The second fraction isolated was composed of two major components that were poorly resolved (Fig. 4). Peak number two coincided with the retention time of a conjugated 18:3 isomer mixture as shown by hydrogenation of this fraction. A small percentage (2.7%) of methyl stearate was observed, indicating overlap between conjugated 18:3 isomers and a major cyclic component. This overlap caused serious problems when evaluating the mass spectra of these components, since the mass spectra of conjugated methyl linolenate and its isomers were identical to those of the component cyclic esters. Several mass spectra were taken during elution of each of the major peaks. The mass spectra were all identical, varying only in intensity, indicating a series of closely related isomers. A typical spectrum, after spectrum averaging and background subtraction, is shown in Figure 6. The mass spectrum shown indicates a base peak at m/e = 121 (C_9H_{13}, 121.1008, calculated 121.1017) for an alkyl ion with a propyl substituent; other ions were present, representing alkyl substituents of up to 10 carbon atoms. Other ions representing a tropilium ion, formed by dehydrogenation, disubstituted ions, as well as a peak at m/e = 261 indicating loss of methanol are present. An ion at m/e = 248, which represented cleavage of the alkyl side chain from the molecular ion of one isomer, was also produced ($C_{16}H_{24}O_2$, 248.1764, calculated 248.1776). This spectrum and other obtained closely resembled those obtained by other workers (8,9,22) for cyclohexadienoid structured and aromatized monomers. This particular spectrum represented a mixture of isomers in which a short alkyl substituent group predominated. In the other major peak of the chromatogram (Fig. 4), the mass spectrum indicated predominance of compounds with other combinations of substituent groups varying in the number of carbon atoms in both the alkyl and carboxyl containing side chains.

Fraction 3, taken from the bottom portion of the band corresponding to the cyclic monomer reaction product as indicated in Figure 3, was subjected to GLC (Fig. 4). Another more complex series of poorly separated isomers was obtained consisting of a large doublet and two other fairly well resolved components. In this case also, there was considerable overlap with both linolenate and its conjugated isomers, and, upon hydrogenation of this fraction, 3.7% stearate was present. The mass spectra of each of the peaks were determined periodically during the elution of the peak, resulting in over 40 spectra. All spectra were identical with only intensity differences, as observed previously. This would be expected, since the gas chromatographic peaks represent a concentration of various isomers in terms of both substituent chain length differences as well as geometrical configuration. The average mass spectrum of the major component of the fraction is reproduced in Figure 7. The mass spectrum indicates the molecular weight as 292 with a peak at 261, representing loss of methoxy (methanol). Peaks are also present for alkyl-substituted cyclohexadiene and aromatic ions at m/e 105, 107, 119, 121, 135, 137, 151 and 165. Ions are present at m/e 78 and 91, formed by dehydrogenation and cleavage of the corresponding ion at m/e 93.

The intense ion at m/e 151 was resolved into three ions as follows. A major ion corresponding to $C_{10}H_{15}O$ (151.1111, calculated 151.1123) and minor ions corresponding to $C_{11}H_{19}$ (151.1463, calculated 151.1486) and $C_9H_{11}O_2$ (151.0748, calculated 151.0759). The second ion represents the alkyl substituted cyclohexadiene ion and the $C_9H_{11}O_2$ ion that fragment from which the alkyl group has been removed. The ion corresponding to $C_{10}H_{15}O$ probably represents a ketene ion. Other higher molecular weight ions consisted primarily of a homologous series of ketene ions.

This spectrum is also in accord with those reported

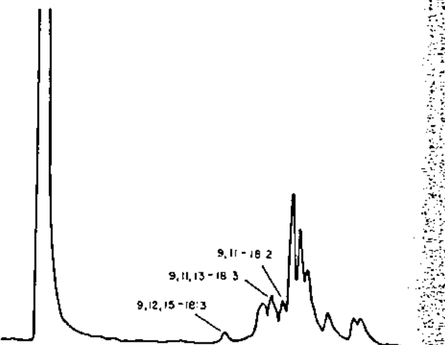
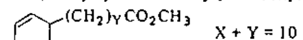


FIG. 8. Capillary gas liquid chromatogram of cyclic monomer ethyl ester reaction product with added 9,11-methyl linoleate, 9,11,13 and 9,12,15-methyl linolenate (150 ft x 0.01 in. capillary) coated with Apiezon L).

aromatic isomers (8,9,21). The mass spectral studies have confirmed the structures of fractions 2 and 3 as mixtures of isomeric ω-(2-alkylcyclohexadienyl) carboxylic acids as shown:



$(CH_2)_Y CO_2CH_3$, $(CH_2)_X CH_3$ $\quad X + Y = 10$

Additional gas chromatographic studies using columns with OV-25 and SE-30 packings yielded inferior separations compared to that obtained with EGS. Results obtained with a 50 ft x 0.02 in. S.C.O.T. column coated with DEGS were not superior to those obtained from the EGS column. However the separation of cyclic monomer was improved considerably using a 150 ft x 0.01 in. capillary column coated with Apiezon L (Fig. 8). As indicated in the gas chromatogram, the separation of the cyclic monomer isomer, as well as octadecadienoate and trienoate samples was enhanced compared to that obtained on the 6 ft EGS column. However this separation is not of practical value for preparative use.

Argentation TLC has therefore allowed the partial removal of the undesirable side reaction products, such as conjugated linolenate and polymeric material, from crude cyclic fatty acid preparations. This method allowed partial fractionation of the isomeric monomers and produced one fraction very low in conjugated linolenate. The amount of material that may be fractionated by this method is low, although in the case of a ^{14}C-labeled substrate this would not be a major factor. Using a microgram scale (10-20 μg), separations are quite good and yielded complex chromatograms which appeared to be composed of separated positional and geometrical isomers of the cyclic monomer. However attempts to increase the plate loadings to preparative scale (20 mg) decreased the separation of the cyclic monomer and conjugated linolenate.

In order to facilitate the preparation of larger quantities of isomeric cyclic monomer mixtures free of conjugated linolenate isomers, argentation column chromatography was investigated. A column packed with acid-washed silicic acid coated with 10% $AgNO_3$, when eluted with 10% diethyl ether in petroleum ether, eluted large amounts of conjugated linolenate and cyclic material. When the ether content of the eluting solvent was decreased to 5% (v/v), less conjugated methyl linolenate isomers eluted. A fraction of pure cyclized methyl linolenate was eluted with 2% v/v ether in petroleum ether. With this solvent, yields of over 70% cyclized material were obtained by direct application of reaction mixture methyl esters to the column. Other reaction products, such as dimeric and higher polymeric

solvent system, the conjugated methyl linolenate was successfully removed from sample sizes of up to 10 g. The eluted material had on the average less than 1% conjugated linolenate isomers as determined by hydrogenation.

Although argentation TLC allowed partial separation of isomeric cyclic monomers and the aromatic isomer, column chromatography yielded a batch type separation of conjugated linolenate from the reaction product. The final purified cyclization product contained ca. 10% of the aromatic isomer, as well as those isomeric cyclohexadienyl products described previously.

ACKNOWLEDGMENTS

G. Rao, E. Mayhood, M. Sullivan and T. Fielder rendered technical assistance. Partial support was obtained from the U.S. Public Health Services Grant FD 00049, the Illinois Agriculture Experiment Station and the Biomedical Support Grant at the University of Illinois at Urbana-Champaign.

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Studies on Peroxidative Hemolysis and Erythrocyte Fatty Acids in the Rabbit: Effect of Dietary PUFA and Vitamin E

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ABSTRACT Clinical signs of vitamin E deficiency were not manifested in the rabbit prior to the onset of dietary PUFA supplementation. PUFA consumption by vitamin E-deficient rabbits tended to promote peroxidative hemolysis by dialuric acid, as well as osmotic fragility when determined in the presence of either hydrogen peroxide or the peroxide-generating system of glucose oxidase and glucose in a continuous salt gradient. Analysis of erythrocyte fatty acids by GLC demonstrated incorporation of the administered PUFA. Peroxidation of RBC PUFA was observed in vitro but not in vivo. Fatty acids of RBC phospholipids were also analyzed for alterations under the experimental conditions. Possible mechanisms of the participation of vitamin E in lipid metabolism and membrane stabilization are discussed. J. Nutr. *104*: 192–201, 1974.

INDEXING KEY WORDS vitamin E · PUFA · rabbits · RBC

In 1947 Hove and Harris (1) observed that vitamin E-deficient animals developed symptoms of muscular dystrophy when dietary polyunsaturated fatty acids (PUFA) were increased. Borgman (2) noted no increase in dystrophic symptomatology when he fed oleic acid to vitamin E-deficient rabbits. However, increased dietary linoleate did cause nutritional muscular dystrophy in E-deficient rabbits, confirming the earlier observation by Hove and Harris (1).

Many investigators have proposed a relationship between the amount of dietary PUFA and the requirement for the antioxidant, vitamin E. Tappel (3) and Pritchard and Singh (4) have postulated a destruction of rat tissue PUFA in the absence of vitamin E which they attribute to in vivo lipid peroxidation. However, Hayes et al. (5), having shown that the canine requirement for vitamin E is directly related to PUFA consumption, associate this phenomenon with lipid metabolism, not solely connected to an antioxidant effect. In their investigation of fatty acid turnover in rat erythrocyte membranes, Oliveira and Nason (6) found no correlation between vitamin E status of the rats and the ability of their erythrocytes to acylate glycerophospholipids. Thus, the role of vitamin E in lipid metabolism is still in question. Although Horwitt (7) has demonstrated an increased requirement for tocopherol in the chick in the presence of an elevated intake of linoleic acid, other workers (8, 9) have been unable to extend these results in studies with rats fed PUFA. Also, El Khatib et al. (10), in their studies of rats and rabbits, found no difference in the amounts of lipid peroxidation in tissues of E-deficient and control animals. The increased requirement for vitamin E in the presence of increased dietary PUFA is therefore still controversial.

The actual mechanism of peroxidative hemolysis of RBC from vitamin E-deficient animals is still in dispute. Studies by Younkin et al. (11) indicate that lipid peroxidation precedes erythrocyte hemolysis. According to Jacob and Lux (12) membrane-bound phosphatidylethanolamine (PE) is selectively attacked by peroxides generated by glucose oxidase and glucose. This observation was contradicted

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by studies performed by Heikkila et al. (13) who found that during peroxidative hemolysis all phospholipids of the erythrocyte membrane were destroyed in approximately equal ratios.

The present study was undertaken to further elucidate the synergistic effect of vitamin E deprivation and increased dietary PUFA upon rabbits. We have noted an effect of increasing consumption of PUFA on the fatty acid composition of RBC membranes and the peroxidative hemolysis of these cells.

MATERIALS AND METHODS

Animals and Diets. White male New Zealand rabbits [1] were raised from weaning on a pelleted vitamin E-deficient or supplemented diet (table 1). The tocopherol-supplemented diet was identical to the deficient diet except for the addition of 225 mg DL-α-tocopherol per kilogram diet. In addition, designated groups of animals were given daily oral supplements of 1 ml cod-liver oil (CLO), 3 ml stripped safflower oil containing 78% linoleic acid [2] or 1 ml 50% ethyl arachidonate.[3]

Procedures. All solvents were reagent grade and were redistilled prior to use. Plasma tocopherol was assayed by the micro method of Hashim and Schuttringer (14).[4] Hemolysis with dialuric acid was performed according to Friedman et al. (15); glucose oxidase–glucose (GO-G) hemolysis was carried out by a modification of the procedure of Jacob and Jandl (16) using a final concentration of glucose of 27.8 mM and 3.0 U/ml glucose oxidase. Direct hydrogen peroxide hemolysis was assayed according to Lubin et al. (17). Erythrocyte density distribution was determined by the method of Danon and Marikovsky (18). Creatine phosphokinase activity was assayed by a modification of the procedure of Kjekshus and Sobel (19). These values have been reported elsewhere by this laboratory.[5]

Osmotic fragility measurements [6] were performed as described by Barker et al. (20). This procedure measures the change in osmotic fragility of RBC in a continuous salt gradient. The fragility curve that is generated as a function of light transmitted through the cell suspension is continuously recorded on a chart. The derivative of the fragility curve is also recorded. A shift to the left is indicative of increased susceptibility to osmotic lysis, while a rightward shift suggests a slight resistance to hemolysis conferred upon the cells by the increased tonicity of the solution (21).

Blood was drawn from the ear vein of the animals or obtained by heart puncture from rabbits anesthetized with Penthrane using EDTA as an anticoagulant. The plasma and buffy coat were removed by centrifugation [7] at 1,000 × *g* for 10 minutes followed by aspiration. RBC were washed three times and hemolyzed according to the procedures of Dodge et al. (22). Membrane ghosts were extracted with chloroform–methanol (2:1). The aqueous phase was removed by aspiration and the remaining chloroform layer was dried with anhydrous sodium sulfate. After evaporation of the sample under nitrogen to dryness, fatty acid methyl esters (FAME) and dimethylacetals (DMA) were prepared by heating the lipid residue with 2 ml methanol–sulfuric acid (9:1) in sealed tubes at

TABLE 1

Synthetic rabbit stock colony diet[1]

Ingredients	g/kg
Casein, GBI Vitamin Free Test	200.00
Cornstarch	260.00
Cane sugar	260.00
Agar, U.S.P.	20.00
Nonnutritive fiber	120.00
Stripped lard	80.00
Salt mix, U.S.P. XIV	60.00
Vitamin mix[2,3]	

[1] General Biochemicals, Chagrin Falls, Ohio. [2] Vitamin mix, contributed the following vitamins per kg diet: (in mg) calcium pantothenate, 45; choline chloride, 6000; i-inositol, 300; menadione, 2.25; niacin, 600; pyridoxine·HCl, 21; riboflavin, 21; thiamin·HCl, 21; DL-α-tocopherol, 225; as well as vitamin A, 4750.2 U/kg and vitamin D, 264.31 U/kg. [3] DL-α-tocopherol omitted from vitamin E-deficient diet.

[1] Edgely Research Farm, Columbus, N. J.
[2] Donated by Dr. Winifred Cort of Hoffmann-LaRoche Inc., Nutley, N. J. The stripping procedure was effected by heating in a molecular still at 270° in a V. L. Watt 10-stage rotary glass evaporator manufactured by Vitamins Ltd., England, at 1 μ vacuum.
[3] Nu-Chek Prep, Elysian, Minn.
[4] DL-α-Tocopherol for standard curves was donated by Dr. Kay Bunnell of Hoffmann-La Roche Inc., Nutley, N. J.
[5] Brin, M., Barker, M. O., Horn, L. & Reed, G. (1973) Analysis of skeletal muscle from vitamin E-deficient and supplemented rabbits fed polyunsaturated fatty acids. Federation Proc. *32*: 943 (abstr.).
[6] Fragiligraph Model D-2, Elron Electronic Instruments, Ltd., Haifa, Israel.
[7] Sorvall RC2-B, HS-4 rotor, Ivan Sorvall, Inc., Norwalk, Conn.

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TABLE 2

Hemolysis of rabbit erythrocytes with glucose oxidase-glucose or hydrogen peroxide

Tocopherol status	Oil	No.	Percentage hemolysis with glucose oxidase		Percentage hemolysis with hydrogen peroxide	
			Mean	Range	Mean	Range
Control	None	(7)	2.1	0–13	0.8	0–1.5
Deficient	None	(13)	10.3	0–27	23.0	0–47
Control	CLO	(11)	0.5	0–6.4	6.3	
Deficient	CLO	(5)	1.1	0–3.1	7.6	
Control	Safflower	(4)	1.7	0–3.7	6.4	4.2–6.6
Deficient	Safflower	(2)	0		53.2	7–99
Control	Arachidonate	(3)	0		5.0	0–7.9
Deficient	Arachidonate	(2)	24.8		97.4	96.8–98

100° for 90 minutes. Following extraction into hexane, FAME and DMA were chromatographed on a 183-cm stainless steel column of 10% ECSS-X on 100/120 mesh Gas Chrom P at 185° to 195°[8] employing helium as carrier gas. Plasma lipids were extracted into 20 volumes of ethanol-isopropyl ether (2:1), filtered, evaporated to dryness, and transmethylated as described above.

Two-dimensional thin-layer chromatography was performed in a glass tank in a Plexiglass box under nitrogen. Lipid extracts were dissolved in chloroform and applied to 20 by 20 cm Silica Gel G plates.[9] Phospholopids were first resolved in a solvent system of chloroform–methanol–50% ammonia (65:27:6) and then in chloroform–methanol–acetone–glacial acetic acid–water (50:20:10:8:4). Following detection with a solution of 0.01% 4',5'-dichlorofluorescein in redistilled absolute ethanol under ultraviolet light, phospholipid spots were scraped off and extracted into 20 volumes chloroform–methanol–50% acetic acid (65:25:14). Extracts were washed, the chloroform layer was dried with sodium sulfate, and FAME and DMA were prepared and analyzed as described above.

TABLE 3

Percentage hemolysis of rabbit erythrocytes after 4 to 6 weeks feeding of oil diets

Tocopherol status	Oil	Percentage hemolysis[1]		
		Glucose oxidase–glucose	Dialuric acid	Hydrogen peroxide
Control	None	0	1.5	0.4
Deficient	None	1	0.1	99
Control	Arachidonate	0	1.4	3
Deficient	Arachidonate	85	13	98
Control	Safflower	4	0	6
Deficient	Safflower	35	0	76

[1] All percentage hemolysis values reading horizontally were obtained from the same rabbit blood sample.

RESULTS

The rabbits were considered to be vitamin E deficient when their plasma tocopherol levels fell below 300 μg/100 ml compared to the control level of at least 1,000 μg/ml. Even after 6 months at this deficient level, the rabbits displayed no signs of muscular weakness when given no oral oil supplement.

In our earlier studies with rats we used susceptibility of RBC to peroxidative hemolysis as an index of vitamin E deficiency. The results shown in table 2 demonstrate that only the blood from the arachidonate-supplemented E-deficient rabbits displayed appreciable hemolysis when treated with the peroxide-generating system of glucose oxidase and glucose. The values presented in table 3 are representative of a typical hemolysis experiment with a second group of rabbits. These data indicate that blood from all E-deficient rabbits, excluding those receiving no oil supplements, exhibited considerable hemolysis following treatment with glucose oxidase and glucose. Some hemolysis with dialuric acid was noted for the blood from arachidonate-supplemented deficients, and in subsequent studies, as much as 59% hemolysis was observed following dialuric acid treatment of

[8] Hewlett-Packard gas chromatograph Model 7620A, Hewlett-Packard Company, Avondale, Pa.
[9] Quanta Gram Q1, Quantum Industries, Fairfield, N. J.

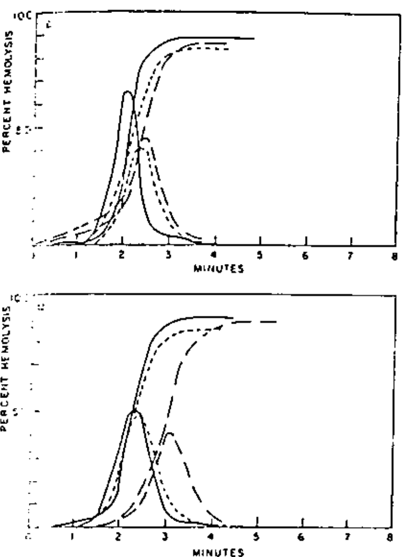

Fig. 1 Fragility curves of blood from control and vitamin E-deficient rabbits given no oil supplements: 1A (top), deficient rabbit; 1B (bottom), control rabbit. Key: ———, fragility curve of blood in phosphate-saline only; — — —, fragility curve of blood in phosphate-saline plus glucose oxidase and glucose; – – – – –, fragility curve of blood in phosphate-saline plus 3% hydrogen peroxide; cells washed, resuspended in phosphate-saline.

blood from safflower oil-fed E-deficient rabbits.

Peroxidative hemolysis of the rabbit blood in an osmotic gradient was studied. Although blood from vitamin E-deficient rats has been shown to be extremely sensitive to this procedure (20), rabbit blood is not. Figure 1 depicts fragility curves from vitamin E-deficient and control rabbits fed no oil supplements. There is no leftward shift of the curve in the presence of either peroxidizing agent, indicating no increased hemolysis under the experimental conditions. Nor did the blood from any of the vitamin E-sufficient rabbits display a leftward shift of the fragility curve of their blood. Figure 2A shows the hemolysis of blood from a vitamin E-deficient safflower oil-supplemented rabbit. Although no leftward shift of the fragility curve was initially noted in the presence of glucose oxidase and glucose, a shift was apparent after a 15-minute incubation of blood with hydrogen peroxide. Blood from a CLO-fed E-deficient rabbit also hemolyzed in the presence of hydrogen peroxide even though it did not with glucose oxidase and glucose (fig. 2B).

Hemolysis profiles of blood from E-deficient rabbits fed ethyl arachidonate exhibit biphasic hemolysis curves (fig. 3), suggesting that there were two populations of erythrocytes, one of which lysed before the other. Density distribution studies further confirmed the presence of two populations as the mean RBC density shifted from 1.110 to 1.102 g/ml within 3 weeks after the onset of ethyl arachidonate supplementation. Reticulocyte staining also demonstrated increased reticulocytosis in the blood of arachidonate-fed control and deficient rabbits, thereby again explaining the biphasic osmotic fragility and the density shift.

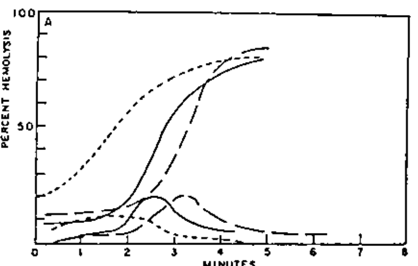

Fig. 2 Fragility curves of blood from vitamin E-deficient rabbits given either safflower oil (15D) or CLO (8D). 2A, rabbit 15D; 2B, rabbit 8D. Key: same as for figure 1.

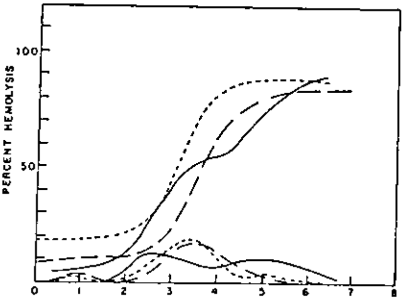

Fig. 3 Fragility curves of blood from a vitamin E-deficient, ethyl arachidonate-supplemented rabbit. Key: same as for figure 1.

Initial investigation of the RBC fatty acid distribution of arachidonate-fed rabbits showed that relatively large amounts of dietary arachidonic acid were incorporated (table 4). RBC from safflower oil-fed rabbits exhibited an increased linoleate content with no concomitant rise in the percentage of arachidonate, presumably due to the rabbits' inefficient conversion of dietary linoleate to arachidonate. The results of a second study, depicted in table 5, also indicate that there was a large increase in the amount of arachidonic acid incorporated into the RBC of ethyl arachidonate-fed rabbits, but no increase for the unsupplemented or safflower oil-fed animals.

On the other hand, the linoleate content of the RBC remained constant in both untreated and safflower oil-fed animals, while decreasing substantially in the arachidonate-supplemented rabbits. The level of eicosadienoic acid, an intermediate in the biosynthesis of arachidonic acid from linoleic acid, was observed to rise in RBC of all animals examined except the E-deficient rabbits fed arachidonate or CLO. No such increases were noted in eicosadienoic acid content of the plasma (table 6), although plasma arachidonic acid content of arachidonate-fed rabbits did increase. Not only did the plasma level of this fatty acid not increase in animals unsupplemented with ethyl arachidonate, but the deficient animals had a consistently lower level of arachidonic acid than the controls.

In vitro peroxidation of blood from unsupplemented rabbits by glucose oxidase and glucose (GO–G) is depicted in table 7. Blood drawn at the same time from the same rabbit was incubated with phosphate-saline buffer (P–S). Peroxidation caused a lowering of the arachidonate content in RBC of the deficients but not in those of the controls. Reduced levels of arachidonic acid are also evident in peroxidized RBC of all the PUFA-fed E-deficient rabbits. Peroxidation also caused a decrease in the relative levels of linolenic and docosahexaenoic acids in RBC from E-deficient rabbits fed CLO.

Table 8 illustrates the fatty acid composition of erythrocyte membrane PE over the time course of the study. There were increased levels of the 16 carbon aldehyde in PE of PUFA-fed deficient rabbits, but not in controls or untreated rabbits. Linoleate content increased significantly in the PE of safflower oil-fed rabbits, but not in that of arachidonate-fed rabbits. Arachidonate content rose substantially in the PUFA-supplemented controls, but to a lesser extent in those given safflower oil than in the arachidonate-fed control rabbits. The level of arachidonic acid in PE from safflower oil-supplemented deficients remained constant.

Analysis of the distribution of fatty acids and fatty aldehydes in erythrocyte phosphatidylcholine (PC) indicates an increased level of the 16 carbon aldehyde in the arachidonate-fed deficient animals (table 9). The relatively high initial level of linoleic acid in the arachidonate-fed deficients returned to the same final value as the controls at the time of sacrifice. Linoleate content increased significantly ($P < 0.005$) in the safflower oil-fed controls, but not in the corresponding deficients. The levels of arachidonic acid decreased in erythrocyte PC of the untreated deficient, but increased in RBC PC derived from both types of PUFA-fed deficient rabbits.

TABLE 4

Fatty acid distribution in rabbit erythrocytes

Animal no.	Tocopherol status	Oil	Area percentage of major fatty acids[1] 16	18	18:1	18:2	18:3	20:4
16C	Control	None	28.5	11.2	25.7	20.0		8.7
8D	Deficient	None	27.2	9.9	27.0	24.9		11.8
18C	Control	Arachidonate	22.3	10.4	30.2	19.6		14.8
2D	Deficient	Arachidonate	20.4	8.9	24.6	18.4		21.7
17C	Control	Safflower	23.5	10.4	30.9	28.0		5.0
7D	Deficient	Safflower	19.6	7.9	33.6	30.6		6.7
18C	Control	CLO	33.5	13.6	33.3	11.0	2.3	2.3
8D	Deficient	CLO	28.8	13.1	31.9	19.0	1.2	5.5

[1] To this and subsequent tables, all fatty acids from 14:0 to 22:6 were analyzed, but, for the sake of simplicity, only the major ones are shown.

TABLE 5

Fatty acid distribution in rabbit erythrocytes

Tocopherol status	Oil	No.	Area percentage of selected fatty acids[1] 18:2 i	18:2 f	20:2 i	20:2 f	20:4 i	20:4 f
Control	None	(4)	18.5	22.1	6.3	10.6	6.7	8.0
Deficient	None	(5)	18.0	23.5	4.1	8.6	5.1	5.2
Control	Arachidonate	(3)	18.5	11.2	6.3	14.3	6.7	14.9
Deficient	Arachidonate	(3)	18.0	11.8	4.1	1.2	5.1	13.3
Control	Safflower	(4)	18.5	17.2	6.3	12.1	6.7	7.7
Deficient	Safflower	(4)	18.0	18.2	4.1	7.7	5.1	7.5
Control	CLO	(8)	18.5	9.3	6.3	11.2	6.7	8.4
Deficient	CLO	(9)	18.0	16.4	4.1	2.4	5.1	7.3

[1] Initial values were obtained from blood drawn prior to onset of PUFA feeding. Final values were obtained from blood drawn at sacrifice of animals 3 to 7 weeks later.

DISCUSSION

After 6 months on a lard-based tocopherol-deficient diet, rabbits did not exhibit characteristic symptoms of muscular dystrophy. These rabbits were judged to be deficient on the basis of RBC hemolysis data, a reduction of muscle creatine phosphokinase activity,[5] as well as the low plasma tocopherol levels. It was only after oil supplementation that the rabbits became fatally dystrophic.

Although blood from the vitamin E-deficient rat is susceptible to dialuric acid hemolysis, it has been reported (23) that blood from the vitamin E-deficient rabbit is not. Other species whose blood is largely refractory to dialuric acid hemolysis include the calf, pig, monkey and human. However, we have demonstrated that, subsequent to PUFA feeding, rabbit blood becomes susceptible to hemolysis by dialuric acid. After ethyl arachidonate ingestion, for

TABLE 6

Fatty acid distribution in rabbit plasma

Tocopherol status	Oil	No.	Area percentage of fatty acids[1] 18:2 i	18:2 f	20:2 i	20:2 f	20:4 i	20:4 f
Control	None	1	16.8	13.4	2.4	3.0	2.0	2.0
Deficient	None	1	14.2	10.7	2.7	0.9	1.1	0.4
Control	Arachidonate	3	16.8	11.1	2.7	5.2	2.0	5.8
Deficient	Arachidonate	3	14.2	11.0	1.9	1.5	1.1	7.3
Control [1]	Safflower	4	16.8	17.4	2.2	2.9	2.0	2.4
Deficient	Safflower	2	14.2	18.8	2.9	2.3	1.1	1.4

[1] See footnote 1, table 5.

TABLE 7

Fatty acid distribution in rabbit erythrocytes following peroxidation

Animal no.	Tocopherol status	Oil	Incubation condition	Area percentage of fatty acids						
				16	18	18:1	18:2	18:3	20:4	22:6
16C	Control	None	P-S	28.5	11.2	25.7	20.0		8.7	
			GO-G	32.0	9.8	23.9	20.7		11.8	
8D	Deficient	None	P-S	27.2	9.9	27.0	24.9		10.7	
			GO-G	27.5	14.1	30.9	23.2		4.4	
18C	Control	Arachidonate	P-S	22.3	10.4	30.2	19.6		14.8	
			GO-G	26.7	10.5	22.2	21.8		14.1	
2D	Deficient	Arachidonate	P-S	24.0	8.9	24.6	18.4		21.7	
			GO-G	34.5	8.0	32.2	21.4		3.9	
17C	Control	Safflower	P-S	23.5	10.4	30.9	28.0		5.0	
			GO-G	23.5	9.2	25.1	33.8		8.4	
7D	Deficient	Safflower	P-S	19.6	7.9	33.6	30.6		6.7	
			GO-G	29.6	9.6	23.8	33.8		4.0	
15BC	Control	CLO	P-S	32.4	13.8	30.2	15.2		2.2	
			GO-G	26.2	13.3	28.5	14.5		4.7	
13D	Deficient	CLO	P-S	25.2	13.4	34.8	13.2	2.0	3.8	7.6
			GO-G	26.5	12.0	47.4	11.4	0.4	1.7	0.4

instance, the rabbit RBC becomes rich in that fatty acid, thereby more closely resembling the rat erythrocyte. The change in rabbit blood susceptibility may therefore be a reflection of the altered composition of rabbit erythrocyte phospholipids. This might also be responsible for the increased osmotic fragility under peroxidizing conditions of RBC from PUFA-fed deficient rabbits as demonstrated by their hemolysis profiles.

Our findings confirm Dinning's observation (24) that there is no difference in hematocrit values between deficient and control animals. In addition, we noted the lack of effect of PUFA feeding on hematocrit values, a finding which is in disagreement with that reported by Hayes and coworkers (5) who noted that, in dogs, hematocrits were significantly depressed by PUFA consumption.

That dietary PUFA were incorporated into erythrocyte membranes was clearly shown by analysis of erythrocyte fatty acids. Other indications of PUFA incorporation included the reticulocytosis exhibited by blood of ethyl arachidonate-fed rabbits. The fatty acid composition of the erythrocyte phospholipids also reflected the fatty acid composition of the diets, especially in the case of ethyl arachidonate supplementation, where a high degree of arachidonic acid was detected in both PC and PE. Despite the apparent lack of increased in-

TABLE 8

Fatty acid distribution in rabbit erythrocyte phosphatidylethanolamine

Tocopherol status	Oil	No.	Area percentage of fatty acids[1]											
			16DMA		16		18		18:1		18:2		20:4	
			i	f	i	f	i	f	i	f	i	f	i	f
Control	None	(1)	1.7	1.4	20.7	25.4	8.4	8.1	31.3	27.1	21.1	20.6	10.2	12.2
Deficient	None	(1)	2.3	1.8	27.2	28.0	3.6	8.2	20.5	27.2	15.4	19.5	8.5	9.9
Control	Arachidonate	(3)	1.2	2.5[c]	23.0	22.2	11.6	10.5	35.0	28.2	12.7	13.0	9.7	18.8
Deficient	Arachidonate	(3)	1.1[d]	4.8[b,c]	25.7	19.3	8.0	7.9	26.5	27.0	18.6	14.3	11.7	14.3
Control	Safflower	(4)	5.5	1.4	25.6	20.4	16.5	8.2	34.1	27.0	12.8[c]	28.3[c]	4.5[a]	9.9[a,b]
Deficient	Safflower	(4)	1.7	4.0	24.3	18.7	9.3	6.5	35.3	37.3	13.5	19.1	4.1	5.4[b]

[1] i = initial values, from blood drawn 2 weeks after the onset of PUFA-feeding; f = final values, from blood drawn at sacrifice of the animals 3 to 7 weeks later. Numbers with matched superscripts were significantly different from each other at the level of $P < 0.01$.

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TABLE 9

Fatty acid distribution in rabbit erythrocyte phosphatidylcholine

Tocopherol status	Oil	No.	Area percentage of fatty acids[1]											
			16DMA		16		18		18:1		18:2		20:4	
			i	f	i	f	i	f	i	f	i	f	i	f
Control	None	(1)	3.0	2.4	35.4	34.3	5.0	8.6	29.4	27.5	20.8	20.4	1.0	2.8
Deficient	None	(1)	4.2	2.1	32.8	35.9	7.0	11.0	26.5	27.5	14.6	21.0	5.2	1.7
Control	Arachidonate	(3)	1.0	2.4[b]	35.2	34.0	9.4	10.9	27.3	29.4	11.9	13.3	6.4	6.6
Deficient	Arachidonate	(3)	0.7[c]	5.6[b,c]	28.8	38.2	9.6	7.7	15.3	25.0	35.5	12.8	3.0	9.0
Control	Safflower	(4)	4.0	1.7	30.7	31.1	5.6	9.3	29.2	28.2	16.9[a]	24.9[a]	1.9	2.5
Deficient	Safflower	(4)	2.4	3.3	33.3	30.5	8.2	8.2	28.1	30.4	17.7	21.1	0.7	2.0

[1] See footnote 1, table 8. [a] $P < 0.005$; [b] $P < 0.02$; [c] $P < 0.05$.

corporation of linoleate into the erythrocyte in the rabbits fed safflower oil in the second study, the phospholipid fraction of the RBC membrane had significantly elevated levels of linoleic acid. It is well known that the β position of both PE and PC is highly specific for PUFA. Thus, it may be postulated that the linoleic acid derived from safflower oil is concentrated in the erythrocyte at the β position of the phospholipids.

The displacement of endogenous fatty acids by dietary fatty acids is generally accepted. Analyses by Century and Horwitt (25) of various tissues of the chick demonstrated the substitution of essential fatty acids, especially arachidonic acid, by the fatty acids native to the administered cod-liver oil. We too have noted the incorporation of fatty acids of administered cod-liver oil into the rabbit erythrocyte.

In vitro peroxidation by the glucose oxidase and glucose system caused a decrease in the arachidonate content in the erythrocytes of all vitamin E-deficient animals. Despite Tappel's postulation of in vivo lipid peroxidation in the absence of vitamin E (3), it is apparent from our analyses of fatty acid distribution in the erythrocyte that there was no in vivo destruction of arachidonic acid in erythrocytes of deficient rabbits. In fact, RBC from deficient animals often contained a higher percentage of arachidonic acid than did control RBC.

There are two major methods for incorporation of dietary fatty acids into RBC: 1. synthesis of erythrocytes in bone marrow, and 2. transacylation of RBC phospholipids by plasma fatty acids. Our data show that plasma from vitamin E-deficient rabbits had a lower level of PUFA than did control plasma, suggesting that circulating RBC deficient in PUFA utilized this source to incorporate the necessary fatty acids into PC. Experiments by Van Deenen et al. (26) have demonstrated that lecithin is the only phospholipid in rabbit RBC that undergoes transacylation by plasma fatty acids. We have observed that PUFA content of RBC PC did not differ between control and vitamin E-deficient rabbits. However, the PUFA content of PE of RBC derived from deficient animals was considerably lower than in controls. (Note especially the values for safflower oil-supplemented rabbits.) Thus, it appears likely that the observed difference reflects alterations occurring during RBC synthesis in the bone marrow, as PUFA deficits of the PC fraction can be corrected by exchange with plasma fatty acids whereas those of the PE fraction cannot.

Further support for this hypothesis may be obtained from the work of Winterbourn and Batt (27) who studied the incorporation of plasma fatty acids into human erythrocytes which had been separated on the basis of age. They found that younger RBC incorporated plasma fatty acids into PE much less efficiently than did older RBC. The considerable amount of reticulocytosis exhibited by the rabbits suggests, therefore, that most of the fatty acids incorporated from plasma into phospholipids would be found in the PC fraction. This effect was apparently great enough to overcome the phenomenon described by Jacob and Lux (12) of greater incorporation of fatty acids into erythrocyte PE by vitamin E-deficient rats than by controls.

The fact that both RBC PE and plasma phospholipids derived from blood of ethyl arachidonate-fed vitamin E-deficient rab-

bits have no PUFA deficit is most likely due to the large excess of PUFA available from the diet. Suggestions that observed differences in PUFA content of PE may reflect in vitro decomposition in the absence of antioxidant can be discounted, as initial values showed no difference in PUFA levels between PE of control and vitamin E-deficient rabbits.

The differences seen most likely reflect changes in lipid biosynthesis in the absence of vitamin E. An interesting observation made by Century and Horwitt (25) in the tocopherol-deficient chick was that fatty acids of the linolenic series found in cod-liver oil inhibit the in vivo conversion of linoleic acid to arachidonic acid in erythrocytes and phospholipids of skeletal muscle and brain mitochondria. We have found that erythrocyte PE from deficient rabbits fed safflower oil contains significantly less arachidonic acid than does that from control animals. Perhaps vitamin E is necessary for the biosynthesis of arachidonic acid from linoleic acid via eicosadienoic acid. From the data presented in table 5 it is apparent that, in all groups, the final values for eicosadienoic acid content are higher in RBC of control rabbits than in RBC of the corresponding deficients. This effect might be due to an inhibition of the conversion of linoleate to eicosadienoate in the absence of vitamin E. Assuming that vitamin E does not itself participate in the reaction, some intermediate produced in the absence of the vitamin might interfere with the biosynthesis of eicosadienoic acid such that an alternate pathway of arachidonic acid synthesis might now be favored. The extent of such inhibition presumably would be enhanced by end product inhibition in the case of ethyl arachidonate feeding.

The inhibition may be a reflection of any of three theoretical functions of vitamin E. The first is the antioxidant effect postulated by Tappel (3), in which some product of lipid peroxidation, such as a free radical, might act as the inhibitor. Another is the lipotropic effect ascribed to tocopherol by Hayes et al. (5), in which tocopherol, in a role not necessarily related to antioxidation, would accelerate the removal of abnormal lipids, such as polymerized PUFA. Any of these polymers might be implicated in the regulatory process. The third role is the physicochemical stabilization of erythrocyte membranes by vitamin E. In a structural model proposed by Lucy (28) the methyl groups of the phytyl side-chain of α-tocopherol interact with the *cis* double bonds of esterified arachidonic acid. In the presence of excess PUFA, membranes are more vulnerable to enzymic degradation by phospholipases (28), leading to a higher metabolic "pool" of free fatty acids, whose accumulation might result in the end product inhibition mentioned above. The elucidation of the complex interactions of vitamin E and PUFA and their role in nutrition indeed merits further study.

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Nutritional Effects of the Cyclic Monomers of Methyl Linolenate in the Rat

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ABSTRACT

Low levels (0.0075, 0.0225, and 0.15%) of cyclic fatty acid methyl esters (>98% pure) were incorporated into diets of weanling rats fed different levels of protein. Animals on low protein diets (8-10% casein) exhibited decreased wt gains and feed consumption with increasing levels of cyclic esters in their diets after 6 weeks. Liver enlargements due to a significant ($P<0.01$) accumulation of liver lipid were noted in animals receiving 0.15% cyclic fatty acid esters in their diets.

INTRODUCTION

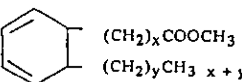
Many studies have been conducted in which a heat-abused fat or fractions from such fats were fed (1-12). Such heated oils produced adverse nutritional and physiological conditions when fed to laboratory animals as parts of normal diets (8-12). Moreover, the non-urea adduct forming fatty acids, a concentrate of the cyclic fatty acids and other polar materials present in such oils, caused a high number of deaths among rats fed this material (11). Repeated studies of the non-urea adduct forming fraction from heated oils have identified disubstituted aromatic and alicyclic fatty acids as major and minor components of these fats (13-16).

Although studies of others (8-12) have shown that cyclic fatty acids in diets cause adverse physiological effects when fed to rats, these diets contained only uncharacterized concentrated fractions fed at relatively high levels rather than the pure cyclic fatty acids themselves. In the present work, the nutritional effects of low levels of highly purified isomeric ω(2-alkyl cyclohexadienyl) carboxylic acids in nutritionally adequate diets containing different levels of protein (casein) were determined.

MATERIALS AND METHODS

Synthesis of Cyclic Fatty Acids

Cyclic fatty acids were synthesized by a modification of the method used by Scholfield and Cowan (17). The crude cyclic fatty acids were converted to the corresponding methyl esters which were further purified by argentation chromatography on silicic acid, with 2% diethyl ether in hexane as eluant. This procedure eliminated all of the dimeric or polymeric material formed and most of the isomers of conjugated linolenic acid as determined by thin layer chromatography. Subsequent gas chromatography after hydrogenation indicated that the isomeric ω(o alkyl cyclohexadiene) carboxylic acid methyl ester mixture made up ca. 90% of the sample; the remainder of the sample was the corresponding aromatic isomer mixture, the general structure of which is:



Experimental Animals and Diets

Male weanling SPF Albino rats (40-50 g) of Sprague Dawley descent, obtained from Murphy Breeding Laboratories (Plainfield, IN), were used for all experiments and housed in galvanized iron wire cages with mesh bottoms. The composition of the diets used (in g/100 g diet) was as follows: salt mixture w (a modification of the Osborne Mendel salt mix containing only inorganic constituents [18]), 3.5; vitamin premix,[3] 1.0; choline chloride, 0.15; corn oil containing either 0, 0.05, 0.15, or 1.0% cyclic fatty acid methyl ester, 15; casein, 8, 10, or 15; dextrose, to 100. All diets were made up in 1 kg batches and were isocaloric containing 4.72 calories/g. Thus, each diet contained 0.0075, 0.0225, and 0.15% cyclic fatty acid methyl ester. The diets were stored under nitrogen in the cold between feedings. Each group of animals was fed the diets for 6 weeks.

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[3] Vitamin mix (mg vitamin/kg diet): retinyl acetate, 20,000 IU; calciferol, 2,000 IU; α tocopherol, 100 IU; ascorbic acid, 22; inositol, 112; menadione bisulfite, 48; para-amino benzoic acid, 22; niacin, 100; Ca Panthothenate, 67; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 100; biotin, 0.45; folic acid, 4; cyanocobalamin, 0.03.

Lipid extractions were accomplished according to the method of Folch et al. (19).

Statistical Treatment of Data

Data mean values are expressed as standard error of the mean (SEM). The F test for significance was employed (20).

RESULTS

Average wt gain, total and average feed consumed, and feed and energy efficiency data for animals in the dietary study are presented in Table I. In diets containing 8 and 10% protein, the average wt gained and feed consumed exhibited a progressively decreasing trend for animals in groups fed with increasing amounts of cyclic fatty acid methyl esters. There is one exception to the trend (group 6) where average wt gains and feed consumption tended to be less than that of a group with a higher content of cyclic esters in the diet. Group 8 gained significantly less wt ($P>0.05$) and consumed less food than did other groups fed the same protein level. Wt gains and feed consumption of animals fed 15% protein diets showed no signficant influence of cyclic monomer level.

The action of the cyclic monomer in the diet can also be observed in the values obtained for energy efficiency. These values measured the efficiency of utilization of food for growth; an increase indicated that the food was not utilized as well for growth. The modified protein efficiency ratios (PER) obtained (Table I) indicated that the lowest ratios were obtained for groups 4 and 8 compared with other groups consuming the same amount of protein. There was not a significant trend for animals fed 15% protein. Values reported for these modified PERs seem to lie on a curve similar to those obtained by Bunce and King (21) in which a maximum PER was obtained between 10-14% protein (casein). Lower PER values were obtained for lower and higher levels of protein.

Liver and Liver Lipid Wts

The percentages of liver:body and lipid:liver wt ratios of rats fed different levels of protein and cyclic fatty acids for different lengths of time are indicated in Table II. Certain experiments took longer to complete than others, and thus the liver:body wt ratios represent average ratios obtained during the period the experiments were conducted. Animals fed the 8% protein diets were fed the diets longer because of their small size upon completion of the 6-week dietary study. Therefore no tests of significance were applied. There were, however, small differences among liver:body wt ratios of animals fed different levels of cyclic acids and protein.

The lipid:liver wt ratios showed a large (probably significant) difference for rats that consumed 0.15% cyclic fatty acids in their diets in groups 1, 2, and 3. The amount of lipid in the liver of animals consuming 0.15% cyclic fatty acids in their diets was slightly higher than the others. The livers removed from rats that consumed 0.15% cyclic fatty acids (groups 1, 2, and 3) were spongy to the touch and white-yellow in color. It was clearly seen that certain lobes exhibited more pronounced fatty infiltration than others.

DISCUSSION

The feed consumption and wt gain data presented in Table I, especially with respect to animals fed 8 and 10% protein, showed trends which were observed by others who reported wt gains among animals fed various heated unfractionated fats (1-3,11,12). Altered organ wts, modified enzyme activities, and an acceleration in the formation of certain types of abnormal tissue have been reported in animals fed heated fats (22). Gottenbos and Thomasson (23) fed rats fish oils which contained low levels of aromatic cyclic fatty acids. Lower wt gains and feed consumption trends were observed in those animals fed increasing levels of cyclized fish oils. In a study comparing body wt changes in toxicity tests, Frazer (24) showed that wt gain and food intake tended to parallel each other. Animals fed small amounts of known toxic compounds also displayed differences in behavior and scattered significantly more food. Animals in the present dietary study also exhibited differences in behavior, and those fed 8% protein were clearly more tense and excitable than rats fed higher levels of protein. Animals fed different levels of protein and increasing amounts of cyclic fatty acids in their diets did not scatter any more food than the control animals. Lower wt gains achieved by these animals were not due to palatability, since preliminary studies in our laboratory indicated that diets containing cyclic compounds seemed to be preferred to diets without them.

A number of investigators have reported wts of and lipid content of livers from experimental animals fed heated fats (1,2,4-6,25,26). The results of liver wts reported varied from highly significant wt differences (5) to no wt changes for rats on heated and fresh oil diets (2,25,26). The protein content in the experimental diets ranged from 18% (5,25) to 30% (4), while others (1,2) provided diets in which the exact

TABLE I

Wt Gained and Feed Consumed by Rats Fed Different Levels of Cyclic Fatty Acids and Protein for 6 Weeks

Group	Protein fed (% by wt)	Number of animals	Cyclic fatty acids in diet (%)	Average wt gain[a] (g)	Average feed consumed (g)	Energy[b] efficiency	Wt gain[c]/wt protein consumed
1	8	8	0.0000	78.6 ± 4.5	448.9 ± 15.4	25.5 ± 1.9	2.06 ± 0.11
2	8	8	0.0075	79.0 ± 4.0	433.6 ± 19.2	25.2 ± 0.9	2.23 ± 0.07
3	8	8	0.0225	70.1 ± 3.2	415.5 ± 13.6	26.5 ± 0.8	2.06 ± 0.08
4	8	8	0.1500	62.0 ± 6.0	382.2 ± 21.6	27.2 ± 0.6	1.96 ± 0.13
5	10	10	0.0000	123.9 ± 10.4	488.0 ± 30.5	19.0 ± 0.7	2.49 ± 0.08
6	10	10	0.0075	102.7 ± 5.8	437.8 ± 11.4	20.0 ± 1.1	2.34 ± 0.11
7	10	10	0.0225	110.9 ± 5.5	451.6 ± 27.9	19.3 ± 0.4	2.44 ± 0.06
8	10	10	0.1500	89.0 ± 7.1[d]	400.9 ± 19.3	21.8 ± 1.3	2.21 ± 0.11
9	15	9	0.0000	213.4 ± 5.2	616.5 ± 8.4	13.5 ± 0.4	2.30 ± 0.06
10	15	10	0.0075	218.1 ± 5.5	640.4 ± 9.0	13.8 ± 0.3	2.26 ± 0.04
11	15	10	0.0225	219.6 ± 7.0	621.7 ± 14.8	13.4 ± 0.3	2.35 ± 0.05
12	15	10	0.1500	212.5 ± 6.6	602.5 ± 10.9	14.9 ± 0.8	2.34 ± 0.07

[a]Mean values ± SEM.

[b] $= \frac{\text{Caloric intake}}{\text{wt gain}}$

[c]A modified protein efficiency ratio.

[d]Significant at $P<0.05$ from groups fed the same protein level.

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TABLE II

Effect of Protein and Cyclic Fatty Acid Level on Liver Size and Liver Lipid Content in the Rat

Cyclic fatty acid in diet (%)	Liver wt (g)	Lipid wt (g)	Lipid wt / Liver wt (%)	Liver wt / Body wt (%)
Group 1: 8% protein[a]				
0.0000	6.49 ± 0.40	0.40 ± 0.14	6.09 ± 0.39	2.91 ± 0.22
0.0075	6.21 ± 0.28	0.39 ± 0.15	6.35 ± 0.83	2.87 ± 0.23
0.0225	6.42 ± 0.16	0.38 ± 0.04	5.86 ± 0.83	3.10 ± 0.04
0.1500	6.31 ± 0.35	0.61 ± 0.22	10.30 ± 1.08	3.18 ± 0.07
Group 2: 10% protein[b]				
0.0000	6.54 ± 0.55	0.38 ± 0.05	6.14 ± 0.48	3.02 ± 0.09
0.0075	5.65 ± 0.27	0.32 ± 0.02	6.30 ± 0.58	2.98 ± 0.65
0.0225	5.98 ± 0.39	0.43 ± 0.07	7.70 ± 1.17	2.97 ± 0.09
0.1500	5.60 ± 0.21	0.51 ± 0.50	10.40 ± 0.15	3.29 ± 0.11
Group 3: 15% protein[c]				
0.0000	8.36 ± 0.48	0.47 ± 0.06	5.52 ± 0.44	3.65 ± 0.16
0.0075	9.45 ± 0.36	0.51 ± 0.05	5.36 ± 0.55	3.74 ± 0.56
0.0225	8.94 ± 0.98	0.49 ± 0.05	5.61 ± 0.32	3.67 ± 0.14
0.1500	10.50 ± 0.78	0.89 ± 0.02	8.46 ± 0.36	4.08 ± 0.15
Group 4: 15% protein[d]				
0.0000	11.60 ± 0.54	0.53 ± 0.03	4.51 ± 0.29	3.32 ± 0.07
0.0075	12.14 ± 1.02	0.57 ± 0.02	4.80 ± 0.46	3.50 ± 0.19
0.0225	12.29 ± 0.43	0.62 ± 0.05	5.06 ± 0.35	3.54 ± 0.15
0.1500	13.00 ± 1.04	0.68 ± 0.09	5.20 ± 0.30	3.44 ± 0.12

[a]Mean ± SEM (8 rats fed diets 9-11 weeks).
[b]Mean ± SEM (10 rats fed diets 6-8 weeks).
[c]Mean ± SEM (4 rats fed diets 5-6 weeks).
[d]Mean ± SEM (5 rats fed diets 7-11 weeks).

protein content was not reported.

The practice of measuring lipid content and composition of livers of rats fed heated oil diets has been repeatedly employed; however, none of the values reported in the literature have been as high as 8-10% of the liver wts as obtained in this study. While not determined in the present study, previous work has indicated that the major lipid accumulated in the liver of rats fed heated fat diets was triglyceride (27,28).

ACKNOWLEDGMENT

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て，式 (6) より $\Gamma_l \gg \Gamma_m$ となり，ほとんどラウリン酸のみが吸着されるはずである。

(b) 一方，表面相における化学平衡が成立していないときには，グリセリンの吸着は無視できるとして，ラウリン酸とモノラウリンの両方が吸着されるであろう。

Fig.-5 において同じ表面圧で一分子の占める面積を比較すると，曲線Ⅰ（A液）はⅡ（ラウリン酸）およびⅢ（1-モノラウリン）よりかなり小さい。表面相における化学平衡が成立しているときには，表面相はほとんどラウリン酸のみであり，曲線Ⅰは曲線Ⅱとほとんど一致するはずであるが，実際には一致していない。したがって曲線Ⅰはラウリン酸とモノラウリンの混合膜であり，水溶液中と同様に表面相におけるモノラウリンの加水分解速度は遅く，化学平衡より吸着平衡が先に成立していることが結論される。

なお一般に混合膜においては，一分子当たりの平均占有面積は占有面積の加成性から期待される値より大きくはならないということが知られている[10]が，この実験でも曲線Ⅰが曲線ⅡとⅢからの混合膜とすれば，混合によって占有面積が減少していることも上述の結論を支持している。

（昭和51年11月19日受理）

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ノート

Studies on the Toxicity of the Autoxidized Oils. VI.

Comparative Toxicity of Secondary Oxidation Products in Autoxidized Methyl Linoleate

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In previous papers[1,2], one of the authors reported that the most toxic compounds in autoxidized methyl linoleate were hydroperoxyalkenals containing 5 to 9 carbon atoms. The 50% lethal dose (LD_{50}) of methyl linoleate hydroperoxyalkenals was determined, however, that of other low molecular weight secondary oxidation products (LMWOP) contained in autoxidized fats were not.

This paper deals with the determination of the LD_{50} of LMWOP. Methyl linoleate was autoxidized at 60°C by bubbling pure oxygen into the ester. LMWOP was separated by molecular distillation and further fractionated by dry column chromatography. Each of the fractions was tested for toxicity. The results indicated that the toxicity pattern according to functional groups was as follows: hydroperoxy-alkenals > hydroxyaldehydes > alkenals > hydroperoxides > alkanals.

The toxic nature of hydroperoxides formed during autoxidation of unsaturated fatty acids has been reported by Kaneda *et al.*[1-3] Many workers have studied the toxicity of autoxidized fats and hydroperoxides have been generally presumed to be major toxic components of autoxidized fats. However, carbonyl compounds, the secondary decomposition products of hydroperoxides, have also to be toxic[4-10].

In previous papers, Yoshioka and Kaneda[1] reported that the longer the autoxidation of unsaturated fatty acids, the more toxic they become. The toxicity of autoxidized fatty acids was likely to be more dependant on the amount of secondary oxidation products than of hydroperoxides. They found the most toxic compounds in autoxidized methyl linoleate were 4-hydroperoxy-2-enals containing 5 to 9 carbon atoms. They determined the 50% lethal dose (LD_{50}) of the hydroperoxyalkenals (HPA) and methyl linoleate hydroperoxides (MLHP) by both oral administration and intraperitoneal injection. HPA was found to be 87 times as toxic as MLHP in the case of oral administration. There was no discrepancies in LD_{50} between oral administration and intraperitoneal injection for HPA, whereas MLHP was found less toxic in oral administration than intraperitoneal injection. They also noticed that some other low molecular weight secondary oxidation products (LMWOP), such as hydroxyaldehydes, hydroxyketones, alkanals and alkenals, separated from autoxidized methyl linoleate showed different toxicity on mice, however, the LD_{50} was not determined.

In the present study, an attempt has been made to determine the LD_{50} of some LMWOP in autoxidized methyl linoleate.

Experimental

Autoxidation of methyl linoleate. Methyl linoleate (ML) of 97% purity was prepared from safflower oil mixed fatty acids by the method of urea adduction[11] and distillation under reduced pressure. ML was autoxidized at 60±2°C by passing filtered oxygen through it. Peroxide value (POV) and carbonyl value (COV) were determined at various time intervals. As the autoxidation time increased, the POV reached a maximum (2550 meq/kg) in about 35 h and then declined, while the COV began to rise rapidly at the same time. When the POV had declined

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to 1300 meq/kg, the oxidation reaction was stopped.

Separation of LMWOP from autoxidized ML. Four kg of autoxidized ML was subjected to molecular distillation at 56°C and 5.0×10^{-3} mmHg. The low molecular weight distillate was collected in a receiver surrounded by dry ice and acetone coolant. The yield of this fraction was about 42 g. Fractionation of the LMWOP by dry column chromatography[13),15)] was carried out at 5°C. About 460 g of silica gel, obtained from Woelm, Eschwege, Germany, 12.5% deactivated with water were packed into a nylon column 230 cm long and 3 cm in diameter and developed with solvent. The solvent system was petroleum ether (bp 30~40°C)-diethyl ether (4 : 1 vol/vol). When the solvent front reached the bottom of the column, the development was stopped and the column was cut into 23 parts (10 cm intervals) and each portion was eluted with ethyl ether. The solvent was evaporated and each portion was analyzed by IR and TLC. The TLC plates were developed in a solvent system consisting of petroleum ether 90, ethyl ether 10 and acetic acid 1.

Properties of the 23 fractions obtained by dry column chromatography are shown in Table-1. Most of the fractions contained carbonyl groups as evidenced by their reaction to 2,4-dinitrophenylhydrazine reagent and IR spectra. Fractions 11 to 13,20 and 21 were positive to the hydroperoxide test. As a sample of alkanal, fraction 3 was extracted with ethyl ether and converted to the 2,4-dinitrophenylhydrazone derivatives (2,4-DNPH). The melting point of 2,4-DNPH was 102°C.

Anal. : Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.76; N, 19.99

Found : C, 52.20; H, 5.78; N, 20.01

The IR spectrum of the compound in fraction 8 indicated a trans double bond and absorption at 990 cm^{-1}. This compound was used as a sample of alkenal. The melting point of the 2,4-DNPH of this compound was 142°C.

Anal. : Calcd. for $C_{12}H_{14}O_4N_4$: C, 51.80; H, 5.07; N, 20.13

Found : C, 51.20; H, 4.95, N, 19.32

From these data, elemental analysis and the results of mass spectrometry, compounds in fractions 3 and 8 were characterized as n-hexanal and 2-*trans*-hexenal derivatives, respectively.

Fractions 14 and 15 exhibited a remarkable resemblance in the IR spectra which suggested that both fractions had the same compound or homologues. These two fractions were analyzed by GC-MS, the mass spectrum of the main compound separated by GLC is shown in Fig.-1. This spectrum was assigned as 2-hydroxyhexanal. The base peak corresponds to m/e 60 which shows α-branching; the characteristic ion series 43,57……and 45,59,73,87……are due to $C_nH^+_{2n+1}$ and $C_nH_{2n}CO^-$ ions, respectively.

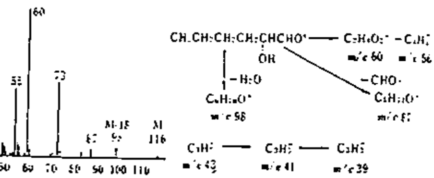

Fig.-1 Mass spectrum of 2-hydroxyhexanal.

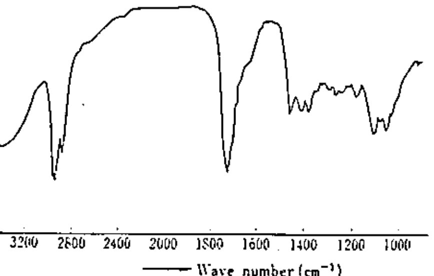

Fig.-2 IR spectrum of fraction 18.

The presence of hydroxyaldehydes was suggested in fraction 18 from the results of IR spectrum (Fig.-2) and TLC analysis. However, due to limited amounts of material obtained from this fraction, not conclusive result on the final structure was possible. Therefore, LD_{50} of this fraction was not determined.

Toxicity test of secondary oxidation products on mice

DD strain male mice with an average body weight of 20 g (5 to 6 weeks of age) were supplied by the Mouse Center, School of Medicine, Tohoku University. ML of 97% purity, POV nil, was used to dissolve each compound. Aliquots of LMWOP were administered orally to mice using a stomach tube following the Behrens method[19] as shown in Table-2. Three groups of 7 mice each were used. The mortality is shown as the total mice killed within 48 h.

Commercial n-hexanal and 2-*trans*-hexenal were used instead of the extracts from autoxi-

Table-1 Fractionation and properties of secondary oxidation products.

Dry column No.	Functional groups by IR	Staining reaction on TLC	
		KI and starch	2,4,D.N.P.[a]
1~2	Ester		
3	Sat. ald.[b]		+++
4~7	Sat. ald., Unsat. ald.[c]		+++
8~10	Unsat. ald.		+++
11	Unsat. ald., Hydroperoxy unsat. ald.	++	+++
12	Hydroperoxy unsat. ald.	++	+++
13	Hydroperoxy unsat. ald., Hydroxy ketone?	+	+
14~15	Hydroxy unsat. ald.		+++
16~17	Hydroxy unsat. ald.		+
18~19	Hydroxy unsat. ald.		++
20~21	Hydroxy, Hydroperoxy acid	+	+
22~23	—		

a 2,4-Dinitrophenylhydrazine + Weak
b Saturated aldeyhde ++ Medium
c Unsaturated aldehyde +++ Strong

Table-2 LD_{50} of secondary oxidation products in autoxidized methyl linoleate. Dose : 0.1 ml/20 g mouse

Progression rate	n	d	h	% Mortality	
n-Hexanal		Initial solution : 2.6 g n-hexanal/ml ML[a]			
1.3^{-4}	0/7	0	12	0.00	
1.3^{-3}	2/7	0	7	41.67	
1.3^{-2}	3/7	2	3	41.67	LD_{50}=82.79 mmol/kg mice
1.3^{-1}	4/7	5	0	75.00	
1.3^{0}	7/7	9	0	100.00	
2-*trans*-Hexenal		Initial solution : 0.5 g 2-*trans*-hexenal/ml ML			
1.6^{-4}	0/7	0	12	0.00	
1.6^{-4}	1/7	0	6	7.70	
1.6^{-3}	3/7	1	2	40.00	LD_{50}= 6.98 mmol/kg mice
1.6^{-2}	5/7	4	0	81.80	
1.6^{-1}.	7/7	9	0	100.00	
2-Hydroxyhexanal		Initial solution : 0.36 g 2-hydroxyhexanal/ml ML			
1.7^{-4}	0/7	0	10	0.00	
1.7^{-3}	2/7	0	5	16.60	
1.7^{-2}	4/7	2	2	54.50	LD_{50}= 5.15 mmol/kg mice
1.7^{-1}	5/7	6	0	84.60	
1.7^{0}	7/7	11	0	100.00	

n Observed mortality % Mortality $=\frac{n-d}{t+h-d}$
d Mortality in lower doses t Number of mice in each group
h Survival in higher doses a Methyl linoleate

Table-3 Comparative LD_{50} of oxidation products formed in autoxidized methyl linoleate.

Compound	LD_{50} mmol/kg mice		LD_{50} mg/kg mice
n-Hexanal[a]	82.79		8292
2-*trans*-Hexenal[a]	6.98		685.0
2-Hydoxyhexanal	5.15		598.2
4-Hydroperoxy-2-alkenals[b]	0.45	as pentenal	52.5
		as nonenal	77.5
Methyl linoleate hydroperoxide[b]	39.10		12760

a Supplied by Tokyo Kasei Industries, further purified by distillation under reduced pressure
b These values were quoted from Yoshioka and Kaneda[1)]

dized ML. These compounds of 95~98% purity were obtained after distillation under reduced pressure. 2-Hydroxyhexanal was prepared fresh from autoxidized ML using the same method. 2-Hydroxyhexanal showed one spot on TLC plate.

Results and Discusson

Results of the toxicity test are shown in Table-2 and-3 with LD_{50} of HPA which reported in a previous paper[2)]. It can be seen in Table-3, LMWOP except *n*-hexanal were much more toxic than MLHP. However, these volatile secondary oxidation products showed less toxicity than HPA. Slow-moving and the difficulty in breathing was noticed in each dying mouse.

Many workers have studied the toxicity of autoxidized fats, but none has reported the LD_{50} of secondary oxidation products in autoxidized fats. These data may contribute to a better understanding of the toxicity of autoxidized fats.

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Autocatalytic Activity in the Low-Polarity Part of Autoxidizing Methyl Linolenate

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A catalytic activity for autoxidation was observed in the methyl linolenate fraction which had been recovered from an autoxidizing methyl linolenate preparation by column chromatography using silicic acid and hexane. It was suggested that there is some catalytically-active material which is liable to contaminate the methyl linolenate recovered, but is unstable when in contact with silicic acid, and is readily lost on reduction with triphenylphosphine.

The autoxidation of higher unsaturated fatty acids which contain three or more double bonds is rapid[1)] and, therefore, attracts the attention of the food and biological chemists. In the present investigation, catalytic products responsible for the rapid oxidation of linolenate were found in the low-polarity part of autoxidizing methyl linolenate.

Linolenic acid was prepared by fractional freezing of 5% acetone solution of the fatty acid mixture of linseed oil at −78°C.[2)] The mother liquor at −78°C contained 80% linolenic acid and 20% linoleic acid, and was converted to methyl esters. The methyl ester sample was purified by passing through silicic acid and charcoal columns as hexane solution, and used in the following experiments. No polar impurities were detected on TLC.

The rate of oxygen uptake was measured with a Warburg's manometer by 2~2.5 h shaking at 30°C as described previously,[3)] using 1 ml lipid samples. For experiments in the presence of copper, copper (II) stearate (0.1ml 10^{-3} M solution in benzene) was added. The rate of peroxide accumulation by prolonged shaking at 27°C was determined by the iodometric titration for peroxide as used in the previous paper.[3)]

Methyl linolenate was shaken at 27°C in the dark place until the peroxide content rose to 58 µeq/ml. A portion (70 ml) was diluted with 150 ml hexane and was applied to a silicic acid column (100 ml bed volume) under a nitrogen atmosphere. The methyl linolenate fraction was eluted with hexane.

The recovered methyl linolenate autoxidized rapidly, although it gave no peroxidic spots on TLC and was not distinguishable from the unoxidized starting material in gas liquid partition chromatographic analysis, IR and UV spectra. The IR absorption near 980 cm^{-1} was not changed at all as shown in Fig.-1, indicating no appreciable increase in *trans*-double bond content in the recovered sample. There was a weak UV absorption peak at 212 nm in the recovered sample, suggesting the presence of conjugated diene impurities, but since its intensity (OD/M=1500) was similar to that of the unoxidized material (OD/M=1800) the impurities can not be responsible for the higher oxidation rate of the recovered sample.

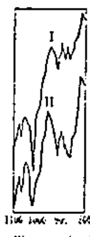

Fig.-1 Infrared spectra of recovered (I) and unoxidized (II) methyl linolenate.

The rate of oxygen uptake in the presence of copper was 0.078 µmol/min (0 µmol/min for the control methyl linolenate). In the absence of copper the oxygen uptake was too low to be observed within 2.5 h measurement. The peroxide accumulation by prolonged shaking was 41 (1 d) and 240 (2 d) µeq/ml, whereas the control linolenate gave 11 (1 d) and 20 (2 d) µeq/ml. This finding suggested that catalysts of low polarity were formed in the linolenate autoxidation and a part of them behaved in the chromatography in a similar manner to the unoxidized linolenate. Catalysts of such a low polarity have not been found in the autoxidation

Lipid Oxidation Products and Chick Nutritional Encephalopathy

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ABSTRACT

Safflower oil and its distilled methyl esters were thermally oxidized and fed to young chicks in a vitamin E deficient diet. At a dietary level of 10%, the oxidized lipids caused more severe nutritional encephalopathy (NE) than the unoxidized methyl esters, indicating that factors other than dietary linoleic acid and vitamin E affect the development of NE. A polar lipid extract from oxidized methyl esters accelerated the induction of NE, as did the synthetic methyl esters of keto-octadecenoic and keto-octadecadienoic acids. Dicumarol exerted a protective action against NE. The possibility is discussed that conjugated keto-polyenoic fatty acids, provided by oxidized oils or formed endogenously in vitamin E deficiency, may play a role in causing NE.

INTRODUCTION

Nutritional encephalopathy (NE), better known as encephalomalacia, is induced in young chicks by diets deficient in α-tocopherol and containing polyunsaturated fatty acids (1). The disease is characterized by degenerative changes mainly in the cerebellum, accompanied by ataxia, prostration, and death. Ultrastructural changes in the cerebella of chicks affected with NE have been described by various authors (for a brief review, see ref. 2). The dietary lipid causing NE is linoleic acid or its esters, whereas derivatives of linolenic acid are inactive (3-5).

Autoxidized polyunsaturated oils have occasionally been used to induce NE in chicks (4,5), and we found thermally oxidized safflower oil to be very effective for that purpose (2,6,7). The α-tocopherol level of such oil is greatly reduced while the linoleic acid content is still high. Since the treatment of the oil results in the accumulation of oxidation products, the question arises whether or not some of these products may play an active role in causing NE. The evidence presented in this report points in this direction.

MATERIALS AND METHODS

Animals and Feeds

Day-old crossbred New Hampshire X White Leghorn male chicks were housed in thermostatically heated battery brooders with raised wire floors and had free access to water and feed.

The compositions of the two vitamin E deficient diets are presented in Table I. The diets contained 4 or 10% lipids, and the composition was adjusted so as to ensure a constant ratio of metabolizable energy to protein. The linoleic acid in these diets was provided by distilled safflower methyl esters or by thermally oxidized safflower oil or methyl esters, prepared as described below. The diets contained 0.005%, 2,6-di-*tert*.-butyl-4-methylphenol (BHT), a level of antioxidant which ensure the stability of the dietary linoleic acid and α-tocopherol for over a week at room temperature. During the experiments, the diets were kept at -18 C and dispensed daily.

[1]Mention of firm names or trade products does not imply endorsement or recommendation by the Department of Agriculture over other firms or similar products not mentioned.

Dietary Lipids

Refined edible safflower oil was purchased from Shemen Ltd., Haifa and from Teth-Beth Ltd., Petah-Tikva. Different batches contained from 71 to 76% linoleic acid and ca. 350 μg α-tocopherol/g. Methyl esters were prepared from the oil by a modification of the transmethylation procedure of Hartman (8), followed by vacuum distillation.

TABLE I

Composition of Diets

Ingredient	Percent	
Lipid[a]	4.00	10.00
Extracted soybean meal	52.00	56.00
Cellulose	1.00	3.00
D,L-methionine	0.14	0.15
Mineral mix[b]	4.00	4.00
Vitamin mix[c]	0.50	0.50
BHT[d]	0.005	0.005
Glucose monohydrate	38.36	26.35

[a]Safflower oil or safflower methyl esters.

[b]Supplying per kg feed: dicalcium phosphate 25 g; limestone 7 g; NaCl 3.5 g; $MnSO_4 \cdot H_2O$ 370 mg; $ZnCO_3$ 145 mg; ferric citrate 165 mg; $CuSO_4 \cdot 5H_2O$ 11.8 mg; KI 2.35 mg; and $CoCl_2 \cdot 6H_2O$ 1.21 mg.

[c]Supplying per kg feed: vitamin A 3000 U; vitamin D_3 200 U; menadione sodium bisulfite 1 mg; thiamine 3.6 mg; riboflavin 7.2 mg; Ca pantothenate 20 mg; niacin 55 mg; pyridoxine 6 mg; biotin 0.2 mg; folic acid 2.4 mg; vitamin B_{12} 0.02 mg-choline chloride 1.3 g. These amounts were premixed with 3.6 g glucose monohydrate.

[d]2,6-di-*tert*. butyl-4-methylphenol.

TABLE II

Effect of Thermally Oxidized Safflower Oil (SO-24)[a] and Freshly Distilled Safflower Methyl Esters (ME-0) on Nutritional Encephalopathy[b]

Dietary lipids	Analysis of lipids		Incidence per 20 chicks	
	α-Tocopherol	Linoleic acid	Ataxia	Mortality
	μg/g	%		
			At 3 weeks	
SO-24 4%	10-20	60-65	7	5
ME-SO 4%	5-12	70-76	15	9
			At 2 weeks	
SO-24 10%			12	11
ME-0 10%			8	1

[a]Safflower oil aerated at 145 C for 24 hr.

[b]The lipids were fed from hatching at the levels indicated.

Thermal oxidation of safflower oil and distilled methyl esters was done by heating batches of 1-2 kg to 145 C ± 2 C under a stream of air (0.5 1/min). The length of the thermal treatment was 24 hr for the oil and 3 hr for the esters. The following abbreviations were used: SO-24 for the oxidized oil; ME-3 for the oxidized methyl esters; and ME-0 for the fresh methyl esters.

A crude extract of polar lipids was prepared from thermally oxidized methyl esters by repeated partition between hexane and 90% (v/v) ethanol, using six separatory funnels arranged in countercurrent fashion. The final ethanolic extract was concentrated under reduced pressure and extracted with ethyl ether. The yield of polar lipids averaged 6%.

Methyl esters of conjugated keto-octadecenoic acid and keto-octadecadienoic acid were prepared from methyl oleate and linoleate, respectively, as described elsewhere (9). The oleate-derived product contained 96% conjugated keto esters consisting of an isomeric mixture of methyl 8-, 9-, 10-, and 11-oxo-octadecenoate. The linoleate-derived product contained 91% keto-dienes consisting mainly of methyl 13-oxo-9,11- and 9-oxo-10,12-octadecadienoate.

For testing in chicks, lipid fractions or synthetic products were dissolved in safflower methyl esters in the amounts indicated.

Quantitative Expression of NE

Chicks were inspected twice daily and the times at which the first signs of ataxia were observed and when death occurred, were recorded. Inspection of the cerebella always confirmed that the affected chicks were stricken with NE. Results were expressed as number of chicks affected per total number of chicks per treatment at the age indicated. Alternatively, curves representing the cumulative incidence of ataxia or death have been plotted.

Analytical Determinations

α-Tocopherol in the dietary lipids was determined by saponification, fractionation of the unsaponifiables by thin layer chromatography on Silica Gel G with hexane/ethyl ether (8:2), and colorimetric reaction of the α-tocopherol fraction with ferric chloride and bathophenanthroline (10).

The fatty acid composition of the lipids was determined after transmethylation of the samples with 3% (w/v) H_2SO_4 in methanol at reflux temperature for 1 hr and extraction of the methyl esters with hexane. The esters were submitted to isothermal gas liquid chromatography at 180 C on Gas Chrom W coated with 15% DEGS. All materials were obtained from Packard Ltd., Jerusalem. Methyl esters prepared for feeding experiments were injected directly into the chromatograph. Glyceryl triheptadecanoate and methyl heptadecanoate were added as internal standards to the oil and methyl ester samples, respectively, for calculation of the true linoleic acid content of the oxidized samples.

RESULTS

In a first trial, two encephalopathogenic diets were compared: thermally oxidized safflower oil, SO-24, and freshly distilled

TABLE III

Effect of Fresh and Thermally Oxidized Safflower Methyl Esters on Nutritional Encephalopathy

Dietary lipids[b]	Analysis of lipids		Vitamin E[a] added to feed	Incidence per 20 chicks at 19 day	
	α-Tocopherol	Linoleic acid		Ataxia	Mortality
	μg/g	%	μg/g		
ME-0	5.0	75.9	0	14	6
			1	13	4
ME-3	0.35	68.5	0	18	14
			1	19	14

[a]D,L-α-Tocopheryl acetate.

[b]ME-0, freshly distilled safflower methyl esters; ME-3, methyl esters aerated at 145 C for 3 hr. The lipids were fed as 10% of the diet from the 8th day, after the chicks received 4% ME-0 during the first week.

safflower methyl esters, ME-0. The oxidized oil had less linoleic acid but no less α-tocopherol than the fresh esters (Table II). When these lipids were fed as 4% of the diet, ME-0 caused a greater incidence of ataxia and mortality than did SO-24. However, at the 10% level, the oxidized oil was more active than the fresh methyl esters.

A similar comparison was made between fresh and oxidized methyl esters, ME-0 and ME-3. Table III shows that ME-3, with less linoleic acid, nevertheless produced a more severe incidence of NE, compared to ME-0. This greater activity of ME-3 was not due to the lower α-tocopherol content of the oxidized vs. the fresh esters, since the difference in tocopherol content between the two diets was no more than 0.5 μg tocopherol/g diet, whereas even the addition of 1 μg DL-α-tocopheryl acetate/g diet had virtually no effect on NE (Table III).

Extracts of polar lipids were prepared from ME-3 by repeated partition between hexane and 90% ethanol. The polar lipids were added to encephalopathogenic diets and their influence on NE was studied. The results of one such experiment, illustrated in Figure 1, show that the polar lipids increased the incidence of NE.

Two synthetic fatty acid oxidation products were tested in the same chick model: a keto monoene prepared from methyl oleate and a keto diene obtained from methyl linoleate. Both compounds accelerated the induction of NE (Figs. 2 and 3).

Fibrin clots have previously been observed in cerebellar capillaries of chicks affected with NE (2). The present chick model was used to study the effect of dicumarol on the incidence of NE. Table IV shows that the anticoagulant exerted a protective effect which increased in direct relation to its concentration in the diet. In this experiment, the diet contained 4% ME-0, but similar results were obtained with diets containing 10% oxidized safflower oil.

DISCUSSION

Several authors (4,5) have reported that the incidence of NE in vitamin E deficient chicks is directly related to the amount of linoleic acid consumed by the chicks. This is seen to be the case in the first experiment in which SO-24 and ME-0 were fed at the 4% level (Tab. II). However, the reversal of activities of the two lipids at the 10% level does not agree with this concept. One possible explanation for the apparently contradictory results is that SO-24 contains oxidation products which are encephalopathogenic and which, at the higher dietary level, are absorbed in sufficient amounts to overcome the opposite reaction expected from the lower linoleic acid content of SO-24 vs. ME-0.

The above explanation receives support from the observation that the oxidized esters, in spite of their lower linoleic acid content, caused a more severe incidence of NE than the fresh esters (Table III), while more direct evidence for the involvement of lipid oxidation products is provided by results obtained with polar lipids extracted from oxidized methyl esters (Fig. 1).

The effects of the two synthetic ketoenoic fatty acid esters (Figs. 2 and 3) are of interest for the following reasons: 12-oxo-9-*cis*-octadecenoic acid was previously reported to increase the severity of NE when given orally with stripped corn oil (11), but not after injection (12). Subsequently, this compound was shown to possess strong prooxidant activity in vitro (13) and eventually its activity on NE was ascribed, according to a communication from the same laboratory (14), to destruction of residual tocopherol in the diet during the tests. The same keto oleate has recently been reinvestigated by Fukuzawa and Sato (15) who reported that it had definite anti-vitamin E activity in the rat and that it specifically combined with bovine serum albumin to form a strongly fluorescent compound (16). These authors postulated that the keto oleate undergoes isomerization to the conjugated 12-oxo-10-*trans*-ene and that the conjugated enone then condenses with albumin amino groups to form fluorescent Schiff base compounds (16). That keto oleate is absorbed by rats when administered by stomach tube was mentioned in a recent review by Perkins (17).

Keto oleate is of little nutritional or pathological significance, because it is unlikely to be found in autoxidized polyunsaturated oils, nor would it be expected to form in vivo. On the other hand, allylic ketoenes have been found in autoxidized methyl oleate (18), and conjugated keto-dienes have been found in autoxidized methyl linoleate (19). Thus, the results of this study with synthetic conjugated ketoenes indicate that this type of compound could be one of the active species contributing to the NE-accelerating effect of oxidized oils rich in linoleic acid.

Moreover, conjugated keto-dienes could be expected to form in vivo, even in the absence of any exogenous supply. In vitamin E deficiency, for instance, polyunsaturated membrane lipids are believed to undergo peroxidation (20,21), and the hydroperoxides formed could yield conjugated keto compounds by several enzymic and nonenzymic reactions, such as those discussed by Gardner (22) for linoleic acid hydroperoxide. In fact, an increase in monocarbonyls has been reported in adipose tissue of vitamin E deficient rats (23). Lipid hydroperoxides are also readily reduced to the corresponding allylic hydroxy compounds by the ubiquitous glutathione peroxidase (EC 1.11.1.9) (24,25); this reaction raises the intriguing question of the possible biological activity on NE of these compounds, or their desaturation to active keto-dienes.

The mechanism by which the synthetic ketoenes enhance the severity of NE is not known. One possibility is suggested by the reaction of 12-oxo-9-octadecenoic acid with albumin, referred to above (16). A similar reaction of conjugated enones or dienones with amino lipids or proteins of cell membranes must result in impaired membrane function, a result usually ascribed to peroxidation of membrane lipids in vitamin E deficiency. The additional possibility that such a condensation reaction might lead to fluorescent products deserves to be investigated, although the fluorophore formed in this case would differ from the 1-amino-3-imino group derived from

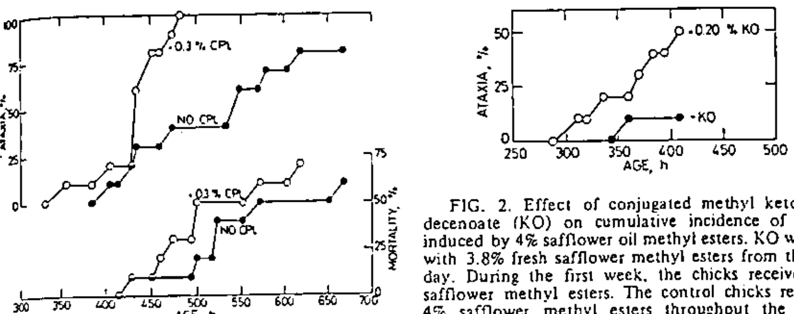

FIG. 1. Effect of crude polar lipids (CPL) on cumulative incidence of encephalopathy induced by 4% safflower methyl esters. The polar lipids were obtained from thermally oxidized safflower methyl esters and were fed with 3.7% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% fresh safflower methyl esters. The control chicks received 4% fresh esters throughout the entire period. There were 20 chicks per treatment.

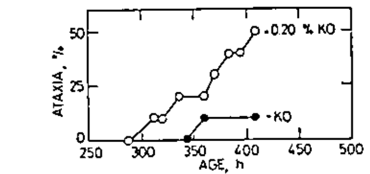

FIG. 2. Effect of conjugated methyl keto-octadecenoate (KO) on cumulative incidence of ataxia induced by 4% safflower oil methyl esters. KO was fed with 3.8% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% safflower methyl esters. The control chicks received 4% safflower methyl esters throughout the entire period. There were 10 chicks per treatment.

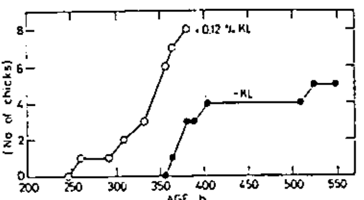

FIG. 3. Effect of conjugated methyl keto-octadecadienoate (KL) on cumulative incidence of ataxia induced by 4% safflower methyl esters. The safflower methyl esters were fed from the first day and KL was added from the 8th day. There were eight chicks per treatment.

TABLE IV

Effect of Dietary Dicumarol on Nutritional Encephalopathy[a]

Dicumarol concentration	Incidence per 20 chicks at 3 weeks	
	Ataxia	Mortality
μg/g		
0	12	8
200	8	7
400	1	1

[a]Chicks received 4% freshly distilled safflower methyl esters from hatching.

malondialdehyde and postulated to form in vitamin E deficiency (26).

The results on the protective effect of dicumarol (Table IV) indicate that the blood coagulation system plays a role in the etiology of NE, in agreement with the histological observation on the presence of fibrin clots in the cerebral vessels (2). Whether or not the process is triggered by thrombocyte aggregation remains to be clarified, but the inability of linolenic acid to induce NE (3-5) would indicate that the cyclo-oxygenase system is involved. For instance, linolenic acid and especially its long chain metabolites are strong inhibitors of prostaglandin formation from arachidonic acid (27), and all *cis*-5,8,11,14,17-eicosapentaenoic acid has recently been reported to be a precursor of a powerful anti-aggregating substance (28). Also, among brain tissues of the rat and guinea pig, the cerebellum has the greatest capacity for PGE_2 formation (29).

The possibility that conjugated keto-enoic fatty acids play a role in the etiology of NE and other syndromes of vitamin E deficiency deserves further study. From a nutritional point of view, attention should be given to the formation and concentrations of conjugated keto-dienes in artificially and commercially heated polyunsaturated oils.

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Cardiopathogenicity of Soybean Oil and Tower Rapeseed Oil Triglycerides when Fed to Male Rats[1]

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ABSTRACT

The triglycerides of soybean oil were purified by molecular distillation and those of Tower rapeseed oil by molecular distillation and adsorption chromatography. The original oils and the purified triglycerides were incorporated in semisynthetic diets at 20% by weight and fed for 16 weeks to weanling male Sprague-Dawley rats to compare the nutritional and pathological effects of the oils and their triglyceride fractions on rats. The study was carried out at two independent laboratories. No significant differences were observed between the results of the two establishments. The incidence of myocardial lesions was significantly higher in rats fed Tower rapeseed oil than in those fed soybean oil. Purification of the triglycerides by molecular distillation and adsorption chromatography appeared to have no major effect on the incidence of myocardial lesions. This supports our previous findings that the cardiopathogenicity of the test oils to rats resides in the triglycerides of these oils.

INTRODUCTION

It has been well documented that male Sprague-Dawley rats fed for at least 16 weeks low erucic acid rapeseed (LEAR) oils develop a higher incidence of myocardial lesions than those fed other vegetable oils (1,2). The increased incidence of lesions in this strain of rat has been attributed to cardiotoxic contaminants (3,4), residual erucic (Δ^{13}-cis-docosenoic) acid (22:1) (5), or a fatty acid imbalance (2,6-9) of LEAR oils. We considered the possibility of cardiotoxic contaminants in LEAR oils and, therefore, fractionated Span rapeseed oil (RSO) containing 4.8% 22:1 by molecular distillation and adsorption chromatography to prepare pure triglycerides and fractions enriched in nontriglyceride components (10). The results of feeding these fractions to rats suggested that the cardiopathogenic properties of Span RSO were associated with the triglycerides of the oil, and not with nontriglyceride contaminants present in the fully refined oil (8).

We decided to repeat the fractionation to further examine the hypothesis that LEAR oil triglycerides are responsible for the increased incidence of cardiac lesions in male Sprague-Dawley rats. With the availability of a LEAR oil which contained only 0.2% 22:1, a fractionation was possible to practically eliminate complicating effects, if any, of 22:1 on myocardial lesions. Soybean oil was similarly fractionated by molecular distillation to evaluate possible effects of the molecular distillation procedure itself. To increase the purity of the triglycerides of the LEAR oil, the purest triglyceride fraction from molecular distillation was further purified by column chromatography, instead of using the two methods independently as performed previously (10). Finally, a protocol was set up to conduct the feeding trial in two independent laboratories (Agriculture Canada, Ottawa and Department of Veterinary Pathology, University of Saskatchewan) with an exchange of histological sections of myocardium before decoding and evaluation of the data.

The results of the fractionation of soybean oil and Tower RSO, the feeding of the oils and their fractions to male Sprague-Dawley rats for 16 weeks, and the cardiopathological findings are presented in this communication.

MATERIALS AND METHODS

Fully refined soybean oil and *Brassica napus* cv. Tower (Tower RSO) were obtained from Canada Packers Ltd., Toronto, Ontario and Cooperative Vegetable Oil Ltd., Altona, Manitoba, respectively.

Molecular Distillation

The distillation was performed by Distillation Products Industries, Rochester, NY, using a CMS-36 molecular still. Prior to distillation, the molecular still was flushed with 50 kg of soybean oil and then by 40 kg of oil to be distilled. A total of 509 kg of soybean oil and 950 kg of Tower RSO were distilled in a similar

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報 文

Absorption of Methyl Linoleate Hydroperoxides in Rabbit

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The absorption of methyl linoleate hydroperoxides (MLHPs) in rabbit was investigated. After oral administration of 700 mg of MLHPs, lymph was collected continuously from the thoracic duct for 2.5 h. The lipids in the collected lymph were extracted and analyzed by high performance liquid chromatography.

The data showed that 0.23% of the dosed MLHPs were contained in the lymph as intact MLHPs. This result indicates that some unchanged hydroperoxides are absorbed into intestinal wall and transported to some organs through lymph.

1 Introduction

In 1953, Kaneda, one of the authors, reported the toxic nature of hydroperoxides formed during autoxidation of unsaturated fatty acids. Kaneda and some other workers reported that the hydroperoxides were found in some tissues after the administration of lipid hydroperoxides, so they concluded that the hydroperoxides might be absorbed from the intestinal wall and transported into tissues[1)~4)]. But exact experiments have not yet been performed to prove this hypothesis. Andrews[5)] noticed that lipid hydroperoxides aministered were not found in the lymph, and consequently, they are decomposed at the intestinal wall. Bergan[6),7)] repoted that hydroxy fatty acids were found in the lymph after administration of methyl linoleate hydroperoxides, but proof was not given on the absorption of intact methyl linoleate hydroperoxides. Nishida and Kummerow[8)] concluded that some peroxide was absorbed from the intestinal wall by the spectral evidence of the conjugated diene in the lymph of rats dosed methyl linoleate hydroperoxide. However, it is difficult to decide whether the diene conjugation is originated from methyl linoleate hydroperoxide or methyl hydroxyoctadecadienoate.

Thus, there are several views concerning the absorption of lipid hydroperoxides, since the absorption mechanism is not clear. If intact MLHPs were assumed to be absorbed from the intestinal wall, they might cause damege to organic tissues or become the initiator of oxidation in the body.

An attempt has been made in the present study to clarify the absorption of MLHPs by high performance liquid chromatography (HPLC).

2 Experimental

2·1 Preparation of MLHPs

Methyl linoleate of 99% purity was prepared from safflower oil by the method of urea adduction and distillation under reduced pressure[9)]. The methyl linoleate was autoxidized at 0°C by passing air through it. When the POV reached 2,000, the autoxidized methyl linoleate was fractionated by dry column chromatography[10)]. The MLHPs thus obtained had a POV of 6,115, 99.8% of theoretical for the pure substance.

2·2 Collection of Lymph

Lymph was collected from a male Japanese White Rabbit, 2,500 g, obtained from the Sendai-Wako Co.. To facilitate the operation, the rabbit was fasted for 24 h. Ligation of the thoracic duct was carried out through an abdominal incision according to the procedure of Bollman[11)], after anesthesia with Nembutal. The animal was maintained in a restraining cage similar to those described by Bollman. Six hours after regaining consciousness, the rabbit was treated via intubation with 700 mg of MLHPs suspended in 50 ml of physiological saline solution containing 2% sodium cholate.

The lymph was collected in tube which was maintained in wet ice and exchanged for a new one every 30 min. The collection of lymph was carried out for 2.5 h. During the collection of lymph, 0.5% NaCl aqueous solution was administered at 10 ml/h to accelerate the secretion of lymph.

2·3 Analytical Method

Lymphatic fluid divided into five fractions was extracted with 20 vol of $CHCl_3/CH_3OH$ (2 : 1). Lipids thus extracted were examined by HPLC. Operating conditions are given as under.

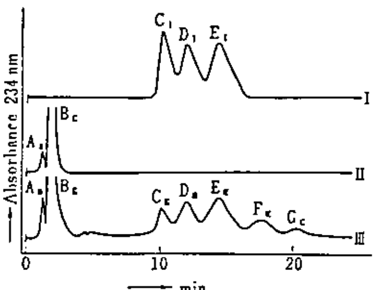
Instrument	Dupont LC-1
Column	Zorbax SIL 2.1 φ mm×250 mm
Ref. Column	Zorbax SIL 2.1 φ mm×250 mm
Column temp.	25.0°C
Mobile phase	Hexane/2-propanol (249 : 1)
Column pressure	20.0 kg/cm²
Flow rate	1.71 ml/min
Detector	UV 234 nm
Sensitivity	0.64 AUFS

The peaks observed from the high performance liquid chromatogram were further investigated by scanning techniqes.

3 Results and Discussion

A total volume of 32.1 ml of lymph was collected. The amount of each collection was, initial : 5.6 ml, second : 5.3 ml, third : 5.6 ml, fourth : 7.2 ml, final : 8.4 ml. Fig.-1 shows the chromatograms by HPLC of MLHPs (I) and extracts from the lymph of rabbits (II, III). The MLHPs administered to the rabbit consist of three isomers, methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate; methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate; and methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate, which were determined from the data obtained by mass spectrometry and infrared analysis[12)].

Line III shows the chromatogram by HPLC of extracts from the lymph of the rabbit administered MLHPs, and line II shows the chromatogram by HPLC of extracts from that of the control rabbit. In line III, peaks A_{III} and B_{III} shows the same retention time as peaks A_{II} and B_{II} in line II. Therefore, the substances presented as peaks A_{III} and B_{III} are endogenous lipids, which consist of mainly triglyceriedes and their related materials. In line III, peaks C_{III},



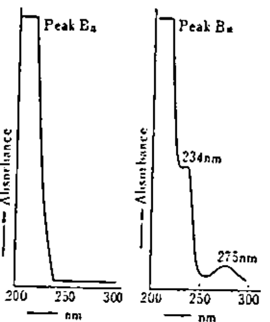
Peak A and Peak B: Triglycerides and their related substance; Peak C: Methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate; Peak D: Methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate; Peak E: Methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate; Peak F and G: Derivatives of MLHPs which are not identified yet.

Fig.-1 Chromatograms by high performance liquid chromatography of original MLHPs (I), extracts from control lymph (II) and extracts from lymph of rabbit dosed MLHPs (III).

D_{III} and E_{III} show the same retention time as those of peaks C_I, D_I and E_I, respectively, in line I. The compounds presented as peaks C_{III}, D_{III} and E_{III} were treated with sodium borohydride and converted to TMS derivatives and analyzed by mass spectrometry. The mass spectra showed ions of high intensity at *m/e* 382 (M), 311 (M-71; loss of $\cdot[CH_2]_4CH_3$) and 225 (M-157; loss of $\cdot[CH_2]_7COOCH_3$), indicating methyl-13-OTMS-9, 11-octadecadienoate and methyl-9-OTMS-10, 12-octadecadienoate. These data indicate that peaks C_{III}, D_{III} and E_{III} represent MLHPs components. From these results it can be interpreted that some of the MLHPs could be absorbed directly from the intestinal wall.

Fig.-2 shows the scanning chart of peaks B_{II} and B_{III} displayed in Fig.-1. There is a λ_{max} at 234 nm in the scanning chart of peak B_{III}, but this peak was not obserbed in peak B_{II}. This fact reveals that the administration of MLHPs broght about the incorporation of MLHPs into the material displayed as peak B. Peak B was identified as triglycerides, so some of the MLHPs which were absorbed from the

* Lymph of the third collection. (60~90 min)



Peak B_{II} : Triglycerides in the control lymph. Peak B_{III} : Triglycerides in the lymph of rabbit dosed MLHPs.

Fig.-2 Scanning chart of Peak B_{II} and Peak B_{III}.

intestinal wall were incorporated into triglycerides. There is also a λ_{max} at 275 nm in the scanning chart of peak B_{III}. This suggests that some of the MLHPs absorbed from the intestinal wall became dienon derivatives and were incorporated into triglycerides. Peaks F_{III} and G_{III} were assumed to be polar derivatives of MLHPs which are not identified yet. Nevertheless, since it was proven that they also have a λ_{max} at 234 nm, these drivatives might have a conjugated double bond structure. Fig.-3 shows the time-courses of absorption patterns of intact MLHPs obtained from the lymph. The lines (II～VI) display the chromatograms by HPLC of extracts from lymph of a rabbit dosed MLHPs. The detction range of each is 0.64 AUFS.

Initial collection (0～30 min)…Line II

When the detection range of 0.64 was employed, it was difficult to discern the peaks of intact MLHPs. But with the sensitivity of the detector increased to 0.04 AUFS, the peaks of the intact MLHPs were easily recognized. From this result it was clear that the intact MLHPs could be absorbed from the intestinal wall within the first 30 minutes after intubation.

Second collection (30～60 min)…Line III

In the case of using the 0.64 AUFS detection range, there was no appearance of the peaks of intact MLHPs. But, when the sensitivity was increased as written above, peaks of the intact MLHPs were recognized also.

Line II…Initial collection (0～30 min),
Line III…Second collection (30～60 min),
Line VI…Third collection (60～90 min),
Line V…Fourth collection (90～120 min),
Line IV…Final collection(120～150 min).
Sensitivity of all, except II' and III', is 0.64 AUFS. Line II' and III' are the midway line of Line II and III, and their sensitivities are heightened to 0.04 AUFS.

Fig.-3 Chromatograms by high performance liquid chromatog raphy of gorinal MLHPs (I) and extracts from lymph of rabbit dosed MLHPs (II-VI).

Third and Fourth collections (60～120 min)…Line IV and V

Peaks of the MLHPs appeared clearly. The proportion of these peaks was, however, different from the original MLHPs. That is to say, compared with the pattern of th original MLHPs, the peak representing methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate was considerably smaller.

Final collection (120～150 min)…Line VI

The pattern of the intact MLHPs is almost the same as the original MLHPs.

Table-1 shows the weight of intact MLHPs in the extracts of lymph of the rabbit dosed MLHPs and the ratio of the intact MLHPs to the dose. The values in **Table-1** were calculated by comparison of HPLC peak areas of control MLHPs*, which weight to peak area

Table-1 Distribution of intact MLHPs in each collecting fraction extracted from the lymph of rabbit dosed MLHPs.

Collecting fraction / Intact MLHPs	Initial (0～30 min)	Second (30～60 min)	Third (60～90 min)	Fourth (90～120 min)	Final (120～150 min)	Total (0～150 min)
Weight (g)	2.48×10^{-5}	2.96×10^{-5}	3.51×10^{-4}	5.65×10^{-4}	6.15×10^{-4}	1.59×10^{-3}
Ratio to the dose* (%)	0.0035	0.0042	0.501	0.0807	0.0879	0.226

* 700 mg of MLHPs

ratio was previously checked, with that of intact MLHPs* in each collection fraction. In the first and second lymph, intact MLHPs were hardly found, but in the third lymph, intact MLHPs was absorbed considerably.

The quantity of intact MLHPs which was absorbed from the intestinal wall appeared to increase rapidly 60 min after the administration of MLHPs. Up to 150 min after the intubation, ca. 0.23% of the dose was absorbed from the intestinal wall as unchanged MLHPs.

From the results obtained, the authors concluded that some unchanged lipid hydroperoxides might be absorbed into intestinal wall and be transported to several organs through lymph; and these MLHPs can damage biomembranes, react with proteins and small metabolites, and initiate oxidation in the body.

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* Each of the three isomers has almost the same λ_{max} at 234 nm. Therefore their peaks' area might approximately represent the ratio of their existence.

うさぎにおけるリノール酸メチルヒドロペルオキシドの吸収について

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うさぎを用い，リノール酸メチルヒドロペルオキシド(MLHPs) の小腸における吸収を検討した。700 mg の MLHPs 投与後，胸管から，30 min ごとのフラクションに分け 2.5h にわたりリンパ液を採取した。集めたリンパ液から脂質を抽出し，高速液体クロマトグラフィーで分析した。

その結果，投与した MLHPs のうち 0.23% が未変化のままリンパ液中に存在することがわかった。このことより，脂質過酸化物のうちのいくらかは未変化のまま小腸壁より吸収されリンパ液に移り，体内の各組織に運ばれることが明らかになった。

5 おわりに

以上，α,β-エポキシシランの化学的性質について合成化学的立場から紹介した[35]。α,β-エポキシシランの反応にほとんど立体及び位置特異的に進行するので，今後より高選択的な分子設計に用いられると思われる。特に α,β-エポキシシランの遷移金属化合物による特異的β開裂は見いだされて間なく，興味ある展開がなされるものと確信する。さらに α,β-エポキシシランの化学的性質が解明されるにつれて工業的応用面も広がってくるものと予測され，新しい素材として脚光を浴びる日が来るのも遠いことではないと思われる。

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報　文

Absorption and Metabolism of Methyl Linoleate Hydroperoxides in Rats

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The intestinal absorption of methyl linoleate hydroperoxides (MLHPs) in rats was studied. After oral administration of MLHPs, lymph was collected continuously from the thoracic ducts for 36 h. The lipids in the collected lymph were extracted and analyzed by high performance liquid chromatography (HPLC).

The resulting data suggest that part of the MLHPs administered was absorbed directly from the intestinal wall and in part converted to some derivatives such as methyl hydroxyoctadecadienoates (MHODs) and methyl oxooctadecadienoates (MOODs) which were also absorbed from the intestinal wall.

1 Introduction

It is known that unsaturated lipids contained in foods are easily autoxidized and form peroxides. Although many workers have studied the nutritive problem of lipid peroxides, little is known about their metabolic fate.

It has been extensively discussed whether fatty acid peroxides are absorbed from the small intestine or not. Andrews *et al.*[1] were unable to find the lipid peroxides in lymph and concluded that they are decomposed on the intestinal wall. Other workers[2)~4)] have also obtained the same result; however, still others[5)~7)] noticed that appreciable amount of peroxides were found in the tissues after the administration of lipid peroxides. Nishida and Kummerow[8)] concluded that some peroxides were absorbed from the intestinal wall, noting the spectral evidence of the conjugated diene in the lymph of rats dosed with methyl linoleate hydroperoxide.

Recently we confirmed that some unchanged MLHPs were certainly absorbed from the intestinal wall in rabbit[9)].

In the present study absorption and metabolism of MLHPs in rats were further studied through the use of reversed phase HPLC.

2 Experimental procedures

2·1 Preparation of MLHPs

MLHPs were prepared according to the conventional method described below. Methyl linoleate of 99% purity was prepared from safflower oil by urea adduction and distillation under reduced pressure[10)]. The methyl linoleate was autoxidized at 5°C by passing dry air through it. When the POV increased beyond 2,000 meq/kg, the autoxidized methyl linoleate was fractionated by dry column chromatography[11)]. The MLHPs thus obtained had a POV of 6,130 meq/kg (99.9% of theoretical value for the pure substance).

2·2 Preparation of MHODs and MOODs

MHODs were prepared by reduction of MLHPs with sodium borohydride[12)]. MOODs were prepared by oxidation of MHODs with chromium trioxide[13)].

2·3 Collection of lymph

Male Wistar rats were fed commercial diets for 30 days until the morning prior to the lymph collection. Rats weighing 347 to 355 g were divided into two groupes of two each. Nembutal (sodium pentobarbital, 40 mg/kg) was injected intracelially prior to surgery. Cannulation of the thoracic duct with a polyethylene tubing (0.86 mm ID, 1.52 mm OD) was carried out through an abdominal incision according to the procedure of Bollman[14)]. After the incision, rats were maintained in a Bollman cage. Diets

and 0.85% physiological saline solution were given ad libitum.

Some anesthetics are known to influence the permeability of lipid membranes. It is quite likely that several hours following the injection of Nembutal some of the anesthetic could have still been remaining in lipid tissues, so the collection of lymph was carried out the next morning. To facilitate the intubation, the diet was removed 6 h prior to giving MLHPs. MLHPs (15.0 mg) suspended in 1.5 ml of physiological saline solution containing 0.2% sodium cholate were given to each rat using a stomach tube. Methyl linoleate (ML) was fed to the control group. Soon after the oral administration of sample esters, lymph collection was initiated. The lymph was collected into a tube which was purged with N_2, surrounded with wet ice, and exchanged for a new tube every 12 h. The collection of lymph was continued for 36 h.

2·4 Analytical method

Lymphatic fluid divided into three fractions (0~12 h, 12~24 h and 24~36 h) was extracted with 20 vol of chloroform/methanol (2 : 1). After removal of nonpolar lipids by use of TLC, the lipids were analyzed by reversed phase HPLC.

The operating conditions were as follows :

Column	LiChrosorb RP-18 (mean particle size : 5 μm) 4.0 φ mm × 250 mm
Precolumn	LiChrosorb RP-18 4.0 φ mm × 50 mm
Mobile phase	Acetonitrile / Water / Tetrahydrofuran (5 : 4 : 1)
Column pressure	200 kg/cm²
Flow rate	0.27 ml/min
Detector	UVIDEC-100-Ⅲ (JAPAN SPECTROSCOPIC Co., LTD)
UV	234.5 nm, 275 nm
Sensitivity	0.02 AUFS

3 Result

In the current study, thoracic lymph flow rates of 1.5 to 2.2 ml/h were obtained in four of the runs (Table-1).

3·1 Absorption of intact MLHPs and MHODs

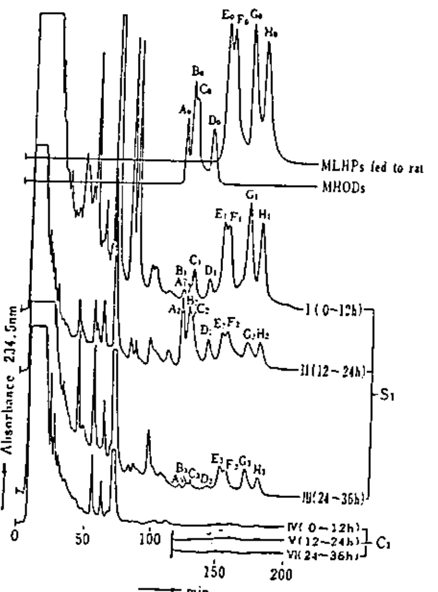
Fig.-1 shows the chromatograms of reversed phase HPLC of lymph lipids and other sample esters. UV 234.5 nm was adopted for the analytical wave length. Chromatograms of MLHPs used as sample ester and reduced products of MLHPs, namely MHODs, are shown on the upper part of Fig.-1.

Table-1 Amount of lymph obtained from rats administered MLHPs or ML. (ml)

Group and No. of rats \ Fraction	1 (0~12 h)	2 (12~24 h)	3 (24~36 h)	Total (0~36 h)	Per hour
S_1	19.3	24.5	25.3	69.1	1.9
S_2	16.3	19.1	19.6	55.0	1.5
C_1	25.0	27.3	26.7	79.0	2.2
C_2	21.5	20.9	23.4	65.8	1.8

S : Subject administered 15 mg of MLHPs.
C : Subject administered 15 mg of ML.

The MLHPs administered to the rats consist



A_{0-3} to D_{0-3} : isomer of methyl hydroxyoctadecadienoate.
E_{0-3} to H_{0-3} : isomer of MLHP.

Fig.-1 High performance liquid chromatogram of extracts from the lymph of rat (S_1) dosed MLHPs (Ⅰ~Ⅲ), extracts from the lymph of rat (C_1) dosed ML (Ⅳ~Ⅵ), MLHPs, and methyl hydroxyoctadecadienoates used as sample oils.

of four isomers, methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate (E_0); methyl 9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate (F_0); methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate (G_0); and methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate (H_0), which were determined from the data obtained by the combination of UV, IR and mass spectrometries[13),14)]. That is to say, after isolation of the four isomers by use of HPLC, UV and IR analyses were carried out.

The molar absorbances and absorption maxima (in ethanol) of the components were : E_0, λ_{max} 236 nm, ε=26,000; F_0, λ_{max} 236 nm, ε=25,900; G_0, λ_{max} 233 nm, ε=28,600; and H_0, λ_{max} 233 nm, ε=28,600. And the IR spectra of the components E_0 and F_0 contained two absorption bands at 949 and 986 cm^{-1} (*cis-trans*), and those of components G_0 and H_0 had a single band 989 cm^{-1} (*trans-trans*).

Peaks A_0 to D_0 show methyl 13-hydroxy-*cis*-9, *trans*-11-octadecadienoate, methyl 9-hydroxy-*trans*-10, *cis*-12-octadecadienoate, methyl 13-hydroxy-*trans*-9, *trans*-11-octadecadienoate, and methyl 9-hydroxy-*trans*-10, *trans*-12-octadecadienoate, respectively, which were also derived from the data obtained by IR, UV and mass spectrometry analysis[13),14)]. These were confirmed by reduction of the individual compounds of hydroperoxides which showed that E_0 gives rise to A_0, F_0 to B_0, G_0 to C_0, and H_0 to D_0.

Lines Ⅰ to Ⅲ show the chromatograms of extracts prepared from the fractionated lymph of rat (S_1) fed MLHPs and lines Ⅳ to Ⅵ show those fed ML (The first part of lines Ⅴ and Ⅵ was omitted.). The chromatograms of HPLC obtained from both rats fed MLHPs were almost the same, so chromatograms from S_1 rat are represented in Fig.-1.

In lines Ⅰ to Ⅲ, peaks E_1 to H_1, E_2 to H_2, and E_3 to H_3 show the same retention time as those of peaks E_0 to H_0, respectively. Consequently, the compounds presented as peaks E_{1-3}, F_{1-3}, G_{1-3} and H_{1-3} were assumed to be MLHPs. In addition, these compounds were collected separately and analyzed for their UV spectra. The spectra of compounds E_{1-3} and F_{1-3} in ethanol were characterized by a maximum at 236 nm, whereas the spectra of compounds G_{1-3} and H_{1-3} gave a maximum at 233 nm. These also suggest that compounds E_{1-3} and F_{1-3} are *cis-trans* isomers of MLHP, and compounds G_{1-3} and H_{1-3} are *trans-trans* isomers of MLHP[16)]. The IR spectrum of the mixture of these compounds showed just the same pattern as that of authentic MLHPs. Mass spectrum analysis was inapplicable to these compounds because of their extreme microquantities.

In lines Ⅳ to Ⅵ, no observable peak exists which shows the same retention time as those of MLHPs. This result indicates that some of the MLHPs administered to the rats were absorbed directly from the intestinal wall and transported to the lymph.

In lines Ⅰ to Ⅲ, peaks A_{1-3} to D_{1-3} show the same retention time as those of peaks A_0 to D_0, respectively. Therefore, the compounds presented as peaks A_{1-3} to D_{1-3} were assumed to be MHODs. In lines Ⅳ to Ⅵ, no observable peak exists which shows a retention time the same as those of MHODs.

By the way, MHODs were found in the digestive tracts of rats dosed MLHPs, while no MHODs were found in those of control rats dosed ML.

The results described above indicate that some of the MLHPs were reduced in the digestive tracts and absorbed from the intestinal wall.

3·2 Absorption of MOODs

Fig.-2 shows the chromatograms of MOODs prepared authentically[12)] and lymph lipids (Lines Ⅰ′ to Ⅵ′). For this experiment, UV 275 nm was used, because the specific absorption of conjugated dienon (R_1-C=C-C=C-C(=O)-R_2) in ethanol was presented at 272 to 278 nm.

Lines Ⅰ′ to Ⅵ′ show the chromatograms of the compounds displayed as lines Ⅰ to Ⅵ in Fig.-1 (The first part of Lines Ⅴ′ and Ⅵ′ was omitted.). In the line of MOODs, peaks I_0 to K_0 show methyl 13-oxo-*cis*-9, *trans*-11-octadecadienoate; a mixture of 9-oxo-*trans*-10, *cis*-12-octadecadienoate and methyl 13-oxo-*trans*-9, *trans*-11-octadecadienoate; and methyl 9-oxo-*trans*-10, *trans*-12-octadecadienoate, respectively. This was confirmed by oxidation of individual compounds of MHODs which showed that A_0 gives rise to I_0, B_0 and C_0 to J_0, and D_0 to K_0.

In lines Ⅳ′ to Ⅵ′, peaks I_1 to K_1, I_2 to K_2,

Fig.-2 High performance liquid chromatogram of extracts from the lymph of rat (S_1) dosed MLHPs (I′~III′), extracts from the lymph of rat (C_1) dosed ML (IV′~VI′), and methyl oxooctadecadienoates.

and I_1 to K_1 show the same retention times as those of peaks I_0 to K_0, respectively. Accordingly, the compounds presented as peaks I_{1-3}, J_{1-3} and K_{1-3} were regarded as MOODs. Furthermore, these compounds were separated for UV spectrum examination. The spectra of compounds I_1 to I_3 in ethanol were characterized by a maximum at 278 nm, J_1 to J_3 at 278 nm and 272 nm, and K_1 to K_3 at 273 nm, respectively. These results also suggest that compounds I_{1-3} to K_{1-3} were isomers of MOOD. Mass spectra of these compounds were not achievable because of their extreme microquantities.

In lines IV′ to VI′, no observable peaks exist which show the same retention times as those of peaks I_{1-3} to K_{1-3}.

By the way, MOODs were found in the digestive tracts of rats dosed MLHPs, while no MOODs were found in those of control rats dosed ML.

The results described above indicate that some of the MLHPs administered were converted to MOODs in the digestive tracts and absorbed from the intestinal wall.

Similar results were obtained on the intestinal absorption of MLHPs, MHODs and MOODs in the case of the other rats (S_2, C_2).

Fig.-3 Distribution of intact MLHPs in each collecting fraction obtained from the lymph of rats (S_1, S_2) dosed 15 mg of MLHPs.

3·3 The quantity of intact MLHPs absorbed from the intestinal wall

Fig.-3 shows the amount of MLHPs which were absorbed from the intestinal wall and transported to lymph. The data in Fig.-3 was calculated by comparison of high performance liquid chromatogram peak areas of control MLHPs, which weight to peak area ratios were previously checked, with that of intact MLHPs in each fraction. Each of the four isomers of MLHPs has a respective λ_{max} at nearly 234.5 nm; therefore, the ratio of their peaks' areas might approximately represent that of their existence.

4 Discussion

During the experiments it was noticed that the amount of lymph was dependent upon the intake of saline. The smaller amount of lymph in each of the first fractions was presumed to be the result of stress by the oral administration of sample esters.

In the first fraction (0~12 h) a fairly large amount of MLHPs appeared (Fig.-1), and in the next 12 h the amount of MLHPs decreased gradually, but some MLHPs were found even after 24 h. These results indicate that after 24 h of intubation, a respectable amount of MLHPs still remained in the intestinal lumen.

The proportion of each peak was different from the original MLHPs dosed to rats, and varied with time (Fig.-4). In the first fraction, the proportion of peaks of *trans-trans* isomer was relatively large. On the contrary, in the second fraction, the content of *cis-trans* isomers was more than that of *trans-trans* isomers (Fig.-5). Similar tendency was found in the case of MHODs or MOODs (Fig.-6, 7). This observation implies that there is some difference in the absorption mechanism between *trans-trans* and *cis-trans* isomers.

Up to 36 h after the intubation, ca, 0.5 to 0.6% of the dose was absorbed from the intestinal wall as unchanged MLHPs (Fig.-3).

In our experiment, a trace amount of MLHPs (15 mg) was given to rats, compared with the lethal dose (over 700 mg)[1], but we recognized that a part of the lipid peroxides could be absorbed from intestinal wall and transported into lymph.

In the first fraction, considerable amounts of MOODs were observed (Fig.-2). In the next fraction the quantity of MOODs decreased extremely. But it is not clear if this result was attributable to the lowering of MOODs in the intestinal lumen.

This time, it was well confirmed that unchanged MLHPs are absorbed, to some extent, from the intestinal wall not only in rabbit[9] but in rats. What is more noteworthy, compared with the preceding study, the animals were administered very small amount* of MLHPs in the present study. The MLHPs were nevertheless found in the lymph of the rats, lately. This observation suggests lipid peroxides exist in daily meal in low concentration are likely to absorbed from intestinal wall. Furthermore,

Fig.-4 Percent of four isomers of MLHP in the lymph collected from the rat (S_1) dosed MLHPs.

Fig.-5 Ratio of (*cis*, *trans* MLHPs)/(*trans*, *trans* MLHPs) found in each collecting fraction of lymph of rats (S_1, S_2) dosed MLHPs (×).

Fig.-6 Ratio of (*cis*, *trans* MHODs)/(*trans*, *trans* MHODs) found in each collecting fraction of lymph of rats (S_1, S_2) dosed MLHPs.

Fig.-7 Ratio of (*cis*-9, *trans*-11 MOOD)/(*trans*-10, *trans*-12 MOOD) found in each collecting fraction of rats (S_1, S_2) dosed MLHPs.

* Less than one fiftieth as much as lethal dose.