A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent

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Summary A method is described for measuring lipid peroxides by means of the color reagent of a commercially available test kit for cholesterol estimation. In principle, this assay makes use of the oxidative capacity of lipid peroxides to convert iodide to iodine, which can be measured photometrically at 365 nm. Calibration curves were obtained using peroxides such as H_2O_2, t-butyl hydroperoxide, and cumene hydroperoxide. A stoichiometric relationship was observed between the amount of organic peroxides assayed and the concentration of iodine produced. Concentrations of lipid peroxides as small as 1 nmol/ml could be measured. The ability to estimate lipid peroxides of isolated low density lipoprotein was demonstrated. — **El-Saadani, M., H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar, and G. Jürgens.** A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid Res.* 1989. **30:** 627–630.

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The major initial reaction products of lipid peroxidation are lipid peroxides and their quantitation serves as a direct and valuable index of the oxidative status of polyunsaturated fatty acid-containing tissues (membranes) or biosystems. Oxidatively modified human serum low density lipoprotein (LDL) has recently gained increasing interest in atherosclerosis research. It might represent a form of LDL by which monocytes/macrophages can be transformed to lipid-laden foam cells, a cell type that is often found in early atherosclerotic lesions (for review see refs 1–3). Several methods currently exist for estimating the oxidation of lipids and some of them have been used to assess lipid oxidation in oxidized lipoproteins. Based on the reaction of malondialdehyde, a break down product of lipid peroxides, with thiobarbituric acid (TBA), the measurement of so-called thiobarbituric acid reactive substances (TBARS) has been used commonly to check lipoproteins for products of lipid peroxidation (4, 5). In this assay the lipoprotein is precipitated by trichloroacetic acid, and only the water-soluble malondialdehyde present in the supernatant is estimated. Thus, this assay is neither

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specific for the hydroperoxy group nor stoichiometric. A modification of the method was described where the lipid peroxides are first precipitated (to separate them from free malondialdehyde) and then degraded to malondialdehyde to be measured fluorophotometrically after reaction with TBA (6). However, this method did not work when applied to lipoprotein solutions as no visible precipitation of lipid peroxides was obtained. This is probably due to the fact that lipid peroxides remain within the lipoprotein particle. Methods making use of the peroxidase activity for the determination of lipid peroxides were reported to have certain limitations when applied to biosystems containing different lipid classes (7); special laboratory equipment may be needed (8). In continuation of our work on oxidation of human serum low density lipoproteins (LDL) (3, 9, 10) there was the need for a quick method, applicable to lipoproteins, for directly measuring lipid peroxides, allowing study of a larger number of samples. This led us to develop an assay using one part of a commercially available test kit for cholesterol estimation. The principle of this assay is based on the oxidative activity of lipid peroxides that will convert iodide to iodine. Iodine can then simply be measured by means of a photometer.

MATERIALS AND METHODS

H_2O_2 was from BDH Limited (Poole, England). T-butyl hydroperoxide and cumene hydroperoxide were from Sigma (Munich, FRG). Human serum LDL was obtained by sequential ultracentrifugation in a density cut from 1.020 g/ml–1.050 g/ml as described earlier (11). Prior to oxidation the lipoprotein fraction was dialyzed extensively against degassed phosphate-buffered saline (PBS) (containing 50 μg chloramphenicol/ml) to remove EDTA and BHT which were present throughout the preparation. As a lipid peroxide-generating system we incubated LDL (1.5 mg/ml) with 5 μM $CuCl_2$ in phosphate-buffered saline containing 2.5 μM ascorbic acid. Samples were withdrawn at different time intervals for analysis. For the peroxide assay we used the color reagent of the commercially available kit for the enzymatic determination of cholesterol (CHOD-iodide, Merck, Darmstadt, FRG). Its composition is listed in **Table 1**. One hundred μl of a lipoprotein solution (containing 1.5 mg/ml of total LDL) was mixed on a vortex mixer with 1 ml of the color reagent. After addition of EDTA and BHT to give final concentrations of 24 μM and 20 μM, respectively, in order to inhibit progress of lipid peroxidation, the sample was allowed to stand for 30 min in the dark at ambient temperature before absorbance was measured at 365 nm against the color reagent only as the blank. Concentrations of LDL up to 1.5 mg/ml did not affect the blank readings. At higher concentrations native LDL was mixed with the color reagent and measured immediately, giving the

TABLE 1. The reagent constituents of the color reagent (Merck, cat. no. 14106)

Potassium phosphate, pH 6.2	0.2 M
Potassium iodide	0.12 M
Sodium azide	0.15 mM
Polyethyleneglycol mono[p-(1,1',3,3'-tetramethyl-butyl)-phenyl]ether	2 g/l
Alkylbenzyldimethylammonium chloride	0.1 g/l
Ammonium molybdate	10 μM

blank of that sample. This reading gives the exact absorbance attributed to LDL only (blank) and not to LDL together with lipid peroxides, which is measured after 30 min. TBARS and conjugated dienes were measured as previously described (refs. 12 and 13, respectively).

RESULTS AND DISCUSSION

Concentrations of lipid peroxides were calculated by the use of the molar absorptivity of I_3 measured at 365 nm (ϵ = 2.46 $\pm$ 0.25 $\times$ $10^4 \cdot M^{-1} \cdot cm^{-1}$). This value was determined by adding known concentrations of I_2 to the assay color reagent. Calibration curves obtained with different peroxides such as H_2O_2, t-butyl hydroperoxide, and cumene hydroperoxide gave values for ϵ of 2.45 $\pm$ 0.04, 2.34 $\pm$ 0.26, and 1.6 $\pm$ 0.15 $\times$ $10^4 \cdot M^{-1} \cdot cm^{-1}$, respectively. A stoichiometric relationship (slope = 1.02) was observed between the amount of organic peroxides assayed and the concentration of I_3 produced.

In **Table 2** the values of lipid peroxides generated upon oxidation of LDL are compared with the conjugated dienes and TBARS measured at different time points. The amount of lipid peroxides estimated by this method was in good agreement with the values of conjugated dienes. However, the values estimated for TBARS given as malondialdehyde equivalents were much lower in the first 24 hr of oxidation. This simply reflects that lipid peroxides are at first the major products formed during oxidation of LDL. Their breakdown to malondialdehyde in order to be measurable by the TBA assay might have been impaired by the presence of EDTA (which was used together with BHT to stop the oxidation process in the sample), as it was reported by Asakawa and Matsushita (14). The steep drop of lipid peroxide levels which occurs later, during oxidation of LDL, is most likely due to a breakdown of lipid peroxides into a variety of secondary products of lipid peroxidation, such as aldehydes, some of which may react in turn with the apoprotein moiety of LDL (3, 9, 15), as well as epoxides and other compounds (16, 17).

The commonly used iodometric procedures are based on the ability of lipid peroxides to oxidize I^- in the reagent to I_2 which in turn reacts with the excess of nonreacted I^-

TABLE 2. Kinetics of the formation of lipid peroxides (LPO), conjugated dienes (CD), and thiobarbituric acid reactive substances (TBARS) during oxidation of LDL

	0 Hr	6 Hr	12 Hr	24 Hr	27 Hr	48 Hr
			nmol/mg LDL			
CD	29.1 ± 2.8	183.3 ± 9.9	203.1 ± 11.9	246.5 ± 14.6	n.e.	n.e.
LPO	5.4 ± 0.3	97.1 ± 6.2	154.2 ± 8.4	288.5 ± 18.9	102.9 ± 8.4	20.0 ± 2.7
TBARS	0.7 ± 0.1	12.6 ± 1.7	15.9 ± 2.1	16.5 ± 1.9	18.5 ± 2.6	16.8 ± 2.3

Values are means of triplicate determinations; LDL concentration: 1.5 mg/ml; incubation temperature: 37°C; n.e., not estimated.

to form I_3. The iodometric techniques normally suffer from their susceptibility to interference by molecular oxygen and light. Moreover they have been applied only to lipid extracts. Several studies have attempted to overcome these disadvantages. Takagi, Mitsuno, and Masumura (18) and Buege and Aust (19) performed the assay under anaerobic conditions and in the presence of cadmium acetate to protect excess I^- from oxygen. Asakawa and Matsushita (20) measured the developed blue color of the iodine–starch complex at 560 nm, but the molar extinction coefficient depended on the chain length of the starch used. Thus, these modified techniques require a considerable amount of material and none of them could fulfill our requirements for a sensitive but simple routine colorimetric method for assaying lipid peroxides, especially in lipoproteins. The color reagent solution contains, apart from the phosphate-buffered potassium iodide, a variety of substances including detergents, furnishing suitable conditions to facilitate a direct interaction between the lipid peroxides in the lipid phase and the color reagent solution. Thus, concentrations of lipid peroxides of less than 1 nmol/ml could be directly measured by this simple assay. Comparing the sensitivity of our assay with estimations by high performance liquid chromatography (HPLC) we found the following values in the literature. Miyazawa, Yasuda, and Fujimoto (21) were able to measure phosphatidylcholine (PC) hydroperoxide on HPLC coupled with chemoluminescence at a detection limit of 7 nmol. Yamada, Terao, and Matsushita (22) using HPLC with electrochemical detection found as their lowest detectable level 200 pmol for PC hydroperoxides. However, those authors used an advanced method for measuring a single class of lipid peroxides, whereas with our simple iodometric procedure we determine the total amount of lipid peroxides.

The major disadvantage of an iodometric assay, namely its sensitivity toward interference by molecular oxygen, is very limited with the reagent used as there was no difference of extinction whether the blank was measured immediately or after 30 min at ambient temperature. Furthermore, no interference from malondialdehyde up to 100 mM, glucose up to 300 mg/dl, ascorbic acid up to 70 mg/ml, uric acid up to 17 mg/dl, reduced glutathione up to 50 mg/dl, hemoglobin up to 300 mg/dl, and bilirubin up to 20 mg/dl could be detected. The assay can also be used to follow oxidation of lipids, i.e., generation of lipid peroxides with other lipoprotein fractions such as high density lipoproteins. ▄▄

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