Arachidonic Acid Autoxidation in an Aqueous Media Effect of α-Tocopherol, Cysteine and Nucleic Acids

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ABSTRACT

The autoxidation of arachidonic acid dispersed in aqueous media was evaluated simultaneously with and without different agents, e.g., α-tocopherol at different concentrations, cysteine, DNA and RNA. The autoxidation rate of arachidonic acid was evaluated by quantitative gas liquid chromatography (GLC) determination of the unoxidized acid and by spectrophotometric measurement of conjugated dienes. α-Tocopherol exhibited a prooxidant activity at concentrations of 1.25×10^{-4} M and 1.25×10^{-5} M and a weak antioxidant activity at a concentration of 1.25×10^{-6} M. Cysteine showed antioxidant activity and greatly reduced the prooxidant activity of α-tocopherol. DNA and RNA had no effect in either case. α-Tocopherol oxidation was followed by high pressure liquid chromatography (HPLC). The prooxidant effect was accompanied by a rapid oxidation of α-tocopherol, except in the presence of cysteine, which prevented the oxidation of α-tocopherol.

INTRODUCTION

Lipid peroxidation occurs in vivo and has important physiological and pathological consequences. Lipid hydroperoxides are produced predominantly by enzymes and by free-radical lipid autoxidation (1-4).

Among the polyunsaturated fatty acids (FA), arachidonic acid plays an essential role in vivo. Its oxidation by enzymes (cyclooxygenase and lipoxygenase) gives products such as prostaglandins, which are linked to inflammation (5,6), thromboxane and prostacyclin, which play a role in platelet aggregation (7,8), and leukotrienes, which are identified as slow reacting substances of anaphylaxis (immediate hypersensitivity reactions) (9-11).

The autoxidation of arachidonic acid (5,8,11,14eicosatetraenoic acid) also leads to the formation of a 5-hydroperoxy-6,8,11,14eicosatetraenoic acid (5-HPETE), which is a proposed intermediate compound in the biosynthesis of the slow reacting substances of anaphylaxis (12). The formation of the 5-HPETE is suppressed when lecithin (1-stearoyl-2-arachidonoyl-phosphatidycholine) is cooxidized at 37 C with α-tocopherol as a homogeneous emulsion (13).

The purpose of this work was to study the autoxidation rate of arachidonic acid dispersed in an aqueous media, the effect of α-tocopherol on its autoxidation rate at different concentrations and, finally, the effect of some synergists on the antioxidant activity of α-tocopherol, e.g., cysteine (14) and nucleic acids (15).

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid was purchased from Sigma Chemical Company, St. Louis, MO (≥99% pure). This acid was dispersed with 0.5% Tween 20 (Merck) in 0.025 M phosphate buffered aqueous solution (pH 9.0) under nitrogen. Arachidonic acid concentration was 10^{-2} M and this stock dispersion was adjusted to pH 6.9 just before use. α-Tocopherol was synthetized and supplied by Hoffmann-La Roche, France. It was dispersed with Tween 20 in phosphate buffer (pH 7.0) according to the same procedure as above. α-Tocopherol concentration was 2.5×10^{-4} M. Cysteine (Merck) was dissolved in phosphate buffer and its concentration was 8×10^{-3} M. Desoxyribonucleic acid (DNA) was a commercial product from calf thymus (Sigma Chemical Company, St. Louis, MO). The DNA (sodium salt) was highly polymerized. Ribonucleic acid (RNA) was a commercial product purchased from Seravac Laboratoires, Paris, France. These nucleic acids were dissolved in phosphate buffer; the concentration was 60 μg/mL (2% with regard to arachidonic acid concentration).

Procedure

Samples (100 mL) were prepared by mixing aliquots of the stock solutions and dispersions, at time zero, in glass tubes and were left in the dark and under air at room temperature. Controls without arachidonic acid were placed under the same conditions.

Measurement of Arachidonic Acid Oxidation

Arachidonic acid oxidation was accompanied in the early stages by an increase of absorption at 234 nm because of conjugated dienes formation. Measurement of the absorbance was performed with a Pye Unicam SP 800 Spectrophotometer. The samples were diluted so that the absorbance was less than or equal to 2.

Simultaneously, the unoxidized arachidonic acid was determined by gas chromatography (GC) using a Pye Unicam 104 Model instrument with a flame ionization detector (FID) and a 2.5 ft × 1/8 in. column, packed with 2.5% FFAP on 80-100 mesh Chromosorb G. The operating conditions were: column temperature, 240 C; injector temperature, 285 C; detector temperature, 250 C; carrier gas, nitrogen; flow rate, 60 mL/mn.

Arachidonic acid was extracted from the aqueous dispersion (1 mL) with a mixture of chloroform/methanol (3.5:2.5, v/v) after the addition of 250 μL of an internal standard (palmitic acid, Merck, dissolved in ethanol at a concentration of 1 mg/mL). After centrifugation, the upper phase was reextracted twice with chloroform. The organic fractions were evaporated to dryness under reduced pressure at low temperature on a rotary evaporator. The residue was dissolved in 1 mL heptane and 10 μL were injected into the chromatograph. Palmitic and arachidonic acids were eluted after 3 min and 15 min.

Measurement of α-Tocopherol

High performance liquid chromatography (HPLC) of α-tocopherol was achieved with an LDC chromatograph (Sopares, Gentilly, France) equipped with a Constametric III pump, a Valco 7000 psi injector, a spectromonitor II UV detector set at 294 nm and a stainless-steel column of Spherisorb ODS C18, 5 μm. The eluting solvent was composed of methanol/water (85:15, v/v) at a flow rate of 2 mL/mn. The aqueous samples and controls containing α-tocopherol were injected directly into the chromatograph, after addition of an internal standard of γ-tocopherol (Hoffmann La Roche, Neuilly/Seine, France) dissolved

in ethanol at concentrations ranging from 0.01 μg to 1 μg/μL. γ-Tocopherol and α-tocopherol were eluted after 17 min and 20 min, respectively.

RESULTS

Arachidonic Acid Oxidation

Effect of α-tocopherol. The addition of α-tocopherol modified the rate of arachidonic acid oxidation. Arachidonic acid oxidation measured by the level of conjugated dienes (A 234 nm) increased with the concentration of α-tocopherol, except when α-tocopherol was used at a concentration of 1.25×10^{-6} M (Fig. 1). These results correlated with the GC quantitative analysis of arachidonic acid, which showed that the concentration of arachidonic acid decreased more rapidly with α-tocopherol at concentrations of 1.25×10^{-4} M and 1.25×10^{-5} M. On the other hand, the level of arachidonic acid decreased more slowly with α-tocopherol at 1.25×10^{-6} M during the first 8 days. After the eighth day, we noted an appreciable decrease in the level of arachidonic acid, especially during 2 days.

Effect of cysteine. The addition of cysteine largely reduced the level of conjugated dienes formed during the autoxidation of arachidonic acid with α-tocopherol at a concentration of 1.25×10^{-4} M and without it (Fig. 2). In both cases, the conjugated diene level remained practically constant during the whole experiment.

Simultaneously, the GC analysis of unoxidized arachidonic acid showed that cysteine slowed down the degradation of arachidonic acid by ca. 20-35% in both cases.

Effect of nucleic acids. The addition of DNA or RNA slightly reduced the level of conjugated dienes formed during the autoxidation of arachidonic acid with α-tocopherol at a concentration of 1.25×10^{-4} M and without it (Fig. 3). However, the GC measurement of arachidonic acid showed that nucleic acids did not modify the level of unoxidized arachidonic acid present in the samples with and without α-tocopherol.

α-Tocopherol Oxidation

During arachidonic acid oxidation, α-tocopherol was rapidly oxidized, and after 3 days, 53% and 49% of α-tocopherol disappeared in the samples containing 1.25×10^{-4} M (Fig. 4) and 1.25×10^{-5} M of α-tocopherol (Fig. 5). In both cases, α-tocopherol was completely oxidized after 10 days.

The addition of nucleic acids did not modify the α-tocopherol oxidation rate (Fig. 4). On the other hand, the addition of cysteine protected α-tocopherol, especially during arachidonic acid oxidation, because after 10 days, ca. 90% of α-tocopherol was still present, as in all controls (Fig. 4).

In spite of its sensibility, the HPLC method, using reverse-phase chromatography did not allow quantitative evaluation of α-tocopherol at the concentration of 1.25×10^{-6} M.

DISCUSSION

Effect of α-Tocopherol Concentration on Arachidonic Acid Oxidation

α-Tocopherol exhibited 2 opposite behavior patterns in the function of its concentration on arachidonic acid oxidation rate. At high concentrations (1.25×10^{-4} M and 1.25×10^{-5} M), it showed a prooxidant effect. In this experiment, the magnitude of the phenomenon increased with the concentration of α-tocopherol. At a low concentration (1.25×10^{-6} M), we noted a weak antioxidant effect during the first 8 days followed by an increase of arachidonic acid oxidation for 2 days.

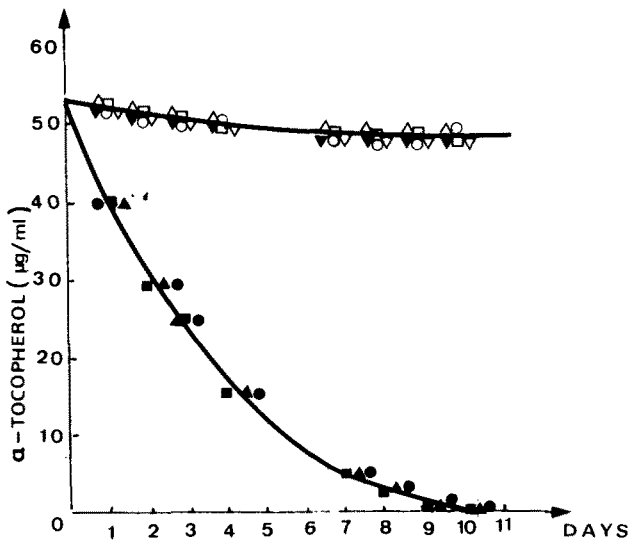

FIG. 1. Measurement of conjugated dienes during arachidonic acid (AA) oxidation in the presence of different concentrations of α-tocopherol (α-T). □: AA 2.5×10^{-3} M; ■: AA 2.5×10^{-3} M + α-T 1.25×10^{-4} M; ●: AA 2.5×10^{-3} M + α-T 1.25×10^{-5} M; ○: AA 2.5×10^{-3} M + α-T 1.25×10^{-6} M.

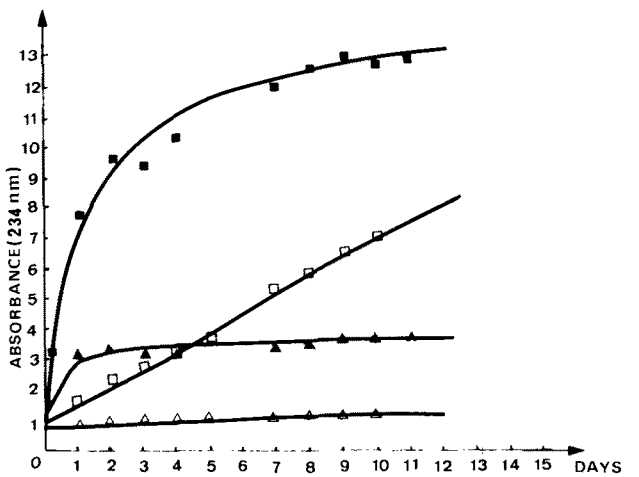

FIG. 2. Effect of cysteine on the conjugated dienes measurement during arachidonic acid (AA) oxidation. □: AA 2.5×10^{-3} M; ■: AA 2.5×10^{-3} M + α-T 1.25×10^{-4} M; △: AA 2.5×10^{-3} M + cysteine 2×10^{-3} M; ▲: AA 2.5×10^{-3} M + α-T 1.25×10^{-4} M + cysteine 2×10^{-3} M.

In a previous paper (16) we showed, in the same manner, that α-tocopherol, was prooxidant or antioxidant depending on its concentration during linoleic acid autoxidation. But at the concentration of 1.25×10^{-6} M, the antioxidant efficiency of α-tocopherol was much greater on linoleic acid oxidation than on arachidonic acid oxidation. Moreover, Witting (17) noted that the relative efficiency of α-tocopherol as antioxidant varied in the ratios 40:1:0.5:0.25 for ethyl oleate, linoleate, linolenate and arachidonate esters, respectively.

Furthermore, in our experimental model, α-tocopherol was rapidly oxidized during the prooxidant reaction because it was not detectable after 9-10 days in samples containing arachidonic acid and α-tocopherol at initial concentrations of 1.25×10^{-4} M and 1.25×10^{-5} M. Unfortunately, by our method we could not evaluate α-tocopherol

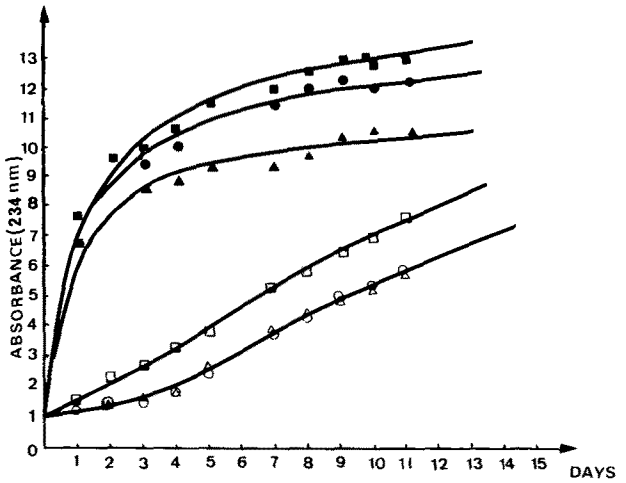

FIG. 3. Effect of nucleic acids on the conjugated dienes measurement during arachidonic acid (AA) oxidation. □: AA 2.5 × 10^{-3} M; ■: AA 2.5 × 10^{-3} M + α-T 1.25 × 10^{-4} M; ○: AA 2.5 × 10^{-3} M + RNA 15 μg/mL; △: AA 2.5 × 10^{-3} M + DNA 15 μg/mL; ●: AA 2.5 × 10^{-3} M + α-T 1.25 × 10^{-4} M + RNA 15 μg/mL; ▲: AA 2.5 × 10^{-3} M + α-T 1.25 × 10^{-4} M + DNA 15 μg/mL.

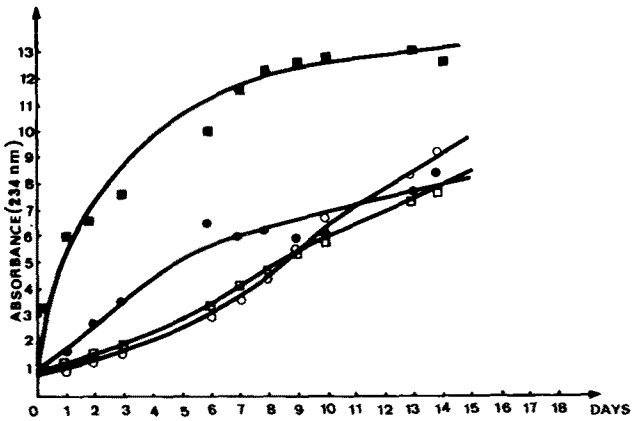

FIG. 4. HPLC measurement of α-tocopherol (α-T) during arachidonic acid (AA) oxidation; effect of cysteine and nucleic acids. α-T 1.25 × 10^{-4} M in a phosphate buffer pH 6.9—□: alone, ▽: with cysteine 2 × 10^{-3} M, ○: with RNA 15 μg/mL; △: with DNA 15 μg/mL; ■: with AA 2.5 × 10^{-3} M; ▼: with AA 2.5 × 10^{-3} M and cysteine 2 × 10^{-3} M; ●: with AA 2.5 × 10^{-3} M and RNA 15 μg/mL; ▲: with AA 2.5 × 10^{-3} M and DNA 15 μg/mL.

oxidation rate at the level of 1.25 × 10^{-6} M. In a previous paper we noted that α-tocopherol as prooxidant was also completely oxidized during linoleic acid autoxidation within the same period as arachidonic acid (18).

These observations agreed with those of Gruger and Tappel (19), who observed that the degree of unsaturation of the lipid hydroperoxides had little effect on the rate of α-tocopherol oxidation.

Effect of Cysteine on Arachidonic Acid Oxidation

Cysteine exhibited an antioxidant behavior during arachidonic acid oxidation in an aqueous media. This result agreed with those of many researchers who noted an antioxidant activity with most amino acids (14,20).

Previously, we had observed an antioxidant behavior of cysteine during linoleic acid oxidation in the same conditions (21).

Nevertheless, Farag et al. (22) found a prooxidant effect of cysteine and other amino acids on linoleic acid dispersed in an aqueous media.

The addition of cysteine to α-tocopherol at a prooxidant concentration (1.25 × 10^{-4} M) significantly reduced the arachidonic acid oxidation rate and protected α-tocopherol from oxidation as nearly 90% of α-tocopherol was still present after 10 days, although 65% of the arachidonic acid was oxidized.

We did not study the degradation of cysteine during this reaction, but Farag et al. (22) have shown that cysteine as prooxidant was converted to a mixture of cystine, cysteic and cystine disulfoxide. Similarly, Gardner and Jursinic (23) found that cysteine with linoleic acid hydroperoxides and $FeCl_3$ (catalyst) at pH values under 6 was oxidized into cystine and oxides of cysteine.

Effect of Nucleic Acids on Arachidonic Acid Oxidation

Nucleic acids are known as synergists of the antioxidant effect of tocopherols. According to Ikeda and Fukuzumi (15), nucleic acids protected tocopherols from oxidation from direct air by forming hydrogen bonds with tocopherols. The addition of nucleic acids exhibited no significant effect on the autoxidation rate of arachidonic acid dispersed in an aqueous media, with and without α-tocopherol, at a prooxidant concentration. We have tested 2

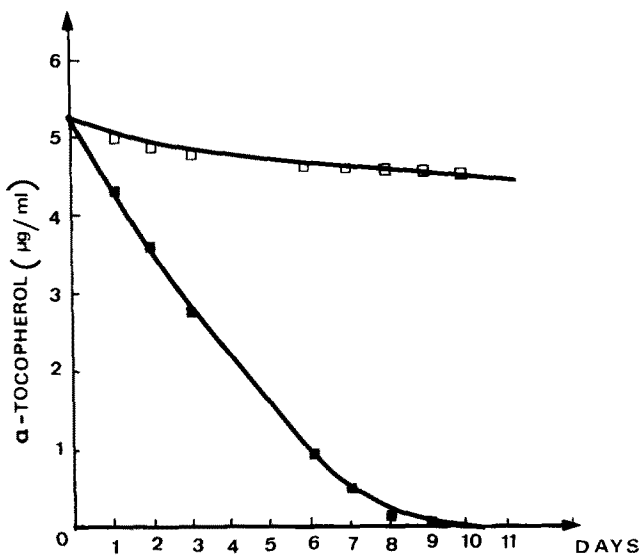

FIG. 5. HPLC measurement of α-tocopherol (α-T) during arachidonic acid (AA) oxidation. α-T 1.25 × 10^{-5} M in a phosphate buffer pH 6.9—□: alone; ■: with AA 2.5 × 10^{-3} M.

desoxyribonucleic acids: one was a standard denaturated DNA, the other a highly polymerized DNA, from calf thymus. Results were the same in both cases. Furthermore, the oxidation rate of α-tocopherol was not modified by the addition of nucleic acids in our experimental model.

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Thin Layer Chromatography/Flame Ionization Analysis of Transesterified Vegetable Oils[1]

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ABSTRACT

A quantitative method was developed for analyzing mixtures containing fatty esters and tri- (TG), di- (DG) and monoglycerides (MG) obtained by the transesterification of vegetable oils. Analyses were performed by thin layer chromatography (TLC)/flame ionization detection (FID) with an Iatroscan TH-10 instrument. Stearyl alcohol served as an internal standard. From plots of area and weight ratios of methyl linoleate and tri-, di- and monolinolein, linear equations were developed from which response factors were calculated. Hydrogen flow rate and developing solvent strongly influenced resolution and baseline stability. Variations in scan speed affected completeness of burning and response factors, but not measured composition. Sample weight also affected response factors but not measured composition. A computerized procedure for data analysis was developed so that 30 samples can be completed in 2-3 hr. Relative standard deviations were 1-2% for major components in the 90-100% range and 6-83% for minor components in the 1-2% range.

INTRODUCTION

Analysis of lipids by thin layer chromatography (TLC) with flame ionization detection (FID) (Iatroscan analyzer) is claimed to be sensitive, linear and reproducible (1), comparable to conventional chemical analyses, superior to gravimetric recovery and less time-consuming than comparable methods (2,3). Others report that: response factors varied with the nature and amount of compound applied and with scan speed (4,5,6); pyrolysis was incomplete at some scan speeds (4); slight deviations in rod geometry with respect to the detector also influenced quantitation (4,5); results varied from rod to rod, and one lot of rods to another (7); and, finally, FID design might affect quantitation (8).

The Iatroscan analyzer appeared to offer a rapid, simple and convenient method to quantitate reaction mixtures from the transesterification of vegetable oils (9,10). Our objectives were to determine the suitability of this technique for quantifying transesterifications and to shed light on the variability of response factors with type of compound, scan speed, completeness of pyrolysis and rod-to-rod variation.

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EXPERIMENTAL

Reagents

Reference materials used in standard solutions in this study were: methyl linoleate; tri-, di- and monolinolein; 1,3- and 1,2-dilinolein; stearyl alcohol; palmitic acid. Reference materials and 2 TLC reference standards (solutions 49 and 50) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN), and were chromatographically pure (>99%). Chloroform was MCB Omnisolv (spectrograde). Developing solvents were A.C.S. reagent grade.

Instrumentation and Operating Conditions

Analyses were performed with an Iatroscan TH-10 Analyzer MK III (Iatron Laboratories, Tokyo, Japan: worldwide distributor, Newman-Howells Assoc., Winchester, Hants, U.K.). The flame ionization detector used hydrogen and air flow rates of 160 mL and 2,000 mL/min. Scan speeds were 2,3 and 4 corresponding to 40 sec/rod, 35 sec/rod and 30 sec/rod. A Linear Instrument Corp. (Irvine, CA) recorder, Model 561, was used at 1 mV full-scale deflection at chart speeds of 10 cm/min, 15 cm/min or 20 cm/min.

Chromarods

Both type S and SII Chromarods were used, but the former predominated; the silica gel particle size is a maximum of 10 in type S and 5 μm in type SII (11). The manufacturer's directions were followed in the storage, use and reactivation of these rods (12). They were stored in a glass chamber in which the atmosphere was saturated with water vapor. Before being spotted, rods were scanned as blanks on the