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)-8)]. Otherwise, abundance of the 5-olefinic unusual acids has been reported for the seed lipids of aquilegia[4),9),10)] 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.pterygusperma*; 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.

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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 + noneluted 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, noneluted 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 noneluted 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 noneluted 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	E[g]	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

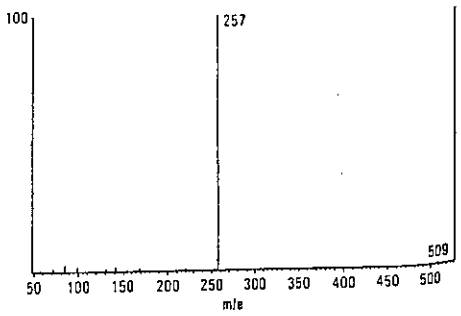

FIG. 1. Daughters of the protonated molecular ion from stearyl palmitate (18:0-16:0) produced by collision-induced dissociation with Ar; collision energy = −15 V.

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 1. 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

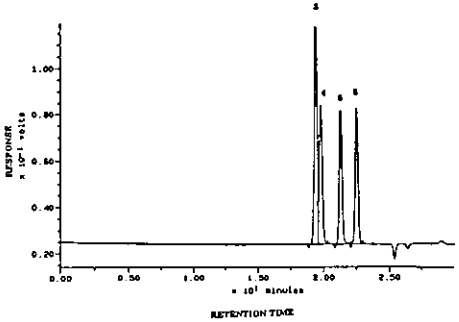

FIG. 1. High-performance size exclusion chromatography of lipid standards. Peak Identity: 3, triacylglycerols; 4, diacylglycerols; 5, monoacylglycerols; 6, free fatty acids.

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

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.

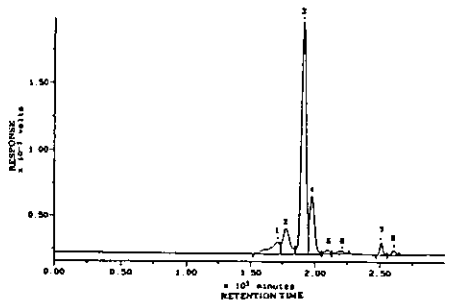

FIG. 2. High-performance size exclusion chromatogram of encapsulated oil (sample F). 1, Triacylglycerol trimer and oligomers; 2, triacylglycerol dimer. Peaks 3-6 are identified in Figure 1. Peaks 7 and 8 are residual solvent and short-chain materials of mol wt *ca.* 100, at the low mol wt exclusion limit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

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

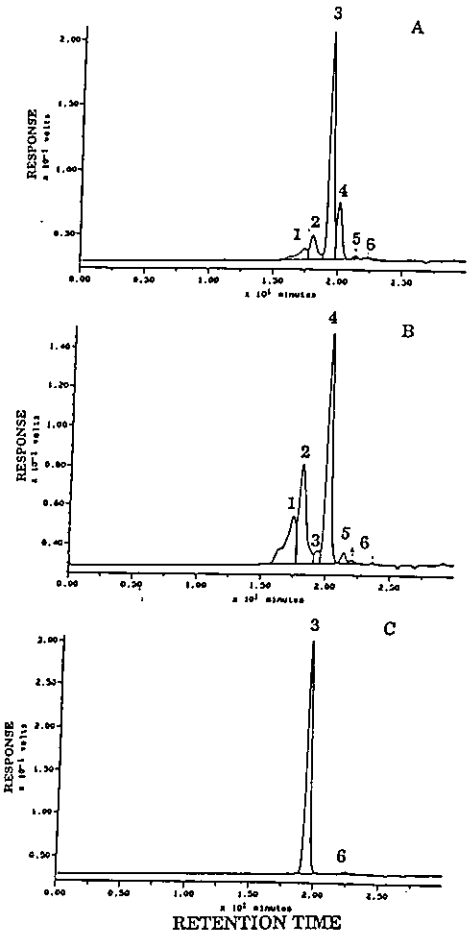

FIG. 3. High-performance size exclusion chromatogram of the polar (B) and nonpolar (C) fractions resulting from silicic acid chromatography of encapsulated oil sample F (A). Peak identity as in Figures 1 and 2.

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.

TABLE 1
Analytical Values of Oils Fed to Animals[a]

	Nonheated PHSBO (NH)	4 Days frying PHSBO (4-DH)	7 Days frying PSHBO (7-DH)
Iodine value	100.4	98.8	94.2
Free fatty acids (%)	0.002	1.39	6.28
Soap value (ppm)	—	21	83
Color	—	27 red 79 yellow	>27 red >79 yellow
GPC data (%)			
Polymeric materials	2.0	6.4	9.9
Triacylglycerols	98.0	81.0	65.8
Diacylglycerols	trace	9.8	13.3
Monoacylglycerols	trace	1.0	4.8
Fatty acid composition (wt%)			
16:0	11.1	12.5	12.9
18:0	7.3	7.1	8.0
18:1n-9	45.9	45.6	47.5
18:2n-6	30.5	30.2	27.1
18:3n-3	1.8	1.7	1.5
Minor components	3.3	2.9	3.0
CFAME (ppm)	<100	1100	1600

Food fried in test oil[b] and treatment

Food fried	Frying temperature (°F)	Frying time (min)	Pounds fried per day
Chicken	350	3	10
Fries	350	3	60
Fish	325	3	100
Vegetables	300	5	25
Hush puppies			

[a]PHSBO, partially hydrogenated soybean oils; GPC, gel-permeation or size-exclusion chromatography; CFAME, cyclic fatty acid methyl esters isomer mixture.
[b]Oil was filtered through filter paper daily to remove particulates.

daily to remove particulate material. Analytical values describing the oils are given in Table 1.

Animals, diets, and procedures. Male Sprague–Dawley weanling rats (50–60 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) and were maintained on nonpurified diet for one week in steel wire mesh cages. All animals were then assigned to three groups of 10 rats each by complete randomization using a random number table (13). One of the groups was considered the control group and was fed the 15% NH diet. Another group was fed the 15% of 4-DH diet while the last group was fed the15% 7-DH diet.

In a pair-feeding experiment, three groups of 10 rats each were fed diets containing 5, 10, and 15% of the 7-DH oil. A control group was designed identical to the previous control rats in this pair-feeding experiment. The composition of the diets fed to animals is described in Tables 2 and 3.

Procedures. All diets were prepared weekly in 1.5-kg batches and the stock diets stored in capped plastic containers under nitrogen in a 4°C cold room. Animals were then switched to their respective diets and were provided with free access to 20 g of food every day for 10 wk. The rats also had free access to tap water. Animal weights and feed intake were recorded daily. At the end of the feeding period, a total of 6–8 rats per day were randomly chosen from each of the different groups and were killed by decapitation after CO_2 inhalation for a short time. Blood was collected under heparin for plasma and the liver was quickly excised and weighed. Two-gram portions of liver were introduced into the appropriate cooled buffer (kept in ice) for the purpose of enzyme activity

TABLE 2
Diet Composition in the Free Access to Food Experiment of Rats[a]

	NH[b]	4-NH[b]	7-DH[b]
Casein[c]	150	150	150
Dextrose anhydrous	650	650	650
Vitamin mixture[c]	10	10	10
Mineral mixture AIN-76[c]	40	40	40
Fat PHSBO (NH)	150	0	0
Fat 4-DH	0	150	0
Fat 7-DH	0	0	150

[a]See Table 1 for abbreviations.
[b]g/kg Diet.
[c]From Harlan Teklad (Madison, WI).

TABLE 3
Diet Composition in the Pair-Feeding Experiment of Rats[a]

	NH[b]	5% 7-DH[b]	10% 7-DH[b]	15% 7-DH[b]
Casein	150	150	150	150
Dextrose anhydrous	600	600	600	600
Cellulose	50	50	50	50
Vitamin mixture	10	10	10	10
Mineral mixture AIN-76	40	40	40	40
Fat PHSBO (NH)	150	100	50	0
Fat 7-DH	0	50	100	150

[a]See Table 1 for abbreviations and Table 2 for company source.
[b]g/kg Diet.

assay and the rest of the liver was frozen by liquid nitrogen and stored at −70°C for future analyses.

Preparation of liver microsomes. Liver microsomes were obtained according to the method described by Lake (14), quickly frozen in liquid nitrogen, and stored at −70°C until enzyme assay the following week.

Measurement of cytochrome P_{450} ($Cyt.P_{450}$), cytochrome b_5 ($Cyt.b_5$), and NADPH-$Cyt.P_{450}$ reductase (EC 1.6.2.4) activity. $Cyt.P_{450}$, $Cyt.b_5$ contents, and NADPH-$Cyt.P_{450}$ reductase (EC 1.6.2.4) activity were determined in liver microsome fractions according to Lake (14).

CPT-I (EC 2.3.1.21) activity. CPT-I activity was assayed according to the "assay II" and using "Method C" described by McGarry *et al.* (15).

G 6-PDH (EC 1.1.1.49) and ICDH (EC 1.1.1.42) activities. About 2 g of fresh liver tissue were homogenized in about 0.04 mL/mg wet weight physiological saline solution containing 0.66 mol/L EDTA, and the enzyme activities determined according to Löhr and Waller (16).

Protein determination. The protein contents of rat liver microsomes, the 31000 × *g* supernatant fluid, and that of liver tissue were determined according to the method described by Lowry *et al.* (17).

Lipid analyses. Lipids were extracted from liver tissue according to the Folch *et al.* (18) method and the total liver lipid content was obtained by gravimetry as described by Lamboni (19). Fatty acid profiles of liver lipids were performed by gas–liquid chromatographic analyses of fatty acid methyl esters prepared according to AOCS Official Method Ce 2-66 (20). Fatty acid profiles were obtained using a model 5730 Hewlett-Packard (Palo Alto, CA) gas chromatograph equipped with a flame-ionization detector. The column used was an Omegawax 250 capillary column (30 m by 0.25 mm with a 0.25-mm film plus a 5-m guard column) (Supelco, Bellefonte, PA). The conditions were: column temperature programmed from 100°C (with a 0 time hold) to 220°C (with an 8-min hold) at a rate of 4°C/ min. The carrier gas was hydrogen at 2–4 psig with a column split ratio of 32:1. The injection temperature was 250°C and the detector temperature was 300°C.

Gel-permeation (size-exclusion) chromatography. Free fatty acids, mono-, di-, and triacylglycerols, and polymeric material were determined by gel-permeation chromatography using a Waters HPLC system with two columns of Ultrastyragel 500 Å pore size, 30-cm in length (Waters Inc., Milford, MA). A mobile phase of tetrahydrofuran at 0.7 mL/min was employed, and the detector was a Waters model 410 differential refractometer.

Glycogen determination. Glycogen content of liver tissue was obtained according to the method described by Siu Lo (21) and modified by Lamboni (19).

Statistical analysis of data. Data were analyzed by analysis of variance for a completely randomized design using StatView statistical software package (StatView SE + Graphics; 1988 Abacus Concepts, Inc., Berkeley, CA). When a significant ($P < 0.05$) F test was detected, pairwise comparisons of means between groups were performed by Fisher's protected least significant difference.

RESULTS

Weight gain, feed efficiency, and liver weight/body weight ratio. No significant differences were found in weight gain, feed efficiency, and liver/body weight ratios in the groups fed the control, 4-DH, and 7-DH diets. Data from studies with both pair feeding and free access to food also showed no significant differences (Table 4).

Protein, lipid, and glycogen. Animals fed 7-DH diet showed a greater ($P < 0.0001$) liver protein content (Table 4) than the control group of rats fed NH diet. The increased content observed when animals were fed 4-DH diet was not significantly different from that of control rats. In comparison to the 4-DH diet group of rats, the increased protein content noticed for the 7-DH diet group was highly significant.

The group of rats fed 10% 7-DH diet in the pair-feeding experiment had a significantly greater liver protein content when compared to the control group. The difference observed in liver protein content when animals were fed 15% 7-DH diet was highly significant when compared to the control group of rats (Table 4).

Liver microsomal protein (Table 4) of rats fed the 7-DH diet was significantly increased ($P < 0.0001$) compared to the control animals fed the NH diet. When rats were fed the 4-DH diet, no significant difference was observed in comparison with a control group of rats fed the NH diet. However, when the 4-DH diet group of rats was compared to the 7-DH diet group, the increased content of microsomal protein measured in the latter group was highly significant.

After 10 wk of pair-feeding rats with 15 or 10% 7-DH diet, liver microsomal protein content was significantly increased compared to the control animals (Table 4).

Liver lipid content of animals fed the 7-DH diet was significantly increased compared to the control group fed the NH diet. The slight increase of liver lipid in rats compared to controls was not significant.

In the pair-feeding experiment, the level of lipid in livers of rats fed the 15% 7-DH diet was greatly increased compared to the control animals fed NH diet. Feeding rats 10% of the

TABLE 4
Several Parameters of Control and Experimental Rats in the Free Access to Food and Pair-Feeding Experiments[a]

	Group						
	NH		4-NH		7-DH (pair-feeding and graded doses)		
Item	Free Acc. Fo. (n = 10)	Pair-feeding (n = 10)	Free Acc. Fo. (n = 10)	Free Acc. Fo. (n = 11)	5% (n = 10)	10% (n = 10)	15% (n = 10)
Liver protein (mg/g)	235.89 ± 22.46	392.65 ± 7.44	242.53 ± 21.05	479.13 ± 16.12[b,c]	398.93 ± 7.92	462.45 ± 14.90[f]	593.22 ± 17.50[b]
Microsomal protein (mg/g)	30.39 ± 1.10	30.51 ± 1.01	30.71 ± 0.66	60.40 ± 1.23[b,c]	30.55 ± 1.00	42.84 ± 1.88[b]	56.47 ± 1.55[b]
Liver lipid (mg/g)	57.73 ± 1.58	63.88 ± 1.69	59.28 ± 0.90	70.80 ± 1.27[b,c]	65.78 ± 1.40	71.72 ± 1.27[f]	79.66 ± 1.86[b]
Lipid/protein ratio	1.06 ± 0.11	0.98 ± 0.02	1.02 ± 0.06	0.60 ± 0.02[b,c]	0.99 ± 0.02	0.94 ± 0.03	0.81 ± 0.04[f]
Liver glycogen (mg/g)	10.65 ± 0.66	11.03 ± 0.78	9.05 ± 0.43	6.77 ± 0.40[b,d]	9.55 ± 0.29[e]	7.00 ± 0.41[b]	6.32 ± 0.34[b]

[a]Values are means ± SEM. Free Acc. Fo. = free access to food; see Table 1 for other abbreviations.
[b]$P < 0.0001$ when compared to the NH group of rats.
[c]$P < 0.0001$ when compared to the group of rats fed diet containing PHSBO used 4 d (4-NH) for frying foodstuffs.
[d]Nonsignificant when compared to the 4-NH group of rats.
[e]$P < 0.05$ when compared to the control group.
[f]$P < 0.01$ when compared to the control group.

7-DH diet resulted in a significant increase of liver lipid content in comparison to the control animals (Table 4). There were no significant differences in the liver lipid or fatty acid composition, regardless of the diet fed.

The lipid/protein ratio (Table 4) of rats fed the 7-DH diet was significantly lower ($P < 0.0001$) than that of the control animals. The low ratio observed when animals were fed the 4-DH diet was not significantly different from the ratio noticed for the control group of rats fed NH diet. However, when the 4-DH diet group of rats was compared to those fed the 7-DH diet, the lowest ratio observed for the latter group was significantly different ($P < 0.0001$) from that of the former group of animals.

Pair-feeding rats with graded doses of 7-DH diets showed that the 15% 7-DH diet induced the lowest lipid/protein ratio with $P < 0.01$, while groups of rats fed 5 or 10% 7-DH diets did not show any significant decrease when compared to the control animals.

Liver glycogen content (Table 4) when rats were fed the 7-DH diet was significantly decreased ($P < 0.0001$) compared to the liver glycogen of those fed the NH diet. When the 4-DH diet group of rats was compared to the 7-DH diet group, the decreased content of liver glycogen observed in the latter group of rats was significantly ($P < 0.05$) different from the liver glycogen content noticed in the former group.

In the pair-feeding experiment, the decreased values of liver glycogen observed for groups fed 10 or 15% 7-DH diets was significantly different ($P < 0.0001$) from the control animals (Table 4).

Microsomal Cyt.b_5 and Cyt.P_{450}. The 7-DH diet induced a significant increase in Cyt.b_5 content in liver microsomes in comparison with a control group of rats fed NH diet (Table 5). The increased content of Cyt.b_5 observed in animals fed the 4-DH diet was also significantly different from the content of Cyt.b_5 measured for the control group. However, the increased content of Cyt.b_5 observed for the 7-DH diet group of rats was significantly different from the level recorded for the group of rats fed 4-DH diet.

Rats pair-fed graded doses of 7-DH diets showed a highly significant increase in Cyt.b_5 when animals were fed the 15% 7-DH in comparison with the control group of rats fed the NH diet. When the 10% 7-DH was fed, the increased value was significant at $P < 0.01$ when compared to the control animals (Table 5).

Cyt.P_{450} content in liver microsomes of rats fed 7-DH diet was highly increased (Table 5) compared to the control group fed the NH diet. The group of animals fed 4-DH diet also had an increased liver Cyt.P_{450} content ($P < 0.01$). Rats fed the 4-DH diet compared to those fed the 7-DH diet exibited the greatest content of Cyt.P_{450} in the latter group, which was significantly different from that noted in the former group of animals.

In the pair-feeding experiment where rats were fed graded doses of the 7-DH diets, the content of liver microsomal Cyt.P_{450} for the group of rats fed 15% 7-DH diet was highly increased compared to the control group of rats fed 15% NH diet. When 10% 7-DH diet was fed to animals, the increase observed was significantly different from the control group (Table 5).

NADPH-Cyt.P_{450} reductase. The activities of NADPH-Cyt.P_{450} reductase (EC.1.6.2.4) measured in liver microsomes of rats fed 4-DH or 7-DH diets were increased when compared to the control group of rats fed NH diet. The increase was significant when animals were fed the 7-DH diet (Fig. 1). A significant increase was also observed when rats were fed the 4-DH diet. The greatest activity measured for the group of rats fed the 7-DH diet was significantly different from that of animals fed the 4-DH diet.

The enzyme NADPH-Cyt.P_{450} reductase (EC 1.6.2.4) when rats were pair-fed with graded doses of 7-DH diets showed an increased level of activity compared to the control group of animals fed the 7-NH diet. The increase was highly significant ($P < 0.0001$) for the 10 and 15% 7-DH diet groups of rats. It was also significant at $P < 0.001$ when rats were fed 5% 7-DH diet in comparison to the control group (Fig. 1).

CPT-I. The activity of CPT-I (EC 2.3.1.21) in liver mitochondrial membrane of rats fed 7-DH diet was significantly decreased ($P < 0.01$) in comparison with a control group of rats fed NH diet (Fig. 2).

A significant decreased activity ($P < 0.01$) of the enzyme CPT-I (EC 2.3.1.21) was observed in the pair-feeding experiment when rats were fed the 7-DH diet in comparison with the control animals. Feeding 5 or 10% 7-DH diets did not result in any significant difference when compared to the control group of rats. (Fig. 2).

ICDH. The activity of isocitrate/NADP-oxidoreductase (EC 1.1.1.42) (decarboxylating) was decreased in liver mitochondria when rats were fed either 4-DH or 7-DH diets in comparison with a control group of rats fed NH diet (Fig. 3). The decreased activity observed for the 7-DH diet group of rats was significantly different from the control group at $P < 0.05$. However, the decreased activity observed when animals were fed 4-DH diet was not significant when compared to the control group. The lowest activity measured for the 7-DH diet group of rats was not significantly different from that of animals fed the 4-DH diet.

TABLE 5
Hepatic Microsomal Cytochromes Contents of Control and Experimental Rats in the Free Access to Food and Pair-Feeding Experiments[a]

	Group						
	NH		4-DH		7-DH (pair-feeding and graded doses)		
Cytochromes	Free Acc. Fo. (n = 8)	Pair-feeding (n = 10)	Free Acc. Fo. (n = 10)	Free Acc. Fo. (n = 11)	5% (n = 10)	10% (n = 10)	15% (n = 10)
Cytochrome b_5 content (nmol/mg microsomal protein)	0.37 ± 0.01	0.46 ± 0.02	0.54 ± 0.02[b]	0.84 ± 0.04[c,d]	0.48 ± 0.03	0.64 ± 0.05[b]	0.75 ± 0.08[c]
Cytochrome P_{450} content (nmol/mg microsomal protein)	1.03 ± 0.03	1.04 ± 0.03	1.47 ± 0.05[b]	1.97 ± 0.08[c,e]	1.13 ± 0.06	1.32 ± 0.09[b]	1.68 ± 0.08[c]

[a]Values are means ± SEM. See Tables 1 and 4 for abbreviations.
[b]$P < 0.01$ compared to the control group of rats fed diet containing NH PHSBO.
[c]$P < 0.0001$ compared to the NH group of rats.
[d]$P < 0.0001$ compared to the group of rats fed diet containing PHSBO used 4 d (4-DH) for frying foodstuffs.
[e]$P < 0.001$ compared to the 4-DH diet group of rats.

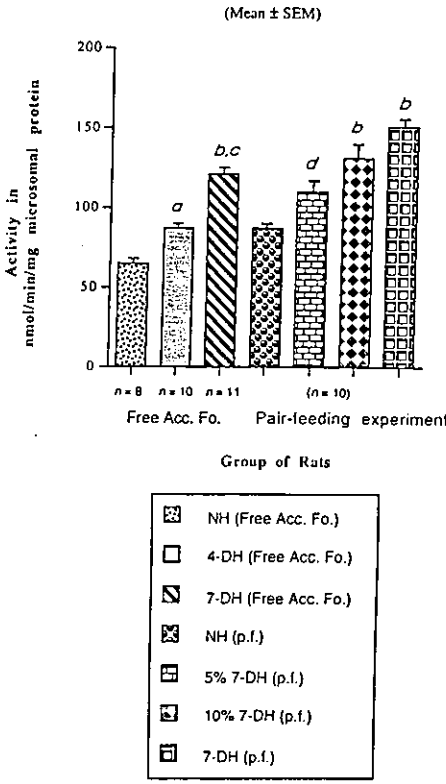

FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 4-DH, and 7-DH diets. NH, control group of rats fed diet containing nonheated (NH) partially hydrogenated soybean oil (PHSBO); 4-DH, group of rats fed diet containing PHSBO used 4 d (4-DH) for frying foodstuffs; 7-DH, group of rats fed diet containing PHSBO used 7 d (7-DH) for frying foodstuffs. [a]$P < 0.001$ when compared to the control group of rats fed the NH diet. [b]$P < 0.0001$ when the 7-DH diet group of rats was compared to the NH group of rats. [c]$P < 0.0001$ when compared to the 4-DH diet group of rats. Free Acc. Fo., free access to food; p.f., pair-feeding.

In the pair-feeding experiment, the decreased activity of the enzyme ICDH (EC 1.1.1.42) observed when rats were fed 15% 7-DH diet was significantly different from the control animals. Feeding 5 or 10% 7-DH diets did not show any significant difference when compared to the control group of rats. (Fig. 3).

G 6-PDH. The activity of (EC 1.1.1.49) D-glucose 6-phosphate/NADP-oxidoreductase measured in the liver of rats fed either 4-DH or 7-DH diets was depressed when compared with a control group of rats fed NH diet (Fig. 4). When rats

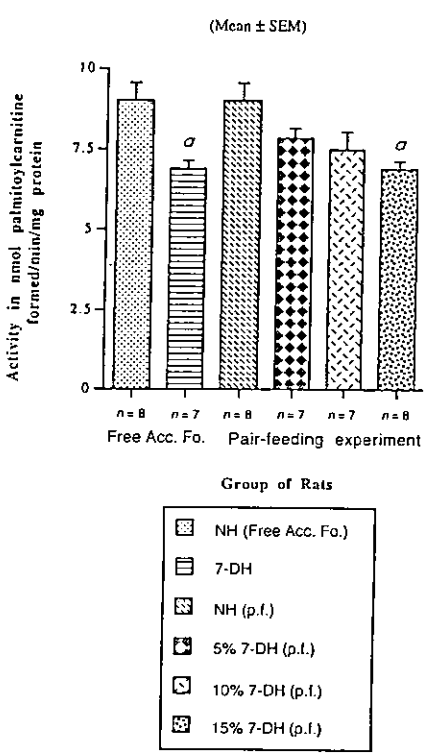

FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH and 7-DH diets. Abbreviations and legend descriptions as in Figure 1. $^{a}P < 0.01$ compared to the control group of rats fed the NH diet.

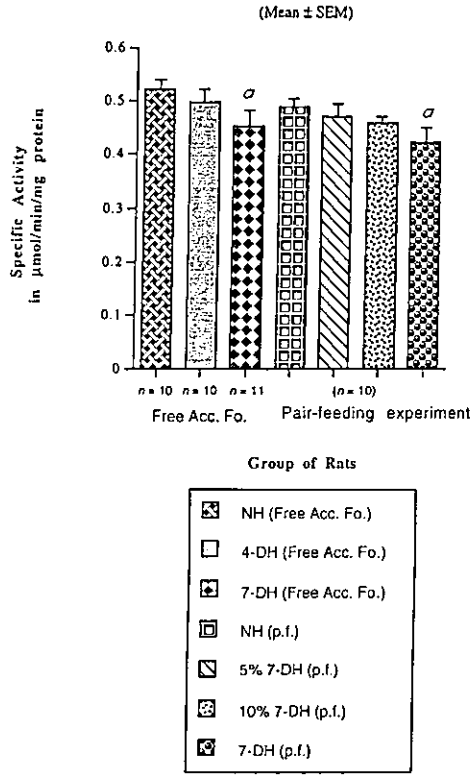

FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 4-DH, and 7-DH diets. $^{a}P < 0.05$ when the 7-DH diet group of rats was compared to the NH group of rats. See Figure 1 for abbreviations and legend descriptions.

were fed the 7-DH diet, the depressed activity observed for the enzyme was highly significant when compared to the control group. Although a depressed activity was observed when animals were fed the 4-DH diet, the difference was not significant when compared to the control group of rats. The lowest activity of G 6-PDH measured for the group of rats fed the 7-DH diet was significantly different from that observed for the group of animals fed the 4-DH diet.

When rats were pair-fed graded doses of the 7-DH diets, a depressed activity of the oxidoreductase (EC 1.1.1.49) was noticed when compared with a control group of rats fed the NH diet. Animals which were fed the 15% 7-DH diet displayed the significantly lowest activity among groups in comparison to the control group. When 10% 7-DH diet was fed to animals, the activity of G 6-PDH was significantly depressed when compared to the control group of rats. Feeding 5% 7-DH diet led to a depressed activity at $P < 0.05$ compared to the control animals (Fig. 4).

DISCUSSION

The weight gain data showed trends that were noted by previous researchers (22–24) who worked with laboratory heated fats. In these studies, feeding rats with commercially heated fats led to growth depression, suggesting that the heated oils may contain substances that prevent animals fed such fats from growing properly. The weight gain declined after 35 d of feeding such a diet to animals. This may suggest that the accumulated deleterious compounds generated during the commercial deep-fat frying process must be ingested at certain levels to exert the adverse nutritional effects of such fats when fed to rats.

Feed efficiency data were not significantly different among groups fed either diets in the free access to food or by pair-feeding experiments, suggesting that the effects observed in this study cannot be attributed to the amount of diet ingested by the rats and may simply reflect the content of the oils fed to animals.

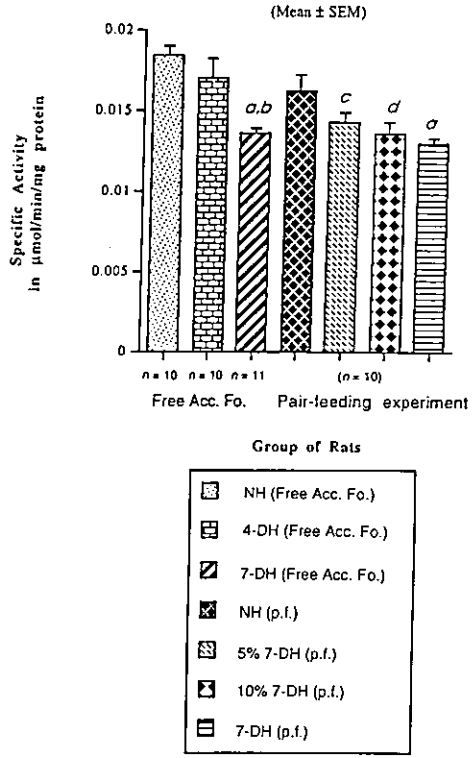

FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 4-DH, and 7-DH diets. See Figure 1 for abbreviations and legend descriptions. $^{a}P < 0.0001$ when the 7-DH diet group of rats was compared to the NH group of rats. $^{b}P < 0.0001$ when the 7-DH diet group of rats was compared to the NH group of rats. $^{c}P < 0.0001$ when compared to the 4-DH diet group of rats. ^{d}Nonsignificant when compared to the 4-NH group of rats.

When rats were fed the 7-DH diet, their liver weight/body weight ratio (Table 3) was not significantly higher than those fed either NH or 4-DH diets, but did suggest an increase in liver size. In addition, the increased level of protein or lipid content in the liver may have contributed to the abnormal liver weight of animals fed the used oils. Pair-feeding rats with the 7-DH diet led to similar results. The increased content of liver protein observed in this study when rats were fed the used oil either *ad libitum* or by pair-feeding agrees with the findings of Miller and Landes (25), who reported an increased content of liver protein when rats were fed laboratory thermally oxidized fats.

The significantly increased level of hepatic lipid obtained when rats were fed the 7-DH diet either in a pair-feeding or free access to food experiment suggests an accumulation of liver lipids in these animals in comparison with the control group of rats fed the diet containing the NH PHSBO. This is probably due to the developing fatty liver previously reported (10). In addition, the lipid/protein ratio (Table 3) of animals fed the 7-DH diet in a pair-feeding or free access experiment clearly confirms the excessive amount of protein being retained or produced when compared to that of lipid in liver tissue of the same group of rats. The ratio was significantly lower in the group of rats fed the 7-DH diet when compared to the control animals fed the NH diet than it was in the group of rats fed the 4-DH diet. A similar lipid/protein ratio was noted in the pair-feeding experiment when the 7-DH diet group of rats was compared to the control group of animals fed the 15% NH diet.

With regard to the effect of the amount of protein on the activity of liver microsomal mixed-function oxidase system (26–28), the highly significant increased content of Cyt.P_{450} (Table 4) in animals fed the 7-DH diet clearly demonstrates an effect of the components generated in the used oil. Rats fed the 4-DH diet also showed an increased level of Cyt.P_{450} in the microsomal fractions at $P < 0.01$. These enzyme systems increase in activity in the presence of xenobiotic materials.

The significantly decreased activity of ICDH observed when rats were fed the 7-DH diet (Fig. 3) suggests an impairment of the trichloroacetic acid cycle enzyme activity. This may have resulted in an accumulation of citrate, which was transferred through the mitochondrial membrane under passive diffusion and regenerated the cytosol with acetyl-CoA under ATP-citrate lyase (EC 4.1.3.6) activity. The excess of acetyl-CoA in the cytosol could then have undergone fatty acid synthesis. The complementary NADPH required for fatty acid synthesis could be supplied by L-malate/NADPH oxidoreductase (malic enzyme) (EC 1.1.1.40). Because a control group of rats was fed the same level (15%) of the NH PHSBO, the decreased activity of ICDH measured in the experimental groups of rats may have other origins that might be attributed to effects of secondary degradation products, such as carbonyl compounds or cyclic fatty acid monomers generated during frying.

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Effect of Dietary Vitamin E Levels on Fatty Acid Profiles and Nonenzymatic Lipid Peroxidation in the Guinea Pig Liver

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ABSTRACT: Guinea pigs were fed for five weeks with three diets containing different levels of vitamin E: LOW (but nondeficient, 15 mg of vitamin E/kg diet), MEDIUM (150 mg/kg diet), and HIGH (1,500 mg/kg diet). Dietary vitamin E supplementation did not change oxidative stress indicators in the hydrophilic compartment but increased liver α-tocopherol in a dose-dependent way and strongly decreased sensitivity to nonenzymatic *in vitro* liver lipid peroxidation. This last effect was already observed in group MEDIUM, and no further decrease in *in vitro* lipid peroxidation occurred from group MEDIUM to group HIGH. The protective effect of vitamin E against *in vitro* lipid peroxidation was observed even though an optimum dietary concentration of vitamin C for this animal model was present in the three different vitamin E diets. Both HIGH and LOW vitamin E decreased percentage fatty acid unsaturation in all phospholipid fractions from membrane origin in relation to group MEDIUM. The results, together with previous information, show that both vitamin E and vitamin C at intermediate concentrations are needed for optimal protection against lipid peroxidation and loss of fatty acid unsaturation even in normal nonstressful conditions. These protective concentrations are higher than those needed to avoid deficiency syndromes.
Lipids 31, 963–970 (1996).

Many studies suggest the involvement of oxidative damage in the etiology of important diseases such as cancer and arteriosclerosis. There is increasing epidemiological evidence (1,2), supported by some longitudinal prospective human studies (3,4), indicating that antioxidants like vitamin E and C are protective against the development of those diseases. This is probably due to their capacity to reduce oxidative damage to lipids (5,6) and other cellular macromolecules (7). Thus, there is great interest in obtaining a better knowledge about the *in vivo* effects of different dietary doses of these vitamins on tissues. Vitamin E or C is suitable for increasing antioxidant capacity safely and easily, because they are susceptible to dietary manipulation, whereas supplementation with antioxidant enzymes or GSH (which is under feedback cellular control) is not feasible in humans.

Vitamin E is considered the principal antioxidant defense against lipid peroxidation in cell membranes in mammals. Lipid peroxidation is a degradative chain reaction of oxygen radicals with unsaturated fatty acids. The most important role of vitamin E in tissues seems to be the protection of membrane polyunsaturated fatty acids (PUFA) against the deleterious effects of oxygen radicals. Specific effects of α-tocopherol that do not involve its antioxidant properties and act upon the architecture of membranes by controlling their lipid profile also have been suggested (8,9). Recent reports have also shown that vitamin E can work as a prooxidant when present in high concentrations *in vitro* (10,11); presumably *via* effects of tocopheroxyl radical.

The notion that the optimum levels of vitamins E and C to protect against *in vivo* oxidative stress are substantially higher than the recommended dietary allowance (RDA) needed to avoid deficiency syndromes is gaining experimental support. The guinea pig is the ideal laboratory animal model to perform diet-controlled *in vivo* experiments since (like higher primates) it cannot synthesize either ascorbate or α-tocopherol. We have recently shown that optimum dietary vitamin C levels in the guinea pig for protection against endogenous oxidative stress in liver are much higher than the minimum daily requirement of this animal, whereas vitamin C megadoses do not bring about further protection and are even detrimental for membrane PUFA (12). We study here the effect of three very different doses of vitamin E—from low but nondeficient levels to a very high dose—using the same guinea pig model. The study was performed at the optimum dose of vitamin C found previously (12) in order to test the efficacy of vitamin E over an adequate vitamin C background. Oxidative stress was studied both for mainly hydrophilic [glutathione redox ratio (GSH/GSSG) and protein oxidation] and lipid-dependent parameters (lipid peroxidation and fatty acid composition). Similar to what was found for vitamin C (12), intermediate dietary levels of vitamin E, sixfold higher than the minimum daily requirement,

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Abbreviations: ANOVA, analysis of variance; DNPH, 2,4-dinitrophenylhydrazine; GSH/GSSG, glutathione redox ratio; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TBA, thiobarbituric acid; TCA, trichloroacetic acid; UI, unsaturation index; U/S, unsaturated/saturated ratio.

The Effects of Cyclic Fatty Acid Monomers on Cultured Porcine Endothelial Cells

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ABSTRACT: The popularity of polyunsaturated oils used in food applications and preparation continues to appreciate as a result of positive health claims. With polyunsaturated oils inherently more susceptible to oxidative and thermal degradation, the formation of new fatty acid species increases considerably. The presence of one species known as cyclic fatty acid monomers (CFAM) has been detected as a component of many oils subjected to various thermal processes including deep-fat frying. The effect of CFAM on metabolic processes has not been fully characterized. In this study, confluent porcine aortic endothelial cells incorporated CFAM into their polar and nonpolar lipid fractions following a 48-h exposure to 31 and 62 ppm CFAM in the culture medium. Subsequently, the influence of CFAM incorporation on various membrane-dependent physical properties and biochemical processes was investigated. CFAM decreased the lipid packing order of the membrane bilayer core but did not alter the lipid packing order of lipid chain segments at or near the lipid–water interface of the membrane. CFAM led to significant reductions in Ca^{2+} ATPase activity and monolayer integrity while eliciting a significant increase of prostacyclin synthesis and secretion.

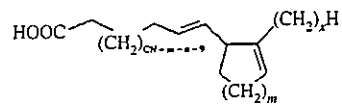
Lipids 32, 925–933 (1997).

During the past two decades, a great deal of progress has been made toward defining the role of dietary fats in the pathogenesis of certain Western diseases. For numerous such conditions including cardiovascular disease and certain cancers, the type and amount of dietary fat have been implicated as positive risk factors (1–4). Several basic pathogenic mechanisms associated with these diseases often can be characterized by altered cell function including aberrant biochemical processes resulting from physical changes at the phospholipid membrane level (5,6). The influence of dietary lipids becomes significant in membrane-dependent properties owing to the importance of fatty acids in membrane phospholipid structure.

In particular, the pathogenesis of vascular disease suggests that dietary fatty acids play an important role especially owing to their influence on the function of the vascular endothelium. Incorporation of polyunsaturated fatty acids (PUFA) into cultured endothelial cells has been shown recently to promote disruption of normal endothelial cell functions (7–9) which would appear to contradict the current hypothesis that PUFA minimize the incidence of atherosclerotic episodes. Furthermore, the inherent instability of PUFA to oxidative and thermal stress can lead to the formation of a host of potential lipid degradation and oxidation products, caused by *in vivo* processes and/or dietary consumption, which subsequently may be presented to endothelial cells .

A popular source of ingested lipid breakdown products is the consumption of deep-fat fried foods. In the case of vegetable oils used in deep-fat frying processes, the formation of lipid oxidation products can be substantial even though a majority of vegetable oils are modified for increased stability by reducing the degree of unsaturation using catalytic hydrogenation. The formation of these partially degraded lipid products can be attributed to the exposure of the frying medium to water (from food), oxygen, metals (from food or equipment), and the high temperature of the frying process (10,11). Various lipid breakdown products have been identified in frying fats and oils derived from both animal and vegetable sources (12,13) and are absorbed by the fried food (14–16). If a frying medium has been abused and overused, accumulation of lipid breakdown products can be significant (17,18).

Cyclic fatty acid monomers (CFAM) are a class of lipid oxidation products generated especially in heated vegetable oils (19–21). A ring structure is formed within the same fatty acyl chain to create a CFAM molecule [Scheme 1 where *x*



SCHEME 1

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Abbreviations: BSA, bovine serum albumin; CFAM, cyclic fatty acid monomer; DPH-PA, 1,6-diphenyl-1,3,5-hexatriene propionic acid; FAME, fatty acid methyl esters; FBS, fetal bovine serum; PUFA, polyunsaturated fatty acids; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene.

represents number of methylene groups determined by the formula: CN (carbon number) $- m - n - 9$] as a result of a new covalent linkage between two nonadjacent carbon atoms. Because of their unique structure, the physical characterization of CFAM has been under investigation since their identification in heated oils and only recently have novel analytical techniques been applied to definitively confirm the structure of CFAM (22–24).

The metabolic and physiologic effects of fatty acids and lipid oxidation products in tissue culture systems have been reported in the literature (25–29). While CFAM from heated linseed oil have specific effects on the electrophysiological function of cultured rat myocardiocytes (30), the focus of this study was to examine the influence of CFAM derived from heated linseed oil on various physical and biochemical cellular parameters of cultured endothelial cells using measurements of membrane lipid packing order, membrane integrity/permeability, prostacyclin synthesis, and ATPase activity.

EXPERIMENTAL PROCEDURES

Preparation and isolation of CFAM. Linseed oil (Cargill, Riverside, ND) was heated at 275°C under nitrogen for 12 h as described previously (21). The protocol for the isolation and purification of CFAM includes saponification of the heated linseed oil, esterification of fatty acids, separation of fatty acid methyl esters (FAME) from polar compounds using silicic acid column chromatography, and isolation of CFAM methyl esters by urea fractionation of the nonpolar FAME fraction (21,31). The urea fractionation step was conducted twice using a 3:1 ratio (w/w) of urea to FAME.

Preparation of primary endothelial cell culture. Endothelial cells were isolated from a 6- to 8-inch section of the descending aortas of freshly slaughtered 5- to 8-mon-old pigs. Two- to three-inch segments of aortas were meticulously trimmed of fat and serosa, branch points ligated, rinsed with modified Hank's buffered salts solution, and then filled with 0.1% collagenase solution (ICN Biomedicals, Costa Mesa, CA). Following incubation for 30 min at 37°C, the enzyme/cell solution was decanted then centrifuged at 200 × *g* for 5 min. The pellet was resuspended in Medium 199 (M-199) containing 10% fetal bovine serum, 100 μg/mL heparin, and 100 μg/mL streptomycin. The cell suspension was plated on 60-mm culture dishes and incubated at 37°C in a humidified 5% CO_2 atmosphere.

After 12 h, the medium was changed to remove any nonadherent material, and the adherent cells were viewed under an inverted microscope equipped with Hoffman modulation. The cells had a cobblestone morphology typical of cultured endothelial cells. The cells were allowed to grow to confluence then were passaged at a dilution factor of 1:3. Further characterization of the endothelial monolayer was performed using factor VIII histochemical staining [1° antibody-goat antihuman factor VIII (ICN Biomedicals); 2° antibody-rabbit antigoat labeled with fluoroscein isothiocyanate (Cappel Labs, Cochransville, PA)], and dil-acetylated low density lipoprotein (Biomedical Technologies Inc., Boston, MA). The fluorescence staining techniques revealed the cultures to be essentially pure endothelial cells. Beginning at passage 4, cells were frozen in the M-199 growth medium containing 10% dimethylsulfoxide; then they were stored in liquid nitrogen until use.

Treatment of endothelial cells with medium containing CFAM. The culture medium containing CFAM was prepared by adding the sodium soaps of CFAM to M-199 containing fatty acid free bovine serum albumin (BSA) (Fraction V; ICN Biomedicals) at a molar ratio of 6:1, respectively. The fatty acid-free BSA was slowly dissolved in M-199 at 37°C under constant stirring. The sodium soaps were prepared by the addition of 6 N NaOH to CFAM (free fatty acid form) dissolved in hexane. The solution was dried under nitrogen then redissolved in warm water. The sodium soaps were added dropwise followed by mild stirring for 3–4 h at 37°C. The CFAM–BSA solution was sterilized by passage through a 0.2-μm cellulose acetate filter under vacuum. This solution was used as stock for dilution with M-199 to the desired levels of CFAM in the medium. Two concentrations of CFAM at 31 and 62 parts per million (ppm) were selected which corresponded to 112 and 224 μM, respectively. These concentrations fall within the spectrum of fatty acid supplementation levels in cell culture systems previously described in the literature (7,8,38,40,41).

Isolation of total cellular lipids and separation of nonpolar and polar fractions. Following incubation with CFAM for 48 h, a total lipid extract was isolated from freshly homogenized endothelial cells using the method described by Folch *et al.* (32) and stored at −80°C until further use. The total cellular lipid extract was separated into nonpolar and polar fractions using silica gel solid phase extraction (33). The extraction was confirmed by silica gel thin-layer chromatography using a mobile phase of hexane/diethyl ether/methanol/acetic acid (90:20:5:2, by vol) and visualized by the application of concentrated sulfuric acid/water (1:1, vol/vol) containing 0.05% potassium dichromate followed by heating at 120°C for 10–15 min.

Determination of phospholipid classes using high-performance liquid chromatography. The relative percentage of the major phospholipid classes present in the polar lipid fraction was determined by high-performance liquid chromatography using the method developed by Christie (34). The separation of phospholipid classes was achieved using a modified tertiary gradient mobile phase system with a Spherisorb silica-3 column (Alltech, Deerfield, IL), and the separated phospholipid components were detected using an evaporative light-scattering detector (Varex, Rockville, MD).

Determination of cholesterol and fatty acid composition using gas–liquid chromatography (GLC). The cholesterol content in the nonpolar lipid fraction was quantitated by GLC analysis using stigmasterol as an internal standard wtih an HP-5 column (Hewlett-Packard, Avondale, PA) installed in an HP 5890 Series II Plus GC (Hewlett-Packard).

The fatty acid composition of the nonpolar and polar lipid fractions as well as M-199 containing 5% fetal calf serum was determined by GLC of native and completely hydrogenated FAME (35). FAME mixtures were subjected to GLC analysis using a CP-Sil 88 column (Chromapack, Raritan, NJ) installed in an HP 5890 Series II GC (Hewlett-Packard).

Hydrogenation. CFAM methyl esters and cellular FAME were catalytically hydrogenated over platinum oxide using a microhydrogenator (Supelco, Inc., Bellefonte, PA) (20). Briefly, 100–150 mg CFAM was added to a thick-walled glass reaction tube, then diluted in 20 mL anhydrous methanol. Platinum oxide (10–15 mg) was added under constant stirring. The reaction chamber was evacuated then pressurized with 10 psi hydrogen. After 30 min, the reaction mixture was filtered through a 25-mm filter disk containing a polytetrafluoroethylene membrane with a pore size of 0.2 μm. The methanol was evaporated in a warm water bath under a stream of nitrogen.

Measurement of CFAM cytotoxicity. Endothelial cells were grown to confluence then exposed to CFAM-containing media for 48 h. The cell number was determined using a hemocytometer, and viablility was assessed by trypan blue dye exclusion.

Measurement of membrane lipid packing order. The measurement of membrane lipid packing order of cultured endothelial cells was accomplished using methods adapted from Block *et al.* (36) and Sheridan and Block (37). Dislodged endothelial cells (1×10^6 cells) were suspended in M-199 with 10% FBS, centrifuged, decanted, diluted in 0.3 mL of ice-cold 50 mM phosphate buffer containing 0.15 M potassium chloride at pH 7.4 (phosphate buffer), and stored on ice until preparation for the membrane polarization determination. The cell suspension was warmed to room temperature and an equal volume of 10 μM 1,6-diphenyl-1,3,5-hexatriene propionic acid (DPH–PA) or 20 μM DPH working solutions in phosphate buffer was added to the cell suspension. Following mixing, the cell-fluorescence probe reaction mixtures were allowed to incubate in the dark for 20 min at room temperature. The cell–probe mixture was centrifuged and rediluted in 1 mL phosphate buffer warmed to 37°C. The cell suspension was placed in a quartz cuvette containing 1 mL of phosphate buffer at 37°C. The anisotropy was determined at 37°C using an ISS spectrofluorometer (ISS, Bellefonte, PA) configured in the L-format. The excitation and emission wavelengths were 366 and 430 nm, respectively, for DPH–PA, 365 and 428 nm, respectively, for 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH), and 362 and 430 nm, respectively, for DPH. A cell suspension prepared without the fluorescence probes was used to correct for background fluorescence. The use of cutoff filters was employed to minimize the effects of light scattering.

Measurement of monolayer integrity. Monolayer integrity was assessed by measuring albumin transfer across a confluent endothelial cell monolayer as described by Henning *et al.* (38). Endothelial cells were seeded at confluence density (1.25×10^5 cells/cm^2) on Cellagen 24-well plate inserts (ICN Biomedicals). The cells were incubated for 8 h to ensure that the cells adhered to the Cellagen inserts then observed for monolayer confluence by microscopic examination. The cells were treated with CFAM media for 48 h. Following the removal of media from the insert (upper) and well (lower) chambers, the concentration of BSA in the media from the lower chamber was detected by measuring the absorbance at 630 nm following reaction with bromocresol green.

Measurement of calcium ATPase activity. Calcium ATPase activity in cultured endothelial cells was assayed using the procedure of Henning *et al.* (8). Briefly, dislodged endothelial cells ($2–3 \times 10^6$ cells) were suspended in M-199 with 10% FBS, centrifuged, decanted, and diluted with 0.25 mL of ice-cold 0.1 M Tris buffer at pH 7.4 and placed on ice. The cell suspension was subjected to ultrasonic homogenization for 15 s in an ice bath. Calcium ATPase activity was assayed following the addition of 50 μL cell homogenate to the following reaction mixture: 500 μL of 0.1 M Tris buffer, pH 7.4; 50 μL of 0.1 M $CaCl_2$; 50 μL of 0.01M ATP. The sample was incubated for 15 min at 37°C, and the reaction was stopped by adding 0.5 mL of cold trichloroacetic acid and the inorganic phosphorus content was determined (39). Total protein was determined by a protein assay kit (Pierce, Rockford, IL).

Measurement of prostacyclin synthesis. Confluent monolayers of endothelial cells in 24-well plates were treated with medium containing CFAM–BSA or BSA alone. Indomethacin (5 μM), a cyclooxygenase inhibitor, was employed to inhibit prostacyclin synthesis, and this incubation served as a positive control. At various times, the medium was removed and was frozen at −20°C until analysis for prostacyclin. The prostacyclin content was determined by measuring the amount of 6-keto-$PGF_{1\alpha}$, its stable metabolite, using an enzyme immunoassay (Amersham, Chicago Heights, IL).

Prior to performing the enzyme immunoassay, media aliquots were subjected to solid-phase extraction. This purification step was employed to isolate a fraction containing 6-prostaglandin $F_{1\alpha}$ free from nonesterified fatty acids which may interfere with the immunoassay determination of prostaglandins (40). Following acidification to pH 3 with 6 N hydrochloric acid, the samples were applied to solid-phase extraction columns containing C_2 packing. The prostaglandin fraction was eluted from the column with 5 mL of methyl formate after washing with 5 mL of methanol, 10% ethanol in water, then hexane, respectively.

Statistical analysis. The influence of CFAM treatment on the mean responses for the various biological parameters was tested for statistical significance using one-way analysis of variance. For each endpoint, the treatment means were compared in pairs using the Student's *t* test. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity and membrane lipid composition. The cytotoxicity of CFAM was determined in cultured endothelial cells. As measured by the exclusion of trypan blue dye, viability of

TABLE 1
Composition of Lipid Materials Isolated from Cultured Porcine Aortic Endothelial Cells Incubated with or without CFAM–BSA (for 48 h)[a]

Parameter	Added CFAM–BSA (ppm) 0	31	62
Total lipid extract (mg/10^6 cells)	0.41 (0.02)	0.38 (0.04)	0.33 (0.04)
Polar lipid fraction (mg/10^6 cells)	0.35 (0.03)	0.33 (0.02)	0.31 (0.02)
Nonpolar lipid fraction (mg/10^6 cells)	0.06 (0.01)	0.05 (0.02)	0.02 (0.02)
Total cholesterol (μmol/10^6 cells)	0.10 (0.01)	0.10 (0.01)	0.10 (0.02)
Cholesterol-to-phospholipid (μmol/mg)	0.28 (0.04)	0.30 (0.03)	0.34 (0.04)
Phospholipid-to-protein (mg/mg)	0.98 (0.08)	0.98 (0.05)	0.80 (0.07)
Phospholipid class (wt)%			
Phosphatidylcholine	62.0 (2.1)	61.6 (1.7)	60.3 (1.6)
Phosphatidylethanolamine	31.5 (1.7)	32.2 (1.0)	34.5 (1.5)

[a]Values represent mean (± SD) for a single determination using two samples per treatment group; CFAM–BSA, cyclic fatty acid monomer–bovine serum albumin.

confluent endothelial cells was not significantly changed following treatment with BSA or CFAM–BSA media during the 48-h incubation interval.

The amount of material from the nonpolar and polar fractions of endothelial cell total lipid extracts was determined along with total cholesterol, enabling the ratios of cholesterol-to-phospholipid, cholesterol-to-protein, and phospholipid-to-protein to be calculated (Table 1). Additionally, the distribution of the major phospholipid classes isolated in the polar lipid fraction was determined (Table 1). The treatment of endothelial cells with CFAM–BSA did not cause significant change in the amount of total, nonpolar or polar lipid fractions, the distribution of phospholipid classes, or the cholesterol-to-phospholipid ratio. However, the ratio of phospholipid-to-protein decreased in cells treated with 62 ppm CFAM–BSA.

The incorporation of CFAM did not result in a substantial change of the relative amounts of unsaturated and saturated fatty acids. Using the ratio of total-unsaturated-to-total-saturated fatty acids, values of 1.6, 1.7, and 1.6 were determined for the control, 31 ppm CFAM–BSA, and 62 ppm CFAM–BSA groups, respectively. Upon CFAM–BSA supplementation, cultured endothelial cells incorporated CFAM into membrane phospholipids as well as neutral lipids. The fatty acid composition of the membrane and neutral lipids from endothelial cells revealed that the relative amount of CFAM within the membrane phospholipids from treatment with 31 and 62 ppm CFAM–BSA reached 3.2 and 4.3%, respectively (Table 2). CFAM containing cyclopentyl rings were preferentially incorporated into both membrane phospholipids and neutral lipids (Table 3). The polar and nonpolar lipid fractions exhibited identical CFAM GLC peak patterns to the pure fraction of hydrogenated CFAM (not shown).

Membrane lipid packing order. The steady-state anistropy (r) of DPH (Fig. 1) was found to decrease significantly in cultured endothelial cells following the exposure to CFAM–BSA at both treatment levels compared to BSA controls ($P < 0.05$). The decrease was more significant by treatment with 62 ppm CFAM–BSA than 31 ppm ($P < 0.05$). Since lipid packing order is directly related to r, the lipid packing order of the hydrophobic core region decreased as a result of CFAM incorporation into endothelial cell membrane lipids as measured by DPH. This perturbation in order of the bilayer core was not reflected in lipid chain segments at or near the lipid–water interface of the membrane bilayer as shown by the lack of change in the r of DPH–PA (Fig. 2) and TMA–DPH (Fig. 3).

Presence of intracellular triglycerides. The presence of intracellular triglycerides using Nile red dye visualization was observed in cultured endothelial cells following incubation with 0, 31, and 62 ppm CFAM–BSA media for 48 h (Fig. 4). The location of intracellular lipid in the 0 ppm CFAM–BSA and BSA controls remained primarily around the nuclei of endothelial cells. Treatment with CFAM–BSA appeared to cause the formation of intercellular lipid droplets, but a loss of lipid around the nuclei of endothelial cells was observed (Fig. 4).

Monolayer integrity. Exposure to CFAM–BSA resulted in an increase of albumin movement across cultured endothelial cell monolayers (Fig. 5). Both levels of CFAM–BSA caused a significant increase in albumin movement vs. the 0 ppm CFAM–BSA and BSA control treatments ($P < 0.05$) with the influence of 62 ppm CFAM–BSA being more pronounced than 31 ppm ($P < 0.05$).

Calcium ATPase function. The activity of calcium ATPase decreased significantly ($P < 0.05$) in cultured endothelial cells treated with CFAM–BSA compared to the respective BSA controls (Fig. 6). The magnitude of the decreased ATPase activity corresponded to an increase in the level of CFAM–BSA in the media.

Prostacyclin synthesis. The production of 6-keto-prostaglandin $F_{1\alpha}$ was determined in cultured porcine aortic endothelial cells as a function of the incubation time and media concentration of CFAM–BSA (Table 4). The presence of CFAM–BSA in the media caused a significant increase in the production of prostacyclin compared to BSA alone. The production of prostacyclin was inhibited significantly owing to

TABLE 2
Fatty Acid Composition (wt%) of M-199 Containing 5% FBS and the Nonpolar (NP) and Polar (P) Lipid Fractions Isolated from Cultured Porcine Aortic Endothelial Cells Incubated with or without CFAM–BSA (for 48 h)[a]

Fatty acid	M-199 + 5% FBS	Added CFAM–BSA (ppm) 0 NP	0 P	31 NP	31 P	62 NP	62 P
14:0	3.1	1.5 (0.2)	0.8 (0.1)	1.3 (0.2)	0.7 (0.1)	1.2 (0.1)	0.7 (0.1)
14:1	1.2	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.3 (0.1)	0.3 (0.1)
16:0	26.6	18.7 (0.5)	19.0 (0.7)	17.3 (0.5)	20.2 (0.8)	16.5 (0.6)	20.5 (0.4)
16:1	1.4	2.1 (0.1)	1.0 (0.1)	2.4 (0.3)	0.6 (0.1)	1.8 (0.1)	0.5 (0.1)
18:0	39.4	23.4 (0.6)	17.1 (0.3)	24.3 (0.4)	14.4 (0.7)	21.4 (0.7)	14.4 (0.5)
Σ18:1	18.1	21.8 (0.5)	34.6 (0.1)	21.2 (0.2)	32.2 (0.6)	20.9 (0.1)	30.3 (0.5)
18:2	1.4	2.0 (0.2)	5.5 (0.3)	4.2 (0.2)	6.2 (0.1)	4.0 (0.2)	7.4 (1.0)
18:3	0.7	2.0 (0.3)	0.6 (0.1)	1.4 (0.1)	0.5 (0.1)	1.3 (0.1)	0.9 (0.1)
20:3	n.d.	2.3 (0.1)	1.1 (0.1)	1.9 (0.2)	1.0 (0.1)	1.4 (0.1)	1.2 (0.1)
20:4	8.7	5.4 (0.2)	7.7 (0.5)	3.2 (0.1)	5.6 (0.2)	2.6 (0.4)	4.8 (0.9)
22:4	n.d.	5.9 (0.5)	3.6 (0.4)	4.0 (0.5)	3.2 (0.2)	2.6 (0.3)	2.8 (0.5)
22:5	n.d.	6.6 (0.2)	3.9 (0.6)	4.2 (0.1)	3.3 (0.1)	2.7 (0.2)	2.9 (0.6)
22:6	n.d.	6.0 (0.4)	3.1 (0.7)	3.0 (0.4)	2.8 (0.1)	1.8 (0.1)	2.2 (0.5)
H_2 CFAM	n.d.	n.d.	n.d.	10.3 (0.6)	3.2 (0.3)	20.5 (0.8)	4.3 (0.4)

[a]Values represent mean (± SD) for a single determination using two samples per treatment group. H_2 represents hydrogenated. n.d. represents not detected. FBS, fetal bovine serum. See Table 1 for other abbreviations.

TABLE 3
CFAM Identified Following Hydrogenation of Lipid Fractions of Cultured Porcine Aortic Endothelial Cells

	Relative proportions of the major CFAM types[a] (% of total CFAM) 1	2	3	4	5	6
H_2 CFAM fraction	17.6	22.8	7.6	22.6	6.9	22.5
Polar lipid fraction	24.6	36.3	10.4	14.1	7.3	7.3
Nonpolar lipid fraction	35.4	38.0	—	16.1	6.3	4.1

[a]Numbers correspond to hydrogenated linseed oil CFAM structures. The structures are: 1 and 3, methyl 9-(2′-butylcyclopentyl)nonanoate; 2 and 5, methyl 10-(2′-propylcyclopentyl)decanoate; 4 and 6, methyl 9-(2′-propylcyclohexyl)nonanoate. Values represent single determinations. See Table 1 for abbreviations.

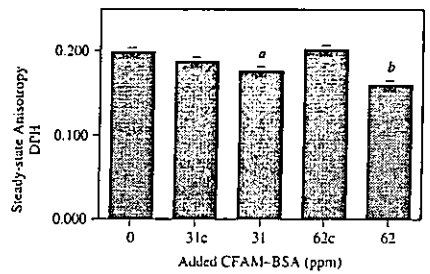

FIG. 1. Steady-state anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in confluent endothelial cells following exposure to media containing cyclic fatty acid monomer–bovine serum albumin (CFAM–BSA) for 48 h. Values represent mean ± SEM for triplicate determinations using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$).

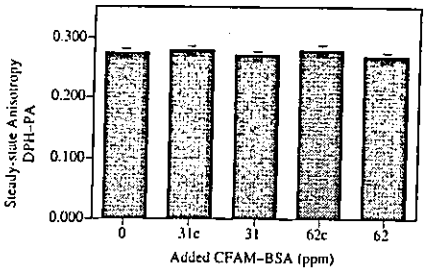

FIG. 2. Steady-state anisotropy (r) of DPH–propionic acid (PA) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media. See Figure 1 for other abbreviations.

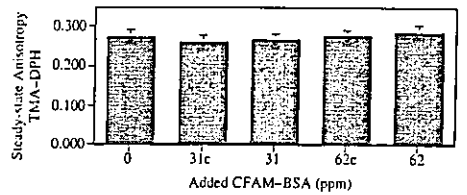

FIG. 3. Steady-state anisotropy (r) of 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group. c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media. See Figure 1 for other abbreviations.

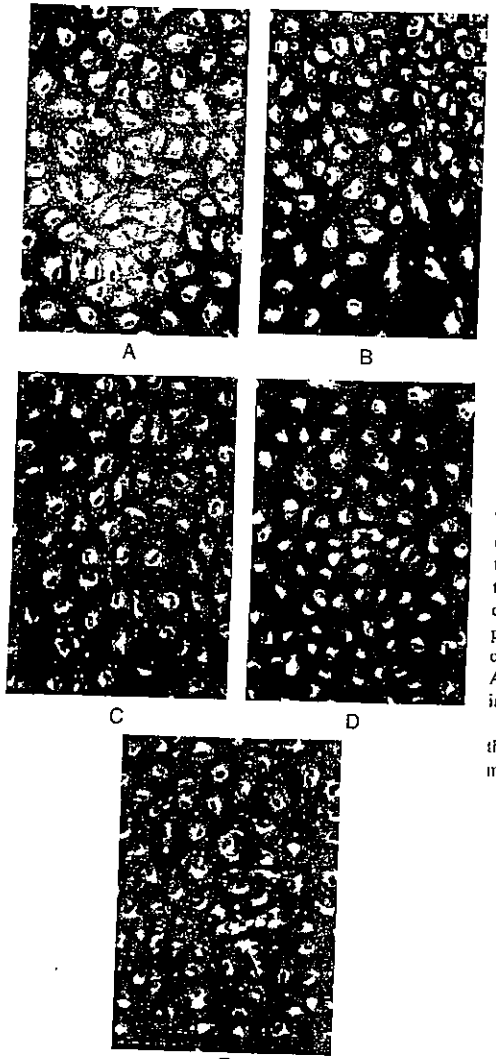

FIG. 4. Visualization by Nile red dye of intracellular lipid droplets in cultured aortic endothelial cells following incubation with 0 ppm CFAM–BSA (A), 31c (B), 31 ppm CFAM–BSA (C), 62c (D), and 62 ppm CFAM–BSA (E) media for 48 h. Magnification equals 200×; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media. See Figure 1 for abbreviations.

the inclusion of indomethacin, a potent cyclooxygenase inhibitor, at a concentration of 5 μM in CFAM–BSA media (data not shown).

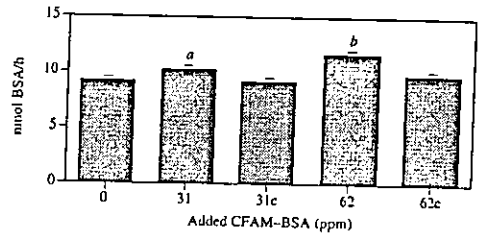

FIG. 5. Movement of BSA across confluent endothelial cell monolayers following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$). See Figure 1 for abbreviations.

DISCUSSION

The incorporation of CFAM into cellular membranes led to changes in several of the biochemical and physical parameters measured in confluent cultured endothelial cells. The uptake of cyclopentyl ring-containing CFAM was favored over cyclohexyl ring-containing CFAM in both the polar and nonpolar lipid fractions. This preferential uptake was observed in cells treated with both 32 and 61 ppm CFAM–BSA for 48 h. A similar trend has been described for the uptake of CFAM in cultured rat myocardiocytes (29).

Though not reported, we observed that CFAM–BSA and the BSA control media inhibited the growth and altered the morphology of cultured endothelial cells at both CFAM concentrations. Inhibition of growth and morphological changes in cultured cells by supplementation with the nonurea-adducting fraction isolated from thermally abused oils have been reported (41). These authors combined the fatty acids from fresh and heated oils with fatty acid-free BSA (1:60 w/w or 4:1 molar ratio) then added the complexed fatty acids–BSA to the media at concentrations of 20, 60, and 100 mg/L (ppm). The fatty acids from fresh oils served as controls for the measurement of cell growth and determination of changes in cell morphology. The use of BSA controls was not reported. It is our contention that BSA should not be used to incorporate fatty acids into media preparations when examining the influence of fatty acids on cell growth and morphology.

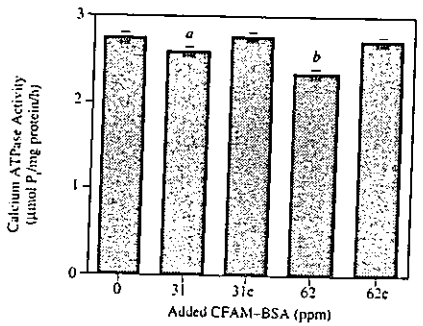

FIG. 6. Calcium ATPase activity of confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$). See Figure 1 for abbreviations.

TABLE 4
CFAM–BSA Time- and Concentration-Induced Release of 6-Keto Prostaglandin $F_{1\alpha}$ from Confluent Cultured Porcine Aortic Endothelial Cells[a]

CFAM–BSA (ppm)	6-Keto Prostaglandin $F_{1\alpha}$ (pg/mL)			
	4 H	8 H	12 H	24 H
0	402	461	531	855
31	308	447	481	796[b]
31c	—	213	417	331[a]
62	318	310[a,c,d]	332[a,d]	539[a,c,d]
62c	—	131	354	120[a]

[a]Values represent mean ± SD using two samples per treatment group. c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media. [a]Treatment group significantly different from 0 ppm CFAM–BSA ($P < 0.05$). [b]31 ppm CFAM–BSA treatment group significantly different from 31c treatment group ($P < 0.05$). [c]62 ppm CFAM–BSA treatment group significantly different from 62c treatment group ($P < 0.05$). [d]62 ppm CFAM–BSA treatment group significantly different from 31 ppm CFAM–BSA treatment group ($P < 0.05$). See Table 1 for abbreviations.

The present experimental evidence suggests that CFAM can alter specific physical characteristics of cellular membranes as well as biological processes involved in the maintenance of homeostatic functions in cultured endothelial cells. In examining factors which influence membrane lipid packing order, the lipid parameters which have undergone measurable change are the phospholipid-to-protein ratio and the presence of CFAM in the membrane lipids. The phospholipid-to-protein ratio and membrane lipid packing order have been observed to be directly proportional (42,43). In addition to further increase in membrane CFAM content compared to the 31 ppm CFAM–BSA treatment, the 62 ppm CFAM–BSA treatment caused a decreased cellular phospholipid-to-protein ratio which may contribute to the observed decrease in membrane lipid packing order.

Another factor to be considered in determining lipid packing order is accumulation of intracellular triglycerides. The use of DPH polarization studies in culture systems often has been precluded owing to the formation of excessive amounts of intracellular triglycerides caused by supplementation of exogenous fatty acids. The uptake of DPH by intracellular lipid droplets in intact cells contributes to a marked decrease in the steady-state anistropy of DPH (44) while the *r* of TMA–DPH and DPH–PA is not influenced by accumulation of intracellular lipid droplets (45). Membrane lipid packing order may not be markedly affected by exposure of cultured endothelial cells to CFAM–BSA as suggested by increased intracellular triglycerides caused by CFAM–BSA exposure as visualized with the use of Nile Red dye. However, the amount of lipid isolated in the nonpolar fraction, which is predominantly triglycerides, was not significantly different between cells treated with and without CFAM–BSA.

The observation that calcium ATPase activity was decreased by exposure of cells to CFAM stands in contrast to the influence of linolenic acid which causes an increase in the activity of total ATPase in lymphocytes (46) and calcium ATPase in endothelial cells (8). This may suggest that the ring structure present in CFAM elicits the same effect on ATPase activity observed for cholesterol incorporation into kidney fibroblast membranes (47). Preliminary results suggest that CFAM treatment also causes a decrease in the total ATPase activity.

The effect of CFAM on monolayer integrity of cultured endothelial cells was identical to the influence of linoleic acid in various mixtures of fatty acids including those prepared from selected edible oils (8). Our result suggests that CFAM disrupt the integrity of the monolayer through changes in cell–cell interactions or transcytosis. Incubation with CFAM–BSA may cause morphological changes in cultured endothelial cells leading to disruption of the monolayer. However, phase-contrast microscopy was the only indicator of endothelial cell morphology used for the observation of the endothelial cell monolayer in culture-well inserts.

CFAM caused greater production of prostaglandin I_2 (PGI_2) (prostacyclin) with respect to the BSA controls, suggesting either that the presence of CFAM was counteracting the inhibitory influence of BSA on PGI_2 production or that CFAM were directly stimulating PGI_2 secretion by cultured endothelial cells. In light of the fact that both BSA and CFAM–BSA caused a decreased production of PGI_2 with respect to the 0 ppm CFAM–BSA media, the presence of CFAM may be compensating for the action of BSA.

Despite the fact that CFAM represented only 8% of the total lipids in the culture medium, the incorporation of CFAM from heated linseed oil into the membrane of cultured endothelial cells caused specific changes in several of the parameters examined in this study. This experimental data indicate that CFAM are by no means inert constituents of biological membranes but can exhibit potent physiological effects *in vitro*. In fact, it is possible that the long-term presence of CFAM in diets may influence the susceptibility of the vascular endothelium toward injury and degeneration. However, having so speculated, the presence of CFAM in membrane phospholipids of vascular endothelia has yet to be confirmed *in vivo*. The present results indicate the need for further consideration regarding the potential impact associated with the presence of CFAM in the diet.

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Cyclic Fatty Acid Monomers from Dietary Heated Fats Affect Rat Liver Enzyme Activity

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ABSTRACT: This study was conducted to investigate the effects of dietary cyclic fatty acid monomers (CFAM), contained in heated fat from a commercial deep-fat frying operation, on rat liver enzyme activity. A partially hydrogenated soybean oil (PHSBO) used 7 d (7-DH) for frying foodstuffs, or 0.15% methylated CFAM diets was fed to male weanling rats in comparison to a control group fed a nonheated PHSBO (NH) diet in a 10-wk experiment. All diets were isocaloric with 15% fat. Animals fed either CFAM or 7-DH diets showed increased hepatic content of cytochrome (cyt.) b_5 and P_{450} and increased activity of (E.C. 1.6.2.4) NADPH-cyt. P_{450} reductase in comparison to the control rats. In addition, the activities of (E.C. 2.3.1.21) carnitine palmitoyltransferase-I and (E.C. 1.1.1.42) isocitrate dehydrogenase were significantly decreased when compared to that of rats fed the NH diet. A significantly depressed activity of (E.C. 1.1.1.49) glucose 6-phosphate dehydrogenase was also observed for these animals compared to the control rats fed NH diet. Moreover, liver and microsomal proteins were significantly increased when CFAM or 7-DH diets were fed to animals in comparison to controls while liver glycogen was decreased significantly in experimental groups of rats. The results obtained in this study indicate that the CFAM in the diet from either synthetic sources or used fats increase the activity of liver enzyme systems that detoxify them.

Lipids 33, 675–681 (1998).

Numerous studies in the literature have indicated that when dietary fats are heated at high temperatures, such as in deep frying, harmful substances are formed (1–4). Among the toxic compounds generated from the deep-fat frying process are carbonyl, cyclic monomer, and dimer derivatives (5,6). Other studies have reported that fats obtained from restaurants and those prepared during frying experiments are not significantly damaged by heating during normal use unless they are abused (7–10).

However, it has been generally accepted that nutritionally harmful materials are present in the oxidized or polymeric portions of heated fats. Iwaoka and Perkins (11) showed that when small quantities (about 1%) of purified methyl ω-(2-alkyl cyclohexyl) carboxylic acids (CFAME) were fed to rats with low-level protein diets (8–10% casein), the CFAME decreased weight gains and feed consumption in these animals compared to controls. Liver enlargement with accumulation of lipid was detected in animals fed 0.15% CFAME in their diets. In addition, there are reports in the literature that toxic compounds formed during deep-fat frying may cause deleterious effects on rats when ingested through heated fats (12–14). The complete isolation and purification of cyclic fatty acid monomers (CFAM) were accomplished by Rojo and Perkins (15,16) as well as Sébédio *et al.* (17).

Even though previous studies have focused on isolating compounds that are generated in fats upon deep frying and feeding them to rats to determine their fate (13,18), little attention has been paid to studying the effects of cyclic compounds contained in heated fats from a commercial deep-frying operation on liver enzyme activity when such fats are fed to rats. Therefore, the present study focused on determining the effects of CFAM present in partially hydrogenated soybean oil (PHSBO) commercially used for 7 d on (7-DH) for frying foodstuffs as well as a diet which contained hydrogenated CFAM methyl esters on rat liver enzyme activity.

MATERIALS AND METHODS

Animals, diets, and procedures. Male Sprague-Dawley weanling rats (50–60 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and were maintained on the standard Harlan Teklab 7001 4% mouse rat diet for 1 wk in steel wire-mesh cages. All animals (10 animals in each group) were then assigned into three groups: a control group of rats [nonheated (NH)] fed a diet containing nonheated PHSBO and two experimental groups fed diets containing either PHSBO used 7 d for frying foodstuffs (7-DH) or CFAME. The animals were randomly assigned as previously described (19). Animals were sacrificed by decapitation with a guillotine after a 12-h fast. The following procedures were employed: protein determination according to Lowry *et al.* (20); total liver lipid extraction according to Folch *et al.* (21); liver glycogen measurement according to Lo *et al.* (22) modified

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Abbreviations: CFAM, cyclic fatty acid monomers; CFAME, methylated CFAM; Cpt-I, carnitine palmitoyltransferase-I; cyt., cytochrome; 7-DH, 7 d for frying foodstuffs; ICDH, isocitrate dehydrogenase; NH, nonheated; PHSBO, partially hydrogenated soybean oil; TCA, tricarboxylic acid.

TABLE 1
Diet Composition (g/kg diet)

	NH	7-DH	CFAM
Casein[a]	150	150	150
Dextrose anhydrous	650	650	650
Vitamin mixture[a]	10	10	10
Mineral mixture[a]	40	40	40
Fat PHSBO (NH)	150	0	148.5
FAT 7-DH	0	150	0
Cyclic fatty acids	0	0	1.5

[a]From Harlan Teklad (Madison, WI). NH = Control group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO); 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs; CFAM = group of rats fed diet containing cyclic fatty acid monomers.

by Lamboni (23); liver mitochondria and microsomes were prepared according to Lake (24); microsomal cytochrome (cyt.) b_5 and P_{450} contents and NADPH-cyt. P_{450} reductase activity were carried out according to Lake (24); carnitine palmitoyltransferase-I (Cpt-I) activity according to McGarry *et al.* (25); glucose 6-phosphate dehydrogenase activity in the 31,000 × *g* supernatant fluid by the use of a Sigma Kit (Sigma Co., St. Louis, MO); and isocitrate dehydrogenase (ICDH) activity in the liver homogenate by the use of a Sigma Kit. The experimental groups of rats (CFAM and 7-DH) had free access to food and tap water as did their controls fed the NH diet.

The CFAM used in the present feeding study were prepared from linseed oil by heating, urea fractionation of the corresponding methyl esters, hydrogenation, and preparative high-performance liquid chromatography (15).

The isolation and determination of CFAM from the used oil were carried out according to the procedure described by Rojo and Perkins (15) as well as by Sébédio *et al.* (17). The CFAM were determined in the PHSBO sample that had been used 7 d for frying foodstuffs. After hydrogenation of the corresponding methyl esters with platinum oxide as catalyst and urea fractionation, the fraction not forming urea adducts was isolated. This fraction is a polar fraction and contained the CFAME materials. An aliquot of 250 µL of phenanthrene solution (0.50 mg/mL) was added to the fraction containing the CFAME as an internal standard. A Hewlett-Packard 5890 (Palo Alto, CA) gas–liquid chromatograph was used with 24 psi H_2 as carrier gas. The column used for the analysis was a CPSIL 88 column (60 m × 0.25 mm × 0.20 µm (Chrompack Inc., Raritan, NJ), and the conditions were as follows: 160°C (0), increased by 2°C/min to 220°C.

The composition of the fats fed as part of the diets was as previously shown (19, Table 1). The diet composition fed to animals for the 10-wk duration of the experiment is described in Table 1. In the present study the animals were fed a fiber-free diet; it has been reported that such diets can increase the toxic effects of a variety of compounds.

Statistical analysis of data. Data were analyzed by analysis of variance for a completely randomized design using the StatView statistical software package (StatView SE + Graphics; 1988 Abacus Concepts, Inc., Berkeley, CA). When *F* tests detected significance ($P < 0.05$), pairwise comparisons of means among groups were performed by Fisher's protected least significant difference.

RESULTS

Feed efficiency, weight gain. In comparison with the control group of rats, the feed efficiency (Table 2) of animals fed a 0.15% CFAME diet did not show any significant difference. However, three rats in the group fed the CFAM diet exhibited considerable hair loss and showed signs of dermatitis. The rats fed the 7-DH diet did not show any significant difference in feed efficiency compared to controls or to the CFAME diet group of rats. When rats were fed CFAM or 7-DH diets, their weight gains were not significantly different from the control group of animals fed NH diet (Table 2).

CFAME. The PHSBO used 7-DH contained 0.16% by weight of CFAM. Gas–liquid chromatography indicated that

TABLE 2
Several Parameters of Control and Experimental Rats[a]

Item	Group		
	NH	7-DH	CFAM
Weight (g)	273.24 ± 25.81	257.23 ± 18.4	270.07 ± 25.18
Feed efficiency (g gain/g feed)	0.234 ± 0.001	0.238 ± 0.001	0.239 ± 0.002
Liver weight/body weight ratio (mg/g)	3.21 ± 0.04	3.2 ± 0.06	3.23 ± 0.04
Liver protein (mg/g)	235.89 ± 22.46	479.13 ± 16.12[b]	460 ± 15.22[b]
Microsomal protein (mg/g)	30.39 ± 1.10	60.40 ± 1.23[b]	63.15 ± 1.34[b]
Liver lipid (mg/g)	57.73 ± 1.58	70.80 ± 1.27[b]	70.17 ± 1.00[b]
Lipid/protein ratio	1.06 ± 0.11	0.60 ± 0.02[b]	0.62 ± 0.02[b]
Liver glycogen (mg/g)	10.65 ± 0.66	6.77 ± 0.40[b]	6.75 ± 0.64[b]

[a]Values are means ± SEM. NH = control group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO) ($n = 10$). 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs ($n = 11$). CFAM = group of rats fed 0.15% cyclic fatty acid monomers diet ($n = 11$).
[b]$P < 0.0001$ when compared to the control animals fed NH diet.

the pattern of cyclic compounds present in the 7-DH was the same as that identified earlier (Rojo and Perkins 1987) with the aid of gas chromatography–mass spectrometry. The retention times of the isomers comprising the mixture of CFAME in the 7-DH were identical to that in the standard CFAME profiles (26).

Lipid, protein, and glycogen. Rats fed 0.15% CFAME or 7-DH diets (Table 2) showed significant increases ($P < 0.0001$) in liver lipid content compared to the control animals. In addition, small fat droplet infiltration in liver cells was extensive in the group of rats fed CFAME diet while only a few (3,4) of the livers of rats fed the 7-DH diet showed the same condition by microscopy.

The protein content of the microsomal fractions (Table 2) increased significantly ($P < 0.0001$) in rats fed the 0.15% CFAME diet as well as those fed the 7-DH diet compared to the control animals. There was no significant difference in microsomal protein when the CFAM diet group of rats was compared to that of the 7-DH group.

The liver glycogen (Table 2) measured in both experimental groups (CFAME and 7-DH) was significantly decreased ($P < 0.0001$) compared to control animals. No significant difference was found in liver glycogen content in either the 7-DH or CFAME group of rats.

Microsomal cyt. b_5 and P_{450}. The contents of microsomal cyt. b_5 and P_{450} (Table 3) were significantly increased ($P < 0.0001$) in rats fed the 0.15% CFAME diet as well as those fed the 7-DH diet compared to control animals fed the NH diet. No significant difference was noticed for the microsomal cyt. contents when the CFAME diet group of rats was compared to those fed the 7-DH diet.

TABLE 3
Hepatic Microsomal Cytochromes Contents of Control and Experimental Rats[a]

Item	Group		
	NH	7-DH	CFAM
Cytochrome b_5 content (nmol/mg micr. protein)	0.37 ± 0.01	0.84 ± 0.04[b]	0.73 ± 0.06[b]
Cytochrome P_{450} content (nmol/mg micr. protein)	1.03 ± 0.03	1.97 ± 0.08[b]	1.86 ± 0.11[b]

[a]Values are means ± SEM; micr. = microsomal. NH = control group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO), ($n = 8$). 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs ($n = 11$). CFAM = group of rats fed 0.15% cyclic fatty acid monomers diet ($n = 11$).
[b]$P < 0.0001$ when compared to the control animals fed NH diet.

Enzymatic activities. (i) NADPH-cyt. P_{450} reductase. The activities of the P_{450} mixed function oxidase enzyme measured in liver microsomes of rats fed either the CFAME or 7-DH diets were significantly increased ($P < 0.0001$) compared to the control group of rats fed the NH diet. The increased activity observed for animals fed the CFAME diet was significantly different ($P < 0.05$) from that measured for the group of rats fed the 7-DH diet (Fig. 1).

(ii) CPT-I. The CPT-I activity measured for rats fed either

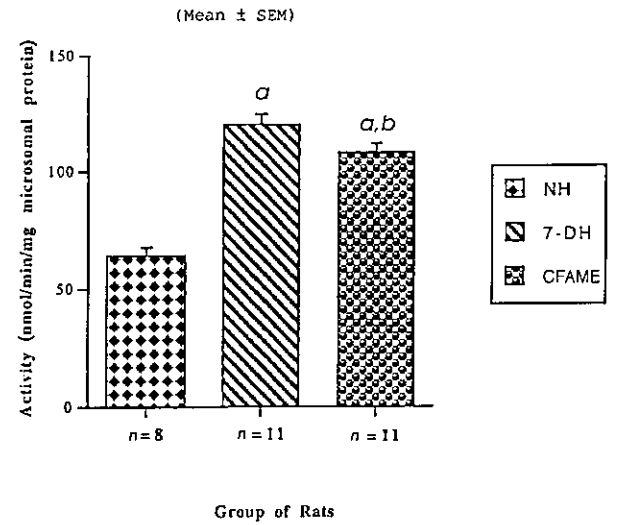

FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 7-DH, and CFAME diets. NH = group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO). 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs. CFAME = group of rats fed diet containing cyclic fatty acid monomer methyl esters. [a]$P < 0.0001$ when compared to the control group of rats fed NH diet. [b]$P < 0.05$ when compared to NH.

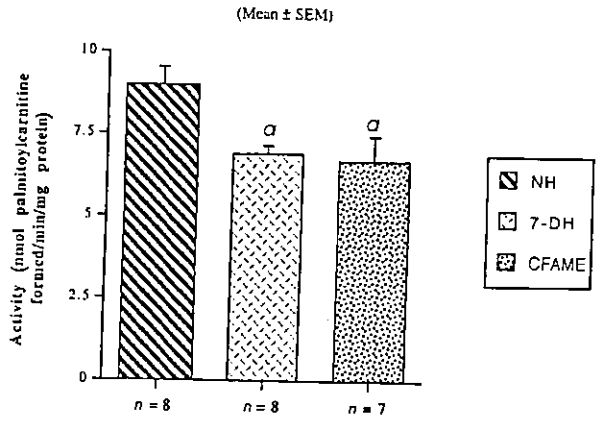

FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.01$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

CFAM or 7-DH diets was significantly decreased ($P < 0.01$) compared to the control group of rats fed the NH diet. The activity of the CFAM group of rats was not significantly different from that measured for the 7-DH diet group of rats (Fig. 2).

(iii) ICDH. The activity of ICDH was significantly decreased ($P < 0.05$) in liver homogenates when rats were fed either CFAM or 7-DH diets in comparison to the control group of rats fed NH diet (Fig. 3). There was no significant difference in the CFAM diet group of rats compared to the 7-DH diet group.

(iv) Glucose 6-phosphate dehydrogenase. The activity of D-glucose 6-phosphate:NADP-oxidoreductase measured in

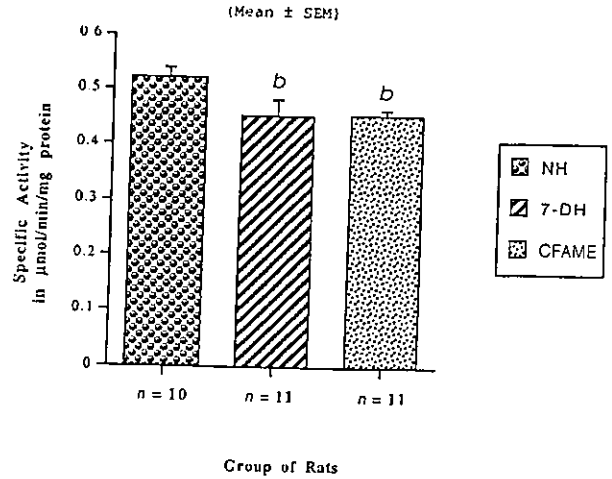

FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{b}P < 0.05$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

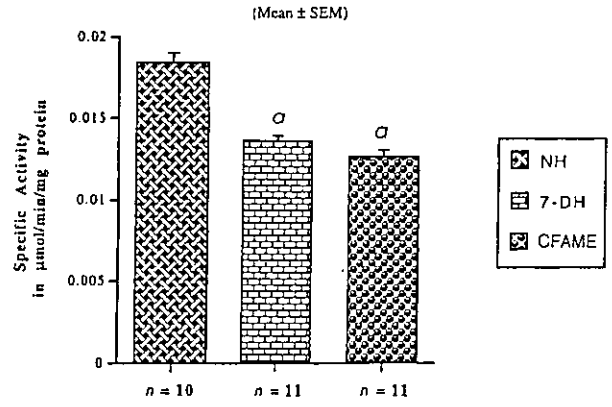

FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.0001$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

the liver of rats fed either CFAME or 7-DH diets showed in each case significantly depressed activity ($P < 0.0001$) compared to the control group of rats fed NH diet (Fig. 4). There was no significant difference when the CFAME diet group of rats was compared to that fed the 7-DH diet.

DISCUSSION

Lamboni and Perkins (19) reported that rats fed the 7-DH diet grew at a slightly slower rate than those fed CFAME. This may be due to the oxidation products generated in the oil upon heating since the CFAM group of rats grew less than control animals but more than the group of rats fed 7-DH diet. The liver weight/body weight ratio was increased in the group of animals fed either CFAM or 7-DH diets. The difference was not significant but did suggest an increase in liver size of those animals fed either CFAME or 7-DH diets. It can be inferred that the used fat contained products that could be attributed in part to CFAM which may have induced an increased liver weight. Indeed, by feeding rats an approximate amount of CFAM as was present in the used oil, similar increases in liver weights were observed. The significantly ($P < 0.0001$) increased liver protein in groups CFAM and 7-DH compared to the control rats fed the NH diet suggests that less protein was being used for somatic growth and more retained in the liver of those animals. This increase of protein in liver of rats fed either CFAM or 7-DH diets may be a response of the body to cope with the adverse metabolic effects of CFAM contained in the used PHSBO diet. The increased content of microsomal protein, discussed previously, suggests an increased rate of protein synthesis to aid in the increased participation of the mixed-function oxidase enzymes involved in xenobiotic detoxification. In addition, it has been reported that there is liver damage in animals fed either CFAME or 7-DH diets compared to the control group of rats (26). This liver damage observed may contribute to the high protein level measured for those animals in liver and microsomal fractions.

Furthermore, the lipid/protein ratio (Table 2) of animals fed either CFAME or 7-DH diets clearly confirmed the large amount of protein being retained or produced compared to that of lipid in liver tissue of the same group of rats. The microsomal cyt. P_{450} and b_5 contents (Table 3) of rats fed either CFAM or 7-DH diets were significantly increased ($P < 0.0001$) in each case compared to control animals fed NH diet. This clearly demonstrates the effects (elevated levels of detoxifying enzymes) of the components generated in the 7-DH oil as well as that of CFAM. Besides the increased content of liver microsomal cyt. P_{450}, the activity of NADPH-cyt. P_{450} reductase was also significantly increased ($P < 0.0001$) in rats fed either CFAM or 7-DH diets compared to the control animals. This suggests an active detoxification in the liver of compounds such as CFAM ingested either in the diet or those generated along with other compounds in the 7-DH oil during the deep-frying process. The decreased NADPH-cyt. P_{450} reductase activity of the CFAM diet-fed rats was compared to those fed the 7-DH diet (Fig. 1). The data clearly suggested that compounds other than CFAM also were generated in the used oil and that they exerted their effects in conjunction with the CFAM. This could explain the highest activity measured for animals fed the 7-DH diet in comparison to those fed the CFAM diet. In addition, these results showed an