Dietary Effects of Conjugated Octadecatrienoic Fatty Acid (9 *cis*, 11 *trans*, 13 *trans*) Levels on Blood Lipids and Nonenzymatic *in vitro* Lipid Peroxidation in Rats

P. Dhar, S. Ghosh, and D.K. Bhattacharyya*

Department of Chemical Technology, University Colleges of Science and Technology, Calcutta University, Calcutta - 700009, West Bengal, India

ABSTRACT: The present study examined the antioxidant activity of conjugated octadecatrienoic fatty acid (9 *cis*,11 *trans*,13 *trans*-18:3), α-eleostearic acid, of karela seed *(Momordica charantia),* fed to rats for 4 wk. The growth pattern of rats and the effect on plasma cholesterol and high density lipoprotein (HDL) cholesterol and peroxidation of plasma lipid, lipoprotein, erythrocyte membrane, and liver lipid were measured. Rats were raised on diets containing sunflower oil mixed with three different levels of conjugated trienoic fatty acid (9*c*,11*t*,13*t*-18: 3) 0.5, 2, and 10% by weight; the control group was raised with sunflower oil as dietary oil as the source of linoleic acid (9*c*,12*c*-18:2). The growth pattern of the three experimental groups of rats showed no significant difference compared to the control group of rats, but the group with 10% 9*c*,11*t*,13*t*-18:3 had slightly higher body weight than the control group of rats. Concentrations of total cholesterol, HDL-cholesterol, and non-HDL-cholesterol in plasma were similar in all four groups. Plasma lipid peroxidation was significantly lower in the case of 0.5% 9*c*,11*t*,13*t*-18:3 group than the control group and the 2 and 10% 9*c*,11*t*,13*t*-18:3 dietary groups as well. Lipoprotein oxidation susceptibility test with 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups was significantly less susceptible to lipoprotein peroxidation when compared with sunflower oil dietary group, and the dietary group with 0.5% 9*c*,11*t*,13*t*-18:3 showed least susceptibility. There was significant lowering in erythrocyte ghost membrane lipid peroxidation in the 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups compared to the sunflower oil groups. Nonenzymatic liver tissue lipid peroxidation was significantly lower in the group of rats raised on 0.5% 9*c*,11*t*,13*t*-18:3, but the groups on 2 and 10% 9*c*,11*t*,13*t*-18:3 acid did not show any significant difference compared with the control group of rats.

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Polyunsaturated fatty acids (PUFA) are susceptible to autoxidation giving rise to peroxyl free radical. Peroxidation takes place both in *in vivo* (1) and *in vitro* (2) conditions. *In vivo* lipid peroxidation takes place either by free radicals or enzymatically (2). Growing evidence exists that the oxidation of lipids plays a significant role in the development of atherosclerosis (3). Free radicals are constantly being generated, and antioxidant defense mechanism neutralizes them, making them ineffective (4). When the free-radical generation exceeds the scavenging capacity of the antioxidant defenses, the result is oxidative stress; there is a preponderance of free radicals that initiate *in vivo* peroxidation of PUFA of membrane lipids (5). There are currently four nutrients—ascorbic acid (5); conjugated linoleic acid (CLA) (6); one food preservative (butylated hydroxytoluene); and one drug (probucol) (7)—that function as *in vivo* antioxidants. The latter two also are antiatherosclerotic in animal models (7,8).

Growing interest exists in dietary conjugated fatty acids, especially CLA, which was shown by several workers to be an effective agent in reducing the incidence of chemically induced cancers and to act as chemoprotective agent (9). Anticarcinogenic effects of CLA might be attributed to their antioxidant activity (10) that serves as an *in-situ* defense mechanism against membrane attack by free radicals. However, recent reports from Van den Berg *et al*. (11) and Chen *et al.* (12) raised some doubts regarding the antioxidant property of CLA.

Conjugated linolenic acid (α-eleostearic acid: 9*c*,11*t*,13*t*-18:3) commonly found in karela seed (*Momordica charantia*) was nutritionally evaluated in our laboratory (13). However, its role as an antioxidant was not established.

In the present study, an attempt was made to determine the antioxidant effects of α-eleostearic acid 9*c*,11*t*,13*t*-18:3 supplemented at three different percentages (0.5, 2, 10%) in the diet by measuring plasma lipid, lipoprotein, erythrocyte membrane (EM), and liver lipid peroxidations. We also studied the growth rate and lipid profile of plasma using the same rat model.

EXPERIMENTAL PROCEDURES

Dietary fat sources. Authentic karela seeds, obtained from the local market of Calcutta, India, were crushed into fine particles,

*To whom correspondence should be addressed at Department of Chemical Technology, Calcutta University, University Colleges of Science and Technology, 92, A.P.C. Road, Calcutta - 700009, West Bengal, India.
E-mail: dkb@cucc.ernet.in

Abbreviations: CLA, conjugated linoleic acid; EM, erythrocyte membrane; HDL, high density lipoprotein; I.P., Indian Pharmacopia; LOS, lipoprotein oxidation susceptibility; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substance.

and the oil was extracted in a Soxhlet apparatus with *n*-hexane (40–60°C boiling point range). The free fatty acids present in the oil were then removed by miscella refining process (14). The extracted oil containing hexane, known as miscella (hexane/oil, 2:1) was mixed with 10% NaOH solution (20% excess of the theoretical amount required) at 40°C for 30 min to neutralize the free fatty acids. The soap formed was removed by centrifugation and the organic phase was washed with water. Deacidified oil was recovered after removing the solvent under vacuum distillation and drying under vacuum.

The refined oil was then bleached with tonsil earth optimum (1% w/w) (15) obtained from P.T. Sud-Chemic (Jakarta, Indonesia) and activated carbon (0.2% w/w), supplied by E. Merck India Pvt. Ltd. (Bombay, India) at 60°C under vacuum for 20 min. After the bleaching operation, the oil was recovered by vacuum filtration and stored at –20°C under nitrogen.

Dietary fat blends. Sunflower oil (trade name Sundrop) was obtained from I.T.C., Ltd. (Hyderabad, India). Sunflower oil was mixed with 1, 4, and 20% karela oil to give a final oil mixture containing 0.5, 2, and 10% by weight eleostearic acid, respectively; see Table 1 for fatty acid composition of the dietary oil mixtures.

Analysis of fat products. Gas chromatography was used as described previously (16). Fatty acid methyl esters were prepared following published procedures (17). Briefly, approximately 50 mg of oil was dissolved in 0.5 mL of diethyl ether, and 1.0 mL of 0.5 N methanolic KOH solution was added. The reaction mixture was shaken for 10 min at room temperature, and shaken vigorously after the addition of 1.0 mL of 1 N HCl. Methyl esters were extracted with 3×1 mL portions of petroleum ether. The extracts were combined and the solvent removed by a flow of nitrogen in a screw-capped test tube.

Conjugation of α-eleostearic acid present in karela seed oil was determined by ultraviolet spectrophotometric analysis at 262, 268, and 274 nm (18).

Feeding experiment. Animal experiments were designed and carried out as reported previously (19). Male albino rats of Charles Foster strain were housed in individual cages and were fed the dietary oils and fresh water *ad libitum*. Daily food consumption and weekly body weight gain were recorded. The feeding experiment was conducted to evaluate the antioxidant effect of α-eleostearic acid supplementation at three different levels.

Thirty-two rats (70–80 g body weight) were divided into four groups each consisting of eight rats having equal average body weight. The rats were fed experimental diets composed of fat-free casein, 18%; fat, 20%; starch, 55%; salt mixture, 4% [composition of salt mixture No. 12 (in g): NaCl, 292.5; KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4 \cdot 7H_2O$, 56.6; KCl, 1.66; $MnSO_4 \cdot 2H_2O$, 9.35; $ZnCl_2$, 0.5452; $CuSO_4 \cdot 5H_2O$, 0.9988; $CoCl_2 \cdot 6H_2O$, 0.0476] (20); cellulose, 3%; one multivitamin capsule [vitamin A I.P. (Indian Pharmacopia) 10,000 units; thiamine mononitrate I.P. 5 mg; vitamin B I.P. 5 mg; calcium pantothenate USP 5 mg; niacinamide I.P. 50 mg; ascorbic acid I.P. 400 units; cholecalciferol USP 15 units; menadione I.P. 9.1 mg; folic acid I.P. 1 mg, vitamin E USP 0.1 mg] per kg of diet. The diets were adequate in all nutrients.

Rats were maintained on the above diets *ad libitum* for 4 wk. For each rat the amount of daily diet consumed and weekly body weight gain were noted. Rats were killed while under mild anesthesia, blood was collected, and liver was immediately excised, blotted, and stored at –40°C before analysis.

Lipid analysis. According to the standard methods, the lipid components such as total cholesterol (21) and high density lipoprotein (HDL)-cholesterol (22) of plasma were analyzed using enzymatic kits supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India).

Plasma lipid peroxide was measured by the assay of thiobarbituric acid-reactive substances (TBARS) according to the standard method (23). The amount of malondialdehyde formed was calculated by taking the extinction coefficient of malondialdehyde to be $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Lipoprotein oxidation susceptibility (LOS) test. Non-HDL-cholesterol oxidation was carried out by precipitating apoB-containing lipoprotein (low density lipoprotein and very low density lipoprotein) according to Bachorik and Albers (24). The LOS test was carried out according to the method described by Phelps and Harris (25).

Preparation and oxidative sensitivity of EM ghost. After plasma separation, the erythrocytes were washed three times with 3 vol of a cooled isotonic solution containing 0.15 M NaCl, 10^{-5} M EDTA. The buffy coat was removed by aspiration after each wash. Finally the EM ghosts were prepared by using hypotonic solution of NaCl according to the method of Rose and Oklander (26).

A modification of the 2-thiobarbituric acid test (27) was used to measure lipid peroxides. A 0.5-mL aliquot of the red blood corpuscle membrane suspension was mixed with 1.0 mL of 10% trichloroacetic acid and 2.0 mL of 0.67% 2-thiobarbituric acid. The mixture was heated at 95°C for 15 min, cooled, and centrifuged. The absorbance of the supernatant was measured at 534 nm in a Shimadzu spectrophotometer (Tokyo, Japan), and the relative amounts of lipid peroxides were expressed in absorbance units, A_{534} nm (28).

Liver tissue lipid peroxidation. For lipid peroxide measurement, *ca.* 1 g of liver tissue was homogenized and extracted using the method of Bligh and Dyer (29).

2-Thiobarbituric acid test for liver lipid peroxidation. The present test was performed according to the method described by Schmedes and Hølmer (30).

RESULTS

Antioxidant property of conjugated octadecatrienoic fatty acid, α-eleostearic (9*c*,11*t*,13*t*-18:3) acid was investigated.

The fatty acid composition of the dietary oils and oil blends are shown in Table 1. The oleic (18:1) and linoleic (18:2) contents were generally similar in all the dietary fats.

The mean body weight gain of rats fed the 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups for 4 wk showed no statistically significant difference when compared with the sun-

TABLE 1
Fatty Acid Composition of the Dietary Oils and Oil Mixtures

Dietary fats	Fatty acid composition (area%)[a]				
	16:0	18:0	18:1	18:2	18:3(9*c*,11*t*,13*t*-18:3)
Sunflower oil	6.5	3.5	31.9	58.1	—
Karela seed oil	2.0	31.7	8.0	7.1	51.1
Sunflower oil + karela seed oil (99:1, w/w)	6.4	3.8	31.7	57.6	0.5
Sunflower oil + karela seed oil (96:4, w/w)	6.3	4.6	30.9	56.1	2.1
Sunflower oil + karela seed oil (80:20, w/w)	5.6	9.1	27.2	47.9	10.2

[a]As determined by gas chromatography. 9*c*,11*t*,13*t*-18:3, α-eleostearic acid. $n = 8$.

TABLE 2
Plasma Cholesterol Profile of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing 0.5, 2, and 10% α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3)

Diets	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	Non-HDL-cholesterol (mg/dL)
Sunflower oil	46.3 ± 7.3[a]	23.3 ± 2.4	23.01 ± 6.7
0.5% 9*c*,11*t*,13*t*-18:3	35.8 ± 2.5	18.1 ± 1.7	17.8 ± 3.2
2% 9*c*,11*t*,13*t*-18:3	40.1 ± 3.7	20.1 ± 2.2	20.0 ± 5.2
10% 9*c*,11*t*,13*t*-18:3	44.3 ± 4.8	19.9 ± 4.6	24.3 ± 2.4

[a]All values are means of eight rats/diet. HDL, high density lipoprotein.

flower oil control group (Fig. 1). Although statistically insignificant, the dietary group with 10% 9*c*,11*t*,13*t*-18:3 showed maximal gain in weight at the end of week 4. In a previous nutritional experiment (13) the mean body weight gain was shown to be significantly more in rats raised on 51.1% 9*c*,11*t*,13*t*-18:3 compared to rats raised on 9*c*,12*c*,15*c*-18:3 dietary fat, which would support our present observation.

The amounts of plasma total cholesterol, HDL-cholesterol, and non-HDL-cholesterol of the rats raised on 0% 9*c*,11*t*,13*t*-18:3 (sunflower oil) and 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diet are included in Table 2. A comparison of the plasma total cholesterol of rats fed 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 and 0% 9*c*,11*t*,13*t*-18:3 (sunflower oil) showed that there was a trend of greater amount of plasma total cholesterol in the 0% 9*c*,11*t*,13*t*-18:3 diet compared to the other three 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diets. The total cholesterol was minimal in the group fed 0.5% 9*c*,11*t*,13*t*-18:3 and increased gradually as the level of 9*c*,11*t*,13*t*-18:3 was raised. There was no significant difference between the 0% 9*c*,11*t*,13*t*-18:3 and three 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups. HDL-cholesterol content in the plasma (Table 2) was maximal in the 0% 9*c*,11*t*,13*t*-18:3 dietary group, but there was no significant difference between the four dietary groups.

Table 3 includes the *in vitro* plasma lipid peroxidation and LOS of the 0, 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diet. It is evident that 9*c*,11*t*,13*t*-18:3 supplementation effectively reduced the susceptibility of the plasma lipid to peroxidation; the minimal susceptibility was achieved with 0.5% 9*c*,11*t*,13*t*-18:3 along with the gradual increase of 9*c*,11*t*,13*t*-18:3 supplementation (2%, 10%). The 0% 9*c*,11*t*,13*t*-18:3 group (sunflower group) showed maximal peroxidation of plasma lipid. Increasing the level of 9*c*,11*t*,13*t*-18:3 in the diet from 0.5 to 2% resulted in 25% increase in sensitivity to *in vitro* plasma lipid peroxidation. When the dietary level of 9*c*,11*t*,13*t*-18:3 was further increased to 10%, the sensitivity to *in vitro* plasma peroxidation increased by an additional 29%, but this change was not significant.

The oxidative sensitivity of the EM ghost and liver lipid

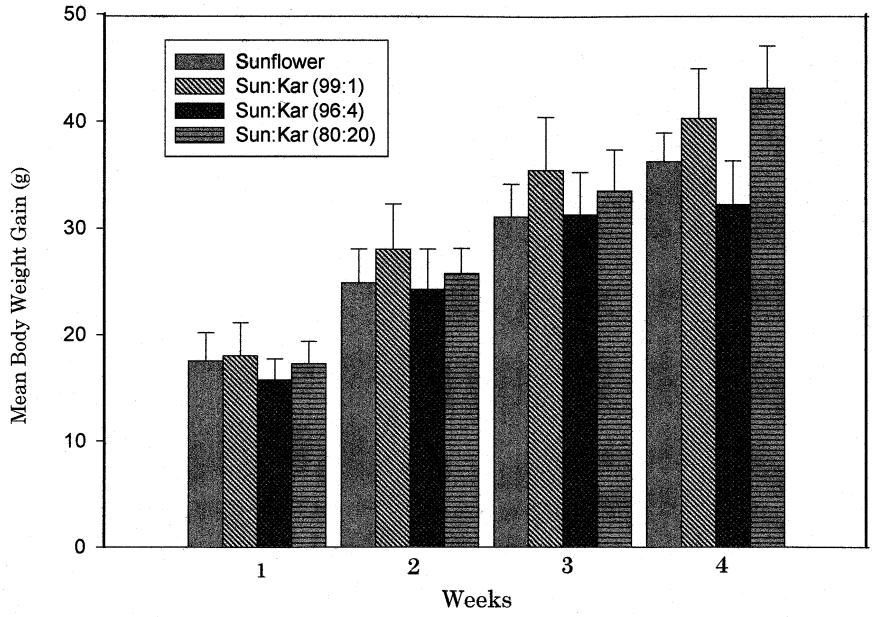

FIG. 1. Mean body weight gain of rats fed sunflower oil and sunflower (Sun):karela seed oil (Kar) blends at different weeks. $n = 8$ rats.

TABLE 3
Plasma Lipid Peroxidation and Lipoprotein Peroxidation of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing Different Levels of α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3) at 20% Fat in Diet

Diets	Plasma lipid peroxidation (nmole of MDA/mL of plasma)	Lipoprotein oxidation susceptibility (LOS) (nmole of MDA/mg of non-HDL-cholesterol)
Sunflower oil	9.3 ± 1.6[a]	51.1 ± 9.8
0.5% 9*c*,11*t*,13*t*-18:3	4.1 ± 0.7[b,c,d]	12.8 ± 2.7[b]
2% 9*c*,11*t*,13*t*-18:3	6.8 ± 1.1	14.7 ± 2.7[b]
10% 9*c*,11*t*,13*t*-18:3	9.0 ± 1.1	16.1 ± 6.6[e]

[a]All values are means of 8 rats/diet. MDA, malondialdehyde. See Table 2 for other abbreviation.
[b]Experimental group vs. control group $P < 0.01$.
[c]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group $P < 0.01$.
[d]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 2% 9*c*,11*t*,13*t*-18:3 group $P < 0.05$.
[e]Experimental group vs. control group $P < 0.02$.

TABLE 4
Lipid Peroxidation of Erythrocyte Membrane (EM) Ghost and Liver Tissue of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing Different Levels of α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3) at 20% Fat in Diet

Diets	EM lipid peroxidation (nmole of MDA/mg of protein)	Liver tissue lipid peroxidation (nmole of MDA/mg of tissue lipid)
Sunflower oil	3.1 ± 0.4[a]	1.8 ± 0.3
0.5% 9*c*,11*t*,13*t*-18:3	1.8 ± 0.2[b]	0.6 ± 0.1[c,d,e]
2% 9*c*,11*t*,13*t*-18:3	2.1 ± 0.3[f]	1.5 ± 0.5
10% 9*c*,11*t*,13*t*-18:3	1.7 ± 0.4[g]	1.5 ± 0.3

[a]All values are means of eight rats/diet. $n = 8$. See Table 3 for other abbreviation.
[b]0.5% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.05$.
[c]0.5% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.01$.
[d]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group.
[e]2% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group $P < 0.01$.
[f]2% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.01$; $P < 0.05$.
[g]10% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.05$.

peroxidation of the four dietary fat groups of rats are presented in Table 4. TBARS production in EM ghosts from the 0% 9*c*,11*t*,13*t*-18:3 group was significantly higher when compared with TBARS production in the EM ghosts of the 0.5, 2, 10% 9*c*,11*t*,13*t*-18:3 dietary groups. But there was no significant difference between the 9*c*,11*t*,13*t*-18:3 supplemented groups.

Liver lipid peroxidation results showed that *in vitro* production of TBARS in liver tissue of dietary group supplemented with 0.5% 9*c*,11*t*,13*t*-18:3 was significantly lower than the 0, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups. Thus the TBARS formation in the *in vitro* condition was lowest in the dietary group that was supplemented with least amount of 9*c*,11*t*,13*t*-18:3.

DISCUSSION

The study showed that 0.5 and 2% 9*c*,11*t*,13*t*-18:3 fatty acid in dietary oil had no effect on the body growth of rats, but there was an improved weight gain with 10% 9*c*,11*t*,13*t*-18:3, though not significant. The previous nutritional experiment (13) showed that 20% 9*c*,11*t*,13*t*-18:3 in the diet improved the body weight gain of rats in comparison with the rats raised on 9*c*,12*c*,15*c*-18:3 dietary fat which supports the present observation. Chin *et al.* (31) studied the effect of CLA on rat growth and development. Pups that continued to receive the CLA-supplemented diet after weaning had significantly greater body weight gain compared to control animals.

For assessment of the effect of 9*c*,11*t*,13*t*-18:3 on atherosclerosis in rats, total cholesterol, HDL-cholesterol, and non-HDL-cholesterol were estimated in plasma. There was no evidence of significant difference in the total cholesterol and various forms of cholesterol between the experimental and the control groups of rats. It can be concluded that the four dietary groups were nutritionally equivalent since the PUFA content of the four dietary fats was more than adequate, generally equal in content, and the levels of 9*c*,11*t*,13*t*-18:3 were low. A diet supplemented with 0.5 g CLA for 12 wk reduced low density lipoprotein-cholesterol and triglycerides markedly (32).

Plasma lipid peroxidation and LOS were maximally reduced at 0.5% of dietary 9*c*,11*t*,13*t*-18:3, and peroxidation increased gradually with the increased levels of 9*c*,11*t*,13*t*-18:3 in the diet group. Therefore, the antioxidant efficacy of 9*c*,11*t*,13*t*-18:3 is maximal at 0.5% in the diet. Ip *et al.* (33) showed that 0.25% CLA in the diet had maximal antioxidant effect, and CLA lowered mammary tissue malonaldehyde formation.

EM lipid peroxidation data indicate that 9*c*,11*t*,13*t*-18:3 had reduced lipid peroxidation possibly after being incorporated into the lipids of red blood cell membrane. Sunflower oil containing high levels of linoleic acid and no 9*c*,11*t*,13*t*-18:3 showed increased membrane lipid peroxidation. Cunningham *et al.* (34) observed that supplementation of linoleic acid increased intracellular lipid peroxide concentrations in normal human mammary epithelial cells. Ha *et al.* (10) from his investigations concluded that CLA might serve as an *in situ* defense mechanism against membrane attack by free radicals which may explain the anticarcinogemic properties of CLA.

Liver tissue lipid peroxidation also reduced at 0.5% 9*c*,11*t*,13*t*-18:3 which may have been due to incorporation of this conjugated linolenic acid. Belury and Kempa-Steczko (35) showed that dietary CLA was incorporated in neutral and phospholipids at the expense of linoleic acid in the diet.

Normally conjugated fatty acids are more rapidly oxidized than the nonconjugated PUFA (11). Various investigators showed that the linolenates oxidize approximately twice as fast as the linoleates (36). According to Swern (37) conjugated triene esters oxidize more rapidly than nonconjugated triene esters. In compounds with more than two conjugated double bonds, conjugation increased the rate of oxidation (38). Thus, in the *in vivo* study conjugated trienoic fatty acids are also likely to be more rapidly oxidized than linoleates by picking up more free radicals, thereby eliminating or reducing the formation of hydroperoxides. CLA in higher concentrations

acts as prooxidant rather than antioxidant (9). At 0.5%, 9*c*,11*t*,13*t*-18:3 showed antioxidant property that decreased with increase in its level. At higher concentrations it could have possibly acted as a prooxidant similar to CLA, also reported to act as a prooxidant at higher concentration (9).

Another possible mechanism could be that oxidation of conjugated trienoic fatty acid resulted in the formation of conjugated dienoic fatty acids (CLA) that acted as antioxidants (9). Brauer and Steadman (38) measured the oxidation of β-eleostearic acid by means of ultraviolet spectrophotometry and observed that the conjugated triene decreased as oxidation proceeded, while the absorption due to conjugated diene increased. This also occurred during oxidation of α-eleostearic acid (39). Allen and Kummerow (40) found that the amount of triene conjugation lost and the amount of diene conjugation formed were proportional to the amount of oxygen absorbed. Thus it may be possible that in the *in vivo* conditions these conjugated linolenic fatty acids may have reduced the formation of hydroperoxides by lowering the generation of free radicals and peroxidation of PUFA occurring in EM and other lipids.

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