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]

Item	Group						
	NH		4-NH		7-DH (pair-feeding and graded doses)		
	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.

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

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

Cytochromes	Group						
	NH		4-DH		7-DH (pair-feeding and graded doses)		
	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.

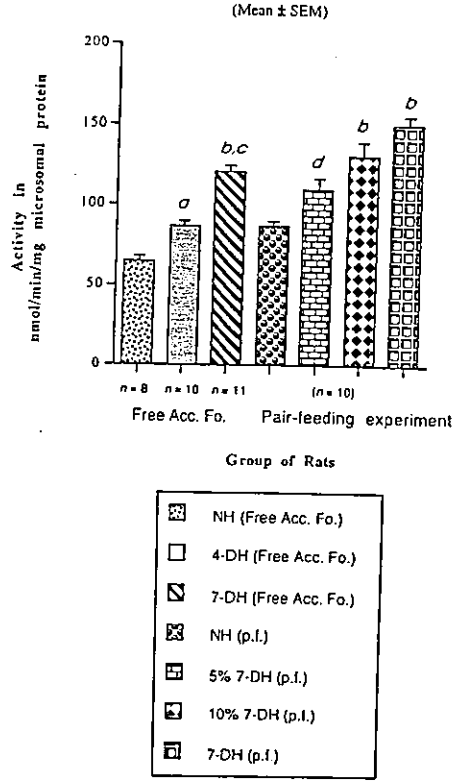


FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 4-DH, and 7-DH diets. NH, control group of rats fed diet containing nonheated (NH) partially hydrogenated soybean oil (PHSBO); 4-DH, group of rats fed diet containing PHSBO used 4 d (4-DH) for frying foodstuffs; 7-DH, 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.

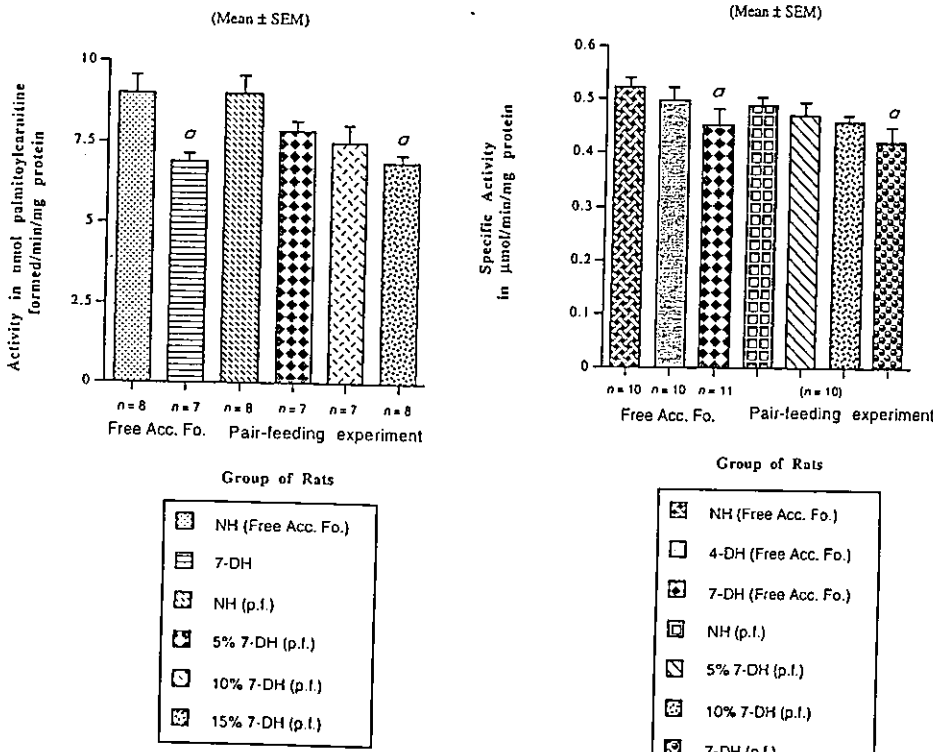


FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH and 7-DH diets. Abbreviations and legend descriptions as in Figure 1. $^{a}P < 0.01$ compared to the control group of rats fed the NH diet.

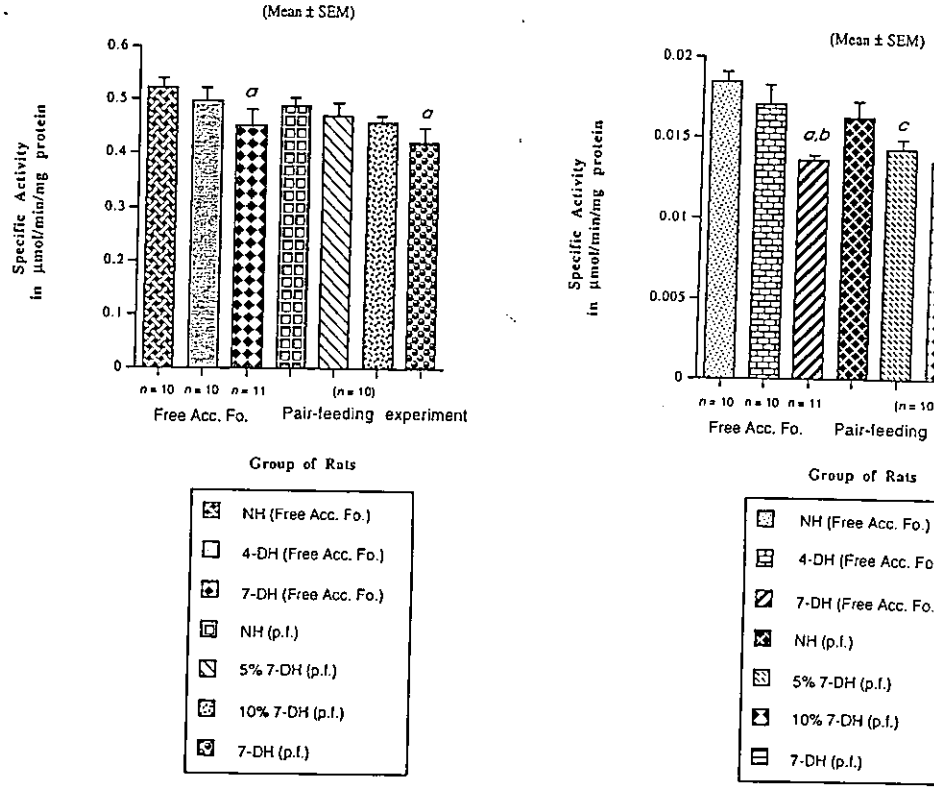


FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 4-DH, and 7-DH diets. $^{a}P < 0.05$ when the 7-DH diet group of rats was compared to the NH group of rats. See Figure 1 for abbreviations and legend descriptions.

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.

ACKNOWLEDGMENTS

We thank the Illinois Agricultural Experiment Station for partial support of this study. Thanks are also given to the Fulbright committee for their financial support of Courdjo Lamboni's graduate career toward Ph.D. degree at the University of Illinois, Urbana–Champaign.

REFERENCES

1. Artman, N.R. (1969) The Chemical and Biological Properties of Heated and Oxidized Fats, in *Advances in Lipid Research* (Pao-

letti, R., and Kritchevsky, D., eds.) Vol. 7, pp. 245–330, Academic Press, New York.
2. Perkins, E.G., and Van Akkeren, L.A. (1965) Heated Fats. IV. Chemical Changes in Fats Subjected to Deep Fat Frying Processes: Cottonseed Oil, *J. Am. Oil Chem. Soc. 42*, 782–786.
3. Perkins, E.G. (1976) Chemical, Nutritional and Metabolic Studies of Heated Fats. I. Chemical Aspects, *Rev. Franç. Corps Gras 23*, 257–262.
4. Alexander, J.C., Valli, V.E., and Chanin, B.E. (1987) Biological Observations from Feeding Heated Corn Oil and Heated Peanut Oil to Rats, *J. Toxicol. Environ. Health 21*, 295–309.
5. Gabriel, H.G., Alexander, J.C., and Valli, V.E. (1979) Nutritional Studies of Fractions from Thermally Oxidized Rapeseed Oil and Lard, *Nutr. Rep. Inter. 20*, 411–422.
6. Artman, N.R., and Smith, D.E. (1972) Systematic Isolation and Identification of Minor Components in Heated and Unheated Fats, *J. Am. Oil Chem. Soc. 49*, 318–326.
7. Iwaoka, W.T., and Perkins, E.G. (1978) Metabolism and Lipogenic Effects of the Cyclic Monomers of Methyl Linolenate in the Rat, *J. Am. Oil Chem. Soc. 55*, 734–738.
8. Crampton, E.W., Common, R.H., Farmer, F.A., Berryhill, F.M., and Wiseblatt, L. (1951) Studies to Determine the Nature of the Damage to the Nutritive Value of Some Vegetable Oils from Heat Treatment, *J. Nutr. 44*, 177–189.
9. Perkins, E.G., and Kummerow, F.A. (1966) The Chemistry of the "Polymers" Resulting from the Thermal Oxidation of Oils, in *Proceedings of the 7th International Congress of Nutrition*, Vols. 1–5; Reprint from Vol. 1, pp. 3–7, Verlag Friedr. Vieweg & Sohn GmbH Braunschweig, Germany.
10. Perkins, E.G. (1976) Chemical, Nutritional and Metabolic Studies of Heated Fats. II. Nutritional Aspects, *Rev. Franç. Corps Gras 23*, 313–322.
11. Andia, A.M.G., and Street, J.C. (1975) Dietary Induction of Hepatic Microsomal Enzymes by Thermally Oxidized Fats, *J. Agr. Food Chem. 23*, 173–177.
12. Yoshioka, M., Tachibana, K., and Kaneda, T. (1974) Studies on the Toxicity of the Autoxidized Oils. IV. Impairments of Metabolic Functions Induced by Autoxidized Methyl Linoleate, *Yukagaku 23*, 327–331.
13. Rohlf, J.F., and Sokal, R.R. (1981) *Statistical Tables*, 2nd edn., pp. 71–75, Freeman & Company, New York.
14. Lake, B.G. (1987) Preparation and Characterisation of Microsomal Fractions for Studies on Xenobiotic Metabolism, in *Biochemical Toxicology. A Practical Approach* (Snell, K., and Mullock, B., eds.), pp. 183–215, IRL Press, Oxford, Washington D.C.
15. McGarry, J.D., Mills, S.E., Long, C.S., and Foster, D.W. (1983) Observations on the Affinity for Carnitine, and Malonyl-CoA Sensitivity, of Carnitine Palmitoyltransferase-I in Animal and Human Tissues, *Biochem. J. 214*, 21–28.
16. Löhr, G.W., and Waller, H.D. (1974) Glucose 6-Phosphate Dehydrogenase, in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., and Gawehn, K., eds.), Vol. 2, pp. 636–643, Verlag Chemie Inter., Deerfield Beach.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement With the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
18. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
19. Lamboni, C. (1983) Etude Expérimentale Chez le Rat des Effets d'un Traitement par un beta-Bloquant, le Pindolol, sur les Caractéristiques Métaboliques de l'Epuisement, Thèse de Doctorat de 3ème Cycle, Université de Dijon, France.
20. *Official Methods and Recommended Practices of the American Oil Chemists' Society* (1973) Method Ce-2-66, American Oil Chemists' Society, Champaign.
21. Lo, S., Russell, J.C., and Taylor, A.W. (1970) Determination of Glycogen in Small Tissue Samples, *J. Appl. Physiol. 28*, 234–236.
22. Alfin-Slater, R.B., Auerbach, S., and Aftergood, L. (1959) Nutritional Evaluation of Some Heated Oils, *J. Am. Oil Chem. Soc. 36*, 638–641.
23. Kaunitz, H., Slanetz, C.A., and Johnson, R.E. (1960) Influence of Feeding Fractionated Esters of Autoxidized Lard and Cottonseed Oil on Growth, Thirst, Organ Weights, and Liver Lipids of Rats, *Acta Chim. Hung. Tomus 23*, 189–199.
24. Poling, C.E., Eagle, E., Rice, E.E., Durand, A.M., and Fisher, M. (1970) Long-Term Responses of Rats to Heat Treated Dietary Fats. IV. Weight Gains, Food and Energy Efficiencies, Longevity and Histopathology, *Lipids 5*, 128–136.
25. Miller, J., and Landes, D.R. (1975) Effects of Feeding Oxidized or Heated Soybean Oil on Tissue Composition and Hematological Status of Rats, *J. Food Sci. 40*, 545–548.
26. Antal, M., Nagy, K., and Bedo, M.B. (1982) Effect of Dietary Protein and Lipid on the Activity of Hepatic Mixed Function Oxidase System in Young and Adult Rats, *Ann. Nutr. Metab. 26*, 393–399.
27. Bidlack, W.R., Brown, R.C., and Mohan, C. (1986) Nutritional Parameters that Alter Hepatic Drug Metabolism, Conjugation, and Toxicity, *Fed. Proc. 45*, 142–148.
28. Campbell, T.C., and Hayes, J.R. (1976) The Effects of Quantity and Quality of Dietary Protein on Drug Metabolism, *Fed. Proc. 35*, 2470–2477.

[Received August 9, 1995, and in final revised form April 11, 1996; Revision accepted June 3, 1996]

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

G. Barja[a,*], S. Cadenas[a], C. Rojas[a], R. Pérez-Campo[a], M. López-Torres[a], J. Prat[b], and R. Pamplona[b]

[a]Department of Animal Biology II (Animal Physiology), Faculty of Biology, Complutense University, Madrid 28040, Spain and [b]Department of Basic Medical Sciences, Faculty of Medicine, Lerida University, Lerida, Spain

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,

*To whom correspondence should be addressed.

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

Brent D. Flickinger[a,1], Robert H. McCusker, Jr.[b], and Edward G. Perkins[c,*]

[a]Division of Nutritional Sciences, [b]Department of Animal Science, Division of Nutritional Sciences, and [c]Department of Food Science, Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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.

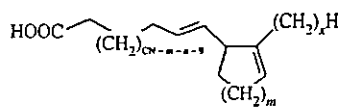
Lipids 32, 925–933 (1997).

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

[1]Current address: Department of Biochemistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760.

*To whom correspondence should be addressed at University of Illinois of Urbana-Champaign, 205 Burnsides Research Laboratory, 1208 West Pennsylvania Ave., Urbana, IL 61801.

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 viability 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		31		62	
		NP	P	NP	P	NP	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 (0.1)	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.

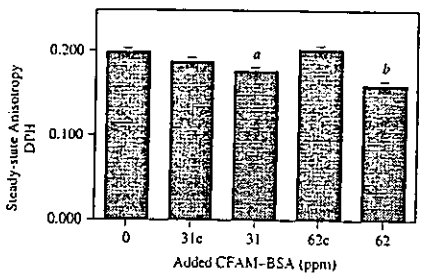


FIG. 1. Steady-state anisotropy (*r*) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in confluent endothelial cells following exposure to media containing cyclic fatty acid monomer–bovine serum albumin (CFAM–BSA) for 48 h. Values represent mean ± SEM for triplicate determinations using three samples per treatment 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 ($P < 0.05$).

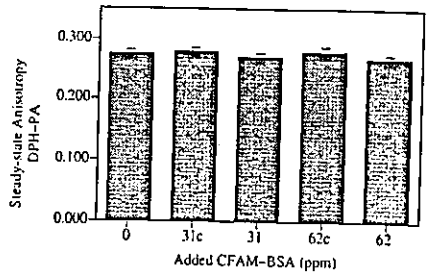


FIG. 2. Steady-state anisotropy (*r*) of DPH–propionic acid (PA) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained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

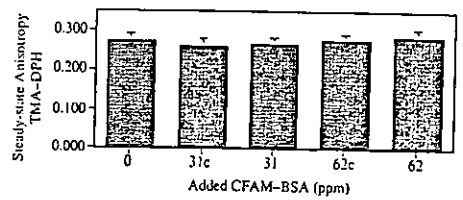


FIG. 3. Steady-state anisotropy (*r*) of 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group. c indicates media containing an equivalent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

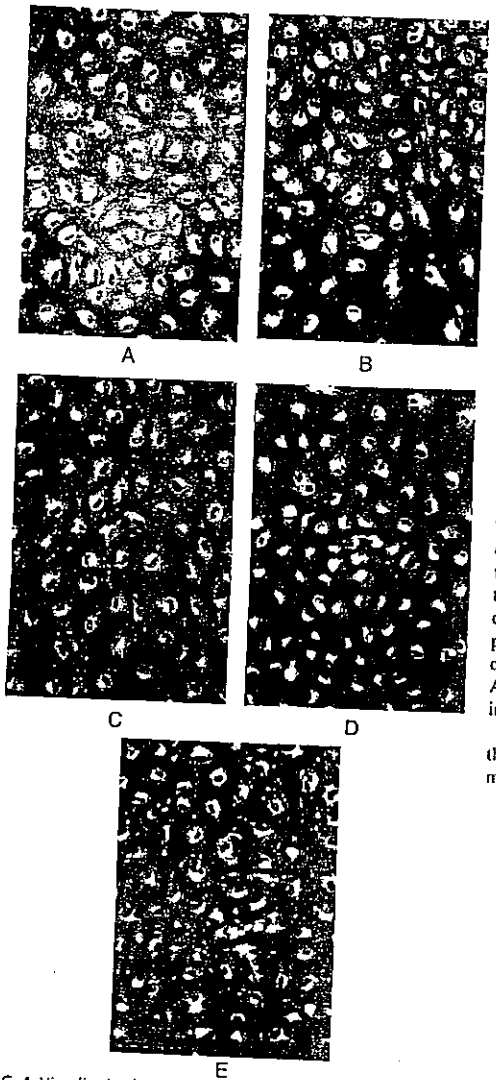


FIG. 4. Visualization by Nile red dye of intracellular lipid droplets in cultured aortic endothelial cells following incubation with 0 ppm CFAM–BSA (A), 31c (B), 31 ppm CFAM–BSA (C), 62c (D), and 62 ppm CFAM–BSA (E) media for 48 h. Magnification equals 200x; c indicates media containing an equivalent 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).

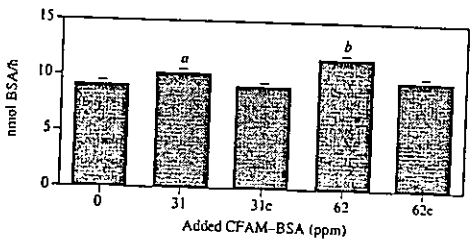


FIG. 5. Movement of BSA across confluent endothelial cell monolayers following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding 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.

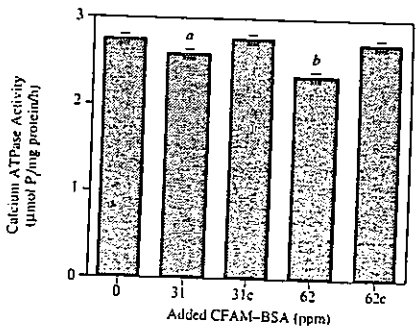


FIG. 6. Calcium ATPase activity of confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media; *a* 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.

ACKNOWLEDGMENTS

The authors thank the Laboratory for Fluoresence Dynamics at the University of Illinois at Urbana-Champaign for the generous use of

their equipment for anisotropy measurements. Special thanks to Chip Hazlett for his advice and assistance in membrane lipid packing order measurements. The authors also thank John P. Jerrell for his technical assistance in gas chromatography of CFAM.

REFERENCES

1. Trowell, H.C. (1981) in *Hypertension, Obesity, Diabetes mellitus and Coronary Heart Disease* (Trowell, H.C., ed.) pp. 3–32, Harvard University Press, Cambridge.
2. Hubert, H.B., Feinleib, M., McNamara, P.M., and Castelli, W.P. (1983) Obesity as an Independent Risk Factor for Cardiovascular Disease, *Circulation 67*, 968–977.
3. Kris-Etherton, P.M., Krummel, D., Russell, M.E., Dreon, D., Mackey, S., Borchers, J., and Wood, P.D. (1988) The Effect of Diet on Plasma Lipids, Lipoproteins and Coronary Heart Disease, *J. Am. Diet. Assoc. 88*, 1373–1400.
4. Glauert, H.P. (1992) in *Dietary Fatty Acids and Cancer* (Chow, C.K., ed.) Vol. 53, pp. 753–768, Marcel Dekker, Inc., New York.
5. Nicolson, G.L. (1976) Transmembrane Control of the Receptors on Normal and Tumor Cells. II. Surface Changes Associated with Transformation and Malignancy, *Biochim. Biophys. Acta 458*, 1–72.
6. Shinitzky, M. (1984) in *Membrane Fluidity and Cellular Functions* (Shinitzky, M., ed.) Vol. 1, pp. 1–51, CRC Press, Boca Raton.
7. Hennig, B., and Watkins, B.A. (1989) Linoleic Acid and Linolenic Acid: Effect on Permeability Properties of Cultured Endothelial Cell Monolayers, *Am. J. Clin. Nutr. 49*, 301–305.
8. Hennig, B., Ramasamy, S., Alvarado, A., Shantha, N.C., Boissonneault, G.A., Decker, E.A., and Watkins, B.A. (1993) Selective Disruption of Endothelial Barrier Function in Culture by Pure Fatty Acids and Fatty Acids Derived from Animal and Plant Fats, *J. Nutr. 123*, 1208–1247.
9. DeCanterina, R., Cybulsky, M.I., Clinton, S.K., Gimbrone, M.A.J., and Libby, P. (1994) The Omega-3 Fatty Acid Docosahexanoate Reduces Cytokine-Induced Expression of Proatherogenic and Proinflammatory Proteins in Human Endothelial Cells, *Arterioscler. Thromb. 14*, 1829–1836.
10. Artman, N.R., and Alexander, J.C. (1968) Characterization of Some Heated Fat Components, *J. Am. Oil Chem. Soc. 45*, 643–648.
11. Aust, S.D., Morehouse, L.A., and Thomas, C.E. (1985) Role of Metals in Oxygen Radical Reactions, *J. Free Radicals Biol. Med. 1*, 3–14.
12. Frankel, E.N., Evans, C.D., Moser, H.A., McConnel, D.G., and Cowan, J.C. (1961) Analysis of Lipids and Oxidation Products by Partition Chromatography: Dimeric and Polymeric Products, *J. Am. Oil Chem. Soc. 38*, 130–137.
13. Artman, N.R., and Smith, D.E. (1972) Systematic Isolation and Identification of Minor Components in Heated and Unheated Fat, *J. Am. Oil Chem. Soc. 49*, 318–326.
14. Artman, N.R. (1969) The Chemical and Biological Properties of Heated and Oxidized Fats, *Adv. Lipid Res. 7*, 245–330.
15. Chang, S.S., Vallese, F.M., Hwang, L.S., Hseih, O.A.L., and Min, D.B. (1977) Apparatus on the Isolation of Trace Volatile Constituents for Foods, *J. Agr. Food Chem. 25*, 450–454.
16. Qian, C., and Perkins, E.G. (1991) Characterization of Deep-Fat Fried Flavor, *INFORM 2*, 323.
17. Poling, C.E., Warner, W.D., Mone, P.E., and Rice, E.E. (1960) The Nutritional Value of Fats After Use in Commercial Deep-Fat Frying, *J. Nutr. 72*, 109–120.
18. Perkins, E.G. (1992) in *Effect of Lipid Oxidation on Oil and Food Quality in Deep Frying* (St. Angelo, A.J., ed.) pp. 310–321, American Chemical Society, Washington, D.C.
19. Frankel, E.N., Smith, L.M., Hamblin, C.L., Creveling, R.K., and Clifford, A.J. (1984) Occurrence of Cyclic Fatty Acid Monomers in Frying Oils Used for Fast Foods, *J. Am. Oil Chem. Soc. 61*, 87–90.
20. Rojo, J.A., and Perkins, E.G. (1987) Cyclic Fatty Acid Monomer Formation in Frying Fats. I. Determination and Structural Study, *J. Am. Oil Chem. Soc. 64*, 414–421.
21. Sébédio, J.L., Prevost, J., and Grandgirard, A. (1987) Heat Treatment of Vegetable Oils. I. Isolation of the Cyclic Fatty Acid Monomers from Heated Sunflower and Linseed Oils, *J. Am. Oil Chem. Soc. 64*, 1026–1032.
22. Mossoba, M.M., Yurawecz, M.P., Roach, J.A.G., Lin, H.S., McDonald, R.E., Flickinger, B.D., and Perkins, E.G. (1994) Rapid Determination of Double-Bond Configuration and Position Along the Hydrocarbon Chain in Cyclic Fatty Acid Monomers, *Lipids 29*, 893–896.
23. Dobson, G., Christie, W.W., Brechany, E.Y., Sébédio, J.L., and LeQuere, J.L. (1996) Silver Ion Chromatography and Gas Chromatography–Mass Spectrometry in the Structural Analysis of Cyclic Dienoic Acids Formed in Frying Oils, *Chem. Phys. Lipids 75*, 171–182.
24. Dobson, G., Christie, W.W., and Sébédio, J.L. (1996) Gas Chromatographic Properties of Cyclic Dienoic Fatty Acids Formed in Heated Linseed Oil, *J. Chromatogr. A 723*, 349–354.
25. Spector, A.A., and Yorek, M.A. (1985) Membrane Lipid Composition and Cellular Function, *J. Lipid Res. 26*, 1015–1035.
26. Hubbard, R.W., Ono, Y., and Sanchez, A. (1989) Atherogenic Effect of Oxidized Products of Cholesterol, *Prog. Food Nutr. 13*, 17–44.
27. Kawamura, K., and Kummerow, F.A. (1992) Effect of 25-Hydroxycholesterol on Cytotoxicity and Prostacyclin Production in Cultured Human Umbilical Arterial Endothelial Cells, *Eicosanoids 5*, 29–34.
28. Sevenian, A., and Peterson, H. (1992) Cytotoxicity of Cholesterol Oxides Associated with Oxidatively Modified LDL, *INFORM 3*, 514.
29. Ribot, E., Grandgirard, A., Sébédio, J.-L., Grynberg, A., and Athias, P. (1992) Incorporation of Cyclic Fatty Acid Monomers in Lipids of Rat Heart Cell Cultures, *Lipids 27*, 79–81.
30. Athias, P., Ribot, E., Grynberg, A., Sébédio, J.-L., and Grandgirard, A. (1992) Effects of Cyclic Fatty Acid Monomers on the Function of Cultured Rat Cardiac Myocytes in Normoxia and Hypoxia, *Nutr. Res. 12*, 737–745.
31. Sébédio, J.-L., and Grandgirard, A. (1989) Cyclic Fatty Acids: Natural Sources, Formation During Heat Treatment, Synthesis and Biological Properties, *Prog. Lipid Res. 28*, 303–336.
32. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 44*, 497–509.
33. Juaneda, P., and Rocquelin, G. (1985) Rapid and Convenient Separation of Phospholipids and Non-Phosphorus Lipids from Rat Heart Using Silica Cartridges, *Lipids 20*, 40–41.
34. Christie, W.W. (1985) Rapid Separation and Quantification of Lipid Classes by High Performance Liquid Chromatography and Mass (Light-scattering) Detection, *J. Lipid Res. 26*, 507–512.
35. Method Cd 3-66 (1990), in *Official Methods and Recommended Practices of the American Oil Chemists' Society*, American Oil Chemists' Society, Champaign.
36. Block, E.R., Patel, J.M., Angelides, K.J., Sheridan, N.P., and Garg, L.C. (1986) Hyperoxia Reduces Plasma Membrane Fluidity: A Mechanism for Endothelial Cell Dysfunction, *J. Appl. Physiol. 60*, 825–835.
37. Sheridan, N.P., and Block, E.R. (1988) Plasma Membrane Fluidity Measurements in Intact Endothelial Cells: Effect of Hyperoxia on Fluorescence Anisotropies of 1-[4-(Trimethylamino)-phenyl]-6-Phenyl Hexa-1,3,5-Triene, *J. Cell Physiol. 134*, 117–123.

38. Henning, B., Shasby, D.M., Fulton, A.B., and Spector, A.A. (1984) Exposure to Free Fatty Acid Increases the Transfer of Albumin Across Cultured Endothelial Monolayers, *Arteriosclerosis 4*, 489–497.
39. Fiske, C.H., and Subbrow, Y. (1925) The Colorimetric Determination of Phosphorous, *J. Biol. Chem. 66*, 375–400.
40. Gold, E.W., and Edgar, P.R. (1978) The Effect of Physiological Levels of Nonesterified Fatty Acids on the Radioimmunoassay of Prostaglandins, *Prostaglandins 16*, 945–952.
41. Bird, R.P., Basrur, P.K., and Alexander, J.C. (1981) Cytotoxicity of Thermally Oxidized Fats, *In Vitro 17*, 397–404.
42. Shinitzky, M., and Inbar, M. (1976) Microviscosity Parameters and Protein Mobility in Biological Membranes, *Biochim. Biophys. Acta 433*, 133–149.
43. Cooper, R.A. (1977) Abnormalities of Cell Membrane Fluidity in the Pathogenesis of Disease, *N. Engl. J. Med. 297*, 371–377.
44. Stubbs, C.D., Tsang, W.M., Belin, J., Smith, A.D., and Johnson, S.M. (1980) Incubation of Exogenous Fatty Acids with Lymphocytes. Changes in Fatty Acid Composition and Effects on the Rotational Relaxation Time of 1,6-Diphenyl-1,3,5-hexatriene, *Biochemistry 19*, 2756–2762.
45. Kuhry, J.-G., Fonteneau, P., Duportail, G., Maechling, C., and Laustriat, G. (1983) TMA–DPH: A Suitable Fluorescence Polarization Probe for Specific Plasma Membrane Fluidity Studies in Intact Living Cells, *Cell Biophys. 5*, 129–140.
46. Szamel, M., and Resch, K. (1981) Modulation of Enzyme Activities in Isolated Lymphocyte Plasma Membranes by Enzymatic Modification of Phospholipid Fatty Acids, *J. Biol. Chem. 256*, 11618–11623.
47. Klein, I., Moore, L., and Pastan, I. (1978) Effect of Liposomes Containing Cholesterol on Adenylate Cyclase of Cultured Mammalian Fibroblasts, *Biochim. Biophys. Acta 506*, 42–53.

[Received October 24, 1996, and in final revised form July 14, 1997; revision accepted August 12, 1997]

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

Courdjo Lamboni[a], Jean-Louis Sébédio[b], and Edward G. Perkins[c,*]

[a]Faculté des Sciences, Département de Biochimie/Nutrition Université du Bénin, Lomé, Togo, [b]I.N.R.A, Station de Recherches sur la Qualité des Aliments de l'Homme, 21034 Dijon Cédex, France, and [c]Burnsides Research Laboratory, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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

*To whom correspondence should be addressed at Department of Food Science, Burnsides Research Laboratory, 1208 W. Pennsylvania Ave., University of Illinois, Urbana, IL 61801. E-mail: eperkins@uiuc.edu

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

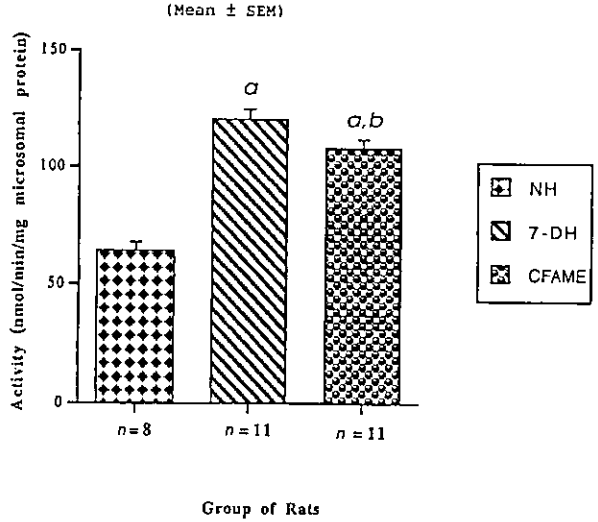


FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 7-DH, and CFAME diets. NH = group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO). 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs. CFAME = group of rats 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.

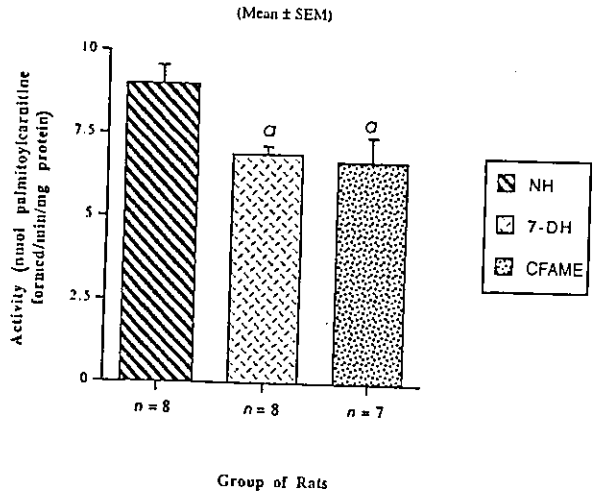


FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.01$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

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

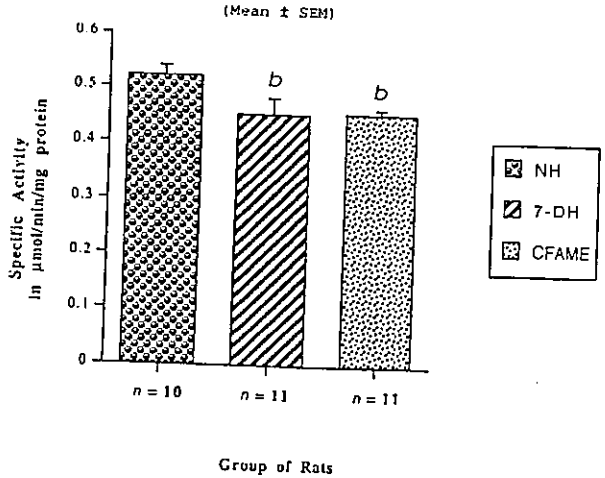


FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{b}P < 0.05$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

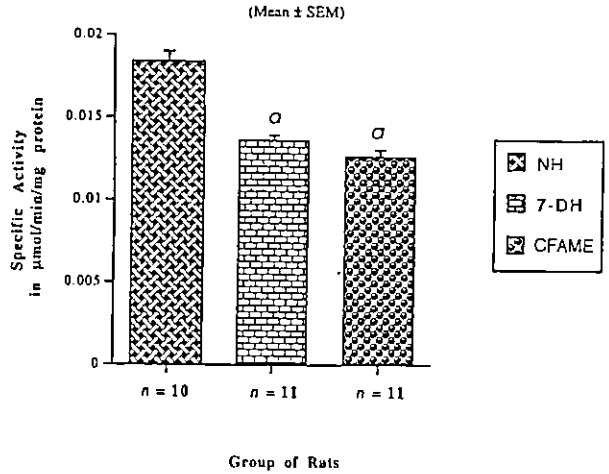


FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.0001$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

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

apparent dose response of the toxic compounds suggesting that liver microsomal content of cyt. b_5 or P_{450} is dependent upon the concentration of the components generated in the oil during heating (19). The reductase is known to receive electrons from NADPH through FAD and FMN. This suggests that NADPH was being used during the detoxification process by the P_{450} mixed-function enzyme. Indeed, the activity of glucose-6-phosphate dehydrogenase measured in the liver 31,000 × *g* supernatant fluid was significantly depressed ($P < 0.0001$) when rats were fed either CFAM or 7-DH diets in comparison to the control group of rats fed the NH diet (Fig. 4). These results indicate that large amounts of NADPH were being produced and that the excess of the reducing equivalent NADPH inhibited the activity of the enzyme discussed previously (19).

The decreased activity of CPT-I measured in rats fed either CFAM or 7-DH diets in comparison to the control animals fed the NH diet (Fig. 2) is compatible with the possibility that less long-chain fatty acids were being degraded *via* the mitochondrial β-oxidation pathway and more fatty acids were being incorporated into triglycerides.

The highly significant decrease in liver glycogen (Table 2), which was measured when rats were fed either CFAM diet or 7-DH diet, suggests increased glycolytic activity, leading to the formation of pyruvate which would subsequently be converted to acetyl-CoA *via* the pyruvate dehydrogenase complex enzyme in the mitochondrial matrix. The acetyl-CoA formed could undergo oxidative degradation through the tricarboxylic acid (TCA) cycle or might be utilized for the biosynthesis of long-chain fatty acids such as palmitic acid *via* fatty acid synthase.

The decreased activity of ICDH, which was measured when rats were fed either CFAM or 7-DH diets (Fig. 3), suggests an impairment of TCA cycle enzyme activity in the presence of CFAM and supports our previous finding (19). If the TCA cycle is impaired, acetyl CoA oxidation would be impaired. It could therefore be inferred that cyclic compounds and other oxidation products which were generated in the fat during the commercial deep-frying process were in part responsible for the impairment of ICDH in the mitochondrial TCA cycle.

Heated fats exert generalized nutritional toxic effects on rat morphology and physiology and these mechanisms are not yet completely understood. Siess *et al.* (27) reported an increased activity of NADPH-cyt. P-450 reductase in female Wistar rats fed for 4 wk a semisynthetic diet containing different quantities of cyclic monomers isolated from linseed oil. In a related study of oil/olestra blends, heated soybean oil was used. No statistical differences were found in the physiological responses to the heated or unheated oil (28).

REFERENCES

1. Firestone, D., Horwitz, W., Friedman, L., and Shue, G.M. (1961) I: Studies of the Effects of Heating on the Chemical Nature of Cottonseed Oil, *J. Am. Oil Chem. Soc. 38*, 253–257.
2. Friedman, L., Horwitz, W., Shue, G.M., and Firestone, D. (1961) The Nutritive Properties of Heated Cottonseed Oil and of Heated Cottonseed Oil Fractions Obtained by Distillation and Urea Adduct Formation, *J. Nutr. 73*, 85–93.
3. Kummerow, F.A. (1962) Toxicity of Heated Fats, in *Symposium on Foods: Lipids and Their Oxidation* (Schultz, H.W., ed.), pp. 294–320, The AVI Publishing Company, Inc., Westport.
4. Perkins, E.G. (1960) Nutritional and Chemical Changes Occurring in Heated Fats: A Review, *Food Technol. 14*, 508–514.
5. Crampton, E.W., Common, R.H., Farmer, F.A., Berryhill, F.M., and Wiseblatt, L. (1951) Studies to Determine the Nature of the Damage to the Nutritive Value of Some Vegetable Oils from Heat Treatment, *J. Nutr. 44*, 177–189.
6. Perkins, E.G., and Kummerow, F.A. (1966) The Chemistry of the Polymers Resulting from the Thermal Oxidation of Oils, in *Proceedings of the 7th International Congress of Nutrition*, Vols. 1–5 (Hamburg), reprinted from Vol. I, pp. 3–7, Verlag Friedr. Vieweg & Sohn GmbH, Braunschweig, Germany.
7. Keane, K.W., Jacobson, G.A., and Krieger, C.H. (1959) Biological and Chemical Studies on Commercial Frying Oils, *J. Nutr. 68*, 57–74.
8. Poling, C.E., Warner, W.D., Mone, P.E. and Rice, E.E. (1960) The Nutritional Value of Fats After Use in Commercial Deep-Fat Frying, *J. Nutr. 72*, 109–120.
9. Poling, C.E., Warner, W.D., Mone, P.E., and Rice, E.E. (1962) The Influence of Temperature, Heating Time, and Aeration upon the Nutritive Value of Fats, *J. Am. Oil Chem. Soc. 39*, 315–320.
10. Rice, E.E., Poling, C.E., Mone, P.E., and Warner, W.D. (1969) A Nutritive Evaluation of Over-Heated Fats, *J. Am. Oil Chem. Soc. 37*, 607–613.
11. Iwaoka, W.T., and Perkins, E.G. (1976) Nutritional Effects of Cyclic Monomers of Linolenic acid, *Lipids 11*, 349–353.
12. Artman, N.R., and Smith, D.E. (1972) Systematic Isolation and Identification of Minor Components in Heated and Unheated Fats, *J. Am. Oil Chem. Soc. 49*, 318–326.
13. Iwaoka, W.T., and Perkins, E.G. (1978) Metabolism and Lipogenic Effects of the Cyclic Monomer of Methyl Linolenate in the Rat, *J. Am. Oil Chem. Soc. 55*, 734–738.
14. Perkins, E.G. (1976) Chemical, Nutritional, and Metabolic Studies of Heated Fats, *Rev. Franç. Corps Gras 23*, 313–322.
15. Rojo, J.A., and Perkins, E.G. (1987) Cyclic Fatty Acid Monomer in Frying Fats. I. Determination and Structural Study, *J. Am. Oil Chem. Soc. 64*, 414–421.
16. Rojo, J.A., and Perkins, E.G. (1989) Cyclic Fatty Acid Monomer: Isolation and Purification with Solid-Phase Extraction, *J. Am. Oil Chem. Soc. 66*, 1593–1595.
17. Sébédio, J.L., Prevost, J., and Grangirard, A. (1987) Heat Treatment of Vegetable Oils I. Isolation of the Cyclic Fatty Acid Monomers from Heated Sunflower and Linseed Oils, *J. Am. Oil Chem. Soc. 64*, 1026–1032.
18. Perkins, E.G., and Taubold, R. (1978) Nutrition and Metabolic Studies of Noncyclic Dimeric Fatty Acid Methyl Esters in the Rat, *J. Am. Oil Chem. Soc. 55*, 632–634.
19. Lamboni, C., and Perkins, E.G. (1996) Effects of Dietary Heated Fats on Rat Liver Enzyme Activity, *Lipids 31*, 955–962.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*, 265–275.
21. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
22. Lo, S., Russell, J.C., and Taylor, A.W. (1970) Determination of Glycogen in Small Tissue Samples, *J. Appl. Physiol. 28*, 234–236.
23. Lamboni, C. (1983) Etude Expérimentale Chez le Rat des Effets d'un Traitement par un beta-Bloquant, le Pindolol, sur les Caractéristiques Métaboliques de l'Epuisement, Thèse de Doctorat de 3ème Cycle, Université de Dijon, France.

24. Lake, B.G. (1987) Preparation and Characterization of Microsomal Fractions for Studies on Xenobiotic Metabolism, in *Biochemical Toxicology. A Practical Approach* (Snell, K., and Mullock, B., eds.), pp. 183–215, IRL Press, Oxford, Washington D.C.
25. McGarry, J.D., Mills, S.E., Long, C.S., and Foster, D.W. (1983) Observations on the Affinity for Carnitine and Malonyl CoA Sensitivity of Carnitine Palmitoyltransferase-I in Animal and Human Tissues, *Biochem. J. 214*, 21–28.
26. Lamboni, C. (1993) Effects of Dietary Heated Fats and Cyclic Fatty Acid Monomers on Rat Liver Enzyme Activity, Doctoral thesis, University of Illinois, Urbana-Champaign, Illinois.
27. Siess, M.H., Vernevaut, M.F., Grandgirard, A., and Sébédio, J.L. (1988) Induction of Hepatic Drug-Metabolizing Enzymes by Cyclic Fatty Acid Monomers in the Rat, *Food Chem. Toxicol. 26*, 9–13.
28. Miller, K.W., and Long, P.H. (1990) A 91-Day Feeding Study with Heated Olestra/Vegetable Oil Blends, *Food Chem. Toxicol. 28*, 307–315.

[Received November 11, 1996, and in final revised form and accepted May 22, 1998]

資料３

Codex 提出書類

(The Hague, the Netherlands, 25-29 April 2005)

codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Viale delle Terme di Caracalla 00100 ROME Tel: 39 06 57051 www.codexalimentarius.net Email: codex@fao.org Facsimile: 39 06 5705 4593

Agenda Item 18

CX/FAC 05/37/37
December 2004

JOINT FAO/WHO FOOD STANDARDS PROGRAMME
CODEX COMMITTEE ON FOOD ADDITIVES AND CONTAMINANTS
Thirty-seventh Session
The Hague, the Netherlands, 25 – 29 April 2005

PRIORITY LIST OF FOOD ADDITIVES, CONTAMINANTS AND NATURALLY OCCURRING TOXICANTS PROPOSED FOR EVALUATION BY JECFA

• COMMENTS (CL 2004/9-FAC)

The following comments have been received from: Japan and ISA

JAPAN

Background

1. In Japan, instant noodles have been regulated by the standards in the Food Sanitation Law; Acid value (AV) of fats and oils contained in instant noodles shall not be more than 3 and peroxide value (PV) shall not be more than 30. This standard was established after we experienced food-poisoning incidents (69 patients) in 1964 caused by the degradation of oil in the instant noodle.
2. The 27th Session of the Codex Alimentarius Commission noted that the Committee on Food Additives and Contaminants (CCFAC) considered that the PV for instant noodles was not a question of safety and therefore was not included in the priority list for JECFA evaluation. The CCFAC noted that there were no data proving a positive correlation between peroxide values of foods and food toxicological parameters. The Commission noted that the draft Standard for Instant Noodles, adopted at Step 5 by its 26th Session, had been circulated for comments at Step 6. A revised text was under preparation to take account of the comments received; the revised draft Standard would be circulated for additional comments and consideration for advancement to Step 8 by the Committee on Cereals, Pulses and Legumes while the list of food additives was to be completed and endorsed by CCFAC.
3. The Delegation of Japan expressed its concern that the CCFAC reply was not based on a risk assessment and reiterated its proposal to include PV in the draft Standard for Instant Noodles.
4. The Commission agreed that the elaboration of the draft Standard should proceed without further delay, with the understanding that the inclusion of PV could be decided by CCFAC in the future in the light of relevant data to be submitted by the Government of Japan to the CCFAC for consideration.

Comments

1. Japan believes that the provisions on PV should be established to protect not only the quality but also the consumers' health, because only the provision of Acid Value is not enough to catch the rancidity of oils and fats. PV holds the amount of peroxide caused by absorption of atmospheric oxygen in fats and shows the producing amount of peroxide as a toxic substance.

2. Japan has been performing the study on the degree of PV and toxicity of fats with degradation in Instant Noodles, and also the study on actual condition of PV in Instant Noodles being distributed in the international markets. Japan herewith submits the results of these studies.
3. According to the results of these studies, it is concluded that oxidation of the oil is very apprehensive phenomenon for food safety because it involves formation of lipid hydroperoxide (indicated by PV) and the secondary oil oxidized product. This change can be measured only by PV, not by AV, because PV and AV do not increase simultaneously. Consequently, measuring PV in oil is very important to grasp the deterioration level of the oil in food from the food safety point of view. The formation of lipid hydroperoxide is slow at first; however, it increases at an explosive pace after the induction period. To prevent this explosive increase of lipid hydroperoxide, keeping the PV at low level, such as 30 meq/kg, is a significant point.
4. Japan strongly proposes that the provision of PV should be considered in the CCFAC based on the evaluation by JECFA (Information on PV to be evaluated by JECFA is attached below).

INFORMATION ON THE CONTAMINANT TO BE EVALUATED BY JECFA

1. Proposal for inclusion submitted by:

   Japan

2. Name of compound; chemical name(s):

   Peroxide Value

3. Identification of (additional) data (toxicology, metabolism) which could be provided to JECFA:

   - Studies on Degradation of Fats in Foods Regarding Relationship between the Degree of Degradation and the Toxicity of Fats in Pre-Cooked Instant Noodles (See Annex 1)
   - List of articles available (See Annex 2)*

4. List of contact persons, including name and address, providing surveillance data with quality assurance information, preferably from three or more regions of the world:

   Codex Contact Point for Japan

* The hard copies of the articles listed in the Annex 2 will be submitted by separate mail.

Studies on Degradation of Fats in Foods Regarding Relationship between the Degree of Degradation and the Toxicity of Fats in Pre-Cooked Instant Noodles

Shun WADA, Ph. D., Prof.

Naohiro GOTOH, Ph. D.

Dept. of Food Science and Technology

Tokyo University of Marine Science and Technology

Introduction

Instant Noodle (instant ramen) was invented in Japan about a half century ago and is now eaten 65.2 billion packages per year (2003 data) by a lot of people all over the world[1]. This food is easy to cook and just boiling it in a hot water for a few minutes (Packaged Noodle) or just pouring a hot water to the noodle (Cup-type Noodle) because the starch of the noodle is pregelatinized. Gelatinization is important process to cook noodle, for instance, the pasta before cook is very hard because the starch in the pasta is not gelatinized. Therefore, the pasta must be boiled to make it soft. This process is "gelatinization" and the way of frying a boiled noodle with oil was hired to fix the pregelatinization condition for instant noodles[1]. Consequently, it would be easily understood that frying the boiled noodle is very important process to prepare Instant Noodle and oil is indispensable ingredient for Instant Noodle.

About 40 years ago, 1964-1965, Japan had terrible food-poisoning incidents caused by the degradation of oil in Instant Noodle[2]. Fortunately, no one died in these incidents, however, many people who ate degraded Instant Noodle had nausea, vomiting, diarrhea, abdominal pain, feeling of weariness and headache. In the severest case, 69 people (men: 38, women: 31) who ate Instant Noodle manufactured by the same company suffered from food-poisoning. The degradation levels of the oil in the noodles were peroxide value (PV): 565-805 meq/kg and acid value (AV): 7.1-28.8. After that, the Ministry of Health and Welfare, current the Ministry of Health, Labor and Welfare, in Japan set standard value for fried type Instant Noodle in Food Sanitation Law to protect the food-poisoning and control the quality of Instant Noodle[3]. In the law, the standard value for PV was set in 30 meq/kg or less than that and AV was set in 3 or less than that. After setting these values, no food-poisoning incidents caused by Instant Noodle have happened so far in Japan.

Japan has proposed the food standard for Instant Noodle to the Codex Committee on Cereals, Pulses and Legumes through the Codex Regional Coordinating Committee for Asia to make it international standard. However, including standard value on PV is still under discussion because many countries believe that measuring only AV is enough to grasp the degradation situation of oil in Instant Noodle. Taking account of food poisoning incident in Japan, measuring PV would be very important from the food sanitation point of view. Consequently, in this review, the relationship between PV and AV in Instant Noodle, how increase PV in Instant Noodle and the relationship between oxidation of the oil and toxicity were discussed.

Relationship between PV and AV in Instant Noodle

The concept for measuring PV and AV are completely different. It is now accepted that the secondary oil oxidized products such as polymerized oil, cyclic fatty acid, hydroperoxy alkenal and hydroxyl alkenal are main cause of toxicity in oxidized oil[4)-14)]. Therefore, the formation of lipid hydroperoxide, the primary oil oxidized product, must be suppressed to prevent the formation of the secondary oil oxidized products in Instant Noodle. In Japan, PV is hired to monitor the formation of the primary oil oxidized product, namely lipid hydroperoxide[15)-17)]. On the contrary, AV is measured to keep the food quality. During the food processing and storage, free fatty acids are formed in the noodle by the hydrolysis of the oil. Free fatty acid itself is not a very toxic compound, however, it becomes a cause of the reduction of flavor and taste. The purpose of measuring AV is to check the free fatty acid level in Instant Noodle[18)19)].

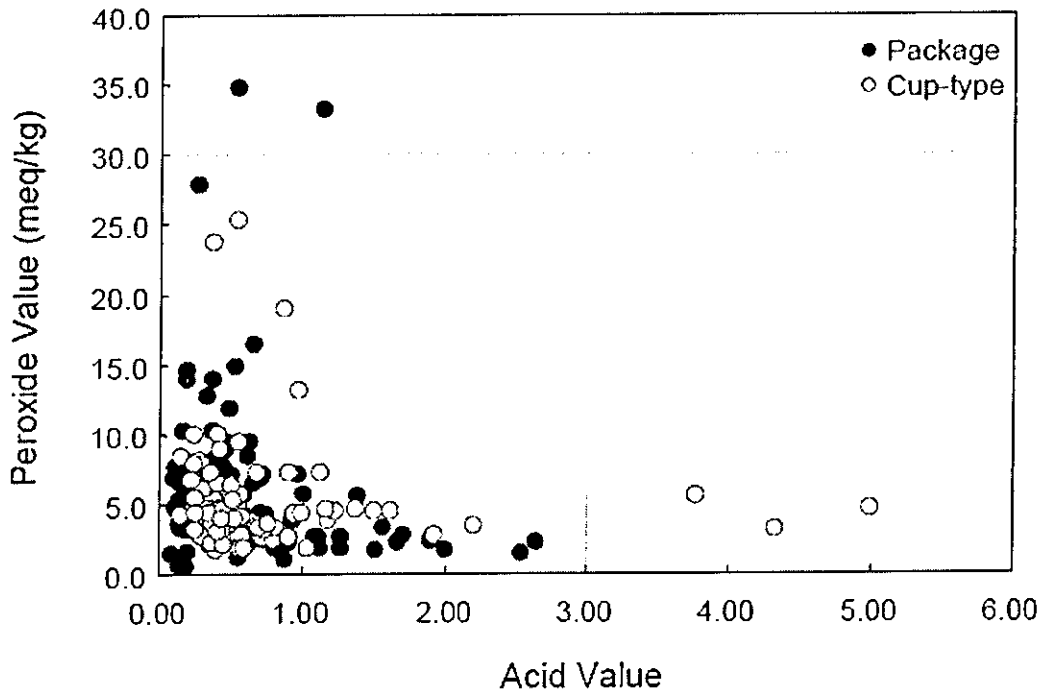


Figure 1. Acid value (AV) vs peroxide value (PV) in 218 Instant Noodles collected from all over the world[20)].

Japan proposed the food standard of Instant Noodle that contains PV and AV to the Codex Regional Coordinating Committee for Asia to make it international standard. However, several representatives of Asian countries did not accept the proposal from Japan. Particularly, including PV in the standard was opposed because they recognized that the PV and AV would increase together during the deterioration of Instant Noodle and measuring AV is enough to keep the food safety and quality. As mentioned above, the concepts for measuring PV and AV are completely different and PV is an essential item to keep the food safety. Consequently, 218 kinds of fried type Instant Noodles were collected from commercial base of all over the world and measured PV and AV of them to grasp the deteriorated situation of Instant Noodles sold in the market[20]. Furthermore, the relationship between PV and AV values was investigated to confirm the truth of other countries opinions. All the measured values on PV and AV are plotted in Figure 1. These results show that the both values are spread to wide range and some of them exceed the criteria (PV: 30 meq/kg and AV: 3) established in Food Sanitation Law in Japan. Since almost all samples were sold in cool condition, the samples exceeding 30 in PV might be exhibited under strong light for a long period. On the other hand, the samples exceeding 3 in AV might be stored under high humidity. Light and moisture strongly affect the degradation of oils. Miura et al.[11][12] made a deteriorated Instant Noodle, which is as same as deteriorated Instant Noodle caused food poisoning in 1964, with sunlight and high temperature and succeeded in reappearing the food poisoning with the sample. Consequently, it would be said that cutting light or sun light is the most important way to preserve Instant Noodle even the material of the package film can suppress the UV and water transmission. In Figure 1, if the both PV and AV increase simultaneously during storage, the approximating curve against these plots must become ever-increasing curve. However, the plots are not scattered like that. The coefficient of correlation for PV and AV was calculated with Pearson's product-moment coefficient of correlation and the result was -0.1083. This value means that the plots are scattered in the downward-sloping and the correlation between PV and AV is poor because the coefficient of correlation is lower than zero and the absolute value is near zero. Consequently, the coefficient of correlation reveals that PV and AV do not form simultaneously in the oil of Instant Noodle during the deterioration. Furthermore, the *P* value was also calculated and the value was 0.1106. This value also explains that the relation between PV and AV is not significant because the value was bigger than 0.05. Therefore, analyzing only AV cannot grasp any deteriorated situation of the oil in Instant Noodle and analyzing both PV and AV has a strong and significant meaning. We conclude that PV is also an indispensable factor to keep the food safety and quality of Instant Noodle.

How increase PV in the oil of Instant Noodle

A great number of studies concerning the oxidation or heating of the oil have been carried out so far. These studies are mainly separated to three types of studies.

(1) The most popular study is that the oil is heated at more than 250 °C under oxygen omitted circumstances such as under nitrogen, carbon dioxide, etc[4)-6)]. This kind of heating forms polymerized oil and cyclic fatty acid without containing oxygen molecular in the structure. These compounds are very toxic, however, these compounds are not oxidized compound. Furthermore, it must be said that these study conditions are not realistic. Consequently, these results are not available when the food toxicity of the oil is discussed.

(2) The oxidations of the oil under atmospheric condition are also carried out[7)-10)]. In this degradation, the oxidation of the oil proceeds by radical chain reaction via lipid peroxyl radical (Figure 2)[21)]. Therefore, the compound formed in this reaction contains oxygen molecular in it. These studies are separated to two types of studies. One is heating the oil over 100 °C and the other is less than that. Taking account of the accumulation of lipid hydroperoxide (PV) in the system, the heating temperature is important point. For instance, the temperature usually hired for deep-fry and stir-fry is around 180 °C and the lipid hydroperoxide is decomposed easily under this condition[22)]. As the result, the PV of the oil does not increase it and the secondary oil oxidized products are formed instead of that. However, it has been reported that the polymerized oil and cyclic fatty acid are not accumulated much in the system. Frankel et al. measured the cyclic fatty acid level in the oil used at fast foods restaurant and found that 0.1-0.5% of total fatty acid was changed to cyclic fatty acid[23)]. The oxidation heated at less than 100 °C accumulates lipid hydroperoxide in the system because the rate of the formation of lipid hydroperoxide is faster than the rate of the decomposition of that. Normally, this kind of oxidation is called "autoxidation". The autoxidation also proceeds under atmospheric condition and accumulates the lipid hydroperoxide (PV) in the system at first (Figure 3)[24)]. The amount of the lipid hydroperoxide finally reaches to the top, after that, it starts to decrement because the rate of the formation of lipid hydroperoxide becomes slower than the rate of the decomposition of that. The reaching level depends on the kinds of fatty acids consisting of the oil, heating temperature, etc. The decomposed lipid hydroperoxide forms aldehyde, ketone, alcohol, alkane, etc. It is now accepted that the hydroxyl alkenal and hydroperoxyl alkenal formed in autoxidation is very strong toxic compounds[14)]. Therefore, the autoxidized oils are also toxic.

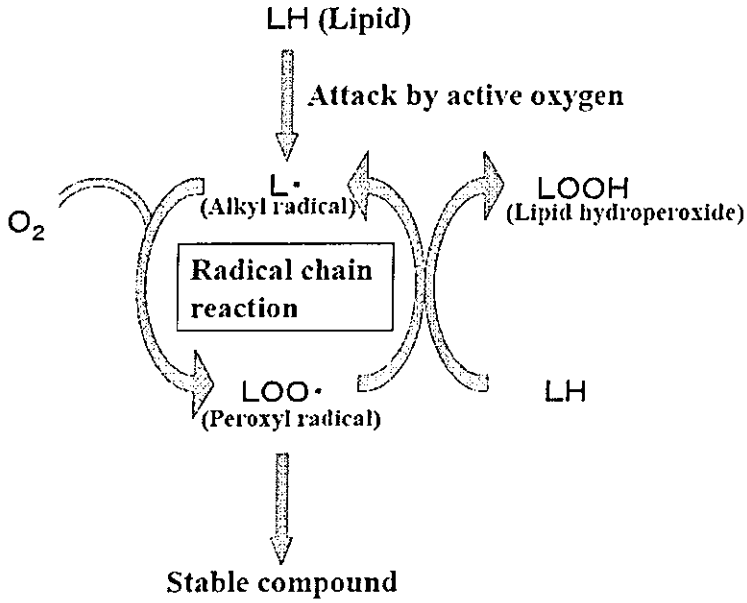


Figure 2. Radical chain reaction on lipid oxidation.

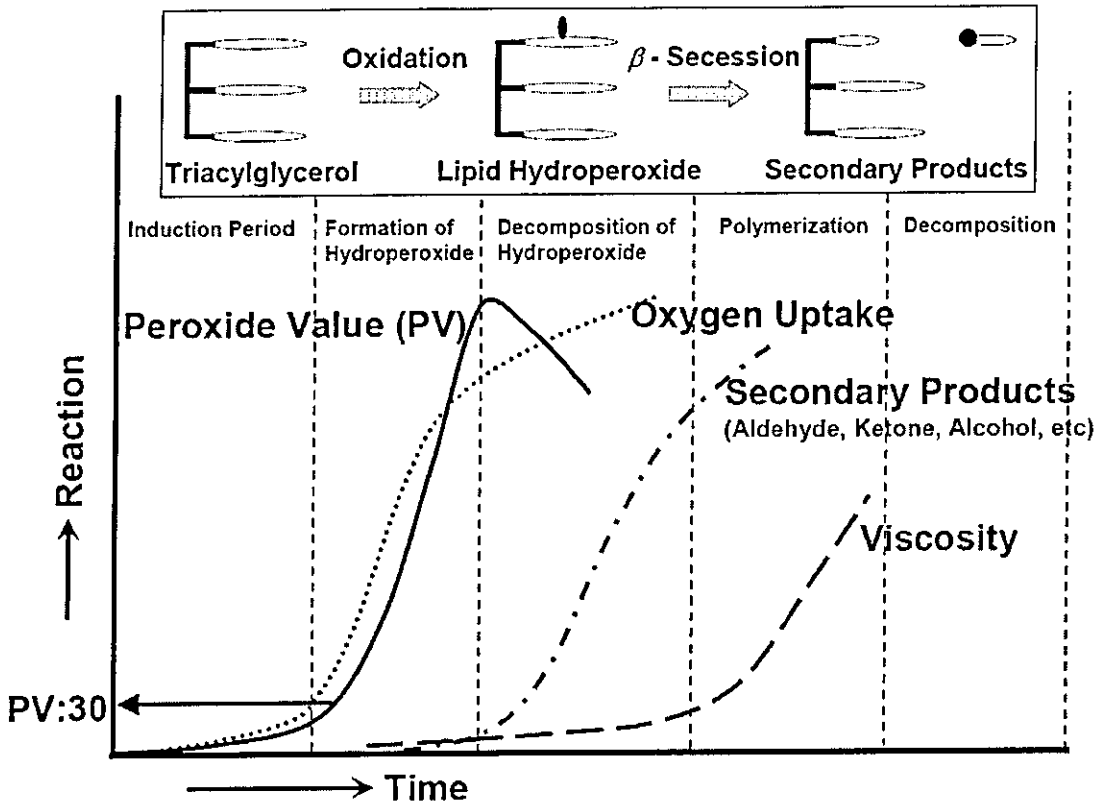


Figure 3. Each stage of autoxidation of the lipids.

(3) The oil degraded by heat and light is also a big problem. This degradation is mixture of autoxidation and photoxidation[11)-13)]. Photoxidation is not radical reaction, but ene reaction[25)]. Therefore, the reaction mechanism is different from radical chain reaction to form the different kinds of degraded oil compounds. The oxidation of the oil in Instant Noodle that caused the food toxicity was developed by these reactions as already mentioned in previous section. It is very difficult to understand how the individual reaction intertwine with each other and finally cause the toxic compounds in this oxidation.

Almost all of the oxidation reactions proceed via formation of lipid hydroperoxides. Therefore, to prevent the formation of lipid hydroperoxides is the best way to keep the food safety and quality. The oil in Noodle is also no exception. In fact there are a few studies which measured how increased PV during the degradation of the oil. Therefore, oxidation of the Instant Noodle was carried out to grasp how increase PV in Instant Noodle. As the results, PV increases slowly till the PV reaches to 50 meq/kg, however, after beyond the 50 meq/kg[26)], explosive increment of PV started in the case of the Instant Noodle is Stored at 60 °C. In Food Sanitation Law of Japan, PV is set in 30 meq/kg or less than that. This would not be a high value. The toxicity of lipid hydroperoxide was investigated by Tovar et al. and the LD_{50} was 12,760 mg/kg mice. On the contrary, LD_{50} of 4-hydroperoxy-2-nonenal, strong toxic compound formed in oil deterioration, is 77.5 mg/kg mice[14)]. Though the deteriorated oil in Instant Noodle which PV is 30 meq/kg does not show any toxicity, the concept of the setting PV in 30 meq/kg is to prevent the oxidation to proceed to the next stage reaction via lipid hydroperoxide and the formation of toxic compounds. Therefore, setting PV in a low value possesses great significance to obtain the quality and safety. Particularly, the low standard value has a great meaning in the case of the oxidation pathway is complicated like the mixture of autoxidation and photoxidation.

The relationship between oxidation of the oil and toxicity

As mentioned above, the lipid hydroperoxides (indicated by PV) are not strong toxic compounds, however, many kinds of studies reveal that the toxicity of the degraded oil increases according to the increase of the lipid hydroperoxide level[10)]. Of course, the toxicity of the oil furthermore increases after decomposition of lipid hydroperoxide starts. In a word, both of lipid hydroperoxide and the secondary oil oxidized products are important toxic compounds[14)].