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 $\Gamma_1 \gg \Gamma_m$ 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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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.*[3)-7)] 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[3)-10)].

In previous papers, Yoshioka and Kaneda[3)] 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 hydroxy=aldehydes, 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[12),13)] 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 $C_{13}H_{18}O_4N_4$: 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 $C_{15}H_{22}O_4N_4$: 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-hydroxy=

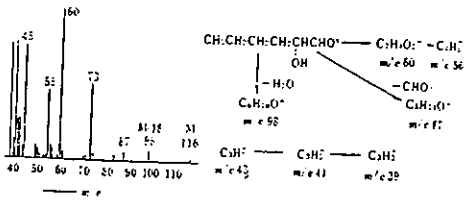

Fig.-1 Mass spectrum of 2-hydroxyhexanal.

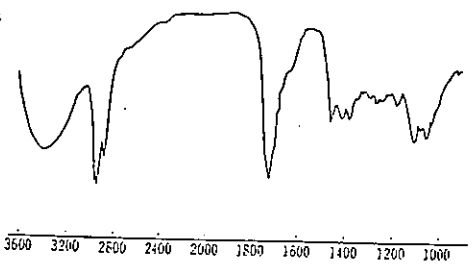

Fig.-2 IR spectrum of fraction 18.

hexanal. The base peak corresponds to *m/e* 60 which shows *c*-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.

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[14)] 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
b Saturated aldeyhde
c Unsaturated aldehyde
+ Weak
++ Medium
+++ 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^{-5}	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
d Mortality in lower doses
h Survival in higher doses
% Mortality $=\frac{n-d}{t+h-d}$
t Number of mice in each group
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.

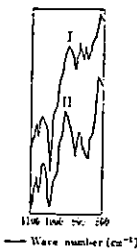

Fig.-1 Infrared spectra of recovered (I) and unoxidized (II) methyl linolenate.

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 ensured 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.

Dietary Lipids

Refined edible safflower oil was purchased

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.

[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.

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: α-Tocopherol	Analysis of lipids: Linoleic acid	Incidence per 20 chicks: Ataxia	Incidence per 20 chicks: 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.

from Shemen Ltd., Haifa and from Teh-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.

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 l/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-3, 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 (Table 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

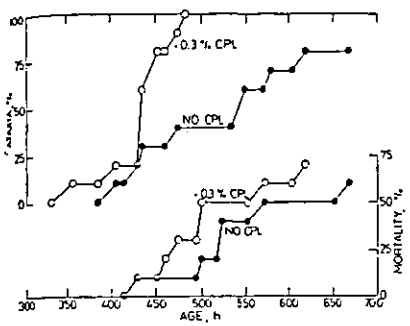

FIG. 1. Effect of crude polar lipids (CPL) on cumulative incidence of encephalopathy induced by 4% safflower methyl esters. The polar lipids were obtained from thermally oxidized safflower methyl esters and were fed with 3.7% fresh safflower methyl esters from the 8th day. During the first week, the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

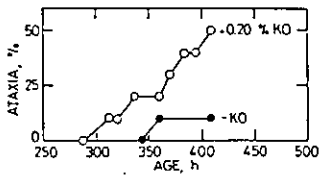

FIG. 2. Effect of conjugated methyl keto-octadecenoate (KO) on cumulative incidence of ataxia induced by 4% safflower oil methyl esters. KO was fed with 3.8% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% safflower methyl esters. The control chicks received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

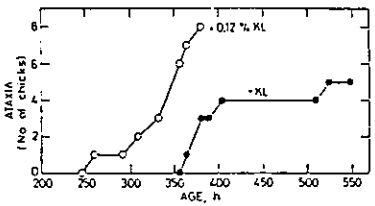

FIG. 3. Effect of conjugated methyl keto-octadecadienoate (KL) on cumulative incidence of ataxia induced by 4% safflower methyl esters. The safflower methyl esters were fed from the first day and KL was added from the 8th day. There were eight chicks per treatment.

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

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.

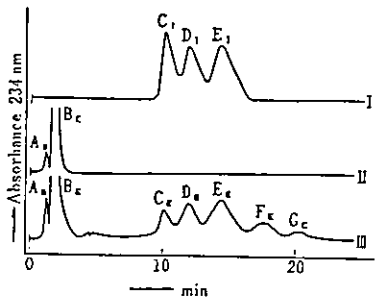
Instrument	Dupont LC-1
Column	Zorbax SIL 2.1 ϕ mm × 250 mm
Ref. Column	Zorbax SIL 2.1 ϕ mm × 250 mm
Column temp.	25.0°C
Mobile phase	Hexane/2-propanol (249 : 1)
Column pressure	20.0 kg/cm²
Flow rate	1.71 ml/min
Detector	UV 234 nm
Sensitivity	0.64 AUFS

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}, 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 ·$[CH_2]_5CH_3$) and 225 (M-157; loss of ·$[CH_2]_7COOCH_3$), 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.

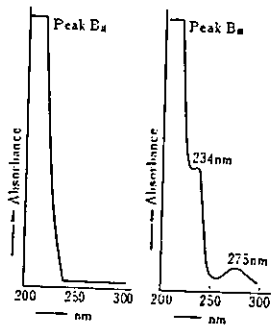


Peak A and Peak B: Triglycerides and their related substance; Peak C: Methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate; Peak D: Methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate; Peak E: Methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate; Peak F and G: Derivatives of MLHPs 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).

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}.

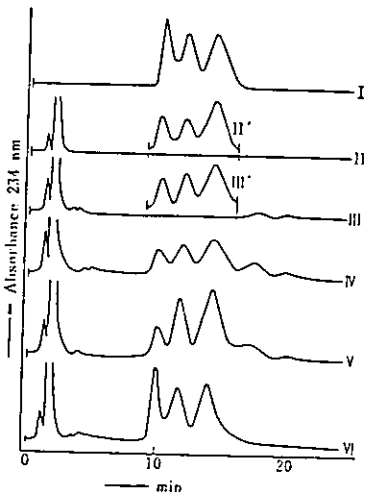
intestinal wall were incorporated into triglycerides. There is also a λ_{max} at 275 nm in the scanning chart of peak B_{III}. This suggests that some of the MLHPs absorbed from the intestinal wall became dienon derivatives and were incorporated into triglycerides. Peaks F_{III} and G_{III} were assumed 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 goriinal 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^{-3}	2.96×10^{-3}	3.51×10^{-4}	5.65×10^{-4}	6.15×10^{-4}	1.59×10^{-2}
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-ニ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。

(昭和 56 年5月 11 日受理)

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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

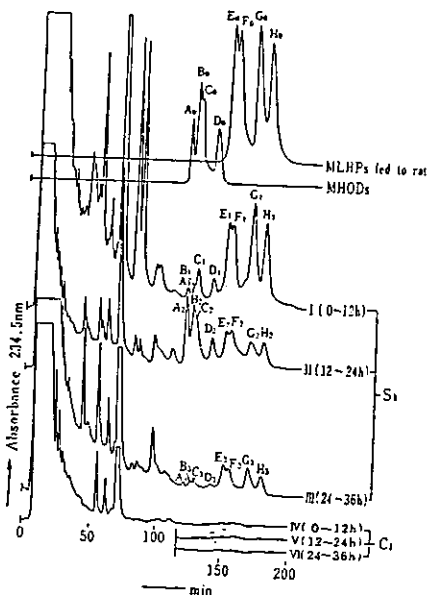
Fig.-1 shows the chromatograms of reversed phase HPLC of lymph lipids and other sample esters. UV 234.5 nm was adopted for the analytical wave length. Chromatograms of MLHPs used as sample ester and reduced products of MLHPs, namely MHODs, are shown on the upper part of Fig.-1.

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



$A_{1\sim3}$ to $D_{1\sim3}$: isomer of methyl hydroxyoctadecadienoate.
$E_{1\sim3}$ to $H_{1\sim3}$: 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[15),18)]. 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),19)]. 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 $E_{1\sim3}$, $F_{1\sim3}$, $G_{1\sim3}$ and $H_{1\sim3}$ were assumed to be MLHPs. In addition, these compounds were collected separately and analyzed for their UV spectra. The spectra of compounds $E_{1\sim3}$ and $F_{1\sim3}$ in ethanol were characterized by a maximum at 236 nm, whereas the spectra of compounds $G_{1\sim3}$ and $H_{1\sim3}$ gave a maximum at 233 nm. These also suggest that compounds $E_{1\sim3}$ and $F_{1\sim3}$ are *cis-trans* isomers of MLHP, and compounds $G_{1\sim3}$ and $H_{1\sim3}$ are *trans-trans* isomers of MLHP[19)]. 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 $A_{1\sim3}$ to $D_{1\sim3}$ show the same retention time as those of peaks A_0 to D_0, respectively. Therefore, the compounds presented as peaks $A_{1\sim3}$ to $D_{1\sim3}$ 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(=O)-C=C-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,

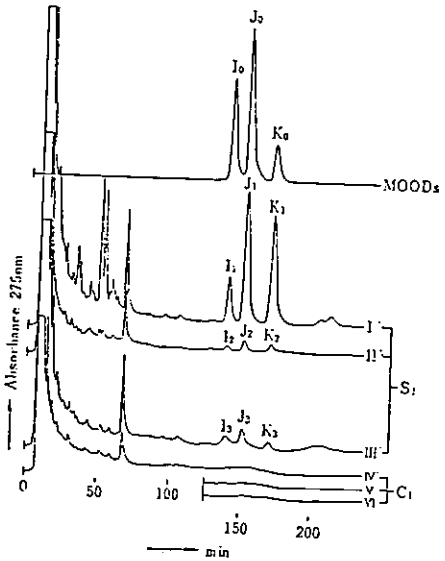


Fig.-2 High performance liquid chromatogram of extracts from the lymph of rat (S_1) dosed MLHPs (I′~III′), extracts from the lymph of rat (C_1) dosed ML (IV′~VI′), and methyl oxooctadecadienoates.

and I, to K, 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, to I, in ethanol were characterized by a maximum at 278 nm, J, to J, at 278 nm and 272 nm, and K, to K, 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).

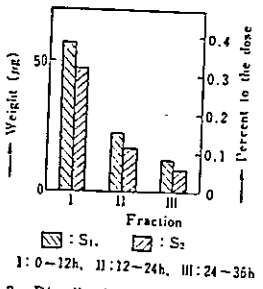


Fig.-3 Distribution of intact MLHPs in each collecting fraction obtained from the lymph of rats (S_1, S_2) dosed 15 mg of MLHPs.

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

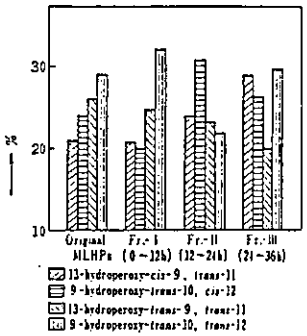


Fig.-4 Percent of four isomers of MLHP in the lymph collected from the rat (S_1) dosed MLHPs.

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

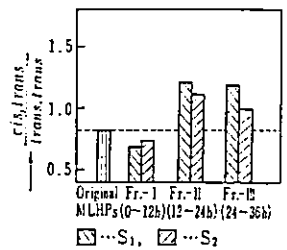


Fig.-5 Ratio of (*cis, trans* MLHPs)/(*trans, trans* MLHPs) found in each collecting fraction of lymph of rats (S_1, S_2) dosed MLHPs (×).

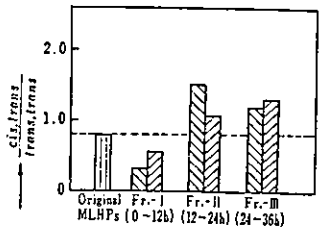


Fig.-6 Ratio of (*cis, trans* MHODs)/(*trans, trans* MHODs) found in each collecting fraction of lymph of rats (S_1, S_2) dosed MLHPs.

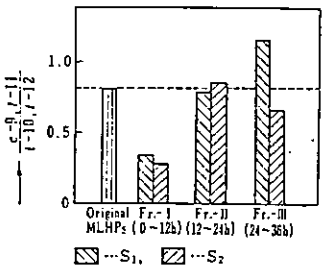


Fig.-7 Ratio of (*cis*-9, *trans*-11 MOOD)/(*trans*-10, *trans*-12 MOOD) found in each collecting fraction of rats (S_1, S_2) dosed MLHPs.

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" 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,

* Less than one fiftieth as much as lethal dose.

we first revealed by use of HPLC that MLHPs dosed to the rats were partly derived to MHODs or MOODs and transported into the lymph.

From the results obtained in this study, the authors concluded that MLHPs administered to rats might be absorbed unchanged into the intestinal wall, whereas they could be changed to MHODs or MOODs. These products and MLHPs absorbed from the intestinal wall would then be transported to organs through lymph and blood, where they could do damage to biomembranes, react with proteins and small metabolites, and initiate lipid peroxidation in the body.

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ラットにおけるリノール酸メチルヒドロペルオキシドの吸収と代謝について

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ラットを用い，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と代謝を検討した。15 mg のMLHPsを経口投与後，胸管リンパ管から，12hごとのフラクションに分け 36 h にわたりリンパ液を採取した。対照のラットには 15 mg のリノール酸メチルを与え同様の操作を行った。集めたリンパ液から脂質を抽出し逆相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 0.5～0.6% が未変化のまま小腸から吸収されるのを始め，ヒドロキシオクタデカジエン酸メチルやオクソオクタデカジエン酸メチルといった誘導体に代謝され，小腸から吸収されることが明らかとなった。

報 文

Trans-5-Olefinic Unusual Fatty Acids in Seed Lipids of Aquilegia

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Open-tubular gas chromatographic analysis (GLC) of fatty acids from the seed lipids of *Aquilegia vulgaris* (I), *A. longissima* (II), *A. hybriba hort* (III), *Delphinium Ajacis* (IV), and *Nigella damascena* (V) belong to Ranunculaceae have been carried out. The unusual fatty acids found in I, II, and III were *trans*-5, *cis*-9, *cis*-12-octadecatrienoic acid (*t* 5, *c* 9, *c* 12-18 : 3) (neutral lipids 56～59%, and polar lipids 35～40% in I and II), *t* 5, *c* 9-18 : 2 (0.5～2.6%), and *t* 5-16 : 1, *t* 5-18 : 1 and *t* 5, *c* 9, *c* 12, *c* 15-18 : 4 (each less than 1%). These unusual acids have not been found in IV and V. The usual acids, 16 : 0, *c* 9-18 : 1 and *c* 9, *c* 12-18 : 2, were found as the major constituents with the minor constituents, 14 : 0, 15 : 0, *c* 7 and *c* 9-16 : 1, 18 : 0, *c* 11-18 : 1, *c* 9, *c* 12, *c* 15-18 : 3, *c* 11-20 : 1 and *c* 11, *c* 14-20 : 2 in common. The structures of the 5-olefinic acids were established by the comparison of the retention data in GLC of the *cis*-5 and *trans*-5 olefinic acids and their partially hydrogenated products on SP 2300 and SP 2340 liquid phases, and ^{13}C-NMR spectra of them. The fatty acid composition of aquilegia seed lipids was a little changed by the germination.

1 Introduction

The occurrence of unusual fatty acids with a 5 : 6 double bond has been found in the lipids of plants[1], marine invertebrates[2),3)] and others. In the previous paper, we reported that fatty acids from the lipids of seeds, arils and leaves of japanese yew, *Taxus cuspidata* contained the 5-olefinic unusual fatty acids such as *c* 5, *c* 9-18 : 2, *c* 5, *c* 9, *c* 12-18 : 3, and *c* 5, *c* 11, *c* 14-20 : 3[4], and that fatty acids from the lipids of seeds of twenty species of Gymnospermae belong to seven families contained more or less contents of the 5-olefinic acids characteristic to each family or species[5]. In this study, the fatty acids from seed lipids of three species of aquilegia and two species of other plants belong to Ranunculaceae have been investigated with particular attention to the 5-olefinic unusual fatty acids.

Some species of Ranunculaceae have been reported to contain high contents of the 5-olefinic acids as acyl groups in their seed lipids[1]. Specially, the fatty acid components from the seed lipids of *Thalictrum sp.* have been investigated by many workers[6)-9)]. Otherwise, abundance of the 5-olefinic unusual acids has been reported for the seed lipids of aquilegia[10)-13)] but their detailed compositions have not been reported. In this study, the fatty acid composition of the seed lipids from three species of aquilegia was obtained by open-tubular GLC. The fatty acids from the neutral lipids of the aquilegia seeds showed ca. 60% content of *t* 5, *c* 9, *c* 12-18 : 3. Aquilegia seeds are a preferable source for the preparation of *t* 5, *c* 9, *c* 12-18 : 3, since the seeds are easily available at a nursery company and the *cis*-5 isomer contents are negligible in the neutral lipids. Collection of *Thalictrum* seed lipids from their wild plants is not easy, and the coexistence of *c* 5, *c* 9, *c* 12-18 : 3 makes it difficult to separate *t* 5, *c* 9, *c* 12-18 : 3 from fatty acids of *Thalictrum* seed oils.

2 Experimental

2·1 Extraction, Fractionation and Methanolysis of Lipids

The species of the samples studied are listed in Table-1. All seeds were air-dried products from a nursery company. Each sample was ground to powder with an electric mill and

TABLE I

Chemical Composition of *Moringa peregrina* Seed (weight of seed, 0.61g)

Assay	Percentage
Kernel	40.0
Moisture	1.8
Fat	54.3
Protein	22.1
Fiber	3.6
Carbohydrate	15.3
Ash	2.5

tion. A Perkin Elmer Model Sigma 2 Gas Chromatograph with flame inoization detecton (FID) was employed for the analysis using nitrogen as the carrier gas. A stainless steel 6-ft long and 1/8-in. od column packed with 15% DEGS on 80-100 mesh Chromosorb W was used under the following conditions: nitrogen flow, 30 mL/min; column temperature, 190 C; temperature of injection port and detector, 200 C.

Identification of each component was made by comparing its retention time with that of the reference sample. Peak areas were calculated using a Perkin Elmer M2 calculating integrator. The gas chromatograph was calibrated with an RM7 standard fatty acid mixture with each peak normalized according to detector response.

RESULTS AND DISCUSSION

The analytical data of *Moringa peregrina* is given in Table I. The percentage of oil was higher than that of other species which have been reported (1-4).

On extraction with petroleum ether, the kernel (40-60 C) gave more yellow-colored oil with characteristic odor. The physicochemical constants of the oil (Table II) were estimated and compared with the oil of other *Moringa* species (1-4). The refractive index of oil agrees with figures previously reported (1-3), but the other physicochemical constants of the oil varied. Fatty acid composition (Table II) is somewhat different from the other species. In *M.-peregrina*, nine fatty acids have been detected; Khan (4) detected only four fatty acids in *M.olifera;* Sengupta (1) detected seven fatty acids in both *M.concanensis* and *M.pterygosperma;* and Verma (3) detected five fatty acids in both *M.olifera* and *M.concanensis*. The difference in the fatty acid composition may be a result of the different species of *Moringa*. *M.peregrina* has a higher percentage of unsaturated fatty acids consisting mainly of oleic acid, which is the predominant fatty acid of the species.

TABLE II

Physicochemical Characteristics and Fatty Acid Composition of *Moringa peregrina* Oil

Determination	Value
Refractive index	1.4610
Saponification number (mg KOH/g)	182.9
Iodin value	69.5
Specific gravity (at 15 C)	0.9095
Peroxide value (meq/kg)	2.3
Acid value (mg KOH/g)	0.04
Unsaponifiable matter (%)	0.3
Fatty acid composition (% by weight by GLC)	
C14:0	trace
C16:0	9.3
C16:1	2.4
C18:0	3.5
C18:1	78.0
C18:2	0.6
C18:3	1.6
C20:0	1.8
C22:0	2.6
Saturated fatty acids	14.7
Unsaturated fatty acids	84.7

M.peregrina therefore has potential as a new source of fat and protein. It is also a source of antibiotic isothiocyanates (7). Further studies of amino acids, vitamins and minerals are in progress.

ACKNOWLEDGMENT

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... Acid Monomers in Frying Oils Used for Fast Foods

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ABSTRACT

Cyclic fatty acid monomers were analyzed by gas chromatography in commercial frying oils obtained in this country and in the Middle East. Samples were obtained from food outlets in California and Illinois after varying periods of usage. The samples from Egypt and Israel were collected from street vendors frying vegetable patties (known as "fallafel") in open-air stands. The United States samples ranged from 0.1 to 0.5% cyclic monomers, and from 1 to 8% polar + nonoluted thermal oxidation materials. The Middle Eastern samples showed significantly more heat abuse, with values for cyclic monomers from 0.2 to 0.7% and polar materials ranging from 2 to 22%.

INTRODUCTION

Cooking and deep-fat frying with polyunsaturated oils result in significant chemical changes that produce flavor deterioration in the fried foods and potential impairment in nutritional value. The literature in this field is extensive and several reviews have appeared (1-3). In the USA, soybean oil and other vegetable oils, intended for frying and other institutional uses, are generally hydrogenated to reduce most or all of the linolenic acid and a large portion of linoleic acid. However, in other countries and in the Middle East, many of the vegetable oils used for cooking and frying are not hydrogenated. If these oils are subjected to extensive heat abuse, their nutritional value may be damaged.

Changes occurring in heated oils have been followed by a large variety of analytical methods including acid, iodine and hydroxyl values, thiobarbituric acid tests, UV absorption, fatty acid composition, nonurea adduct-forming material, triglyceride content, nonoluted material by gas chromatography (GC), polar-polymeric material by column chromatography, octanoate content, dielectric constant, color and viscosity (4-18). Much of the methodology reported for the analysis of thermal changes has been used without valid basis and may explain some of the controversy in the literature on the effect of heating unsaturated oils. Despite limitation of the methodology, there is now evidence that a measurable amount of cyclic monomers accumulate in heated vegetable oils (19,20).

No precise information has been available concerning which specific compounds are nutritionally harmful (1). Previous work of Iwaoka and Perkins (21) with rats showed that incorporation of 0.15% cyclic fatty acid methyl esters in low-protein diets containing 15% corn oil caused accumulation of liver lipids. Because of the markedly increasing consumption in this country of fried foods prepared in fast-food establishments, it is important to assess the amount of cyclic monomers actually formed in such commercial operations.

A GC procedure was previously developed (20) to determine quantitatively cyclic monomers formed in hydrogenated and unhydrogenated soybean oils heated at 195 C intermittently for 52 hr or continuously for 104 hr with frying of potato slices. In the present study, the same method for analyzing cyclic monomers was applied to a variety of samples of oils obtained from actual commercial frying operations. In the Middle East, vegetable patties known as "fallafel" are commonly deep-fat fried in vegetable oils by street vendors. Samples of oils used in such operations in Egypt and in Israel were obtained to compare their cyclic acid content with those from the USA.

EXPERIMENTAL

Samples

Fresh and used cooking oil samples were collected from several fast-food outlets in Peoria, Illinois, and in Davis, California, after varying periods of usage and just prior to being discarded. In the USA outlets, frying oil losses on foods were replenished daily. The types of foods fried included doughnuts, chicken, chicken liver, French fries, seafood, tofu and mixed substrates alternating between meat, pork cutlets and shrimp. Information on the number of frying cycles could not be obtained. History of usage of the Middle East oils was unknown. These Middle Eastern samples were collected at random from street vendors and transported to the USA by air. Length and conditions of usage varied widely. The Egyptian and Israeli samples were derived, respectively, from unhydrogenated cottonseed and soybean oils. All samples were stored under nitrogen at 4 C until analyzed.

Fatty Acid Composition

Major fatty acids were determined by GC of methyl esters prepared by the procedure of Metcalfe et al. (22). Instrumentation and conditions for GC included a Hewlett Packard Model 5711A chromatograph with flame ionization detector and a 610 x 0.257 cm id stainless steel column packed with 15% OV-275 (Supelco Inc., Bellefonte, PA) on high performance Chromosorb W. The column was operated isothermally at 220 C with injector and detector temperatures at 250 C. Nitrogen carrier gas flow rate was 10 mL/min. Standard mixtures of simple triglycerides were used to obtain relative retention times and response factors.

Cyclic Fatty Acid Monomers

The GC method used for cyclic monomers was that of Meltzer et al. (20). Briefly, the oil samples were saponified, hydrogenated and crystallized in acetone (−47 C) to remove 77-97% of the saturated linear fatty acids. The concentrated cyclic acids were then methylated and analyzed by GC using methyl heptadecanoate as an internal standard. The group of peaks assigned to cyclic monomers, which were eluted between methyl stearate and polar materials, was based on previous identification by comparison with a reference standard and by gas chromatography-mass spectrometry.

Thermal Oxidation Materials

Extent of oxidative degradation was estimated by measuring nonoluted materials from a GC column according to the method of Waltking et al. (23). This procedure was adapted to methyl esters by using ca. 20% by weight methyl heptadecanoate as internal standard and by using their equation to calculate nonoluted material.

TABLE I

Fatty Acid Composition (Weight Percent) of Fresh Cooking Fats[a]

Fatty acid	Vegetable shortenings (VS)[b]			Animal-vegetable shortenings (AVS)[c]				Partially hydrogenated vegetable oil (PHV)	Cottonseed oil (CSO)[d]		Soybean oil (SBO)
	D[e]	D	P[f]	D	D	P	P	P	D	Eg	I[h]
12:0	–	–	1.3	0.1	0.1	–	0.1	0.1	–	–	–
14:0	0.1	0.1	0.6	3.7	3.5	3.9	4.3	0.3	0.8	0.8	0.1
16:0	11.4	11.0	15.2	26.8	26.4	27.7	27.2	13.5	20.3	23.0	7.4
16:1	0.2	0.2	–	5.5	5.2	–	–	–	0.7	0.6	–
18:0	11.8	13.0	9.8	18.1	18.8	21.1	22.9	9.8	2.8	2.8	5.4
18:1	74.0	72.3	70.6	42.6	41.7	44.9	42.4	45.6	19.6	19.5	26.8
18:2	2.3	2.9	2.5	2.4	3.4	2.4	3.1	29.9	55.0	52.8	53.6
18:3	0.2	0.2	–	0.5	0.6	–	–	0.8	0.3	0.5	6.7
20:1	–	0.3	–	0.3	0.3	–	–	–	0.5	–	–

[a]GC analyses are average of duplicate determinations.
[b]Hydrogenated soybean oil for "heavy-duty frying."
[c]Beef fats and cottonseed oil with dimethyl polysilane, BHA and BHT.
[d]Winterized cottonseed oil.
[e]Davis, CA.
[f]Peoria, IL.
[g]Egypt.
[h]Israel.

RESULTS AND DISCUSSION

Fats used by the restaurants sampled included vegetable shortenings (VS), animal-vegetable shortenings (AVS), partially hydrogenated vegetable oil (PHV) and cottonseed oil (CSO). Fats used in the Middle East included CSO in Egypt and soybean oil (SBO) in Israel. Fatty acid compositions were determined on the fresh unused fat sampled when available. Analyses in Table I show that monoenes vary from 46 to 74% and dienes from 2.3 to 29.9 in VS, AVS and PHV. In unhydrogenated SBO and CSO, the monoenes ranged from 20 to 27%, dienes from 54 to 55% and trienes from 0.3 to 7%. Only samples of AVS used were reported to include additives such as methyl polysilane, BHA and BHT.

GC analyses for cyclic monomers and polar + noneluted thermal oxidation materials varied widely with the origin, type and usage of the fats (Table II). The commercial USA samples ranged from 0.1 to 0.5% cyclic monomers and from 1 to 8% polar + noneluted materials. The corresponding fresh unused fats gave a value for materials with same GC retention ranging from 0.02 to 0.06% as cyclic monomers and from 0.6 to 1.9% as polar + noneluted materials. The lowest level of cyclic monomers was found in the fats used for doughnut frying (0.06-0.08%) and the highest level was found in the fats used for French fries (0.4-0.5%) and when CSO was used for frying (0.3-0.4%). The differences suggest that the cooking of French fries causes greater deterioration of the fats than cooking of the other foods. However, this assumes that comparable percentages of make-up fat were added daily to the different fryers used for each type of food.

The Middle Eastern samples showed significantly more heat abuse, with values for cyclic monomers from 0.2 to 0.7% and polar + noneluted materials from 2 to 22%. It is remarkable that, in all samples examined, the cyclic monomer content was less than 1%, even in the most abused samples that contained relatively large amounts of polar + noneluted materials. The low values for cyclic monomers may be accounted for by the dilution effect of make-up fat during the frying operation. No meaningful correlation was apparent between the level of cyclic monomer and polar + noneluted material.

These results confirm our previous study (20) with soybean oil and hydrogenated soybean oils heated in the laboratory under conditions analogous to those common in deep-fat frying operations. Although the level of cyclic monomer was in the same range (0.3-0.6%), the level of polar + noneluted material was generally lower in the commercial US samples (1-8%) than in the laboratory samples (18-21%). This difference would indicate a larger amount of thermal oxidation products in the laboratory samples. In another study, Guillaumin (24) showed the presence of cyclic monomers (0.07-0.2%) in all unsaturated fats examined by GC analysis. After 15 heatings at 200 C, cyclic monomers showed an increase of only 0.02-0.10%. To analyze for cyclic monomers, these workers used a direct gas chromatographic procedure without preliminary concentration (19). However, we found that at the low levels of cyclic monomers found in heated fat, it was necessary to concentrate the hydrogenated fatty acids prior to gas chromatography (20), and this observation was recently confirmed by Grandgirard and Julliard (25). Billek et al. (26) established an arbitrary level of 25-30% oxidized polar materials (by silica gel chromatography) to indicate that a frying oil is deteriorated to the discard point.

Caution must be exercised in interpreting the literature on nutritional effects of heated fats. Early reports (27-29) indicated that fats heated under frying conditions had no harmful effects. However, the diets used in these studies contained excessively high protein levels, which are known to lessen the effects of heated fats (30). In a more recent study, synthetic cyclic monomeric compounds, similar to those isolated from heated fats, have been shown to depress growth in rats and cause hepatomegaly or fatty livers due to accumulation of lipid (21). In this study, the diets were low in proteins to stress the animals, and the mineral mix used was deficient in important trace elements including zinc, cobalt, selenium and molybdenum. Additional data are needed on the level of cyclic monomers and polar materials in fats extracted from fried foods. Since pan frying is most commonly used in the world and may be more abusive to fats (24), further work is needed on the effect of this treatment on the extent of formation of oxidation product. Future work should also focus on the analysis of those polar and noneluted materials that were significant components of most of the heated samples examined in this work.

TABLE II

Cyclic Fatty Acid Monomers and Polar + Noneluted Materials in Commercial Cooking Oils[a]

Origin[b]	Oil Type[c]	Usage[d]	Heating Temperature[e]	hr/day	Days	Relative percent Cyclic monomers	Polar + noneluted
USA-D1	VS	Fresh	–	–	–	0.02	0.6
		Doughnuts	181	8	7	0.06	1.2
		Doughnuts	181	8	7	0.08	2.8
USA-D2	VS	Fresh	–	–	–	0.06	0.7
		Chicken	191	9	28	0.24	0.9
		Chicken livers	177	9	2	0.42	3.7
		French fries	177	9	2	0.40	3.7
		Chicken livers	177	9	2	0.37	1.3
		French fries	177	9	2	0.50	8.2
USA-P1	VS	Mixed	180	10	7	0.10	2.0
		Mixed	180	10	7	0.15	4.9
		Mixed	180	10	7	0.11	8.0
USA-P2	VS	Mixed	190	8	7	0.12	3.8
USA-D3	AVS	Fresh	–	–	–	0.06	1.1
		Seafood/ French fries	191	10	3.5	0.15	4.0
		Seafood/ French fries	191	10	7	0.14	2.3
USA-P3	AVS	Mixed	195	10	7	0.15	2.9
		Mixed	195	10	14	0.45	4.1
		Mixed	195	10	7	0.12	3.2
USA-P4	PHV	Fresh	–	–	–	0.03	1.9
		Mixed	190	8	7	0.34	4.2
USA-D4	CSO	Fresh	–	–	–	0.26	2.6
		Tofu	177	5	15	0.37	8.3
		Tofu	177	5	15	0.38	7.3
		Tofu	177	5	15	0.40	6.6
Israel	SBO	Fallafel				0.50	22.2
		Fallafel				0.44	2.3
		Fallafel				0.38	2.3
		Fallafel				0.20	4.5
Egypt	CSO	Fallafel				0.39	7.4
		Fallafel				0.66	10.1
		Fallafel				0.17	2.4
		Fallafel				0.48	9.9

[a]Method of Meltzer et al. (20).
[b]USA-D: Davis, CA; USA-P: Peoria, IL.
[c]See Table I; VS = vegetable shortening; AVS = animal-vegetable shortening; PHV = partially hydrogenated vegetable oil; CSO = cottonseed oil; SO = soybean oil.
[d]Mixed = mixture of substrates including meat, pork and shrimp. Fallafel = Middle Eastern vegetable patties—no exact history available on usage. Daily make-up with fresh oil in Davis and Peoria outlets.
[e]Temperature ± 5 C.

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Compositional Analysis of Natural Wax Ester Mixtures by Tandem Mass Spectrometry

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ABSTRACT

Tandem mass spectrometry is particularly suited for the analysis of complex, natural wax ester mixtures R_1-CO_2-R_2. Reduction of the mixture with deuterium provides species that are separable (through mass spectrometry) based on the number of original double bonds. Chemical ionization with isobutane produces high yields of protonated molecular ions and very little further fragmentation. These ions are separated by the first mass filter and then dissociated through collisions with argon. The positively charged dissociation products are almost exclusively the protonated acid ions $(R_1\text{-}CO_2H_2)^+$ that can then be separated by the second mass filter before detection and quantitation. The technique overcomes many of the obstacles previously faced during wax ester analysis. Results from this method are compared with those obtained by previous work, and the isomer composition of a new wax ester oil, orange roughy oil, is given.

INTRODUCTION

The analysis of naturally occurring wax ester mixtures for the relative abundances of isomers within each chain length is a somewhat formidable task. Since Aasen et al. showed that electron impact (EI) mass spectra could be used to quantitate saturated wax ester mixtures (1), mass spectrometry has become the method of choice of many workers (2-4). Complications encountered in these analyses include the difficulties associated with gas chromatographic separation of these relatively high molecular weight compounds and the problem of quantitatively introducing them into the mass spectrometer. Further, EI gave a great deal of nonspecific fragmentation with a relatively small percentage of the total ion current attributed to diagnostically important ions.

Recently, we showed that chemical ionization (CI) with isobutane gave spectra with intense protonated molecular ions and very little further fragmentation (5). Although this feature was not beneficial for structural information, it appeared to be particularly advantageous for mass spectrometry/mass spectrometry (MS/MS), because a high yield of ions representing the molecular species could be formed. When subsequent experiments showed that these ions could be dissociated to yield essentially one daughter ion per acyl radical (the protonated acid), a method was needed to identify and quantitate unsaturated isomers and analogs. Tris(triphenylphosphine)chlororhodium(I) catalyzes the reduction of double bonds with very little exchange between substrate and reagent (6), which results in saturates that include two atoms of deuterium per original double bond. Therefore, the protonated molecular ion and associated protonated acid ion from unsaturated compounds have m/z values 2 units greater per double bond and, although they behave chemically like their fully protonated counterparts, they are easily distinguished by mass spectrometry. Thus conditions were available to conduct the analysis in a single MS/MS experiment, because the entire wax ester mixture could be reduced with deuterium and the protonated molecular ions separated by the first mass filter. Following dissociation, the ions arising from the component acids could be analyzed in the second mass filter. In this paper we describe methods used to conduct such analyses and the results obtained from four natural wax ester mixtures.

EXPERIMENTAL

Purified wax ester standards were prepared from appropriate alcohols and acyl chlorides (2). Saponification (and recovery of unsaponifiables) was carried out essentially as prescribed by the AOCS (Method Ca 6b-53); the combined aqueous layers were then acidified and the free acids were recovered by extraction with diethyl ether. Free acids were methylated with diazomethane and alcohols were acetylated in pyridine/acetic anhydride (1:2). Analysis of the wax esters and their hydrolysis products by gas chromatography has been described previously (2).

The sample (5 mg) and internal standard, palmityl $[^2H]_6$-stearate (0.5 mg), were dissolved in ca. 2 mL of $CHCl_3$ in a 30-mL test tube equipped with a side-arm to which a small balloon was attached. Ca. 20 mg of tris-(triphenylphosphine)chlororhodium(I) catalyst (6) was added and the flask was stoppered. A three-way stopcock with one arm through the stopper, one arm to vent and one arm connected to a deuterium cylinder facilitated saturation of the atmosphere with deuterium. Flushing was accomplished by repeatedly inflating the balloon with deuterium and then exhausting through the vent. After 8-10 cycles, the system was assumed to be saturated. The reduction medium was vigorously stirred (with the balloon inflated) for 7-8 hr; by this time, some insolubles had formed. The solution volume was reduced to a minimum

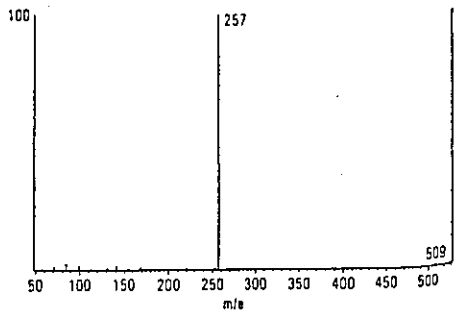

FIG. 1. Daughters of the protonated molecular ion from stearyl palmitate (18:0-16:0) produced by collision-induced dissociation with Ar; collision energy = ~15 V.

TABLE I

Comparison of Isomer Compositions Obtained by Different Methods

Chain length: unsaturation	Alkoxy-acyl groups	Isomer composition (%) Present work MS/MS	Sample (method, ref.)
			Sperm whale oil (EI/MS, 2)
32:0	12-20	tr	4
	13-19	–	4
	14-18	8	6
	15-17	tr	1
	16-16	64	63
	17-15	4	2
	18-14	20	19
	19-13	tr	1
	20-12	4	2
34:1	14:0-20:1	4	4
	14:1-20:0	tr	–
	16:0-18:1	61	47
	16:1-18:0	1	15
	18:0-16:1	6	9
	18:1-16:0	25	23
	20:0-14:1	tr	–
	20:1-14:0	2	2
36:1	14:0-22:1	–	4
	14:1-22:0	2	6
	16:0-20:1	67	46
	16:1-20:0	1	6
	18:0-18:1	16	17
	18:1-18:0	8	11
	20:0-16:1	1	5
	20:1-16:0	4	4
	22:0-14:1	tr	1
	22:1-14:0	1	–
36:2	14:1-22:1	–	2
	16:1-20:1	9	9
	18:1-18:1	86	87
	20:1-16:1	4	3
			Jojoba oil (HPLC-GC, 9)
40:2	16:1-24:1	tr	1
	18:1-22:1	1	4
	20:1-20:1	89	82
	22:1-18:1	10	12
	24:1-16:1	tr	1
42:2	18:1-24:1	tr	2
	20:1-22:1	25	21
	22:1-20:1	74	75
	24:1-18:1	1	2
			Spermaceti (HPLC-GC, 10)
30:0	14:0-16:0	9	12
	16:0-14:0	84	79
	18:0-12:0	7	9
32:0	14:0-18:0	1	3
	16:0-16:0	81	82
	18:0-14:0	16	15

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The Presence of Oxidative Polymeric Materials in Encapsulated Fish Oils

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Encapsulated health food oils such as fish oils are readily available in health food stores, pharmacies, and supermarkets. They are popular in the United States as well as in the European countries. However, such oils, because of their high degree of unsaturation, are easily oxidized and form complex mixtures of high molecular weight oxidation products. The present work reports the application of high-performance size exclusion chromatography to the determination of these materials in encapsulated fish oils. Of the six samples studied, five showed from 1-10% of dimeric triacylglycerols and one contained 6.3% trimeric triacylglycerols and 3.1% oligomeric triacylglycerols. Further investigation of this sample with silicic acid chromatography indicated that it contained a total of 36.3% polar material.

Lipids 26, 23-26 (1991).

Fish oils have been extensively studied in recent years due to their antiatherosclerotic (1-3) and hypotriglyceridemic effects (4). Recent reports have discussed the oxidative deterioration of fish oils and have suggested that ingestion of unstabilized fish oils entails a risk of exposure to potentially toxic products of n-3 fatty acid peroxidation. The resultant oxidation products may play a role in carcinogenesis (5,6), inhibit prostacyclin production (7) and may exert other adverse biological effects (8).

Unless extreme care is taken, fish oils, which contain a high percentage of long-chain polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids in triacylglycerol form, are very susceptible to autoxidation (9,10). Such autoxidation will initially form hydroperoxy free radicals which become stabilized by addition of hydrogen to hydroperoxides or by reaction with triacylglycerols to form dimeric and trimeric triacylglycerols linked *via* peroxidic-type bonds (10).

Encapsulated fish oils are now readily available for purchase. It would be useful to ascertain the presence or absence of such oxidation products and high molecular weight materials in such oils. The technique of high-performance size exclusion chromatography (HPSEC) is well established and has been employed repeatedly to separate triacylglycerols from higher molecular weight oxidation products such as the dimeric, trimeric, and oligomeric materials formed as a result of lipid oxidation (10-20).

It was the purpose of this work to examine several different brands of encapsulated fish oils in order to ascertain the presence or absence of higher molecular weight oxidation products.

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Abbreviations: DG, diacylglycerol; FFA, free fatty acids; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusion chromatography; MG, monoacylglycerol; TG, triacylglycerol; THF, tetrahydrofuran.

EXPERIMENTAL

Various brands of encapsulated fish oils were purchased locally. Three capsules of fish oil from each of different bottles from different batches were opened. The oil from each capsule was individually taken up into tetrahydrofuran (THF) to make a 20% solution by weight of oil. This solution was injected directly into each of the high-performance liquid chromatography (HPLC) column systems described below. Two different sets of columns were used as follows: Set one was a series of connected polystyrene-divinyl benzene polymer packed columns, in the order 500 Å, 100 Å, and 100 Å. Each column was 30 cm × 4.7 mm stainless steel (PL Laboratories, Shropshire, England) and the packing was 5 microns in diameter. Column set two was a series connected G 2000, to a G 1000 column, prepared with the same general type of polymeric packing as above, 5 microns diameter, 30 cm × 7 mm diameter (Supelco Inc., Bellefonte, PA). Detectors used were the Waters Model 410 differential refractometer and a Du Pont infrared detector set at 1735 cm^{-1} to monitor the ester carbonyl absorption. The remainder of the HPLC system and data handling system was as previously described (21). In both column systems, tetrahydrofuran (THF) was used as the eluting solvent at a flow rate of 1 mL/min. The THF used as mobile phase was kept under an atmosphere of nitrogen gas, and contained 0.027% butylated hydroxytoluene to minimize peroxide formation. This did not interfere with the HPSEC since it eluted in the low molecular weight exclusion volume peak at the end of the run. Polymeric standards as well as lipid standards were obtained from Supelco Inc.

Fractionation of oils into polar and nonpolar components was carried out according to the International Union of Pure and Applied Chemistry (IUPAC) method for the determination of polar components in fats using silicic acid column chromatography (22). The determination of peroxide, iodine, and anisidine values was carried out according to the IUPAC methods (21). Each sample was analyzed in triplicate and the range of values obtained reported. The fatty acid composition of the fish oils was determined according to the procedure of Ackman *et al.* (23). Tocopherols were determined according to the method described by Shukla (24).

RESULTS AND DISCUSSION

Data for six encapsulated oil samples which were analyzed are shown in Table I. A considerable range of quality is shown as evidenced by the peroxide value and anisidine value. The range of peroxide values found as well as the anisidine values, which are a measure of aldehyde content of a fat, indicate that both hydroperoxides and other types of peroxide linkages as well as their decomposition products are present in these oils. The fatty acid composition of the encapsulated material is given in

TABLE 1

Analytical Values of Encapsulated Fish Oils[a]

Product	Peroxide value	Anisidine value	Iodine value
A[a]	3.2-3.2	29.8-30.5	190.5-190.4
B[b]	2.7-2.6	14.2-14.4	196.2-196.9
C[c]	3.2-2.6	16.9-17.2	203.9-204.8
D[d]	2.2-2.2	29.8-28.6	197.2-201.7
E[e]	20.6-20.9	17.4-18.3	242.4-241.0
F[f]	1.6-1.5	27.5-27.6	289.8-290.6

[a]Range of values from analyses of three different capsules' contents. Expiration date: [a]10/89, [b]11/89, [c]7/91, [d]9/89, [e]10/89, [f]9/90. Analyzed: 3/1/89-5/1/89.

TABLE 2

Fatty Acid Composition of Various Encapsulated Oils

Fatty acid	A	B	C	D	E	F
14:0	7.0	5.8	5.7	6.4	1.0	1.0
15:0	0.6	0.5	0.6	0.8	0.1	0.1
16:0	15.8	15.6	14.3	16.4	2.6	2.7
16:1n-7	9.8	8.8	8.1	8.7	1.5	5.3
17:0	0.4	0.4	0.7	0.6	0.2	0.6
18:0	4.4	4.1	3.8	4.5	2.5	0.5
18:1n-9	15.6	14.9	14.4	13.0	10.7	9.7
18:2n-6	3.7	4.3	4.0	3.4	1.5	3.7
18:3n-3	1.0	1.5	1.2	1.1	1.1	1.2
18:3n-6	—	—	—	—	—	—
18:4n-3	2.4	3.5	4.0	2.7	2.7	5.8
20:0	—	0.6	—	0.6	0.6	—
20:1n-9	2.2	3.3	3.3	2.1	12.7	3.2
20:2n-6	—	—	—	—	—	—
20:4n-3	0.8	1.1	1.3	0.9	1.8	1.6
20:4n-6	0.9	0.7	0.8	1.1	1.2	1.5
20:5n-3	17.3	17.3	18.9	16.2	24.2	33.7
22:0	—	0.1	—	0.2	0.1	—
22:1n-9	—	—	—	—	—	—
22:1n-11	1.7	2.3	3.0	0.9	10.5	1.4
22:4n-3	0.7	0.7	0.7	0.6	1.2	1.3
22:5n-3	2.8	1.9	2.0	3.1	4.1	3.7
22:6n-3	12.3	11.9	12.6	15.8	18.9	22.8
24:0	—	0.1	—	0.1	Tr.	—
24:1n-9	—	—	—	—	—	—
24:1n-11	0.5	0.5	0.6	0.7	0.7	0.1

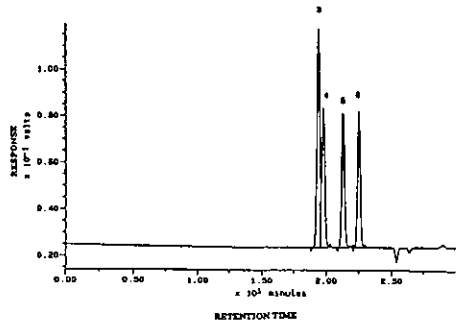

FIG. 1. High-performance size exclusion chromatography of lipid standards. Peak Identity: 3, triacylglycerols; 4, diacylglycerols; 5, monoacylglycerols; 6, free fatty acids.

TABLE 3

Composition of Encapsulated Fish Oils[a]

Sample	FFA[b]	MG[c]	DG[d]	TG[e]	Dimer TG[f]	Trimer TG[g]	Oligomers[h]
A	0.8	0.8	2.9	94.5	2.0	Tr	—
B	0.6	1.2	4.5	92.4	1.3	Tr	—
C	—	0.5	—	98.4	1.0	Tr	—
D	0.8	0.4	1.2	96.3	1.3	Tr	—
E	80.7[i]	9.3	—	10.0	—	—	—
F	0.9	1.1	14.6	64.1	10.0	6.3	3.1

[a]Wt % determined by HPSEC using weighed mixtures of standards to determine response factors.
[b]FFA, free fatty acids, mol wt ca. 313 dalton (Da).
[c]MG, monoacylglycerol, mol wt ca. 402 Da.
[d]DG, diacylglycerol, mol wt ca. 715 Da.
[e]TG, triacylglycerol, mol wt ca. 1028 Da.
[f]Dimer TG, dimeric triacylglycerol, mol wt ca. 2056 Da.
[g]Trimer TG, trimeric triacylglycerol, mol wt ca. 3084.
[h]Oligomers, polymers of triacylglycerols with multiple mol wts of $(1028)_{x+3}$ mol wt calc'd using 22:5 as average chain length and unsaturation (313 Da).
[i]Ethyl esters; the sample is a concentrate of fatty acid ethyl esters.

Table 2. The values appear to be typical of those obtained from fresh oils (23).

Further analysis of each of the encapsulated oils by high-performance size exclusion chromatography (HPSEC) with two different column systems and comparison with standard mixtures of lipids (Fig. 1) indicated that all of the samples contained varying percentages of mono- and diacylglycerols as well as dimeric and higher molecular weight triacylglycerols (Table 3). The appearance of free fatty acids, mono- and diacylglycerols may be a result of enzyme action on the oils prior to encapsulation. As an example of the separation obtained, a chromatogram of sample F obtained from one of the chromatographic systems is shown in Figure 2. High molecular weight thermal and oxidative products may have been formed as a result of thermal treatment of the oil such as in deodorization or as a result of autooxidation prior to encapsulation. High molecular weight oxidation products may have also been formed in the oils after encapsulation. It has been shown previously that soft gelatin capsules are somewhat permeable to oxygen and that this is dependent upon the plasticizer content as well as storage conditions (25). Therefore a certain amount of autooxidation may have taken place within the capsule, at a more increased rate considering the increased susceptibility of fish oils toward oxidation as indicated by Cho *et al.* (26). These authors indicated that the oxygen uptake of eicosapentaenoic and docosahexaenoic acid was 5.2 and 8.5 times faster, respectively, than of ethyl linolenate.

The oils examined in the present study contained from 164 to 7,965 ppm of tocopherols (Table 4), presumably added to stabilize the oils toward oxidation. However, tocopherols are known to be rather poor antioxidants when used in edible oils. They are even less efficient when used with oils containing polyunsaturated fatty acids (27). Furthermore, depending upon their level in an oil, tocopherols can exhibit prooxidant effects. In soybean oil containing 1,500 ppm of mixed tocopherols, optimum stability was found when the tocopherol levels were reduced to 400-600 ppm (27). The products examined in this work contained, with the exception of one sample, high levels of tocopherols (Table 4). In view of the prooxidant effects of high levels of tocopherols, and the known permeability of gelatin capsules toward oxygen (25), autooxidation may be expected to continue within an encapsulated oil.

In order to determine the general types of materials found as dimeric, trimeric and oligomeric triacylglycerols, further chromatography was carried out. Oils can contain materials resulting from both thermal and oxidative stress. Thermal stress, occurring in the absence of oxygen produces a variety of high molecular weight materials which elute from HPSEC columns with the same elution volumes as those materials shown in Figures 1 and 2. These materials, because of their elution volumes, can also be described as dimers, trimers and oligomers of triacylglycerols. Ratnayake *et al.* (13) have recently indicated that there were significant levels of materials defined as "polymers" in 10 of 16 samples of encapsulated fish oils they examined. However these authors did not further define the composition of the "polymeric" material. Furthermore the same types of materials may

TABLE 4

Tocopherol Composition of Encapsulated Fish Oils

Product	Tocopherols (total) ppm	Alpha T[a] ppm	Gamma T[b] ppm	Delta T[c] ppm	Alpha TT[d] ppm	Gamma TT[e] ppm
A	164	141	—	—	23	—
B	7965	1380	4349	2236	—	—
C	2475	173	1391	911	—	—
D	859	851	8	—	—	—
E	1744	630	737	376	—	1
F	4399	492	2346	1558	—	3

[a]Alpha tocopherol. [b]Gamma tocopherol. [c]Delta tocopherol. [d]Alpha tocotrienol. [e]Gamma tocotrienol.

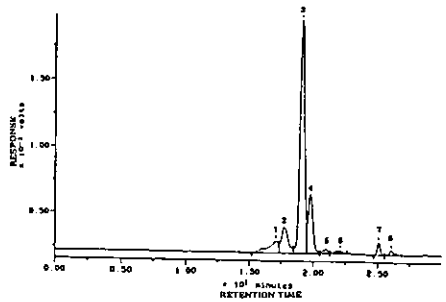

FIG. 2. High-performance size exclusion chromatogram of encapsulated oil (sample F). 1, Triacylglycerol trimer and oligomers; 2, triacylglycerol dimer. Peaks 3-6 are identified in Figure 1. Peaks 7 and 8 are residual solvent and short-chain materials of mol wt ca. 100, at the low mol wt exclusion limit of the columns.

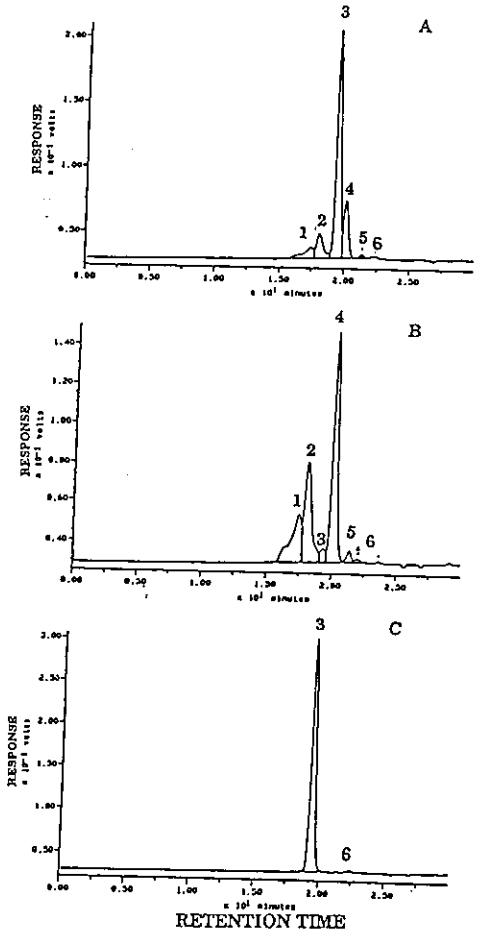

FIG. 3. High-performance size exclusion chromatogram of the polar (B) and nonpolar (C) fractions resulting from silicic acid chromatography of encapsulated oil sample F (A). Peak identity as in Figures 1 and 2.

be formed as a result of oxidative stress and usually contain peroxide linked triglyceride moieties. These materials also exhibit the same elution volumes on the HPSEC columns since their resolution is such that high molecular weight materials differing in molecular weight by one or more extra oxygen atoms cannot be separated. However, it is possible to separate oils which may contain high molecular weight compounds with the aid of adsorption chromatography on silicic acid. The oils can be easily separated into a fraction containing the nonpolar components of the oil and another containing polar and polymeric material (22). Separation of the oil sample F into its nonpolar and polar fraction with the aid of a silica column using the method specified in the IUPAC manual (22) indicated that it contained 36.3 wt % of polar material (by gravimetric determination of the eluted fractions from the IUPAC method). Subsequent HPSEC analysis of both the nonpolar and polar fractions is shown in Figure 3. HPSEC indicated that the polar fraction consisted of materials corresponding to the molecular weights and agreeing with the elution volumes of dimeric and higher polymeric materials, mono- and diacylglycerols and free fatty acids. The nonpolar fraction appeared to contain only triacylglycerol. The data indicated that the higher molecular weight material present is likely to be composed of triacylglycerol bonded *via* peroxy bridges. Heating this oil will lead to the destruction of the peroxidic linkages and the formation of both volatile and nonvolatile degradation products.

Further research on the chemical structure of the products formed in these oils as well as on their nutritional and physiological effects is currently under way in our laboratories.

It appears that because of the unknown health effects of the oxidative polymeric materials, and their high level in some encapsulated oils, caution may be advised when ingesting fish oil capsules on a regular basis.

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Effects of Oleic, Arachidonic and 5,8,11,14-Nonadecatetraenoic Acids on Lipid Secretion and Ketogenesis in Perfused Rat Liver

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The effects of perfused oleic (18:1n-9), arachidonic (20:4n-6) and 5,8,11,14-nonadecatetraenoic (19:4n-5) acids on triglyceride and cholesterol secretion and ketone body production were studied in isolated rat liver. As compared to oleic and 19:4n-5 acids, both ketone body production and triglyceride secretion were significantly lowered when arachidonic acid was perfused. The concentration of triglyceride in the post-perfused liver was lower upon perfusion with arachidonic acid than upon perfusion with oleic acid or 19:4n-5 acid. Cholesterol secretion in the liver perfused with arachidonic acid or 19:4n-5 acid was significantly higher than with oleic acid. The concentration of cholesterol in the post-perfused liver was slightly but significantly higher with 19:4n-5 acid than with the other fatty acids. The results suggest that 19:4n-5 acid when compared with arachidonic acid affects lipid metabolism in liver differently.
Lipids 26, 27-30 (1991).

Polyunsaturated n-6 fatty acids (PUFAs) and particularly linoleic acid exert a hypocholesterolemic effect in experimental animals and humans (1,2). Although several mechanisms have been proposed for this cholesterol-lowering effect (2), the precise mechanism has not been elucidated as yet. Fatty acids are secreted from the liver to varying degrees in very low density lipoprotein (VLDL) and are subsequently metabolized in the blood stream to form intermediate and low density lipoproteins. A decrease in VLDL secretion may thus result in a reduction of serum cholesterol concentration. Beynen and Katan (3) suggested that PUFAs, when compared to saturated fatty acids, are more readily oxidized to ketone bodies in the liver rather than secreted as VLDL-triglyceride.

Studies *in vitro* showed that the rate of oxidation of individual PUFAs depends on the number of double bond and chain length of the PUFA (4-6). In order to gain insights into the effect of various fatty acids on lipid metabolism, it is, therefore, important to study the effect of PUFAs on the secretion of lipids and the production of ketone bodies in the liver. Although arachidonic acid has been shown to have a more pronounced hypocholesterolemic effect than linoleic acid (7), only limited information is available on the metabolic fate of 20:4n-6 in the liver.

In the present study, the effect of infused arachidonic acid on lipid metabolism was compared with that of oleic acid in isolated perfused rat liver. The metabolic effect of 5,8,11,14-nonadecatetraenoic (19:4n-5) acid was also examined because it is of interest to establish structure-function relationships of PUFAs as regulators of lipid metabolism.

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Abbreviations: 19:4n-5, 5,8,11,14-nonadecatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5,12-HETE, 5,12-dihydroxyeicosatetraenoic acid; PUFAs, polyunsaturated fatty acids; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. Oleic acid (99% purity) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Arachidonic acid (99% purity) and 5,8,11,14-nonadecatetraenoic acid (99% purity) were microbial products (8). Bovine serum albumin fraction V and 3-hydroxybutyrate dehydrogenase were obtained from Boehringer Mannheim GmbH (Germany).

Animals and diets. Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 300 g-330 g were housed in a temperature-controlled room and fed commercial rat chow (type CRF-1, Japan Charles River, Kanagawa, Japan) *ad libitum*. Liver was isolated under sodium pentobarbital anesthesia and perfused with recirculating 120 mL Krebs-Henseleit buffer (pH 7.4) containing 0.1% glucose, 1.5% bovine serum albumin and 25% washed bovine erythrocytes at a rate of 20 mL/min at 37°C. The procedure was described in detail previously (9). At the beginning of circulation, 5 mL of 20 mM fatty acid sodium salt (100 μmole) was added and the solution was continuously infused at the rate of 5 mL/hr (100 μmole/hr). At 1-hr intervals, 15 mL perfusate was removed for analysis of ketone bodies and lipids. The perfusion was continued for 4 hr.

Analysis of ketone body. Acetoacetate and β-hydroxybutyrate were measured enzymatically in a deproteinized sample of the perfusate as described previously (9).

Analysis of lipids. Lipids in perfusate and post-perfused liver were extracted and purified by the method of Folch *et al.* (10). Triglycerides (11) and cholesterol (12) were analyzed by procedures described elsewhere. Triglyceride in the perfusate and liver was separated by silica gel G thin-layer chromatography (petroleum ether/diethyl ether/acetic acid, 82:18:1, v/v/v). The triglyceride fraction was visualized with fluorescein, scraped off the plate, and treated with 14% boron trifluoride/methanol (1:1, v/v) (13). Fatty acid methyl esters were extracted with hexane and analyzed by gas-liquid chromatography on a 10% SILAR 10C column (13).

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test to evaluate the significant difference between a pair of means (14).

RESULTS

Ketone body production in perfused rat liver. Ketone body production in liver perfused with arachidonic acid was significantly lower than in liver perfused with either oleic

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Effects of Dietary Heated Fats on Rat Liver Enzyme Activity

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ABSTRACT: The objective of this study was to evaluate the effects of dietary heated fats from a commercial deep-fat frying operation on rat liver enzyme activity. The fats, partially hydrogenated soybean oil (PHSBO) used for four days and for 7 days (7-DH) for frying foodstuffs in a commercial restaurant, were fed to rats in either free access to food or by pair-feeding graded doses. All diets were isocaloric and contained 15 g/100 g of diet. Experiments were conducted with control rats fed nonheated (NH) PHSBO diet. Animals fed 7-DH diet in each set of experiments had larger amounts of cytochromes P_{450} and b_5 and greater activity of NADPH-cytochrome P_{450} reductase when compared to controls. The activities of carnitine palmitoyltransferase-I and isocitrate dehydrogenase were significantly lower in rats fed test diets in comparison to controls. A significantly depressed activity of glucose 6-phosphate dehydrogenase was also noticed for these animals when compared to those fed NH. In addition, liver and microsomal protein concentrations were significantly greater in rats fed the used oils in comparison to controls, and liver glycogen was significantly lower.
Lipids 31, 955–962 (1996).

Deep-fat fried foods, such as French fries, fried chicken, fried fish, or potato chips, are typical fast foods served in restaurants. The heated fat from a deep-fat fryer is absorbed into the fried foods, and are eventually ingested through that food. In the deep-fat frying process, the temperature of the oil is at least 180°C and the oil is used repeatedly, with many cooling and reheating cycles. It has been noted in the literature (1–3) that during deep-fat frying, moisture and air are mixed into the hot fat and that intermittent heating is more damaging to the fat than continuous heating. Under these conditions, both thermal and oxidative deterioration of the fat takes place during the heating process.

Other studies (3–5) have reported many adverse biological effects of heated and oxidized fats, such as retarded growth, enlargement and/or fatty necrosis of the liver, hair loss, and dermatitis when such fats are fed to rats. It also has been reported (3,6,7) that during deep-fat frying many toxic compounds are formed in the heated oil which may cause the deleterious effects observed when ingested by rats. Among the toxic compounds generated during deep-fat frying are carbonyl, cyclic monomer, and dimer derivatives (8,9).

Even though previous studies have focused on biochemical indices and histopathological evaluations (4,5,10), little attention has been paid to the effects of fats used for frying foodstuffs under restaurant conditions on the activity of several metabolic enzymes in rat liver.

Andia and Street (11) reported increased activity of the enzyme S-adenosylmethionine:phosphatidylethanolamine methyltransferase when thermally oxidized oil was fed to rats. Yoshioka *et al.* (12) reported low activities of thiokinase and succinate dehydrogenase when autoxidized safflower oil was fed to rats. They associated the observed metabolic effects with elevated carbonyl values of the oxidized oils.

The present study focused on the effects of partially hydrogenated soybean oil (PHSBO) that had been used for four days (4-DH) or seven days (7-DH) for frying foodstuffs. In addition, rats were pair-fed diets containing graded dose of the 7-DH oil to evaluate the effects of the amounts of used oil ingested by animals on their liver enzyme activity. The oil was obtained from a commercial fast food restaurant deep-fat frying operation producing French fries, fried fish, and fried chicken (Table 1). The following rat liver enzymes were investigated: carnitine palmitoyltransferase-I (CPT-I), isocitrate dehydrogenase (ICDH), glucose 6-phosphate dehydrogenase (G 6-PDH), and NADPH-cytochrome P_{450} reductase. The observed effects from the experimental groups of rats were compared with those of control groups of animals (in free access to food and by pair-feeding) fed diets containing nonheated (NH) PHSBO.

MATERIALS AND METHODS

The oils used in this study were obtained from a fast food restaurant which served French fries, deep fried chicken, fried fish, onion rings, mushrooms, and hush puppies (Table 1). The control was NH PHSBO and the used oil fed was taken from fryers at 4 days (4-NH) and 7 days (7-NH). The 4-NH oil was about halfway through its useful life and the 7-NH oil, although near the throwaway point, still produced good quality fried food. The oils were filtered through a paper filter

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Abbreviations: CPT-I, carnitine palmitoyltransferase-I; Cyt.b_5, cytochrome b_5; Cyt.P_{450}, cytochrome P_{450}; 4-DH/7-DH, oil heated for 4 and 7 d; G 6-PDH, glucose 6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; PHSBO, partially hydrogenated soybean oil; NH, nonheated; 4-NH/7-NH, nonheated oil at 4 and 7 d.