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Is malonaldehyde a valuable indicator of lipid peroxidation?

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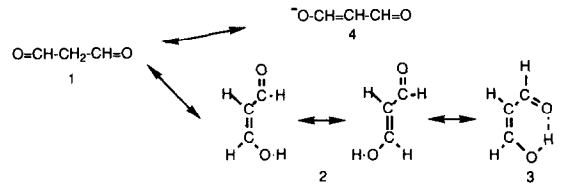
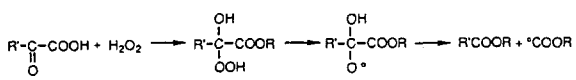
Abstract—Malonaldehyde (MDA), a decomposition product of lipid hydroperoxides which is used as an indicator of oxidative damage to cells and tissues, reacts, *in vitro*, with hydrogen peroxide to form undetermined degradation products. Since human polymorphonuclear leukocytes (PMNs) release reactive oxygen species including hydrogen peroxide when stimulated with phorbol myristate acetate (PMA), we incubated specific amounts of MDA with resting PMNs and PMA-stimulated PMNs. The amount of MDA recovered after 30 min incubation with stimulated cells, as determined by MDA–thiobarbituric acid assay, was 25% lower than that recovered with resting cells. In the presence of catalase 18% of MDA disappeared and in the presence of superoxide dismutase 15% disappeared. This indicates that measurements of MDA production in living systems, in the presence of reactive oxygen species, could be underestimated.

Malonaldehyde (MDA*), which is believed to form as a decomposition product of certain lipid hydroperoxides, is one of the most studied lipid hydroperoxidation products [1]. MDA formation occurs after unsaturated fatty acids are decomposed by free radicals formed by sunlight, by metal ions such as Cu or Fe, or by enzymes such as lipoxygenases and cyclo-oxygenases which are involved in eicosanoid synthesis. Thus, MDA levels have been used to estimate oxidative damage to living tissues [2], free radical production in biological systems [3] or as diagnostic indicators of the freshness of lipid-rich foods [4].



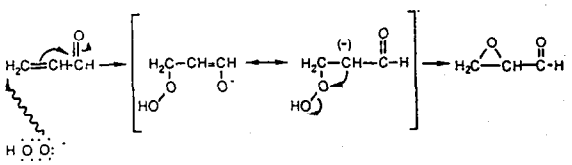
(1)

MDA occurs in aqueous solutions in various resonance forms (Eqn 1), depending on the pH [5]. The pK value of the enolic group is 4.5 [6]; therefore, the enolate anion (**4**) is the predominant form in neutral and alkaline media and undissociated forms (**1**, **2** and **3**) occur at acidic pH [7]. These forms show that MDA is closed to very reactive compounds such as α-keto acids of α,β-unsaturated ketones and aldehydes. Bernardi *et al.* [8] prepared °COOR radicals, by treatment of esters of α-keto acids with hydrogen peroxide in acid media, to carbalkoxylate protonated nitrogen heterocycles (Eqn 2). Whereas, in



(2)

basic media, α,β-unsaturated ketones and aldehydes react with hydrogen peroxide (Eqn 3) to form oxacyclopropane from propenal by the Michael-type 1,4-addition reaction [9].



(3)

The hydroperoxide ion being the nucleophile agent, this type of reaction is known to provide 1:1 and 1:2 adducts between the β-hydroxyacrolein form of MDA and amino acids [10].

MDA is also a good substrate for peroxidases, as recently shown by Mottley *et al.* [11].

As a bifunctional reagent, MDA can cross-link proteins or nucleotides [12, 13] to form Schiff-base products by condensation with primary amino groups of proteins, nucleic acids or amino acids which have been observed as fluorescent "lipofuscin" pigments, in tissues of animals under stressful conditions. Lipofuscin levels have been used as cellular aging indicators [14].

The reactivity of MDA and its extensive use as an indicator of *in vivo* peroxidation therefore prompted us to study its reactivity toward hydrogen peroxide which is produced in many living tissues [15].

Moreover, it has been shown for a long time that aldehydes are substrates for xanthine oxidase [16] and have superoxide radical anion-generated capacities. Since xanthine oxidase is widely used *in vitro* as a superoxide anion source or is generated from xanthine dehydrogenase during ischemic strokes [17], we studied the ability of xanthine oxidase to oxidize MDA.

Materials and Methods

MDA was prepared by hydrolysis of 1,1,3,3-tetramethoxypropane as described previously [18]. The MDA concentrations were checked by measuring the UV absorbance at 247 nm (ε = 13,700) and by thiobarbituric acid (TBA) assay (ε = 153,000 at 532 nm) [19]. Hydrogen peroxide was obtained from Merck (Darmstadt, F.R.G.).

HPLC analysis was carried out on a Spherisorb NH_2 analytical column (25 × 1 cm) (Shandon, Paris). The mobile phase consisted of 10% acetonitrile in Tris buffer 0.03 M, pH 7.4. The flow rate was 0.8 mL/min, the effluent was monitored at 267 nm with a Linear UVIS 204 detector. In dilute neutral or alkaline solutions, the maximum absorbance of MDA is shifted to 267 nm (ε = 31,500) [20]. Fluorescence spectra were performed with a Perkin–Elmer

*Abbreviations: MDA, malonaldehyde; TBA, thiobarbituric acid; PMN, human polymorphonuclear leukocyte; PMA, phorbol myristate acetate; BSA, bovine serum albumin.

MPF3 spectrofluorometer. UV spectra were recorded with a double beam UVICON spectrometer.

Measurements of the effects of phorbol myristate acetate (PMA)-stimulated human polymorphonuclear leukocytes (PMNs) on MDA. PMNs were prepared from the blood from antecubital veins of healthy human volunteers as described previously [21], PMNs (10^6 cells/200 μL) in Krebs–bicarbonate solution were added with synthetic MDA (2–8 μM) and stimulated when necessary by the addition of PMA (80 ng). After 30 min incubation, the tubes were chilled and centrifuged at 4° for 20 min (27,000 *g*). MDA was measured in the supernatant fractions by TBA assay.

Results

Effect of H_2O_2 on the determination of MDA in buffered solutions. Figure 1 shows the HPLC elution profile for a solution of synthetic MDA in 0.12 M phosphate buffer (pH 8), recorded at different times after the addition of an equimolar H_2O_2 concentration. A slow decrease of the sharp peak at RT = 6.5 min was observed. The decrease rate was not affected by 1 μM Cu(II) or 1 μM Fe(II) or 100 μM Mn(II) in Tris buffer but was strongly enhanced by addition of 100 μM Mn(II) in HCO_3^-/CO_2 buffer, pH 7.5 (Fig. 2).

Table 1 compares the absorbance at 532 nm of MDA–TBA red pigment in MDA solutions containing various amounts of H_2O_2. The results for each tested sample were expressed, as the ratio of its absorbance to the absorbance of the corresponding free MDA solution, this last value being taken as 100.

Inhibition of lipofuscin formation by H_2O_2. The effect of H_2O_2 on MDA cross-linking proteins was studied by recording the fluorescence emission at 460 nm (excitation at 400 nm) in MDA solutions (2×10^{-2} M) containing 8 mg/mL bovine serum albumin (BSA) with and without H_2O_2 (10^{-2} M) in phosphate buffer (pH 8). As shown in Fig. 3, H_2O_2 inhibited the formation of fluorescent pigments by reaction of MDA with BSA.

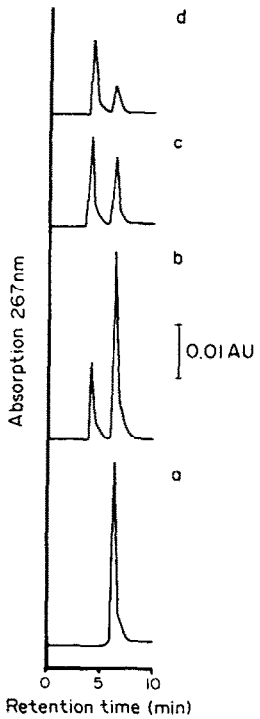


Fig. 1. Time course of HPLC chromatograms for MDA solutions (0.1 mM) with the addition of equimolar concentrations of H_2O_2. (a) MDA alone; (b) MDA + H_2O_2 at $t = 0$; (c) $t = 20$ min and (d) $t = 1$ hr.

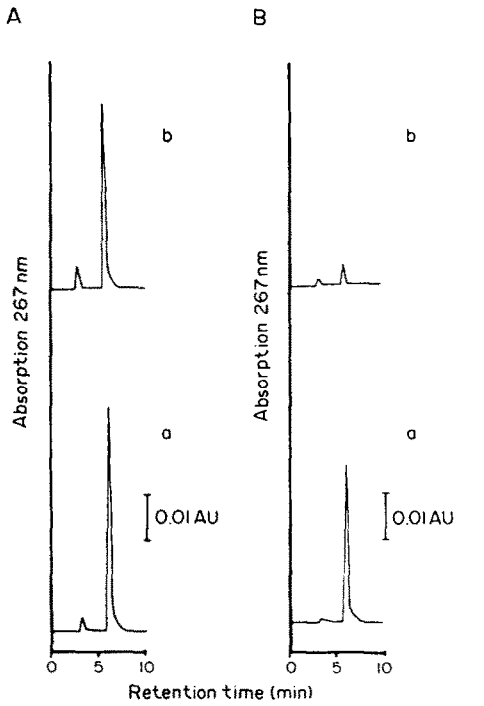


Fig. 2. Time course of HPLC chromatograms for MDA solutions (0.1 mM) H_2O_2 (0.1 mM) in the presence of 100 μM Mn(II). (A) In 0.03 M Tris buffer pH 7.5; (B) in 0.03 M sodium bicarbonate buffer (pH 7.5); (a) $t = 1$ min; (b) $t = 20$ min.

Effect of activated oxygen species generated by PMA-stimulated PMNs on MDA concentrations determined by the TBA assay. MDA concentrations contained in PMN suspensions mixed with specific amounts of synthetic MDA were estimated by TBA assay before and after cell stimulation with PMA (Table 2). The role of H_2O_2 and $O_2^{\cdot}$ were tested by adding catalase or superoxide dismutase before stimulation.

Reduction of cytochrome c by superoxide radical anions generated in the reaction of xanthine oxidase with MDA. Figure 4 compares the cytochrome *c* reduction observed by addition of MDA to xanthine oxidase solution with the reduction promoted by xanthine in the same experimental conditions.

Discussion

It is well established that MDA occurs in many biological systems such as peroxidized tissues, cells and cell fractions,

Table 1. Absorbance at 532 nm of MDA–TBA red pigment from solutions of MDA with the addition of increasing amounts of H_2O_2

	MDA (μM)				
H_2O_2 (μM)	34	46	72	86	115
0	100	100	100	100	100
100	95	97	98	99	99
500	88	88	91	91	91
1000	86	86	88	86	86

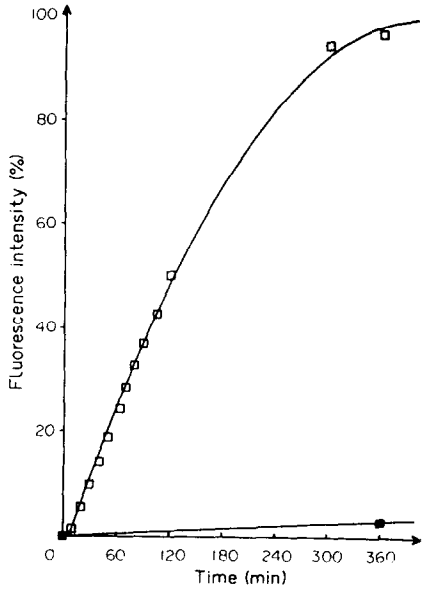


Fig. 3. Time course of the effect of hydrogen peroxide on lipofuscin formation. Increase of the fluorescence emission (λ_{em} = 460 nm, λ_{ex} = 400 nm) of BSA (40 mg) in 5 mL of 0.12 M phosphate buffer (pH 8). (□) In the presence of 2×10^{-2} M MDA; (■) in the presence of 2×10^{-2} M MDA and after addition of 10^{-2} M H_2O_2.

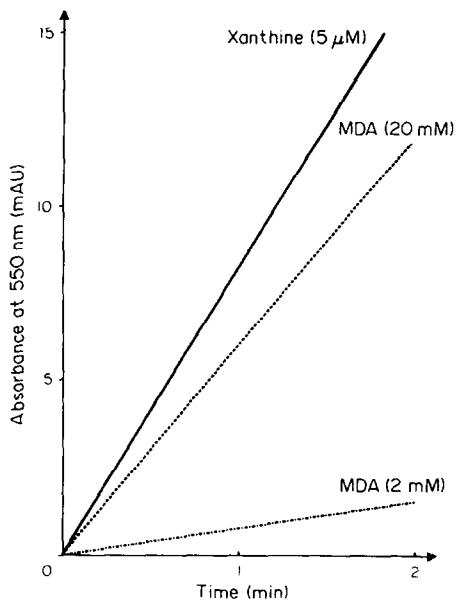


Fig. 4. Reduction of cytochrome *c* by superoxide radical anions generated in the reaction of xanthine oxidase with MDA. One millilitre of reaction mixture contained 4 μM cytochrome *c* in 50 mM sodium phosphate (pH 7.5). The reaction was initiated by adding 50 mU of desalted xanthine oxidase. The oxidation rates were determined as the rates of reduction of cytochrome *c* by following the increase in absorbance at 550 nm.

foods from plant and animal sources, fats and dairy products. Many procedures have been successfully used to follow the rate or extent of MDA formation in various biological samples. However, MDA is not an inert structure; in the presence of equimolar concentrations of H_2O_2, MDA peaks detected by HPLC at 270 nm (Fig. 1) decreased as a function of time and disappeared after 1 hr incubation. In the presence of Mn(II) and HCO_3^-, the decreased rate of MDA was strongly enhanced (Fig. 2), whereas Cu(II) and Fe(II) had no effect. Then it seemed that H_2O_2 reacted as a hydroperoxide ion rather than as a hydroxyl radical generator. The effect of Mn(II) is related to the production of $CO_3^{\cdot-}$ radicals [22].

We attempted to identify the reaction product of MDA with H_2O_2 by recording the ^{13}C NMR spectra of equimolar mixtures of MDA and H_2O_2 in D_2O, but the spectra contained too many ^{13}C signals to be interpreted.

Since the TBA assay is widely used to determine MDA content in living tissues, we compared the MDA–TBA red pigment formed in MDA solutions incubated for 5 min with various amounts of H_2O_2. Table 1 shows that in the presence of H_2O_2, the measured MDA contents were always lower than expected.

In biological samples, TBA assays generally detect substances other than MDA, including other aldehydes such as 4-hydroxynonenal, then we incubated solutions of 4-hydroxynonenal with H_2O_2, in the conditions used for MDA studies and observed no decrease of the 4-hydroxynonenal HPLC peak at 223 nm [20].

The reaction between MDA and H_2O_2 should also be taken into account when the lipofuscin content in tissues is used as an index of ageing, as shown in Fig. 3. We observed that the presence of H_2O_2 inhibited the formation of fluorescent material by reaction of MDA with BSA.

Since these results were obtained *in vitro* with concentrations of H_2O_2 higher than concentrations present in living tissues, we incubated specific amounts of MDA with human PMNs which are known to release reactive oxygen species: $O_2^{\cdot-}$, $HO^{\cdot}$, $^1O_2$ and H_2O_2 when stimulated. Table 2 shows that the amounts of MDA recovered after stimulation of the cells with PMA were lower than the amounts recovered after incubation with resting cells.

To test the effects of reactive oxygen species produced simultaneously with H_2O_2 on MDA, we added catalase or

Table 2. Effect of PMA-stimulated PMNs on MDA: absorbance at 532 nm of MDA–TBA red pigment formed by incubation of MDA with 10^6 PMNs before and after PMA stimulation

MDA (μM)	Resting PMNs	PMA-stimulated PMNs		
		—	+ SOD	+ Catalase
2	0.407	0.297	0.334	0.346
4	0.784	0.607	0.640	0.666
8	1.300	0.996	1.000	1.100

superoxide dismutase to cell suspensions before stimulation. Table 2 shows that in the presence of catalase, which inhibited the effect of H_2O_2, 18% of the MDA disappeared, whereas, in the presence of superoxide dismutase, which inhibited the effect of $O_2^{\cdot-}$, 15% of the MDA disappeared. Thus, it seemed that $O_2^{\cdot-}$ as well as H_2O_2 reacted with MDA. As mentioned above, the MDA decrease rate in the presence of H_2O_2 was not affected by addition of either Cu(II) or Fe(II) showing that $HO^{\cdot}$ did not react with MDA.

It thus appears that MDA production measured in living systems in the presence of reactive oxygen species could be underestimated. Similarly, our results show that the *in vivo* formation of xanthine oxidase during ischemic stroke or its addition to biological samples studied *in vitro* could undergo an oxidation of MDA, leading to an underestimation of the MDA content of the biological material under study.

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