

Inhibition of Benzo(a)pyrene-induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid¹

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ABSTRACT

Grilled ground beef contains factors that inhibit the initiation of mouse epidermal carcinogenesis by 7,12-dimethylbenz(a)anthracene. Previously we isolated an active principal and characterized it as an isomeric mixture of conjugated dienoic derivatives of linoleic acid (CLA). We now show that synthetic CLA inhibits the initiation of mouse forestomach tumorigenesis by benzo(a)pyrene. Four and 2 days prior to p.o. treatment with benzo(a)pyrene, female ICR mice were given (a) CLA in olive oil, (b) linoleic acid in olive oil, or (c) olive oil alone or plus 0.85% saline (control groups). Three days later the cycle was repeated for a total of 4 times. At 30 wk of age, the mice were sacrificed. In three independent experiments, mice treated with CLA developed only about half as many neoplasms/animal as mice in the control groups ($P < 0.025$); in two of the experiments tumor incidence was also reduced ($P < 0.05$). There were no significant differences in food intake or body weight among the groups. High-performance liquid chromatography/gas chromatography analysis established that, following intubation, only the c-9, t-11 CLA isomer was incorporated into forestomach phospholipids. In studies aimed at elucidating the mechanism of action, we found that CLA is an effective antioxidant. Under the conditions of the test CLA was more potent than α -tocopherol and almost as effective as butylated hydroxytoluene. These observations indicate that CLA might serve as an *in situ* defense mechanism against membrane attack by free radicals and may, at least in part, explain the anticarcinogenic properties of CLA.

INTRODUCTION

A precise understanding of the role of dietary fat in carcinogenesis is complicated by numerous factors, including caloric effects and specific fatty acid effects (1). Among the fatty acids of which lipid is comprised, only the essential fatty acid, linoleic acid, has been unequivocally shown to enhance carcinogenesis (2). The often discussed differences in tumor yield observed when isocaloric diets containing different fats are fed appear to be almost solely a function of linoleic acid content. Hence, feeding a diet containing corn oil will have a greater enhancing effect on mammary carcinogenesis than feeding an isocaloric diet containing olive oil, etc. (3).

Given this important positive effect of linoleic acid on carcinogenesis, it is of interest that an anticarcinogenic principal which we isolated from extracts of grilled ground beef was identified as CLA³ (4, 5). CLA (mostly esterified) is present in grilled ground beef at a concentration of about 1 g/kg and in dairy products in amounts varying from 0.03 to 1.8 g/kg (5).

The purpose of this study was to investigate the mechanism whereby CLA acts as an anticarcinogen. We established that

CLA reduces BP-induced mouse forestomach neoplasia, found that following intubation of synthetically-prepared CLA containing 9 isomers only the c-9,t-11 CLA isomer becomes incorporated into forestomach cell PL, and demonstrated that CLA is an effective antioxidant.

MATERIALS AND METHODS

Materials. Linoleic acid (99%), saline (0.85% sodium chloride solution, w/v), silicic acid (300 to 200 mesh), and BP were purchased from Sigma Chemical Company (St. Louis, MO). Organic solvents (acetone, acetonitrile, chloroform, and methanol; all HPLC grade) were obtained from Burdick and Jackson Laboratory (Muskegon, MI). Ferrous ammonium sulfate, HCl (gas, 99.99%), hexadecanoic acid, methanol (anhydrous), phenylnonane, sodium methoxide (25% in methanol), and thiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Olive oil was obtained from a local grocery. All other chemicals used were ACS grade.

CLA was synthesized from linoleic acid and analyzed for purity by a capillary GC method (5). The principal isomers in the mixture were c-9,t-11-, t-10,c-12-, t-9,t-11-, and t-10,t-12-octadecadienoic acids, which accounted for about 90% of the material. The remaining minor contributors were c-9,c-11-, t-9,c-11-, c-10,c-12-, c-10,t-12-, and c-11,c-13-octadecadienoic acids.

Treatment of Mice. Three independent experiments were conducted according to the protocol described by Benjamin *et al.* (6). For each, 75 female ICR mice (Sprague-Dawley, Madison, WI), 6 to 7 wk of age, were housed in polycarbonate cages (5 mice/cage) in a temperature- and humidity-controlled facility and permitted free access to water and food (TD86348 semipurified diet; Teklad Test diets, Madison, WI). Two wk later the animals were randomized by body weight and divided into 3 groups (25 mice/group). They were then subjected to a forestomach tumorigenesis initiating regimen as follows. On Monday and Wednesday each animal was given by gavage (a) 0.1 ml of CLA plus 0.1 ml of olive oil; or (b) 0.1 ml of linoleic acid plus 0.1 ml of olive oil; or (c) 0.1 ml of olive oil alone (Table 1, Experiment 1) or plus 0.1 ml of saline (Table 1, Experiments 2 and 3); on Friday all animals were given 2 mg of BP in 0.2 ml of olive oil. This sequence was repeated for 4 wk. Beginning with the first intubation and continuing thereafter, body weights and food intake were recorded once and twice weekly, respectively. All surviving mice were sacrificed 22 wk after the first dose of BP.

To investigate the tissue distribution of CLA, 75 female ICR mice, 6 to 7 wk of age, were subjected to the forestomach tumorigenesis regimen as described above in Experiment 2 or 3 without BP treatment. Five mice from each group were sacrificed at 0, 1, 2, 3, and 4 wk after the first CLA intubation (p.o.), and forestomachs were removed and stored frozen (-57°C) until analysis.

Tumor Histology. The forestomachs were fixed for 24 h at room temperature in an expanded state produced by i.g. instillation of 4% neutral buffered formalin. Forestomachs were split longitudinally, and presumed tumors 1 mm or larger were counted using a dissecting microscope, followed by histological examination (7) for the confirmation of neoplasia. Thus, histological examination of the BP-induced forestomach tumors (Table 1) revealed that they were papillomas with or without focal areas of epidermal hyperplasia.

CLA Analysis. Total fat of forestomach samples (150 to 200 mg) was extracted by chloroform:methanol (2:1) (8) and fractionated into phospholipids by silicic acid column chromatography (9). Phenylnonane (12 mg/0.1 ml of methanol; internal standard for HPLC) and hexadecanoic acid (78 μg /0.1 ml of methanol; internal standard for

Received 8/4/89; revised 11/6/89; accepted 11/9/89.

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¹ This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison; USPHS Training Grant 5-T32CA-08451 from the Division of Extramural Activities, National Cancer Institute, Department of Health and Human Services; a grant from the Wisconsin Milk Marketing Board; and gift funds administered through the Food Research Institute, University of Wisconsin-Madison.

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³ The abbreviations used are: CLA, isomeric derivatives of linoleic acid containing a conjugated double bond system; BP, benzo(a)pyrene; BHT, butylated hydroxytoluene; PL, phospholipid; HPLC, high-performance liquid chromatography; GC, gas chromatography.

GC) were added to the phospholipids and then interesterified with methanol-HCl solution (10). Fatty acid methyl esters were extracted with hexane (3 × 3 ml). Pooled hexane extracts were washed with water (3 × 3 ml), dried over anhydrous sodium sulfate, and concentrated to 2-ml volume under nitrogen. Aliquots of the hexane concentrate were used for the determination of UV absorbance at 268 nm against the control prepared as a reference and for the quantification of linoleic and oleic acids by capillary GC (5). The remaining hexane concentrate was then dried under nitrogen, dissolved in acetone, and analyzed by reversed-phase HPLC for total CLA methyl esters. Total CLA methyl esters pooled from several HPLC runs of a given sample were extracted with hexane (3 × 3 ml), and then individual CLA isomers were quantified by capillary GC (5).

Assay for Antioxidant Activity. Antioxidant activity of CLA was determined by the thiocyanate method (11), using synthetically prepared CLA. Linoleic acid (375 μmol) was incubated for 15 days at 40°C alone or with 375 nmol of ascorbic acid, α-tocopherol, CLA, or BHT in a reaction mixture consisting of 10 ml of 0.2 M phosphate buffer (pH 8), 4.5 ml of double-distilled water, and 10.5 ml of ethanol. The degree of linoleic acid oxidation (peroxide value) was then determined by adding ferrous ammonium sulfate and thiocyanate and measuring absorbance at 480 nm. Benzoyl peroxide was used as a standard, and data were reported relative to the reaction volume (25 ml).

Instrumentation. HPLC analysis of the total CLA (methyl ester) was carried out on an Ultrasphere-C₁₈ reversed-phase column (25 × 0.46 cm; inner diameter, 5 μm; Beckman). Mobile phase [80% acetonitrile in water:acetic acid (150:1)] was delivered (2 ml/min) by a Beckman Model 110A pump. Eluents were monitored at 245 nm using a Beckman Model 163 UV detector and recorded by a Spectra Physics Model 4270 integrator. GC conditions for the analysis of individual CLA isomers, linoleic acid, and oleic acid were previously described (5). UV absorbance was measured by a Beckman Model DU-7 spectrophotometer.

Statistical Analysis. Data were analyzed for statistical significance using, where appropriate, the least significant difference test, one-way analysis of variance, or the χ^2 test.

RESULTS

Effect of CLA on Forestomach Neoplasia. The effect of CLA on BP-induced neoplasia of the forestomach in female ICR mice is shown in Table 1. In three experiments, CLA treatment (total dose, 800 mg/mouse) significantly reduced the number of tumors/mouse, whereas linoleic acid had no such effect. Tumor incidence was also reduced by CLA in Experiments 1 and 3. Body weight and food intake were not affected by CLA;

therefore calorie restriction, known to reduce tumor risk (1, 3), was not a factor in the reduction of forestomach neoplasia by CLA.

Incorporation of CLA into Forestomach PL. We investigated the fate of CLA following intubation and found that CLA is incorporated into the PL fraction of tissue cell membranes. Fig. 1 shows the incorporation of CLA into forestomach PL (a subsequent paper will detail the uptake of CLA into the PL of cells of other tissues). Uptake exhibited a biphasic response, possibly the result of CLA being incorporated into different cell populations with differing PL turnover rates. As indicated in "Materials and Methods," the synthetic CLA used in this study consisted of a mixture of 9 isomers. However, only one CLA isomer, the *c*-9,*t*-11 isomer, was identified in the PL fraction (Fig. 2), indicating that this isomer was preferentially incorporated.

Table 2 shows the concentrations of oleic acid, linoleic acid, and CLA in forestomach PL as a function of fatty acid intake. CLA content varied widely. The lowest CLA level was found when olive oil alone was intubated. When linoleic acid was intubated, CLA in the PL was 3 times higher, whereas when CLA was intubated, the difference was 60-fold. These wide swings in PL CLA content were not at the expense of linoleic or oleic acid content.

Antioxidant Activity of CLA. Fig. 3 shows that CLA is much more resistant to oxidation than linoleic acid, in agreement with our previous findings (4). Hence, peroxides are not readily formed from CLA (as opposed to linoleic acid).

Given that many anticarcinogens are also antioxidants (12), we tested CLA for antioxidant activity (Fig. 4). The most effective dose was the lowest tested (0.375 μmol); at higher concentrations CLA was less efficacious as an antioxidant. This is not surprising as the balance between antioxidant and prooxidant activity is known to be a complex function dependent on concentration and oxygen partial pressure (13).

The antioxidant potential of CLA was compared with several known antioxidants (Fig. 5). Under the conditions of the test, CLA was more potent than α-tocopherol and almost as effective as BHT, a synthetic antioxidant widely used as a food preservative. It should be noted that the ratio of CLA to oxidizable substrate (linoleic acid) was 1 to 1000. Hence, CLA exhibits true antioxidant activity, not just a sparing effect.

The observation that CLA is an antioxidant is notable in that

Table 1 Inhibition of BP-induced forestomach neoplasia in female ICR mice by CLA

CLA or linoleic acid (0.1 ml) was administered p.o. with 0.1 ml of olive oil twice weekly for 4 wk, and other mice were treated similarly with 0.1 ml of olive oil in Experiment 1, or 0.1 ml of olive oil plus 0.1 ml of saline in Experiments 2 and 3. All mice were administered p.o. with 2.0 mg of BP in 0.2 ml of olive oil once weekly for 4 wk.

Experiment	Treatment	No. of mice/treatment	Tumor incidence (%)	Forestomach tumors ^a			Food intake (kcal/wk/mouse)
				Tumors/mouse	Tumors/tumor-bearing mouse	Body wt (g/mouse) ^b	
1	Olive oil	22	90.9	3.6 ± 0.5 ^c	3.8 ± 0.5	31.5 ± 0.7	87.1 ± 3.00
	CLA	19	70.9 ^d	1.4 ± 0.5 ^d	2.0 ± 0.3 ^d	33.2 ± 0.9	90.7 ± 3.25
	Linoleic acid	14	78.9	3.5 ± 1.3	4.5 ± 1.3	32.7 ± 0.8	95.7 ± 3.39
2	Olive oil	24	95.8	5.8 ± 0.8	6.0 ± 0.7	30.8 ± 0.8	96.3 ± 2.19
	CLA ^e	24	95.8	3.1 ± 0.6 ^d	3.2 ± 0.6 ^d	29.3 ± 0.6	89.0 ± 1.86
	Linoleic acid	22	100.0	6.3 ± 1.3	6.3 ± 1.3	30.6 ± 0.7	95.0 ± 2.30
3	Olive oil	22	100.0	5.0 ± 0.6	5.0 ± 0.6	33.1 ± 0.9	86.9 ± 1.44
	CLA	24	70.8 ^d	1.7 ± 0.4 ^d	2.5 ± 0.3 ^d	30.0 ± 0.6	90.5 ± 1.32
	Linoleic acid	20	90.0	3.7 ± 0.7	4.1 ± 0.6	31.8 ± 0.8	79.2 ± 1.53

^a Twenty-five mice intubated with saline but not BP developed no forestomach tumors nor any abnormal forestomach pathology.

^b Mean body weight over a period of 22 wk.

^c Mean ± SEM.

^d Significantly different from the other column values in the same experiment at $P < 0.025$ (Tukey's w test) for tumors/mouse and tumors/tumor-bearing mouse; and at $P < 0.05$ (χ^2 test) for tumor incidence (in Experiment 1, the incidence of the CLA group is only significantly different from that of the olive oil group).

^e Tumors that developed in 3 mice (of 24) accounted for 38% of total tumors.

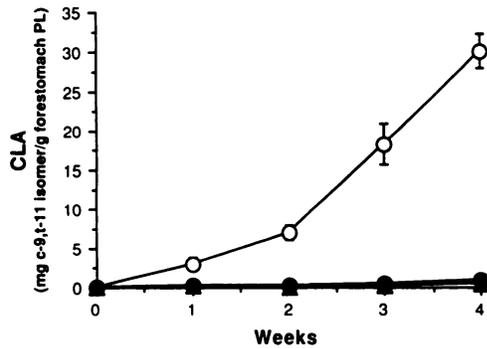


Fig. 1. Incorporation of the c-9,t-11 CLA isomer into forestomach PL of mice given CLA (O), linoleic acid (●) or olive oil (▲).

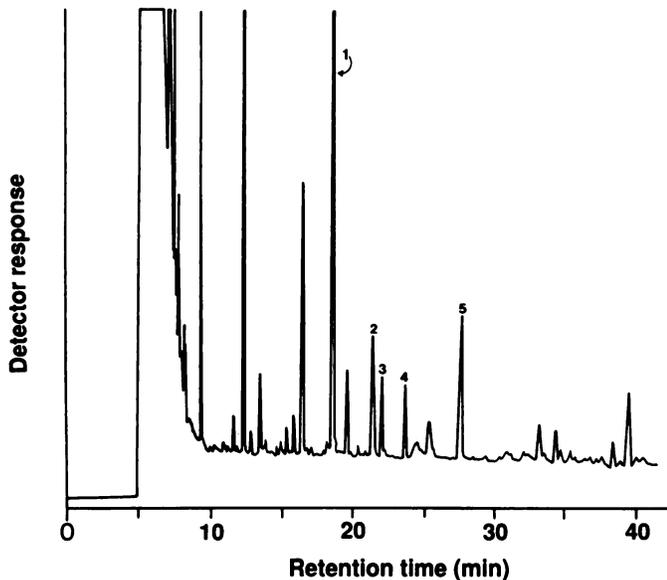


Fig. 2. Capillary GC chromatogram of the CLA methyl esters isolated from forestomach PL at 4 wk in Fig. 1. Peak identification: 1, hexadecanoic acid (internal standard); 2, stearic acid; 3, oleic acid; 4, linoleic acid; and 5, c-9,t-11 CLA methyl ester. The attenuation setting on the integrator was as follows: 0 to 26 min, 16; 26 to 42 min, 4.

Table 2. Fatty acid concentrations (mg/g) in forestomach PL 4 wk after treatments

Treatment ^a	c-9,t-11 CLA	Linoleic acid	Oleic acid
Olive oil	0.51 ± 0.01 ^b	38.92 ± 3.25	43.51 ± 2.60
Linoleic acid	1.30 ± 0.10 ^c	54.13 ± 3.69 ^c	44.25 ± 3.52
CLA	30.1 ± 2.10 ^c	31.40 ± 5.36	31.80 ± 8.50

^a Olive oil, 0.8 ml of olive oil; linoleic acid, 0.8 ml of olive oil plus 0.8 ml of linoleic acid; and CLA, 0.8 ml of olive oil plus 0.8 ml of CLA (p.o.) over a period of 4 wk. Control group taken before treatment reported in Table 3.

^b Mean ± SEM.

^c Significantly different from the other values in the same column at $P < 0.05$ (least significant difference test).

there is no obvious structural feature within the molecule to account for such activity. However, UV spectral analysis of a hexane extract from a reaction mixture containing CLA plus linoleic acid and incubated in air under the same conditions as used for Figs. 4 and 5 revealed decreased absorption at 234 nm and increased absorption at 268 nm (Fig. 6, *solid line*). These changes have previously been reported for CLA and were interpreted as indicating that a ketone group had been introduced into the CLA molecule (14–16). However, one might expect the UV absorbance maximum of CLA containing a ketone and a carbon double bond to be less than 268 nm (17). One possibility that would satisfy the electronic requirements for such a spectral shift is the transformation of the conjugated dienoic

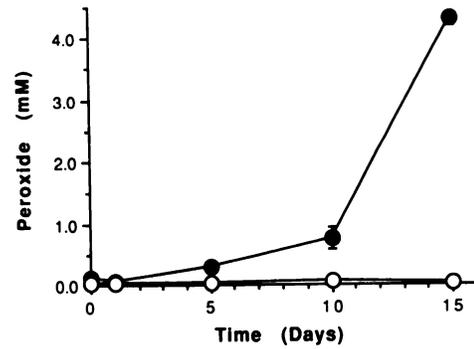


Fig. 3. Peroxide formation from CLA (O) or linoleic acid (●). Test fatty acid (375 μmol) was incubated at 40°C in a reaction mixture consisting of 10 ml of 0.2 M phosphate buffer (pH 8), 4.5 ml of double-distilled water, and 10.5 ml of ethanol. Points, mean of three determinations; bars (shown when they exceed the size of the symbols), SEM.

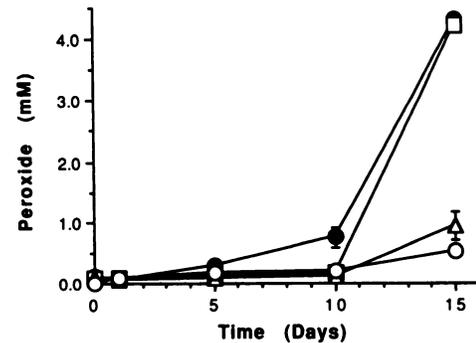


Fig. 4. Antioxidant activity of CLA. Linoleic acid (375 μmol) was incubated alone (●) or with CLA (37.5 μmol, □; 3.75 μmol, Δ; or 0.375 μmol, ○) under the conditions described for Fig. 3.

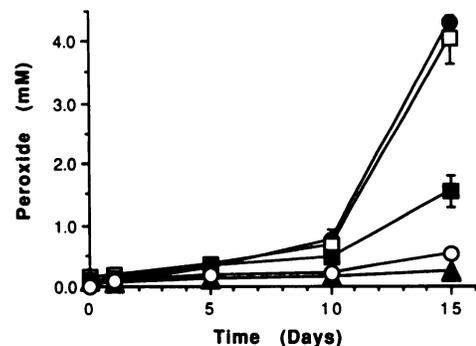


Fig. 5. Comparisons of CLA with other antioxidants. Linoleic acid (375 μmol) was incubated alone (●) or with 0.375 μmol of ascorbic acid (□), α-tocopherol (■), CLA (○), or BHT (▲) under the conditions described for Fig. 3.

function into a β-hydroxy acrolein moiety (17).

Table 3 shows that the UV absorbance at 268 nm was increased in PL extracted from forestomach tissue of mice treated with CLA. Hence, the change reported in Fig. 6 appears to occur *in vivo* as well as *in vitro*. However, the increased UV absorbance was not dependent on the absolute concentration of CLA in PL (Table 3). The CLA in PL increased about 300-fold, whereas the absorbance at 268 nm increased only 3-fold.

DISCUSSION

The present study shows that CLA effectively reduces BP-induced forestomach neoplasia. This finding complements our previous report that CLA reduced mouse epidermal neoplasia induced by 7,12-dimethylbenz(*a*)anthracene (4). Results of

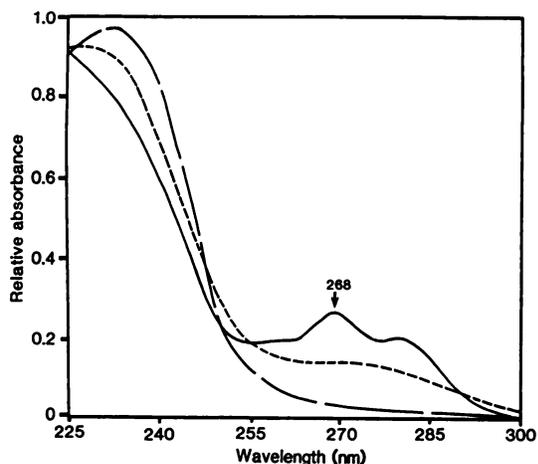


Fig. 6. UV absorption profile of CLA plus linoleic acid (—), CLA alone (---), or linoleic acid alone (· · · ·) following incubation for 15 days at 40°C under the conditions described for Fig. 3. One-ml samples from the 25-ml reaction volumes were extracted with hexane and analyzed by UV spectroscopy.

Table 3 Incorporation of the *c*-9,*t*-11 CLA isomer into forestomach PL
Eight 0.1-ml CLA doses were given p.o. over a period of 4 wk.

	<i>c</i> -9, <i>t</i> -11 CLA esterified in PL (mg/g) ^a	Absorbance at 268 nm ^b
Before treatment	0.1 ± 0.05 ^c	0.05 ± 0.01
After treatment	30.1 ± 2.10 ^d	0.15 ± 0.03

^a PL extracts analyzed for specific CLA isomers; more than 95% was the *c*-9,*t*-11 isomer. Hence, even though the CLA preparation used for treatment contained nine isomers ("Materials and Methods"), the *c*-9,*t*-11 isomer was preferentially incorporated.

^b Value was obtained from the fatty acid methyl esters isolated from forestomach PL (1.0 mg/ml of hexane).

^c Mean ± SEM for 3 determinants.

^d Also reported in Table 2.

mechanistic studies indicate that the *c*-9,*t*-11 isomer of CLA is preferentially incorporated into the forestomach membrane PL, suggesting that this CLA isomer may be the most important isomer in terms of biological activity. Incorporation of the *c*-9,*t*-11 CLA isomer into PL did not produce a gross disturbance in the PL oleic or linoleic acid content. We also established that CLA is an effective antioxidant, an observation that may in part explain the anticarcinogenic effects of CLA given the evidence for the possible involvement of oxygen radicals in BP activation (18–25), tumor promotion (26–28), and oncogene activation (29).

The mechanism whereby CLA acts as an antioxidant is not yet clear, but the UV changes shown in Fig. 6 and Table 3 provide a possible explanation. For example, formation of a β -hydroxy acrolein moiety within the CLA molecule would satisfy the electronic requirements for such a spectral shift (17). Malondialdehyde, a secondary product of lipid peroxidation, is present as a β -hydroxy acrolein above pH 7 and exhibits a UV maximum at 267 nm (30). Such a structure could arise from reaction of CLA with peroxy or hydroxyl radicals, followed by molecular oxygen, as proposed in Fig. 7. A similar moiety is present in a novel antioxidant isolated from *Eucalyptus* leaves (31). The data of Table 3, which confirm an earlier study of Reiser (32), indicate that the putative β -hydroxyl acrolein moiety may also form *in vivo*, in CLA incorporated into PL.

Assuming that the scheme proposed in Fig. 7 is correct, it is then necessary to ask why CLA containing a β -hydroxyl acrolein moiety should act as an antioxidant. There appear to be two possibilities: antioxidant activity may result from resonance enolization of the β -hydroxy acrolein moiety; and/or the β -hydroxy acrolein moiety may chelate iron, which would in turn

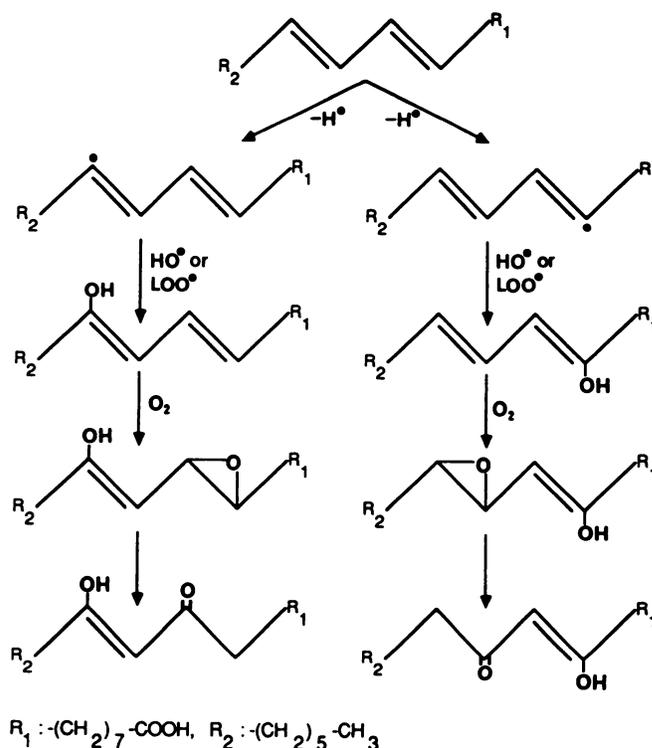


Fig. 7. Proposed model for the conversion of CLA to an active antioxidant. The sequence involves reaction of either carbon bordering the conjugated diene system with hydroxyl or peroxy radical, followed by reaction of the distant double bond with molecular oxygen, to produce a β -hydroxy acrolein moiety. Antioxidant activity most likely results from the chelation of iron by the β -hydroxy acrolein moiety (thereby interfering with the Fenton reaction) (33, 34) and perhaps also from resonance enolization.

lead to inhibition of the Fenton reaction (the Fenton reaction is responsible for generating hydroxyl radicals in the model system that we used). The latter possibility is particularly attractive since similar diketones are known to chelate iron (33).

The effective chelation of iron by β -hydroxy acrolein derivatives of CLA could be of great relevance to the inhibition of membrane peroxidation *in vivo*. For example, the report of Schaich and Borg (34) indicates that significant amounts of iron, hydrogen peroxide, and reducing agents (reactants of the Fenton reaction) do in fact partition into the lipid phase of model membrane systems; that the Fenton reaction "goes" in a lipid, hydrophobic environment; and that Fenton-type reactions occurring within lipid phases initiate the chain reactions of lipid autoxidation. Hence, by effectively chelating iron within microenvironments of membrane lipid, CLA (and particularly the *c*-9,*t*-11 isomer which can be incorporated into PL) could prevent the occurrence of Fenton-type reactions and subsequent damage by hydroxyl radicals of membrane sites that are critically involved in the induction of various pathological conditions, including the initiation and promotion of carcinogenesis. In this way CLA that is esterified to PL within the cell membrane could serve as a unique *in situ* antioxidant. (Very recently obtained data, which we will report elsewhere, indicate that rat liver microsomes containing esterified CLA are in fact considerably more resistant to iron-dependent oxidation than control liver microsomes).

Interestingly CLA is known to be present in human tissue, blood, and body fluids (35, 36). In addition to dietary sources (5, 36), there is evidence that the *c*-9,*t*-11 CLA isomer is produced *in vivo* via carbon-centered free radical oxidation of linoleic acid (this apparently involves interaction with certain serum proteins) (35). The concentration of the phospholipid-

esterified *c*-9,*t*-11 isomer in blood is modulated by oxidative stress (e.g., it is increased in the serum of alcoholics and returns to normal following alcohol withdrawal) (35). Hence, the formation and action of CLA may be seen in terms of a feedback loop that serves to protect the cell from damage by active oxygen. In this model CLA would act in concert with α -tocopherol, which is thought to be the only significant lipid-soluble chain-breaking antioxidant in blood (37). CLA would prevent the generation of hydroxyl radicals, whereas α -tocopherol would directly inactivate peroxy (and other) oxygen radicals.

ACKNOWLEDGMENTS

We thank Dr. John Beery for histological analysis.

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