Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes

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Abstract The alteration of structural and biological properties of human plasma low density lipoprotein (LDL) exposed to oxidative conditions is in part ascribed to lipid peroxidation. The objective of this investigation was to measure quantitatively several parameters in oxidizing LDL indicative for lipid peroxidation. Exposure of freshly prepared EDTA-free LDL to an oxygen-saturated buffer led to a complete depletion of alpha- and gamma-tocopherol within 6 hr, thereafter lipid peroxidation commenced as indicated by the kinetics of the loss of linoleic (18:2) and arachidonic (20:4) acids, the formation of aldehydic lipid peroxidation products and fluorescent apoB. Within 24 hr of oxidation, on average 79 nmol of 18:2 (initial 345) and 12.8 nmol of 20.4 (initial 25.6) were oxidized per mg of LDL and the sample contained in total 7.1 nmol of aldehydes with the following molar distribution: 36.6% malonaldehyde, 25% hexanal, 8.9% propanal, 8.2% 4-hydroxynonenal, 7.6% butanal, 4.1% 2.4-heptadienal, 3.4% pentanal, 3.4% 4-hydroxyhexenal, and 2.5% 4-hydroxyoctenal. Malonaldehyde was predominantly (93%) in the aqueous phase, whereas the other aldehydes remained mostly (34–98%) within the LDL particle, where the total aldehyde concentration was in the range of 12 mM. Oxidized LDL exhibited a 1.6-fold enhanced electrophoretic mobility. Similarily, native LDL incubated for 5 hr with aldehydes showed increased electrophoretic mobility. At equal concentrations (5 mM) 4-hydroxynonenal was most effective, followed by 2,4-heptadienal, hexanal, and malonaldehyde. This study reports for the first time the rate and extent of the change of LDL constituents occurring during lipid peroxidation.—**Esterbauer, H., G. Jürgens, O. Quehenberger, and E. Koller.** Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* 1987. **28:** 495–509.

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The low density lipoprotein (LDL) of human serum consists of a glycoprotein (apoB), free and esterified cholesterol, phospholipids, triglycerides, and lipid-soluble vitamins. LDL delivers cholesterol to the peripheral cells by means of a receptor-mediated endocytosis (1). Modification of LDL by oxidative processes results in an enhanced uptake by the scavenger receptor of macrophages (2, 3). This altered functionality might, in vivo, affect cholesterol metabolism and lead to a conversion of macrophages into the lipid-laden foam cells that are constituents characteristic of the atherosclerotic plaques (4). In vitro oxidative modification of LDL can be mediated by exposure to cultured endothelial cells (5, 6), smooth muscle cells (6, 7), or to monocytes and neutrophils (8). This cell-modified LDL contains increased levels of thiobarbituric acid-reactive substances, which has been taken as evidence that lipid peroxidation and possibly also phospholipase A_2 activity are essentially involved in the modification process (2, 6–9). Most of the cell-mediated effects can be mimicked by incubation of LDL solely in a cell-free medium under oxidative conditions (2, 3). Moreover the addition of antioxidants (BHT, vitamin E, glutathione) or chelators for transition metal ions (EDTA, desferrioxamine, diethylenetriamine pentaacetic acid) prevented the modification of LDL by cells or in a cell-free medium which further strengthens the possible involvement of free radical chain reactions (5–7).

The molecular mechanisms of the oxidation processes and the reaction pathways leading to a modification of LDL are largely unknown. It has been proposed that aldehydes such as malonaldehyde (10, 11) or 4-hydroxynonenal (12) generated by lipid peroxidation from polyunsaturated fatty acids contained in the LDL phospholipids interact with apoB and specifically modify lysine residues important for the interaction of LDL with the apo B/E receptor of fibroblasts (13, 14).

The aim of this investigation was to study the oxidation of LDL in a cell-free medium by quantitative analysis of

Abbreviations: LDL, low density lipoprotein; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; BHT, butylated hydroxytoluene; HPLC, high performance liquid chromatography; TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid.

the fatty acids, vitamin E, and aldehydic lipid peroxidation products, which can interact with apoB and thereby modify the structural and biological properties of LDL.

MATERIALS AND METHODS

4-Hydroxyalkenals were prepared as previously described (15); hexanal and 2,4-heptadienal were purchased from Aldrich, Steinheim; all other aldehydes were from Merck, Darmstadt. The fatty acid methyl esters used for GLC references were from Supelco, Bellefonte. TLC was performed on precoated silica gel 60 plates (Merck). The dialysis bag was from Serva Heidelberg and was kept for 30 min in boiling water prior to use. If not otherwise stated, all other chemicals and solvents were of analytical grade and were purchased from Merck or Sigma, Munich.

Isolation and autoxidation of LDL

Human plasma LDL was prepared by step-wise ultracentrifugation within a density cut-off of d 1.020–1.050 g/ml (16). Two preparations were from single female donors (LDL-1, LDL-2), and two were from pooled plasma of two male and female donors (LDL-3, LDL-4). The donors were healthy volunteers under 25 years of age. Whole blood was obtained by venipuncture after 12 hr of fasting. EDTA (1 mg/ml) was present throughout all steps of the preparation. Butylated hydroxytoluene (20 μM) was added to the plasma. For the oxidation experiments, LDL was dialyzed for 48 hr at 4°C in the dark against vacuum-degassed 0.01 M phosphate buffer, pH 7.4, containing 0.16 M NaCl and 0.1 mg/ml chloramphenicol. LDL samples were stored until use at 4°C for not longer than 12 hr. These samples are designated as "freshly prepared or native LDL." As judged by SDS-PAGE in 3.75% gels and staining with Coomassie blue R 250, only the apoB 100 band was detectable (12). Autoxidation of LDL was performed essentially according to Schuh, Fairclough and Haschemeyer (17). The dialyzed LDL solution was diluted with the dialysis buffer to a final concentration of 1.5 mg/ml, transferred into the dialysis bag, and immersed in a threefold volume of dialysis buffer. The system was kept in the dark at room temperature for 24 hr and oxygen was continuously bubbled through the external buffer. For the kinetic measurements, samples were withdrawn at 0, 3, 6, 12, 18, and 24 hr.

Fatty acid, vitamin E and BHT analysis

The LDL solution (3 ml) was spiked with 0.5 mg of BHT and 0.1 mg of heptadecanoic acid as internal standard and extracted four times with 2 ml of a mixture of chloroform–methanol 2:1 (v/v). The pooled extract was brought to a volume of 3 ml. Two ml of this lipid solution was dried with anhydrous sodium sulfate and evaporated to dryness under nitrogen in a centrifuge tube equipped with a Teflon-lined screw-cap. The residue was dissolved in 0.5 ml of benzene; 1 ml of boron trifluoride–methanol reagent (20%) was added and the solution was kept for 90 min at 110°C. After the addition of 1.5 ml of water, the fatty acid methyl esters were extracted three times with 1.3-ml portions of benzene. Phase separation was achieved by centrifugation. The pooled extracts were brought to a small volume and separated by TLC (benzene), to remove most of the BHT. The zone of fatty acid methyl ester was scraped off and eluted with dichlormethane. The solution was brought to 0.3 ml and the esters were separated by capillary GLC on a 50-m glass column with CP Sil 88 (Chrompack) as described (18).

For vitamin E estimation (19) in LDL, aliquots of the lipid extracts (0.5 ml) were evaporated to dryness under nitrogen. The residue was dissolved in methanol, centrifuged, and analyzed by HPLC on a Spherisorb ODS column (4.5 × 250 mm) with methanol as mobile phase and a fluorescence detector set at an excitation of 292 nm and an emission of 335 nm.

To determine the amount of BHT possibly retained in LDL after dialysis, 3 ml of a 48-hr dialyzed sample was worked up as described above for the fatty acid analysis, except that in this case supplementation with additional BHT and TLC separation were omitted. The final solution was analyzed by capillary GLC on the 50-m glass CP Sil 88 column with the same program used for the fatty acid separation (Fig. 1, 2) except that the attenuation was increased to 4 × 1. Under these conditions BHT should give two peaks in the GLC chromatogram, the major one (free form of BHT) immediately prior to the 14:0 peak and the second one (probably the methoxy form of BHT produced during methylation with BF_3/methanol) between 17:0 and 18:0. The position of the BHT peaks and the detection response factor for the first peak relative to the internal standard 17:0 were determined by analyzing a reference sample containing 0.1 mg of BHT and 0.1 mg of 17:0 under exactly the same conditions as described for the LDL sample. The detection response factor BHT/17:0 was 1.22. The threshold level of BHT that could be determined in this way was 10 ng/mg of LDL. The GLC chromatogram of the dialyzed LDL did not show the two BHT peaks.

Identification and quantification of aldehydic lipid peroxidation products

At the end of the 24-hr autoxidation period (for the kinetic measurements at the indicated time points) the LDL was withdrawn from the dialysis bag and 4 ml of this LDL solution plus 12 ml of the external buffer were mixed with 4 ml of 1.8 mM dinitrophenylhydrazine in 1 N HCl. For the native LDL (4 ml), 12 ml of freshly prepared 0.01 M phosphate buffer was used. The mixture was sonicated and kept for 2 hr at room temperature in the dark and an additional hour at 4°C. The dinitrophenylhydrazones

were extracted with dichlormethane (3 × 7 ml); phase separation was achieved by centrifugation. The pooled extract was stored for 1 hr at − 20°C and filtered. The dinitrophenylhydrazones were preseparated by TLC (silica gel 60, 20 × 20 cm, Merck) into three classes as described (20). Final analysis of the classes was by HPLC on a Spherisorb ODS 5 μ (4.6 × 250 mm) column essentially as previously described (20). In one experiment, aldehydes in the LDL solution and the external dialysis buffer were derivatized and then analyzed separately.

For the analysis of 4-hydroxynonenal in its underivatized form, 3 ml of the autoxidized LDL solution or 9 ml of the external dialysis buffer were worked up as described (21), using extraction on Extrelut-column followed by a purification step with a 1-ml C 18 Bond Elut column. The purified extract was separated by HPLC on a Sepherisorb ODS 5-μ column with acetonitrile–water 9:10 (v/v). The effluent was monitored at 223 nm. In one case the on-line spectrum of the peak assumed to be 4-hydroxynonenal was recorded.

Fluorescence measurements

For fluorescence measurements, the freshly prepared or oxidized LDL was diluted with the dialysis buffer to a final concentration of 1.5 mg of LDL/ml and the emission spectra were recorded with an excitation of 360 nm. The lipid fraction was prepared by extracting 3 ml of LDL solution (1.5 mg/ml) four times with 2 ml of a mixture of chloroform–methanol 2:1 (v/v). Phase separation was achieved by centrifugation. The pooled extracts were brought to a volume of 3 ml by gassing with nitrogen and the fluorescence spectra (Ex 360 nm) were scanned. The aqueous phase of the chloroform–methanol extract was removed, the precipitated apoB was washed twice with 3 ml of water, dried under nitrogen, and redissolved in 3 ml of 3% aqueous SDS solution. This solution of apoB was then used for fluorescence spectroscopy. For incubation experiments of LDL with various aldehydes such as hexanal, 2,4-heptadienal, malonaldehyde, and 4-hydroxynonenal, LDL was dialyzed for 48 hr against the above-mentioned dialysis buffer containing 1 mg/ml EDTA. Thereafter, LDL was incubated for 4 hr at 37°C with the aldehydes (1.0 mM) and then separated into the lipid fraction and apoB. The fluorescence spectra were scanned on a Jobin Yvon JY3D spectrofluorimeter. A slit width of 10 nm was used. The instrument was standardized with quinine sulfate (0.1 μg/ml in 0.1 N H_2SO_4) to give a fluorescence intensity of 100 at 450 nm, when excitation was done at 350 nm. All spectra were recorded with an excitation of 360 nm.

Other analyses

The concentration of LDL was estimated gravimetrically. Agarose gel electrophoresis of LDL was performed with the Lipidophor system kindly supplied by Immuno AG (Vienna, Austria).

For determining the effect of aldehydes on the electrophoretic mobility of LDL, 1 ml of chloroform solutions containing 5 or 8 μmol of the respective aldehydes was brought to dryness with nitrogen and 1 ml of freshly prepared LDL (2.5 mg/ml) in 0.01 M phosphate buffer, pH 7.4, containing 1 mg EDTA/ml was added. The mixture was kept at 37°C for 5 hr and then applied directly to the agarose gel.

RESULTS AND DISCUSSION

Effect of autoxidation on the fatty acid composition and vitamin E content of LDL

Four preparations of human plasma LDL were used for this study, two were isolated from plasma of single donors (LDL-1, LDL-2) and two were obtained from pooled plasma (LDL-3, LDL-4). The fatty acids contained in the freshly prepared LDL samples in bound and free form were extracted by the method of Folch, Lees, and Sloane Stanley (22), converted to their methyl esters, and separated by capillary GLC (18). A typical profile of such a separation is shown in **Fig. 1,** and the amount of the individual fatty acids of the four LDL samples are listed in **Table 1.** The major fatty acids were linoleic (18:2), palmitic (16:0), and oleic acid (18:1) comprising about 85% of the total fatty acids, followed by stearic (18:0), arachidonic (20:4), myristic (14:0), and pamitoleic (16:1) acid. In addition to these main fatty acids, traces of other fatty acids could be detected in LDL when larger samples were analyzed and when the detector attenuation was increased **(Fig. 2).** Based on the retention times and chromatograms of reference fatty acid mixtures, the minor peaks were tentatively identified as 20:0, 22:0, 24:0, 18:3, 20:3, 22:5, and 22:6. The total amount of these trace fatty acids was not more than 2% in any of the four LDL samples and they were therefore not considered in the quantitative analysis. The reproducibility of the fatty acid analysis was checked with LDL-2 and for all fatty acids, except arachidonic acid; the standard deviation was less than 5%. The somewhat higher standard deviation for arachidonic acid (± 8%) was likely due to its instability. The variation of the percentage of the individual fatty acids within the four LDL samples (Table 1) is much higher than the standard deviation of the method and most likely reflects the effect of different dietary conditions on the fatty acid distribution of LDL (23, 24). The total amount of fatty acids, as calculated on the basis of heptadecanoic acid as internal standard (Table 1), ranged from 219 to 273 μg/mg of LDL; the amount of 18:2 and 20:4 varied from 76 to 118 μg/mg of LDL and from 6 to 9 μg/mg of LDL respectively. The Folch extracts were also

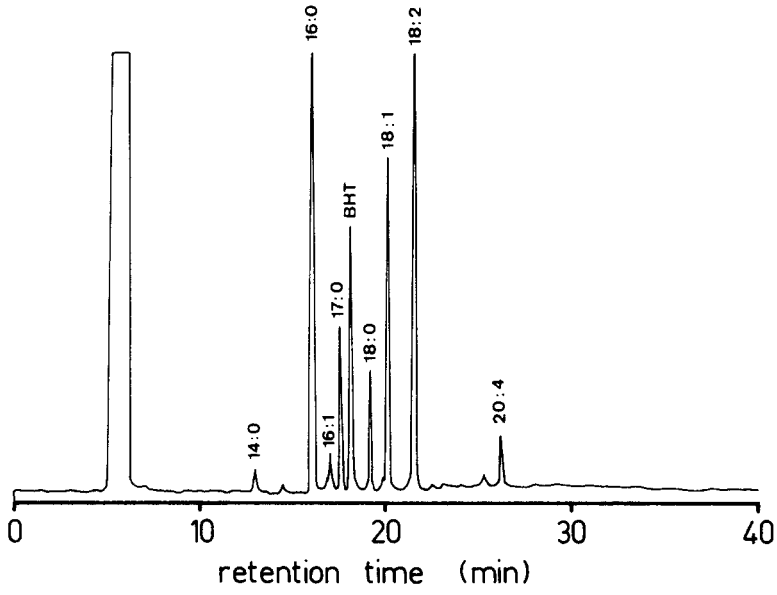

Fig. 1. Fatty acid profile of human plasma LDL. The fatty acid methyl esters equivalent to 3 mg of LDL were dissolved in 0.3 ml of dichloromethane, 0.5 μl of this solution was separated by GLC on a 50-m glass capillary column. The attenuation was 16 × 1; 17:0 was the internal standard for quantification. The BHT peak solely results from the BHT added to the LDL solution prior to lipid extraction.

analyzed by HPLC for the vitamin E content of the LDL samples (Table 1). The values varied for alpha-tocopherol from 0.695 to 1.015 μg/mg of LDL and for gamma-tocopherol from 0.047 to 0.089 μg/mg of LDL.

To study the effect of autoxidation, solutions (1.5 mg/ml) of the four LDL preparations were placed in a dialysis bag which was immersed, in the dark, in a buffer continuously gassed with oxygen. This autoxidation procedure was previously described by Schuh et al. (17) and it has, among others, the advantage of avoiding foaming, which occurs when oxygen is bubbled directly into the LDL solution. After a 24-hr exposure to the oxygen-saturated buffer, all LDL samples were still clear; the color of the solutions, however, had changed from yellow to colorless. The LDL solutions were then removed from the dialysis bag and the fatty acid content and distribution were again analyzed (Table 1). The 24-hr exposure to oxygen-saturated buffer resulted in decrease of linoleic and arachidonic acid by 22% and 50%, respectively, whereas the other fatty acids remained more or less constant or slightly increased. This confirms reports in the literature (18, 25) that major changes caused by oxidative

TABLE 1. Fatty acids and vitamin E in native and autoxidized LDL

	LDL-1		LDL-2		LDL-3		LDL-4		Mean ± SD	
	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized	Fresh	Oxidized
						μg/mg of LDL				
14:0	5.9	5.5	9.3	8.2	4.1	2.5	7.3	7.1	6.7 ± 2.2	5.9 ± 2.5
16:0	68.2	62.2	72.4	68.9	63.1	65.5	79.7	75.1	70.8 ± 7.0	67.9 ± 5.5
16:1	3.7	3.5	8.4	7.8	4.0	3.4	4.1	4.1	5.1 ± 2.2	4.7 ± 2.1
18:0	11.2	9.9	11.8	11.6	12.1	12.9	14.2	12.9	12.3 ± 1.3	11.8 ± 1.4
18:1	36.6	34.6	41.1	40.2	41.2	35.3	41.5	40.5	40.1 ± 2.3	37.6 ± 3.2
18:2	87.7	74.9	76.3	65.0	104.6	59.0	117.7	99.3	96.6 ± 18.3	74.6 ± 17.8
20:4	5.9	3.9	7.7	4.5	9.1	2.4	8.5	4.9	7.8 ± 1.4	3.9 ± 1.1
Total fatty acids	219.3	194.5	227.0	206.3	238.2	181.0	273.0	243.9	239.4 ± 23.7	206.4 ± 27.0
Total PUFA[a]	93.6	78.8	84.0	69.5	113.6	61.4	126.1	104.1	104.3 ± 19	78.5 ± 18.5
α-Tocopherol	0.695	0.00	n.e.[b]	n.e.	0.969	0.00	1.015	0.00	0.893 ± 0.173	0.00
γ-Tocopherol	0.047	0.00	n.e.	n.e.	0.068	0.00	0.089	0.00	0.068 ± 0.021	0.00

[a]Polyunsaturated fatty acids.
[b]Not estimated.

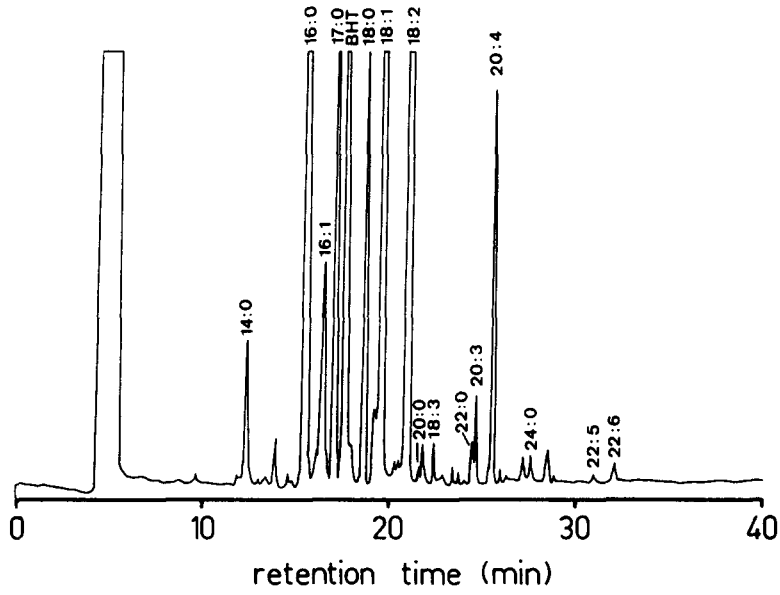

Fig. 2. Fatty acid profile of LDL showing the minor fatty acids. Sample and separation was as in Fig. 1. The esters were dissolved in 0.15 ml of dichloromethane, and 1 μl was injected and attenuation 8 × 1.

conditions are in the highly unsaturated fatty acids. The total amount of polyunsaturated fatty acids (18:2, 20:4) consumed in the 24-hr experiment was on average 25.6 μg/mg of LDL, about 85% of which was 18:2 and 15% 20:4. This clearly shows that in LDL linoleic acid is the major source for lipid peroxidation products, although it is less susceptible to autoxidation. The kinetics of the disappearance of 18:2 and 20:4 was followed in one experiment with LDL-4 **(Fig. 3).** Within the first 3 hr of exposure to the oxygen-saturated buffer, no decrease of the polyunsaturated fatty acids occurred, which indicates that 18:2 and 20:4 withstood autoxidation completely during this period. This initial lag phase was then followed by a rapid consumption of both fatty acids, lasting for about 9 hr, the average rate of disappearance being 4.5 nmol/hr for 18:2 and 1.0 nmol/hr for 20:4. Thereafter the rate of consumption slowed down again, although only fractions of about 10% (18:2) and 30% (20:4) of the potentially oxidizable fatty acids were consumed. This result suggests that in LDL only a rather small portion of the polyunsaturated fatty acids is easily attacked by oxygen, whereas the major fraction is in fact highly resistant to oxidative degradation. The decrease of polyunsaturated fatty acids was exceptionally high in LDL-3, in which the linoleic acid decreased within 24 hr from 104.6 to 59 μg/mg of LDL and the arachidonic acid from 9.1 to 2.4 μg/mg of LDL. After 48 hr the sample still contained 59 μg/mg of LDL linoleic acid, and the arachidonic acid decreased to 1.3 μg/mg of LDL.

As mentioned in the experimental section, all plasma samples were supplemented with BHT to prevent oxidation during the isolation procedure. The question therefore arises as to whether the resistance of the LDL fatty acids against oxidation is due to BHT retained in the LDL and not completely removed by the dialysis step. To check this, a GLC method was developed to measure the BHT content of LDL. This method is highly sensitive and would allow detection of BHT at a level above 10 ng/mg of LDL. The GLC trace of a 48-hr dialyzed LDL sample did not show a BHT peak, even when the chromatogram was recorded at a high attenuation of 4 × 1. In the dialyzed LDL, as used in our oxidation studies, the amount of retained BHT, if present at all, must therefore be less than 10 ng/mg of LDL. When compared to the amount of 18:2 and 20:4 and the tocopherols, this is an extreme low value and it is highly unlikely that such minute amounts could have a protective effect against oxidation.

The vitamin E contained in LDL is highly sensitive towards oxygen. (Fig. 3). Within the first 6 hr the alpha-tocopherol dropped by 97% from 1.020 to 0.030 μg/mg of LDL; similarly gamma-tocopherol decreased from 0.090 to 0.020 μg/mg of LDL. Vitamin E is known to be one of the most potent lipophilic antioxidants and the comparison of the time-dependence of the decrease of fatty acids and vitamin E indicates that the oxidative degradation of the polyunsaturated fatty acids in LDL only starts when the vitamin E is largely consumed. Nevertheless, the fatty acid autoxidation does not proceed faster under conditions (12–24 hr) of a nearly complete depletion of vitamin E.

EDTA (1 mg/ml), added to the buffer in which the LDL-containing dialysis bag was immersed, completely

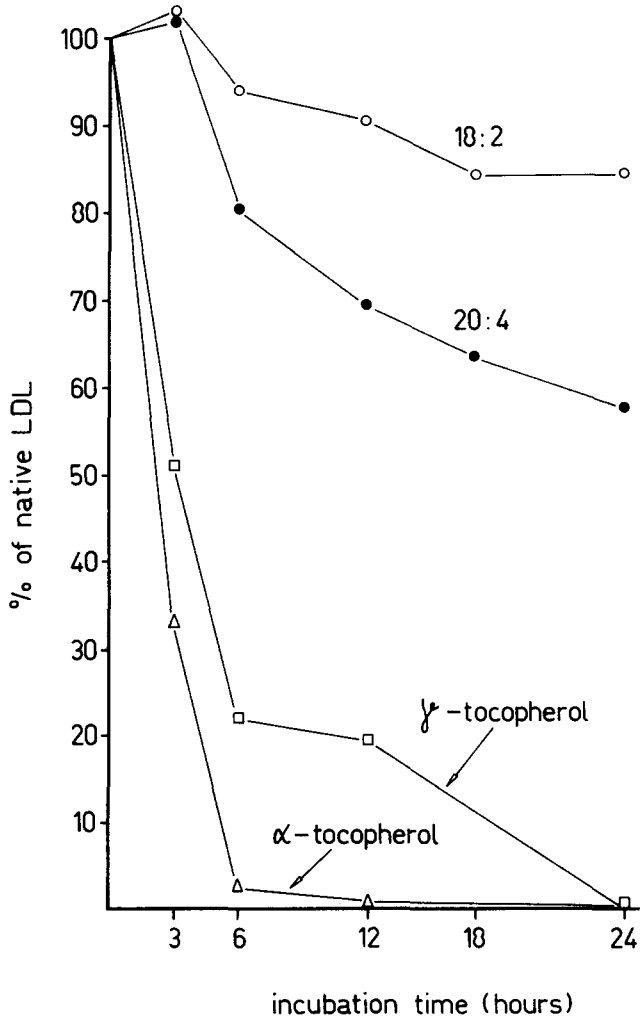

Fig. 3. Kinetics of the decrease of linoleic acid, arachidonic acid, and vitamin E during autoxidation of LDL. LDL (1.5 mg/ml) in a dialysis bag was exposed for 24 hr to an oxygen-saturated buffer. At the indicated time points the fatty acids were analyzed by GLC and vitamin E was analyzed by HPLC. The analysis was performed with the sample LDL-4 (Table 1).

prevented the loss of fatty acids and vitamin E for 24 hr. This strongly suggests that bivalent transition metal ions present as contaminants in the phosphate buffer or in the dialysis bag (kept for 30 min in boiling double-distilled water) are involved in the initiation of the lipid peroxidation process.

Identification and quantitative measurement of aldehydic lipid oxidation products

It is known from many other investigations (26–29) that the oxidative degradation of polyunsaturated fatty acids leads to the formation of a complex variety of aldehydes, including malonaldehyde, alkanals, 2-alkenals, 2,4-alkadienals, and 4-hydroxyalkenals. We examined the aldehydes present in the samples LDL-2, LDL-3, and LDL-4 before and after the 24-hr oxidation. The principle of the method employed included derivatization of the aldehydes to the 2,4-dinitrophenylhydrazone derivatives, preseparation into three different aldehyde classes by TLC, and final separation by HPLC (20). **Fig. 4** shows, as an example, the HPLC profile of class I, which consists mainly of the hydroxyaldehydes, of a 24-hr oxidized LDL sample. Based on the retention times of authentic aldehydes separated under identical conditions, the following aldehydes were identified in class I: 4-hydroxyhexenal, 4-hydroxyoctenal, and 4-hydroxynonenal. The former two aldehydes were present only in the oxidized LDL, whereas the latter one was also present in small amounts in the freshly isolated LDL. The rather large peaks eluting prior to 4-hydroxyhexenal are not yet identified and the ascertainment of their nature will be the subject of future studies. The two larger peaks in this group are not necessarily products of autoxidation since they were

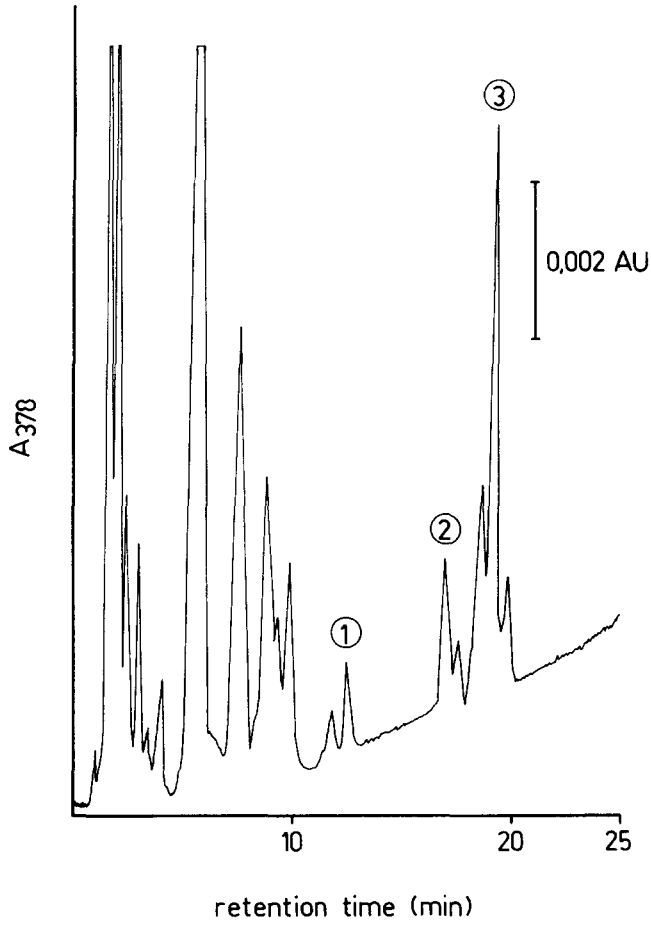

Fig. 4. Separation of the class I aldehydes in oxidized LDL by HPLC. LDL was oxidized in a dialysis bag for 24 hr, a volume equivalent to 6 mg of LDL was derivatized with dinitrophenylhydrazine. The hydrazones were preseparated by TLC into three classes, the class I aldehydes (hydroxyaldehydes) were then separated on a 5-μ Spherisorb ODS column with a linear gradient of 55% methanol-45% water to 100% methanol. Flow rate 1 ml/min, detection 378 nm. The numbered peaks are: 1, 4-hydroxyhexenal; 2, 4-hydroxyoctenal; 3, 4-hydroxynonenal.

found in nearly equal intensity in both the native and oxidized LDL.

The LDL samples contained only traces of the aldehydes belonging to class II (ketoaldehydes) and autoxidation did not change the HPLC pattern; therefore, the separation is not shown.

The separation of the corresponding class III, which consists of alkanals, 2-alkenals, and alkadienals, is shown in **Fig. 5.** In class III we could identify propanal, butanal, pentanal, hexanal, and 2,4-heptadienal; the latter two were only present in the oxidized LDL, and hexanal was, in all three oxidized LDL samples investigated, by far the major aldehyde. In all LDL samples, varying amounts of butanone were found also. This ketone could be derived principally from oxidized cholesterol (30). Varying concentrations of butanone were also found (31) in freshly prepared hepatocytes, where it appears to be a natural metabolite not related to lipid peroxidation. Similarly, it is unlikely that butanone in LDL results from lipid oxidation, since no increased values were found in the oxidized LDL.

The measurements of malonaldehyde as thiobarbituric acid reactive substances (TBARS) are most frequently used to demonstrate lipid peroxidation in biological systems including LDL (32, 33). This test is not specific for malonaldehyde, since many other compounds also give the pink pigment under the assay conditions. With dinitrophenylhydrazine, malonaldehyde gives a pyrazol derivative and not a hydrazone (34), which means that malonaldehyde cannot be estimated as a hydrozone, nor does it interfere with the HPLC estimation of the other aldehydes as dinitrophenylhydrazones. To show whether free malonaldehyde is formed during LDL oxidation, we employed the direct HPLC method previously developed (32), which allows the qualitative and quantitative detection of malonaldehyde without derivatization. The chromatogram of the buffer in which the LDL (LDL-3)-containing dialysis bag was immersed for 24 hr clearly showed the peak for malonaldehyde. Some free malonaldehyde was also present in the oxidized LDL solution in the dialysis bag itself. The quantitative determination revealed that of the total free malonaldehyde 70% escaped from the dialysis bag and diffused into the outside buffer, whereas only 30% remained associated with the LDL solution. The TBA assay performed on the same sample gave only slightly (± 15%) higher malonaldehyde values, which shows that most of the so-called TBARS is in fact free malonaldehyde.

One aldehyde of particular interest is 4-hydroxynonenal. This aldehyde is highly reactive towards proteins (18, 28) including LDL (35), cytotoxic to certain cells (36), and can modify LDL by binding to lysine residues (12). It seemed, therefore, desirable to support the chromatographic findings by additional and independent experiments to prove the existence of this aldehyde in LDL.

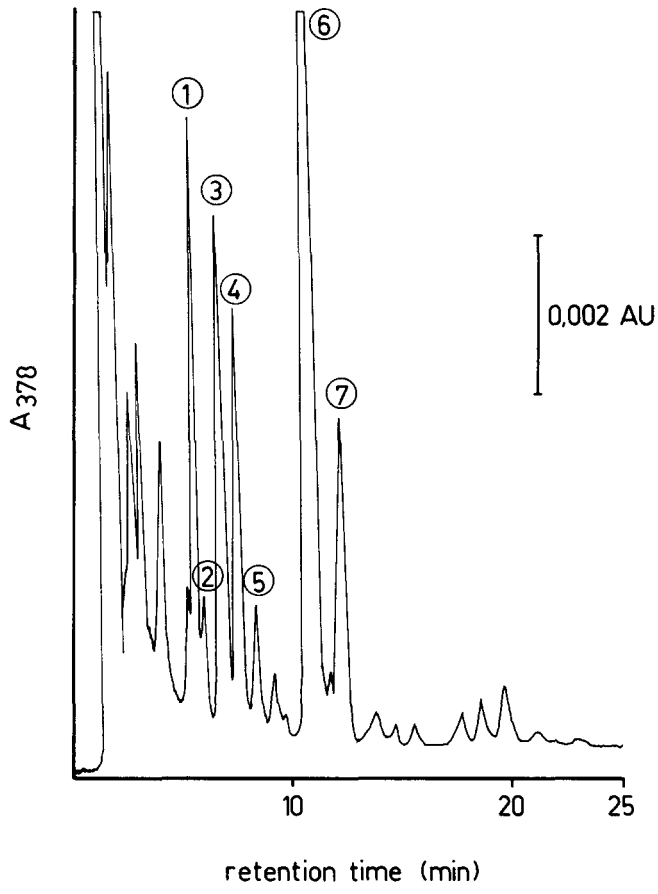

Fig. 5. Separation of the class III aldehydes in oxidized LDL by HPLC. Sample preparation was as in Fig. 4. The class III aldehydes recovered by TLC were separated on a 5-μ Spherisorb ODS column with 77.5% methanol–22.5% water, flow rate 1 ml/min, detection at 378 nm. The numbered peaks are: 1, propanal; 2, acetone; 3, butanal; 4, butanone; 5, pentanal; 6, hexanal; 7, 2,4-heptadienal.

The peak material from the HPLC separation (Fig. 4), assumed to be the dinitrophenylhydrazone of 4-hydroxynonenal, was collected and investigated by mass spectroscopy. Chemical ionization with methane as reactant gas gave, in agreement with authentic 4-hydroxynonenal-dinitrophenylhydrazone, the following ions indicative for the correct molecular weight: 377 (M + 41), 365 (M + 29), 337 (M + 1) and 319 (M + 1–18). The electron impact spectrum showed all fragments including the molecular ion (m/e = 336) reported previously (27) for the 4-hydroxynonenal-dinitrophenylhydrazone. To prove that 4-hydroxynonenal is present in oxidized LDL in its free form and not artifactually created from a precursor during the acidic derivatization with dinitrophenylhydrazine, the aldehyde was also directly analyzed. For that, the oxidized LDL was worked up to by two solvent extraction steps as previously described (21) and the 4-hydroxynonenal-enriched extract was separated without derivatization by HPLC. The chromatogram clearly showed the peak of free 4-hydroxynonenal, moreover, the UV

TABLE 2. Aldehydes in native LDL and LDL autoxidized 24 hr

	LDL-1		LDL-2			LDL-3		LDL-4		Mean ± SD	
	Native	Oxidized	Native	Oxidized[a]	Oxidized	Native	Oxidized	Native	Oxidized	Native	Oxidized
					nmol/mg of LDL						
Propanal	0.27	n.e.[b]	0.23	0.45	1.19	0.14	0.36	0.10	0.38	0.24 ± 0.14	0.64 ± 0.47
Butanal	0.27	n.e.	0.24	0.58	1.08	0.42	0.24	0.14	0.30	0.33 ± 0.17	0.54 ± 0.46
Pentanal	0.00	n.e.	0.12	0.22	0.27	0.05	0.18	0.06	0.28	0.09 ± 0.08	0.24 ± 0.05
Hexanal	0.00	n.e.	0.00	0.00	3.24	0.00	0.77	0.00	1.36	0.00 ± 0.00	1.79 ± 1.28
2,4-Heptadienal	0.00	n.e.	0.00	0.00	0.44	0.00	0.15	0.00	0.29	0.00 ± 0.00	0.29 ± 0.14
4-Hydroxyhexenal	0.00	n.e.	0.00	0.00	0.29	0.00	0.21	0.00	0.22	0.00 ± 0.00	0.24 ± 0.04
4-Hydroxyoctenal	0.00	n.e.	0.00	0.00	0.31	0.00	0.00	0.00	0.22	0.00 ± 0.00	0.18 ± 0.16
4-Hydroxynonenal	0.06	n.e.	0.49	0.47	0.97	0.23	0.31	0.23	0.51	0.29 ± 0.18	0.59 ± 0.33
Malonaldehyde	0.31	1.39	0.45	0.43	1.66	0.64	4.83	n.e.	n.e	0.46 ± 0.13	2.62 ± 1.90
Butanone	2.08	n.e.	1.14	2.90	1.15	0.32	0.63	1.88	1.10	1.35 ± 0.80	0.96 ± 0.28
Total aldehydes, excluding malonaldehyde	0.60		1.08	1.25	7.79	0.84	2.22	0.53	3.56	0.92 ± 0.31	4.52 ± 2.9

The four LDL samples analyzed are those reported in Table 1. The aldehydes, except malonaldehyde, were estimated by HPLC as dinitrophenylhydrazones.

[a]Autoxidation in the presence of EDTA (1 mg/ml).

[b]Not estimated.

spectrum of the peak recorded on line was identical to that recorded from a separation of an authentic 4-hydroxynonenal, with a maximum at 225 nm. The quantitative evaluation of the chromatograms from the separations of the dinitrophenylhydrazone and the free aldehyde gave the same amount of 4-hydroxynonenal per mg of LDL, which indicates that both methods are equally suited for its measurement. It should be noted here, that we usually prefer the hydrazone method because it is more sensitive and also gives the whole range of the other aldehydes, both qualitatively and quantitatively. Thus, all values for the individual aldehydes (except malonaldehyde) listed in **Table 2** are those obtained by HPLC separation of the dinitrophenylhydrazones. In the experimental design used for the LDL oxidation, the aldehydes generated within the LDL can, when they escape from the LDL particle, diffuse through the wall of the dialysis bag into the external buffer. That this occurs was shown in one example where the aldehyde concentration was determined inside the dialysis bag and in the external buffer separately **(Table 3).** It is evident that the aldehydes are most unequally distributed and in general are more concentrated inside the LDL-containing dialysis bag. It seems reasonable to asume that the distribution reflects the different lipophilicity of the aldehydes. The more lipophilic an aldehyde is, the more pronounced should be its tendency to dissolve and remain in the lipid phase of the LDL particle and thus not be able to diffuse freely across the dialysis membrane into the buffer phase. The situation is very similar to equilibrium dialysis ex-

TABLE 3. Distribution of the aldehydes of autoxidized LDL between the dialysis bag and the external buffer

	Inside Dialysis Bag	Outside Dialysis Bag	Total[a]	Associated with LDL	Approximate Concentration in LDL
	μM	*μM*	*nmol*	*% of total*	*mM*
Propanal	1.26	0.173	1.78	61	1.44
Butanal	1.08	0.180	1.62	55	1.18
Pentanal	0.30	0.033	0.40	67	0.35
Hexanal	4.54	0.103	4.85	91	5.88
2,4-Heptadienal	0.65	0.003	0.66	98	0.86
4-Hydroxyhexenal	0.22	0.073	0.44	34	0.19
4-Hydroxyoctenal	0.32	0.043	0.45	62	0.36
4-Hydroxynonenal	1.19	0.083	1.44	77	1.48
Malonaldehyde	0.75	0.580	2.48	7	0.15

Twenty ml of LDL-2 solution (1.5 mg/ml) in a dialysis bag was immersed in 60 ml of oxygen-saturated phosphate buffer (0.01 M, pH 7.4) for 24 hr. The concentration of the aldehydes inside and outside the dialysis bag was then estimated. The approximate concentration of the aldehydes within the LDL particle was calculated on the assumption that the aldehydes are dissolved in the lipid phase which constitutes about 75 weight % of total LDL and has a density of 1.0 g/ml.

[a]Based on 1 ml inside and 3 ml outside.

periments for determining receptor–ligand interactions and the concentration of the aldehyde associated with the LDL is the difference between the concentration inside and concentration outside. It is assumed for this calculation that the aldehyde molecules not associated with LDL move freely across the membrane and are in equilibrium distribution between inside and outside. Control dialysis experiments with 20 μM 4-hydroxynonenal in the absence of LDL led to an equilibrium distribution within 1 hr and it is certainly reasonable to suppose that the other aldehydes listed in Table 3 behave similarly. The rate of the generation of aldehydes by the oxidizing LDL particle is slow during the late phase (12–24 hr) of oxidation (see below) and its influence on the equilibrium distribution can be neglected. 2,4-Heptadienal, hexanal, and 4-hydroxynonenal remained 98, 91, and 77% associated with the LDL, indicating that they are the most lipophilic aldehydes generated in the oxidation process. On the contrary, malonaldehyde is the least lipophilic oxidation product and only 7% remained in the LDL particle. Additional important information that can be derived from the results of the distribution experiments is the approximate concentration of the aldehydes within the lipid phase of LDL. This calculation is based on the assumption that the aldehydes are uniformly distributed in the lipid phase which amounts to about 75 weight % of LDL and has a density of about 1.0 g/ml. According to this calculation, the total concentration of the aldehydes in the 24-hr-oxidized LDL-2 was approximately 13 mM, and 90% was due to aldehydes other than malonaldehyde.

As mentioned above, the reported quantitative estimation of the aldehydes inside and outside the dialysis bag was performed only once with the 24-hr-oxidized LDL-2. For routine analysis, pooled samples of three volumes of buffer and one volume of LDL were analyzed. Similarly, the freshly prepared LDL was diluted with three volumes of fresh buffer and then quantitatively analyzed by the dinitrophenylhydrazone method. The amounts of the individual aldehydes thus found in the native and 24-hr-oxidized LDL samples are given in Table 2. On the average, the oxidized LDL samples contained five times more aldehydes as compared to the fresh LDL (4.52 vs. 0.92 nmol/mg LDL). There was, however, a remarkable difference between the three samples. For example, LDL-3 gave 0.97 nmol of hexanal/mg whereas LDL-2 gave 3.24 nmol/mg. The respective values for 4-hydroxynonenal were 0.31 and 0.77 nmol/mg. Malonaldehyde, on the other hand, was highest in oxidized LDL-3. This is additional support for our previous statements (20, 31) that malonaldehyde or TBARS are not reliable indications to describe fully the extent of lipid peroxidation.

The kinetics of the development of the various aldehydes in a 24-hr-oxidation experiment **(Fig. 6)** showed a lag phase of approximately 3 hr for 4-hydroxynonenal, 4-hydroxyhexenal, 2,4-heptadienal, pentanal, and butanal, whereas the generation of propanal, hexanal, and 4-hydroxyoctenal had already commenced in the first 3 hr. The initial rates were highest for hexanal (0.132 nmol/hr) and propanal (0.058 nmol/hr); this was, however, about 50 to 100 times lower than the rate of the consumption of the polyunsaturated fatty acids. Similar to the decrease of the fatty acids, the increase of most aldehydes tended to level off after a 12-hr oxidation period. The reasons for the decrease of butanal and 4-hydroxyoctenal are unclear. When EDTA (1 mg/ml) was added to the external oxygenated buffer, virtually the same aldehyde values were found after 24 hr as were already present in the freshly prepared LDL. This indicates that EDTA does not affect the basal level of aldehydes but completely blocks their additional formation.

Effect of autoxidation and aldehyde treatment on the fluorescence properties and the electrophoretic mobility of LDL

Native plasma LDL exhibited a weak and diffuse fluorescence in the region 400–600 nm when excitation was performed at 360 nm; after 24 hr oxidation the fluorescence intensity was strongly increased in the 420–460 nm region **(Fig. 7).** It is known from previous studies (37) performed with three-dimensional fluorescence spectroscopy, that LDL contains at least 13 different chromophores that contribute to the LDL fluorescence in the visible range, 6 of them are ascribed to apoB and 7 to the lipid fraction. In agreement with reported preliminary results (38), it was found that autoxidation mainly affected the fluorescence properties of apoB and led to a strong emission maximum at 430 nm. This enhancing effect of autoxidation on the apoB fluorescence was observed in all four LDL samples studied **(Table 4).** The relative changes, however, were remarkably different and ranged from 135 to 280% as compared to the native LDL (= 100%). Much less pronounced was the increase of the 430 nm fluorescence in the LDL lipids (Table 4). From this one can conclude that the fluorescence increase of total LDL is largely due to apoB. The change of the apoB fluorescence during autoxidation is a time-dependent process. No increase of the initial fluorescence (17 relative fluorescence units) was observed during the first 3 hr. This initial lag phase was then followed by a rapid increase to 26 (6 hr), 29 (12 hr), and 31 (24 hr) relative fluorescence units. Thereafter (24–48 hr, LDL-3) the generation of new fluorophors nearly ceased, indicating that autoxidation had led to a maximum fluorescence of apoB that could not be exceeded. The most likely explanation is that the protein contains only a certain number of functional groups with a potential to be converted to fluorophors in the autoxidation process and that after about 24 hr the LDL is depleted of such groups. The assumption of a maximum value for the 430 nm fluorescence intensity of apoB would also explain why LDL possessing a priori a

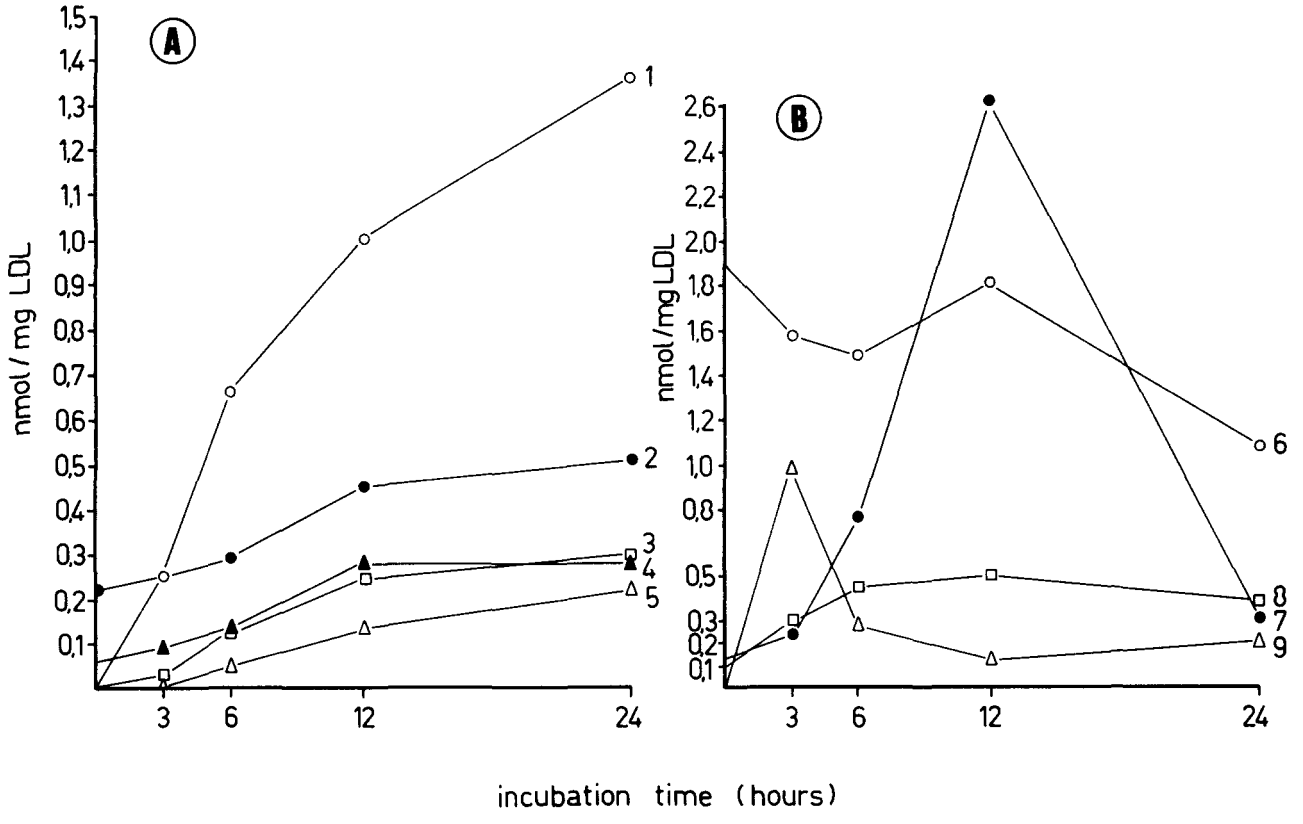

Fig. 6. Kinetics of the formation of aldehydes during autoxidation of LDL. The experiment was performed as described in Figs. 4 and 5, and the aldehydes present at the indicated time points were estimated by HPLC as dinitrophenylhydrazones. A: 1, hexanal; 2, 4-hydroxynonenal; 3, 2,4-heptadienal; 4, pentanal; 5, 4-hydroxyhexenal. B: 6, butanone; 7, butanal; 8, propanal; 9, 4-hydroxyoctenal.

high fluorescence (LDL-1) responds only weakly to autoxidation with the formation of new fluorophors (Table 4).

The mechanisms leading to the formation of the fluorophors in apoB or in other biological samples exposed to oxidative conditions are largely unknown. The most frequently accepted assumption is that aldehydic lipid oxidation products condense with lysine residues to form Schiff's bases which are further rearranged yielding fluorescent structures. In this way malonaldehyde could form, by consecutive condensation with two neighbouring amino groups, the fluorescent amino-imino-propene structure. However, the fluorescence properties in terms of emission and of excitation maxima of proteins (39) and of LDL (38) treated with malonaldehyde are significantly different from those found in oxidized biological samples (40, 41). It is, therefore, unlikely that the 430 nm fluorescence of apoB is due to modification by malonaldehyde generated in the oxidation process. We have shown (38) that incubation of LDL with 4-hydroxynonenal reveals an apoB with exactly the same spectral characteristics (excitation maximum 358 nm, emission maximum 430 nm) as apoB isolated from autoxidized LDL. If 4-hydroxynonenal is, in fact, involved in the formation of the fluorophor, one would expect some correlation between the amount of 4-hydroxynonenal detectable in native or oxidized LDL and the fluorescence intensity of the respective apoB. Such a plot is shown in **Fig. 8.** The values used for the construction of this curve were obtained from LDL-2, LDL-3, and LDL-4 prior to and after 24 hr oxidation as well as from the kinetic experiment with LDL-4. The data points fit fairly well in a hyperbolic curve. Nonlinear regression analysis gave a maximum fluorescence intensity of 60.4 ± 12.5 and a constant K for half-maximum fluorescence of 0.48 ± 0.25 nmol of 4-hydroxynonenal/mg of LDL. The hyperbolic curve can be interpreted as successive saturation of a limited number of potential fluorogenic groups with 4-hydroxynonenal. Moreover, one must assume that a certain fraction (about 50%) of these groups is already saturated by 4-hydroxynonenal in native LDL. In agreement with this is the observation that 4-hydroxynonenal was found in all freshly prepared LDL samples, and in all preparations the Ex 358/Em 430 nm fluorophor was clearly detectable by three-dimensional fluorescence spectroscopy (37). It should be emphasized here that no linear or hyperbolic correlation exists between fluorescence intensity and any of of the other individual aldehydes (hexanal, propanal, etc.) or the total amount of carbonyls.

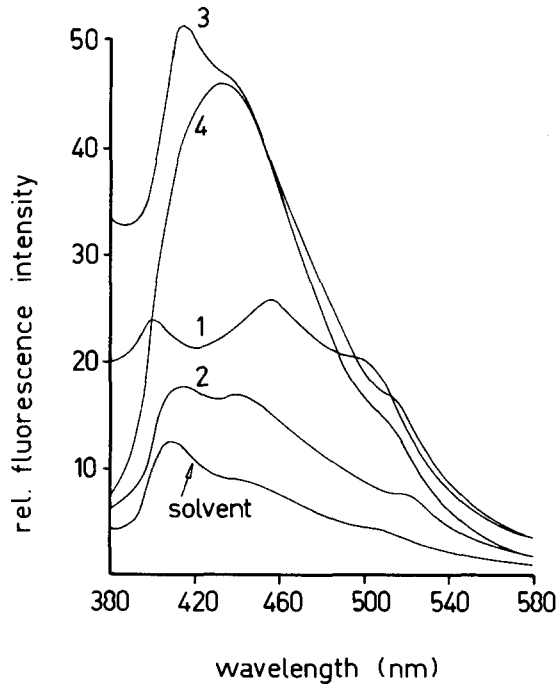

Fig. 7. Emission fluorescence spectra at 360 nm excitation of native LDL and LDL oxidized 24-hr. 1, Native LDL (1.5 mg/ml) in 0.01 M phosphate buffer pH 7.4. 2, ApoB from 3.0 mg of native LDL dissolved in 2 ml of 3% aqueous SDS solution. 3, LDL 24-hr-oxidized (1.5 mg/ml) in 0.01 M phosphate buffer, pH 7.4. 4, ApoB from 3.0 mg of LDL 24-hr oxidized dissolved in 2 ml of 3% aqueous SDS solution. Solvent, fluorescence of the 3% SDS solution.

Treatment of freshly prepared LDL with hexanal did not lead to a fluorogenic apoB; 2,4-heptadienal, on the other hand, produced a fluorescent apoB with an excitation maximum at 345 nm and an emission maximum at 425 nm.

In agreement with others (2, 3, 6), we also found that autoxidation renders the LDL more negatively charged and thereby increases the anodic electrophoretic mobility on agarose gels. Compared to the native LDL, the relative electrophoretic mobility of oxidized LDL was about 1.6. It has been suggested (11) that the change of the electrophoretic mobility is caused by the condensation of malonaldehyde with positively charged ε-amino groups of lysine residues, since LDL incubated with high concentrations of this aldehyde (100–200 mM) exhibited an increased electrophoretic mobility (10, 11). Such an effect, however, is not restricted to malonaldehyde. It has been shown previously (12, 34), and confirmed in this study, that 4-hydroxynonenal treatment also increases the relative electrophoretic mobility **(Table 5).** With 5 mM 4-hydroxynonenal, the electrophoretic mobility increased to 1.87. Somewhat less effective, compared on a molar base, were hexanal and 2,4-heptadienal; at a concentration of 5 mM, these aldehydes increased the relative electrophoretic mobility to 1.13 and 1.31. At a concentration of 8 mM, these aldehydes gave relative electrophoretic mobilities of 1.31 and 1.44, respectively. 4-Hydroxynonenal could not be tested at a concentration of 8 mM because the LDL solution became very turbid and finally the LDL precipitated. Obviously all types of aldehydes, i.e., n-alkanals, 2,4-alkadienals, 4-hydroxyalkenals, malonaldehyde, are capable of increasing the electrophoretic mobility of LDL to some extent. This, together with the finding that all of these aldehydes are produced during autoxidation of LDL, suggests that the modification of LDL to a more negatively charged particle results from a concerted action of more than one aldehyde. By their high reactivity, the 4-hydroxyalkenals are predominantly involved in this process, but their effect could be enhanced in an additive fashion or synergistically by the many other aldehydes concomitantly formed during lipid oxidation processes.

CONCLUSION

Several aspects important for the critical evaluation of the hypothesis that lipid peroxidation can alter the biological functionality of LDL have received little attention. Most important, in our opinion, are the stoichiometry and the kinetics of the processes in cells and in cell-free media; in other words, which and how many molecules of fatty acids are degraded, how fast is the process, and how many metastable or stable endproducts are formed.

In most studies regarding LDL oxidation, the measure-

TABLE 4. Effect of autoxidation on the fluorescence of total LDL, LDL lipids, and apoB

	Total LDL		Lipid Fraction		ApoB		
	Native	Oxidized	Native	Oxidized	Native	Oxidized	% Increase (Native ApoB = 100%)
LDL-1	n.e.[a]	n.e.	17	19	27	36	135%
LDL-2	n.e.	n.e.	8	13	18	37	216%
LDL-3	22	48	14	21	17	47[b]	280%
LDL-4	25	38	15	19	17	32	183%
Mean ± SD	23.5 ± 1.5	43.0 ± 5.0	13.5 ± 3.8	18.0 ± 3.4	19.7 ± 4.8	38.0 ± 6.3	(203 ± 61%)

Lipids and apoB from native LDL and LDL autoxidized for 24 hr were prepared and dissolved in chloroform–methanol and 3% SDS, respectively. The emission spectra were recorded at an excitation of 360 nm. The values given are the fluorescence intensities at 430 nm, relative to the quinine standard.

[a]Not estimated.

[b]After 48 hr oxidation, the relative fluorescence intensities were 21 and 48 for the lipid fraction and apoB, respectively.

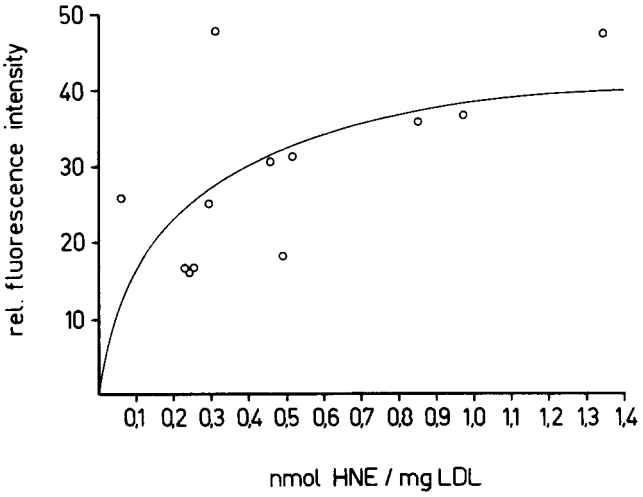

Fig. 8. Relationship between the fluorescence intensity of apoB at 430 nm and the 4-hydroxynonenal content of LDL. The data points were from native and 24-hr-oxidized LDL (Table 5) and from kinetic experiments where the change of the fluorescence was followed over an autoxidation period of 48 hr.

ment of malonaldehyde by the thiobarbituric acid assay is employed to demonstrate that lipid peroxidation has or has not occurred (2, 3, 6, 7, 42). The problems with this assay are manifold, e.g., the low specificity and the lack of correlation with the degree of fatty acid oxidation (43). Malonaldehyde, or its immediate precursor (cyclic endoperoxides), can only be formed from polyunsaturated fatty acids possessing three or more methylene-interrupted double bonds, but not from linoleic acid (43, 44), which constitutes 92% of the polyunsaturated fatty acids in LDL (Table 1). This means that linoleic acid in LDL could be completely oxidized without being recognizable by the thiobarbituric acid assay. We have, as shown in this report, measured quantitatively the effects of autoxidation in a cell-free medium on the fatty acids and vitamin E content of LDL and the formation of specific aldehydes derived from oxidative degradation of lipids. Also investigated were the effects of autoxidation and aldehydic autoxidation products on the fluorescence properties and electrophoretic mobility of LDL. The conclusions we wish to draw from our findings are as follows.

1) For one of us (H.E.), who has in the past studied lipid peroxidation in many other biological systems, the most surprising result was the high resistance of the polyunsaturated fatty acids (18:2, 20:4) in LDL against oxidation. In microsomes, isolated hepatocytes, and homogenates of many tissues the fatty acids are quickly oxidized within minutes or a few hours (18, 20, 25, 27, 45). On the contrary, in LDL the polyunsaturated fatty acids completely withstand autoxidation for at least 3 hr and, even when the lipid peroxidation process is finally initiated, the rate of the disappearance of the polyunsaturated fatty acids is slow. Thus, after 24 hr exposure to an oxygen-saturated buffer, the LDL still contains 78% of linoleic and 50% of arachidonic acid originally present in the samples (Table 1). Obviously lipid peroxidation does not proceed in LDL in an autocatalytic chain reaction but rather in a retarded mode. The cause for this resistance against oxidation certainly does not rely on BHT retained in LDL, since not a trace of BHT was detected in the dialyzed LDL. From studies with rat liver microsomes (46), it is known that lipid peroxidation primarily attacks the unsaturated fatty acids contained in the two positions of phosphatidylethanolamine and it is intriguing to speculate that in LDL a certain type of polyunsaturated fatty acids is also attacked by lipid peroxidation.

2) The initiation of lipid peroxidation in LDL is obviously intimately linked with its vitamin E content as can be concluded from the different kinetic experiments. Within the first hours of exposure of LDL to the oxygenated buffer, the alpha-tocopherol (initial 1.015 μg/mg of LDL) dropped by 70% (3 hr) and 98% (6 hr) and only the vitamin E-depleted LDL was able to undergo lipid peroxidation as evidenced by the time course of the loss of fatty acids (Fig. 3), formation of fluorescent material (see text), and aldehydic products (Fig. 6). The estimation of the exact threshold level of vitamin E that could protect LDL against oxidation was not within the scope of this work and would afford the measurements of many more time points within the 3- and 6 hr time periods. From this one can assume that cell-mediated oxidation of LDL (2, 5–8) is either preceeded by a destruction of vitamin E or that cells possess mechanisms that circumvent the protective action of vitamin E.

3) Lipid peroxidation in LDL is accompanied by the formation of various aldehydes arising from chain cleavages of polyunsaturated fatty acids. This was anticipated from many other studies with peroxidizing biological systems (for review see 18, 28). In oxidized LDL the identified aldehydes are in sequence of the relative molar distribution after 24 hr of oxidation: malonaldehyde, hexanal, propanal, 4-hydroxynonenal, butanal, 2,4-heptadienal, 4-hydroxyhexenal, pentanal, and 4-hydroxy-

TABLE 5. Electrophoretic mobility of native, autoxidized, and aldehyde-treated LDL

Treatment	Electrophoretic Mobility
Stored at 4°C	1.00
Stored at 37°C, 5 hr	1.00
Autoxidized, 24 hr	1.55–1.65
5 mM 4-Hydroxynonenal, 37°C, 5 hr	1.87
5 mM Malonaldehyde, 37°C, 5 hr	1.10
5 mM 2,4-Heptadienal, 37°C, 5 hr	1.31
5 mM Hexanal, 37°C, 5 hr	1.13
8 mM 2,4-Heptadienal, 37°C, 5 hr	1.44
8 mM Hexanal, 37°C, 5 hr	1.31

All incubation buffers, except for autoxidation, contained EDTA (1 mg/ml).

octenal (Table 2). The total amount of these aldehydes was 7.14 nmol/mg of LDL compared to 1.38 nmol/mg of LDL already present in the freshly prepared LDL sample. Thus, 4.76 nmol/mg of LDL was formed during autoxidation and remained in the system in a detectable form. In order to get some realistic impression of what this figure means in relation to the loss of the polyunsaturated acids (on average 90.8 nmol/mg of LDL) it is reasonable to base the stoichiometry on μg of carbon atoms. From Table 1 it would appear that, on the average, 19.8 μg of fatty acid carbon atoms/mg of LDL disappeared, whereas, as calculated from Table 2, only 0.355 μg of C-atoms appeared in the aldehydes, that is only 2% of the total lost fatty acid carbon atoms. This figure would not be very much different if the calculation was based on the individual experiments, i.e., LDL-2 fresh versus LDL-2 oxidized, etc. The conclusion that must be drawn from this quantitative comparison is that either the major part (98%) of the polyunsaturated fatty acids was converted to products other than aldehydes or that most of the aldehydes had reacted with LDL constituents (apoB, lipids) and were therefore not detectable in our assay.

That such reactions do occur is clearly evident from the increase of the fluorescence of apoB and also from the effect of aldehydes on the electrophoretic mobility of LDL. Nevertheless, it does not fully explain the fate of the fatty acid oxidation products. Malonaldehyde accounted in our experiments for only 0.5% of the lost fatty acid carbon atoms. In the literature, we could not find any absolute figures for fatty acid loss, only the relative distribution (3, 23, 47). For example, Ball et al. (3) exposed LDL to 100 μM Cu^{2+} for 24 hr and found 23 nmol of malonaldehyde per mg of protein, and the 20:4 and 18:2 decreased by 75 and 50% relative to 18:0. Assuming that their LDL preparation had about the same fatty acid content and distribution as ours, these figures would translate to 39.5 μg of carbon atoms lost as fatty acids and 0.21 μg of carbon recovered as malonaldehyde (0.5% of total loss), which is fully consistent with our results.

From all this, it seems reasonable to assume that the main pathway of lipid peroxidation in LDL is not chain cleavage reactions (which would mainly yield aldehydes) but oxidation reactions where the fatty acid chain remains intact.

4) The aldehydes, although minor products, could nevertheless play an important role in the modification of the apoB. Most aldehydes remain associated with the LDL particle, most likely dissolved in the lipid phase, where they reach a total concentration of approximately 12 mM (Table 3). Such concentrations are certainly high enough for chemical reactions with amino acid side groups of apoB embedded in, or in close vicinity to, the lipid phase. Malonaldehyde is a hydrophilic compound and most of it is released from the LDL particle into the outer aqueous phase, where the concentration is only in the 1-μM range. The other aldehydes also do not build up significant concentrations (range 0.04–0.18 μM) in the aqueous phase in which the LDL particle is dissolved and such concentrations are not high enough to modify proteins in a reasonable time (28). In fact, incubation of LDL with aldehyde concentrations of about 1 μM does not enhance the fluorescence or the electrophoretic mobility (34). It should be stressed that the peculiar situation of generating the aldehydes within the LDL particle cannot be reproduced in experiments with aldehydes added externally in low concentratrions, an aspect that has been already discussed in some detail for peroxidizing microsomes (48). Our results strongly suggest that the strong fluorophor (Ex 358 nm/Em 430 nm) in apoB of oxidized LDL results from a reaction of the lipid peroxidation product 4-hydroxynonenal with certain amino acid residues of the protein. It has also been shown that modification of LDL with 4-hydroxynonenal (5 mM) reduces the recognition of LDL by the apoB/E receptor of fibroblasts and increases its negative surface charge (14). Modification of LDL with malonaldehyde (100–200 mM) leads to an enhanced uptake of LDL by human monocyte macrophages followed by cholesteryl ester accumulation (10). Haberland, Fogelman, and Edwards (11) demonstrated that the uptake of malonaldehyde-modified LDL by the scavenger receptor of monocyte macrophages occurred only above a threshold neutralization of 60 mol of lysine residues/mol of LDL by malonaldehyde. However, it was suggested that the recognition by the scavenger receptor not only depended on the pure net charge modification of LDL but probably involved critical lysine residues (49).

5) The most characteristic and significant chemical parameters in which oxidized LDLs are different from freshly prepared LDL are: complete depletion of vitamin E, presence of hexanal, 2,4-heptadienal, 4-hydroxyhexenal, and 4-hydroxyoctenal, increased electrophoretic mobility, and strongly enhanced apoB fluorescence at 430 nm with 360 nm excitation.

Careful and extended quantitative chemical analysis could reveal many more characteristic changes and improve our knowledge of the basic chemistry of the lipid peroxidation process in LDL.

We dedicate this article to Professor Dr. Anton Holasek in recognition of his merits and continuous efforts to stimulate basic lipoprotein research in Austria. This work was supported by the Österreichischen Fonds zur Förderung der wissenschaftlichen Forschung Projektnummer P6176B and by the Jubiläumsfonds der Österreichischen Nationalbank. We appreciate the technical assistance of Gerhard Ledinski, Josef Stangl and Gerd Kager.

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