and its derivatives. Formation of dienals also begins at early stages of oxidation and their concentration in the system approaches a constant level after peroxide destruction. Enals appear to begin forming only at later stages of oxidation and later reach a steady state level after approximately 200 min. It must, however, be mentioned that since the contribution of cholesterol, absorbing at the same range, is altered by its oxidation,^{8,10} the results for enals are distorted by this contribution. This may explain the 'negative' values of absorbance in the spectral range attributed to enals at the initial stages of oxidation (not shown in Figure 5). Consequently, the accumulation of enals, as evaluated by the method presented here, is only qualitative.

The kinetics of oxidation can be evaluated even when part of the spectral range is covered by extra strong absorption of other components present in biological systems. Absorption of albumin, for example, makes it impossible to use the absorption at 234 nm for studies of LDL oxidation in albumine-containing samples (e.g. in plasma). However, using either the derivative technique for hydroperoxides or absorption coefficients for hydroperoxides, dienals and 7-ketocholesterol, the reaction pathway can be monitored (Schnitzer *et al.*, in preparation). As obvious from Figures 1 and 5 the 'degree of oxidation of LDL' as determined from the absorbance at 234 nm, may be

quite misleading since samples containing different contents of oxidation-products can give similar absorbance at 234 nm.

The proposed methods for evaluation of the time-dependence of the content of oxidation products is *a priori* less direct than chemical analysis of products at various times by chromatographic techniques such as HPLC. However, HPLC determinations are also not devoid of shortcomings, the most serious of which is the need for extraction, fractionation and derivatisation of the reaction products prior to actual analysis.^{1,6,8} The method based on determination of absorbance at four wavelengths is simple, continuous and direct as it requires neither isolation of the reaction products nor derivatisation. This is of special importance with respect to determination of the time-dependence of accumulation of peroxides, which are relatively unstable yet important intermediates of oxidation.

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