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A NEW FLUORESCENCE METHOD FOR THE CONTINUOUS DETERMINATION OF SURFACE LIPID OXIDATION IN LIPOPROTEINS AND PLASMA

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We report on a new method for the determination of lipid oxidation in lipoproteins and plasma. The biological lipid system is preloaded with a fluorescent analog of phosphatidylcholine containing diphenylhexatriene (DPH) propionic acid covalently linked to the *sn*-2 position. When externally added, the respective phospholipid label (DPHPC) localizes to the surface monolayer of a lipoprotein. Under oxidative conditions (e.g. in the presence of Cu^{2+} ions) the fluorophore undergoes decomposition, resulting in a continuous decrease of fluorescence intensity which reflects the oxidation of a chemically defined phospholipid molecule with well defined localization. When incorporated into LDL particles, the kinetics of the decrease in DPHPC fluorescence intensity upon exposure to Cu^{2+} is very similar to that of conjugated diene accumulation. Furthermore, our assay can be applied to follow the oxidation of lipids in diluted serum and may also be developed into a suitable test system for clinical studies of susceptibility of plasma lipids to oxidation.

KEY WORDS: Low density lipoprotein (LDL), Phospholipids, Diphenylhexatriene, Atherosclerosis, Antioxidants

Abbreviations: EDTA, ethylenedinitrilo tetraacetic acid disodium salt; LDL, low density lipoprotein; PBS, phosphate buffered saline; DPH, diphenylhexatriene; PC, phosphatidylcholine; SM, sphingomyelin; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride

INTRODUCTION

Peroxidation of polyunsaturated lipids plays an important role in the pathogenesis of many diseases and especially in atherosclerosis[1]. Therefore, knowledge of the kinetics and mechanism of lipid peroxidation is crucial for the understanding of the onset of such processes. Several methods have been previously developed to study susceptibility of lipids in lipoproteins towards peroxidation under various conditions. These include measurements of consumed oxygen[2], as well as production of lipid peroxides[3], conjugated dienes[4] and thiobarbituric acid-reactive substances (TBARS)[5]. Methods based on fluorescence measurement, both of intrinsic fluorescence[6] and an extrinsic marker (parinaric acid)[7,17] were also used as oxidation parameters. All these methods show the

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same characteristic time course for the initial steps of lipid peroxidation, except the technique using parinaric acid which is much more sensitive towards oxidation compared to normal polyunsaturated lipids.

In general, there are three time phases in lipid peroxidation that can be seen with all methods described above: lag phase, propagation phase and decomposition phase[8]. During the lag time preceding oxidation due to exposure of LDL to any oxidizing agent, the naturally occuring, LDL-associated, antioxidants (mostly vitamin E) are being oxidized. Accordingly, the length of this phase reflects the "oxidation resistance" of LDL. In view of the significant role of lipid oxidation in the pathogenesis of lipid-associated diseases, the dependence of the "lag time" on the composition and structure of LDL and on dietary, environmental as well as genetic factors and various pathogenic factors, gained much attention. In most of these studies, the oxidation induced by Cu^{2+} was either followed continuously by monitoring the accumulation of conjugated dienes or by measuring TBARS at various times. These, as well as most other techniques (oxidation of parinaric acid excluded), yielded quantitatively similar results with respect to the initial time course. Nonetheless, all these methods can be used only for fractionated lipoproteins. Studying "lipid oxidizability" in plasma or serum could have the benefit of being independent of the details of the fractionation procedure. However, lipid oxidation in plasma is inhibited by various components (mostly albumin) and its dependence on the extent of dilution and on the concentration of the oxidizing agent is very complex. Recently, it became evident to us that diluted plasma is readily accessible to Cu^{2+}-induced oxidation since the protective effect of albumin is markedly reduced. When diluted 100 fold, the plasma lipids can be oxidized by 10–100 μM Cu^{2+} with a "well behaved" kinetics, that can be monitored by the absorbance of conjugated dienes at 234 nm[15]. However, the sensitivity of this techniques for such diluted samples is quite low.

We have now developed a new method on a fluorescence basis, measuring the decrease in fluorescence intensity of a lipid conjugate of diphenylhexatriene (DPH), which is also highly sensitive towards oxidation. DPH oxidation seems to be comparable to the oxidation of natural polyunsaturated lipids as determined by the diene method. Being covalently linked to (phospho-) lipids (by means of DPH-propionic acid) it reflects the relative oxidation susceptibility of the respective lipid classes. Here we describe the phosphatidylcholine derivative of DPH (DPHPC) (Figure 1) as an example of a lipoprotein surface component that is subject to oxidation initiated by copper *in vitro*. For this purpose, LDL was preloaded with the fluorophore at high lipid to label ratios in order to leave the lipoprotein surface structure undisturbed. Oxidation was determined continuously from the decrease in DPHPC fluorescence intensity. The data obtained by this continuous and sensitive method are in very good agreement with those obtained by the diene method. It can be expected that the new technique will open new possibilities in the analysis of lipid oxidation not only in lipoproteins but also in biomembranes and food chemistry.

MATERIALS AND METHODS

Standard laboratory chemicals were purchased from Merck, Germany, or Sigma, USA, and were of analytical grade or better. 1-Palmitoyl-2-((2-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl)-*sn*-glycero-3-phosphocholine (DPHPC) (Figure 1) was prepared as described[9].

Human serum was prepared from blood drawn from healthy donors. LDL was

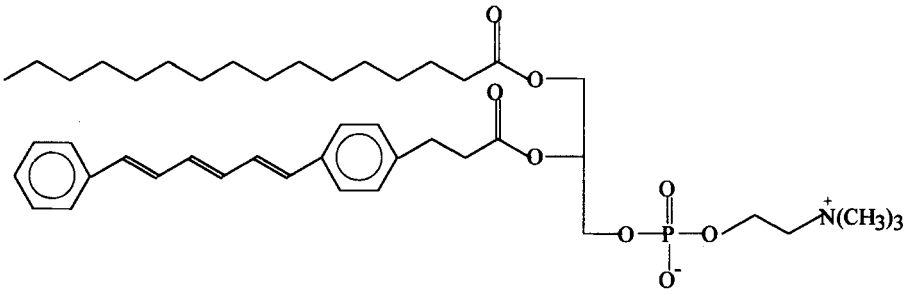

FIGURE 1 Chemical structure of DPHPC.

prepared as described[10] by sequential ultracentrifugation. LDL cholesterol was determined enzymatically using the Chol MPR 2 kit from Boehringer-Mannheim, Germany. LDL concentration was estimated assuming an LDL molecular weight of 2500 kDa and a cholesterol content of 31,6%. For phospholipid analysis, total lipids were isolated from LDL by chloroform/methanol (2/1 v/v) extraction [11] and separated by TLC on silica gel plates, using chloroform/methanol/water (65/25/4, v/v/v) as a solvent. Spots containing ethanolamine glycerophospholipids, choline glycerophospholipids or sphingomyelin were scraped off the plate and their phosphorus content was determined according to Broekhuyse[12].

LDL samples isolated by sequential ultracentrifugation in the presence of NaBr, EDTA and NaN_3, were diluted with argon-saturated PBS buffer (PBS: 8 g NaCl, 200 mg KCl, 200 mg KH_2PO_4, 1,43 g Na_2HPO_4; dissolved in 1 l H_2O, pH 7,4) to give a total cholesterol concentration of 2mg/ml. An ethanolic solution (10 μl) of labeled lipid (18 nmol/ml LDL suspension) is added to the LDL suspension under gentle stirring. This mixture is kept under an argon atmosphere at 37°C for 12 hours. For the oxidation experiments, NaBr, EDTA and NaN_3 were removed from the labeled LDL preparations by desalting into PBS by gel filtration in Econo columns supplied by Bio-Rad, USA. The PBS solution has to be stirred before with Chelex 100 from Bio-Rad to remove contaminating transition metal ions usually contained in sodium phosphate. For the oxidation assay, LDL is diluted with PBS-buffer to give a final volume of 3.0 ml and final concentrations of total phospholipid, labeled phospholipid and cholesterol in the assay mixture are 55 μg (73 nmol) /ml, 0.73 nmol/ml, and 79 μg (204 nmol)/ml, respectively. Oxidation is started by the addition of an appropriate amount of a 0,1 mM stock solution of copper sulfate. The reaction is monitored by the time-dependent decrease in fluorescence intensity at 430 nm (excitation at 360 nm) on a Shimadzu RF-540 fluorometer.

An ethanolic solution (10 μl) of labeled lipid (20 nmol/ml serum) is added to 1 ml of argon-saturated human serum (2 mg total cholesterol/ml). This mixture is kept under argon at 37°C for 12 hours. A 30 μl aliquot of labeled serum is diluted with 2,97 ml PBS. Final concentrations of phospholipid, label and cholesterol in the assay mixture are 30 μg (40 nmol)/ml, 0.6 nmol/ml, and 20 μg/ml, respectively. Oxidation is started by the addition of an appropriate amount of a 10 mM stock solution of copper sulfate. The oxidation reaction is monitored as described above.

To study the effect of vitamin E on lipid oxidation, LDL samples (2 mg total cholesterol/ml) were labeled with DPHPC after addition of appropriate amounts of vitamin E dissolved in 10 μl ethanol. Vitamin E concentrations in the incubation mixtures were 2 μM, 4 μM, and 6 μM, respectively. Samples were desalted and diluted for the oxidation assay as described above.

RESULTS

Previous studies of our laboratory[13] have shown that incorporation of DPH-phospholipids into apo B-containing lipoproteins is almost completed in 6 hours. Therefore, we incubated LDL with vesicles of fluorescent phospholipids[13] for at least 6 hours at 37°C under argon. Proper handling of LDL samples under an inert atmosphere does not affect the oxidation status of LDL[13]. We confirmed this by a control experiment, in which a fluorophore-containing LDL sample, made by 6 hours of incubation with DPHPC yielded the same lag time and propagation phase (in the diene method) as the non-incubated LDL (data not shown). In the present study, we have used a DPHPC to phospholipid ratio of 1/100 (mol/mol). Thus, if DPHPC is incorporated quantitatively into the LDL, the overall phospholipid composition of the particle has only changed by 1%.

Upon oxidation of DPHPC, its fluorescence decreases, and the time course of DPH-lipid degradation can be determined from the decrease of the initial fluorescence intensity. Figure 2 shows a typical experiment performed under "standard conditions" with 250 μg total LDL mass/ml (corresponding to 50 μg protein/ml, 79 μg total cholesterol/ml and 55 μg phospholipid/ml, see methods) and 1,67 μM Cu^{2+} as a prooxidant at 37°C. From this data we can obtain the lag time graphically from the intercept of the tangents to the slow and fast decrease of fluorescence intensity[14]. Furthermore, we can obtain the half time of the time-dependent decrease of fluorescence ($t_{1/2}$), which reflects the oxidation susceptibility of the fluorescent PC, depending on concentrations of prooxidant or antioxidant[14]. For any given LDL sample, both the latency and half life determined by this method were equal to the corresponding values as determined by diene accumulation (Figure 3). A similar correlation was obtained when lipid oxidation was initiated by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) (data not shown). Table 1 shows that the lag times for the oxidation of LDL samples from different donors may vary considerably.

Furthermore, the influence of the antioxidants vitamin E and vitamin C on the oxidation of LDL could also be easily quantified from the prolongation of the lag phase. For this purpose vitamin E was incubated together with the fluorescent DPHPC and LDL whereas vitamin C was added to LDL shortly prior to the addition of Cu^{2+} (Figure 4).

One of the main advantages of the fluorescence assay over the diene method is its high sensitivity. Thus, it is also applicable to complex lipoprotein systems such as diluted serum (see discussion). As expected, no oxidation occurred in full serum in the presence of Cu^{2+} or AAPH within 6 hours but after a 100 fold dilution, oxidation could

TABLE 1
LDL-oxidation by Cu^{2+}

LDL sample	lag time [min]	
	DPH-method	Diene-method
1	25	24
2	46	47
3	134	130
4	135	134
5	146	144

LDL samples from five different donors were oxidized in the presence of Cu^{2+} (1,67 μM). Lag times were determined as described in the legend to figure 2 and are indicated as means of three experiments (± 5% S.D.). Similar lag times were found in all cases if 2 mM AAPH was used as a prooxidant instead of Cu^{2+}.

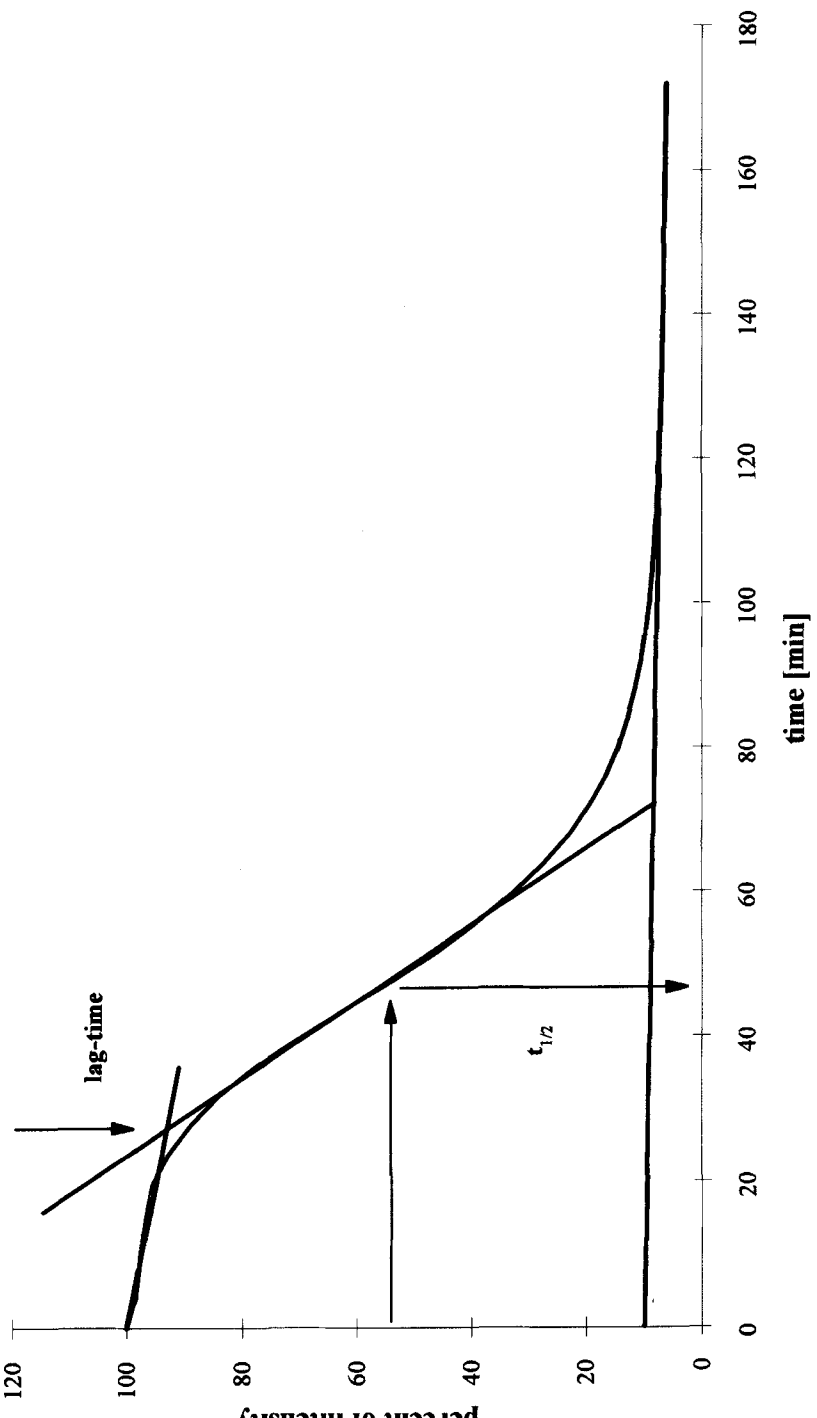

FIGURE 2 Oxidation assay. Cu^{2+}-mediated oxidation of DPHPC in LDL was monitored by the decrease of fluorescence intensity at 430 nm. The LDL sample (750 μg total mass, 220 nmol phospholipid, 613 nmol cholesterol) contained 2,2 nmol DPHPC in 3 ml PBS buffer. Oxidation was started by addition of 50 μl of an 0,1 mM $CuS0_4$ solution (final concentration: 1,67 μM). The fluorescence intensity at 430 nm was recorded in intervals of 4 min at 37°C. The data were graphed using Microsoft Excel spread sheet program by normalizing all intensity values to an initial value of 100%. The values of t_{lag} and $t_{1/2}$ were determined graphically as indicated.

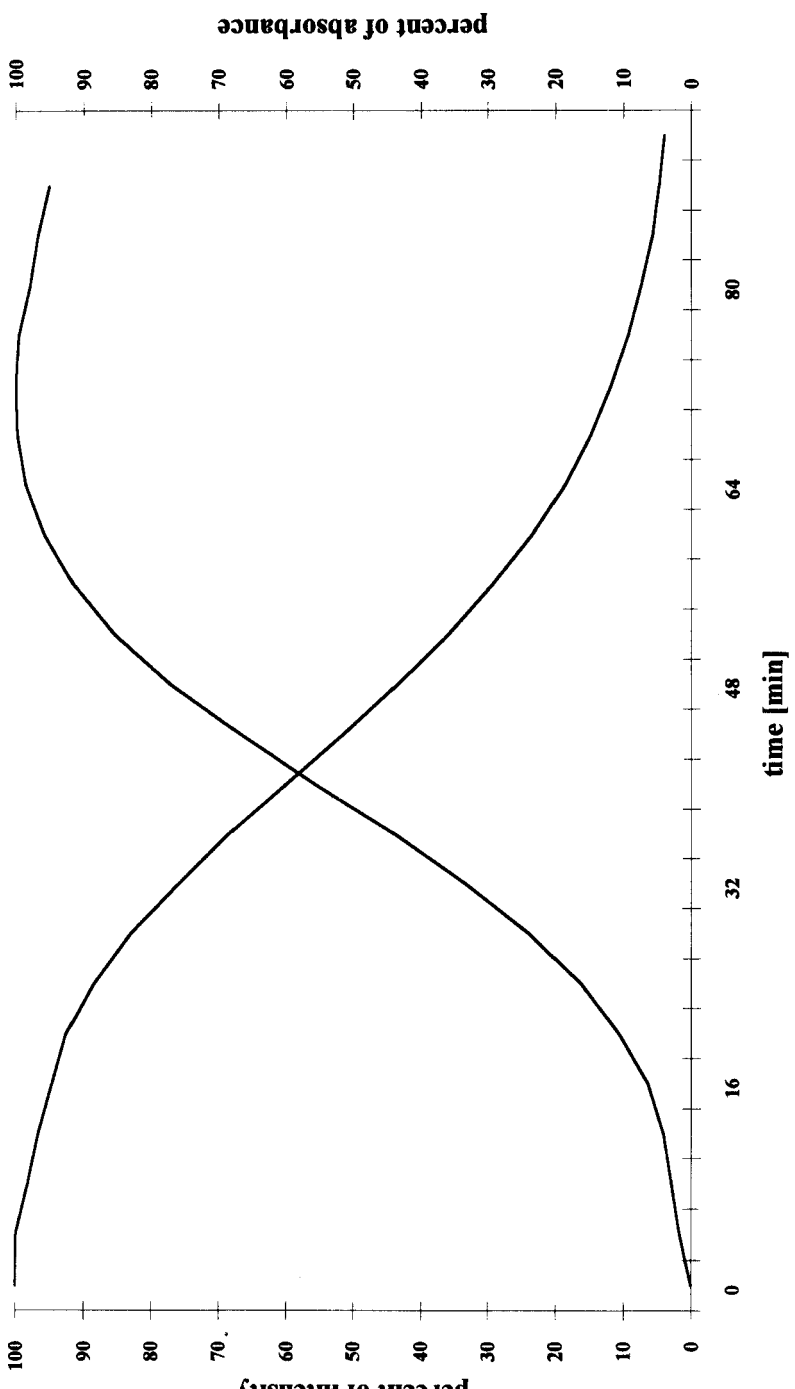

FIGURE 3 Comparison between the DPH method and the diene method. An LDL sample was oxidized in the presence of Cu^{2+} as described in figure 2 and progress of oxidation was studied following the diene absorption at 234 nm and the fluorescence intensity at 430 nm at 37°C. Both curves were normalized to 0% and 100% as starting values, respectively. Lag times for both methods are nearly identical.

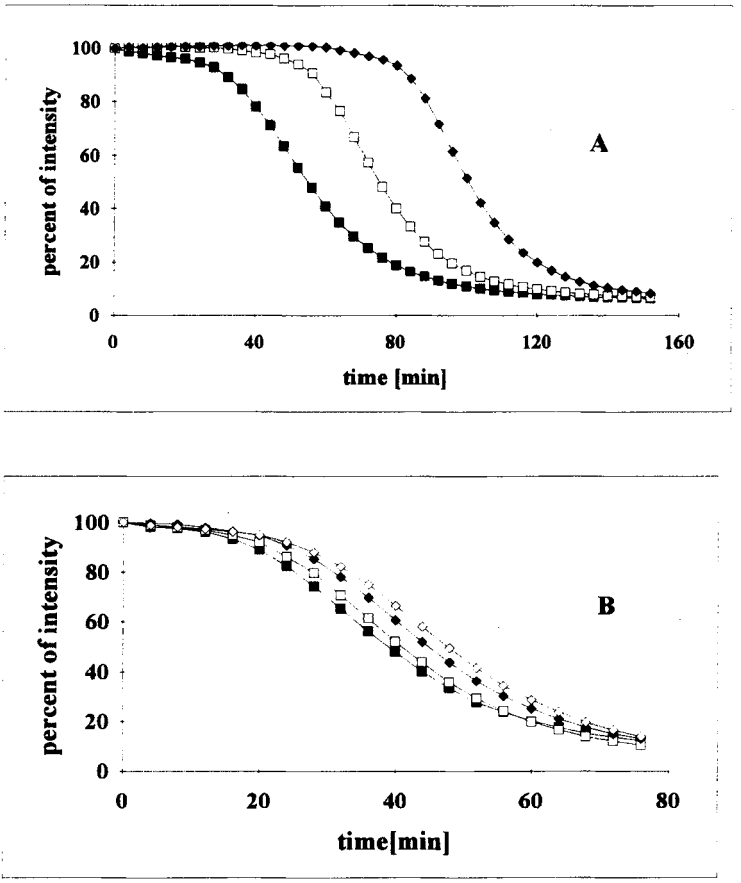

FIGURE 4 Influence of antioxidants on oxidation of DPHPC in LDL. In panel A ascorbic acid was added to the reaction mixture (final concentrations: ■ 0 μM, □ 2,5 μM, ♦ 5 μM). In panel B α-tocopherol was present in the incubation mixture (see materials and methods) (final concentrations: ■ 0 μM, □ 80 nM, ♦ 160 nM, ◊ 240 nM). The progress of oxidation at 37°C was measured as indicated in Figure 2.

be detected with varying concentrations of 10–100 μM copper (data not shown). In this case the observed kinetics should not only reflect lipid degradation and protection by lipid-soluble antioxidants but also the effect of all the water-soluble antioxidants contained in serum[15]. Figure 5 shows data for the oxidation of DPHPC in serum samples (diluted 100 fold) from three different donors. The observed lag times vary considerably, indicating a broad spectrum of oxidation susceptibilities that can be detected by our new method and might be of relevance in clinical studies.

DISCUSSION

Lipid peroxidation is currently being considered one of the most important factors in atherogenesis. For related studies, simple and well reproducible methods for the measurement of lipid oxidation are required. Although several methods have been developed, most of them lack specificity for distinct lipid classes and/or sensitivity. The new fluorescence method presented here is specific with respect to lipid classes, more sensitive than measurements of light absorption, continuous and very easy to use both for lipoproteins or diluted serum. The fluorescent probe (DPH-phosphatidylcholine), whose fluorescence is sensitive towards oxidative degradation, can be easily incorporated into lipoprotein samples with a well defined localization in the particle surface layer[16] containing phosphatidylcholine (PC) and sphingomyelin (SM) as the main phospholipid classes. Hence, DPHPC may be considered as a fluorescent reporter for the behaviour of unsaturated phosphatidylcholines in lipoproteins under various environmental conditions, e.g. oxidative stress. Our new technique provides the means to follow directly oxidative decomposition of such an appropriate marker lipid. Upon oxidation, the fluorogenic DPH system is destroyed and the time-dependent progress of oxidative lipid damage can easily be monitored via the consequent decrease in fluorescence intensity. This method is therefore superior to other methods in that it measures the oxidative degradation of a single and defined lipid molecule as compared to parameters originating from the overall lipid oxidation in biological lipid assemblies.

Noteably, but not surprising, our fluorescence method when applied to a given lipoprotein class (e.g. LDL) leads to very similar lag times to those obtained by the diene method. The latter parameter depends on the time-dependent consumption of antioxidants such as vitamin E and should therefore only depend on the type of lipoprotein but not on the lipid class contained therein. If on the other hand (phospho-) lipid fluorophors are used as marker systems that are more susceptible towards oxidation (e.g. parinaric acid), artificially shortened lag times are observed as compared to the diene method[7,17].

Apart from the high sensitivity of fluorescence detection, the main advantage of the proposed method is the observation of defined lipid classes under oxidative stress. This approach is therefore not only restricted to the use of fluorogenic PC; preliminary results have, in fact, indicated its applicability to SM (data not shown). As a consequence, one would be in the position to analyse the relative oxidation susceptibilities of different lipids towards different prooxidants acting on lipoprotein lipids and/or proteins by different mechanisms. For instance, AAPH is a source of radicals approaching randomly a lipoprotein particle. In contrast, copper ions are supposed to initiated oxidation in a protein-bound state on a lipoprotein[18]. The question as to how the fate of different lipids depends on the presence of various prooxidants can be easily studied by the use of DPH derivatives of various lipids.

For clinical purposes, stability of serum or plasma towards oxidation might become a useful parameter if it can be causally related with atherogenesis and/or coronary heart disease and stroke. Degradation of lipids in undiluted plasma cannot be observed *in vitro* and the contribution of various plasma components to this stability has yet to be elucidated. Recent preliminary studies (E. Schnitzer and D. Lichtenberg, in preparation) indicate that the major contributor to this protective effect is albumin and that much of this effect is due to copper-binding to albumin, which is primarily a function of albumin concentration rather than of the albumin/LDL ratio. As a consequence, lipid oxidation in unfractionated plasma can be induced if the plasma is first diluted

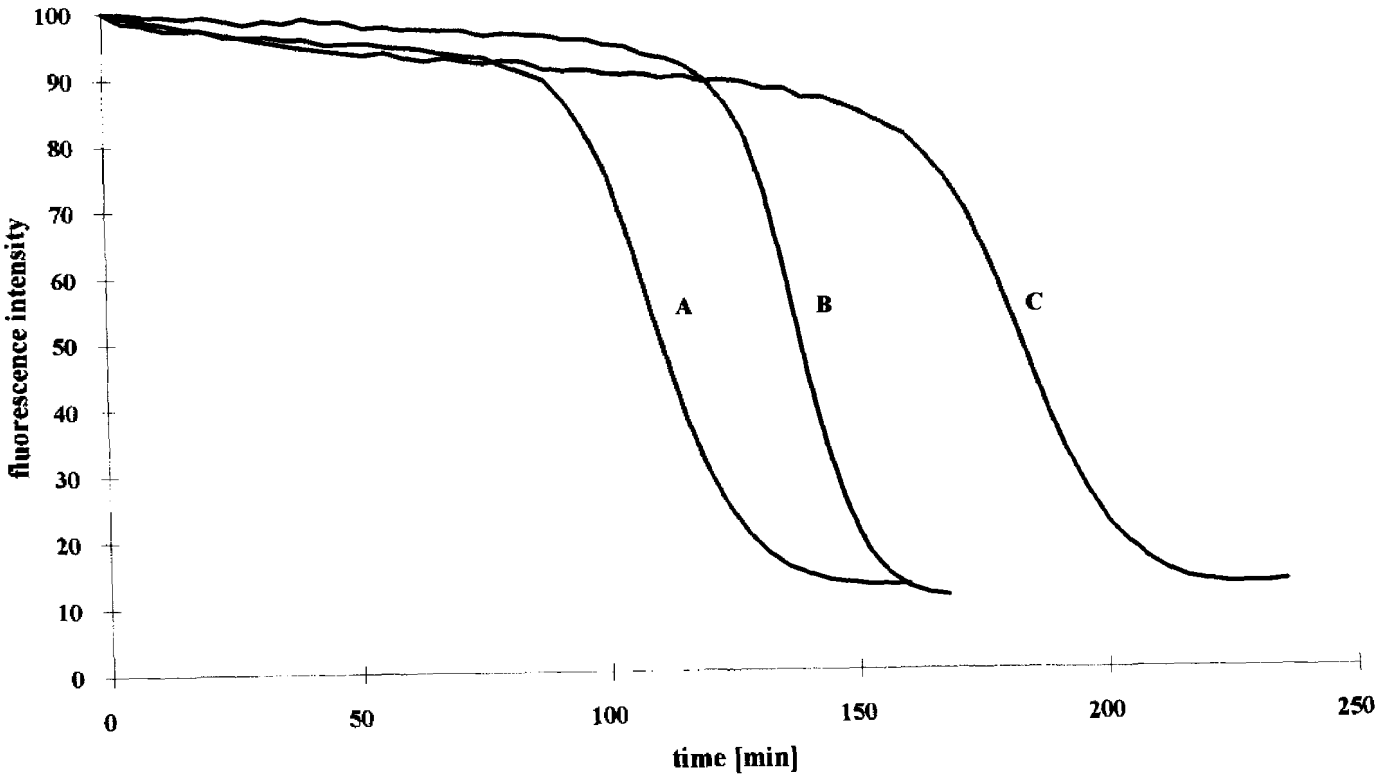

FIGURE 5 Oxidation of DPHPC in 1% serum. Human sera from three different donors (A, B, C) were labeled as described in materials and methods and oxidation was initiated by addition of a 10 mM stock solution of copper sulfate (final concentration: 50 μM). Lag times and progress of oxidation were determined as indicated in figure 2. Lag times: A (94 min), B (124 min), C (164 min).

(prior to copper addition). However, the sensitivity of conventional optical methods is not sufficiently high to enable studies of oxidation of very diluted plasma. With DPH-lipids, this problem can be circumvented, since the fluorescence background of the sample in the presence of the labeled lipid analog is appropriately low, and any changes in the DPH-signal can be measured with high precision. First results obtained with our new method have already shown that serum susceptibility towards oxidation may vary considerably between different individuals (Figure 5). The clinical potential of these findings is currently being subject of further investigations.

Another promising field of DPH lipids as tracers of lipid oxidation should be membrane biochemistry. So far, investigation of membrane systems has been more difficult as compared to lipoproteins. In general the use of defined DPH-lipids should open new possibilities also in many other fields of lipid oxidation (e.g. food chemistry) and enable the researcher to study defined lipid components in complex biological systems.

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