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INVESTIGATION OF LIPID PEROXIDATION IN HUMAN LOW DENSITY LIPOPROTEIN

O. QUEHENBERGER, E. KOLLER, G. JÜRGENS[†] and H. ESTERBAUER[‡]

Institute of Biochemistry, University of Graz, Schubertstraße 1, [†]Institute of Medical Biochemistry, University of Graz, Harrachgasse 21/III, Austria

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Human plasma low density lipoprotein (LDL) exposed to oxygen saturated buffer becomes depleted of alpha-tocopherol within 3 to 6 hours. Thereafter, lipid peroxidation commences as evidenced by the loss of 18:2 (67 nmol/mg LDL) and 20:4 (12 nmol/mg LDL) and the concomitant formation of 4-hydroxy-nonenal (0.28 nmol/mg LDL) and fluorescent compounds. The major fluorophor in apo B of oxidized LDL has an excitation maximum at 355 nm and an emission maximum at 430 nm. A fluorophor with the same spectral properties is produced in apo B, if LDL is incubated with 4-hydroxynonenal, whereas malonaldehyde gives a fluorophor with excitation and emission maxima at 400/470 nm. Three-dimensional fluorescence spcetroscopy proved to be an useful tool in analysing the complex fluorescence of apo B.

KEY WORDS: LDL, autoxidation, lipid peroxidation, 4-hydroxynonenal, malonaldehyde, fluorescence

INTRODUCTION

A number of reports appeared recently which suggest that modification of plasma low density lipoprotein (LDL) by oxygen free radical reactions and lipid peroxidation is causally involved in the development of atherosclerosis.[1–3] LDL plays a central role for the transport and metabolism of cholesterol and structural changes of its lipid and/or apo B moiety could have far reaching effects on its functional properties. Thus it was shown[3,4] that LDL incubated with cultured cells (human umbilical vein endothelial cells, aortic smooth muscle cells, activated macrophages) becomes oxidized as evidenced by the accumulation of thiobarbituric acid reactive substances (TBARS). Moreover such cell modified LDL had an increased negative surface charge,[4] was cytotoxic to fibroblasts[5] and was no longer recognized by the classical LDL receptor,[6] but only be the scavenger (acetyl LDL) receptor on the plasma membrane of macrophages.[7]

This alteration of LDL seems not to be an unique property of cells, since more or less the same modification of LDL takes place when incubated in a cell free medium under prooxidative conditions i.e. oxygenated buffer, addition of Cu^{++} and absence of antioxidants (BHT, vitamin E) or metal chelators such as EDTA. Moreover, it was found that incubation of LDL with the lipid peroxidation product malonaldehyde also yields a lipoprotein with an increased negative surface charge which has no affinity for the LDL receptor but an enhanced recognition through the macrophage scavenger receptors.[8]

‡ Correspondence should be addressed to Prof. Dr. H. Esterbauer, Institute of Biochemistry, University of Graz, Schubertstraße 1, A-8010 Graz, Austria

Since the malonaldehyde concentrations necessary to produce this modification are in the range of 100 to 200 mM, which is extremely high from the biological point of view, we have questioned the possibility that malonaldehyde generated within the LDL particle in the course of lipid peroxidation could be responsible for the structural and functional alterations.[9]

We have shown[10] that a great variety of aldehydes other than malonaldehyde are formed in many biological systems during lipid peroxidation. 4-Hydroxynonenal (HNE) is one of the major aldehydes detectable in peroxidized samples, this aldehyde most likely arises from the degradation of ω-6 polyunsaturated fatty acids such as 18:2 and 20:4. HNE is highly reactive towards proteins[11] and can already at concentrations of 1 mM alter the chemical and biological properties of LDL very similar to malonaldehyde or lipid peroxidation.[9]

The immediate question is, whether HNE is generated by peroxidizing LDL and if so, whether this in situ formed HNE does react and thereby modify the apo B. In this report we present results which clearly prove the occurence of HNE in oxidized LDL. Moreover, through the application of three dimensional fluorescence spectroscopy (fluorescence intensity topograms = FIT), we have obtained some good indications, that the fluorescence of apo B in oxidized LDL is due to modifications caused by HNE. Particular emphasis is given to the FITs, since this new technique could have a very broad application for the investigation of lipid peroxidation in biological systems.

MATERIALS AND METHODS

All solvents, buffers and detergents were of analytical grade and purchased from Merck. Other chemicals required for reference and/or identification purposes were the fatty acid methylesters (Supelco), the tocopherols (Merck) and various aldehydes (Merck). 4-Hyroxyalkenals were prepared in our laboratory.[11] Fresh whole blood was obtained by venipuncture from healthy human volunteers under 25 years of age. Human plasma LDL was prepared by step-wise ultracentrifugation within a density cut-off of $d = 1.020 - 1.050\ g/cm^3$ as described.[9]

The LDL was immediately dialyzed for 48 h at 4°C against degassed 0.01 M phosphate buffer (pH 7.4) containing 0.16 M NaCl and 0.1 mg/ml chloramphenicol to remove EDTA added during the isolation procedure. Autoxidation was essentially performed as described by Schuh *et al.*[12] The LDL was adjusted to 1.5 mg/ml, transferred to a dialysis bag and placed in a 3-fold volume of the above described dialysis buffer which was continuously gassed with oxygen. After 0, 3, 6, 12, 18 and 24 hours aliquots were withdrawn and investigated. For modification of the lipoprotein, LDL (1.5 mg/ml in the above mentioned buffer, containing in addition EDTA in a concentration of 1 mg/ml) was incubated with HNE (1 mM) and MDA (1 mM) at 37°C for 4 h as described.[9] For fluorescence measurements 3 ml of the LDL solution (1.5 mg/ml), prepared as described above, was extracted 4 times with 2 ml of a mixture of chloroform/methanol (2:1, v/v). The pooled extracts were concentrated to 3 ml by gassing with nitrogen and this lipid fraction was used for fluorescence measurements. The aqueous phase of the chloroform/methanol extraction was removed, the precipitated apo B washed with water and redissolved in 3 ml of 3% aqueous SDS and the fluorescence spectroscopy was performed.

Fluorescence spectra were scanned on a Jobin Yvon JY3D spectrofluorimeter (Ex 360 nm). 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. For three-dimensional fluorescence spectroscopy the samples were diluted 1:1 with the respective solvent. The contour plots (= fluorescence intensity topograms, FITs) were obtained on a Aminco SPF-500 spectrofluorimeter as described previously.[13] Band passes of the instrument were 10 nm in excitation and emission and the elements of the excitation/emission matrix were acquired with 5 nm increments of the emission and excitation wavelengths. Solvent background was subtracted and the contour lines were from 5 to 95% in 5% increments of the highest peak.

The fatty acids were extracted according to Folch[14] and analysed on g.l.c. as fatty acid methylesters. For vitamin E estimation aliquots of the Folch extraction were evaporated to dryness. The residue was redissolved in methanol, centrifuged and separated on h.p.l.c. as described.[15] The aldehydes were separated on h.p.l.c. as their dinitrophenylhydrazone derivatives,[16] malonaldehyde was estimated as thiobarbituric acid reactive substances and as free aldehyde.[17]

RESULTS AND DISCUSSION

Exposure of LDL (1.5 mg/ml) to an oxygen saturated buffer led to a time dependent decrease of alpha-tocopherol and polyunsaturated fatty acids (18:2, 20:4) and to an increase of HNE and the fluorescence intensity of apo B. The result of a typical experiment is shown in Fig. 1 and the various data measured in the fresh and oxidized

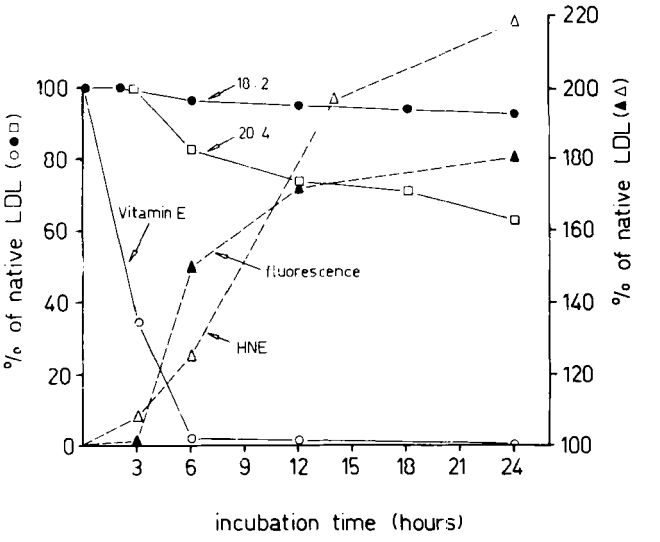


FIGURE 1 Change of the content of vitamin E, 18:2, 20:4, HNE and the fluorescent compounds during autoxidation of freshly prepared LDL. The fluorescence was measured at Ex 360/Em 430 nm on the apo B (0.37 mg/ml) in SDS 3%.

TABLE I

Vitamin E, polyunsaturated fatty acids, aldehydes and fluorescence of native and 24 hours oxidized LDL. The fluorescence was measured at 430 nm with excitation at 360 nm, the values given are % of a quinine standard (= 100%)

	Native LDL	Oxidized LDL
18:2 (nmol/mg LDL)	420	353
20:4 (nmol/mg LDL)	28	16
α-Tocopherol (nmol/mg LDL)	2.35	0.00
γ-Tocopherol (nmol/mg LDL)	0.21	0.00
4-Hydroxynonenal (nmol/mg LDL)	0.23	0.51
Malonaldehyde (nmol/mg LDL)	0.46	2.62
Other aldehydes (nmol/mg LDL)	0.30	3.05
Fluorescence of the lipid fraction	15	19
Fluorescence of apo B	17	32

LDL sample are summarized in Table I. The effects were reproducible with LDL prepared from the plasma of different donors, the extent of the changes and kinetics however varied from sample to sample. The HNE present in the 24 hour oxidized LDL samples varied from 0.31 to 0.97 nmol/mg LDL. In addition to HNE, the oxidized LDL contained a great variety of other aldehydes, most prominent were propanal, butanal, pentanal, hexanal, 2,4-heptadienal, 4-hydroxyhexenal and 4-hydroxyoctenal. The samples contained, as anticipated, also malonaldehyde like substances (1.6–4.8 nmol/mg LDL), most of which (80%) was in fact free malonaldehyde as evidenced by the previously developed direct h.p.l.c. method.[17] The freshly prepared LDL contained small amounts of propanal, butanal, pentanal and 4-hydroxynonenal.

The molecular mechanism leading to the fluorescent products in the course of lipid peroxidation and the chemical structure of the fluorophors thereby formed are largely unknown. Proposed were 1-amino-3-iminopropene derivatives formed by condensation of malonaldehyde with two neighbouring aminogroups[18] and dihydropyridine derivatives.[19] Newer reports[20,22] emphasize that the spectral properties (excitation maxima/emission maxima) of biological samples which had undergone lipid peroxidation are significantly different from those obtained with malonaldehyde treatment. This is consistent with the results which we have obtained by a thorough study of the fluorescence properties of LDL.

Apo B from freshly prepared human plasma LDL showed, as anticipated from previous studies,[21] in addition to the strong tryptophan fluorescence (Ex 287/Em 340 nm) also a weak and diffuse fluorescence in the visible range (400–600 nm) if excitation is performed at 360 nm (Fig. 2). This fluorescence was strongly increased in apo B isolated from oxidized LDL and HNE or malonaldehyde treated LDL. The spectrum of the oxidized LDL was very similar to that of the HNE treated LDL, but clearly different to the malonaldehyde treated LDL, which suggests that the fluorophor(s) in apo B probably does (do) not arise from malonaldehyde but rather from HNE. This vague statement can be made much more precise through three dimensional spectroscopy. We have shown[21] that native LDL contains at least 13 constituents contributing to the fluorescence in the visible region, 6 are contained in the apo B and 7 in the lipid moiety.

It is for theoretical reasons not possible to determine in such a fluorescent multicomponent system with some accuracy the position of the excitation and emission maxima of the individual fluorophors. In three dimensional fluorescence spectroscopy

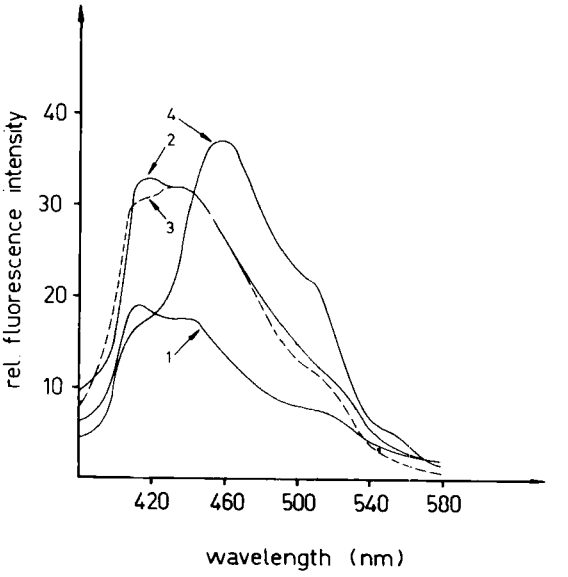


FIGURE 2 Fluorescence emission spectra at 360 nm excitation of apo B from native LDL (1); 24 h autoxidized LDL (2); HNE (1 mM) modified LDL (3); MDA (1 mM) modified LDL (4). The concentration of apo B was 0.37 mg/ml in SDS 3%.

the emission spectra (intensity as the function of the emission wavelength) are measured as a function of the excitation wavelength in 5 nm increments. The data are stored in a file and then used to compute three dimensional plots as shown in Figs. 3 and 4 or the fluorescence intensity topograms as shown in Figs. 5, and 6. The three dimensional graphic plots are highly impressive since they show very clearly the

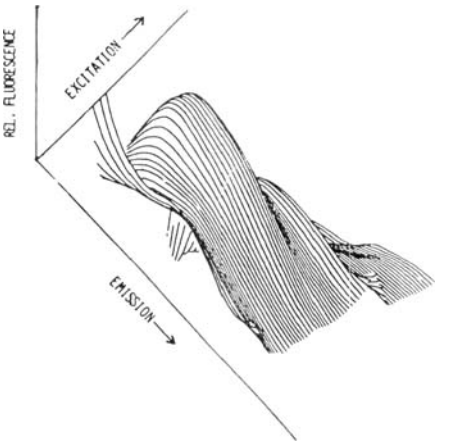


FIGURE 3 Three dimensional fluorescence spectrum of the apo B from 24 h autoxidized LDL. The data used for this plot were those accumulated for the FIT shown in Fig. 5; the viewer's perspective is indicated by the arrow A in Fig. 5.

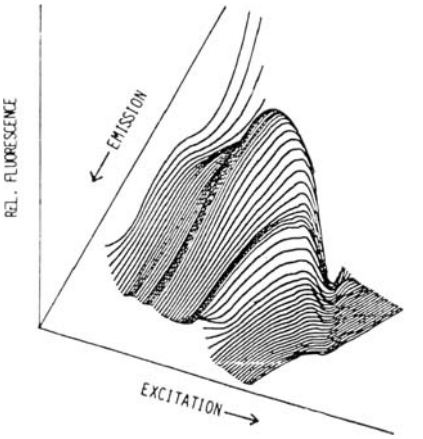


FIGURE 4 Three dimensional fluorescence spectrum of the apo B from 24 h autoxidized LDL. This spectrum was constructed with the same data file as in Fig. 3, yet with a different perspective i.e. arrow B in Fig. 5.

fluorescence with the plateaucs, hills, shoulders and valleys. However these fluorescent "landscapes' are very much dependent from the line of sight as can be seen by comparison of Figs. 3 and 4, which are graphs from one and the same data file obtained from fluorescent measurement of apo B from oxidized LDL. (The situation is very much similar to a hiker in the Alps who gets a very different view from the surrounding landscape, depending on where he stands and in which direction he looks. In front of a hill he can not see a high mountain behind it and vice versa). This

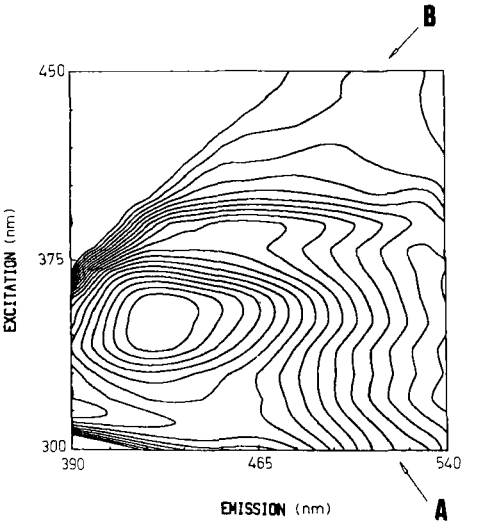


FIGURE 5 Fluorescence intensity topogram (FIT) of the apo B from 24 h autoxidized LDL. The arrows A and B indicate the viewer's perspective from Fig. 3(A) and 4(B). The FIT is solvent background corrected. The apo B concentration was 0.19 mg/ml in SDS 3%.

problem is circumvented by the fluorescence intensity topograms (FIT), in which all points with the same fluorescence intensity are connected by contour lines as shown in Fig. 5.

In our computer program the highest fluorescence intensity is set to 100% and the contour lines are plotted in 5% increments. Also considered in our plots is the solvent background which is substracted in all FITs. In the FIT plot a single fluorophor would give symmetrical, circular or ellipsoidal contour lines and any divergence from that indicates an overlapping with one or more additional fluorophors. The assymetric 96% contour line in Fig. 5 covering the range of 340–365 nm excitation and 413–440 nm emission indicates the presence of two fluorophors, one of which with an excitation and emission maximum at about 355/430 nm and one at 345/425 nm. Also recognizable in the FIT is the shoulder (see also Fig. 3) at about 385/465 nm, the intensity of which is about half of the main fluorescent peak.

The interpretation of the FIT needs some experience and in case of doubts the three dimensional plots with different perceptions as well as the numerical raw data are helpful. The various apo B from autoxidized LDL investigated so far gave more or less the same FITs as shown in Fig. 5, not in all cases however could the double peak clearly be resolved into the two overlapping fluorophors. Such an example is shown in the FIT of Fig 6D. The fluorophor of apo B from HNE treated LDL gave an excitation and emission maximum at 355/430 nm (Fig. 6B), which is identical to one of the main fluorophors found in oxidized LDL.

Malonaldehyde modification on the other hand gave a fluorophor at 400/470 nm (Fig. 6C) and the large difference to the oxidized LDL can be taken as a clear evidence that malonaldehyde is not involved in the formation of the major apo B fluorophor during autoxidation of LDL.

The native LDL (Fig. 6A) exhibits a very complex FIT in the visible range.[21] The main flourophors have excitation and emission wavelengths at 330/390 nm (not shown in Fig. 6A), 355/430, 365/460 nm, additional weak fluorophors are at 380/540, 400/530 and 420/490 nm. The 355/430 nm fluorescent chromophor probably arises from HNE which has been formed in LDL *in vivo*. This assumption is supported by the fact, that all native LDL samples contained some HNE and that the 355/430 nm fluorescence was always high in samples with high HNE content (data not shown).

CONCLUSION

Several lines of research suggest that modification of LDL through lipid peroxidation is a crucial event in the multistep process of the development of atherosclerosis.[1-4] A weak point in this hypothesis is, that evidences for oxidation in LDL are mainly based on the presence of increased levels of malondehyde-like materials as assayed by the rather unspecific TBA assay. The significance of this report lies in the more precise description of oxidized LDL through quantitative measurement of several parameters indicative for lipid peroxidation.

Exposure of LDL to oxygen-saturated buffer leads within three to six hours to a depletion of alpha-tocopherol, and only then lipid peroxidation commences as evidenced by the decrease of 18:2 and 20:4 and the concomitant evolvement of the lipid peroxidation product HNE and other aldehydes and the increase of the fluorescence of apo B (Fig. 1). Compared with other systems (microsomes, hepatocytes, free fatty acids and others) LDL should be considered as highly resistant against lipid peroxida-

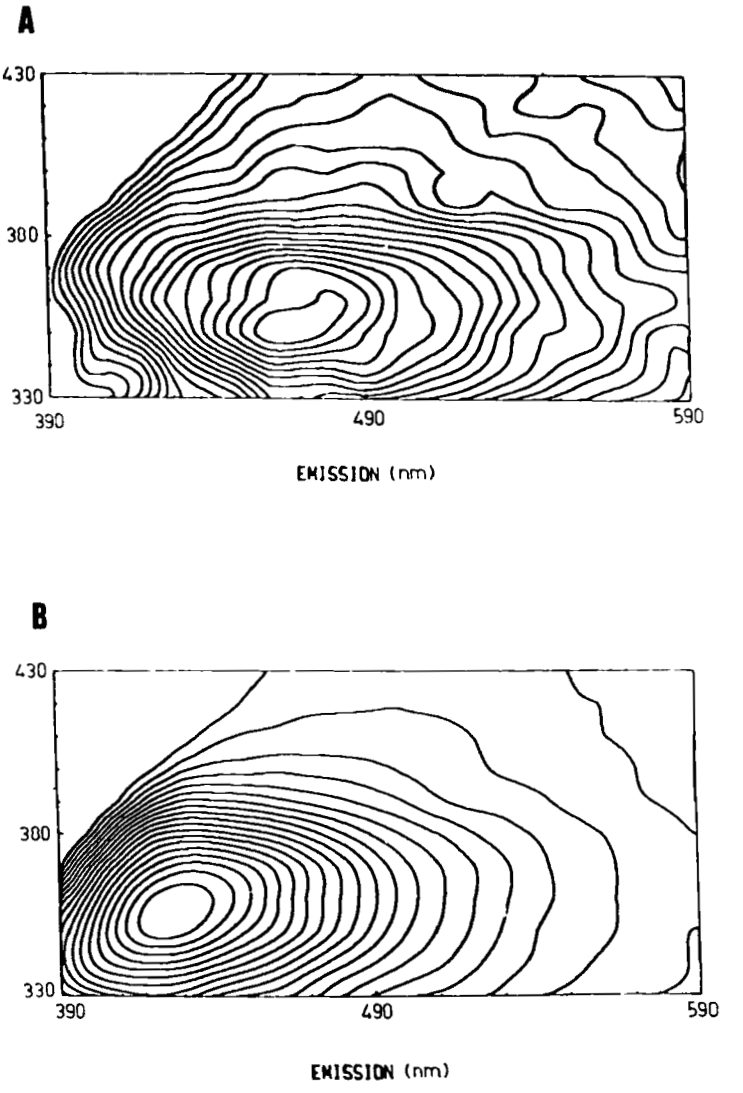
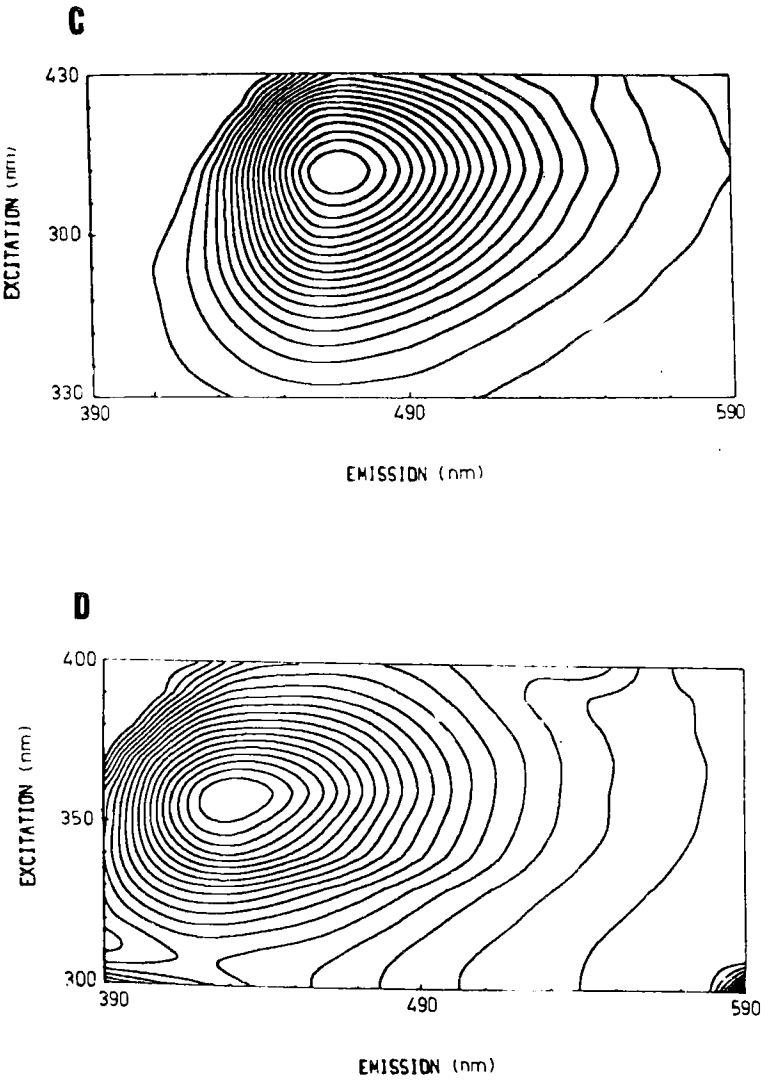


FIGURE 6 FIT of apo B from native LDL (A); 1 mM HNE modified LDL (B); 1 mM MDA modified LDL (C); 24 h autoxidized LDL (D) The FIT is solvent background corrected. The concentration of apo B was 0.19 mg/ml in SDS 3%.

tion and obviously vitamin E very effectively prevents the initiation of lipid peroxidation. But even after complete vitamin E depletion the process is not very fast and has not at all the kinetics characteristic for an autocatalytic process (Fig. 1).

Based on the figures in Table I and the molecular weight of 2.55×10^6 dalton for LDL it can be calculated that the LDL molecule lost during 24 h oxidation 6 molecules vitamin E, 171 molecules 18:2 and 31 molecules 20:4; present in the native



LDL molecule were 6 (alpha-tocopherol), 1070 (18:2) and 71 (20:4) molecules. In total, only 16 aldehyde molecules could be detected in the oxidized LDL molecule, about one of them was HNE, 7 malonaldehyde and 8 other aldehydes. In this comparison it should be considered that these aldehyde molecules are only the remnants of the lipid peroxidation process, since most of them could have reacted with LDL constituents and therefore not be detectable in our assay.

Clear evidence that such reactions occur comes from the increase of the fluorescence of apo B, which is mainly due to a fluorophor with an excitation and emission maximum at 355/430 nm. This fluorophor most likely arises from interaction with

HNE with reactive groups in apo B, but certainly not from malonaldehyde. The demonstration of the exact coordinates of the various fluorophors in fresh and oxidized LDL was possible through three dimensional fluorescence spectroscopy.

We believe that this new technique could also be helpful for the more precise characterisation of the fluorescence of other biological samples which had undergone lipid peroxidation.

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