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THE CHEMILUMINESCENCE ASSAY OF LIPID PEROXIDATION PRODUCTS IN HUMAN BLOOD PLASMA LIPOPROTEINS

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A chemiluminescence (CL) flash kinetics on the addition of Fe^{2+} ions into oxidized low density lipoprotein (LDL) suspension has been studied. LDL oxidation was carried out at 37°C without and in the presence of 5 or 50 μM of $Cu.^{2+}$ It has been found that under certain experimental conditions (the addition of excess iron ions, more than 1 mM) the amplitude of CL flash depended almost linearly (1) on the concentration of oxidized LDL and (2) on the extent of LDL oxidation measured as diene conjugates (DC) and 2-thiobarbituric acid-reactive substance (TBARS) accumulation. The corresponding correlation coefficients were: for TBARS – 0.94 and for DC – 0.97, in the case of LDL autooxidation; 0.72 and 0.98, in the case of copper-induced LDL oxidation. A sensitivity of the CL method was shown to be significantly enhanced (by more than two orders) in the presence of CL sensitizer – 2,3,5,6-1H,4H-tetrahydro-9-(2′-benzoimidazolyl)-quinolizin-(9,9a,1-gh)coumarin.

KEY WORDS: Lipid peroxidation, hydroperoxides, iron (II) ions, copper (II) ions, chemiluminescence, blood plasma lipoproteins.

Abbreviations
·OH – hydroxyl radical;
BHT – butylated hydroxytoluene;
HDL – high density lipoproteins;
LDL – low density blood lipoproteins,
CL – chemiluminescence;
C-525 – 2,3,5,6-1H,4H-tetrahydro-9-(2′-benzoimidazolyl) – quinolizin-(9,9a,1-gh) coumarin;
H_2O_2 – hydrogen peroxide;
EDTA – ethylene diamine tetraacethyc acid;
LPO – lipid peroxidation;
MDA – malondialdehyde;
$O_2\cdot^-$ – superoxide anion, dioxide radical;
TBA – 2-thiobarbituric acid;
TBARS – TBA-reactive substances;
DC – diene conjugates;
LOOH – lipid hydroperoxides.

INTRODUCTION

Oxidized blood lipoproteins have recently been implicated as causal factor in atherosclerosis development.[1-3] In particular, it has been shown in our earlier experiments

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that oxidation of blood plasma lipoproteins alters the cholesterol transfer between LDL (or HDL) and membranes of erythrocytes or monocytes.[4-6]

There are several biochemical methods to determine the degree of blood plasma lipoprotein oxidation, among which diene conjugation and accumulation of compounds reacting with 2-thiobarbituric acid (TBARS) are most commonly used.[7] The latter method, though being rather sensitive, is not very specific as far as many other substrata can readily react with TBA to produce similar color product.[8] In addition, the major component of TBARS, malondialdehyde (MDA) is a minor product of chain lipid peroxidation that is produced, according some estimations,[7] in amounts less than 2 molar percents of peroxidized unsaturated fatty acids in LDL.

Lipid hydroperoxides are apparently the primary products of lipid peroxidation reaction (LPO). In the presence of transient metal ions lipid hydroperoxides may initiate new chains of LPO in lipoproteins and so give rise to further LPO product accumulation.[7,9,10] A wide-spread technique for measurement lipid hydroperoxides is spectrophotometric determination of diene conjugation at 231 nm.[7] This method is neither very specific (alcohols formed under hydroperoxide reduction also are diene conjugates) nor particularly sensitive. Hydroperoxide assay with high-pressure liquid chromatography and subsequent CL measurement in the presence of peroxidase and luminol derivatives is reported to be the most sensitive method.[11-14] It is, however, comparatively expensive and time-consuming, for which reason it has not yet been widely recognized in clinical investigations. Meanwhile, it has been reported that the addition of transient metal salts to fatty acid solutions and biomembrane suspensions, containing oxidized lipids, was accompanied with a chemiluminescence flash, the amplitude of which correlated, under certain conditions, with the concentration of hydroperoxides in the samples.[9,10,15]

It was the purpose of our study to determine whether chemiluminescence response on the addition of Fe^{2+} can be used for estimation of lipid oxidation degree in blood low density lipoproteins.

MATERIALS AND METHODS

Tris-buffer and 2-thiobarbituric acid were obtained from Merck (Germany), heparin from Spofa (Checho-Slovakia), butylated hydroxytoluene and $FeSO_4 \times 7H_2O$ from Sigma (USA). 2,3,5,6-1H,4H-tetrahydro-9-(2′-benzoimidazolyl)-quinolizin-(9,9a, 1-gh) coumarin (C-525) was obtained from NIOPIK Moscow Scientific Production Association, Russia. Other chemicals were of reagent grade from Reakhim (Russia).

Lipoproteins were isolated from blood serum of healthy donors by preparative ultracentrifugation in NaBr solution of different densities in the presence of 0.01% EDTA.[16] The lipoprotein fractions were dialysed against 500 volumes of 100 mM NaCl + 10 mM Tris-HCl, pH 7.4, for 18 hours at 4°C. The lipoproteins obtained were kept at 4°C and used within next day (24 hours). The protein concentration in LDL preparations was measured by the method described in.[17]

The chemiluminescence measurements were performed with the luminometer CLM-3M (Ecoton Ltd, Russia). The luminescence intensity was measured with a sensitive photomultiplier FEU-100 (Russia), possessing spectral sensitivity region 170–830 nm. The samples in final volume 2 ml were introduced into thermostated cuvette placed above and close to the photocathode and supplied with mechanical stirrer (See[9,10] for more detail). The dark current of the photomultiplier was measured during 1 min, then the shutter was opened and the solution of $FeSO_4$ was

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introduced through black plastic tube by means of dispenser. A chemiluminescence signal was integrated for 50 ms time intervals and CL kinetics was recorded within 10 s using IBM PC 386-AT.

The measurement of TBA-reactive substances (TBARS) was performed in 0.25 ml aliquots as described in.[18] To prevent lipid peroxidation during the procedure of TBARS assay, BHT in the final concentration 0.5 mM was added to the incubation medium. The absorbancy of TBARS at 532 nm, D(532), was measured on Beckman DU-7 spectrophotometer (USA), the absorption at 600 nm, D(600), being taken as a background. The final value D(532/600) = D(532) − D(600) is used in this paper.

The concentration of hydroperoxides was estimated in 0.1 ml LDL aliquots. The diene conjugates absorption at 231 nm of LDL heptane extracts was measured,[19] the absorption at 300 nm being taken as a background: D(231/300) = D(231) − D(300).

RESULTS

To choose conditions for CL measurements LDL were dialysed with following incubation for 4 h at 37°C. This is a conventional method of lipoprotein oxidation resulting in a considerable accumulation of LPO products in LDL.[4-6] However, susceptibility of LDL oxidation varies strongly between individuals. Although we carried out experiments with LDL from different donors, data are presented only for individual LDL preparations for each series of experiments. Figure 1a presents the kinetics of CL flashes occurring on the addition of different iron concentrations to oxidized LDL suspension containing 0.01 mg of protein per ml. At all iron concentrations used (from 0.01 mM to 5 mM) a quick CL flash of approximately similar shape was observed whose duration did not exceed 2–3 s. Along with the growing iron concentration from 0.01 to 0.5 mM a maximal flash amplitude enhanced and did not practically change on a further increase of Fe^{2+} concentration, as it is seen from the corresponding concentration dependence presented in Figure 1b. This result pointed to the fact that at Fe^{2+} concentration of more than 0.5 mM the intensity of flashes is not already limited by the amount of added iron and is evidently determined by the content of another reagent - lipid peroxides of oxidized LDL.

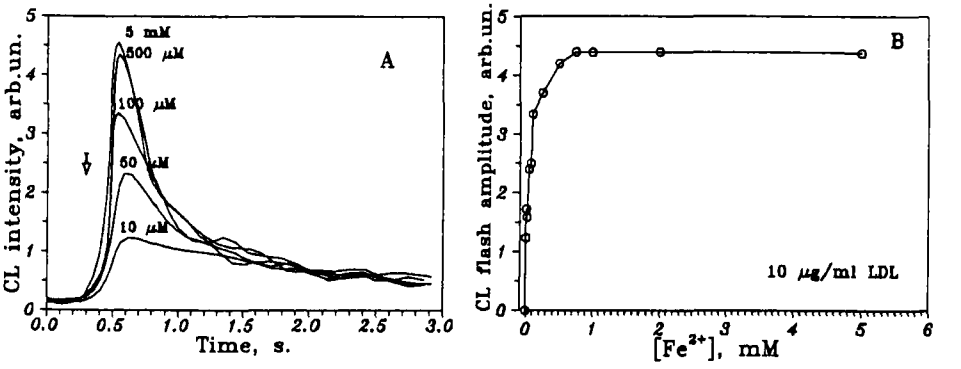

FIGURE 1 A - Effect of the concentration of Fe^{2+} added to LDL suspension (10 μg/ml of protein in 0.1 M NaCl, 0.02 M Tris-HCl, pH = 7.4) on CL flash kinetics. The moment of Fe^{2+} addition is indicated by the arrow. Fe^{2+} concentrations are presented at the curves. B - The dependence of the maximal CL flash amplitude on Fe^{2+} concentration.

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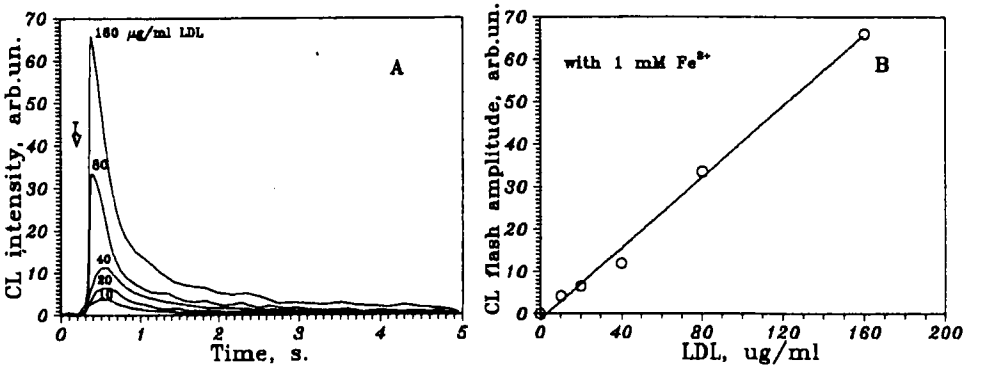

FIGURE 2 A - Effect of LDL concentration on CL flash kinetics. The moment of 1 mM Fe^{2+} addition is indicated by the arrow. LDL concentrations (μg/ml) are presented at the curves. All other conditions are the same as in legend to Figure 1. B - The dependence of the maximal CL flash amplitude on LDL concentration.

Figures 2a, b presents the kinetics and concentration dependence of CL flash amplitude on the addition of 1 mM Fe^{2+} to a suspension containing different concentrations of oxidized LDL. It is seen that with the increase of LDL concentration from 0.01 to 0.16 mg/ml, no significant changes in the character of CL kinetics took place, and the maximal amplitude of the flash depended practically linearly on the LDL concentration in the sample. The changes in CL flash amplitude at different LDL concentrations in samples (as well as it has been observed previously in the case of unsaturated fatty acid solutions and mitochondria suspensions[15] can be supposed to be associated with changes in lipid peroxide concentrations.

To testify this assumption, we investigated changes of CL quick flash amplitude in samples containing the same concentrations of LDL with a different extent of oxidation. To oxidize LDL wide spread methods were used: LDL autooxidation at 37°C and LDL oxidation in the presence of copper ions which sharply accelerate LPO process.[20,21] Figure 3 presents the kinetics of LDL autooxidation at 37°C for 4h; as a control, the kinetics of non-dialysed LDL preparation autooxidation are also presented (dashed lines in Figure 3). It is seen that in the latter case no significant LDL peroxidation was observed. The rate of LDL oxidation was recorded by measuring of TBA-reactive product (MDA) concentrations and diene conjugate (DC) concentrations. The CL flash amplitude turned out to be in a good accordance with the concentration of LPO products, TBARS and DC, in LDL (0.94 and 0.98, correspondingly) under changes of their content 2-3-fold (Figure 4A, B).

LDL oxidation in the presence of copper ions did allow to enhance the amount of LPO products accumulated in LDL. Figure 5 presents the kinetics of TBARS and DC accumulation, and CL quick flash amplitudes on LDL oxidation in the presence of two different $CuSO_4$ concentrations of 50 and 5 μM (Figure 5 and Figure 6, correspondingly). After 6h of such an oxidation we observed a more than 10-fold increase of the amount of primary and secondary LPO products. It is seen in the figures that it is also in these cases that the amplitude of CL flash reflects well enough the development of LPO process in LDL, especially for DC accumulation. The corresponding coefficients of correlation between the amplitude of CL flash and concentrations of TBARS and DC, calculated from the data of Figure 5 and Figure 6, were:

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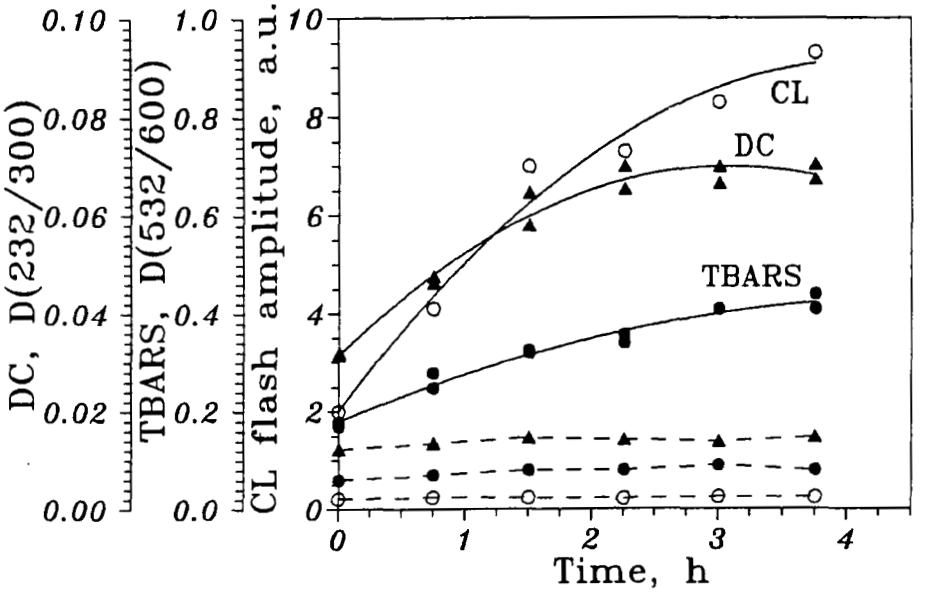

FIGURE 3 Kinetics of LDL (0.5 mg/ml of protein in 0.1 M NaCl, 0.02 M Tris-HCl, pH = 7.4) autooxidation at 37°C. CL - chemiluminescence flash amplitude on the addition of 1 mM Fe^{2+} into aliquots containing in final volume 10 μg/ml of LDL protein; TBARS accumulation was measured as D(532/600); DC - diene conjugate accumulation measured as D(231/300). The corresponding kinetics for fresh LDL preparation (before dialyzing) are indicated by dashed lines.

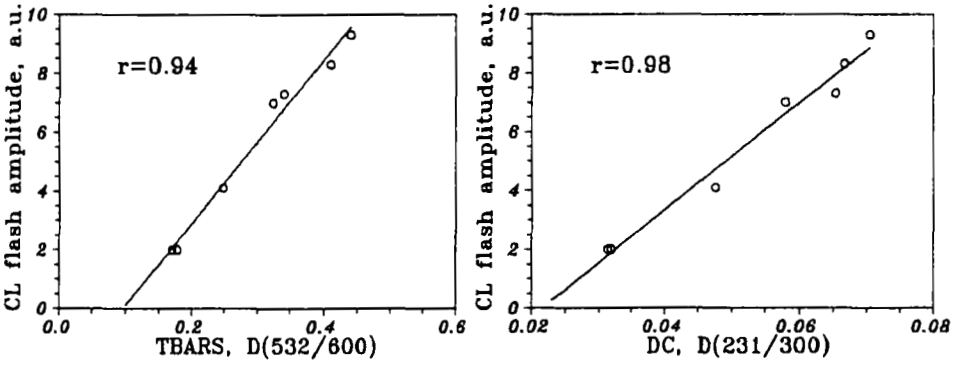

FIGURE 4 The correlations between CL flash amplitude and LPO product content (A - TBARS, B - diene conjugates) in autooxidized LDL, obtained from the data of Figure 3. R - a coefficient of linear correlation.

for MDA - 0.72 (Figure 7A), and for DC - 0.97 (Figure 7B).

The sensitivity of the CL method can be estimated, in particular, as a minimal LDL concentration in a sample, giving a measurable CL flash. The dependence of CL flash on LDL concentration in double logarithmic axes is presented in Figure 8 (curve 1). The crossing point of this straight line with abscissa provides the minimal value of

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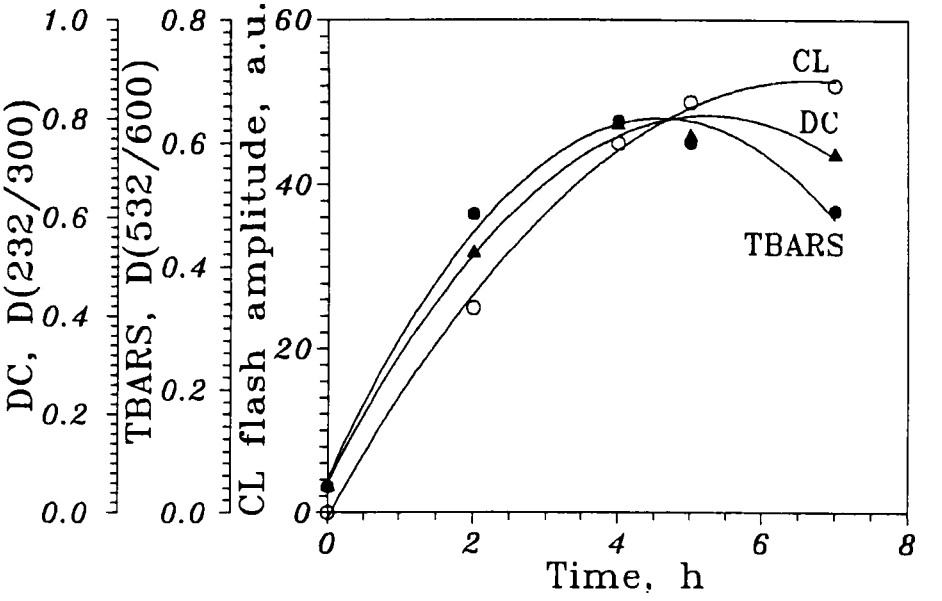

FIGURE 5 Kinetics of LDL oxidation at 37°C in the presence of 50 μM $CuSO_4$. Other conditions were as indicated in the legend to Figure 3.

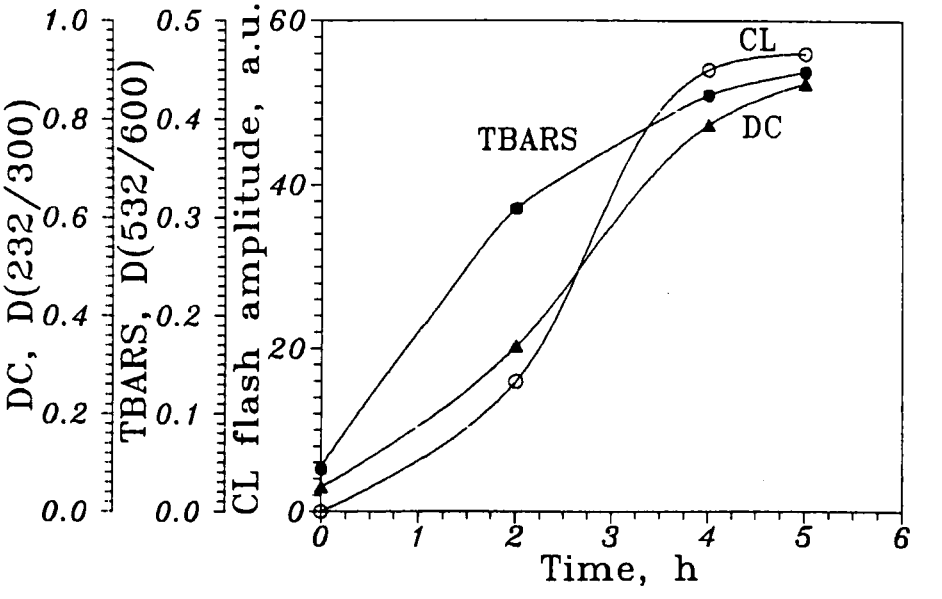

FIGURE 6 Kinetics of LDL oxidation at 37°C in the presence of 5 μM $CuSO_4$. For other conditions see the legend to Figure 3.

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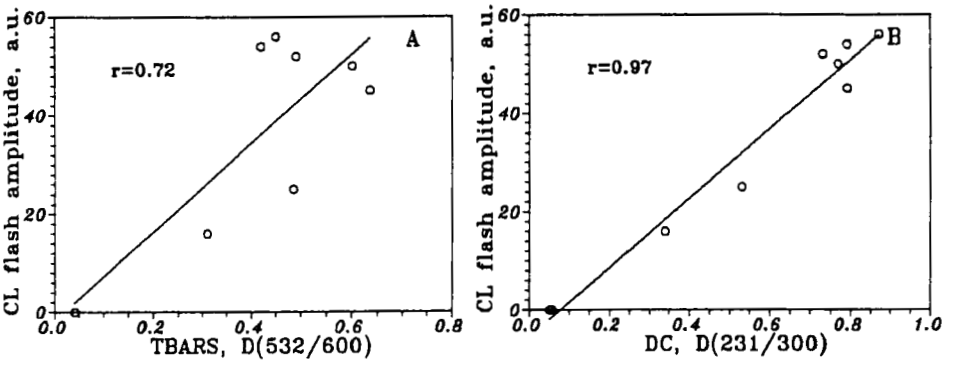

FIGURE 7 The correlations between CL flash amplitude and LPO product (A - TBARS, B - diene conjugates) content obtained from the data of Figure 5 and 6. R - a coefficient of linear correlation.

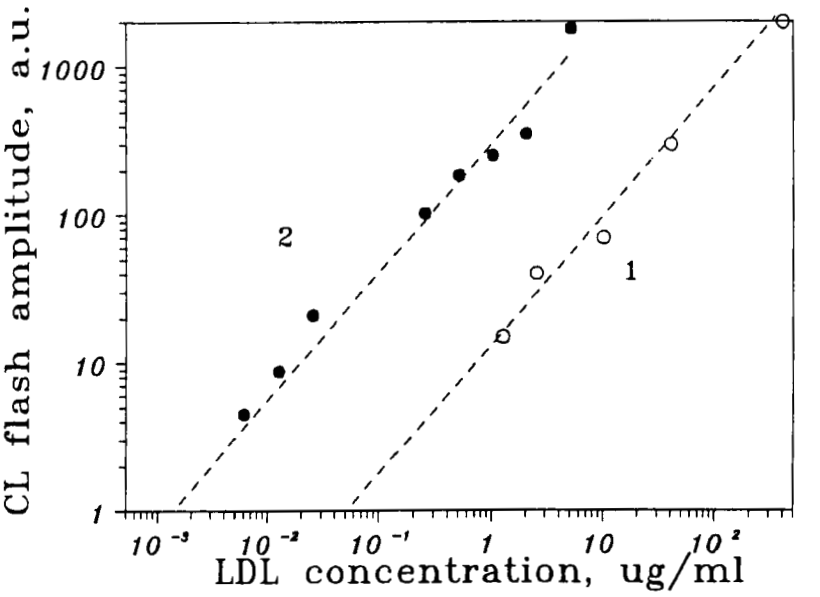

FIGURE 8 The dependence of the maximal CL flash amplitude on LDL concentration. 1 - without sensitizer; 2 - in the presence of C-525 sensitizer (10 μM). All other conditions are the same as in legend to Figure 1.

LDL concentration, which can be used for CL flash determination by our procedure. This value is about 1×10^{-1} μg of protein per ml (see Figure 8). Recently we have reported that a number of coumarin derivatives was found to be a perfect sensitizers of CL accompanying lipid peroxidation reactions in liposomes.[26] The most potent of them, C-525, was used for CL flash enhancement in our experiment.

The coumarin dye was dissolved in ethanol and added to the LDL suspension; the final concentration of ethanol did not exceed 4%. It was shown in control experiments that ethanol did not influence the chemiluminescence kinetics and intensity in

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this concentration (the data are not presented). It is seen in Figure 8, curve 2, that C-525 at concentration of 10 μM both significantly amplified CL flash intensity and decreased the minimal LDL concentration about two orders (for 10^{-3} μg/ml).

DISCUSSION

As the result of long-term experiments and mathematical modelling of Fe^{2+}-induced LPO kinetics in membranes and other lipid systems the leading role of hydroperoxides in the development of the process was established.[9,10,22] The interaction of hydroperoxides with Fe^{2+} ions, depending on their concentration, determines either self-accelerating (at Fe^{2+} concentration below critical for this system), or pulse (at higher Fe^{2+} concentrations) form of LPO and accompanying CL kinetics. In case of the addition of excess Fe^{2+} to lipid solutions containing hydroperoxides, a maximal amplitude of CL quick flash was proportional to the squared hydroperoxides concentration:[15]

$$I = v\,[LOOH]^2 \qquad (1)$$

according to a simplified scheme of the of the reactions[9,10]

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO + HO^-$$

$$LO\cdot + LH + O_2 \rightarrow LO_2\cdot + LOH$$

$$LO_2\cdot + LO_2\cdot \rightarrow LO + LOH + O_2 + h\nu \text{ (chemiluminescence)}$$

Nevertheless, such an approach is appropriate only for homogeneous system. In real biological systems, the mentioned reactions proceed under conditions of two-phase separation of reagents. This is why the kinetics of the process is determined by real local concentrations of the reagents next to the surface of the water/LDL interfaces which, in turn, must depend on the physical properties of hydrophobic phase (for LOOH and $LO_2\cdot$) and of the surface of LDL (for Fe^{2+})[22-24]. We investigated the kinetics of the CL flash arousing on addition of Fe^{2+} to a suspension of LDL in Tris-buffer, in a wide range of Fe^{2+}, LDL, and LPO product concentrations. The existence of a plateau on the dependence of CL flash amplitude on the Fe^{2+} concentration allowed to determine a working Fe^{2+} concentration (1 mM) at which the form and intensity of CL flash does not practically depend on the amount of the added iron but is determined by the content of LDL and the products of their oxidation. However, the dependence of the flash amplitude on the concentration of oxidation products in the sample (which was changed in our experiments by two ways - by variation of the content of oxidized LDL and LPO product accumulation) was almost linear, i.e. it did not correspond to Equation 1. This effect may apparently be accounted for by the fact that in the course of LPO increase of lipid hydroperoxides concentration in the LDL volume take place; but their interaction with Fe^{2+} occurs in the thin layer next to the LDL surface,[25] and, apparently, not all lipid peroxide molecules become accessible for the participation in the reaction. It may lead to underestimating of a squared dependence of the CL flash intensity on LOOH concentration which, as a result, becomes quasi linear. Without going into details of the mechanism of this phenomenon, it should be admitted that such a situation is rather usable for practical application of the CL method. The following should be attributed to the advantages of this method:

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1. quickness of carrying-out of the analysis (which is determined by a short-time measurement of CL kinetics and by the absence of sample preparation procedures - lipid extraction, incubation with some reagents, etc.);

2. a rather high sensitivity which allows to work with relatively low LDL concentrations (without sensitizers - micrograms of protein per ml of LDL suspension, and in the presence of C-525 - nanograms of protein per ml). All these allow to hope that the CL method of hydroperoxide determination may be successfully applied as an express-micrometer of estimation of lipoprotein oxidation in biological and clinical investigations.

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