Specific Susceptibility of Docosahexaenoic Acid and Eicosapentaenoic Acid to Peroxidation in Aqueous Solution

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In order to evaluate the peroxidation process, we have measured both fatty acid loss in the medium and the formation of malonaldehyde (MA). MA is a secondary product of the peroxidative degradation process and is of considerable biological significance (6). Most of the methods involving an MA derivatization procedure before chromatography require a heat and/or acid treatment (7-11), which can lead to artificial production of MA, and thus to an overestimation of the MA levels that were initially present in the sample. Some high performance liquid chromatographic (HPLC) methods (12-14) do not require derivatization and permit direct analysis of MA, but they are less specific and less sensitive (15). In the present study, we combined a gas chromatographic (GC) method (8), using a derivatization procedure with a direct HPLC method (12) in order to ensure accurate measurement of MA production in peroxidized fatty acid solutions, as well as to identify those fatty acids which are most susceptible to MA formation during peroxidation. The peroxidation process was initiated by UV photoirradiation under aerobic conditions at room temperature. It has recently been demonstrated by electron paramagnetic resonance studies that photoirradiation produces superoxide radicals (O_2^-) under aerobic conditions (16), which may (via Haber-Weiss type reactions) yield highly reactive oxygen species (OH^·), and hence contribute to initiation processes leading to the autooxidation of PUFA.

The present work was intended to study the peroxidation of fatty acids as a function of degree of unsaturation, number of carbon atoms and metabolic series (n-3, n-6). Particular attention was paid to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) because of their assumed beneficial effects on platelet aggregability and in atherosclerosis (5,17).

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**MATERIALS AND METHODS**

**Reagents.** Stearic (18:0), linoleic (18:2n-6), α-linolenic (18:3n-3), γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3n-6), arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), and docosahexaenoic (22:6n-3) acids were purchased from Sigma France, Ltd. Ammonium iron (II) sulfate hexahydrate was obtained from Prolabo, France. 2-Hydrazinobenzothiazole (HBT) was obtained from Eastman Kodak, and 1,1,3,3-tetramethoxypropane (TMP) from Merck (West Germany). 2-(Pyrazol-1-yl)benzothiazole (HBT-MA) and 2-(3',5'-dimethylpyrazol-1-yl)benzothiazole (HBT-AA) were prepared by the reactions of HBT saponified by IN NaOH, and dissolved at a concentration...
of 2 mM in a 25 mM borate buffer, pH 6.9, under nitrogen to minimize autoxidation, following a modification of the method described by Cillard and Cillard (18). Five ml of aqueous fatty acid solution was placed in an open polystyrene flask (3 cm i.d.), and then irradiated under aerobic conditions at a distance of 40 cm from a 30 W-254 nm UV lamp (Bioblock Scientific, intensity of 1780 μW/cm² at 15 cm from the filter), at 23°C, in a closed reaction chamber (40 cm x 60 cm x 70 cm) for various time periods. The peroxidation process was stopped by cooling the samples on ice in the dark.

Fatty acid analysis. Fatty acids were analyzed as methyl esters on a Varian model 3300 GC equipped with a flame ionization detector (FID), using a spira wax capillary column (25 m x 0.2 mm i.d.) and temperature programming (150-210°C; 1.5°C/min). GC peak areas were measured with a Merck model D 2000 integrator. After extraction of free fatty acids according to the method of Folch et al. (19), fatty acid methyl esters were prepared according to Hagenfeldt (20). Before methylation, nonadecanoic acid was added to the mixture as an internal standard. Fatty acid loss after irradiation was estimated by calculating the difference between the initial fatty acid level (T₀) and the level after 24 hr exposure to UV light (T₂₄).

MA analysis by gas chromatography. Malonaldehyde (MA) was measured on a Packard model 437 GC equipped with a nitrogen phosphorus detector (NPD) using a glass column (3 m x 3 mm i.d.) packed with 5% OV 17 on 80/100 mesh chromosorb, which was maintained at 215°C. GC data were recorded with a Packard model 641 recorder. MA quantities were determined according to the procedure of Beljern-Leymarie and Bruna (8). 0.6 ml of 0.1 M citrate buffer, pH 2.5, and 0.3 ml of 5 mM HBT solution were added to 0.1 ml of the fatty acid solution. The mixtures were placed in capped glass tubes and were heated in a water bath for 30 min at 70°C. The HBT-MA derivative was extracted with 1 ml of hexane containing 2.5 μM HBT/AA as internal standard.

MA analysis by high pressure liquid chromatography. MA was analyzed using Varian model 500 liquid chromatograph equipped with a TSK G 2000 PQ column (70 mm x 7.5 mm i.d.) which was perfused with a mobile phase of 0.1 M sodium phosphate buffer, pH 8.0, at a flow rate of 0.3 ml/min. Free MA in the PUFA aqueous solution was separated and quantified within 10 min. Absorbance was monitored at 267 nm and HPLC peaks were measured with a Merck model D 2000 integrator. MA levels were determined according to the procedure by Csallany et al. (12). Fifty μl of fatty acid solution was directly injected onto the HPLC column. To prepare the same calibration curves for MA analysis by GC and by HPLC, aliquots of a 6.3 mM stock solution of an acid hydrolyzed TMP solution [prepared as previously described (8)] were diluted with 0.01 M phosphate saline buffer solution, pH 7.0, to give final concentrations of standards ranging from 0.5-60 μM.

Analysis of metal catalysts. Iron assays were performed using a Perkin Elmer 300 atomic absorption spectrophotometer equipped with an acetylen-oxygen burner. The detection limit of the assay was 1 μM. Calibration curves for iron assays were prepared by successive dilution of a 180 μM iron solution. Copper was assayed using a Perkin Elmer 380 atomic absorption spectrophotometer equipped with an HGA 500 electrothermal atomizer. The detection limit was 0.02 μM.

RESULTS

Analytical features of MA determination in fatty acid solutions. In order to achieve precise measurement of MA formation, we have compared two analytical methods. Arachidonic acid, a well known source of MA (21), was the PUFA used for these comparative studies. MA that was produced from 2 mM arachidonic acid solutions after six hr irradiation was simultaneously estimated by GC and HPLC and expressed as the difference between MA concentrations produced at six hr and MA concentrations at time 0 (prior to exposure). The results were 9.9 ± 1.1 μM (mean ± SD, n = 17) by GC, and 10.3 ± 1.5 μM (mean ± SD, n = 17) by HPLC. Reproducibility was tested using samples of 5 μM MA in aqueous solution (prepared by successive dilution of a 6.3 mM MA stock solution with 25 mM borate buffer, pH 6.9) and of 2 mM arachidonic acid in aqueous solution, prepared according to the experimental procedure described in Materials and Methods and photo-irradiated by UV light for 24 hr. The coefficients of variation for the GC method were 1.9% (n = 17) and 6.0% (n = 17), respectively, and for HPLC, 6.9% (n = 17) and 11.0% (n = 17), respectively. The statistical comparison of the relative accuracy of the two methods was performed with Student's paired t-test (22)—a series of 16 different samples of 2 mM arachidonic acid was analyzed for MA production after 24 hr UV-induced peroxidation, and the difference between each pair of results was calculated and compared. There was no significant