Complementary procedures for pro- and anti-lipoperoxidant activity measurements

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Summary. Evaluation of lipoperoxidation is performed by 2 methods, the first one measuring the amount of peroxy-function (−OOH) at a given time, and the second one measuring the accumulation of a degradation product against time. The combination of the 2 methods allows us to investigate the kinetics of lipoperoxidation and to demonstrate, for example, the accelerating effect of lidocaine on autoxidation processes.

For several years, studies of lipid peroxidation have been increasingly important in biology and in medicine[1,2]. A possible role of lipoperoxides has been suggested in the development of pathological processes such as atherosclerosis[3], toxic and radiobiological phenomena[1], oncological processes[4], and in development of the adult respiratory distress syndrome[5]. In lipoperoxidation studies, it is not only important to detect the presence of peroxides, but also to evaluate the rate of their generation and relate it to the biochemical, biophysical, pharmacological and physiological factors present.

The mechanism of lipoperoxidation may be divided into 4 distinct steps (fig. 1):

a) Initiation: a polyunsaturated organic chain is activated to a radicalar form, by deshydrogenation; after that, triplet oxygen can react with this newly formed doublet molecule. The deshydrogenation may be performed either by an enzymatic process, such as lipoxygenasic activity[6], or by small radicals arising from oxygen metabolism, such as ·OH or alkoxy radicals.

b) Autoxidation: peroxidation is propagated by autocatalysis, forming an autoxidation cycle.

c) Decomposition of hydroperoxide: the hydroperoxides produced during the autocatalytic step are broken off, giving a lot of decomposition products, particularly aldehydes for lipoperoxides of polyunsaturated fatty acids[7].

d) Termination of the autoxidation cycle: the radicals are trapped, or form dimers[1,7].

Autoxidation appears to be the most important step from the point of view of biochemists and physicians; it is during these cycles that the greatest membrane damage occurs. Therefore, it is important to detect the pro- or anti-lipoperoxidant properties of drugs and reagents, by studying their action on this propagation cycle. The autoxidation rate may be followed either by measuring the −OOH level or by measuring the final concentration of lipoperoxide decomposition products (for example, by the classical TBA technique).

Some unsatisfactory techniques have been proposed in the literature, to evaluate the rate of autoxidation itself. Oxygen consumption monitoring is not specific for peroxidation, in biological systems. Chemiluminescence, which needs sensitive and complicated apparatus, may be due to other reactions than peroxidation[1]. We propose, in this study, a fast procedure to obtain a standardized cycle of autoxidation. A direct −OOH and OO· estimation is given by methods using N,N′-dimethylparaphenylene diamine

Effect of nor-dihydroguiairetic acid (NDGA) on lipoperoxidation measured by DMPD and TBA

Incubation time (min)	Control	NDGA 10^{-5} M	NDGA 10^{-4} M
a) DMPD: Δ[−OOH] in μeq/ml			
1	0.02	0.1	0.05
5	0.18	0.2	−0.40
60	0.70	0.3	−0.60
b) TBA: Δ[MDA] in μeq/ml			
1	–	18.5	13
5	26	22.5	22
60	46	36	34

Δ, Difference between lipoperoxides obtained in presence of NDGA and starting lipoperoxide concentration. The OD-values are converted into lipoperoxides μeq, referring to the curve of figure 2.

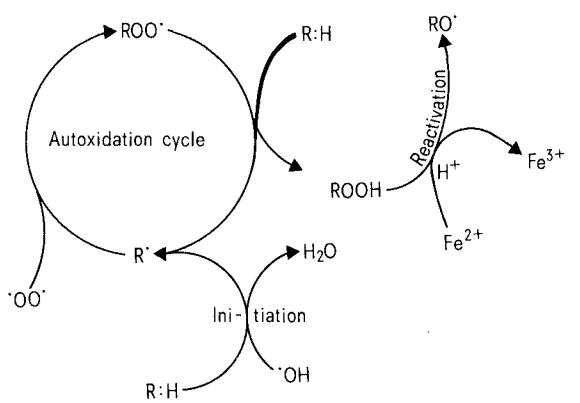

Figure 1. Lipoperoxidation cycle: R:H, polyunsaturated lipid; R·, polyunsaturated lipid radical; ·OO·, triplet molecular oxygen (O_2); ROO·, lipoperoxy radical; ROOH, lipid hydroperoxyde; RO·, alkoxy radical (see introduction).

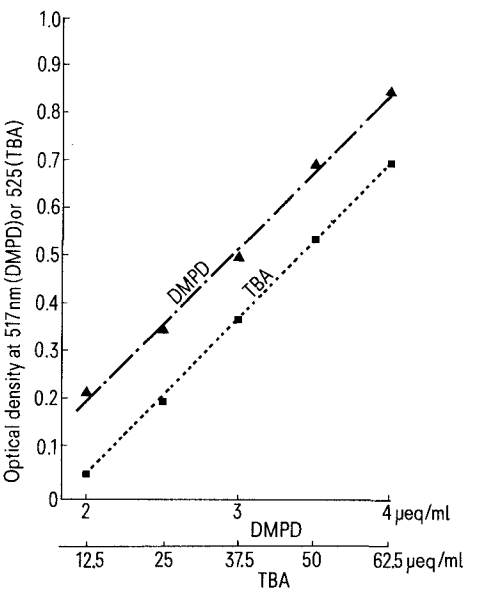

Figure 2. Standard absorption curves for DMPD and TBA methods. Abscissae: concentration of lipoperoxides estimated in microequivalents (μeq) per ml, by iodometry.

dichloride (DMPD); the malonaldehyde (MDA) accumulation resulting from peroxide decomposition is measured by the classical thiobarbituric acid (TBA) method.

The experiments described in the present paper demonstrate that the 2 methods are complementary.

Methods. Autoxidation technique. Sodium arachidonate solutions (10^{-3} M) are incubated at 56 °C in the presence of peroxidized lineolate (10^{-5} M), in phosphate buffer (pH: 6.4) with Fe^{2+}($FeSO_4 \cdot 7\ H_2O$: 10^{-4} M) and EDTA (5×10^{-5} M). Reactions are performed in sealed vials filled with pure oxygen and constantly stirred by a Dubnow device, for various time periods.

Peroxide estimation (−OOH content). The technique published by Deby et al.[8] is used; this is a modified procedure of the method proposed by Ceriotti and Spandrio[9], and applied to TLC by Vioque and Holman[10]. In a 1st step, lipoperoxides oxidize Fe^{2+} to Fe^{3+}, which, in turn, oxidizes DMPD, to form a pink colored product. The lipoperoxides are extracted by chloroform, the solutions are then evaporated under N_2, in glass stopped tubes (10 ml); the following reagents are then added: ethanol 300 µl (dissolving the peroxidized lipids); $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (1% in H_2O): 50 µl; N,N′-dimethyl-p-phenylene (0.1% in the following solution: $CHCl_3$/CH_3COOH/H_2O; 5:5:1). The tubes are closed under nitrogen. After 20 min, the absorbance is measured at 517 nm, against a blank.

A standard curve is made using several dilutions of peroxidized linoleate solution. Peroxide levels are measured in the concentrated solution by iodometry. Extinction coefficient was $7.2 \times 10^4\ M^{-1}\ cm^{-1}$.

Iodometry. This method is performed according to Olcott et al.[11], without modification. 50 mg of peroxidized linoleate are dissolved in 3 ml of $CHCl_3/CH_3OH$ mixture (3:1). 1 ml of saturated KI solution is then added, under N_2. After 120 sec, 10 ml of starch solution (1% in water) are added. After 1 min the free iodine is titrated by 0.005 N $Na_2S_2O_3$. Iodometry is only used for the standardization of the concentrated peroxide mixtures.

Thiobarbituric acid method. To an aliquot of the reaction mixture the following are added: 2 ml of phosphate buffer (0.2 M; pH 7.0), 1 ml of ethanol, 1 ml of trichloracetic acid (20% in water), and 1 ml of thiobarbituric acid (0.67% in water). The mixture is heated at 100 °C for 20 min. After cooling and centrifugation, the absorbance is measured at 525 nm. The extinction coefficient found was $1.5 \times 10^5\ M^{-1}\ cm^{-1}$ (Sinnhuber and Yu[12]).

Reagents used in this study. Linoleic and arachidonic acids were from Sigma; N,N′-dimethyl-p-phenylene diamine dichloride and soluble starch from Merck; Nor-dihydroguaiaretic acid (NDGA) from Fluka; lidocaine was kindly supplied by Astra-Nobel Pharma Laboratories (Brussels, Belgium).

Calculation of autoxidation rate. The content of peroxides is determined by both methods, before incubation, and is substracted from the value measured after the different times of incubation: the difference measures the increment in −OOH and TBA reactive material and is represented by Δ(−OOH) and Δ(MDA), respectively.

Results. a) Comparison of the sensitivity of the 2 methods. In figure 2, the DMPD test appears to be 10 times more sensitive than the TBA assay is measuring lipoperoxides.

b) Chemical functions measured by DMPD. Not only lipoperoxides, but also hydrogen peroxide give a positive reaction with DMPD, as shown in figure 3.

c) Interest of the 2 combined methods. In figure 4, each point is obtained by substraction of the initial lipoperoxide value found at time zero from each measured value. It represents the variation of −OOH or MDA concentration at a given time [Δ(−OOH) and Δ(MDA)]. As malonaldehyde cannot be destroyed under these experimental conditions, its concentration rises more or less rapidly. But the −OOH concentration, at a given time, is the result of peroxide neoformation, balanced by peroxide decomposition. Thus, great fluctuations can be observed, particularly at the onset of reaction.

d) NDGA and lidocaine effects. In the presence of a strong antioxidant, such as NDGA, Δ becomes negative (table).

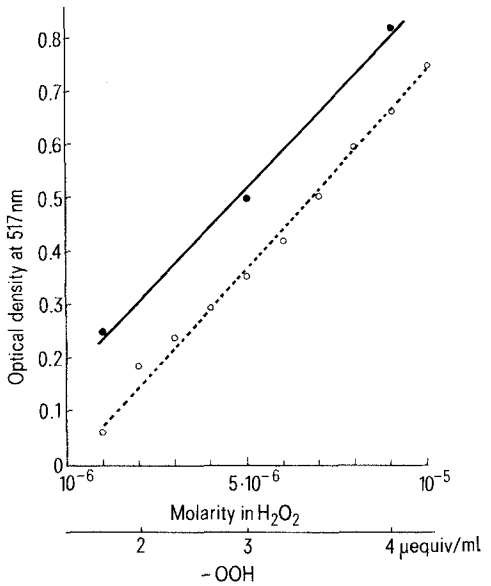

Figure 3: Hydrogen peroxide (dotted line) and linoleyl peroxides (continuous line) measurements by the DMPD technique. Abscissae: Upper scale; molar concentrations in hydrogen peroxide. Lower scale; concentrations of linoleyl peroxides expressed in microequivalents per ml.

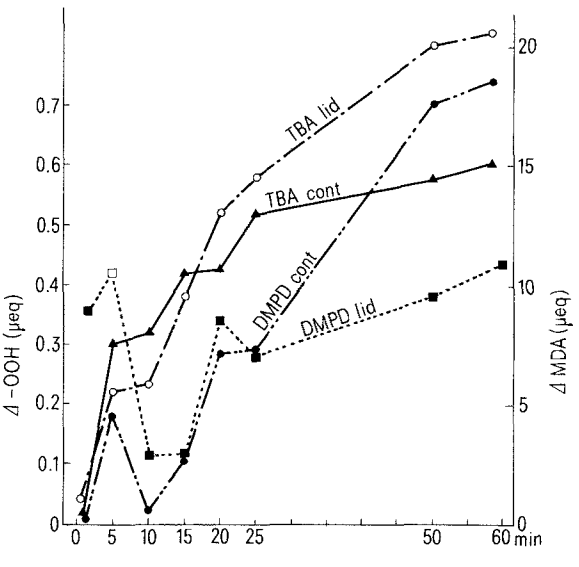

Figure 4. Effects of lidocaine on lipid autoxidation against time. Left ordinates: Increase of lipoperoxide (−OOH) concentration of lidocaine (DMPD lid) vs control (DMPD cont). Right ordinates: Increase in TBA measurable products (aldehydes: −CHO) in the presence of lidocaine (TBA lid) and in the controls (TBA cont).

The pro-oxidant activity of lidocaine is particularly clear with DMPD assays in the 1st min following the starting of autoxidation (fig. 4). The acceleration of autoxidation results in a rapid rise, followed by a fall of [−OOH], with a simultaneous increase in TBA reacting products. If we consider the DMPD results only after 1 h, we might infer that lidocaine impairs the autoxidation cycle. However, the measure of the peroxide level at the onset of autoxidation, by the DMPD test, shows on the contrary an accelerating effect of lidocaine. The TBA test clears up this problem, since it detects a progressive accumulation of peroxide decomposition products, which is more important in the presence of lidocaine.

On the other hand, used alone, TBA is unable to demonstrate the starting activation by lidocaine. The discrepancies between the TBA and DMPD methods disappear when autoxidation is inhibited by efficient anti-oxidants (table).

Discussion. The pro- or anti-oxidant properties of a substance can be measured by its ability to inhibit or to accelerate the propagation reaction, during the autoxidation cycle. However, many artefacts may occur, which are sometimes difficult to avoid.

a) Autoxidation cycle. The use of preformed hydroperoxides and of Fe^{2+} for cycle reactivation (fig. 1) is substituted for the classical initiation by hydroxyl radicals (generated by a Fenton reaction or by the system xanthine/xanthine-oxidase). With this method the modifications observed after application of anti-lipoperoxidants cannot be attributed to a hydroxyl scavenging effect. Addition of EDTA as ligand is required for a better peroxidation rate, maintained by Fe^{2+}; ligands orientate and stabilize Fe^{2+} in an active configuration[2]. The use of EDTA is critical; above an optimal concentration of $5 \cdot 10^{-5}$ M, it becomes a powerful inhibitor of lipoperoxidations. A great variability in the peroxidation rate is generally observed in the case of irregular oxygenation; the time and the intensity of oxygen bubling must therefore be carefully standardized.

b) Significance of DMPD test. This method, applied to lipid chloroform extracts, measures directly the lipoperoxide concentration at the time of extraction. This concentration is always dependent on the balance between peroxide formation and decomposition into smaller fragments[7]. Artefacts may arise, due to Fe^{3+} or Cu^{2+} in the determination medium. Only fresh solutions of $(NH_4)_2FeSO_4$, prepared under N_2 (to avoid Fe^{2+} oxidation), are suitable for DMPD reactions. Accidental bringing of transition cations arising from the aqueous peroxidation medium is avoided by the $CHCl_3$ extraction, which moreover eliminates the water-soluble oxidants able to oxidize Fe^{2+} to Fe^{3+}.

c) Conditions of TBA test. This technique is used directly in the aqueous peroxidation medium. The addition of Fe^{++} was recently demonstrated to be necessary[13]; Fe^{++} was present in the autoxidation cycle medium at 10^{-4} M, which appears to be the optimal concentration[14]. EDTA, required for a good autoxidation cycle, is present at 5×10^{-5} M, and does not induce the inhibiting effect observed at 3×10^{-4} at 3×10^{-4} M, in the presence of Fe^{2+} (Asakawa and Matsushita[13]). The pH of the reaction was near the value of 4.0 considered to the optimal[14].

d) Significance of TBA test. This method measures malonaldehyde arising from the spontaneous breakdown of lipoperoxides. Contrary to the assertions of Dahle et al.[15], Asakawa and Matsushita[13] demonstrated recently that linoleate hydroperoxides can form TBA color, as well as more desatured fatty acids. Therefore, the TBA test estimates the lipoperoxidation processes indirectly by measurement of decomposition products, which progressively accumulate. Artefacts may be due to preexistent aldehydes or 2-dioxy-sugars.

e) Examples of the complementarity of the 2 methods. 1. Pro-oxidant effect of lidocaine. The −OOH concentration is significantly enhanced in the 1st min of incubation. Breakdown of lipoperoxides results in a fall of DMPD values, whilst TBA values begin to rise, by a progressive accumulation of decomposition products. New rises in DMPD are accompained by a reduction in the slope of the TBA curve. If DMPD is used alone, with only measurements after 60 min of incubation, false conclusions may be drawn, leading to the misconception that lidocaine is an anti-lipoperoxidant; DMPD values in the control are indeed higher than the values of lidocaine test. The TBA test, measuring the total number of peroxide molecules formed and decomposed, shows a higher value in the presence of lidocaine, demonstrating a prolipoperoxidant activity of this substance. However, TBA is unable to reveal the acceleration of lipoperoxidation observed with DMPD at the onset of incubation with lidocaine.

2. Anti-oxidant effect of NDGA. The 2 methods are in concordance, leading to the conclusion that NDGA is a strong inhibitor of lipoperoxidation.

Thus, the complementarity appears to be general in the case of agents accelerating autoxidation (pro-lipoperoxidants).

Conclusions. The direct estimation of hydroperoxy functions (−OOH) measures the balance between −OOH formation and decomposition. The thiobarbituric acid method estimates the decomposition products which progressively accumulate. But the TBA test cannot give a good view of the early kinetic aspect of lipoxidation. DMPD, measuring the actual −OOH content, analyzes, with greater sensitivity, the early rise in −OOH production. But the DMPD technique, at the decomposition phase of autoxidation, will lead to false estimations, which are corrected by the TBA method. The 2 techniques are thus complementary; the first one is better for a kinetic study, and the second is good for a total measurement.

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