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Hematological and Lipid Changes in Newborn Piglets Fed Milk-Replacer Diets Containing Erucic Acid

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ABSTRACT: Canola oil is not presently permitted in infant formulations in the United States because of lack of information concerning the effects of feeding canola oil to the newborn. We have previously reported a transient decrease in platelet counts and an increase in platelet size in newborn piglets fed canola oil for 4 wk, and have confirmed this in the present study. In canola oil-fed piglets, changes in platelet size and number were overcome by adding either long-chain saturated fatty acids from cocoa butter (16:0 and 18:0), or shorter-chain saturates from coconut oil (12:0 and 14:0). Feeding a high erucic acid rapeseed (HEAR) oil, with 20% 22:1n-9, led to an even greater platelet reduction and increased platelet size throughout the 4-wk trial. Bleeding times were longer in piglets fed canola oil or HEAR oil compared to sow-reared and soybean oil-fed piglets. There were no other diet-related changes. Diet-induced platelet changes were not related to platelet lipid class composition, but there were fatty acid changes. The incorporation of 22:1n-9 into platelet phospholipids of piglets fed canola oil was low (0.2–1.2%), and even for the HEAR oil group ranged from only 0.2% in phosphatidylinositol to 2.4% in phosphatidylserine. A much greater change was observed in the concentration of 24:1n-9 and in the 24:1n-9/24:0 ratio in platelet sphingomyelin (SM). The 24:1n-9 increased to 49% in the HEAR oil group compared to about 12% in animals fed the control diets (sow-reared piglets and soybean oil-fed group), while the 24:1n-9/24:0 ratio increased from about 1 to 12. Even feeding canola oil, prepared to contain 2% 22:1n-9, led to a marked increase in 24:1n-9 to 29% and had a 24:1n-9/24:0 ratio of 5. The canola oil/cocoa butter group, which also contained 2% 22:1n-9, showed a lower level of 24:1n-9 (20%) and the 24:1n-9/24:0 ratio (3) compared to the canola oil group. The results suggest that the diet-related platelet changes in newborn piglets may be related to an increase in 24:1n-9 in platelet SM, resulting from chain elongation of 22:1n-9. The inclusion of canola oil as the sole source of fat in the milk-replacer diets of newborn piglets resulted in significant platelet and lipid changes.

Lipids 33, 1–10 (1998).

The present U.S. Food and Drug Administration regulations do not permit the inclusion of canola oil in infant formulations (1) because of lack of experimental evidence concerning the effects of feeding canola oil, and specifically erucic acid (22:1n-9), to the newborn. We have recently shown that piglets fed milk-replacer diets containing canola oil showed a transient delay in the rise of platelet numbers and an associated increase in platelet volume (2). In addition, some vegetable oils high in oleic acid (18:1n-9), such as high-oleic acid sunflower oil (HOAS), a mixture of oils that mimicked canola oil, and olive oil, also showed these platelet changes. These platelet changes were overcome by the third week on continuous feeding of these diets, or immediately by adding coconut oil to canola oil (2). There was no evidence that the lower platelet counts were associated with increased bleeding times in piglets fed these diets compared to sow-reared piglets. However, the question remained as to whether 22:1n-9 could further decrease platelet counts in the newborn piglet, and possibly affect bleeding times. The inclusion of these oils into infant formulations with resultant low platelet count and a prolonged bleeding time may have serious implications for the infant, since thrombocytopenia is known to occur in premature and term infants (3,4).

Previous studies with human test subjects showed that normal young men given diets containing high-erucic acid rapeseed (HEAR) oil (39% 22:1n-9) for 22 d had a pronounced drop in platelet count with a return to normal after discontinuation of the diet for 1 wk (5). A similar decrease in platelet counts was observed in patients suffering from X-linked adrenoleukodystrophy (ALD) who had received a dietary mixture of triolein and trierucin (4:1) in order to reduce their level of plasma very long chain saturated fatty acids, particularly 26:0 (6–8). These patients showed minor bruising, but no clinical symptoms of bleeding (6), and their platelet counts returned to normal after discontinuation of the diet (6,7). It is of interest to note that lower platelet counts are not limited to

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Abbreviations: ALD, adrenoleukodystrophy; CE, cholesterol esters; FFA, free fatty acids; Hb, hemoglobin; HEAR, high erucic acid rapeseed; HOAS, high oleic acid sunflower; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SM, sphingomyelin.

22:1n-9 consumption. Eating fish and/or fish oil diets that contain high levels of cetoleic acid (22:1n-11), a positional isomer of erucic acid (22:1n-9), also caused a decrease in platelet counts and an increase in bleeding times (9–12).

The present study was undertaken to further evaluate the platelet changes associated with the feeding of milk-replacer diets containing canola oil. Newborn piglets were either left with the sow or fed milk-replacer diets containing soybean oil, canola oil, a canola oil/cocoa butter mixture, or a HEAR oil for 4 wk. The HEAR oil diet was included to determine the effect of high levels (20%) of 22:1n-9 on platelets and bleeding times in newborn piglets. The canola oil/cocoa butter mixture was included to determine if the long-chain saturated fatty acids caused the same ameliorating effect on platelets as the short-chain fatty acids derived from coconut oil (2).

MATERIALS AND METHODS

Animals and diets. Newborn Yorkshire male and female piglets were left with the sow for 1 d prior to allocating them to one of five dietary groups, balanced for litter and sex. Four groups of six piglets/diet were fed milk-replacer diets in specially designed cages (13), and one group of five piglets was left cross-fostered with one sow. Body weights were measured at 1 d of age and every 3 d thereafter, and the feed (dry matter) was adjusted to 7% of body weight/day.

The following dietary oils and oil mixtures were fed: soybean oil obtained from CanAmera Foods Inc. (Toronto, Ontario, Canada); a mixture (92:2) of canola oil (0.8% 22:1n-9) and HEAR oil (42.9% 22:1n-9) (both oils obtained from CanAmera Foods Inc., Altona, Manitoba, Canada) to increase the level of 22:1n-9 to 2%, the maximum permissible level of 22:1n-9 in canola oil in the United States (1); a mixture of canola oil, cocoa butter (26% 16:0, 29% 18:0, 38.5% 18:1n-9; Hershey Canada Inc., Smith Falls, Ontario, Canada), HEAR oil, and linseed oil in the ratio of 52:40:4:4, which contained about 2% 22:1n-9 and 8% linolenic acid (18:3n-3), similar to canola oil; and a mixture of HEAR oil and canola oil (46:54), prepared with a final content of about 20% 22:1n-9. The fatty acid compositions of the dietary oils are shown in Table 1.

The milk-replacer diets were prepared as described previously (14) with 25% dietary oil, and all vitamins were added after the spray-drying process. The vitamin E content was 150 IU/kg diet to avoid vitamin E deficiency in the canola oil-fed group (15). The dry-milk powders were stored at –20°C until used. The milk replacers were prepared daily to include 21% solids and 16% γ-globulin (2). The digestible energy content of the final milk replacer was 953 kcal/L (16).

Pathology. After 4 wk of experiment, each piglet was anesthetized within 2 h of removal from the diet with a 5:1 mixture of ketamine hydrochloride (Rogarsetic-Rogar/STB, Montréal, Québec) and acepromazine maleate (Atravet-Ayerst Lab., Montreal, Québec) and killed by exsanguination. A complete gross pathology was performed on each piglet, and 20 different tissues were sampled for histological analysis. All tissues were fixed in formalin.

TABLE 1
Fatty Acid Composition of the Dietary Oils

Fatty acids	Sow milk	Soybean oil	Canola oil[a]	Canola/cocoa[b]	HEAR oil[c]
≤14:0	2.1	0.1	0.1	0.1	0.1
16:0	23.2	9.9	5.1	13.1	4.2
16:1n-7	4.6	0.2	0.3	0.4	0.3
18:0	6.1	3.6	2.2	12.6	1.7
18:1n-9	32.4	23.8	50.8	43.0	36.1
18:1n-7	2.8	1.5	2.6	2.2	1.8
18:2n-6	22.9	50.0	22.5	15.0	18.9
18:3n-3	1.1	8.7	9.9	7.7	9.3
20:0	0.2	0.4	0.8	0.8	0.8
20:1n-9	0.6	0.4	2.1	1.4	4.2
22:0	0.1	0.4	0.5	0.3	0.6
22:1n-9	0.1	0	2.0	2.2	20.2
24:0	0.1	0.1	0.2	0.1	0.2
24:1n-9	0.1	0	0.3	0.2	0.7
n-6 PUFA[d]	1.8	0	0	0	0
n-3 PUFA[e]	0.7	0	0	0	0

[a]Mixture of canola oil (0.8% 22:1n-9) and high-erucic acid rapeseed (HEAR) oil (42.9% 22:1n-9) in a ratio of 98:2 to give a final oil with 2% 22:1n-9.
[b]Mixture of canola oil, HEAR oil, linseed oil (49% 18:3n-3), and cocoa butter in a ratio of 52:4:4:40.
[c]Mixture of canola oil and HEAR oil in a ratio of 54:46 to give a final oil with 20% 22:1n-9.
[d]n-6 PUFA, polyunsaturated fatty acids, includes 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6.
[e]n-3 PUFA, includes 20:5n-3, 22:5n-3, and 22:6n-3.

Blood measurements. Blood (0.5 mL) for hematological measurements was taken from the piglets by orbital sinus puncture (17) in tubes containing EDTA as anticoagulant. The blood was throughly mixed with the anticoagulant, transported at room temperature, and analyzed within 1–2 h for complete blood count using a Technicon H-2 analyzer (Technicon Instrument Corp., Tarrytown, NY). Bleeding time measurements were taken using a Simplate II device as described previously (2).

Platelet isolation. At time of kill, 15 mL of blood was taken from each piglet in vacutainer tubes containing EDTA. The whole blood was centrifuged at 150 × *g* at room temperature for 15 min. The plasma was adjusted to pH 6.5 with 1 M citric acid, and 2 drops of EDTA were added. Traces of red and white cells were removed by centrifuging the plasma again at 150 × *g* at room temperature, followed by 2500 × *g* at 4°C for 15 min to remove the platelets. The platelets were suspended in 1 mL of buffer (20 mM Na_2HPO_4, 130 mM NaCl, and 2 mM EDTA at pH 6.5) containing inhibitors (10 μL 4 mM iodoacetamide, 20 μL 4 mM *N*-ethylmaleimide in 50% ethanol, and 10 μL aprotinin containing 10,000 units/mL) and centrifuged at 2500 × *g* at 4°C for 15 min. The platelets were then stored at –70°C until analyzed.

Lipid analysis. Total platelet lipids were extracted using the Bligh and Dyer mixture (18). The total platelet lipids from each individual piglet were not sufficient to perform a complete lipid class analysis, and therefore, the lipids from two piglets on the same diet were pooled. All the lipid classes

were separated by three-directional thin-layer chromatography (TLC) using silica gel H plates (19). The spots were visualized with ultraviolet light after spraying the plates with 2′,7′-dichlorofluorescein. The spots were scraped off, methyl heptadecanoate was added as internal standard, and the lipids were methylated in the presence of the silica gel using anhydrous HCl/methanol (5% by wt) at 80°C for 1 h. The resulting methyl esters and dimethyl acetals were analyzed by gas–liquid chromatography using a 30-m Supelcowax-10 capillary column (Supelco Inc., Bellefonte, PA) (20).

Statistics. The blood data were analyzed using the general linear models procedure of SAS (21), with terms for litter and diet as major effects, while the fatty acid data were analyzed with diet as the major effect.

RESULTS

Growth and health. The piglets fed the milk-replacer diets gained significantly ($P < 0.05$) more weight over the 4-wk period (from 5.0–6.8 kg) than piglets left with the sow (4.0 ± 0.4 kg). There were some differences in weight gain among the four milk-replacer diets. After 9 and 12 d on diet, the HEAR oil group showed the best (1.3 ± 0.08 and 1.9 ± 0.1 kg) and canola oil the lowest weight gain (0.9 ± 0.08 and 1.3 ± 0.1 kg), but after 4 wk on diet, the soybean oil group showed best (6.8 ± 0.5 kg) and HEAR oil the lowest weight gain (5.0 ± 0.4 kg). No diet-related gross or histological abnormalities were observed among the piglets. One animal fed the canola/cocoa butter mixture was euthanized at 12 d of age because of congenital rectal stricture, and three animals died of unrelated causes: two fed soybean oil, on days 24 and 27, and one fed HEAR oil, on day 28.

Platelets. One-day-old piglets had a relatively low platelet count ranging from 167 to 372 × 10^9/L with an overall mean of 285 ± 21 × 10^9/L ($n = 29$). The platelet counts in the sow-reared piglets doubled after 1 wk, and remained high thereafter (Table 2). The platelet counts agreed with published data for normal newborn (337 ± 79 × 10^9/L) and adult (578 ± 128 × 10^9/L) pigs (22,23). The lifetime of pig platelets was reported to be 9–11 d (22). Piglets fed soybean oil showed a somewhat slower rise in platelet counts after the first 2 wk, which was, however, not significantly different compared to sow-reared piglets (Table 2).

Piglets fed canola oil with 2% 22:1n-9 showed an increase in platelet counts after the first week, but thereafter the counts were significantly lower throughout the 4-wk trial compared to the sow-reared control group (Table 2). The addition of cocoa butter to canola oil enriched the long-chain saturated fatty acids 16:0 and 18:0 in the dietary oil mixture, resulting in a significantly higher platelet count than seen with canola oil alone (Table 2).

The HEAR oil-fed group with 20% 22:1n-9 showed markedly lower platelet counts throughout the 4-wk trial (Table 2). Generally, the platelet counts for this group did not increase above the levels observed at 1 d of age.

Mean platelet volume. The platelet volume decreased in the sow-reared piglets from 14 to 10 fL per platelet (Table 2). The decease was slower in the soybean oil and the canola oil/cocoa butter groups, but at 4 wk on diet, the values were similar to the sow-reared controls. The platelet volume remained elevated for the canola oil group throughout the 4-wk trial, and increased significantly in piglets fed the HEAR oil diet. The platelet volume for the HEAR oil-fed group after 4 wk on diet was greater than 22 fL, the upper limit of discrimination of the H-2 analyzer. There was a significant negative correlation of platelet size to platelet counts (–0.64; $n = 135$).

Other hematological parameters. The packed cell volume, hemoglobin (Hb), and red blood cell counts were significantly higher in the sow-reared piglets compared to piglets fed the milk-replacer diets (Table 2). There were no significant differences among the piglets fed the milk-replacer diets, except for piglets fed the HEAR oil diet. The HEAR oil group showed lower values for all these three parameters, and which were significant for packed cell volume and Hb. There were generally no significant diet differences in mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, prothrombin time, and activated thromboplastin time (Table 2).

Bleeding times. The bleeding time increased in all piglets with age (Table 3). The increase was most rapid in the HEAR oil group. After 4 wk on diet, only the HEAR oil and canola oil groups gave significantly higher bleeding times compared to the other groups.

Platelet lipids. The platelet lipids were not significantly different between the dietary groups, and for that reason the mean for all diets for each of the platelet lipid classes is shown in Table 4. The major phospholipids were phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with intermediate levels of sphingomyelin (SM) and phosphatidylserine (PS), a smaller amount of phophatidylinositol (PI), and trace amounts of diphosphatidylglycerol. In addition, the neutral lipids cholesterol, cholesterol esters (CE), and free fatty acids (FFA) were present. The results were expressed as percentage of total lipids and compared to results from human and dog platelets (Table 4).

Platelet phospholipid fatty acids. Each of the four phospholipids showed characteristic fatty acid profiles (Table 5). Alk-1-enyl ethers were present mainly in PE. All the phospholipids contained about 50% saturated fatty acids, except PE which had about 30%. PC had the highest concentration of 16:0, while 18:0 predominated in the other phospholipid classes. The highest levels of arachidonic acid (20:4n-6) were present in PE and PI, and the highest levels of C_{22} polyunsaturated fatty acids (PUFA) (22:4n-6, 22:5n-3, and 22:6n-3) were found in PE and PS.

Sow's milk consisted mainly of 16:0, oleic acid (18:1n-9), and linoleic acid (18:2n-6), with small amounts of preformed long-chain n-6 (1.6%) and n-3 (0.7%) PUFA (Table 1), which were reflected in the fatty acid profile of the phospholipids (Table 5). Piglets fed soybean oil in their milk-replacer diets generally showed increased levels of 18:0 and 18:2n-6 compared to sow-reared piglets, at the expense of 16:0 and 18:1n-9. Despite the fact that soybean oil was rich in 18:2n-6

TABLE 2
Hematological Variables of Piglets Left with the Sow or Fed Milk-Replacer Diets (for 4 wk)[a]

Diets	Time on diet (wk) 0	1	2	3	4	Time on diet (wk) 0	1	2	3	4
	PCV (g/L)					PLT ($\times 10^9$/L)				
Sow-reared	0.27	0.33	0.36[a]	0.37[a]	0.36[a]	281	567[a]	776[a]	661[a,b]	586[a]
Soybean oil	0.32	0.31	0.32[b]	0.34[a,b]	0.32[b]	264	465[a]	685[a]	758[a]	597[a]
Canola oil	0.31	0.34	0.31[b]	0.33[b]	0.33[a,b]	292	498[a]	497[b]	538[b]	431[b]
Canola/cocoa[b]	0.29	0.33	0.32[b]	0.32[b]	0.34[a,b]	298	517[a]	775[a]	607[a,b]	609[a]
HEAR[c] oil	0.32	0.31	0.31[b]	0.30[b]	0.30[b]	289	224[b]	321[c]	340[c]	229[c]
SEM[d]	0.017	0.009	0.011	0.017	0.014	21	51	57	92	41
ANOVA[e]	NS	NS	**	*	*	NS	**	***	*	***
	Hb (g/L)					MPV (fL)				
Sow-reared	9.3	10.8[a,b]	12.1[a]	12.4[a]	12.1[a]	14.4	12.2[b]	9.6[c]	9.6[c]	10.3[c]
Soybean oil	10.7	10.2[b,c]	10.4[b]	11.0[b]	10.3[b,c]	16.7	11.8[b]	12.2[b,c]	13.6[b]	10.9[c]
Canola oil	10.6	10.9[a]	10.1[b]	10.6[bc]	10.6[b,c]	16.1	13.5[a,b]	13.9[b]	13.8[b]	14.3[b]
Canola/cocoa	10.0	10.8[a,b]	10.6[b]	10.6[b,c]	11.2[a,b]	15.8	11.7[b]	10.6[b,c]	11.5[b,c]	10.4[c]
HEAR oil	10.6	10.0[c]	10.2[b]	9.6[c]	9.7[c]	15.5	15.9[a]	19.4[a]	20.7[a]	>22[a,f]
SEM	0.6	0.3	0.3	0.4	0.4	1.2	0.6	1.6	1.7	0.5
ANOVA	NS	*	**	**	**	NS	*	***	***	***
	RBC ($\times 10^{12}$/L)					MCV (fL)				
Sow-reared	4.6	5.3	6.1	6.7[a]	6.6[a]	59.1	61.4	59.1	55.3	55.0
Soybean oil	5.4	5.2	5.5	6.0[a,b]	5.6[b]	59.3	60.2	58.6	56.7	56.4
Canola oil	5.3	5.8	5.4	5.8[b]	5.9[a,b]	58.5	58.6	57.0	56.7	55.6
Canola/cocoa	4.9	5.5	5.5	5.5[b]	6.0[a,b]	60.0	60.8	58.4	57.3	57.1
HEAR oil	5.4	5.5	5.5	5.5[b]	5.5[b]	58.1	59.0	55.8	54.4	54.7
SEM	0.3	0.2	0.2	0.2	0.2	1.1	0.9	1.1	1.5	1.1
ANOVA	NS	NS	NS	*	*	NS	NS	NS	NS	NS
	MCH (pg)					PT (s)				
Sow-reared	20.1	20.3	19.8	18.6	18.4	11.5	9.9	10.2	10.3[a]	10.1
Soybean oil	19.9	19.8	19.1	18.3	18.3	10.9	10.7	10.3	9.5[c]	9.5
Canola oil	20.0	18.7	18.7	18.5	18.1	10.7	10.3	10.7	9.5[c]	10.0
Canola/cocoa	20.4	19.8	19.4	19.2	18.7	11.0	10.2	10.5	10.1[a,b]	9.9
HEAR oil	19.6	19.1	18.6	17.5	17.8	10.5	10.3	10.0	9.7[b,c]	11.8
SEM	0.4	0.4	0.4	0.4	0.4	0.6	0.2	0.3	0.2	0.8
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	*	NS
	MCHC (g/L)					APTT (s)				
Sow-reared	341	331	335	336	334	20.5	17.7	15.9	16.5[b]	18.1
Soybean oil	335	330	326	325	325	20.0	18.5	18.3	20.7[a,b]	19.1
Canola oil	341	319	328	326	326	20.3	18.7	17.7	15.8[b]	17.8
Canola/cocoa	341	325	332	335	327	21.8	16.8	17.7	17.2[a,b]	17.4
HEAR oil	339	324	332	322	325	20.3	17.2	17.8	22.2[a]	20.4
SEM	4	4	3	5	3	2.1	0.6	0.7	2.0	0.8
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	*	NS

[a]Means with different superscript letters a–c are significantly different ($P < 0.05$). PCV, packed cell volume; Hb, hemoglobin; RBC, red blood cell; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; PLT, platelets; MPV, mean platelet volume; MCV, mean corpuscular volume; PT, prothrombin time; APTT, activated thromboplastin time.
[b]A mixture of canola oil and cocoa butter; see Table 1.
[c]HEAR, high-erucic acid rapeseed.
[d]SEM, pooled standard error of the mean.
[e]ANOVA, analysis of variance testing for diets; NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[f]Out of range for the Technicon H-2 analyzer (Technicon Instrument Corp., Tarrytown, NY) settings.

and 18:3n-3, the concentration of PUFA derived from these precursors (i.e., 20:4n-6, 22:4n-6, and 20:5n-3, 22:5n-3, and 22:6n-3, respectively) was generally similar to, or lower than, that found in sow-reared piglets.

Both canola oil and HEAR oil were high in 18:1n-9 (36 and 51%, respectively), and contained similar levels of 18:2n-6 (~20%) and 18:3n-3 (~9%), but these two groups had marked differences in their concentration of long-chain monounsaturated fatty acids of the oleic acid family (n-9) (Table 1). The feeding of canola oil and HEAR oil generally resulted in similar changes in platelet phospholipids, except for the significantly higher levels of 20:1n-9, 22:1n-9, and

TABLE 3
Bleeding Times (min) of Piglets Left with the Sow or Fed Milk-Replacer Diets (for 4 Wk)[a]

	Time on diet (wk)				
Diets	0	1	2	3	4
Sow-reared	5.1	3.5[c]	7.0[c]	8.7[c]	10.5[b]
Soybean oil	3.9	5.6[b,c]	8.7[b]	10.8[b]	10.2[b]
Canola oil	4.8	6.1[b]	8.7[b]	12.0[a]	11.9[a]
Canola/cocoa[b]	4.0	5.5[b,c]	7.9[b,c]	10.5[b]	11.4[a,b]
HEAR[c] oil	4.8	9.6[a]	10.4[a]	11.3[a,b]	12.2[a]
SEM[d]	0.6	0.7	0.5	0.4	0.5
ANOVA	NS	***	***	***	*

[a]Means with different superscript letters (a–c) are significantly different ($P < 0.05$).
[b]A mixture of canola oil and cocoa butter; see Table 1.
[c]HEAR, high erucic acid rapeseed.
[d]SEM, pooled standard error of the mean. NS, $P > 0.05$; *$P < 0.05$; ***$P < 0.001$.

24:1n-9 in piglets fed HEAR oil (Table 5). The highest accumulation of 22:1n-9 in the platelet phospholipids of the HEAR oil group was found in PS (2.4%), intermediate in PC and PE (~1%), and lowest in PI (0.2%). With few exceptions, these two rapeseed oils resulted in increased levels of 18:1n-9, total monounsaturated fatty acids, 18:2n-6 and 18:3n-3 in all platelet phospholipid classes compared to sow-reared piglets, and decreased levels of 16:0 and total saturated fatty acids only in PC and PI. Furthermore, long-chain PUFA derived from 18:2n-6 (18:3n-6, 20:3n-6, 20:4n-6, and 22:4n-6) were lower in PE and PS, while long-chain PUFA derived from 18:3n-3 (20:5n-3, 22:5n-3, and 22:6n-3) were higher in all phospholipid classes.

The canola oil/cocoa butter mixture had similar dietary levels of 22:1n-9 and 18:3n-3 as canola oil, except for the much higher levels of long-chain saturated fatty acids, 16:0 and 18:0 (Table 1). Feeding this oil to piglets resulted in increased saturated fatty acids in platelet PC and PI, and lower levels of long-chain n-9 monounsaturated fatty acids in all phospholipid classes (Table 5). However, the n-6 and n-3 PUFA profile was similar to canola oil, but there was no reduction in the levels of 20:4n-6 in platelet PE as observed with the feeding of canola oil.

The ratio of n-6 to n-3 PUFA metabolites (from 18:2n-6 and 18:3n-3), in all phospholipids, was highest in sow-reared piglets, intermediate in piglets fed soybean oil, and lowest in piglets fed the three rapeseed oil type diets (Table 5). The rapeseed diets included canola oil, the canola oil/cocoa butter mixture, and HEAR oil. There were no significant differences in the n-6 to n-3 ratios between the three rapeseed oil-containing groups (Table 5).

Platelet SM fatty acids. Platelet SM consisted mainly of long-chain saturated fatty acids from 16:0 to 24:0, and n-9 monounsaturated fatty acids, of which nervonic acid (24:1n-9) was the major fatty acid (Table 6). The total saturated fatty acid content in platelet SM was about 66% in sow-reared and soybean oil-fed piglets, and decreased progressively with the feeding of canola oil (52%) and HEAR oil (38%), but remained unaffected with the feeding of the canola oil/cocoa butter mixture (66%). The decrease in total saturated fatty acids in the canola oil and HEAR oil group was in response to the marked increase in 24:1n-9 (Table 5). Compared to sow-reared piglets, the relative concentration of 24:1n-9, the elongation product of 22:1n-9, increased by 16 and 35%, respectively, for the canola oil and HEAR oil group. The ratio of 24:1n-9 to 24:0 increased from about 1 in the sow and soybean oil group to 3 in the canola/cocoa butter group, to 5 in the canola group, and to 11 in the HEAR oil group.

Platelet CE and FFA. These two minor neutral lipid classes closely reflected the dietary fatty acid composition, and contained only small amounts of C_{20} and C_{22} PUFA (data not included). The accumulation of 22:1n-9 in these two lipid classes was 5.8 and 10.0% in the HEAR oil group, 1.0 and 1.4% in the canola oil group, and 1.0 and 1.2% in the canola/cocoa butter group, respectively. In addition, the HEAR oil group showed significant amounts of 24:1n-9 in both CE (3.9%) and FFA (2.2%).

TABLE 4
Interspecies Comparison (% total lipids) of Platelet Lipids

Lipid class[a]	Piglet (4-wk-old)	Human[b]	Dog[c]
PC	27.3	21.5–28.2	29.1
PE	19.3	13.2–19.1	18.1
PS	9.2	7.9–9.2	9.4
PI	4.0	4.8–8.3	5.0
SM	12.5	9.8–11.4	14.6
DPG	0.4	—[d]	—
C	23.7	18.9–22.2	19.7
CE	1.9	—	0.9
FFA	1.7	—	—

[a]Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; DPG, diphosphatidylglycerol; C, cholesterol; CE, cholesterol esters; FFA, free fatty acids.
[b]Calculated from data in Reference 25; range found between three countries.
[c]Calculated from data in Reference 26; also reported are lyso PC 1.7%, phosphatidic acid 0.2%, and unknown 1.0%.
[d]No values reported.

DISCUSSION

This report is a continuation of studies to evaluate canola oil in milk-replacer diets of newborn test animals to determine if this oil is safe for inclusion into infant formulations. We previously reported that newborn piglets fed milk-replacer diets containing canola oil as the only source of fat in the diet showed a transient delay of 3 wk in the rise of platelet counts, increased platelet volume, but no increased bleeding times (2). The results of the present study indicated that newborn piglets fed canola oil had lower platelet counts and increased platelet size (Table 2) which were negatively correlated, increased bleeding times (Table 3), and these changes persisted to 4 wk of age. These platelet changes need clarification and further testing to determine if the presence of canola oil in milk replacers constitutes a potential risk for infants. Throm-

TABLE 5
Fatty Acid Composition (area %) of Platelet Phospholipid from Piglets Fed Sow Milk or Milk-Replacer Diets (for 4 wk)

	Saturated fatty acids				Monounsaturated (Mono.) fatty acids						Polyunsaturated fatty acids (PUFA)								
	DMA[a]	16:0	18:0	Σ Sat.	18:1n-9	18:1n-7	20:1n-9	22:1n-9	24:1n-9	Σ Mono.	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:4n-6	22:5n-3	22:6n-3	n-6[b] PUFA	n-6/n-3 PUFA[c]
Phosphatidylcholine																			
Sow milk	0.3[d]	52.1	5.9	58.6	20.8	2.6	0.1	trace[e]	0.1	25.6	7.3	0.1	6.4	0.1	0.2	0.1	0.1	7.4	19.2
Soybean oil	0.2	38.5	13.1	52.3	19.5	2.5	0.3	trace	0.1	23.1	16.4	0.4	4.9	0.2	0.1	0.1	0.1	6.5	11.5
Canola oil	0.3	32.7	9.8	43.2	30.0	4.3	1.2	0.2	0.2	37.1	10.5	0.6	5.4	0.6	0.1	0.2	0.2	6.9	6.1
Canola/cocoa[f]	0.3	36.0	12.1	48.7	27.2	2.3	0.5	0.1	0.1	31.1	10.3	0.6	6.3	0.7	0.1	0.2	0.2	7.4	6.1
HEAR[g] oil	0.2	31.8	10.8	43.4	26.3	3.6	2.3	1.3	0.6	35.5	10.5	0.6	5.9	0.7	0.1	0.2	0.2	7.4	5.6
SEM[h]	0.04	1.1	0.2	1.1	0.7	0.2	0.2	0.1	0.07	1.1	0.7	0.03	0.7	0.03	0.02	0.02	0.02	0.8	0.2
ANOVA[i]	**	***	***	***	***	***	***	***	***	***	* **	***	NS	***	*	*	*	NS	***
Phosphatidylethanolamine (PE)																			
Sow milk	20.6[j]	12.6[k]	15.4	28.5	19.9	2.4	0.1	trace	0.1	24.4	4.9	0.3	33.8	0.3	3.6	0.7	0.5	39.1	19.2
Soybean oil	9.2	9.6	21.3	31.5	18.4	2.3	0.2	trace	0.1	22.4	13.3	0.6	23.6	0.8	2.8	1.1	0.6	28.4	10.6
Canola oil	12.4	12.3	15.9	28.7	27.3	3.9	0.7	0.2	0.2	34.1	7.5	0.9	18.7	1.6	2.3	1.7	0.7	22.9	5.2
Canola/cocoa	21.4	6.1	15.6	22.1	23.2	1.5	0.3	0.1	0.1	26.5	7.4	1.0	29.8	3.9	2.2	2.7	1.2	33.8	4.2
HEAR oil	13.2	13.3	19.5	33.7	25.9	3.3	1.6	1.0	0.5	34.2	7.0	0.7	15.4	1.4	1.8	1.4	0.5	19.3	5.4
SEM	2.6	0.5	0.9	1.2	1.1	0.2	0.2	0.1	0.08	1.3	0.5	0.1	1.3	0.3	0.2	0.2	0.1	1.2	0.7
ANOVA	*	***	*	*	**	***	***	***	***	**	***	*	**	**	**	*	*	***	***
Phosphatidylserine																			
Sow milk	0.2	1.5	43.9	45.8	28.6	1.4	0.1	0.1	0.2	31.3	6.3	0.1	9.0	0.1	2.3	0.7	0.3	13.7	11.0
Soybean oil	0.1	1.9	45.4	47.9	21.7	0.9	0.1	0.1	0.1	23.5	14.9	0.2	6.9	0.2	1.3	0.8	0.4	10.9	7.4
Canola oil	0.2	1.8	43.6	46.0	28.6	1.7	0.5	0.5	0.3	32.3	8.2	0.2	6.8	0.5	0.8	1.0	0.4	10.1	5.1
Canola/cocoa	0.2	1.2	47.0	48.6	27.9	0.8	0.2	0.4	0.2	30.2	8.6	0.2	6.3	0.5	0.7	1.0	0.4	9.4	4.6
HEAR oil	0.1	1.7	46.6	49.1	26.4	1.4	0.9	2.4	1.3	33.2	6.7	0.1	5.4	0.3	0.6	0.7	0.3	8.4	5.5
SEM	0.04	0.1	0.8	0.8	1.0	0.05	0.07	0.2	0.2	0.9	0.4	0.02	0.5	0.05	0.1	0.1	0.03	0.7	0.4
ANOVA	NS	*	NS	NS	*	***	***	***	***	**	***	**	*	**	NS	NS	NS	*	***
Phosphatidylinositol																			
Sow milk	0.4	7.0	43.0	50.6	8.2	2.4	0.1	trace	0.4	12.2	1.2	trace	32.9	0.1	0.4	0.1	0.1	34.0	80.1
Soybean oil	0.2	3.6	46.4	50.8	7.8	1.4	0.1	trace	0.5	10.5	3.3	0.1	33.0	0.2	0.2	0.1	0.1	34.0	67.4
Canola oil	0.2	2.6	41.7	45.0	11.0	1.9	0.5	0.1	0.7	14.8	1.6	0.1	35.6	0.4	0.2	0.2	0.1	36.5	42.4
Canola/cocoa	0.4	3.4	50.2	54.3	8.4	1.0	0.2	trace	0.6	10.9	1.3	0.1	30.5	0.3	0.2	0.2	0.1	31.0	41.1
HEAR oil	0.2	2.8	46.2	49.7	9.0	1.6	0.9	0.2	1.6	14.1	1.2	0.1	32.0	0.5	0.1	0.2	0.1	32.8	37.3
SEM	0.7	0.3	1.5	1.7	0.6	0.04	0.1	0.03	0.2	0.9	0.2	0.01	2.1	0.02	0.02	0.02	0.01	2.1	2.3
ANOVA	NS	***	*	*	*	***	***	***	***	*	***	**	NS	***	*	*	*	NS	***

[a]DMA, dimethylacetals.
[b]n-6 PUFA, total n-6 polyunsaturated fatty acids minus 18:2n-6.
[c]Ratio of all n-6/n-3 PUFA excluding 18:2n-6 and 18:3n-3.
[d]All values are means of two pooled samples of two piglets each, except canola which is a mean of three pooled samples of two piglets.
[e]Trace (<0.05%).
[f]Canola/cocoa, a canola oil/cocoa butter mixture.
[g]HEAR, high-erucic acid rapeseed containing 20% 22:1n-9.
[h]SEM, pooled standard error of the mean.
[i]ANOVA, analysis of variances testing for diet. NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[j]All PE DMA expressed as total area percentage of all peaks.
[k]All PE fatty acids expressed as area percentage of only the fatty acid peaks.

TABLE 6
Fatty Acid Composition of Platelet Sphingomyelin from Piglets Fed Sow Milk or Milk-Replacer Diets (for 4 wk)

Fatty acid	Sow milk	Soybean oil	Canola oil	Canola/cocoa[a]	HEAR[b] oil	SEM[c]
14:0	0.4[d]	0.4	0.4	0.4	0.3	0.1
16:0	29.7	28.5	23.8	26.0	18.6	1.6
16:1n-7	0.5	0.4	0.5	0.4	0.3	0.1
18:0	10.5	8.8	10.5	17.7	8.4	2.0
18:1n-9	2.2	1.4	3.0	3.0	2.3	0.5
18:1n-7	0.3	0.2	0.5	0.3	0.3	0.1
18:2n-6	0.5	0.5	0.5	0.5	0.4	0.1
20:0	2.2	2.2	1.9	2.8	1.0	0.2
20:1n-9	0.1	0.1	0.1	0.1	0.2	0.04
20:4n-6	1.3	1.0	2.6	4.3	3.3	1.0
22:0	13.9	13.4	8.4	12.1	4.6	1.0
22:1n-9	0.5	0.3	1.2	1.1	1.6	0.2
22:2	0.8	0.4	0.6	0.3	0.3	0.1
23:0	0.6	1.0	0.6	0.6	0.3	0.1
23:1	0.1	0.1	0.2	0.1	0.1	0.02
24:0	9.9	10.6	6.4	6.4	4.6	1.0
24:1n-9	13.6	10.6	29.1	19.7	48.9	2.5
24:1n-7	1.9	0.9	0.9	0.3	0.4	0.1
24:2	7.3	14.4	5.1	2.1	2.0	1.0
25:0	0.2	0.7	0.5	0.2	0.2	0.04
26:0	0.5	0.9	0.3	0.1	0.1	0.06
∑ Sat.	67.5	65.2	52.2	66.0	38.0	2.2
∑ Mono.	19.3	14.1	35.7	25.1	54.3	2.6
24:1n-9/24:0	1.4	1.0	4.6	3.1	12.3	1.2

[a]A mixture of canola oil and cocoa butter; see Table 1.
[b]HEAR, high-erucic acid rapeseed.
[c]SEM, pooled standard error of the mean.
[d]Values are means of two pools of two animals each.

bocytopenia is know to occur in premature and term infants (3,4).

The results of our previous study appeared to indicate that the platelet changes in canola oil-fed piglets were related to the high content of 18:1n-9, since similar results were obtained with other oils high in 18:1n-9, such as HOAS oil (76.1% 18:1n-9), a mixture of nonrapeseed oils which mimicked the fatty acid profile of canola oil (54.1% 18:1n-9), and occasionally olive oil (75.5% 18:1n-9) (2). On the other hand, the addition of cocoa butter to canola oil raised the level of long-chain saturated fatty acids (16:0 and 18:0), and retained a high level of 18:1n-9 (43%) (Table 1), while normalizing the platelet count and size (Table 2). We previously observed that the addition of coconut oil (rich in 12:0 and 14:0) to canola oil was effective in restoring normal platelet parameters in piglets, despite the high 18:1n-9 content of 54% (2). These results suggest that factors other than 18:1n-9 were involved in platelet changes. There appeared to be a lack of dietary saturated fatty acids when canola oil was fed, since the platelet changes were corrected by addition of saturates. The correction also occurred naturally, at 3–4 wk of age, when piglets were able to synthesize fatty acids *de novo* (14). In our previous study we were unable to determine the contribution of 22:1n-9 (present at about 2% in the canola oil and oil mixture) to the observed platelet changes, and for this reason we now included an experimental diet containing an HEAR oil with 20% 22:1n-9 to test the effect of 22:1n-9.

The HEAR oil group showed marked reduction in platelet counts, increased platelet size, increased bleeding times, and decreased hemoglobin throughout the 4-wk trial (Tables 2 and 3). These newborn piglets fed HEAR oil consumed about 3 g of 22:1n-9/kg body weight/day (16). Similar results were obtained when healthy young men consumed a HEAR oil that contained 39% 22:1n-9 as the only source of fat in the diet (5). In these experiments, a pronounced drop in platelet number was observed with the consumption of about 1.4 g 22:1n-9/kg body weight/day. After 22 d, the diet was discontinued for health concerns, because the platelets dropped below 120×10^9/L. In that same report, low-erucic acid rapeseed oil with less than 2% 22:1n-9 did not cause a reduction in platelet numbers (5). Platelet size was not reported. A similar thrombocytopenia was also observed in patients suffering from ALD, a sex-linked disorder in which very long-chain saturated fatty acids accumulate in all tissues, after treatment with "Lorenzo Oil," a 4:1 mixture of glycerol triolein and glycerol trierucin (6–8). The consumption of 22:1n-9 in these children was up to 18 g glycerol trierucin/d (7) or about 0.5 g 22:1n-9/kg body weight/day based on a body weight of 35 kg. Decreased platelet counts and increased size were directly attributed to dietary 22:1n-9, since its removal from the diet restored platelet numbers and size to normal (7). The authors performed confirmatory studies to show that these platelet changes were not spuriously related to EDTA (8). ALD patients given a high triolein diet did not show symptoms of thrombocytopenia (7,8). There was no evidence of increased bleeding times in these patients, but a few patients showed a tendency to bruise easily (6). Furthermore, platelet reduction and increased platelet size were not restricted to long-chain monounsaturated fatty acids of the n-9 family such as erucic acid (22:1n-9). Consumption of fish, high in oil content and cetoleic acid (22:1n-11), a positional isomer of 22:1n-9, gave similar results. Dyerberg and Bang (9) also observed lower platelet counts, higher platelet volume, and increased bleeding times in adult Eskimos compared to control subjects from Denmark. Subsequent studies have shown that a high consumption of fish, rich in oil and 22:1n-11, produced similar platelet changes in healthy human subjects (10–12).

The evidence indicates that 22:1n-9 is involved in platelet changes; however, the etiology is not known. Bone marrow appeared to be normal on histological examination. This is in agreement with our previous piglet study (2) and in ALD patients (7,8). In addition, Zierz *et al*. (7) reported that the bone marrow of ALD patients revealed no evidence of megakaryocyte reduction.

Diets were prepared to include 150 IU of vitamin E per kg diet to ensure that the canola oil- and HEAR oil-fed piglets had sufficient vitamin E, since we previously observed that milk-replacer diets containing canola oil required additional vitamin E (15). The vitamin E levels in liver and heart were found to be lower in the canola oil and the canola oil/cocoa butter mixture compared to soybean oil, but the differences

were generally not significant (results published elsewhere, 24). On the other hand, the HEAR oil group showed significantly lower tissue vitamin E levels compared to the soybean oil group. However, the high dietary vitamin E level included in the diets of this study ensured adequate tissue vitamin E levels even in the HEAR oil group when compared to the sow-reared controls (24). These results support the conclusion of the previous study (15) that rapeseed oils (HEAR more than canola) appear to contain substances that induce oxidative stress in the piglets. This oxidative stress may have damaged platelets and reduced the Hb content, which would account for their lower levels in rapeseed oil-fed piglets. Further studies will be required to determine the mechanism of platelet changes in the newborn piglet using specific indicators (4).

The platelet lipids were investigated in this study to see if the platelet changes could be related to specific lipid or fatty acid changes. The relative proportions of the platelet lipid classes were not significantly affected, and the composition was similar to that found in adult humans (25) and dogs (26) (Table 4). Blomstrand *et al.* (27) also reported no differences in platelet lipid class composition between rats fed trierucin (22% 22:1n-9) or herring oil (12% 22:1n-11) diets for 10 wk, although they did not present any values.

The accumulation of 22:1n-9 into the structural platelet lipids was found to be relatively minor even when piglets were fed the HEAR oil diet which contained 20% 22:1n-9 (Tables 5 and 6). It ranged from 0.2 (PI) to 2.4% (PS). The minor lipids CE and FFA were an exception. They accumulated larger amounts of 22:1n-9, but these lipids are not considered to be structural platelet components. In addition, the accumulation of 22:1n-9 into platelet lipids was negligible in groups fed oils containing about 2% 22:1n-9, and absent when oils were devoid of 22:1n-9. Therefore, the level of 22:1n-9 in the platelet lipids does not appear to explain the observed thrombocytopenia in newborn piglets.

The greatest change in the fatty acid composition of structural platelet lipids was observed in the 24:1n-9 content of platelet SM. The 24:1n-9 is derived by chain elongation of 18:1n-9 (28,29), or from dietary 22:1n-9 (30–33), when present. The accumulation of 24:1n-9 reached 49% of the total platelet SM fatty acids in the HEAR oil-fed piglets, compared to 14% in sow-reared and 11% in soybean oil-fed piglets (Table 6). Canola oil-fed piglets also showed a marked increase of 24:1n-9 to 29%, while the canola oil/cocoa butter group showed an intermediate increase to only 20%. Our data suggest that dietary 22:1n-9 (and 20:1n-9) was effectively incorporated into the 24:1n-9 of the SM fraction (30-33) after an apparent control step, possibly the first step in the chain elongation of 18:1n-9 to 20:1n-9. This could explain why canola oil showed very high levels of 24:1n-9 despite only 2% 22:1n-9 in the diet. An increase in dietary saturates (canola oil/cocoa butter group) showed a moderation in the elongation of 22:1n-9 to 24:1n-9.

To test this hypothesis, one should evaluate the platelet SM composition of piglets fed an oil that gave similar platelet changes as canola oil but was devoid of 22:1n-9, such as HOAS oil in our previous study (2). We had only reported the total, not the platelet SM composition in our previous paper (2), now selectively presented in Table 7. The canola oil-fed group showed an increase in 24:1n-9 and the 24:1n-9/24:0 ratio in both platelet SM and red blood cell membranes SM, while the HOAS oil-fed group showed a similar response only in the red blood cell membranes SM. It should be noted that the HOAS platelet SM (Table 7) may not be entirely reliable because of the limited sample size. The sample size of red blood cell membrane SM was much larger, and it showed the increase in 24:1n-9 and the 24:1n-9/24:0 ratio (Table 7),

TABLE 7
Selected Fatty Acids of Platelet and Red Blood Cells Membrane Sphingomyelin (SM) of Sow-Raised Piglets, and Piglets Fed Milk-Replacer Diets Containing Different Vegetable Oils (for 4 Wk)[a]

Diets	Platelets			Red blood cell membranes		
(% 22:1n-9 in diet)	22:1n-9	24:1n-9	24:1n-9/24:0	22:1n-9	24:1n-9	24:1n-9/24:0
Sow milk	0.5[a]	13.6	1.4	0.1[b]	13.7	0.8
Soybean oil	0.3	10.6	1.0	0.1	9.2	0.5
Canola oil (2.0%)	1.2	29.1	4.6	0.2	29.4	2.2
Canola/cocoa (2.2%)	1.1	19.7	3.1	0.2	23.6	1.4
HEAR[b] oil (20.2%)	1.6	48.9	12.3	0.7	49.5	9.5
Sow milk[c]	0.8[c]	21.8	1.4	0.1[d]	15.6	0.6
Soybean oil	0.4	14.9	1.2	tr	10.4	0.5
HOAS[d] oil	1.1	10.2	0.6	0.1	30.1	1.6
Canola oil (1.6%)	0.9	37.6	3.6	0.2	31.3	2.1

[a]All values (a–d) are means of [a]two pools of two animals each, [b]four animals/diet, [c]one pool of three animals, and [d]three animals/diet.
[b]HEAR, high-erucic acid rapeseed.
[c]This experiment was published previously (Ref. 2), but neither the platelet or red blood cell SM results were presented.
[d]HOAS, high-oleic acid sunflower.

which suggests that one can use these fatty acid changes as a predictor of platelet changes in newborn piglets.

It was not possible to evaluate the changes in platelet SM due to dietary 22:1n-9 or 22:1n-11 in the other studies, since the fatty acid composition of platelet SM was either not reported in piglets (34), rats (27) or humans (5–8,10–12,35,36), or the total platelet fatty acid composition did not include either 24:0 or 24:1n-9 (8,12,27,34–36). Dyerberg and Bang (9) were the only ones who reported 24:0 and 24:1 in total platelet lipids, and they confirmed the higher values of 24:1 (4.4 vs. 1.1%) and the 24:1/24:0 ratio (14.7 vs. 1.6) in adult Eskimos compared to Danish control subjects. A recent study by Jensen *et al*. (36) in which term infants were fed formulas containing up to 40% canola oil for 120 d was of interest. They reported the fatty acid composition of plasma and erythrocyte phospholipid, but not that of platelets, and did not include 24:0 and 24:1 in their analysis.

The platelet lipids of ALD patients fed the glycerol triolein/trierucin mixture were reported to have a negative correlation of platelet count to total platelet 18:1n-9 (–0.49; P = 0.01), 22:1n-9 (–0.56; P = 0.003) and 24:1n-9 (–0.65; P = 0.0003) (8). Based on these results, the authors concluded that 22:1n-9, but not 18:1n-9, affected the platelet biology, since a diet high in glycerol triolein did not show changes in platelet number. The 24:1n-9 was not considered, despite the evidence which showed that both the concentrations and correlations of 24:1n-9 were greater than for 22:1n-9 (Table 1, Ref. 8). It would be expected that the changes would have been much greater if platelet SM from ALD patients, instead of total platelet lipids, had been investigated. It was of interest to note that the plasma SM of the ALD patients showed a marked increase in 24:1 (from 16.0 to 46.8%), and in the 24:1/24:0 ratio (from 0.6 to 6.1) after consumption of glycerol trierucin, which was much greater than the incorporation of 22:1 (from 1.38 to 6.21%) into plasma SM (37). This would support the hypothesis that thrombocytopenia appears to be related to 24:1n-9, the elongation product of 22:1n-9. Furthermore, one cannot rule out an additive effect of 24:1n-9 in animals fed a diet low in 18:2n-6 to 18:3n-3 (canola 2.3). Jensen *et al.* (38) recently showed that infants fed a formula with an 18:2n-6 to 18:3n-3 ratio of 4.8 had reduced weights, and their plasma levels had lower 20:4n-6 and higher 22:6n-3 levels at 120 d of age compared to a formulation having an 18:2n-6 to 18:3n-3 ratio of 44.

In conclusion, a reduction in platelet number and an increase in platelet size and bleeding time were evident in newborn piglets fed milk-replacer diets containing canola oil as the only source of fat in the diet. The platelet changes with canola oil were overcome by the addition of long-chain saturated fatty acids to the diet, which were just as effective as the shorter-chain fatty acids from coconut oil. HEAR oils showed marked and consistent platelet and hemoglobin reduction, and increased platelet size and bleeding times. The greatest lipid changes were observed in platelet SM fatty acid composition, which showed a significant increase in 24:1n-9 and in the 24:1n-9/24:0 ratio both for canola oil and HEAR oil. The addition of cocoa butter to canola oil moderated these fatty acid changes in platelet SM. The results show significant platelet and lipid changes in newborn piglets fed milk-replacer diets containing canola oil as the sole source of fat in the diet, which was corrected by mixing canola oil with saturated fats in a 3:2 ratio. Canola oil should be evaluated as part of infant formula preparations which mimic mother's milk that contains about 45% saturates (39), similar to that undertaken by Jensen *et al.* (38) to test if these adverse effects are removed.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Robert C. Fouchard and the staff at the swine barn at Agriculture Canada, Mary Calvert and her staff at the Ottawa Civic Hospital, and the staff at the Ontario Veterinary Laboratory Services. The study was partially supported by a grant from the Canola Council of Canada. Contribution number 2437 from the Center of Food and Animal Research.

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[Received June 25, 1997, and in final revised form October 1, 1997; revision accepted November 11, 1997]

Effect of Modified Dairy Fat on Postprandial and Fasting Plasma Lipids and Lipoproteins in Healthy Young Men

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ABSTRACT: Fatty acid profile of milk fat can be modified by cow feeding strategies. Our aim was postprandially and after 4 wk to compare the effect of a modified milk fat (M diet) [with 16% of the cholesterolemic saturated fatty acid (C12–16) replaced by mainly oleic and stearic acids] with the effect of D diet, including a conventional Danish milk fat on plasma lipids and lipoproteins. A side effect of the cow feeding regime was a 5% (w/w) increase in *trans* fatty acid in M diet. Eighteen subjects were fed for two periods of 4 wk strictly controlled isoenergetic test diets with 40% of energy from total fat and the same content of dietary cholesterol in a randomized study with cross-over design. Contrary to expectations, fasting low density lipoprotein (LDL) cholesterol concentration did not differ after the experimental periods. However, M diet resulted in a higher fasting total triacylglycerol concentration compared to D diet ($P = 0.009$). Postprandial samples were taken at two different occasions (i) at day 21, after breakfast and lunch and (ii) on the last day of the study 2, 4, 6, and 8 h after a fat load. Postprandial plasma triacylglycerol and chylomicron triacylglycerol showed higher peak values after D diet than M diet (interaction effect, diet × times $P < 0.05$). In conclusion, M diet did not lower LDL cholesterol compared to D diet. Thus any cholesterol-lowering effect of oleic and stearic acids may have been obscured by the high content of cholesterol-raising saturated fatty acids in milk fat. A higher content of the *trans* fatty acids in M diet might have counteracted the cholesterol neutral/decreasing effect and increased plasma triacylglycerol.
Lipids 33, 11–21 (1998).

Dairy products are important nutrient sources contributing protein of high quality, vitamins, calcium, and zinc. However, a high intake of milk fat is considered cholesterol-raising as it contains a large proportion of saturated fatty acids (SFA). Fats high in SFA raise the plasma cholesterol concentration, although the individual SFA do not contribute equally to this effect. Fatty acids with a chain length of 4 to 10 carbon atoms and stearic acid (18:0) previously have not been considered to affect cholesterol concentrations (1–5). Recent research has demonstrated a cholesterol-raising effect of 8:0 and 10:0 (6,7). SFA with 20 or more carbon atoms are poorly absorbed (6). In contrast, palmitic acid (16:0), myristic acid (14:0), and lauric acid (12:0) are considered cholesterol-raising (2–5, 8–10). Oleic acid is demonstrated to be either neutral or cholesterol-lowering (9,11,12).

Numerous studies have investigated the effect of extreme changes of fatty acid composition of the diet, whereas only a few studies have included realistic changes in fatty acid composition of the conventional diet. The purpose of the study was to monitor the effect on lipoproteins, fasting and postprandially in healthy young men, of a modified milk fat compared to a conventional milk fat.

It is well known that the fatty acid composition of milk fat can be markedly influenced by the diet, especially by the amount and fatty acids profile of the fat in the cow's diet (13,14). The milk fatty acid profile generally reflects a balance between fatty acids synthesized *de novo* in the mammary gland on the basis of acetic acid originating from ruminal fermentation and fatty acids fed in the diet for the cows and perhaps modified in the rumen by biohydrogenation. Despite this complexity, it recently has been shown that the fatty acid profile of milk fat with regard to the major fatty acids can be forecast with good precision on the basis of fatty acids in the cow's diet (15). In this study the modification of the milk fat was a partial substitution of palmitic acid (and to a smaller extent myristic acid) with oleic acid (and to a smaller extent stearic acid) by means of a realistic, cost neutral modification of the feed of the cows.

SUBJECTS AND METHODS

Eighteen young men were recruited for the study. Their ages ranged from 21 to 28 yr (mean 25 yr), body weights from 63 to 104 kg (mean 80 kg), and body mass index from 19 to 27

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Abbreviations: apo, apoprotein; CE, cholesterol esters; CLA, conjugated linoleic acid; D diet, Danish milk fat; 20:3n-6, dihomo-γ-linolenic acid; 22:6n-3, docosahexaenoic acid; 22:5n-3, docosapentaenoic acid; E%, energy percentage; HDL, high density lipoprotein; 12:0, lauric acid; LDL, low density lipoprotein; M diet, modified milk fat; MUFA, monounsaturated fatty acids; 14:0, myristic acid; 16:0, palmitic acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acid; 18:0, stearic acid; TAG, triacylglycerol; 18:1n-7, vaccenic acid; VLDL, very low density lipoprotein.

kg m^{-2} (mean 24 kg m^{-2}). The subjects had no history of atherosclerotic disease, and all were apparently healthy as assessed by a medical questionnaire. They were all nonsmokers. None took medicine. Most subjects had a moderate physical activity level (training max. 1–2 h twice a week and/or daily biking to work). They continued with the same physical activity throughout the trial. The participant's habitual diet was assessed from a 7-d weighed food record. We calculated energy intake and dietary composition using a national database (National Food Agency, Dankost, Denmark). Habitual energy intake ranged from 9 to 19 MJ (mean 14 MJ) with 26–42% of energy (E) from fat (mean 32 E%), the ratio of polyunsaturated fatty acid (PUFA) to SFA was 0.2–0.8 (mean 0.4); intake of cholesterol was 175–665 mg/10 MJ (mean 371 mg/10 MJ), and dietary fiber intake ranged from 1.4 to 3.2 g/MJ (mean 2.4 g/MJ). Two different diets were served in randomized order for 4 wk each. The two intervention periods were separated by a 2-mon period on habitual diet.

Diets. We served a diet high in conventional Danish butter (D), which was high in palmitic and low in oleic acid, and a modified butter (M), which was low in palmitic and high in oleic acid (for details of the fatty acid composition of the test butters, see Table 1). The butter was manufactured using two different bulks of milk produced at the Danish Institute of Animal Science (Foulum, Denmark). Forty cows were given a basal diet *ad libitum* (mixture of beet roots, grass silage, crushed barley, and straw) and supplemented with two different concentrate mixtures. By use of prediction formulas of Hermansen (15), the two concentrate mixtures were composed in such a way that they were expected to result in either a normal milk fat composition or in a milk fat high in oleic acid. The normal concentrate mixtures consisted of 94% soybean meal and 6% saturated fats and were given in an amount of 4.7 kg/cow/day. The experimental concentrates consisted of 50% soybean meal and 50% crushed double-low rape seeds (low in glucosinates and erucic acid) given in an amount of 3.2 kg/cow/day. After 3 wk of normal concentrates feeding, the milk was collected for butter production for the D diet high in palmitic acid. Subsequently, the cows were given the experimental concentrate for a period of 7 wk, in which the milk was collected for butter production of the M diet high in oleic acid.

The fatty acid composition of each test fat was determined by gas–liquid chromatography (Table 1). The experimental diets contained 40 E% of fat (30 E% derived from butter and 6.5% from grapeseed oil. The remaining fat content of 3.5% was contributed by other food items, which were constant and identical in the two test diets. The analyzed dietary cholesterol and macronutrient content of the diet high in palmitic acid (D diet) and the diet high in oleic acid (M diet) are shown in Table 2.

The experimental meals consisted of natural and common food items that were prepared and cooked in customary ways. The two kinds of test butter were served as spread and incorporated in bread, rolls, and cakes. On day 21 of the dietary periods, the effect of two consecutive realistic meals, breakfast and lunch with mean 25 g milk fat in each meal, including ingredients as just described, was investigated in the 18 subjects.

TABLE 1
Fatty Acid Composition of the Two Test Milk Fats

	% Fatty acid of total fatty acids	
Fatty acids	Modified milk fat (M)	Danish milk fat (D)
4:0	3.46	3.83
6:0	1.88	2.34
8:0	1.08	1.40
10:0	2.30	3.14
10:1	0.22	0.37
12:0	2.86	4.09
14:0	10.67	12.07
14:1	1.83	1.81
15:0[a]	1.01	1.45
16:0[a]	21.13	36.82
16:1n-7	1.71	2.25
17:0[a]	0.89	1.17
17:1	0.24	0.31
18:0	10.88	7.23
18:1 *trans*	6.4	1.1
18:1n-9	24.95	15.33
18:1n-7	1.40	0.55
18:2n-6	1.93	1.61
20:0	0.22	0.14
18:3n-3	0.50	0.42
Conj. 18:2[b]	1.57	0.52

[a]16:0, 15:0, and 17:0 also include the branched isoforms typical for milk fat.
[b]Conjugated 18:2 also included 0.20–0.25% 20:0.

Dinner dishes, in which grapeseed oil was included, consisted of cooked vegetables and lean meat with pasta, rice, or mashed potatoes. Rolls, marmalade, orange juice, and tea or coffee were served for breakfast. Lunch was a cold meal consisting of lean roast beef or fish paté, salad dressing without fat. Fruit juice, fresh fruit, cakes, and candy were served as snacks.

Meal tests. On day 21 of the dietary periods the effect of two consecutive meals of the test diet, breakfast and lunch with mean 25 g milk fat in each meal, was investigated in the 18 subjects.

On the last day of the dietary periods, effect of a single high-fat meal, the butter load, was investigated in eight subjects. The meal consisted of butter, rolls, and cake with butter incorporated, orange juice, and marmalade. The fat intake of each test meal was fixed to 1.2 g milk fat/kg body weight, 55% of the energy contribution was derived from fat, 41% from carbohydrates, and 4% from protein. The energy content of the test meal ranged from 5.7 to 8.7 MJ (mean 6.7 MJ), and the content of fat in each test meal ranged from 83 to 126 g (mean 98 g).

Energy intake was individualized according to the food records of the subjects. To avoid bias from underreporting, the calculated energy intake from the records was compared with the estimated energy requirements for the subjects' basal metabolic rate according to body weight, sex, age, and level of physical activity (16). The experimental diet was com-

TABLE 2
Analyzed Mean Daily Energy Content and Nutrient Composition of the Experimental Diets[a]

	M diet	D diet
Protein (g/10 MJ)	68	66
(% of energy)	11.6	11.3
Carbohydrate		
(g/10 MJ)	281	283
(% of energy)	47.7	49.2
Total fat		
(g/10 MJ)	107	107
(% of energy)	40.5	41.5
Saturated fatty acids (% of total fatty acids)		
4:0	2.42	2.56
6:0	1.54	1.76
8:0	0.97	1.13
10:0	2.05	2.69
12:0	2.22	3.12
14:0	8.90	9.73
15:0	0.76	0.95
16:0	22.26	36.09
17:0	0.19	
18:0	11.12	7.25
20:0		
Monounsaturated fatty acids (% of total fatty acids)		
14:1	1.47	1.50
15:1		
16:1	1.51	1.12
17:1		
18:1 *trans*	4.28	0.30
18:1	28.88	17.85
20:1		
Polyunsaturated fatty acids (% of total fatty acids)		
18:2	11.87	13.77
18:3	0.56	0.20
20:2		
Cholesterol (mg 10 MJ)	373	386

[a]Analyzed values are means of two duplicate portions. Diet D, high in palmitic acid; Diet M, high in oleic acid collected in two different periods. Variations between periods were negligible. There was good agreement between analyzed and calculated values (data not shown). See Table 1 for abbreviations.

posed to reflect the higher of the two energy-intake scores whenever the two were not identical.

Duplicate portions of each test diet were collected during 1 wk in each period of the study. For each test diet, portions were pooled per diet and analyzed by the National Food Agency of Denmark (Table 2). Analyzed energy content and nutritional composition of the diets were in good agreement with calculated values (data not shown). The analyses showed a general tendency to a lower fatty acid content in the experimental diets than calculated from the fatty acid composition in the test butters. Lower values in fatty acid content in mixed diets are a common finding of The National Food Agency of Denmark. This is probably due to a lower fatty acid content in some fats [like phospholipids (PL)] than in triacylglycerol (TAG), degradation of fat, and loss during handling and storage (T. Leth, The National Food Agency of Denmark, personal oral communication). This is obviously due to degradation and loss during handling and storage. All foods were prepared and weighed in individual servings in the experimental kitchen of the Department of Human Nutrition of the Royal Veterinary and Agricultural University (Frederiksberg, Denmark). Lunch was served on weekdays at the Department. All other dishes were provided daily as a package with instructions for its preparation. Meals for the weekend were provided on Fridays. Body weight without heavy clothing was recorded before lunch three times a week. Physical activity was restricted, and daily records of tea and coffee intake, physical activity, illness, and deviation from the protocol were monitored as described earlier (5,15). The subjects were highly motivated.

Blood analysis. Fasting samples: Morning blood samples were taken before each of the two study periods (two samples before the first and one sample before the second dietary period) and on day 21, 26, and 28 of each dietary period. Sampling was performed after at least 12 h of fasting and after 15 min of supine rest. Postprandial samples were taken on two different occasions in the study periods: (i) on day 21, at 3 h after breakfast and 3 h after lunch; (ii) on the last day of the dietary period (day 28) 2, 4, 6, and 8 h after a high butter load (1.2 g milk fat/kg body weight) of the test fats. The subjects refrained from heavy physical activity or alcohol intake for at least 24 h before the sampling. The plasma concentration of C-reactive protein was assessed to rule out infectious diseases of the subjects at the time of blood collection. Values were in the normal range (<5 mg/L) except for one subject. Data from this occasion were excluded from analysis of plasma lipids and lipoproteins. Blood for lipid and lipoprotein analysis was collected in tubes containing EDTA, which were immediately placed on ice and centrifuged at $3000 \times g$ for 15 min at 4°C. Plasma for apolipoprotein and fatty acid analysis was stored at −80°C. Plasma for lipoprotein analysis was stored at 4°C and analyzed within 48 h. Chylomicrons, very low density lipoprotein (VLDL) + chylomicron remnants, and low density lipoprotein (LDL) + high density lipoprotein (HDL) fractions were separated by ultracentrifugation. Chylomicrons were isolated by carefully overlayering 3 mL of plasma with 2.5 mL of salt solution of density 1.006 kg/L. The ultracentrifuge tubes (13 × 64 mm; Polyallomer Bell-Top Quick-Seal Centrifuge Tubes; Beckman Instruments, Palo Alto, CA) were centrifuged for 23 min at 20°C at $100,000 \times g$ in an ultracentrifuge (L7-55; Beckman Instruments) using a fixed-angle rotor (r = 96.1 mm) (50.4 Ti; Beckman Instruments). The tubes were sliced 45 mm from the bottom, and the top fraction ($S_f > 400$) was transferred and adjusted to a total volume of 5 mL with saline (17). The bottom fraction $S_f < 400$ was transferred to another UC tube, adjusted to 5.5 mL with saline of density 1.006 kg/L, and centrifuged for 16 h at 4°C at $105,000 \times g$. After tube slicing 30 mm from the bottom, the top fraction containing VLDL and chylomicron remnants and the bottom fraction containing LDL + HDL were transferred to separate tubes and adjusted to a final volume of 5 mL.

Cholesterol and TAG concentrations were assessed by enzymatic procedures (Boehringer Mannheim GmbH, Mannheim, Germany) on a Cobas Mira analyzer (Roche, Basel,

Switzerland). Total HDL and HDL_3 cholesterol concentrations were measured enzymatically after precipitation with polyethylene glycol (Quantolip; Immuno AG, Vienna, Austria) (16). HDL_2 was calculated by subtracting HDL_3 cholesterol from the total HDL cholesterol. LDL cholesterol was calculated from the difference of cholesterol in the infranatant and HDL cholesterol. The concentrations of plasma apolipoproteins (apo) B and A-1 were determined by immunoturbidimetry in a Monarch apparatus (Instrumentation Laboratories, Lexington, MA) using monospecific polyclonal antibodies against apo B and A-1 (kits from Orion Diagnostica, Espoo, Finland). Precision was determined by the analysis of internal plasma pool which gave run-to-run coefficient of variation percentages: TAG 1.75%, total cholesterol 1.6%, HDL cholesterol 3.61%, HDL_3 cholesterol 6.83%, apo A-1 1.46%, and apo B 3.11%. Accuracy was checked using a control serum of certified value (Quantolip control serum) (Immuno AG, Vienna, Austria) (18).

Fatty acid pattern in cholesterol esters (CE) and PL was determined at the end of each period. The plasma lipids were extracted into chloroform/methanol (1:1, vol/vol), and the PL and CE were separated by thin-layer chromatography on precoated silica gel 60 plates (Merck, Darmstadt, Germany) with hexane, diethyl ether, and acetic acid (80:20:1, by vol) as the mobile phase. Methyl esters were prepared by transmethylation catalyzed by BF_3 (19). The fatty acid composition was determined by GC using an HP5880A instrument (Hewlett-Packard, Palo Alto, CA) with split injection and a 30 m × 0.32 mm fused silica column with a 0.2 µm film of SP2380 (Supelco Inc., Bellefonte, PA). The oven was programmed to increase the temperature at a rate of 2°C/min from 140 to 160°C and after 2 min to 200°C at 3°C/min. The fatty acids were identified by comparing retention times with standards of fatty methyl esters.

Ethics. The protocol and the aim of the study were fully explained to the subjects, who gave their written consent. The research protocol was approved by the Scientific Ethics Committee of the municipalities of Copenhagen and Frederiksberg (01-487/93).

Statistical analysis. Because there were no significant differences in blood lipids and lipoproteins between the three samplings (day 21, 26, and 28) from each experimental period, a mean value was calculated and used in the statistical analysis. A paired *t*-test was used for fasting blood lipid values to compare the effect of the two diets. Owing to differences in VLDL cholesterol in habitual values before the two study periods, a paired *t*-test comparing differences between habitual and experimental values was done. To analyze postprandial data, repeated measures of analysis of variance (SPSS Inc., Chicago, IL) with Huynh-Feldt adjustment of degrees of freedom were used to assess effect of time, difference in effect of the experimental fats, and interaction between effects of time and type of fat during the 0 to 8 h period of the day. If a significant interaction between effects of time and type of fat is found, it means that the mean difference between the two fats varies with time. The graphs of the time course will illustrate how the means differ. In the case of a significant interaction, the tests for type of fat and time effects are not meaningful, and therefore not reported. If, however, there is no significant interaction, it means that the two time courses can be regarded as parallel, and the effects of time and type of fat can be tested separately.

RESULTS

Effect of experimental diet after 4 wk (fasting samples) follows: The body weights of the volunteers did not differ significantly at the end of the two experimental periods; the body weight was 79.5 ± 2.5 kg (mean ± SEM) on the D diet, 79.4 ± 2.5 kg on the M diet. The fasting mean plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, HDL_2-, HDL_3- cholesterol, VLDL cholesterol, ratio LDL/HDL cholesterol, TAG, and the plasma concentrations of apo A-1, and apo B, and ratio apo A-1 to HDL cholesterol and ratio apo B to LDL cholesterol for the two diets are given in Table 3.

Diet M compared with diet D resulted in 14% (0.14 mmol/L) higher plasma TAG ($P < 0.008$). No significant difference was observed between diet D and M for plasma total cholesterol, LDL, HDL, HDL_2, HDL_3, LDL/HDL cholesterol ratio, VLDL cholesterol, and apo A-1, apo B concentrations. There was no significant difference between the ratios apo A-1 to HDL cholesterol or ratio apo B to LDL cholesterol after the test diets, indicating that the size of the HDL and LDL particles did not differ after the two milk test fats.

TABLE 3
Fasting Plasma Lipids and Lipoprotein Concentration in 18 Men at Habitual Diet and After Four-Week Period on Two Test Diets[a]

	Habitual diet[b]	M diet[c]	D diet[d]
Total cholesterol[e]	4.01 ± 0.17	4.24 ± 0.17[f]	4.31 ± 0.19[f]
LDL cholesterol	2.67 ± 0.16	2.83 ± 0.15	2.89 ± 0.19[f]
HDL cholesterol	1.15 ± 0.07	1.13 ± 0.06	1.20 ± 0.06
HDL_2 cholesterol	0.21 ± 0.02	0.20 ± 0.03	0.26 ± 0.04
HDL_3 cholesterol	0.95 ± 0.04	0.93 ± 0.04	0.93 ± 0.04
VLDL cholesterol	0.19 ± 0.02	0.23 ± 0.03[f]	0.19 ± 0.02
Total triacylglycerol[e]	0.85 ± 0.06	0.99 ± 0.09	0.85 ± 0.07
LDL:HDL cholesterol	2.47 ± 0.21	2.64 ± 0.15	2.55 ± 0.24
Apo A-1 (mg/L)	1490 ± 47	1401 ± 43[f]	1437 ± 37
Apo B (mg/L)	903 ± 48	926 ± 45	914 ± 47
Apo A			
HDL-cholesterol (mg/mmol)	1330 ± 164	1284 ± 135	1268 ± 134[f]
Apo B			
LDL-cholesterol (mg/mmol)	340 ± 23	332 ± 23	322 ± 28[f]

[a]M = diet with milk fat high in oleic acid.; D = diet with milk fat high in palmitic acid; apo, apoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein.
[b]Mean of three samplings, two before the first period and one before the second period.
[c]Mean of three samplings (except for one case, samples on one occasion, from one person were excluded).
[d]Mean of three samplings.
[e]Plasma lipids and lipoproteins are mmol/L.
[f]x ± SEM, significantly different from habitual diet $P < 0.05$.
[g]x ± SEM, significantly different from D diet $P < 0.05$.

Diet M compared with habitual diet resulted in 5% (0.23 mmol/L) higher total cholesterol ($P = 0.006$) and a 17% higher VLDL cholesterol concentration ($P = 0.014$). No other significant differences were observed.

Diet D compared with habitual diet (mean of three determinations) resulted in 7% (0.30 mmol/L) higher total cholesterol ($P = 0.009$) and 8% higher LDL cholesterol concentration ($P < 0.035$); a corresponding lower ratio apo A-1 to HDL cholesterol ($P < 0.029$) and a lower ratio apo B to LDL cholesterol ($P < 0.002$) were observed after D diet, indicating a larger size of the HDL and LDL particles. No other significant differences were observed.

The blood lipid levels on habitual diet before the two dietary periods (means of one and two samplings, irrespectively) did not differ with exception of VLDL cholesterol ($P = 0.007$), which was 36% (0.09 mmol/L) lower. However, no carry-over effect was observed on habitual samples collected prior to the second period.

The fatty acid composition of plasma CE and PL in the end of the test periods is presented in Table 4. In CE diet M resulted in a 59% higher content of 18:1,*trans* ($P = 0.039$) and a tendency to a higher content of 18:1n-7 ($P = 0.05$) than diet D. In CE there were no significant differences in 16:0, 18:0, 18:2, or the major fatty acid 18:1n-9 comprising about 56% after both diets. Conjugated 18:2n-6 was not incorporated in CE and PL. In PL an 11% lower concentration of the minor SFA with 15 carbon atoms and a higher 22:6n-3 after the M diet compared with D diet ($P < 0.05$) were found. The major fatty acids were 16:0 (about 25%) and 18:2 (about 20%) and there were moderate amounts of 18:0 (15–19%), 18:1, and 20:4 (9–10%). In contrast to the CE, other PUFA, dihomo-γ-linolenic acid (20:3n-6), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3) were also present in the PL fraction. The PUFA comprised totally about 50%, corresponding to the occupancy of the *sn*-2 position in the PL molecule.

Postprandial lipemia 3 h after breakfast and 3 h after lunch. Intake of breakfast and lunch with realistic amounts of butter (mean 25 g milk fat in each meal) resulted in an increase in total plasma TAG and chylomicron-TAG after 6 h (Fig. 1A,B) after both D or M diets. From 0 to 6 h there was an increase in VLDL–TAG ($P < 0.001$) and in LDL–TAG ($P < 0.005$), and a slight decrease in LDL cholesterol and HDL_3 cholesterol ($P = 0.03$) (Fig. 1C–F). According to the repeated-measures analysis of variance, there was a difference in effect of the test butters over time on plasma total TAG concentration and chylomicron TAG (interaction effect) ($P < 0.05$). From Figure 1A and B it appears that M diet caused a higher plasma total TAG and chylomicron TAG concentration than the D diet in the mean level.

Postprandial lipemia after fat load. The butter load either from D or M diet gave a rise in total plasma TAG and chylomicron and VLDL, LDL, HDL TAG, with the highest measured value 4 h after the load except for LDL TAG, which showed a somewhat different pattern (Fig. 2A–E). VLDL cholesterol increased up to 4 h and fell to lower concentra-

TABLE 4
Fasting Fatty Acid Composition of Cholesterol Esters and Phospholipids After Four Weeks on Experimental Diet[a]

	Cholesterol ester		Phospholipid	
Fatty acid	M diet	D diet	M diet	D diet
12:0	0.16 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
14:0	1.10 ± 0.05	1.11 ± 0.04	0.55 ± 0.04	0.50 ± 0.02
15:0	0.30 ± 0.10	0.30 ± 0.01	0.30 ± 0.01[a]	0.34 ± 0.02
16:0	10.93 ± 0.21	11.13 ± 0.26	25.10 ± 0.44	25.40 ± 0.34
16:1n-7	2.05 ± 0.10	2.10 ± 0.11	0.74 ± 0.02	0.71 ± 0.02
17:0	0.18 ± 0.01	0.18 ± 0.01	0.45 ± 0.01	0.46 ± 0.01
18:0	1.30 ± 0.06	1.28 ± 0.05	13.66 ± 0.20	13.72 ± 0.15
18:1 *trans*	0.37 ± 0.04[a]	0.15 ± 0.05	0.68 ± 0.11	0.62 ± 0.11
18:1n-9	16.00 ± 0.44	15.36 ± 0.40	8.87 ± 0.26	8.43 ± 0.26
18:1n-7	1.06 ± 0.02	1.00 ± 0.02	1.34 ± 0.04	1.30 ± 0.03
18:2n-6	55.85 ± 0.44	56.50 ± 0.40	25.79 ± 0.25	26.30 ± 0.37
18:3n-6	0.49 ± 0.03	0.50 ± 0.03	—	—
18:3n-3/20:0	0.47 ± 0.01	0.47 ± 0.12	0.17 ± 0.01	0.17 ± 0.02
20:1	—	—	0.14 ± 0.01	0.14 ± 0.02
20:2	—	—	0.39 ± 0.01	0.39 ± 0.01
20:3n-6	0.71 ± 0.03	0.72 ± 0.03	3.18 ± 0.13	3.22 ± 0.12
20:4n-6	5.66 ± 0.19	5.57 ± 0.17	9.69 ± 0.22	9.67 ± 0.19
20:5n-3	0.47 ± 0.03	0.47 ± 0.03	0.71 ± 0.04	0.70 ± 0.03
22:6n-3	0.64 ± 0.03	0.62 ± 0.03	—	—
22:4n-6	—	—	0.41 ± 0.01	0.43 ± 0.02
22:5n-6/22:4n-3	—	—	0.25 ± 0.02	0.25 ± 0.02
22:5n-3	—	—	1.15 ± 0.04	1.15 ± 0.03
22:6n-3	—	—	4.56 ± 0.14[a]	4.36 ± 0.13

[a]M = diet with milk fat high in oleic acid; D = diet with milk fat high in palmitic acid. [a]Significantly different from D diet $P < 0.05$.

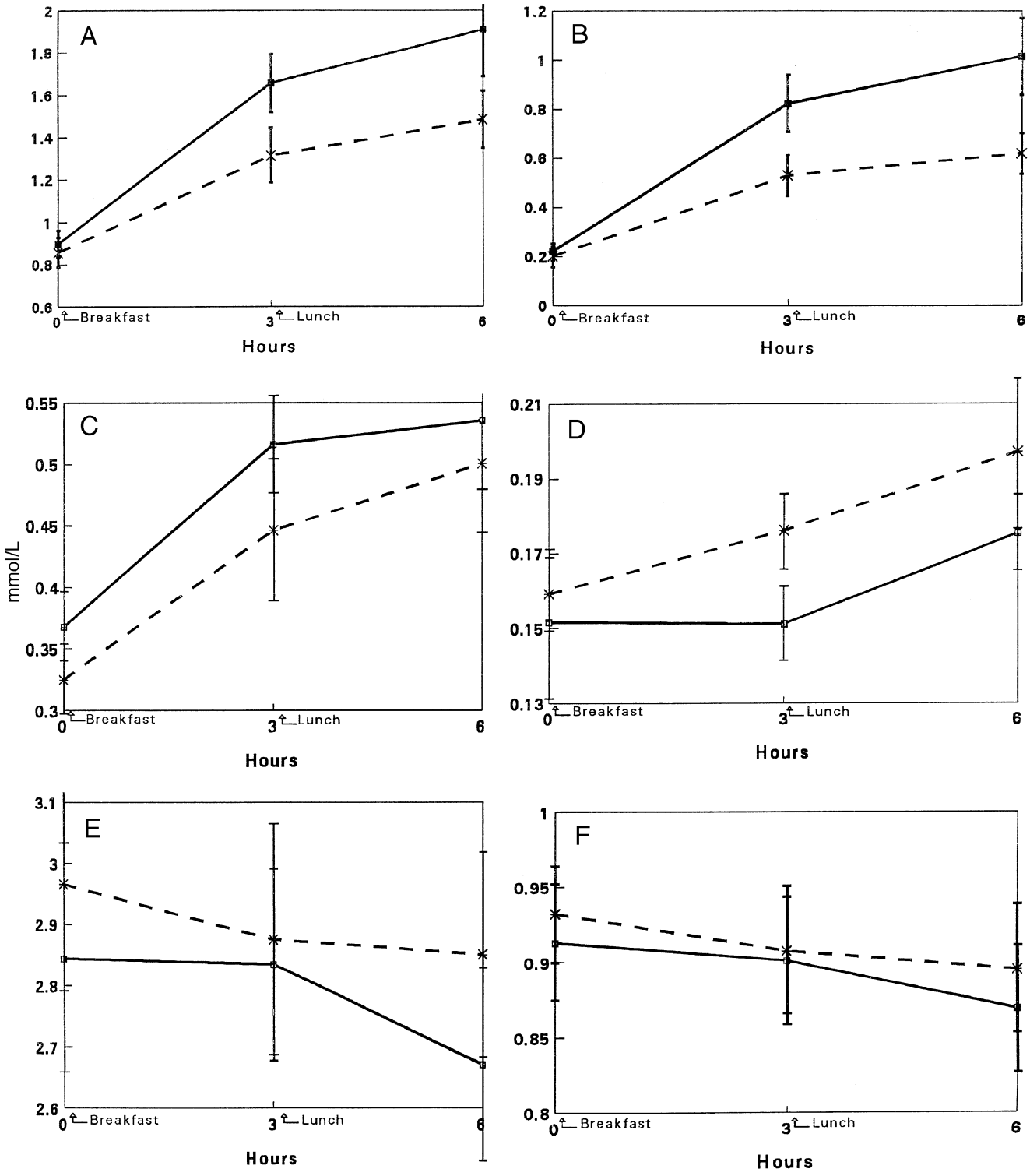

FIG. 1. Entry values before and changes after intake of breakfast and lunch, both meals each containing realistic amounts of milk fat (mean 25 g milk fat) in each meal. M diet contained modified milk fat (solid lines and squares) and D diet (dashed lines and crosses) contained Danish milk fat. Samplings at hours 0 are fasting values; other values are postprandial. Values are means ± SEM and $n = 18$. For details of fatty acid composition of the test fats, see Table 1. A, total (TAG); B, chylomicron TAG; C, very low density lipoprotein (VLDL)–TAG; D, low density lipoprotein (LDL)–TAG; E, LDL cholesterol; F, high density lipoprotein (HDL_2) cholesterol.

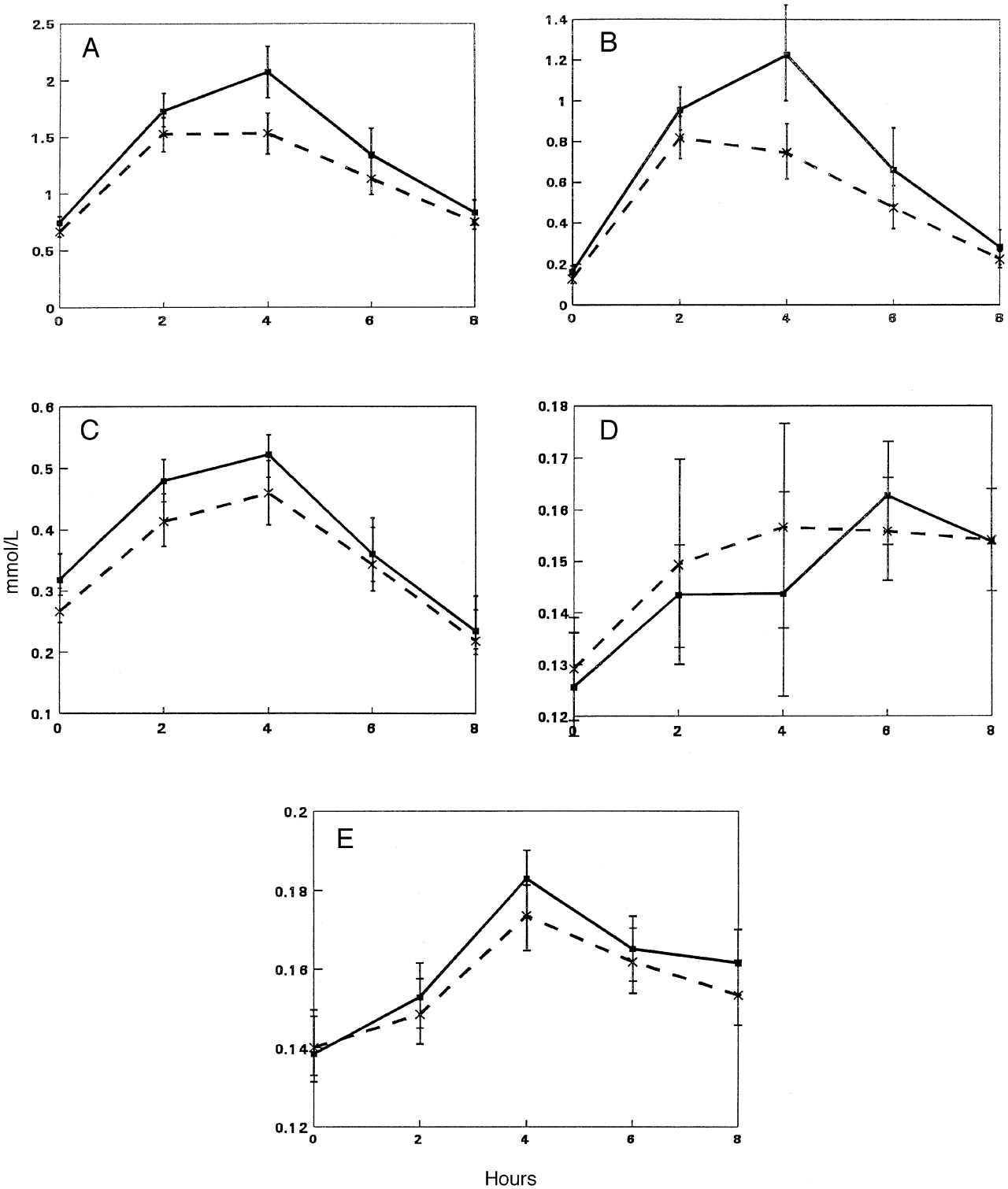

FIG. 2. Entry values before a high fat load (1.2 g/milk fat/kg body weight) and changes after intake of M diet (solid lines and squares) and D diet (dashed lines and crosses). Samplings at hours 0 are fasting values; other values are postprandial. Values are means ± SEM and $n = 8$. For details of fatty acid composition of the test butters, see Table 1. See Figure 1 for abbreviations. A, total TAG; B, chylomicron TAG; C, VLDL–TAG; D, LDL–TAG; E, HDL–TAG.

tions than fasting values in 8 h ($P < 0.001$) (Fig. 3A). LDL, HDL, and HDL_3 cholesterol concentrations decreased with the lowest value measured at 4 h and increased thereafter (Fig. 3B–D). According to the repeated measures analysis of variance, there was a difference in effect of the test butters over time on plasma total TAG concentration and chylomicron TAG (interaction effect) ($P < 0.005$). From Figure 2A and B it appears that M diet resulted in higher values of

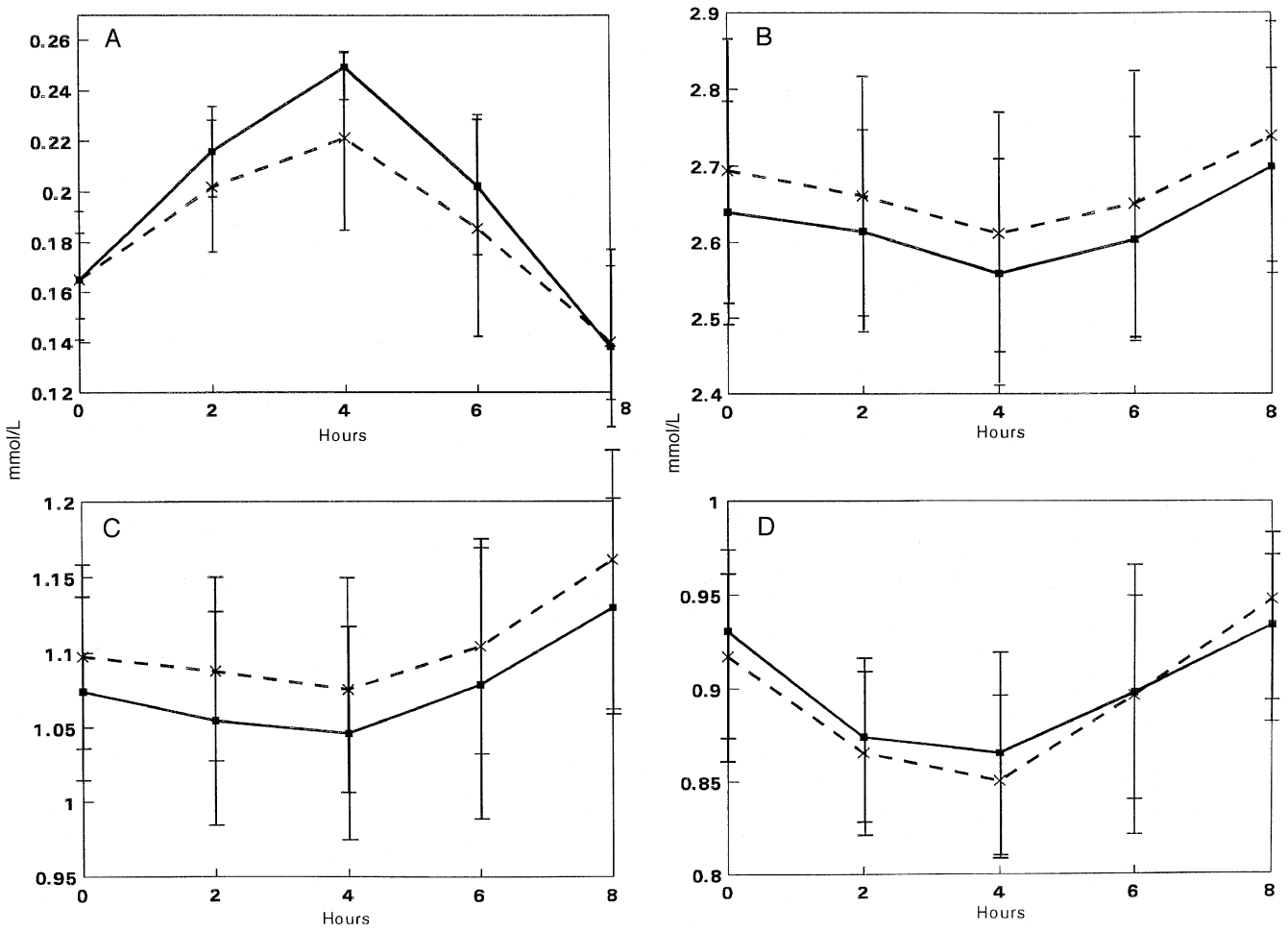

FIG. 3. Entry values before a high fat load (1.2 g/milk fat/kg body weight) and changes after intake of M diet (solid lines and squares) and D diet (dashed lines and crosses). Samplings at hours 0 are fasting values; other values are postprandial. Values are means ± SEM and $n = 8$. For details of fatty acid composition of the test butters, see Table 1. See Figure 1 for abbreviations. A, VLDL cholesterol; B, LDL cholesterol; C, HDL cholesterol; D, HDL_3 cholesterol.

plasma total TAG concentration and chylomicron TAG than D diet after 4 h.

A comparison of the plasma TAG elevating effect after 6 h after the butter load (1.2 g milk fat/kg body weight) with the effect of two meals with normal amounts of butter did not show any significant differences.

There was a positive significant relation between initial TAG levels and postprandial response after 6 h at the two meals' monitoring and after 4 h after the fat load indicated by positive regression coefficient [$\beta = 1.6$ ($P < 0.007$) and $\beta = 4.3$ ($P < 0.02$), respectively].

DISCUSSION

This study was designed specifically to compare the effect of a milk fat with a modified fatty acid composition (higher content of oleic and stearic acids and lower content of the cholesterol-raising SFA, especially palmitic acid) with conventional Danish milk fat on postprandial and fasting plasma lipids and lipoproteins in young healthy men. The change in fatty acid composition in the modified milk fat was obtained by natural feeding procedures, and the conventional Danish milk fat had a fatty acid composition typical for the winter period.

Contrary to our expectations, the results indicate that M diet high in oleic acid, enriched in stearic acid, and low in palmitic acid, and D diet high in palmitic and low in oleic acid had a similar effect on total cholesterol, LDL cholesterol, and apo B concentrations.

Nutritionally a decrease in the cholesterolemic SFA (C12–C16) and an increase in monounsaturated oleic acid and eventually stearic acid are assumed desirable (4,5,20). In our study the fatty acid composition in M butter had a 1.5-fold lower content of C12–C16 (from 53 to 35 wt%), a 5% higher 18:1n-7*trans,* and a 1.6-fold higher content of C18 + C18:1n-9 (from 23 to 36 wt%) compared to normal Danish butter (D butter). According to Keys' equation (21), the expected change in total plasma cholesterol when changing from diet D to diet M was −0.35 mmol/L (the energy from *trans* fatty acid was not included in equation owing to lack of

knowledge on effect of vaccenic acid, the *trans* fatty acid formed in the rumen). However, a relevant statement reported by Kris-Etherton's group is that when C_{12}-C_{16} SFA are high, the cholesterol-lowering effect of monounsaturated fatty acid (MUFA) is obscured (12). In this way there may be a threshold value for the ratio between cholesterolemic and beneficial fatty acid which has to be passed to obtain reduction in plasma total cholesterol. This might have been the case in other studies in which a substitution of cholesterolemic SFA with oleic acid did not result in a decrease in LDL cholesterol (22,23). In addition, a dietary fatty acid threshold has been suggested by K.C. Hayes *et al.* (24), who stated that sensitivity of plasma cholesterol to palmitic acid is based on the dietary C18:2, or C14, i.e., the 18:2/14:0 ratio.

Interestingly, a lowering of plasma lipids by modified milk fats (protected unsaturated fatty acids were fed to the cows) was demonstrated by an Australian group (25). Although the relative percentage modification was rather similar in the Australian study compared to our study, the absolute oleic acid and linoleic acid were higher and palmitic acid lower in the Australian study than in our study (25). In this way the relative lower SFA may be one of the reasons why the effect of oleic acid was successfully "expressed" in the above-mentioned study.

An additional explanation of the finding that M diet did not decrease LDL cholesterol compared with D diet may be the increased content of *trans* fatty acids, mainly vaccenic acid (18:1n-7) in M diet. This increase was a side effect of the cow-feed manipulation. It has been reported that elaidic acid, (18:1 *trans* n-9), a *trans* fatty acid formed mainly in partial hydrogenation of vegetable oils, is cholesterolemic (26–28). However, the effect of the *trans* fatty acids formed in rumen, mainly vaccenic acid (18:1 *trans* n-7) on lipoproteins, is to our knowledge not examined in experimental studies.

A side effect of the cow-feed manipulation was an increased content of conjugated linoleic acid (CLA). The effects of CLA on blood lipids have not been studied specifically. CLA possess apparent anticarcinogenic and antioxidant properties (29). Although M diet resulted in significantly higher CLA postprandially in total TAG than D diet (data not shown), the CLA were not found in the CE and PL fraction. This is unexpected as CLA are reported to be readily incorporated in PL (29).

The M diet resulted in higher, fasting total TAG concentration compared with D diet. A TAG-raising effect of beef tallow compared to olive oil on fasting plasma TAG has been demonstrated in men (30). Beef tallow has a similar content of oleic and *trans* fatty acids as M butter in this study. Oleic acid, however, has not been shown to increase fasting plasma TAG. The content of *trans* FA was 5.3% higher in the milk fat in M compared to D. For this reason it may be suggested that the increase in *trans* fatty acids may have resulted in the higher plasma TAG after M diet which agrees with observations by others (27,28,31,32). Although the increase in plasma TAG does not exceed the range for normal concentration in plasma, a TAG-increasing effect of dietary fat might be critical in risk groups in regard to coronary heart disease (33,34).

A higher postprandial response in total and chylomicron TAG was observed after M than D diet. It has been suggested that unsaturated fatty acids generally appear to produce a greater postprandial TAG response than do saturated fats (35). This observation agrees with unpublished results by us and others. However, reports on postprandial lipemea are not consistent (36–38). Dietary intake prior to the postprandial monitoring (37,39), amount (40), absorption time (41), and physical properties of test fat may play a role (36). Finally interaction processes may take place. Although a higher postprandial TAG response associated with reduced clearance is found in coronary heart disease patients compared to healthy people, a relatively high response may not be considered unbeneficial *per se*.

Comparing the two different postprandial experiments, the massive butter load and the two consecutive meals with butter in more realistic amounts, there was overall the same tendency in the effect on blood lipid parameters. The fat load but not the two meal events increased total HDL TAG and decreased total HDL cholesterol (HDL_3 significantly, and HDL_2 borderline significantly). We speculate if this may be due to a higher cholesteryl ester transfer protein activity during high butter load in comparison to intake of normal amounts of butter.

Postprandial fatty acid composition of plasma TAG after a butter load resembled as expected the fatty acid composition of test fats (data not shown). Except for a higher increase in 18:1 *trans* after the M diet, fatty acid composition of plasma CE did not reflect the difference in fatty acid composition of the test diets. We suggest that the equal proportion of palmitic acid in CE in spite of the different proportion in the test butters may be due to a higher rate of endogenous synthesis of palmitic as suggested previously by us (42) and by others (43). The rather equal proportion of oleic acid in CE may be due to elongation and desaturation of palmitic acid to oleic acid. Although there was a high content of 18:2n-6 in CE after both diets, conjugated 18:2n-6 was not found in CE or PL fraction. The somewhat higher content of C_{15} in D diet was reflected in the PL.

In regard to feeding strategy the increased content of *trans* fatty acids in the modified milk, a side effect of the cow-feed manipulation, could have counteracted the cholesterol-decreasing effect of MUFA. Thus to optimize milk fat in a nutritional way, a different feeding regime has to be used. In this experiment we fed the cows rapeseed oil rich in unsaturated fatty acids for the production of modified butter. Obviously the ability of the cow to completely hydrogenate these fatty acids in the rumen was exceeded. We hypothesize that a reduction of SFA without an increase in *trans* fatty acids could have been obtained if we had fed the cows stearic acid instead of rapeseed oil or rapeseed oil in the form of calcium soaps. These possibilities should be examined further.

In conclusion, a diet containing milk fat in which part of the cholesterol-raising SFA was substituted by MUFA and stearic acid did not lower total plasma cholesterol and LDL

cholesterol compared to typical Danish milk fat. The modified milk fat had an increasing effect on mean postprandial and fasting plasma TAG concentration. One possible reason that modified milk fat did not lower cholesterol compared to Danish milk fat may be that the decreasing effect of MUFA was obscured by the relatively high content of SFA in milk fat. The increase in fasting plasma TAG after modified milk fat might well be explained by the increase of *trans* fatty acids. Additional studies will be necessary to clarify effects of *trans* fatty acids, especially vaccenic acid, on lipemia and lipoproteins.

ACKNOWLEDGMENTS

We thank Aarhus Oliefabrik A/S, Oils and Fats Division, R&D, Denmark, for assistance with advice, analysis, and delivery of test fats. We are also very grateful to our dietitian Hanne Jensen and to Ingerlise Grönfeldt and the other staff of the metabolic kitchen. We thank Pia Lund, M.Sc. for the GC analysis and our technicians, and especially Kirsten Ebbesen and Klara Jörgensen for their conscientious and excellent work. Supported by grant JD-93 S 2464 Å9300085, The Danish Dairy Research Foundation and the Danish Research Development Program for Food Technology (FØTEK).

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[Received April 21, 1997, and in final revised form November 19, 1997; revision accepted November 25, 1997]

Regulation of Very Low Density Lipoprotein Apo B Metabolism by Dietary Fat Saturation and Chain Length in the Guinea Pig

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ABSTRACT: Studies investigated the effects of dietary fatty acid composition and saturation on the regulation of very low density lipoprotein (VLDL) apo B flux, clearance, and conversion to low density lipoprotein (LDL) in guinea pigs fed semipurified diets containing 15% (w/w) corn oil (CO), lard (LA), or palm kernel oil (PK). Plasma cholesterol levels were highest with dietary PK (3.1 ± 1.0 mmol/L) followed by LA (2.4 ± 0.4 mmol/L) and CO (1.6 ± 0.4 mmol/L) intake. VLDL particles were larger ($P < 0.05$) in the LA (78 ± 7 nm) and PK (69 ± 10 nm) groups compared to animals fed CO (49 ± 5 nm). VLDL-apo B fractional catabolic rates (FCR) were highest in guinea pigs fed the LA diet ($P < 0.05$) and VLDL apo B flux, estimated from VLDL ^{125}I-apo B turnover kinetics, were higher in LA compared to PK or CO fed guinea pigs. In the case of PK consumption, the kinetic estimates of VLDL apo B flux significantly underestimated rates compared to direct VLDL apo B secretion measurements and LDL turnover analyses. These data demonstrate that differences in the composition and amount of saturated fatty acids have differential effects on VLDL apo B flux, catabolism, and conversion to LDL which, together with changes in LDL receptor-mediated catabolism, determine plasma LDL cholesterol levels in guinea pigs. The data also indicate that kinetic analysis of VLDL metabolism in PK fed animals is inaccurate possibly due to the presence of a small, nonequilibrating pool of newly synthesized VLDL which is rapidly converted to LDL.

Lipids 33, 23–31 (1998)

Very low density lipoprotein (VLDL) particles are secreted by the liver and function primarily in the transport of endogenous triacylglycerol (TAG). Elevated VLDL concentrations lead to hypertriglyceridemia and, in addition, VLDL is the precursor of low density lipoprotein (LDL), which is the major carrier of plasma cholesterol. Both hypertriglyceridemia and hypercholesterolemia are major risk factors for cardiovascular disease (1,2).

Numerous epidemiological studies suggest that dietary fat saturation modulates hepatic cholesterol metabolism and plasma lipoprotein concentrations, which in turn modify an individual's cardiovascular disease risk profile. There is a well-documented association between the intake of saturated fat (SFA) in the diet, elevated plasma LDL cholesterol levels, and increased risk of cardiovascular disease (3,4).

Metabolic studies have shown that the type of dietary fat has significant effects on LDL synthesis and intravascular remodeling as well as apo B/E (LDL) receptor-mediated catabolism (3). Decreased hepatic apo B/E receptor activity with intake of SFA, as compared to polyunsaturated fatty acids (PUFA), has been reported in guinea pigs (5–7), as well as other animal models (8,9). Furthermore, intake of diets containing palm kernel oil (PK) as the source of fat leads to increased LDL flux compared to intake of PUFA corn oil (CO) (5,6). This increased LDL flux mediated by SFA could be due to increased VLDL production, increased rates of VLDL conversion to LDL, or both. The only reported human study ($n = 4$) suggests that dietary SFA increases VLDL apo B flux as well as LDL production, but has a minimal effect on VLDL and LDL catabolism (10). In contrast, animal studies have shown that in gerbils (11) and in squirrel and cebus monkeys (12) SFA decreases VLDL–TAG secretion compared to intake of PUFA, and Groot *et al.* (13) reported no significant differences in VLDL–TAG secretion in rats fed palm oil vs. sunflower oil.

In a study using rhesus monkeys, an attempt was made to better define the specific effects of different polyunsaturated/saturated (P/S) ratios on lipoprotein metabolism (14). The authors found that monkeys fed the diet with the lower P/S ratio exhibited a significant increase in the VLDL-independent transport of LDL apo B compared to those fed the higher P/S ratio indicating the complexity of fatty acid composition in regulating LDL apo B transport (14).

Studies in guinea pigs demonstrated that the composition and amount of SFA alter the rates of hepatic VLDL–TAG and apo B secretion in the guinea pig by different mechanisms (15).

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Abbreviations: CO, corn oil; FCR, fractional catabolic rate; LA, lard; LDL, low density lipoprotein; PK, palm kernel oil; P/S, polyunsaturated/saturated; PUFA, polyunsaturated fatty acids; RID, radial immunodiffusion; SFA, saturated fat; TAG, triacylglycerol; VLDL, very low density lipoprotein.

Intake of lard (LA) (rich in long-chain SFA) increased hepatic VLDL–TAG secretion rates, whereas PK in the diet (rich in short-chain SFA and higher concentrations of SFA) increased nascent VLDL apo B production, suggesting increased number of VLDL particles being secreted by the liver (15).

Since the majority of LDL apo B in normal humans is derived from VLDL (16), it is expected that SFA will affect VLDL metabolism. It has been shown that the mass of VLDL converted to LDL vs. the amount of VLDL cleared directly from the plasma compartment varies among normal and hypertriglyceridemic individuals (17). It also has been shown that the presence of apo E in the VLDL affects the rate of VLDL removal in rabbits (18). Based on these reports on the factors that influence VLDL channeling (14–18), the present studies were undertaken to further define the role of dietary SFA on the synthesis, intravascular remodeling, and catabolism of VLDL by using an animal model, the guinea pig, that, similar to humans, transports the majority of cholesterol in LDL (5,6).

The objectives were to determine whether the observed changes in plasma LDL cholesterol concentrations and flux (5,6) with intake of SFA are due to increased VLDL production and increased fractional conversion of VLDL to LDL, and to investigate how changes in VLDL metabolic channeling determine plasma LDL cholesterol concentrations.

MATERIALS AND METHODS

Materials. Agarose was from Gibco-BLR (Grand Island, NY). Agarose immunodiffusion tablets were purchased from Sigma Chemical (St. Louis, MO). LDL–Direct column chromatography was obtained from Isolab (Akron, OH). Radial immunodiffusion kit was from Bio-Rad (Richmond, CA). Rapid Silver Staining electrophoresis kit and cyanogen bromide activated sepharose were purchased from Sigma Chemical.

Diets. Diets were prepared and pelleted by Research Diets, Inc. (New Brunswick, NJ). All diets were isocaloric (15.9 Kj/g) and contained identical compositions except for the fat type. Fat content was 15% (w/w) as either CO, LA, or PK. The fatty acid compositions of the experimental diets were determined by gas chromatography (Table 1). The diets were formulated to meet Nutrition Research Center-specified nutritional requirements of guinea pigs (5). Plant sterols (0.9 mg sitosterol/g diet) and cholesterol (0.1 mg/g diet) content were normalized for all diets (5,6).

Animals. Male Hartley guinea pigs from Sasco Sprague-Dawley (Omaha, NE) weighing between 450–500 g were randomly assigned to one of the three diet groups for 4 wk prior to *in vivo* VLDL kinetic studies or isolation of plasma for electron microscopic studies. Previous studies have shown that this time period is sufficient to attain constant plasma cholesterol levels and a metabolic steady state. All animals consumed equal amounts of the diets, and there were no differences between growth rates or final body weights (Table 2). All animal procedures were conducted in accordance with U.S. Public Health Service and U.S. Department of Agriculture guidelines, and experimental protocols were approved by the University of Arizona Institutional Animal Care and Use Committee.

Electron microscopy. Four animals per dietary group were fasted overnight and used to isolate plasma VLDL for measurements of VLDL particle size. Plasma VLDL was isolated by ultracentrifugation at a density of 1.006 g/mL, and washed once at that density. Centrifugation was performed in a Ti-50.2 rotor (Beckman Instruments, Palo Alto, CA) for 24 h at 40,000 × *g* at 15°C. Freshly prepared VLDL was used 1–2 d after preparation to avoid deterioration of the intact particles and aggregate formation. Prior to microscopic examination, samples were dialyzed against a volatile buffer (0.125 M ammonium acetate, 2.6 mM ammonium carbonates, and 0.26 mM EDTA, pH 7.4) (19). VLDL particles were observed by negative stain electron microscopy with 2% sodium phosphotungstate, filtered through 0.45-mm filter, and adjusted to pH 7.0–7.5. Transmission electron microscope was used (Jeol 100-CX TEM, Tokyo, Japan) with a condenser aperture of 200 mm and accelerating voltage of 80 kV. Negative staining of the lipoprotein was done on formol-coated copper mesh grids (200 mesh). Images were taken at 20,000 × (20), and random photographs within the same grid squares and random grid squares were taken. At least one hundred particle diameters were examined from the negatives of the photographs, and the size of the VLDL particles was measured by a ruler under magnifying lens and normalized to the magnification factor of the electron microscope. The distribution of VLDL particle sizes, as well as the mean diameters, were calculated for all three dietary fat groups.

Preparation and purification of polyclonal antibodies against apo B-100. Guinea pig LDL was isolated by sequen-

TABLE 1
Fatty Acid Composition of Semipurified Diets

Fatty acid (%)	Experimental diets		
	Corn oil (CO)	Lard (LA)	Palm kernel (PK)
12:0	0	0	52.4
14:0	0.2	1.6	18.0
16:0	11.6	24.0	8.5
18:0	2.2	13.6	14.0
18:1	25.0	42.3	4.1
18:2	58.4	10.7	1.4
18:3	1.4	1.2	0
20:0	0.4	2.4	0
P/S[a]	4.11	0.29	0.02

[a]P/S, ratio of polyunsaturated to saturated fatty acids

TABLE 2
Characteristics of Guinea Pigs Fed Test diets[a]

Diet (*n*)	Body weight (g)	Growth (g/d)	Plasma lipids (mmol/L)	
			Triacylglycerol	Cholesterol
CO (12)	741 ± 159	9 ± 2	1.12 ± 0.37	1.62 ± 0.44[a]
LA (12)	754 ± 169	9 ± 2	1.29 ± 0.49	2.41 ± 0.64[b]
PK (14)	733 ± 220	10 ± 2	1.12 ± 0.54	3.12 ± 1.0[c]

[a]Data are presented as the mean ± SD for *n* (number in parentheses) animals fed CO, LA, or PK. See Table 1 for abbreviations. [b]Values in the same column with different superscripts are significantly different ($P < 0.01$) as determined by analysis of variance and the Student-Newman-Keuls Multiple comparisons tests.

tial ultracentrifugation within a 1.023–1.075 g/mL density range. LDL was collected following tube slicing, washed once at d = 1.075 g/mL, dialyzed against 0.9% NaCl- 0.01% EDTA buffer, and purified by agarose-heparin column chromatography. The purity of LDL was checked by denaturing polyacrylamide gel electrophoresis (21). Purified LDL (antigen) was concentrated to 1 mg/mL apo B-100 protein. The recipient sheep was immunized subcutaneously with the antigen (300 mg in complete Freund's adjuvant) in one dose, followed by two booster doses (200 mg in incomplete Freund's adjuvant) every 10 d. After each injection blood samples were taken to check production of apo B antibodies using radial immunodiffusion kits (RID; Biorad). Four weeks after the initial injection, the animal was bled to collect 1 L of blood. The blood was allowed to clot at room temperature and the serum kept frozen at −20°C.

To prepare sheep antiguinea pig apo B-100 antibody, serum from immunized sheep was passed through a guinea pig LDL-sepharose column. The anti-apo B 100 antibodies, which were bound to the matrix after washing with 0.1 M phosphate buffered saline, pH 7.4 were eluted by lowering the pH to 3 using elution buffer (0.2 M glycine/HCL, pH 3) (22).

Apo B quantification by radial immunodiffusion (RID). Lipoprotein apo B mass values were determined by RID. Affinity purified sheep antiguinea pig antibody was incorporated in 1% Agarose at 50°C and cast as an open gel on an Agarose (0.2%) precoated glass plate (100 × 100 mm). Each gel contained approximately 250 mg of affinity column purified antibody for the RID assay. The polymerized gels were cured on ice, and 50 µL sample wells were created with a 4-mm gel punch. Calibrator apo were purified by affinity chromatography from ultracentifugally isolated guinea pig LDL, and the apo concentrations were determined by a modified Lowry procedure (23). The apoprotein calibrators and samples were diluted with phosphate buffered saline, loaded into the wells, and incubated in a humidified 37°C chamber for 24–72 h, depending on the sample concentrations and the lipoprotein fraction. Nonspecific proteins were removed by overlaying the gel with 1-cm stack of filter paper and a glass plate and applying pressure for 1 h. Gels were washed overnight with buffer using gentle agitation at room temperature, pressed, and rewashed with 5% glycerol, rinsed, and dried under a forced beam of warm air and stained with either Coommassie blue or silver nitrate (silver-enhanced RID) (24). Diameters of the immunoprecipitate rings were measured using an RID reader (Bio-Rad). Linear regression equations were generated for the standard calibrator curve with X as the concentration of standards (µg/mL) and Y the diameter squared of the precipitation rings (mm^2). Linear regression correlations were used to calculate apo B concentrations (µg/mL).

In vivo *VLDL kinetics.* Guinea pig plasma VLDL was isolated from animals fasted for 12 h by ultracentrifugation at density 1.006 g/mL, dialyzed against 0.9% NaCl-0.01% EDTA for 24 h, and concentrated to 0.5 mg/mL protein. VLDL iodination was carried out according to the methods of Goldstein *et al.* (25). ^{125}I-VLDL was used within 2 d after iodination of lipoproteins to minimize possible radiation oxidation (26). Guinea pigs were fasting 12 h before surgery and during the first 10 h of the study to avoid interference of intestinal chylomicrons with isolated VLDL. Measurements of VLDL-associated radioactivity indicated that only 3–5% of the radioactivity was accounted for by free iodine, while 95–97% was bound to the VLDL particle as determined by trichloroacetic acid precipitation. The percentage of labeled TAG and phospholipids ranged between 15–24% among the VLDL particles isolated from guinea pigs fed the autologous diet. Approximately 30–33% of the ^{125}I radioactivity was associated with apo B-100, and 45–55% of the radioactivity was associated with non-apo B-100 apo consistent with other reports (27). Animals were injected with labeled VLDL (35 µg) through an indwelling catheter inserted in the internal carotid artery, and blood samples were collected for the analysis of plasma VLDL and LDL specific radioactivities. Approximately 1 mL of blood was collected at 3 (zero time), 10, 20, and 40 min and at 1, 3, 6, 10, 22, and 27 h. Plasma was separated by low-speed centrifugation, VLDL and LDL isolated by ultracentrifugation, and an aliquot of isolated VLDL and LDL used to measure apo B mass by RID and radioactivity following isopropanol precipitation (28). Aliquots (170 µL) of VLDL or LDL and unlabeled LDL carrier (30 µL) were precipitated with an equal volume of 100% isopropanol (200 µL) and were incubated overnight at 4°C. Samples were centrifuged for 30 min at 1000 × g, and radioactivity in the apo B pellet was measured in a gamma counter. Apo B mass of VLDL and LDL was measured by RID as described above.

Model selection and kinetic analysis. The selected model for plasma VLDL turnover was the two pool model as described by Matthews (29). We used this model because the two-pool model is routinely used for analysis of plasma lipoprotein turnover studies in small laboratory animals (5,30,31).

VLDL apo B specific radioactivity was determined as cpm per µg apo B protein, and plasma VLDL apo B specific activity decay curves were resolved into two exponential functions. VLDL apo B fractional catabolic rate (FCR) values were measured using a curve-fitting program (JANA, SCI Software, Lexington, KY), which indicated that the data were best fit with a two-pool model. Apo B VLDL pool size was determined by multiplying VLDL apo B (mg/mL), calculated by RID, × plasma volume per unit of weight (mL/kg). Plasma volume is equivalent to 4% total body weight in guinea pigs (32).

The area under the plasma VLDL apo B specific radioactivity vs. time curve was calculated using the linear trapezoidal rule method (33). When the time interval between 2 points was long (e.g., from 10 to 22 h), the logarithmic trapezoidal rule was used (33). Total apo B specific radioactivity was calculated by the summation of all the measured individual areas under the specific radioactivity curves.

VLDL apo B production rates were calculated by two different methods. In one method, the production rate (µg/kg·h) was calculated as a function of VLDL apoB FCR (h^{-1}) × VLDL apo B pool size (µg/kg), and the second method VLDL apo B production rates were calculated by dividing the

total dose of injected radioactivity (cpm) by the area under the plasma VLDL apo B specific activity time curve (cpm/μg per h) and adjusting the value per kg body weight (34).

Statistics. Data are presented as mean ± SD. One-way analysis of variance was used to assess differences in plasma cholesterol, TAG, and apo B concentrations among diet groups; and between VLDL apo B FCR and production rates. Differences among mean values were evaluated by student-Newman-Keuls Multiple Comparison Tests and were considered significant at $P < 0.05$. Statistical analysis of the kinetic model was best fitted using a two-pool model (JANA). Linear regression analysis was used to determine correlation coefficients between variables.

RESULTS

Dietary fat effects on plasma lipids and growth rates. There were no significant differences in the final body weights of guinea pigs fed the experimental diets (Table 2). Guinea pigs fed the three test diets had similar growth rates, indicating equal dietary intakes among groups. There were significant differences in plasma cholesterol levels with animals fed the PK diet having the highest levels followed by animals fed the LA diet, and the lowest plasma total cholesterol concentrations occurred in animals fed the CO-based diet (Table 2). No differences were observed for plasma TAG concentrations among dietary treatments.

VLDL particle size. The size distribution of VLDL particles (Fig. 1) differed for VLDL isolated from the guinea pigs fed the three types of dietary fat. VLDL particles isolated from animals fed the CO diet had a smaller average diameter (49 ± 5 nm) compared to VLDL from LA (78 ± 7 nm) or PK fed guinea pigs (69 ± 10 nm) (Fig. 1) ($P < 0.05$). In addition, the frequency distribution of particles from CO-fed animals was shifted toward a smaller size and skewed to the left. In contrast, VLDL isolated from animals fed the PK- and LA-based diets were more heterogeneous.

In vivo *VLDL apo B-100 kinetics.* Analysis of VLDL apo B decay curves (Fig. 2) indicates that the turnover kinetics could be resolved into biexponential curves. Plasma ^{125}I-VLDL exhibited a significantly faster turnover rate with LA intake, followed by the PK and CO diet groups (Fig. 2). The metabolic parameters for guinea pig VLDL kinetics are summarized in Table 3**.** LA intake resulted in a faster VLDL apo B FCR compared to animals fed CO- and PK-based diets. VLDL apo B pool sizes and flux calculated by multiplying FCR × pool size were not statistically different among groups (Table 3).

A positive correlation was found between the mean VLDL apo B FCR and the mean VLDL particle size for the three diet groups ($r = 0.99$, $P < 0.05$) (Fig. 3), suggesting that the larger VLDL particles are more rapidly removed from the plasma VLDL lipoprotein density range compared to smaller particles.

There was a positive correlation ($r = 0.73$, $P < 0.002$) between VLDL apo B flux and VLDL apo B pool size (Fig. 4) for animals from all dietary fat groups. Comparable values were obtained for VLDL apo B flux determined by multiplying FCR × pool size (Table 3), and VLDL flux determined by dividing the total injected VLDL apo B radioactivity by the area under the curve (±10%). Values were 1.03 ± 0.40, 2.28 ± 0.93, and 0.88 ± 0.41 mg/kg–h for animals fed CO, LA, and PK, respectively.

VLDL–LDL precursor–product relationships. In addition to analysis of plasma VLDL turnover using a two-pool model, the turnover curves were analyzed to determine VLDL–LDL precursor–product relationships by analysis of the areas under the respective specific activity decay curves. Examination of the precursor–product relationship between plasma VLDL

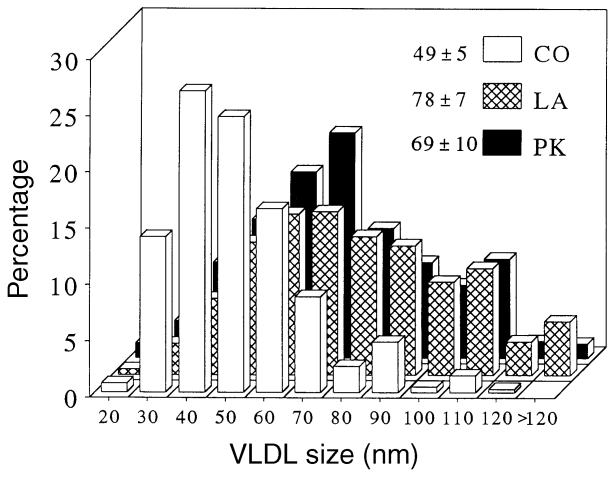

FIG. 1. Very low density lipoprotein (VLDL) particle size distribution of guinea pigs fed semipurified diets containing 15% (w/w) corn oil (CO: 49 ± 5 nm), lard (LA: 78 ± 7 nm), or palm kernel oil (PK: 69 ± 10 nm). Values represent percentage distribution of at least 100 particles counted directly from the negative of electron microscopic images of isolated mature VLDL particles (CO < LA = PK, $P < 0.05$).

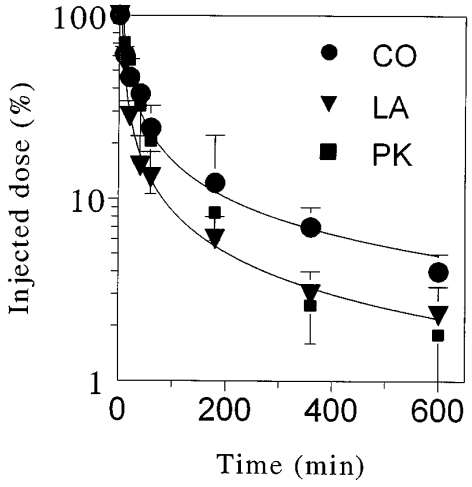

FIG. 2. Plasma disappearance curves of homologous VLDL isolated from guinea pigs fed semipurified diets containing 15% (w/w) CO, LA, or PK injected into animals fed the same diets. The values represent the mean of VLDL apoprotein (apo) B specific activity presented as percentage of injected dose for $n = 5$ determinations for animals fed CO and PK and $n = 4$ for LA fed guinea pigs. See Figure 1 for abbreviations.

TABLE 3
Metabolic Parameters of ^{125}I-VLDL Turnover Kinetics (two-pool analysis) in Guinea Pigs Fed Diets Containing 15% CO, LA, or PK Diets (for 4 wk)[a]

	VLDL kinetic parameters		
Diet (*n*)	FCR (pools/h)	Pool size (mg/kg)	Flux (mg/kg-h)[b]
CO (5)	0.65 ± 0.08[a]	2.11 ± 0.86	1.39 ± 0.67
LA (4)	1.22 ± 0.37[a]	1.91 ± 0.98	2.26 ± 1.33
PK (5)	0.98 ± 0.02[b]	1.09 ± 0.39	1.11 ± 0.50

[a]Data are presented as the mean ± SD for (*n*) animals. Values in the same column with different superscripts are significantly different ($P < 0.01$) as determined by analysis of variance and the Student-Newman-Keuls Multiple comparisons test

[b]Very low density lipoprotein (VLDL) apoprotein (apo) B flux calculated as VLDL apo B fractional catabolic rate (FCR) × VLDL apo B pool size. See Table 1 for other abbreviations.

and LDL–apo B specific radioactivity (Fig. 5) indicated that plasma LDL apo B specific activity reached its peak value at the intersect with the VLDL specific activity curve, consistent with LDL apo B being derived from VLDL apo B (35).

VLDL apo B secretion rates calculated from studies using guinea pigs fed the same diets and treated with Triton WR 1339 (18) were compared to VLDL apo B flux determined by ^{125}I-apo B turnover to determine whether there were significant differences between methods (Table 4). Animals from the CO and LA groups had similar values for apo B VLDL flux calculated from either method while the values for animals from the PK group were significantly lower when flux was calculated from the VLDL apo B turnover data (Table 4). These results suggest that apo B flux calculated from turnover studies is underestimated in animals fed PK diet, and this underestimation could be due to the presence of a rapid turnover pool of VLDL, which cannot be detected by use of isotopes as discussed below.

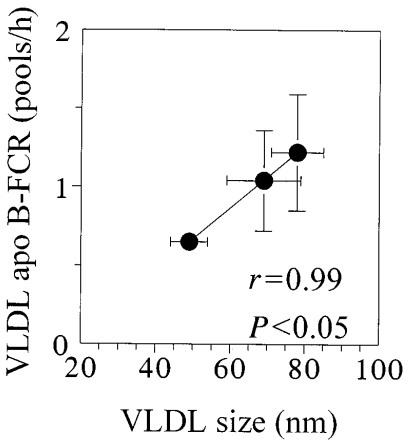

FIG. 3. Correlation between mean VLDL particles size and mean VLDL apo B fractional catabolic rate (FCR) values for guinea pigs fed semipurified diets containing 15% (w/w) CO, LA, or PK ($r = 0.99$, $P < 0.05$). See Figure 1 for other abbreviations.

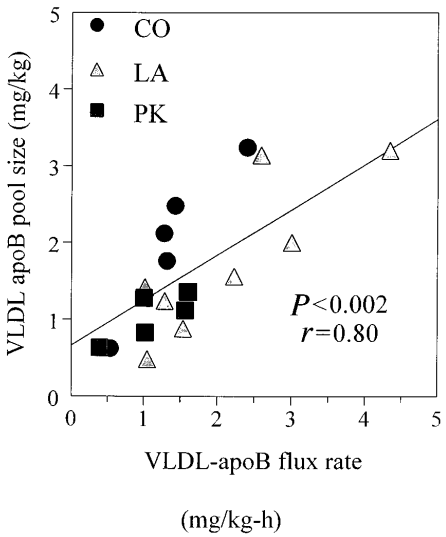

FIG. 4. Correlation between VLDL apo B flux rates (mg/kg·h) and VLDL apo B pool size (mg/kg) for guinea pigs fed semipurified diets containing 15% (w/w) CO (●), LA (▲), or PK (■) ($r = 0.80$, $P < 0.002$). See Figure 1 for abbreviations. See Figure 1 for abbreviations.

DISCUSSION

The present study tested two SFA with different SFA concentration and different proportions of fatty acid chain length: LA, rich in palmitic and stearic acids; and PK, rich in lauric and myristic acids, and compared their effects on VLDL metabolism to intake of polyunsaturated CO, rich in linoleic acid. The objective of this study was to determine the effects of fats varying in fatty acid composition and SFA amount on VLDL synthesis, intravascular remodeling, and catabolism.

Dietary fat saturation effects on plasma lipids and VLDL size. The results indicate that intake of PK and LA diets resulted in significantly higher plasma cholesterol levels compared to a CO-based diet. Plasma cholesterol levels decreased as the proportion of short-chain fatty acids decreased and the proportion of PUFA increased, similar to reports in humans (4) and in guinea pigs (5,6). In the guinea pig these effects are specific to plasma LDL with no significant effects on plasma HDL cholesterol concentrations (7).

Previous studies have shown that intake of SFA alters LDL composition and peak density resulting in larger particles compared to LDL isolated from animals fed CO (5,7). These changes in LDL composition with changes in dietary fat saturation are similar to the dietary fat-induced changes in VLDL, the precursor of LDL, observed in this study. Electron microscopic measurements demonstrated that intake of SFA resulted in larger VLDL particles. In addition, the particles were more heterogeneous as indicated by the frequency distribution. It has been reported that larger (65 ± 20 nm) VLDL particles are generated with a lipid-rich diet (15% CO, 1.6% cholesterol) in guinea pigs (36). VLDL can exist as a multidisperse population with particles size ranging between 30 and 80 nm (37), and heterogeneity of VLDL metabolism is thought to be affected by differences in VLDL particle size. These reports are consistent with the present observation of

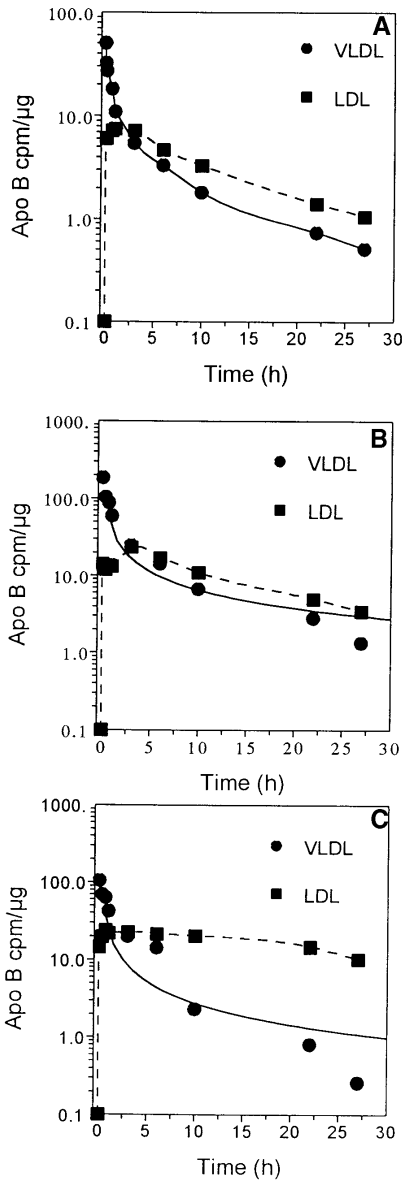

FIG. 5. A representative curve for the precursor–product relationships between VLDL and LDL as determined from VLDL apo and LDL apo B specific activity time course for animals fed CO (A), LA (B), or PK (C). See Figures 1 and 2 for abbreviations.

variability in VLDL particle size and indicate that variations in fat quantity as well as dietary fatty acid composition lead to significant changes in the size distribution of VLDL particles. These findings suggest that changes in fatty acid composition related to chain length and unsaturation can significantly affect the interactions of VLDL with the various enzymes involved in the delipidation of VLDL and on the intrinsic metabolic properties of these particles.

In addition, VLDL particle size has a significant effect on the metabolic fate of VLDL (35). Shames and Havel (38) employed the concept of kinetic heterogeneity to account for the fact that in some *in vivo* kinetic measurements of exogenously labeled VLDL kinetics the data are interpreted as indicating direct hepatic LDL secretion which can be due to rapidly metabolized nonequilibrated pools of VLDL subpopulations. As discussed below, data from the present study suggest that such heterogeneity due to differential effects of saturated fatty acids affects these kinetic determinants.

TABLE 4
Comparison of VLDL Flux Determined from VLDL Apo B secretion Measurements and from ^{125}I-VLDL Turnover Kinetics of Guinea Pigs Fed Diets Containing CO, LA, or PK Diets[a]

Diet	VLDL apo B secretion (mg/kg·h)[b]	VLDL ^{125}I-apo B flux (mg/kg·h)[c]
CO	1.14 ± 0.63[b] (15)	1.21 ± 0.54 (5)
LA	1.53 ± 0.83[b] (12)	2.27 ± 1.13 (4)
PK	3.11 ± 1.84[a] (12)	0.99 ± 0.46 (5)

[a]Data presented as mean ± SD for (*n*) animals per diet group. Values in the same column with different superscripts are significantly different as determined by analysis of variance and the Newman Keules *post hoc* test ($P < 0.01$).
[b]Data for VLDL apo B secretion rates in Triton WR 1339 treated guinea pigs fed the test diets have been previously reported (Ref. 15) and are presented here for comparative purposes only.
[c]Values for VLDL apo B flux are the average flux values calculated from the two-pool analyses and from the area under the specific activity curve analyses presented in Table 3. See Figures 1 and 3 for abbreviations.

Dietary fat saturation effects on in vivo *VLDL kinetics.* SFA of different chain length have differential effects on LDL metabolism (5–7), as well as on VLDL apo B synthesis and catabolism (9). The present study measured rates of VLDL production, catabolism, and intravascular conversion to LDL to determine effects of dietary fat chain length and saturation on plasma VLDL metabolism and intravascular remodeling. From our studies we concluded that, compared to the CO diet, intake of the LA diet increases VLDL apo B FCR. Increased direct hepatic removal of VLDL with intake of diets rich in 16:0 has been reported in rhesus monkeys (14). In our study, VLDL particle size was significantly correlated to VLDL apo B FCR, suggesting that the larger the VLDL particle, the shorter residence of the apo B in the VLDL density range: an observation previously noted for VLDL metabolism in guinea pigs treated with lovastatin (35).

Several investigators have suggested that dietary SFA have differential effects on VLDL–TAG secretion compared to VLDL apo B production, and we have previously shown that feeding LA increased VLDL–TAG secretion whereas intake of PK increased VLDL apo B secretion compared to CO intake (15). Grundy and Denke (3) suggested that the higher LDL apo B flux with intake of SFA could be due to increased VLDL apo B production, decreased VLDL catabolism, or increased conversion of VLDL to LDL due to decreased apo B/E receptor activity. Data from human studies suggest that SFA intake increases VLDL synthesis with little effect on VLDL apo B catabolism (10).

Human and animal studies have consistently demonstrated that dietary PUFA compared to SFA intake results in higher plasma LDL turnover (59,39); however, some discrepancies exist regarding the effects on LDL apo B flux (40). These studies suggest that intake of SFA decreases LDL catabolism but does not consistently increase LDL flux.

Studies in guinea pigs have shown that dietary SFA have

significant effects on LDL flux, composition, and catabolism (5,6). These data are consistent with the findings of the present study in that the calculated effects of PK intake on VLDL production were different from LA. PK intake did not increase the calculated VLDL production; however, the higher plasma LDL cholesterol levels suggest that there is an increased conversion to LDL in agreement with our previous data on the effects of PK on LDL flux (5,6). In contrast, LA intake led to a higher VLDL apo B flux and an apparently higher proportion of direct VLDL catabolism without conversion to LDL which explains the lower plasma LDL cholesterol concentrations in animals fed LA vs. PK diets.

LA feeding resulted in VLDL particles with faster FCR values than those VLDL isolated from CO- or PK-fed guinea pigs. It is possible that VLDL particles from LA-fed guinea pigs are enriched with apo E which has a greater affinity for cellular receptors (41), and thus the percentage of VLDL directly removed from the circulation is greater than the percentage converted to LDL. Apo E is necessary for TAG-rich lipoprotein receptor-mediated uptake and the LDL-receptor related protein binds apo E-enriched β-VLDL (42). These observations suggest that VLDL can be catabolized directly from the circulation through alternative routes, and are in agreement with the results from the present study in which a large proportion of VLDL secreted in response to LA feeding apparently was not converted to LDL but was rather removed from the circulation although LA intake reduces hepatic LDL receptors (5,7).

De novo *LDL production.* Analysis of VLDL conversion to LDL indicates that VLDL apo B flux in the PK group was less than LDL apo B flux, suggesting input of LDL apo B from a source not in equilibrium with the ^{125}I-VLDL apo B pool. There are two potential explanations to account for this observation. The first hypothesis is that there is direct *de novo* LDL secretion by the liver as proposed from some studies in animal models (14,43) as well as from lipoprotein kinetics studies in humans with familial hypertriglyceridemia (16). However, we have previously shown that there is no direct LDL secretion in guinea pigs fed the test diets used in this study (15). Interestingly, Goldberg *et al.* (44) reported that there was no direct LDL secretion in cynomolgus monkeys when the conversion of VLDL to LDL was blocked using lipoprotein lipase antibodies, even though these authors had previously suggested the existence of *de novo* LDL production in the same animal model based on *in vivo* VLDL turnover studies (45). In liver perfusion studies of cholesterol-fed guinea pigs, Guo and colleagues (46) found no evidence of direct LDL secretion, consistent with the results of our previous *in vivo* VLDL secretion studies (15). Shames and Havel (38) have suggested that the apparent dilution of plasma LDL apo B specific activity relative to VLDL apo B specific activity could be attributed to kinetic heterogeneity of VLDL apo B without the requirement of *de novo* LDL production. Based on the available evidence (15,38,46), there is no indication of direct LDL secretion by the liver in the guinea pigs.

The most likely explanation for the observation that LDL apo B flux is greater than VLDL apo B flux in guinea pigs fed the PK diet is the presence of a small pool of VLDL particles with a very rapid FCR that is not detected using the conventional isotope tracers, and consequently the production rate of VLDL apo B is underestimated (38). In normal and hyperlipidemic rabbits, Yamada *et al.* (18) reported that a large portion of VLDL particles were metabolized very rapidly and did not contribute to the measured VLDL mass which leads to underestimation of VLDL apo B flux. In agreement with this thesis, studies in humans have suggested that a portion of LDL is produced from a pool of VLDL which is very rapidly converted to LDL (47,48). These findings in part account for the fact that nascent VLDL apo B secretion was increased with PK feeding in the guinea pig (15), whereas in the present studies increased nascent VLDL apo B flux with intake of the PK diet was not detected with exogenously labeled VLDL. PK feeding may also be associated with the synthesis of TAG-poor VLDL particles which are preferentially converted to LDL and not directly removed from the liver. Oschry *et al.* (49) reported that large TAG-rich VLDL particles are removed by direct hepatic uptake, while smaller TAG-poor particles are converted to LDL. We have previously shown that hepatic nascent VLDL from animals fed PK diet had less TAG and more protein compared to nascent VLDL from the CO group (15), resulting in the formation of cholesterol-enriched mature VLDL particles which possibly are preferentially converted to LDL, leading to increased LDL production compared to animals fed CO- or LA- based diets. Interestingly, the relationship between VLDL apo B secretion rates, measured using Triton WR 1339 blockage, and VLDL apo B flux, measured using ^{125}I-VLDL, are comparable for CO- and LA-fed animals.

These and previously reported data demonstrate that intake of SFA increases VLDL flux relative to intake of PUFA; and that varying the amount and composition of SFA has differential effects on VLDL apo B and VLDL–TAG secretion. A higher amount of SFA with higher proportion of short-chain fatty acids (PK diet) increases hepatic VLDL apo B secretion and increases conversion of VLDL to LDL, resulting in elevated plasma LDL cholesterol concentrations. Compared to CO, intake of a higher proportion of long-chain SFA as in the case of LA was associated with an increase in VLDL flux and possibly an increase in VLDL direct removal with less transport to LDL; therefore, LA invoked a less-significant cholesterolemic effect compared to PK feeding. In either case, it is evident that effects of the amount of SFA and differences in the composition of fatty acids on plasma LDL cholesterol levels are in part due to effects on VLDL production, catabolism, and conversion to LDL. These modifications in VLDL metabolism together with changes in LDL receptor-mediated FCR (5–7) are major determinants of dietary fatty acid-mediated changes in plasma cholesterol levels in the guinea pigs.

ACKNOWLEDGMENTS

The authors would like to thank Stephanie Cameron for performing the animals' surgeries, Dr. Mark Wise and Anita Dunn for providing the sheep serum, and David Bentley for his assistance in the electron micro-

scopic facilities, Dr. Michael Mayerson for his helpful discussions and suggestions, and Henrik Steinberg for his excellent technical assistance.

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[Received October 1, 1997, and in final revised form December 1, 1997; revision accepted December 2, 1997]

Enhancement of Low Density Lipoprotein Binding to Both Low Density Lipoprotein Receptor-Positive and -Negative Cells by Tetracycline Antibiotics

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ABSTRACT: Some tetracycline (TC) antibiotics, including TC and anhydrotetracycline, have been found to enhance specific binding of low density lipoprotein (LDL) to both LDL receptor-positive and -negative cells at relatively higher concentrations. When incubated at 37°C, the ability of LDL receptor-negative human fibroblasts to bind ^{125}I-LDL was increased from <2 to 45 ng/mg by 170 μM TC. In normal human fibroblasts and Hep G2 cells, ^{125}I-LDL binding was elevated 1.4- to 2-fold by 113 μM TC. The ^{125}I-LDL binding in the presence of TC was diminished by both heparin and EDTA. The enhancement by TC was not observed when ^{125}I-LDL binding was assayed at 4°C. TC enhanced LDL binding to paraformaldehyde-fixed Hep G2 cells, excluding LDL receptor induction in the mechanism. These results demonstrated that TC enhanced cellular LDL binding through a process not involving functional LDL receptors.
Lipids 33, 33–38 (1998).

Extensive epidemiologic studies have shown that increased levels of plasma cholesterol are associated with an increased risk of cardiovascular diseases, especially coronary artery diseases (1,2). Most of the plasma cholesterol exists as lipoproteins, of which low density lipoprotein (LDL) predominates in humans. LDL supplies peripheral cells, as well as the liver, with cholesterol through its binding to specific receptors (LDL receptors) and subsequent internalization and lysosomal degradation (3,4). In experimental animals, approximately 70% of the total-body uptake of radiolabeled LDL takes place in the liver by the LDL receptor-dependent pathway (5,6). Pharmacologically, 3-hydroxy-3-methylglutaryl CoA reductase inhibitors raise the number of hepatic LDL receptors and lower plasma cholesterol levels (4,7–9).

The present study has been undertaken to find an agent that can enhance cellular LDL binding capacity. The searching from microbial culture extracts has led to the identification of some tetracycline (TC) antibiotics as active compounds. In this paper, we describe the effects of several TC antibiotics on the binding of LDL to cultured cells, including normal and LDL receptor-negative human skin fibroblasts.

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Abbreviations: HDL, high density lipoprotein; KOK, receptor-negative form of familial hypercholesterolemia; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; MN, internalization-defective form of familial hypercholesterolemia; TC, tetracycline.

MATERIALS AND METHODS

Materials. TC antibiotics except anhydrotetracycline and isotetracycline, which were chemically synthesized from TC, were obtained from Sigma (St. Louis, MO). Dimethylsulfoxide or methanol solutions of these agents were used for assays with solvent concentration of <1% (vol/vol). Human skin fibroblasts from a normal subject and from two patients with homozygous familial hypercholesterolemia (strains MN and KIK) were generous gifts from Drs. Akira Yamamoto and Yasuko Miyake, the National Cardiovascular Center, Japan. The strain MN is defective in the internalization of the surface-bound LDL (internalization-defective) (10–12), and the strain KIK produces neither functional nor immuno-detectable LDL receptors (LDL receptor-negative) (12). Hep G2 cells were obtained from the Riken Cell Bank, Tsukuba Science City, Japan. Macrophage J774 cells were provided by the Japanese Cancer Resources Bank (Tokyo, Japan). Human LDL and bovine lipoprotein-deficient serum (LPDS) were prepared by ultracentrifugation as described by Goldstein *et al.* (13). LDL was radioiodinated by the iodine monochloride method (13) to a specific activity of 200–500 cpm/ng protein. Oxidized LDL was prepared by incubating LDL with 5 μM $CuSO_4$ as described previously (14). The concentration of the lipoproteins is expressed in terms of protein.

Cell culture. Human fibroblasts were maintained in a humidified 5% CO_2 incubator at 37°C in dishes (90 mm) containing 6 mL medium A [Eagle's minimum essential medium containing 100 units/mL penicillin G, 100 μg/mL streptomycin, 1% (vol/vol) nonessential amino acids (Irvine Scientific, Santa Ana, CA)] supplemented with 10% (vol/vol) fetal bovine serum. Both Hep G2 cells and J774 macrophages were grown in medium B (Dulbecco's modified Eagle's medium containing 100 units/mL penicillin G and 100 μg/mL streptomycin) supplemented with 10% fetal bovine serum. For assays, human fibroblasts were seeded into six-well tissue culture plates at a density of 2×10^4 cells/well in 1.5 mL of antibiotic-free medium A (day 0). On days 3 and 5, medium was replaced with 1.5 mL of fresh medium A containing 10% fetal

bovine serum. On day 7, each monolayer was used for assay. Hep G2 cells were seeded into 24-well plates at a density of 3 $\times 10^5$ cells/well in 0.3 mL of antibiotic-free medium B supplemented with 10% fetal bovine serum. After growth for 24 h, the cells were used for assays. The macrophage J774 cells were seeded into 24-well plates at a density of 1.5×10^5 cells/well in 0.4 mL of medium B. The next day, medium was replaced with 0.3 mL of medium B containing 10% LPDS. After incubation for an additional 24 h, the cells were used for assays.

Surface binding, internalization, and degradation of ^{125}I-LDL at 37°C. Each monolayer of fibroblasts was rinsed with buffer A (137 mM NaCl, 2.7 mM KCl, and 7.9 mM sodium phosphate, pH 7.4), and then received 1 mL of medium A containing 10% LPDS and 10 μg/mL ^{125}I-LDL. After incubation at 37°C for 3 h, a portion of the medium was removed to determine trichloroacetic acid-soluble noniodide radioactivity (degraded ^{125}I-LDL). The remaining monolayer was sequentially washed as described by Goldstein *et al.* (13). Surface binding of ^{125}I-LDL was determined by incubating washed cells with 1 mL of 10 mg/mL heparin in 10 mM HEPES, pH 7.4, and 50 mM NaCl at 4°C for 60 min, followed by removing an aliquot of the buffer for counting. The remaining cells were dissolved in 1 mL of 0.2 M NaOH to determine internalized ^{125}I-LDL. In some experiments, washed cells were directly dissolved in 1 mL of 0.2 M NaOH for determination of cell-associated ^{125}I-LDL. To determine nonspecific binding, internalization, cell association and degradation, cells were incubated with ^{125}I-LDL in the presence of 400 μg/mL of unlabeled LDL. The specific values were calculated by subtracting the radioactivity obtained in the presence of excess unlabeled LDL from that obtained in its absence. Nonspecific values were usually <10% of total values. For determination using monolayers of Hep G2 cells which had been grown in 24-well plates, cells were incubated with 0.2 mL of medium B containing 10% LPDS and 10 μg/mL ^{125}I-LDL, and the volume of buffers used was reduced to 1/5.

Binding of ^{125}I-LDL to paraformaldehyde-fixed Hep G2 cells at 37°C. Each monolayer of Hep G2 cells grown in 24-well dishes was treated with 0.2 mL of 1.6% (wt/vol) paraformaldehyde in buffer A at 4°C for 45 min. This treatment renders cells unable to internalize surface-bound LDL at 37°C (15). After washing four times with 0.3 mL of 50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 2 mg/mL bovine serum albumin, the fixed cells received 0.2 mL of medium B containing 10% LPDS and 10 μg/mL of ^{125}I-LDL. Cells were incubated at 37°C for 2 h, washed as described above, and then dissolved in 0.2 mL of 0.2 M NaOH. Nonspecific binding was determined in the presence of an excess of unlabeled LDL as described above.

Other methods. The incorporation of [^{14}C]oleate into cholesteryl esters and the binding of ^{125}I-LDL at 4°C were determined as described (13). Cell association and degradation of oxidized ^{125}I-LDL in macrophage J774 cells were determined as described previously (14). Protein was determined by the method of Lowry *et al.* (16) with bovine serum albumin as a standard. Antibacterial activity was determined turbidimetrically using *Staphylococcus aureus* IFO 12732 (17).

RESULTS

When normal human fibroblasts that had been grown in a medium containing fetal calf serum were incubated with ^{125}I-LDL at 37°C for 3 h, the cells specifically bound, internalized, and degraded 15, 130, and 164 ng of ^{125}I-LDL per mg cell protein, respectively. TC enhanced this activity 1.6- to 2.5-fold at a concentration of 113 μM (Fig. 1). Under these conditions, cholesteryl ester formation, as determined by

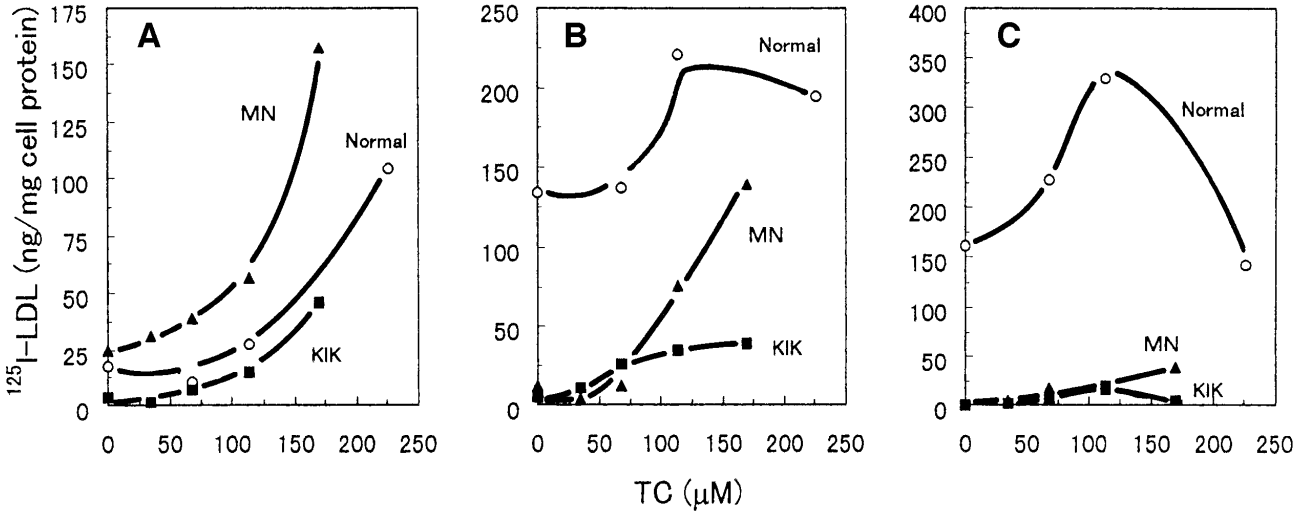


FIG. 1. Effects of tetracycline (TC) on the surface binding (A), intracellular accumulation (B), and degradation (C) of ^{125}I-low density lipoprotein (LDL) in three strains of human fibroblasts. Each monolayer of fibroblasts received ^{125}I-LDL (10 μg/mL) and the indicated concentrations of TC. After incubation at 37°C for 3 h, the amounts of ^{125}I-LDL specifically bound (A), internalized (B), and degraded (C) were determined. Fibroblasts used were from: ○, a normal subject; ▲, a patient with the internalization-defective form of familial hypercholesterolemia (MN); ■, a patient with the receptor-negative form of familial hypercholesterolemia (KIK). Each value represents the average of duplicate determinations.

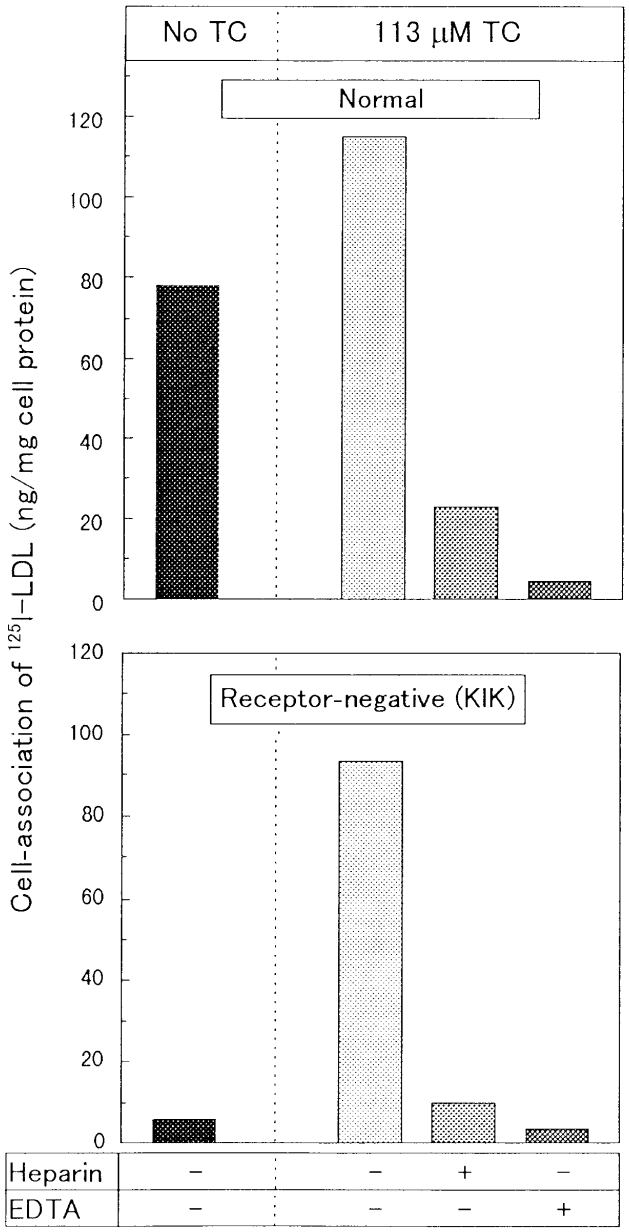


FIG. 2. Effects of heparin and EDTA on the cell association of ^{125}I-LDL in the presence of TC in normal and LDL receptor-negative fibroblasts. Each monolayer of fibroblasts received ^{125}I-LDL (10 μg/mL) and indicated concentrations of TC. After incubation at 37°C for 3 h in the absence or presence of heparin (10 mg/mL) or EDTA (5 mM), the amounts of ^{125}I-LDL specifically associated to the cells were determined. Each value represents the average of duplicate determinations. See Figure 1 for abbreviations.

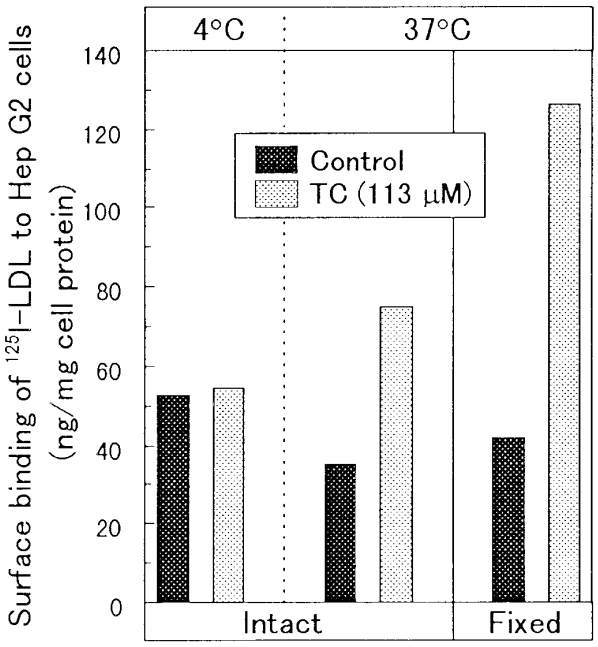


FIG 3. Effects of TC on the binding of ^{125}I-LDL to intact and paraformaldehyde-fixed Hep G2 cells at 4 and 37°C. Each monolayer of Hep G2 cells, either intact or fixed with paraformaldehyde, received ^{125}I-LDL (10 μg/mL) and the indicated concentrations of TC. After incubation at either 4 or 37°C for 2 h, the amounts of ^{125}I-LDL specifically bound were determined. Each value represents the average of duplicate determinations. See Figure 1 for abbreviations.

[^{14}C]oleate incorporation, was elevated 1.4-fold, from 0.261 to 0.369 nmol/3 h/mg cell protein. Similar elevation of ^{125}I-LDL binding was observed with Hep G2 cells (data not shown). TC also enhanced ^{125}I-LDL binding to two strains of mutant fibroblasts: one (MN) with a defect in the internalization of receptor-bound LDL and the other (KIK) producing neither functional nor immunodetectable LDL receptors (Fig. 1). ^{125}I-LDL binding to KIK fibroblasts was increased from <2 to 45 ng/mg cell protein by TC at a concentration of 170 μM. In both MN and KIK cells, the level internalization of ^{125}I-LDL was as low as the limit of detection, while in the presence of 113 μM TC the amounts of the internalized ^{125}I-LDL were increased to 75 and 37 ng/mg cell protein, respectively. The degradation of ^{125}I-LDL was enhanced by TC in MN fibroblasts but was not prominent in KIK cells (Fig. 1).

EDTA and heparin inhibited cell association (surface binding plus internalization) of ^{125}I-LDL in both normal and KIK fibroblasts incubated with TC (Fig. 2). The binding of ^{125}I-LDL to Hep G2 cells was unaffected by TC at 4°C. This is a striking contrast to the incubation at 37°C (Fig. 3). When Hep G2 cells were treated with paraformaldehyde and then subjected to ^{125}I-LDL binding at 37°C, TC enhanced the binding of ^{125}I-LDL to the fixed cells as well as to intact cells (Fig. 3), suggesting that the TC effect does not involve LDL receptor induction. In addition, the effect of TC to enhance LDL binding was reversible. Thus, when paraformaldehyde-treated Hep G2 cells were pretreated with 113 μM TC for 2 h at 37°C and then washed with buffer to remove TC, ^{125}I-LDL binding was 15.8 ng/mg cell protein, a value comparable to that of the fixed cells undergoing no pretreatment (15.0 ng/mg cell protein). When ^{125}I-LDL (50 μg/mL) was pretreated with 113 μM TC at 37°C for 2 h followed by being subjected to gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) to remove TC, the binding of the pretreated ^{125}I-LDL to Hep G2 cells was 18.5 ng/mg cell protein, which

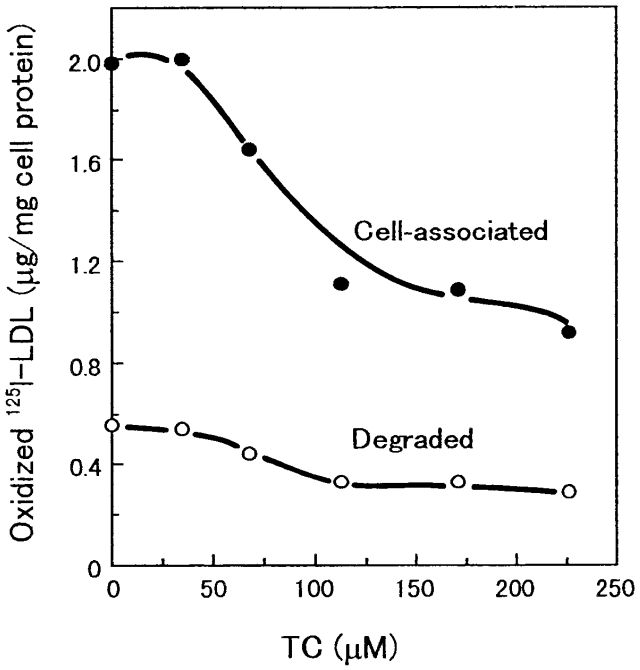


FIG. 4. Effects of TC on the cell association and degradation of oxidized ^{125}I-LDL in J774 macrophages. Each monolayer of J774 macrophages received oxidized ^{125}I-LDL (10 μg/mL) and the indicated concentrations of TC. After incubation at 37°C for 3 h, the amounts of oxidized ^{125}I-LDL specifically associated to the cells (●) and degraded (○) were determined. Each value represents the average of duplicate determinations. See Figure 1 for abbreviations.

was comparable to the value for the binding of untreated ^{125}I-LDL (17.0 ng/mg cell protein).

Unlike native LDL, oxidized LDL binds predominantly to scavenger receptors which recognize a wide variety of negatively charged molecules (18). When the macrophage-like J774 cell line, which expresses scavenger receptors, was incubated with 113 μM TC, the amounts of cell-associated and degraded oxidized ^{125}I-LDL were reduced by 40% (Fig. 4), excluding the possibility that TC enhances the scavenger receptor pathway.

Several TC analogs were tested for their ability to enhance ^{125}I-LDL binding to paraformaldehyde-fixed Hep G2 cells. The results indicated that compounds with R_4-hydrogen, TC, minocycline, chlortetracycline and anhydrotetracycline, were effective in enhancing the binding (Fig. 5A). On the other hand, R_4-hydroxy compounds, doxycycline, methacycline and oxytetracycline, failed to increase the binding. Isotetracycline, which has R_4-hydrogen but lacks the complete ring structure, was less active than TC. Anhydrotetracycline, which is 17 times less active than TC in antibacterial activity, was slightly more potent than TC in enhancing LDL binding (Fig. 5B).

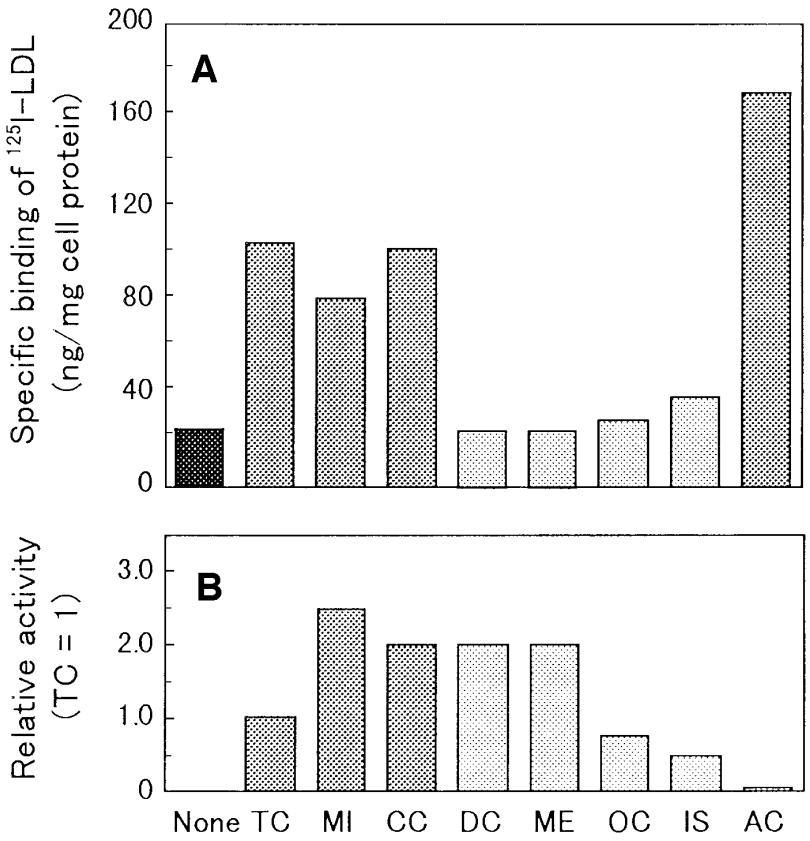
DISCUSSION

In the present study, we have shown that some TC antibodies enhance the binding of LDL to both LDL receptor-positive and -negative cells. This effect was observed even with the paraformaldehyde-treated cells, but it required incubation of the cells in the presence of TC and LDL at the same time at 37°C but not at 4°C. Neither preincubating cells with TC nor preincubating LDL with TC enhanced the binding. The activity of TC analogs was dependent on the substituent of C-5 (see below). These results suggest that the LDL binding activation by TC is mediated by a specific, temperature-dependent process. One of possible mechanisms would be that a reversible modification of LDL (or a cell surface molecule) by TC leads the LDL (or the molecule) to bind cells (or LDL). These features are quite different from the effects of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, which induce LDL receptor expression at a transcriptional level (4,9).

The present experiments suggested that the TC enhancement of LDL binding involved a receptor other than the LDL receptor. There are two classes of multiligand lipoprotein receptors, the scavenger receptors and LDL receptor-related protein (19). These receptors bind nonlipoprotein ligands as well as lipoproteins and appear to participate in a wide variety of biological processes. Recently, Acton *et al.* (20,21) have identified the class B scavenger receptor (SR-BI), which can bind unmodified, native LDL and high density lipoprotein (HDL) as well as oxidized LDL and acetylated LDL. Like TC-induced LDL binding, the HDL binding to SR-BI does not accompany degradation of the protein moiety, whereas cholesteryl esters of the bound HDL is efficiently transferred to the cells (21). In addition, some proteoglycans bind lipoproteins including apoE-enriched lipoproteins, which are then internalized by the LDL receptor-related protein (19). While there would be the possibility that one (or some) of these molecules is involved in the TC-promoted LDL binding, additional studies are required to explore the detailed mechanism.

The antibacterial activity of TC antibiotics relates to oxygen-bearing groups: ketone or hydroxy at C-1, C-3, C-10, C-11, and C-12 is essential for antibacterial activity (22). The carbamoyl group at C-2 and the configuration at C-4, C-5a, and C-6 positions are also required for the activity. The present experiments demonstrated that the C-5 substituents played a crucial role in enhancing LDL binding. Thus, the analogs having hydrogen at C-5 were active, while the hydroxy-substituted compounds having substantial antibacterial activity were inactive. These findings demonstrated that the elevation of LDL binding by TC analogs was not related to their antibacterial activity. This conclusion was also supported by the fact that anhydrotetracycline, which was far less active than TC in antibacterial activity, showed LDL binding-promoting activity comparable to that of TC.

In preliminary experiments with normal dogs, oral administration of TC and anhydrotetracycline (3–10 mg/kg, twice daily) for 2–4 wk caused 10–25% reduction in plasma cholesterol levels. It is likely that cholesterol-lowering effects of these agents could be explained by the elevation of LDL binding. The relation between these two events is to be studied further in detail.



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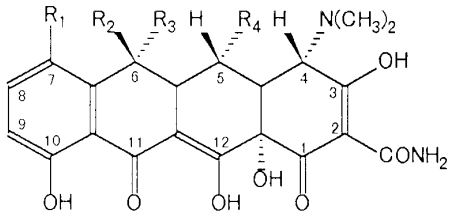
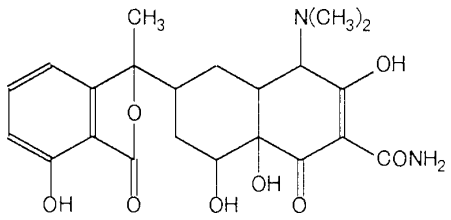
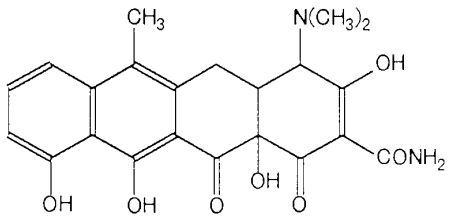


Compound	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	OH	CH_3	H
Minocycline (MI)	$N(CH_3)_2$	H	H	H
Chlortetracycline (CC)	Cl	OH	CH_3	H
Doxycycline (DC)	H	H	CH_3	OH
Methacycline (ME)	H	= CH_2		OH
Oxytetracycline (OC)	H	OH	CH_3	OH



Isotetracycline (IS)



Anhydrotetracycline (AC)

FIG. 5. Antibacterial activity of TC analogs and their effects on the binding of ^{125}I-LDL to paraformaldehyde-fixed Hep G2 cells. (A) Each monolayer of paraformaldehyde-treated Hep G2 cells received ^{125}I-LDL (10 μg/mL) and the indicated compounds (110 μM). After incubation at 37°C for 2 h, the amounts of ^{125}I-LDL specifically bound were determined. Each value represents the average of duplicate determinations. (B) The minimum concentrations of the indicated compounds to inhibit the growth of *Staphylococcus aureus* were determined in duplicate in brain heart infusion medium supplemented with 10% (vol/vol) fetal calf serum at 37°C. The activities of the compounds were expressed by taking the activity of TC as 1. Structures of the compounds tested and abbreviations used for them are shown in (C). See Figure 1 for other abbreviations.

ACKNOWLEDGMENTS

The authors thank H. Yahagi for technical assistance and M. Furuta for blood drawing.

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[Received May 5, 1997, and in final revised form November 19, 1997; revision accepted November 25, 1997]

Stereochemistry-Dependent Inhibition of RAS Farnesylation by Farnesyl Phosphonic Acids

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ABSTRACT: This investigation compares the effects of three farnesyl pyrophosphate analogs on selected aspects of isoprenoid metabolism. *E,E*-α-Hydroxyfarnesylphosphonate was prepared by an improved variation on a literature synthesis, which also gave access to the new *Z,E*-α-hydroxyfarnesyl- and α-hydroxygeranylphosphonates. A striking find is that only *E,E*-α-hydroxyfarnesylphosphonate induces alteration of RAS processing in intact human-derived leukemia cells and inhibits farnesyl protein transferase in enzyme assays, while the *Z,E*-α-farnesyl- and geranylphosphonates are inactive. The inhibitory activity of *E,E*-α-hydroxyfarnesylphosphonate is greater in enzyme than intact cell assays. This active compound does not significantly inhibit geranylgeranyl protein transferase I or squalene synthase, nor does it diminish cholesterol synthesis. These results indicate that the length of the terpenoid chain and olefin stereochemistry allow selective inhibition of critical enzymes of terpenoid metabolism. Discrimination was observed between inhibition of farnesyl protein transferase and squalene synthase by *E,E*-α-hydroxyfarnesylphosphonate, even though both enzymes utilize farnesyl pyrophosphate as their natural substrate.

Lipids 33, 39–46 (1998).

Mutated *RAS* genes that encode for activated and therefore transforming RAS proteins are present in a significant percentage of human tumors (1). Farnesyl protein transferase (FPTase) catalyzes the posttranslational farnesylation of both wild-type and mutated RAS as well as RAS-related proteins (2–4). RAS isoprenylation is important because this modification promotes RAS localization to the cell membrane, which is essential for RAS's biological activity (5–7). Cytoplasmic RAS proteins are devoid of signal transduction activity: transformed cells in which activated RAS is subsequently localized to the cytoplasm revert to the nontransformed phenotype. Thus impairment of RAS farnesylation is one strategy to block the transforming activity of mutated *RAS* genes and activated RAS proteins.

FTPase inhibitors are being developed by a number of laboratories (8–14). Development of these agents has been facilitated by the understanding that RAS and RAS-related proteins contain a consensus carboxy terminus that binds to the active site of FPTase (15,16). Tetrapeptides homologous to the carboxy terminus of the RAS proteins are extremely potent competitive inhibitors of FPTase and of RAS farnesylation in *in vitro* biochemical assays (8–13,17). However, without modification such oligopeptides are ineffective when added to intact cells because they are taken up inefficiently and/or because they are rapidly degraded (8–13). Development of FPTase inhibitors also has focused on natural products, such as chaetomellic acid and zaragozic acids, which have been shown to inhibit FPTase and RAS processing *in vitro* but not in intact cells (9).

An alternative approach to inhibit FPTase utilizes nonhydrolyzable analogs of farnesyl pyrophosphate that cannot participate in the transferase reaction. This strategy has resulted in the synthesis of α-hydroxyfarnesylphosphonic acid, a compound that inhibits FPTase and has been utilized to determine the *in vitro* kinetics of the farnesylation process (18). Expanding upon these such studies (9,18), we have synthesized two stereoisomeric α-hydroxyfarnesylphosphonic acids and the corresponding methyl esters and have examined the stereochemical requirements for these compounds to inhibit RAS processing in intact cells. Specifically, we have assessed the role of two isomeric farnesyl phosphonates and a geranyl phosphonate on RAS processing in human-derived leukemia cell lines. We observed discrimination between inhibition of FPTase and squalene synthase, which both utilize farnesyl pyrophosphate as their natural substrate. We have shown that the most active compound can selectively impair RAS farnesylation without greatly interfering with other aspects of isoprene metabolism, such as geranylgeranyl protein transferase I (GGPTase I) or cholesterol synthesis.

EXPERIMENTAL PROCEDURES

Chemical syntheses. Tetrahydrofuran (THF) was distilled from sodium/benzophenone while dichloromethane was distilled from calcium hydride, immediately prior to use. All reactions in these solvents were conducted under a positive pressure of an inert gas. Flash column chromatography was done on Merck (Rahway, NJ) grade 62 silica gel (230–400 mesh). Nuclear magnetic resonance (NMR) spectra (^{1}H at 300 MHz and ^{13}C at 75 MHz) were recorded with $CDCl_3$ as

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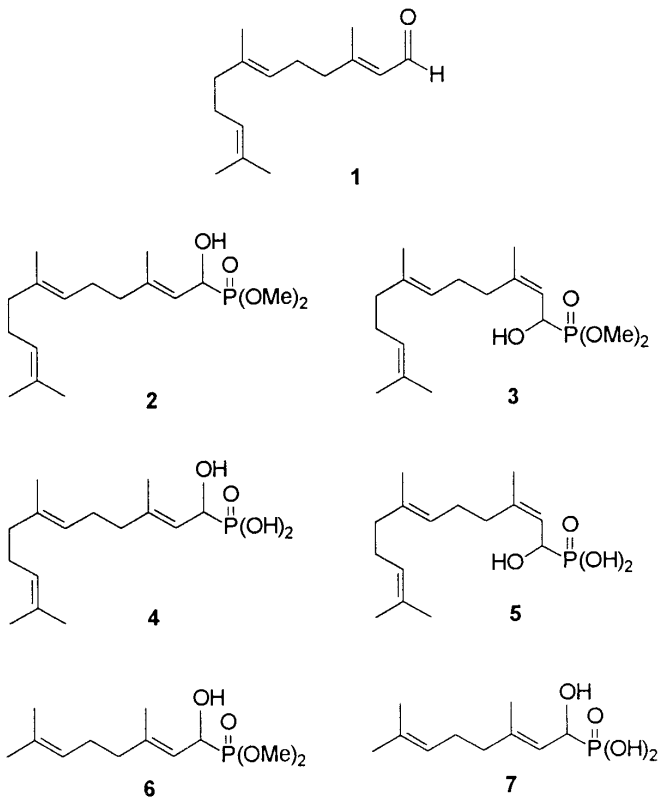
Abbreviations: FPP, farnesyl pyrophosphate; FPTase, farnesyl protein transferase; GGPTase I, geranylgeranyl protein transferase I; IC_{50}, 50% inhibitory concentration; NMR, nuclear magnetic resonance; THF, tetrahydrofuran.

solvent and $(CH_3)_4Si$ (^1H) or $CDCl_3$(^{13}C, 77.0 ppm) as internal standard. ^{31}P NMR shifts are reported in ppm relative to 85% H_3PO_4 (external standard). High-resolution and fast atom bombardment (FAB) mass spectra were obtained on a ZAB-HF reversed geometry mass spectrometer at the University of Iowa Mass Spectrometry facility. Elemental analyses were performed by Supersun Technology (Stony Brook, NY).

Chemical syntheses of the isoprenoid phosphonates. Scheme 1 shows the structures of the (α-hydroxyfarnesyl) phosphonate esters (**2**,**3**) and phosphonic acids (**4**,**5**), along with the geranyl analogs (**6**,**7**).



SCHEME 1

(E,E)-*3,7,11-Trimethyl-2,6,10-dodecatrienal (**1**).* Farnesol (2.18 g, 9.80 mmol) was added dropwise over 10 min to a stirred solution of pyridinium dichromate (19) (5.36 g, 14.3 mmol) and molecular sieve powder (20) (5.36 g) in dichloromethane (38 mL) under nitrogen at room temperature. After the resulting brown solution was stirred at room temperature for 2 h, the reaction mixture was diluted with ether (20 mL) and filtered through a pad of Celite. The orange filtrate, combined with repeated ether washings of the pad, was concentrated *in vacuo* to a volume of 100 mL. The solution was passed through silica gel under vacuum filtration, the silica was rinsed repeatedly with ether, and the filtrate was concentrated *in vacuo* to give compound **1** (1.86 g, 86%) as a yellow oil. This oil was a 10:1 mixture of the 2*E*,6*E* and 2*Z*,6*E* isomers, respectively. For the major isomer: ^1H NMR δ 10.00 (*d*, 1H, *J* = 8.0 Hz), 5.89 (*dd*, 1H, *J* = 8.0, 1.2 Hz), 5.05–5.15 (*m*, 2H), 2.20–2.28 (*m*, 4H), 2.17 (*d*, 3H, *J* = 1.2 Hz), 1.95–2.10 (*m*, 4H), 1.68 (*s*, 3H), 1.61 (*s*, 3H), 1.60 (*s*, 3H). Most resonances for the minor isomer overlap with those of the major isomer, but the ratio could be established by integration of the aldehyde resonances at δ 10.00 and δ 9.92 (*d*, 1H, *J* = 8.2 Hz).

(R,S)-*Dimethyl [1-hydroxy-3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienyl] phosphonate (**2**) and (*R,S*)-dimethyl [1-hydroxy-3,7,11-trimethyl-2*Z*,6*E*,10-dodecatrienyl]phosphonate (**3**).* To a stirred solution of dimethyl phosphite (2.4 mL, 26 mmol) in THF (25 mL) was added lithium hexamethyldisilazane (26 mL, 1 M in THF) at −78°C. After the resulting mixture was stirred for 1 h, compound 1 (5.29 g, 24 mmol) in THF (105 mL) was added *via* cannula, and the mixture was stirred at −78°C for 2 h. The reaction was quenched by addition of 1 M acetic acid in ether (150 mL) and filtered through a pad of Celite. The filtrate, combined with repeated ether washings of the pad, was concentrated *in vacuo* to give an orange oil. Purification by flash column chromatography on silica gel (9:1, ethyl acetate/hexanes) provided a mixture of the isomeric phosphonates (6.38 g, 80%), and repeated chromatography gave pure *E,E*-phosphonate **2** and *Z,E*-phosphonate **3**, each as a yellow oil.

E,E-phosphonate **2**: (18) ^1H NMR ($CDCl_3$) δ 5.34 (*m*, 1H), 5.05–5.15 (*m*, 2H), 4.69 (*ddd*, 1H, *J* = 8.7, 8.7, 5.5 Hz), 3.82 (*d*, 3H, *J* = 10.4 Hz), 3.80 (*d*, 3H, *J* = 10.4 Hz), 2.88 (*dd*, 1H, *J* = 7.4, 5.5 Hz), 1.90–2.20 (*m*, 8H), 1.73 (*dd*, 3H, *J* = 3.1, 1.3 Hz), 1.68 (*s*, 3H), 1.60 (*s*, 6H); ^{13}C NMR δ 143.5 (*d*, J_{CP} = 13.8 Hz), 135.6, 131.4, 124.3, 123.5, 119.0 (*d*, J_{CP} = 3.1 Hz), 65.7 (*d*, J_{CP} = 164.2 Hz), 53.6 (*d*, J_{CP} = 5.8 Hz), 53.5 (*d*, J_{CP} = 5.5 Hz), 39.7, 39.7, 26.7, 26.2, 25.7, 17.7, 17.1, 16.0; ^{31}P NMR 26.35.

Z,E-phosphonate **3**: ^1H NMR ($CDCl_3$) δ 5.37 (*m*, 1H), 5.05–5.15 (*m*, 2H), 4.68 (*ddd*, 1H, *J* = 9.1, 8.7, 5.2 Hz), 3.82 (*d*, 3H, *J* = 10.4 Hz), 3.80 (*d*, 3H, *J* = 10.4 Hz), 2.65 (*dd*, 1H, *J* = 6.9, 5.3 Hz), 1.95–2.20 (*m*, 8H), 1.81 (*dd*, 3H, *J* = 3.8, 1.3 Hz), 1.68 (*s*, 3H), 1.60 (*s*, 6H); ^{13}C NMR δ 144.2 (*d*, J_{CP} = 14.4 Hz), 136.1, 131.5, 124.2, 123.4, 119.5 (*d*, J_{CP} = 2.8 Hz), 65.2 (*d*, J_{CP} = 165.7 Hz), 53.6, 53.5, 39.7, 32.6, 26.7, 26.4, 25.7, 23.5, 17.7, 16.0; ^{31}P NMR 26.43; high-resolution mass spectrum calculated for $C_{17}H_{31}O_4PNa$ 353.1858 $(M + Na)^+$, found 353.1845.

(R,S)-*[1-Hydroxy-3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienyl]phosphonic acid (**4**).* According to the published procedure (18), phosphonate **2** (110 mg, 0.33 mmol) was treated with trimethylsilyl bromide (0.195 mL, 1.48 mmol) and 2,4,6-collidine (0.195 mL, 1.48 mmol) in dichloromethane (5 mL) to obtain the corresponding phosphonic acid **4** (54 mg, 54%): ^1H NMR [dimethyl sulfoxide (DMSO)] δ 5.00–5.25 (*m*, 3H), 4.23 (*t*, 1H, *J* = 9.5 Hz), 1.80–2.20 (*m*, 8H), 1.62 (*d*, 3H, *J* = 0.4 Hz), 1.60 (*d*, 3H, *J* = 0.6 Hz), 1.56 (*s*, 3H), 1.55 (*s*, 3H); ^{31}P NMR 25.10.

(R,S)-*[1-Hydroxy-3,7,11-trimethyl-2*Z*,6*E*,10-dodecatrienyl]phosphonic acid (**5**).* Through extension of the procedure described for the *E,E* isomer (18), phosphonate **3** (67 mg, 0.20 mmol) was treated with trimethylsilyl bromide (0.107 mL, 0.81 mmol) and 2,4,6-collidine (0.107 mL, 0.81

mmol) in dichloromethane (3 mL) to give phosphonic acid **5** (45 mg, 74%) as an orange oil: ^{1}H NMR ($CDCl_3$) δ 7.20 (*br*, 3H), 5.27 (*m*, 1H), 5.00–5.15 (*m*, 2H), 4.59 (*m*, 1H), 1.80–2.20 (*m*, 8H), 1.73 (*s*, 3H), 1.64 (*s*, 3H), 1.56 (*s*, 6H); ^{31}P NMR 23.51; high-resolution mass spectrum calculated for $C_{15}H_{26}O_4P$ 301.1569 $(M - H)^-$, found 301.1565.

(R,S)-*Dimethyl [1-hydroxy-3,7-dimethyl-2*E*,6-octadienyl]phosphonate (**6**).* In a manner similar to that described for preparation of phosphonate **2**, lithium hexamethyldisilazane (3.70 mL, 1 M in THF) was added to a solution of dimethyl phosphite (0.340 ml, 3.70 mmol) in THF (3.5 mL) at −78°C. After the resulting solution was stirred for 1 h, geranial (500 mg, 3.28 mmol) in THF (15 mL) was added *via* cannula and the mixture was stirred at −78°C for 2.75 h. Standard workup, as described above, and purification by flash column chromatography on silica gel (97:3 ethyl acetate/methanol) provided phosphonate **6** (751 mg, 87%) as a white crystalline solid: ^{1}H NMR ($CDCl_3$) δ 5.33 (*m*, 1H), 5.05–5.13 (*m*, 1H), 4.69 (*ddd*, 1H, *J* = 9.1, 4.6, 4.4 Hz), 3.83 (*d*, 3H, *J* = 6.7 Hz), 3.79 (*d*, 3H, *J* = 6.7 Hz), 2.74 (*dd*, 1H, *J* = 6.1, 0.4 Hz), 2.05–2.18 (*m*, 4H), 1.73 (*dd*, 3H, *J* = 3.2, 1.3 Hz), 1.68 (*s*, 3H), 1.61 (*s*, 3H); ^{31}P NMR 26.50; high-resolution mass spectrum calculated for $C_{12}H_{24}O_4P$ 263.1421 $(M + H)^+$, found 263.1420.

(R,S)-*[1-Hydroxy-3,7-dimethyl-2*E*,6-octadienyl]phosphonic acid (**7**).* Phosphonate **6** (71 mg, 0.27 mmol) was treated with trimethylsilyl bromide (0.160 mL, 1.21 mmol) and 2,4,6-collidine (0.160 mL, 1.21 mmol) in dichloromethane (3.2 mL) at 0°C for 30 min and then the reaction was allowed to warm to room temperature. After 17 h, toluene (5 mL) was added and all volatiles were removed *in vacuo* to give a white oily solid. Water (10 mL) and 1 M HCl was added until pH 2 was obtained. The mixture was extracted with ethyl acetate (3 × 20 mL), the combined organic extracts were dried over $MgSO_4$, and then concentrated in vacuo to afford compound **7** (57 mg, 88%) as a white solid: ^{1}H NMR (dimethylsulfoxide) δ 9.0–11.5 (*br*, 1H), 5.05–5.23 (*m*, 2H), 4.24 (*dd*, *J* = 10.1 Hz, 1H), 3.0–3.8 (*br*, 2H), 1.91–2.10 (*m*, 4H), 1.64 (*s*, 3H), 1.60 (*d*, *J* = 2.1 Hz, 3H), 1.57 (*s*, 3H); ^{31}P NMR 21.54. Analysis calculated for $C_{10}H_{19}O_4P$: C, 51.26; H, 8.18. Found: C, 51.13; H, 8.30.

Cell culture. RPMI-8402 cells were grown in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum. Cells were maintained in TI-75 culture flasks at 37°C in humidified 5% CO_2 atmosphere. Trypan blue exclusion was measured by incubating cells for 5 min with a 1:1 0.4 mg/dL trypan blue solution and counting clear viable and blue nonviable cells with a hemacytometer. All studies with the phosphonic acids and esters contained a final dimethylsulfoxide concentration of 0.4% which does not alter the parameters measured in these studies (data not shown).

Measurement of total RAS. Cells (1×10^7 cells at 1×10^6 cells/mL) in logarithmic phase growth were incubated in RPMI-1640 media with 10% fetal calf serum supplemented with the test compounds for 24 to 72 h. Cells were pelleted by centrifugation at 800 × *g* for 10 min and washed twice with cold phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Cells were resuspended in 0.1 mL lysing buffer (20 mM Tris pH = 7.5, 0.1 M NaCl, 1.0% Nonidet, 0.05 g/dL Na deoxycholate) and vortexed intermittently for 30 min. The cell pellets were dounced 10 times and centrifuged at 10,000 × g for 30 min. Protein content of the supernatant was assayed according to the method of Lowry *et al.* (21). Cell lysate containing 100 µg protein was subjected to electrophoretic fractionation on denaturing 15% polyacrylamide gels (SDS-PAGE). An H-ras standard was loaded to one lane on each gel. The gel slab was transblotted to Immobilon-Polyvinylidene fluoride (Millipore, Bedford, MA) prior to blocking for 2 h with blocking buffer [5 mg/dL skim milk, 1 mg/dL bovine serum albumin, 0.01% antifoam A in phosphate buffered saline]. The membrane was then sequentially incubated with NCC-004 anti-RAS antibody (22,23) in hybridoma supernatant for 2 h, biotinylated rabbit antimouse IgG for 2 h, and avidin-linked horseradish peroxidase (Vectastain ABC, Vector Labs, Burlingame, CA) for 30 min. The membrane was washed five times with 0.1% Tween 20 in phosphate buffered saline between each incubation. The RAS bands were visualized by incubation of the membrane with 0.7 mM 3,3-diaminobenzidine and 0.006% H_2O_2 in 10 mM Tris (pH = 7.5).

Measurement of newly synthesized RAS. Cells were washed and incubated at 3×10^6 cells/mL in methionine RPMI-1640 media for 1.5 h with or without test compounds. [^{35}S]Methionine/cysteine (1180 Ci/mM) was added to a final concentration of 0.045 mCi/mL. After incubation for 4 h, the cells were harvested and lysed as described above in the measurement of total RAS section with the exception that 1 g/dL bovine serum albumin was added to the lysing buffer. Radiolabeled cell lysate containing 200 µg of protein was precleared, immunoprecipitated with Y13-259 rat anti-RAS IgG, and electrophoretically fractionated as described previously (23). After fixation the gels were dried and exposed to Eastman Kodak (Rochester, NY) XAR5 (X-OMAT) film for 48 h. With development, the density of the bands corresponded to the amount of radiolabeled RAS proteins.

FPTase and GGPTase I assay. Partially purified FPTase and GGPTase were isolated from bovine brain white matter by homogenization at 4°C in lysis buffer (50 mM Tris-HCl (pH = 8.0), 1 mM EGTA, 1 mM $MgCl_2$, 5 mM dithiothreitol, 10 µg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 2 µg/mL leupeptin) (16). The homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was removed and centrifuged at 100,000 × *g* for 60 min at 4°C. The cytosolic protein fraction was removed and stored at −80°C (24).

Inhibition of FPTase and GGPTase was determined using the method of Harwood (25) with some modifications. Reaction mixtures, 15.3 µL containing 0.5 µM ^{3}H[FPP] or ^{3}H[GGPP] (12770 dpm/pmole), 5 mM $MgCl_2$, 20 mM Tris-HCl (pH = 7.5), 2 mM dithiothreitol, 8 µM $ZnCl_2$, 4 µM of H-ras/wild type (FPTase assay) or 8 µM of H-ras/CVLL (GGPTase I assay), without or with selected farnesyl phosphonates are set up in a 96-well plate. Reactions are started with

the addition of 9.7 μL of the bovine brain cytosol containing either 3.0 mg/mL (FPP) or 18.8 mg/mL (GGPP) of protein and incubated at 37°C for 60 min. Reactions are terminated with the addition of 200 μL of 10% HCl in EtOH. Samples are incubated for an additional 15 min at 37°C to ensure protein precipitation and enzyme hydrolysis. Samples are filtered through P-81 filters (Whatman, Maidstone, England) using a Brandel harvester (Biomedical Research and Development Laboratories Inc., Gaithersburg, MD). Filters are washed with EtOH and dried. Scintillation counting enabled determination of ^{3}H-FPP or ^{3}H-GGPP incorporation into RAS using 3A70 (Research Products International Corp., Mt. Prospect, IL) cocktail.

Cholesterol synthesis. Radiolabeled acetate incorporation into cholesterol was measured according to our previously described methods (26,27). In brief, RPMI-8402 cells were cultured in 20% lipoprotein-depleted serum. The cells were incubated for 18 h without or with the desired farnesyl phosphonate before the addition of ^{14}C-acetate. Four hours later the amount of radiolabeled cholesterol in the cultures was determined by thin-layer chromatography fractionation of cell lysates. The amounts of acetate metabolized to cholesterol were calculated for each cell culture and expressed as percentage observed in control cultures. Lovastatin, an inhibitor of hydroxymethylglutaryl CoA reductase and therefore cellular cholesterol synthesis, was used as a positive control.

Squalene synthase. Inhibition of squalene synthase activity was determined using the method of Ciosek *et al.* (28) with some modification. Microsomes were isolated from Sprague-Dawley rats (Harland, Indianapolis, IN) that had been fed a diet containing 10% cholestyramine for 1 wk. Microsomes were diluted to 2 mg/mL protein in 275 mM KH_2PO_4/K_2HPO_4 (pH = 7.4). Each reaction contained 0.16 mg microsomal protein in a final volume of 0.5 mL buffer (50 mM KH_2PO_4/K_2HPO_4 (pH = 7.4), 10 mM KF, 1.62 mM NADPH, and 5 mM $MgCl_2$) containing a final concentration of 22 μM farnesyl pyrophosphate (FPP) that includes 1.0 μCi of [1-^{3}H]FPP (15–30 Ci/mM) without and with selected farnesyl phosphonates. Reactions are started with the addition of [1-^{3}H]FPP and incubated at 30°C for 60 min under nitrogen. Reactions are terminated with the sequential addition of 0.5 mL 40% KOH and 0.5 mL ethanol containing internal standard ^{14}C-squalene (1000 dpm) with carrier squalene (15 μg) and lanosterol (25 μg). The samples were saponified by heating to a boil followed by gradual cooling. The mixtures were extracted twice with 2 mL hexane and the hexane evaporated to dryness. The residue was dissolved in 30 μL chloroform and subjected to thin-layer chromatographic separation on LK5D (Whatman) silica plates with hexane/chloroform/methanol (30:10:1). Lipids were visualized with iodine vapor. Squalene and lanosterol bands were scraped and counted in Budget Solve (Research Products International Corp.) cocktail. Dual label scintillation counting enabled determination of [1-^{3}H]squalene and lanosterol synthesis adjusted for extraction efficiency. (*E,E*)-(4,8,12-Trimethyl-3,7,11-tridecatrienylidene)bisphosphonic acid, a known inhibitor of mammalian squalene synthase (IC_{50} 32 nM), was employed as a positive control (28).

Materials. Farnesol (>99% pure) and geraniol (98% pure) were obtained from Aldrich (Milwaukee, WI). RPMI-1640 media and fetal calf serum were obtained from the Hybridoma Facility at the University of Iowa. Lovastatin was kindly provided by Alfred Alberts of Merck, Sharp & Dohme (Darmstadt, Germany) and was converted to the open acid prior to use. NCC-004 anti-RAS IgG was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo), H-ras standard was kindly provided by Dr. Robert Deschenes (University of Iowa), Y13-259 anti-RAS IgG was from Oncogene Science (Uniondale, NY), and rabbit antimouse IgG, goat antirabbit IgG, and Vectastain ABC kits were from Vector Labs.

RESULTS

Effects of the isoprenoid phosphonates on RAS processing in intact cells. To determine the effects of the isoprenoid phosphonates on RAS processing, RPMI-8402 cells were incubated with 0, 100, and 200 μM of the phosphonates (Fig. 1) for 24 h prior to harvesting and lysing. Control cultures contained 0 or 100 μM lovastatin without and with 10 mM mevalonic acid. Figure 1 is a Western blot of cell lysate demonstrating that in control cells without lovastatin there is a couplet of RAS bands that corresponds to farnesylated RAS proteins. This couplet is a consequence of RAS phosphorylation and has been described (9). After incubation with 100 or 50 μM lovastatin for 24 h prior to harvesting, there is accumulation of increasing amounts of unmodified RAS protein that migrates with an apparent higher molecular weight than does the farnesylated RAS proteins. The effects observed with lovastatin are reversed with the addition of 5 mM mevalonic acid. Incubation of cells with the *E,E*- (**4**) and *Z,E*-α-hydroxy-

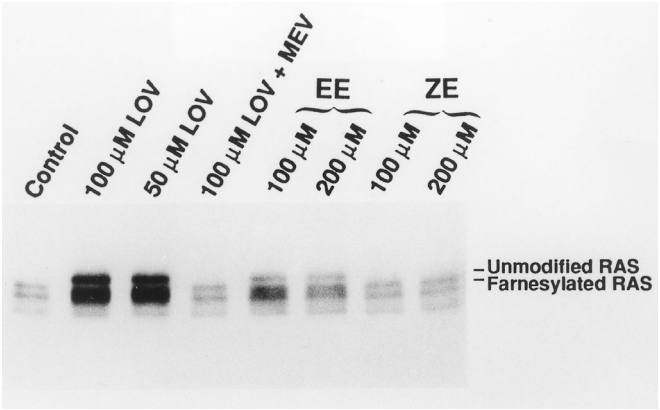


FIG. 1. Effects of *E,E*- and *Z,E*-α-hydroxyfarnesylphosphonates on RAS levels in RPMI-8402 cells. This Western blot was developed as described in the Experimental Procedures section. Above each lane is listed the chemical added to the incubation mixture. Cells were incubated without additive chemicals (Control), with lovastatin (LOV) without and with 10 mmolar mevalonic acid (MEV), and the *E,E*- and *Z,E*-isomers of α-hydroxyfarnesylphosphonate. Each lane contains 100 μg of protein from cell lysate.

farnesylphosphonate (**5**) isomers for 24 h reveals that the *E,E*-isomer **4**, but not the *Z,E*-isomer **5**, results in the accumulation of unmodified RAS protein. Mevalonic acid reverses the effect of *E,E*-α-hydroxyfarnesylphosphonate in a concentration-dependent fashion (data not shown).

Incubation of cells with up to 20 μM of either dimethyl *E,E*- or dimethyl *Z,E*-α-hydroxyfarnesylphosphonate (**2** and **3**) does not result in the accumulation of unmodified RAS protein (data not shown). Cells maintain viability, assessed as trypan blue exclusion, to above 90% when incubated with up to 200 μM of the *E,E*- (**4**) and *Z,E*-α-hydroxyfarnesylphosphonates (**5**). In contrast, above 20 μM concentrations, dimethyl *E,E*- or dimethyl *Z,E*-α-hydroxyfarnesylphosphonate (**2** and **3**) are nonspecifically toxic to these cells.

Neither α-hydroxygeranylphosphonate (**7**) nor its dimethyl ester (**6**) displays inhibitory activity for RAS farnesylation in intact RPMI-8402 cells (data not shown). Both compounds were tested at up to 200 μM concentrations because toxicity, assessed by trypan blue exclusion, does not occur.

Effects of isoprenoid phosphonates on newly synthesized RAS. Cells were incubated with 0.2 to 20 μM *E,E*-α-hydroxyfarnesylphosphonate (**4**) or 25 μM lovastatin prior to and during pulsing with radiolabeled methionine. Figure 2 demonstrates that radiolabeled RAS is detected as both unmodified and farnesylated forms after coincubation of cells with [^{35}S] methionine for 4 h without other added chemicals (control). Cells treated with 25 μM lovastatin contain only unmodified RAS protein because of the induced depletion of FPP that impairs RAS posttranslational processing. Cells treated with 0.2 μM α-hydroxyfarnesylphosphonate (**EE**) contain similar amounts of unmodified and farnesylated RAS as do untreated cells. In contrast, cells treated with either 2.0 or 20 μM α-hydroxyfarnesylphosphonate (**EE**) contain only unmodified RAS as do lovastatin-treated cells. This autoradiograph indicates that treatment of cells with between 0.2 and 2.0 μM α-hydroxyfarnesylphosphonate (**EE**) would decrease farnesylation of newly synthesized RAS by 50%.

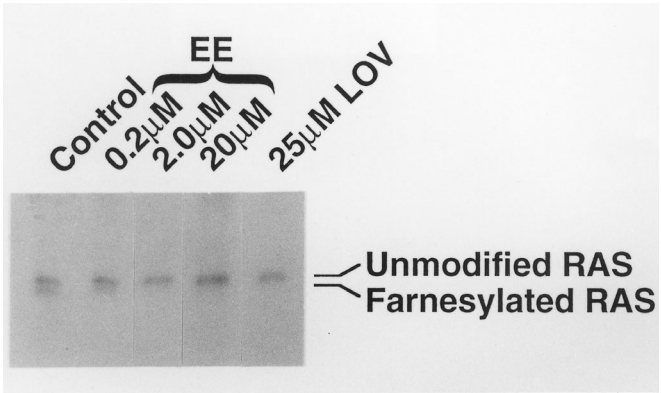


FIG. 2. Effects of *E,E*-α-hydroxyfarnesylphosphonate on newly synthesized RAS levels. RPMI-8402 cells were radiolabeled and 200 μg of protein from cell lysate immunoprecipitated as described in the Experimental Procedures section. For this autoradiogram, each lane is labeled as either Control or with the concentrations of *E,E*-α-hydroxyfarnesylphosphonate (EE) or lovastatin (LOV) used in the incubations.

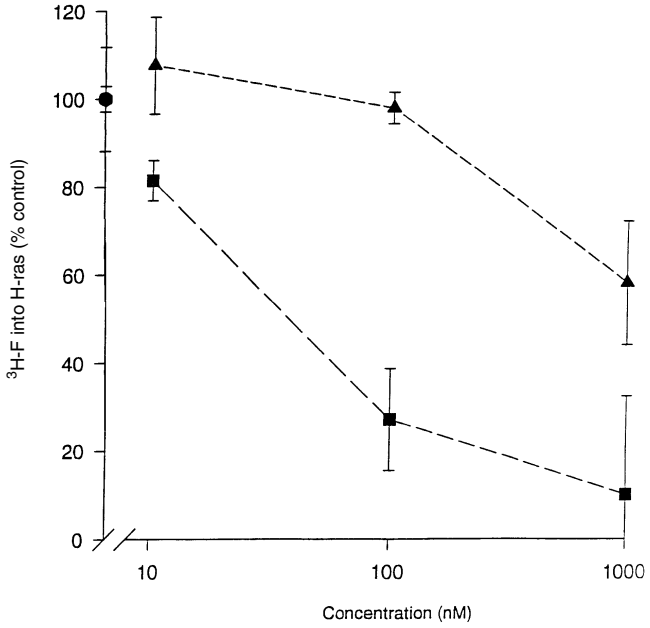


FIG. 3. Effects of (■), *E,E*- and (▲), *Z,E*-α-hydroxyfarnesylphosphonates on FPTase activity. This assay was performed as described in the Experimental Procedures section. The points are the mean of three experiments and the error bars indicate ±1 standard deviation.

Effects of the isoprenoid phosphonates on FTPase. Figure 3 displays the effects of increasing concentrations of compounds **4** (■) and 5 (▲) on *in vitro* FTPase activity. The abscissa indicates the concentration of the phosphonates in the FTPase reaction, and the ordinate indicates dpm of radiolabeled farnesyl from FPP incorporated into precipitable H-ras protein as percentage control. Compound **4** inhibits FTPase with IC_{50} of approximately 65 nM, whereas compound **5** displays no FTPase inhibitory activity up to 100 nM concentrations. Compounds **2**, **3**, **6**, and **7** do not inhibit FPTase, at least up to as high as 500 nM concentrations.

Effects of the isoprenoid phosphonates on GGPTase I. Figure 4 displays the effects of increasing concentrations of compound **4** on *in vitro* GGPTase I activity. The abscissa indicates the concentration of the phosphonate in the GGPTase I reaction, and the ordinate indicates dpm of radiolabeled geranylgeranyl from geranylgeranyl pyrophosphate incorporated into precipitable mutant H-ras/CVLL protein as percentage control. Compound **4** slightly inhibits GGPTase I although an IC_{50} is not reached even at 50 μM concentrations.

Effects of the isoprenoid phosphonates on cholesterol synthesis and squalene synthase. Table 1 displays the effects of *E,E*- (**4**) and *Z,E*-α-hydroxyfarnesylphosphonate (**5**) on cholesterol synthesis in intact cells and squalene synthase in enzymatic assay. Neither compound greatly decreased cholesterol synthesis nor squalene synthase activity in comparison to control experiments. Lovastatin was used as a positive control for the cholesterol inhibition assay and (*E,E*)-(4,8,12-trimethyl-3,7,11-tridecatrienylidene)-bisphosphonic acid was used as a positive control for the squalene synthesis assay.

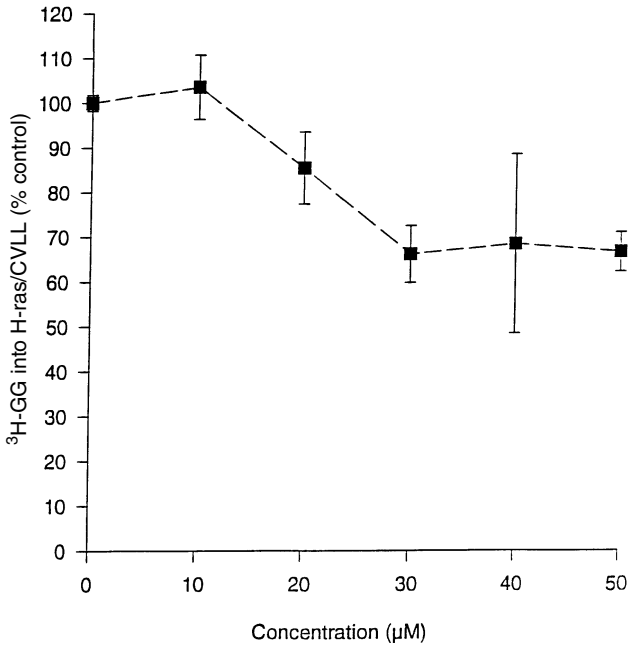


FIG. 4. Effects of *E,E*-α-hydroxyfarnesylphosphonate on geranylgeranyl protein transferase I activity. This assay was performed as described in the Experimental Procedures section. The points are the mean of three experiments and the error bars indicate ±1 standard deviation.

DISCUSSION

This preparation of the α-hydroxyfarnesylphosphonic acids employed a sample of farnesal (**1**) that was known to consist of a 10:1 mixture of the *E,E*- and *Z,E*-isomers. Condensation of this mixture with the anion of dimethyl phosphite according to an improved variation (29) of the procedure reported by Pompliano *et al.* (18) (80 vs. 38% yield), gave the expected mixture of the dimethyl *E,E*- and dimethyl *Z,E*-α-hydroxyfarnesylphosphonates (**2** and **3**). Because these olefin isomers could then be separated by column chromatography, it was possible to obtain both of the desired olefin stereoisomers of the farnesyl chain length. Each was then independently hydrolyzed to the corresponding phosphonic acid (**4** and **5**). Preparation of the geranyl derivatives was accomplished by a parallel set of reactions, giving the dimethyl α-hydroxygeranylphosphonate (**6**) and α-hydroxygeranylphosphonic acid (**7**) in very good yields. For the dimethyl esters, purity was established most readily by analysis of the ^{1}H NMR spectra and the purity of the phosphonic acids was verified by analysis of the ^{31}P NMR spectra.

TABLE 1
Effects of Isoprenoid Phosphonates on Cholesterol Synthesis and Squalene Synthase[a]

	Cholesterol Synthesis (% control)	Squalene Synthase (% control)
Control	100	100
Compound **4**	114	94
Compound **5**	113	80
Lovastatin	32	
Squalene synthase Inhibitor		9.3

[a]Cholesterol synthesis: Compound **4** and Compound **5** were tested at 200 μM. Lovastatin was tested at 1 μM. Squalene synthase: Compound **4** and Compound **5** were tested at 1 μM. Squalene synthase inhibitor refers to (*E,E*)-(4,8,12-trimethyl-3,7,11-tridecatrienylidene)bisphosphonic acid that was tested at 100 nM. Compound **4** is (*R,S*)-[1-hydroxy-3,7,11-trimethyl-2*E*,6*E*,10-dodecatrienyl]phosphonic acid. Compound **5** is (*R,S*)-[1-hydroxy-3,7,11-trimethyl-2*Z*,6*E*,10-dodecatrienyl]phosphonic acid.

The biological assay results for these compounds demonstrate that in intact cells, only the *E,E*- (**4**) but not the *Z,E*-α-hydroxyfarnesylphosphonate (**5**) impairs RAS processing. Inhibition of FPTase by compound **4** is consistent with that reported by Pompliano *et al.* (18), but a more dramatic effect is observed when [^{35}S]methionine incorporation into RAS in intact cells is measured. The *E,E*-isomer is a potent inhibitor of the farnesylation of newly synthesized RAS. Furthermore, the IC_{50} for the *E,E*-isomer (**4**) to inhibit RAS processing in intact cells is between 0.2 and 2.0 μM (Fig. 3), whereas IC_{50} for the *E,E*-isomer to inhibit bovine FPTase in enzyme assay is approximately 65 nM (Fig. 4), suggesting that cells may either impede intracellular localization, metabolically inactivate, or actively extrude compound **4**. Alternatively, the mechanisms that regulate RAS expression in intact cells (30) may compensate for compound **4** FPTase inhibitory activity. Our further expansion of the original data (18) with our demonstration that the *Z,E*-isomer **5** is less active in both intact cell and enzyme assays also indicates that competitive interaction with the site of the FPP binding to FPTase is dependent upon the olefin stereochemistry.

To assess the effect of alkyl chain length on inhibition of RAS processing in intact cells and in direct FPTase assays, a C-10 analog (**7**) of the active *E,E*-isomer **4** was tested for activity. If recognition of the α-hydroxyfarnesyl phosphonate is dominated by interactions with the phosphoryl group, one might expect smaller α-hydroxyalkylphosphonates to serve as competitive inhibitors as well. However, α-hydroxygeranylphosphonate (**7**) did not alter RAS expression in intact cells nor inhibit FPTase in enzyme assay. Thus competitive interaction of inhibitors with the site of FPP binding to FPTase is dependent upon alkyl chain length. A parallel impact of chain length is found with the enzyme GGPTase I, where the *E,E*-(α-hydroxyfarnesyl)phosphonate is not an inhibitor.

The dimethyl esters of active FTPase inhibitors should have increased cell permeability relative to the phosphonic acids because the esters remain neutral while the acids would be ionized under assay conditions. If hydrolysis to the parent acid were to occur within the cells, the dimethyl esters could interfere with RAS processing to a greater degree than the parent phosphonic acids. However, in our assays, the dimethyl ester **2** did not impair RAS processing in intact cells nor did it inhibit FPTase in enzyme assay despite the marked toxicity of this compound with respect to severely decreasing cell viability. The dimethyl ester **3** was similarly toxic to cells, raising the possibility that such toxicity is a consequence of the esterification. At the same time, the C-10 dimethyl ester **6** was nontoxic to cells. These results do not exclude the possibility that toxicity might be a consequence of detergent activity, as has been described for other similar compounds, (9) and that the longer alkyl chain is required for a detergent effect.

Ultimately, any clinical application of FPP analogs as FPTase inhibitors will depend on their selectivity for inhibition of FPTase as compared to the other enzymes in the isoprenoid/cholesterol metabolic pathway (9). The greatest concern is that isoprenoid phosphonates will inhibit other enzymes that utilize FPP as a substrate, such as squalene synthase. Our experiments clearly show that the active inhibitor **4** is specific for FPTase over squalene synthase and displays only minimal inhibition of GGPTase I. The limited inhibition of GGPTase I is particularly important because GGPTase I and FPTase share α-subunits (31), and one might speculate that there would be cross-inhibition of these two enzymes. To ensure valid assays of squalene synthase inhibition, (*E,E*)-(4,8,12-trimethyl-3,7,11-tridecatrienylidene)bisphosphonic acid was employed as a positive control. Our finding that the *E,E*-isomer **4** does not inhibit squalene synthase is contrary to the unpublished observation by others (9) that this compound inhibits squalene synthase with an IC_{50} of 0.63 μM.

Of these findings, the most significant result is that the *E,E*-phosphonate **4** completely blocks RAS farnesylation *via* inhibition of FPTase, while not reducing overall cholesterol synthesis in intact cells and not inhibiting squalene synthase in enzyme assay. Because both FPTase and squalene synthase utilize FPP as their natural substrate, it may have been reasonable to assume that both enzymes would be affected by this compound in a parallel fashion. Because this is not the case, phosphonate analogs of FPP are valuable as selective inhibitors of the various enzymes of isoprenoid metabolism.

ACKNOWLEDGMENTS

This work was supported by grants from the Roy J. Carver Charitable Trust, Leukemia Society of America, and University of Iowa Cancer Center. R. Hohl is a recipient of an American Society of Clinical Oncology Young Investigator Award and a Pharmaceutical Research and Manufacturers of America Foundation Faculty Development Award in Clinical Pharmacology. We thank Sarah Holstein, Department of Chemistry at the University of Iowa, for preparation of (*E*,*E*)-(4,8,12-trimethyl-3,7,11-tridecatrienylidene)bisphosphonic acid. The RPMI-8402 cells were provided by M. Diaz, Loyola University.

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[Received September 4, 1997, and in final revised form and revision accepted December 12, 1997]

Stereospecific Metabolism of Isomeric Epoxyoctadecanoic Acids in the Lactone-Producing Yeast *Sporidiobolus salmonicolor*

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ABSTRACT: The metabolic course of four isomeric epoxyfatty acids derived from oleic-, elaidic-, (*Z*)-, and (*E*)-vaccenic acids in the lactone-producing yeast, *Sporidiobolus salmonicolor,* was studied by using the deuterium-labeled precursors. Dihydroxy-, hydroxyoxo-, and hydroxy fatty acids as well as γ-lactones were identified as metabolic intermediates. Quantitative analysis of the label content and estimation of the enantiomeric composition of the lactones established that, in the first step, the racemic epoxyfatty acids were enantiospecifically hydrolyzed by an epoxide hydrolase. During the subsequent metabolism, the stereochemical orientation of the hydroxy groups of the dihydroxyfatty acids were modified by an oxidation/reduction step.
Lipids 33, 47–58 (1998).

Epoxyfatty acids can be found in many plants as constituents of the cutine polymers in green leaves where they are involved in the defense mechanisms of green plants (1–4). Epoxyfatty acids are derived from unsaturated fatty acids by double-bond epoxidation, catalyzed either as a main or a side reaction by a variety of metallo-enzymes, mostly of the cytochrome P-450 type. Enzymes which utilize hydroperoxides as oxygen source are summarized under the terms "epoxygenases" or "peroxygenase" in the literature. Their activity was first discovered in spinach (5), but have been found in a variety of edible plants (6–8). Some authors also have confirmed the ubiquitous appearance of this kind of enzymatic activity indirectly while identifying epoxyfatty acids as products of bacteria, fungi, and mammals (9–13).

Epoxyfatty acids are chiral compounds. Stereochemical investigations of their formation, performed with microsomes or purified epoxygenases from Jerusalem artichokes, soybeans and broad beans, revealed that enzymatic epoxidation reactions are enantioselective (14–16). However, the subsequent metabolism of epoxyfatty acids is initiated in general by the hydrolytic cleavage of the oxirane ring, catalyzed by epoxide hydrolases. These enzymes, active on carboxylic epoxyfatty acids, were first discovered in a cell-free extract of the flax rust uredospores (*Melampsora lini*) (17). The stereospecificity of this enantioselective reaction toward both configural isomers of 9,10-epoxystearic acids was first established with an enzyme preparation from a *Pseudomonas* strain (18,19). The most detailed studies concerning the regio- and enantiospecificities of epoxyfatty acid hydrolases were performed by Blée and Schuber (20–23), using purified soybean enzyme.

Recently, Schöttler and Boland (24,25) have investigated the importance of a saturated epoxyfatty acid acting as a precursor in the biosynthesis of γ-dodecanolactone, an important flavor compound in many fruits. They proved the crucial role of the enantioselectivity of epoxyfatty acid hydrolases for the enantiomeric distribution of this γ-lactone in strawberries, peaches, and nectarines.

In this paper, we report the stereochemical aspects of the metabolism of epoxyfatty acids in the lactone-producing yeast *Sporidiobolus salmonicolor*. *Sporidiobolus salmonicolor* was chosen for the following reasons: The yeast shows a diverse fatty acid metabolism including a variety of interesting enzymes and is easy to cultivate. Therefore, the yeast has been used as an appropriate model for metabolic studies in the field of fatty acids for many years (26–31). The organism accumulates chiral lactones during its cultivation, which are in general biosynthesized from unsaturated fatty acids. These compounds are easy to obtain and to analyze. By using suitably labeled precursors, here double-bond labeled racemic epoxy fatty acids, these lactones will conserve stereochemical information by its configuration and labeling patterns about those enzymes which are involved in the metabolic pathways of the organism. Starting from deuterium-labeled racemic epoxides of (*Z*)- and (*E*)-oleates and vaccenates (9,10- and 11,12-epoxystearic acid esters), we are able to give a detailed insight into the β-oxidative course of these compounds with respect to the biosynthesis of the important aroma compounds, γ-decalactone and γ-

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Abbreviations: ee, Enantiomeric excess; GC–MS, gas chromatography–mass spectrometry; ^{1}H NMR, proton nuclear magnetic resonance; TMS, trimethylsilyl.

dodecalactone. The few examples given above show the widespread natural occurrence of γ-lactones in popular fruits. They may demonstrate the important contribution of C_{10}–C_{12} γ-lactones to the flavor of many foodstuffs. For this reason they are produced in ton-scale as flavor enhancers (fruity notes, such as coconut, apricot, peach, etc.) for use in flavor industry (for a recent review, see Ref. 32).

EXPERIMENTAL PROCEDURES

Syntheses. All four deuterium-labeled epoxyfatty acid isomers were synthesized by chemical epoxidation (*m*-chloroperbenzoic acid in CH_2Cl_2 for 18 h at room temperature) from the corresponding double-bond labeled monounsaturated fatty acids or their esters, respectively. The (*Z*)- and (*E*)-monoenoic acids were obtained by reductive deuteration of the 9-, 11-octadecenoic acids or the 9-, 11-octadecen-1-ols, respectively, which were synthesized by a C–C-bond formation between OH-protected ω-alkyn-1-ols and alkylbromides, followed by deprotection and/or oxidation to the corresponding acids.

The preparation of the [$^2H_2$]-(*Z*)-epoxyoctadecanoic acids was described elsewhere (26). The synthesis of the (*E*)-isomers was performed as follows:

To 0.625 g (14.88 mmol) of $LiAlD_4$ (99% isotopic purity), dissolved in 25 mL of anhydrous diethylenglycoldimethylether (diglyme) at 0°C, 1.0 g (3.71 mmol) of the octadecen-1-ol was added, and the reaction mixture was refluxed for 72 h. After cooling to 0°C, the reaction was hydrolyzed with 5 mL of D_2O (99% isotopic purity), acidified with 2 M HCl, and extracted three times using, in total, 100 mL of diethylether. The combined organic phases were washed with brine, dried over Na_2SO_4, and the solvent was evaporated in vacuum. The product was purified on silica gel to obtain 0.883 g (3.15 mmol) of the pure [$^2H_2$]-(*E*)-octadecen-1-ol. The alcohol was oxidized to the acid in a two-step procedure, using pyridinumchlorochromate followed by $HClO_2$ as oxidants. The overall yield was regularly higher than 70% for all isomeric fatty acids.

Analytical data. [9,10-$^2H_2$]-Methyl-(*Z*)-9,10-epoxyoctadecanoate: Proton nuclear magnetic resonance (^{1}H NMR) ($CDCl_3$): δ = 0.86 (*t, J* = 7 Hz, 3 H, CH_3CH_2), 1.17–1.48 (*m,* 22 H, CH_2), 1.57 (quint, *J* = 7 Hz, 2 H, $CH_2CH_2COOCH_3$), 2.28 (*t, J* = 7 Hz, 4 H, $CH_2CD(O)CDCH_2$), 3.63 (*s*, 3 H, CH_2COOCH_3). Mass spectrum (70 eV), *m/z* (%): 314 (<1) [M^+], 283 (2) [$M^+ - CH_3O$], 265 (1), 247 (<1), 215 (7), 186 (11), 169 (7), 157 (26), 156 (59), 140 (23), 125 (17), 110 (33), 101 (58), 96 (35), 88 (100), 69 (71), 55 (99), 43 (70), 41 (74); d-content at C(9)/C(10): averaged 93.1%.

[9,10-$^2H_2$]-Ethyl-(*E*)-9,10-epoxyoctadecanoate: ^{1}H NMR ($CDCl_3$): δ = 0.85 (*t, J* = 7 Hz, 3 H, CH_3CH_2), 1.20–1.54 (*m,* 22 H, CH_2), 1.23 (*t, J* = 7 Hz, 3 H, CH_3CH_2O), 1,58 (quint, *J* = 7 Hz, 2 H, $CH_2CH_2COOCH_2CH_3$), 2.25 (*t, J* = 7 Hz, 4 H, $CH_2CD(O)CDCH_2$) 4.09 (*q, J* = 7 Hz, 2 H, $CH_2COOCH_2CH_3$). Mass spectrum (70 eV), *m/z* (%): 329 (<1), 328 (<1) [M^+], 311 (<1) [$M^+ - H_2O$], 300 (<1), 283 (4) [$M^+ - C_2H_5O$], 265 (2), 247 (2), 223 (1), 215 (16), 186 (19), 157 (37), 156 (100), 153 (32), 140 (32), 125 (18), 110 (40), 101 (49), 96 (37), 88 (92), 83 (41), 69 (56), 55 (70), 41 (48); d-content at C(9)/C(10): averaged 93.9%.

[11,12-$^2H_2$]-Ethyl-(*Z*)-11,12-epoxyoctadecanoate: ^{1}H NMR ($CDCl_3$): δ = 0.87 (*t, J* = 7 Hz, 3 H, CH_3CH_2), 1.18–1.49 (*m,* 22 H, CH_2), 1.21 (*t, J* = 7 Hz, 3 H, CH_3CH_2O), 1.58 (quint, *J* = 7 Hz, 2 H, $CH_2CH_2COOCH_2CH_3$), 2.22 (*t, J* = 7 Hz, 4 H, $CH_2CD(O)CDCH_2$) 4.06 (*q, J* = 7 Hz, 2 H, $CH_2COOCH_2CH_3$). Mass spectrum (70 eV), *m/z* (%): 328 (<1), 283 (2) [$M^+ - 45$], 265 (2), 243 (8), 225 (2), 214 (11), 197 (4), 184 (28), 150 (13), 129 (26), 101 (82), 98 (40), 88 (100), 82 (41), 69 (66), 55 (99), 41 (71); d-content at C(11)/C(12): averaged 94.5%.

Synthesis of [11,12-$^2H_2$]-Ethyl-(*E*)-11,12-epoxyoctadecanoate: ^{1}H NMR ($CDCl_3$): δ = 0.85 (*t, J* = 7 Hz, 3 H, CH_3CH_2), 1.22–1.56 (*m,* 22 H, CH_2), 1.23 (*t, J* = 7 Hz, 3 H, CH_3CH_2O), 1.60 (quint, *J* = 7 Hz, 2 H, CH_2CH_2-$COOCH_2CH_3$), 2.24 (*t, J* = 7 Hz, 4 H, $CH_2CD(O)CDCH_2$) 4.10 (*q, J* = 7 Hz, 2 H, $CH_2COOCH_2CH_3$). Mass spectrum (70 eV), *m/z* (%): 328 (<1) [M^+], 310 (<1) [$M^+ - H_2O$], 283 (3) [$M^+ - C_2H_5O$], 265 (2), 243 (7), 225 (2), 214 (9), 197 (4), 184 (28), 165 (10), 150 (11), 129 (27), 101 (80), 98 (40), 88 (100), 69 (68), 55 (99), 41 (63); d-content at C(11)/C(12): averaged 94.1%. The enantiomeric excesses (ee) were calculated as in Equation 1:

$$ee = \frac{E_1(\%) - E_2(\%)}{E_1(\%) + E_2(\%)} \quad [1]$$

(concentration of enantiomer E_1 > enantiomer E_2).

The microorganism *S. salmonicolor,* a carotinoid pigment-producing yeast, was obtained from the Deutsche Stammsammlung (Braunschweig, Germany). It is identical to the yeast ATCC 24259 of the American strain collection. The organism was cultured and stored (4°C) on wort agar-agar-slants. For metabolic experiments, the cells of the agar slants were transferred to a 1-L Erlenmeyer flask under sterile conditions, containing 200 mL of the following medium: 45 g/L sucrose, 5 g/L lactose, 3.0 g/L $MgSO_4 \cdot 7\,H_2O$, 2.5 g/L L-alanine, 2.5 g/L K_2HPO_4, 2.5 g/L $(NH_4)_2SO_4$, 0.1 g/L $CaCl_2 \cdot 2\,H_2O$ in deionized water. The cultures were incubated at 22–24°C on a horizontal shaker (100 rpm). After 96–120 h incubation (stationary growth phase of the organism tested by estimating the dry weight of the organisms), 250 mg (0.79 mmol) of racemic 9,10-d_2-methyl-(*Z*)-9,10-epoxyoctadecanoate and 250 mg (0.76 mmol) 9,10-d_2-ethyl-(*E*)-9,10-epoxyoctadecanoate were administered to different flasks of cultured *S. salmonicolor* during the stationary growth phase of the organisms. At certain times 10-mL aliquots of the culture broths were taken and extracted. The formation of degradation products was monitored by gas chromatography–mass spectrometry (GC–MS) analysis after methylation and silylation of the etheral extracts. Additional information to the feeding experiments, the sample work-up as well as the

NMR, achiral and chiral GC–MS studies were previously described (26,27).

RESULTS

Experiments with [9,10-$^2H_2$]-(Z)- and [9,10-$^2H_2$]-(E)-9,10-epoxyoctadecanoates. During the course of the fermentation, both substrates were metabolized to several deuterated dihydroxy-, hydroxyoxo-, hydroxyfatty acids and labeled γ-dodecalactone. These compounds were identified by their prominent fragment ions of their MS-spectra [α-cleavage before and after trimethylsilyl (TMS)-group rearrangement] and compared with available reference data (Table 1) (33). Except for the precursor, no epoxyfatty acids were detected.

The quantitative course of the metabolite formation is summarized in Figures 1 and 2. Both substrates were completely metabolized by 168 h. As main metabolic products, C_{14} and C_{12} dihydroxyfatty acids temporarily accumulated in the culture broths in concentrations of up to 52 mg/L. There were apparent differences between the product spectra obtained from both isomeric substrates. After adding the (*Z*)-isomeric precursor, significant amounts of labeled 5,6-, and 3,4-hydroxy-oxo fatty acids were formed by the yeast (up to 20 mg/L), carrying the oxo-functionality always in the latter position of the vicinal hydroxy/oxo group in the alkyl chain of the acids. After the addition of the (*E*)-isomer, these acids could only be detected as trace components (<100 μg/L). However, only in the experiment starting with the (*E*)-precursor, up to 30 mg/L of 9,10-dihydroxyoctadecanoate could be found in the fermentation broth of the organism, representing the product of the first metabolic step, the hydrolysis of the oxirane ring of the 9,10-epoxyoctadecanoate.

Labeled γ-dodecalactone was formed from both isomers of 9,10-epoxyoctadenoate by the yeast. This chiral compound was suitable to study stereochemical aspects of the present metabolic pathway(s) in more detail. The deuterated part of the lactone conserves stereochemical information in its labeling patterns and its enantiomeric composition and was accordingly analyzed by both achiral and chiral GC–MS (26,27).

As shown in Figures 1B and 2B as well as in Table 2, compared to the (*Z*)-configured isomer, the (*E*)-configured substrate was the better precursor of γ-dodecalactone [yield: (*Z*) = 0.2 mol% vs. (*E*) = 1.26 mol% after 168 h]. The relative amounts of one- and twofold deuterated γ-dodecalactone, which were significantly different from both experiments, appear to be even more interesting. The formation of high quantities of monodeuterated γ-lactone [about 40–50% of the labeled part after addition of (*Z*)-, and 20–25% after administration of (*E*)-epoxyoctadecanoate] reflects the partial loss of one deuterium during the biotransformation process of the epoxy acids to the lactone. The reason for this deuterium re-

TABLE 1
Identified Metabolic Products of d_2-Methyl-(*Z*)-9,10-epoxyoctadecanoate and d_2-Ethyl-(*E*)-9,10-epoxyoctadecanoate as Their Mass Spectra (as methyl esters and as silylated TMS-derivatives), After Incubation of 50 mg (250 mg/L) of Each Precursor in Cultures of *Sporidiobolus salmonicolor*

Precursor Products	EI–MS spectra[a] *m/z* (relative intensity)
d_2-Methyl-(*Z*)-9,10-epoxyoctadecanoate	
[5,6-$^2H_2$]5,6-Dihydroxytetradecanoic acid	389(1),388(2),318(<1),277(1),216(38),215(20),205(15),204(90),172(22),147(17),130(20),114(21),100(21),83(22),73(100)
[5-$^2H_1$]5-Hydroxy-6-oxotetradecanoic acid	330(2),314(4),279(1),256(1),204(100),172(31),130(37),114(26),100(30),73(43)
[3,4-$^2H_2$]3,4-Dihydroxydodecanoic acid	377(<1),361(<1),316(<1),287(2),286(3),271(1),249(30),216(89),147(13),104(21),83(18),73(100)
[4-$^2H_1$]4-Hydroxy-3-oxododecanoic acid	302(1),286(<1),270(<1),196(1),176(52),134(36),132(28),73(100)
[3,4-$^2H_2$]γ-Dodecalactone[b]	180(<1),128(3),110(1),102(2),101(3),100(2),98(2),97(3),87(22),86(10),85(100),83(5),75(7),74(8),57(14),56(15),55(16)
[2-$^2H_1$]2-Hydroxydecanoic acid[b]	260(32),232(4),217(18),216(100),104(24),89(49),73(97)
d_2-Ethyl-(*E*)-9,10-epoxyoctadecanoate	
[9,10-$^2H_2$]9,10-Dihydroxyoctadecanoic acid	390(<1),389(1),388(2),319(<1),318(<1),314(<1),277(1),216(38),215(20),205(15),204(90),172(22),147(17),130(20),114(21),100(21),83(22),73(100)
[5,6-$^2H_2$]5,6-Dihydroxytetradecanoic acid	389(1),388(2),318(<1),277(1),216(38),215(20),205(15),204(90),172(22),147(17),130(20),114(21),100(21),83(22),73(100)
[3,4-$^2H_2$]3,4-Dihydroxydodecanoic acid	377(<1),361(<1),316(<1),287(2),286(3),271(1),249(30),216(89),147(13),104(21),83(18),73(100)
[3,4-$^2H_2$]γ-Dodecalactone[b]	180(<1),128(3),110(1),102(2),101(2),100(2),98(2),97(3),87(24),86(9),85(100),83(5),75(7),74(10),57(16),56(17),55(19)
[2-$^2H_1$]-2-Hydroxydecanoic acid[b]	260(32),232(4),217(18),216(100),104(24),89(49),73(97)

[a]EI–MS, electron ionization–mass spectrometry.
[b]Occurred accompanied with its unlabeled isotopomer.

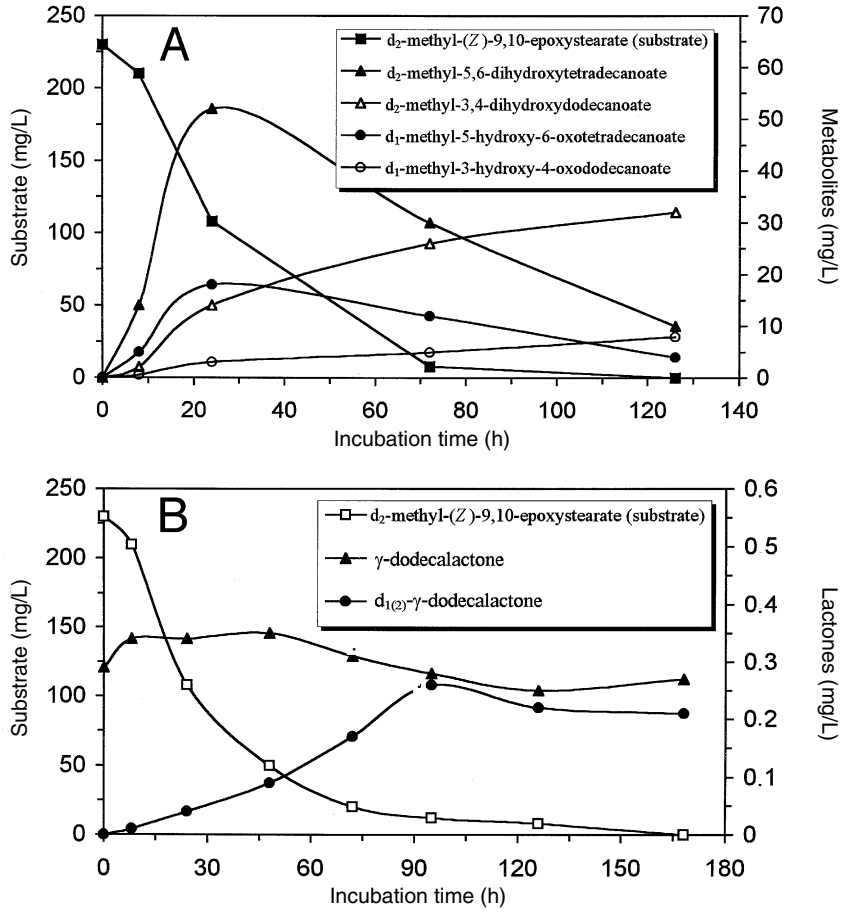

FIG. 1. Time-dependent course of the product formation after addition of 50 mg (250 mg/L) [9,10-$^2H_2$]-methyl-(*Z*)-9,10-epoxyoctadecanoate to 250-mL shaking-flask cultures of *Sporidiobolus salmonicolor* (stationary growth phase). (A) Substrate and hydroxyfatty acids as products; (B) substrate and lactonic products.

moval could be an oxidation/reduction step at the chirality center C-4 of the resulting lactone, comparable to the biosynthesis of δ-decalactone in *S. salmonicolor* (28). This hypothesis was confirmed by the finding that only this regioisomeric hydroxyoxofatty acid was formed by the yeast which carried the oxo-group at the latter C-atom of the vicinally arranged functional group in the carbon chain (Table 1).

More detailed information about the stereochemical course of this metabolic pathway to the γ-dodecalactone was obtained by the chiral GC–MS analysis of the isotopomeric lactones (Table 3). The enantiomeric distribution of the d_2-labeled lactone represents the initial stereochemical orientation of the latter hydroxy group (at C-10) after the ring-opening of the epoxide hydrolase and the subsequent β-oxidative degradation. Coming from the (*Z*)-configured substrate, the d_2-lactone was biosynthesized in 77.4% ee (*S*). When derived from the (*E*)-9,10-epoxystearate, the lactone occurred in the opposite configuration [86% ee (*R*)].

The only reasonable explanation of the loss of the label at the chirality center at C-4 of the lactone is the abstraction of a deuterium by a dehydrogenase or oxidase which formed the oxofatty acid. After another reduction step at this carbon, finally leading to the d_1-4-hydroxydodecanoic acid, the excreted d_1-labeled lactone product conserved the stereochemical specificity of this reductive enzymatic activity [The required cofactor(s) must have been protonated and not deuterated since the medium was based on H_2O. Consequently, one can discriminate between the stereospecificity of the two different pathways to d_2- and d_1-lactone]. Hence, the (*Z*)-precursor was metabolized to the (*R*)-configured d_1-γ-dodecalactone (47.2% ee) opposite to its d_2-isotopomer which occurred in the (*S*)-configuration. The d_1-labeled γ-dodecalactone from the (*E*)-configured substrate, on the other hand, was estimated to possess nearly the identical enantiomeric composition as its d_2-isomer [90.6% ee (*R*)], isolated from the same culture at the same time.

*Experiments with deuterated [11,12-$^2H_2$]-(*Z*)- and [11,12-$^2H_2$]-(*E*)-11,12-epoxyoctadecanoates*. The experiments with the racemic d_2-(*Z*)- and d_2-(*E*)-vaccenoates were performed in the same manner as described above. Again dihydroxy-, hydroxyoxo-, and hydroxyfatty acids as well as γ-decalactone as the corresponding lactone were identified as labeled metabolites from both incubation experiments (Table 4). The courses of the product formation are presented in Figures 3

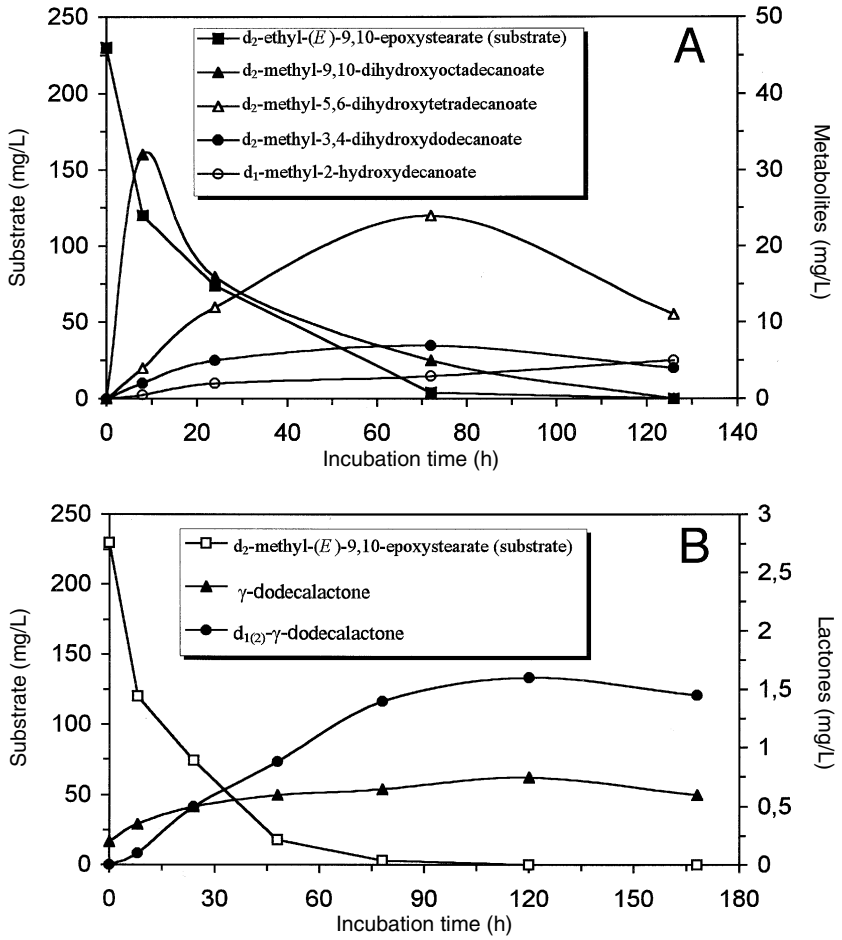

FIG. 2. Time-dependent course of the product formation after addition of 50 mg (250 mg/L) [9,10-$^2H_2$]-ethyl-(*E*)-9,10-epoxyoctadecanoate to 250-mL shaking-flask cultures of *Sporidiobolus salmonicolor* (stationary growth phase). For (A) and (B) see Figure 1.

and 4. The results are roughly comparable to those obtained from the epoxidized oleic and elaidic acid experiments. Once more the 5,6-dihydroxyfatty acids were detected in the highest concentration of all metabolic products excreted by the yeast (up to 64 mg/L); even hydroxy-oxo compounds could be identified in the cultures. As a consequence of the position of the epoxy group (C11/12) of the utilized precursors, this time labeled γ-decalactone was produced by *S. salmonicolor* as the lactonic compound. Comparable to the above-presented experiments, the (*E*)-configured epoxy acid was again the far better substrate for the production of γ-decalactone [yield: (*Z*) 0.46 mol%; (*E*) = 2.92 mol%) (Table 2)]. Accurate analytical estimation of the isotopomeric composition revealed that from both precursors mainly the double-labeled γ-decalactone was produced by the yeast. Nevertheless, the part of d_1-labeled γ-decalactone was obvious. The results of the chiral GC–MS analysis of the isotopomeric γ-decalactones are shown in Table 3. In contrast to the unlabeled γ-decalactone which was biosynthesized enantiomerically pure in its (*R*)-configuration, the deuterated isomers only occurred with moderate optical purity, each with an excess of the (*R*)-enantiomer.

DISCUSSION

The presented results allow us to gain a detailed insight into the metabolism of isomeric epoxyfatty acids in a yeast for the first time. Both enantiomers of all four isomeric substrates were metabolized, and their pathways could be successfully traced. Some common aspects of their degradation are obvious: (i) No chain-shortened epoxyfatty acids could be detected. Therefore, the first metabolic step of the pathway is the hydrolytic opening of the oxirane ring (accompanied by the hydrolysis of the ester group of the precursors), resulting in the formation of dihydroxyfatty acids. Consequently, this indirectly proves the existence of at least one epoxyhydrolase in *S. salmonicolor* which accepts epoxyoctadecanoates as substrates. (ii) Once included in the β-oxidation cascade, the C_{18}-dihydroxyfatty acids were successfully degraded by the separation of two, respectively three acetyl-CoA molecules and then released into the medium again (5,6- and 3,4-dihydroxyfatty acids accumulated in the highest concentration of all metabolites). For this reason, β-oxidation of these compounds appears to be the "bottleneck" of the metabolism in total. In contrast to the degradation of 4,5-dihydroxyalkanoic

TABLE 2
Concentration and Relative Amounts of One- and Twofold Labeled γ-Dodecalactone or γ-Decalactone, Respectively, Found After Addition of 250 mg/L [9,10-$^2H_2$]-Methyl-(*Z*)-9,10-epoxyoctadecanoate, [9,10-$^2H_2$]-Ethyl-(*E*)-9,10-epoxyoctadecanoate, [11,12-$^2H_2$]-Ethyl-(*Z*)-11,12-epoxyoctadecanoate, or [11,12-$^2H_2$]-Ethyl-(*E*)-11,12-epoxyoctadecanoate to Cultures of *Sporidiobolus salmonicolor* [achiral GC–MS coupling (capillary column: DB wax™)][a]

	Substrate							
	Time (h)							
Product	0	8	24	48	72	95	126	168
γ-Dodecalactone	d_2-Methyl-(*Z*)-9,10-epoxyoctadecanoate							
Concentration (μg/L)	249	347	379	409	480	543	468	480
Unlabeled (%)	100	98.9	86.7	76.0	63.8	51.8	53.0	56.0
d_1-Labeled (%)	0	0.4	8.3	12.4	16.7	22.6	22.2	21.0
d_2-Labeled (%)	0	0.7	5.0	11.6	19.5	25.6	24.8	23.0
	d-Ethyl-(*E*)-9,10-epoxyoctadecanoate							
Concentration (μg/L)	285	410	920	1507	2019	2209	2402	2058
Unlabeled (%)	100	95.8	52.6	36.7	34.3	32.3	31.9	30.5
d_1-Labeled (%)	0	1.2	8.8	10.3	12.2	12.9	12.9	13.4
d_2-Labeled (%)	0	5.0	38.6	53.0	53.5	54.8	55.2	56.1
γ-Decalactone	d_2-Ethyl-(*Z*)-11,12-epoxyoctadecanoate							
Concentration (μg/L)	560	620	990	1420	2010	2530	3040	4520
Unlabeled (%)	100	97.7	90.1	84.1	82.3	81.5	80.5	83.8
d_1-Labeled (%)	0	1.0	2.9	4.1	4.5	4.7	5.1	4.6
d_2-Labeled (%)	0	1.3	7.1	11.8	13.2	13.8	14.4	11.6
	d_2-Ethyl-(*E*)-11,12-epoxyoctadecanoate							
Concentration (μg/L)	660	790	1920	6930	8210	8790	9360	9820
Unlabeled (%)	100	95.6	63.6	50.5	55.2	56.2	58.4	61.3
d_1-Labeled (%)	0	0.7	6.4	8.1	7.9	7.2	6.9	6.4
d_2-Labeled (%)	0	3.7	31.0	41.4	36.9	36.6	34.7	32.3

[a]GC–MS, gas chromatography–mass spectrometry.

acids, which strictly accumulate in the culture broth as their 5-hydroxy-γ-lactones (no further metabolization) (30), obviously the 5,6- and the 3,4-dihydroxy compounds were introduced again in the β-oxidation cycle and were completely metabolized by *S. salmonicolor* at last. (iii) All of the four substrates served as precursors in the biosynthesis of deuterated γ-decalactone or γ-dodecalactone, respectively. The corresponding γ-lactones were excreted in relatively low amounts by *S. salmonicolor* (yields between 0.2–2.9 mol%). Apparently, the (*E*)-configured substrates were always the better precursors with respect to the lactone formation. (iv) No intermediates of the pathway from the 3,4-dihydroxy acids, the potential metabolic precursors of the γ-lactones, to the corresponding lactones could be detected [this is in contrast to the results of Albrecht and Tressl (29), who identified γ-dec-2-enolactone as an intermediate in the γ-decalactone formation from ethyl-3,4-epoxydecanoate in *S. salmonicolor*]. Here, we are not able to provide more information about the involved enzymatic steps from the 3,4-dihydroxy acids to the saturated γ-lactones.

Some additional remarks need to be made about points (iii) and (iv). The formation of the direct γ-lactone precursor 4-hydroxyfatty acid from the 3,4-dihydroxyfatty acid strongly depends on the metabolic sequence and the substrate affinity of the involved enzymes. A dehydration step of the 3-hydroxy group, leading to (*E*)-4-hydroxy-dec-2-enoic acid is known as a standard reaction step of the fatty acid biosynthesis. For that, the 3,4-dihydroxy compound must be transferred to an acyl carrier protein of the fatty acid biosynthesis cycle. After one enzymatic step (dehydration), it must be released again. Apart from this, the reduction of the Δ^2-double bond is not a very common reaction in yeasts. Consequently, it seems more likely that the cyclization of the 3,4-dihydroxy compounds, leading to the 3-hydroxy-γ-lactone, competes directly with the further β-oxidation of these compounds which will yield α-hydroxy acids.

The possibility of the enzyme-catalyzed dehydration of 3-hydroxy-γ-lactones to (*Z*)-γ-alk-2-enolactones and the further reduction to the saturated γ-lactone was established for yeasts by Fronza *et al.* (34) and Gatfield *et al.* (35). Therefore, it seems likely that *S. salmonicolor* uses the same biosynthetic pathway. One cycle of β-oxidation of the 3,4-dihydroxyfatty acid leads to 2-hydroxyacids (they could be detected in the broth of *S. salmonicolor* after adding the epoxyfatty acids). These compounds are not substrates for further β-oxidation. Fulco (36) could establish a decarboxylation (α-oxidation) of long-chain 2-hydroxyfatty acids in a *Saccharomyces* strain. Hence, α-oxidation in *S. salmonicolor* seems possible even

TABLE 3
Relative Distribution of Both Enantiomers of the Deuterium-Labeled γ-Dodecalactone and γ-Decalactone Isotopomers Formed 168 h After Addition of [9,10-$^2H_2$]-Methyl-(*Z*)-9,10-epoxyoctadecanoate, [9,10-$^2H_2$]-Ethyl-(*E*)-9,10-epoxyoctadecanoate, [11,12-$^2H_2$]-Ethyl-(*Z*)-11,12-epoxyoctadecanoate, and [11,12-$^2H_2$]-Ethyl-(*E*)-11,12-epoxyoctadecanoate to Cultures of *Sporidiobolus salmonicolor* [chiral GC–MS coupling (capillary column: Lipodex E™)][a]

	Relative amount (%)	Configuration (R) (%)	(S) (%)	ee[b] (%)
γ-Dodecalactone	d_2-Methyl-(*Z*)-9,10-epoxyoctadecanoate			
Unlabeled	54.8	42.8	57.2	14.4 (*S*)
d_1-Labeled	21.9	73.6	26.4	47.2 (*R*)
d_2-Labeled	23.3	11.3	88.7	77.4 (*S*)
	d_2-Ethyl-(*E*)-9,10-epoxyoctadecanoate			
Unlabeled	29.4	78.4	21.6	56.8 (*R*)
d_1-Labeled	12.5	95.3	4.7	90.6 (*R*)
d_2-Labeled	58.1	93.0	7.0	86.0 (*R*)
γ-Decalactone	d_2-Ethyl-(*Z*)-11,12-epoxyoctadecanoate			
Unlabeled	83.5	99.0	1.0	98.0 (*R*)
d_1-Labeled	4.5	73.0	27.0	46.0 (*R*)
d_2-Labeled	12.0	50.3	49.7	0.6 (*R*)
	d_2-Ethyl-(*E*)-11,12-epoxyoctadecanoate			
Unlabeled	63.1	98.8	1.2	97.6 (*R*)
d_1-Labeled	6.8	74.1	25.9	48.2 (*R*)
d_2-Labeled	30.1	71.2	28.8	42.4 (*R*)

[a]Lipodex E (Machery & Nagel, Düren, Germany).
[b]ee = Enantiomeric excess; see Table 2 for other abbreviation.

though there is no knowledge of α-oxidation occurring in microorganisms. Further examination is therefore still needed to explain the metabolic pathway of unusual oxygenated fatty acids.

However, besides the aspects of lactone biosynthesis, the presented results give access to a better understanding of the stereospecificity of the enzymes which are involved in the degradation of epoxyfatty acids. The stereochemical analysis of accumulated chiral lactones served as an advantageous approach for this purpose and is well established using chiral GC capillary columns with chemically modified cyclodextrin phases (37,38). The interpretation of the analytical data revealed the enantioselectivity of the epoxide hydrolase and the subsequent β-oxidation to the epoxyfatty acids. Therefore, it is worthwhile to discuss the results in detail here.

The d_1- and d_2-labeled parts of both γ-lactones were optically active, and each part was obtained having a different enantiomeric excess. These results prove the existence of at least one enantiodiscriminating step in the metabolic pathways of the racemic epoxides. Interestingly, the d_2-γ-dodecalactone synthesized from d_2-(*Z*)-9,10-epoxystearic acid had the opposite configuration [77.4% ee (*S*)] to the d_2-γ-dodecalactone isolated after adding d_2-(*E*)-9,10-epoxystearic acid [86.0% ee (*R*)]. This finding indicates that the enantioselectivity of this metabolic pathway depends on the stereochemical specificity of the epoxyhydrolase (provided that the β-oxidation enzymes involved react in the same stereochemical manner for both substrates). It seems that this enzyme depends strongly on the initial configuration (*E*- or *Z*-) of the

TABLE 4
Identified Metabolic Products of d_2-Ethyl-(*Z*)-11,12-epoxyoctadecanoate and d_2-Ethyl-(*E*)-11,12-epoxyoctadecanoate as Their Mass Spectra (as methyl esters and as silylated TMS derivatives), After Incubation of 50 mg (250 mg/L) of Each Substrate to Cultures of *Sporidiobolus salmonicolor*

Substrate	EI–MS Spectra
Products	*m/z* (relative intensity)
d_2-Ethyl-(*Z*)-11,12-epoxyoctadecanoate	
[5,6-$^2H_2$]5,6-Dihydroxydodecanoic acid	377(<1),361(3),277(6),217(2),204(71),188(66),172(18), 147(15),130(18),114(16),104(14),100(15),98(12),73(100)
[5-$^2H_1$]5-Hydroxy-6-oxododecanoic acid	302(2),286(19),274(2),204(80),172(23),158(11),130(38), 114(20),100(22),73(100)
[3,4-$^2H_2$]3,4-Dihydroxydecanoic acid	349(<1),333(1),316(<1),288(1),279(1),249(20),188(80), 146(10),104(17),98(13),73(100)
[3,4-$^2H_2$]γ-Decalactone[a]	152(<1),129(1),128(7),114(<1),113(1),111(1),110(1),86(43),85(100),55(5),43(4),41(5)
[2-$^2H_1$]2-Hydroxyoctanoic acid[a]	232(14),188(48),104(16),98(15),89(50),73(100)
d_2-Ethyl-(*E*)-11,12-epoxyoctadecanoate	
[11,12-$^2H_2$]11,12-Dihydroxyoctadecanoic acid	461(<1),445(1),391(<1),361(6),347(1),301(1),288(60),271(4), 188(78),147(21),130(12),104(12),73(100)
[5,6-$^2H_2$]5,6-Dihydroxydodecanoic acid	377(<1),361(3),277(6),217(2),204(71),188(66),172(18), 147(15),130(18),114(16),104(14),100(15),98(12),73(100)
[3,4-$^2H_2$]3,4-Dihydroxydecanoic acid	349(<1),333(1),316(<1),288(1),279(1),249(20),188(80), 146(10),104(17),98(13),73(100)
[3,4-$^2H_2$]γ-Decalactone[a]	152(<1),129(1),128(8),114(<1),113(1),111(1),110(1),86(47),85(100),55(6),43(4),41(5)
[2-$^2H_1$]-2-Hydroxyoctanoic acid[a]	232(14),188(48),104(16),98(15),89(50),73(100)

[a]Occurred accompanied with its unlabeled isotopomer; TMS, trimethylsilyl. See Table 1 for other abbreviation.

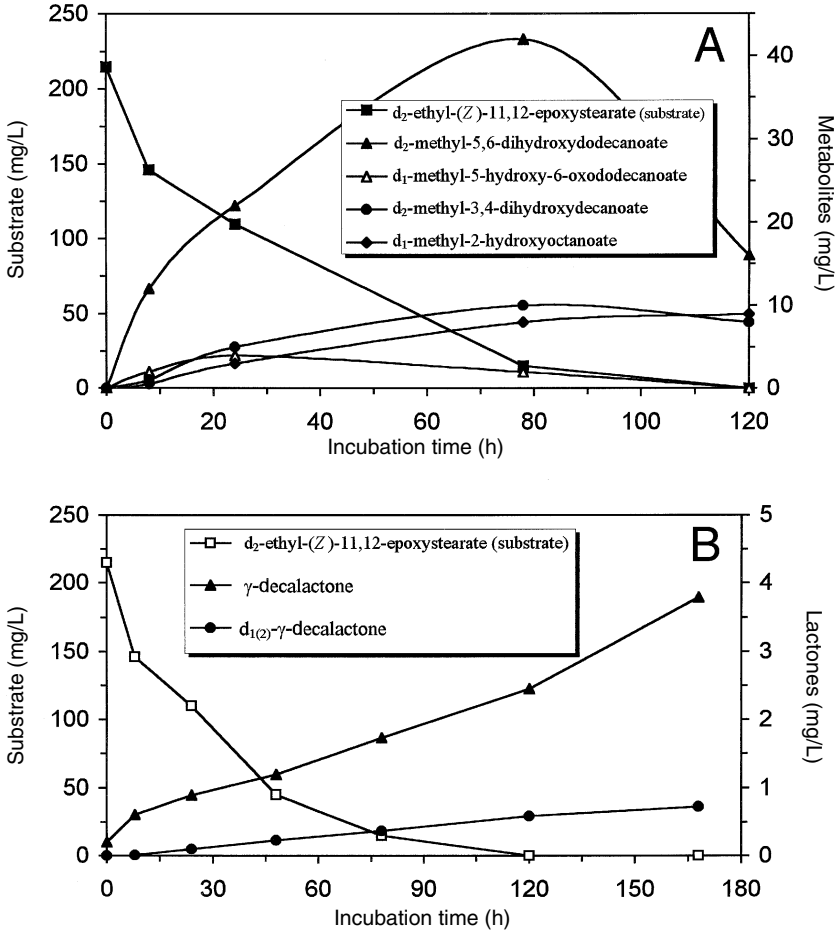

FIG. 3. Time-dependent course of the product formation after addition of 50 mg (250 mg/L) [11,12-$^2H_2$]-ethyl-(*Z*)-11,12-epoxyoctadecanoate to 250-mL shaking-flask cultures of *Sporidiobolus salmonicolor.* For (A) and (B) see Figure 1.

epoxy group in the substrates. Therefore, the epoxy groups of both enantiomers of the (*Z*)-substrate (*R*,*S*- or *S*,*R*-, respectively) were hydrolytically cleaved by a highly stereoselective attack of water at the (*R*)-9- or (*R*)-10-carbons to yield the (*S*,*S*)-9,10-dihydroxystearic acid enantiomer as intermediate. In the (*E*)-configured substrate, on the other hand, the water mainly either attacked the (*S*)-10- or the (*R*)-9-chirality center to produce the (*R*,*S*)-9,10-dihydroxystearic acid. This clearly demonstrates that in the case of the 9,10-epoxyfatty acids only one of the two stereocenters of each enantiomeric epoxy acid was attacked by a water molecule to cleave the oxirane ring (Fig. 5). Interestingly, these results are comparable with those of Schöttler and Boland (25) and Blée and Schuber (22) who found that the epoxide hydrolases of strawberries and soybeans introduce the water molecule (*S*)-specifically to racemic (*Z*)-9,10-epoxystearates. Therefore, epoxide hydrolases of different organisms seem to share common stereochemical features.

Discussing the results of the experiments with the epoxidized (*Z*)-, and (*E*)-vaccenic acids, one can say that these precursors follow the same metabolic sequence as the derivatives of oleic and elaidic acids. The 11,12-epoxystearates are also precursors for an important lactonic flavor compound in *S. salmonicolor*, the γ-decalactone. However, the analysis of the enantiomeric composition of the lactone revealed that these epoxyfatty acids are not the natural endogenous precursors of γ-decalactone. Unsupplemented cultures of *S. salmonicolor* and cultures fed with oleic acid produce exclusively the (*R*)-configured lactone [*via* an (*R*)-12-hydroxylation of oleic acid, followed by β-oxidation of the intermediate ricinoleic acid] (26). Therefore, the degradation of added epoxidized vaccenoates should be regarded as an "exogenously induced" pathway to both enantiomers of γ-decalactone in this yeast. Both biochemical pathways (*via* hydroxylation and epoxidation) to lactones should be discussed for future investigations, especially concerning the γ-decalactone biosynthesis in fruits and other microorganisms.

Nevertheless, these results obtained from the 11,12-epoxy isomers do not allow such an unambiguous interpretation of the stereochemical orientation of the metabolic pathway as for the 9,10-epoxy stearates. The d_2-γ-decalactone derived from (*Z*)-11,12-epoxystearic acid was analyzed to be nearly racemic [0.6% ee (*R*)], whereas the d_2-γ-decalactone isolated from cultured *S. salmonicolor,* fed with the (*E*)-isomer,

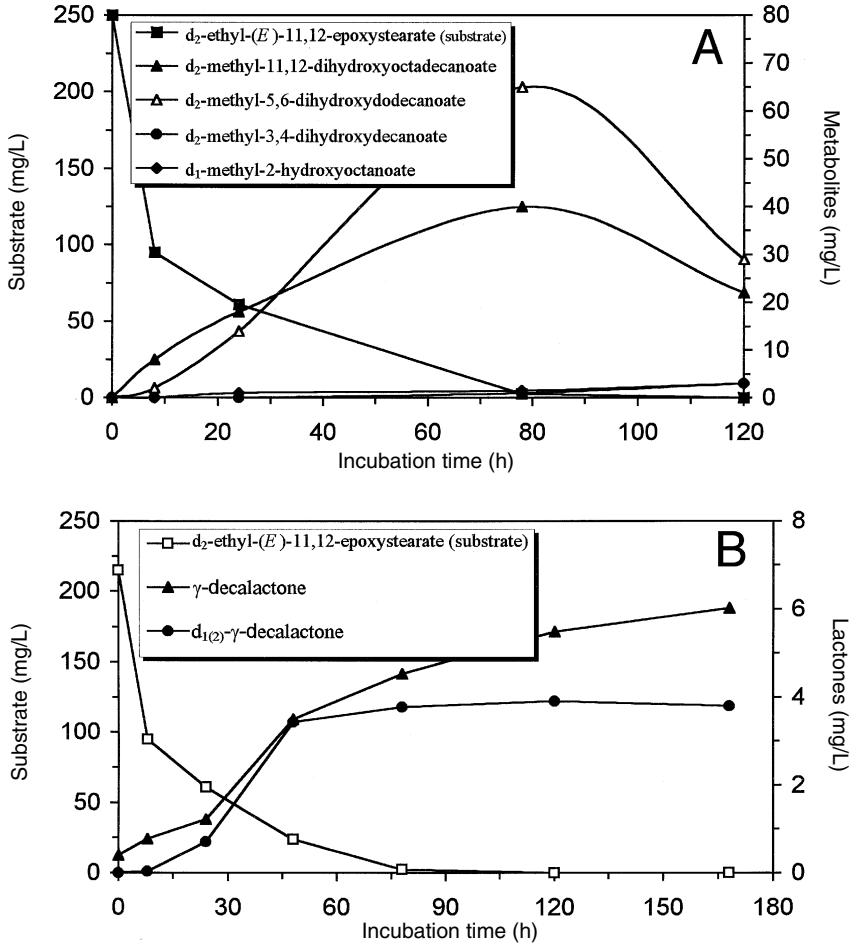

FIG. 4. Time-dependent course of the product formation after addition of 50 mg (250 mg/L) [11,12-$^2H_2$]-ethyl-(*E*)-11,12-epoxyoctadecanoate to 250-mL shaking-flask cultures of *Sporidiobolus salmonicolor*. For (A) and (B) see Figure 1.

showed only moderate optical purity [42.4% ee (*R*)]. Additional to the findings for the 9,10-epoxides, these results also clearly mark the dependency of the enantioselectivity of the epoxide hydrolase on the position of the epoxy group in the fatty acid chain.

The metabolism of the isomeric 9,10- and 11,12-epoxyoctadecanoic acids in the yeast *S. salmonicolor* is summarized in Scheme 1.

ACKNOWLEDGMENTS

The work was supported by AIF (Arbeitsgemeinschaft Industrieller Forschung), Köln, Germany. We thank Hans Köppler for his exceptional work in doing mass spectrometric analysis. We are also very grateful to Dr. R. Schäfer and Prof. Dr. D. Rewicki (Institut für Organische Chemie, Freie Universität Berlin) for their support in NMR spectroscopic analysis.

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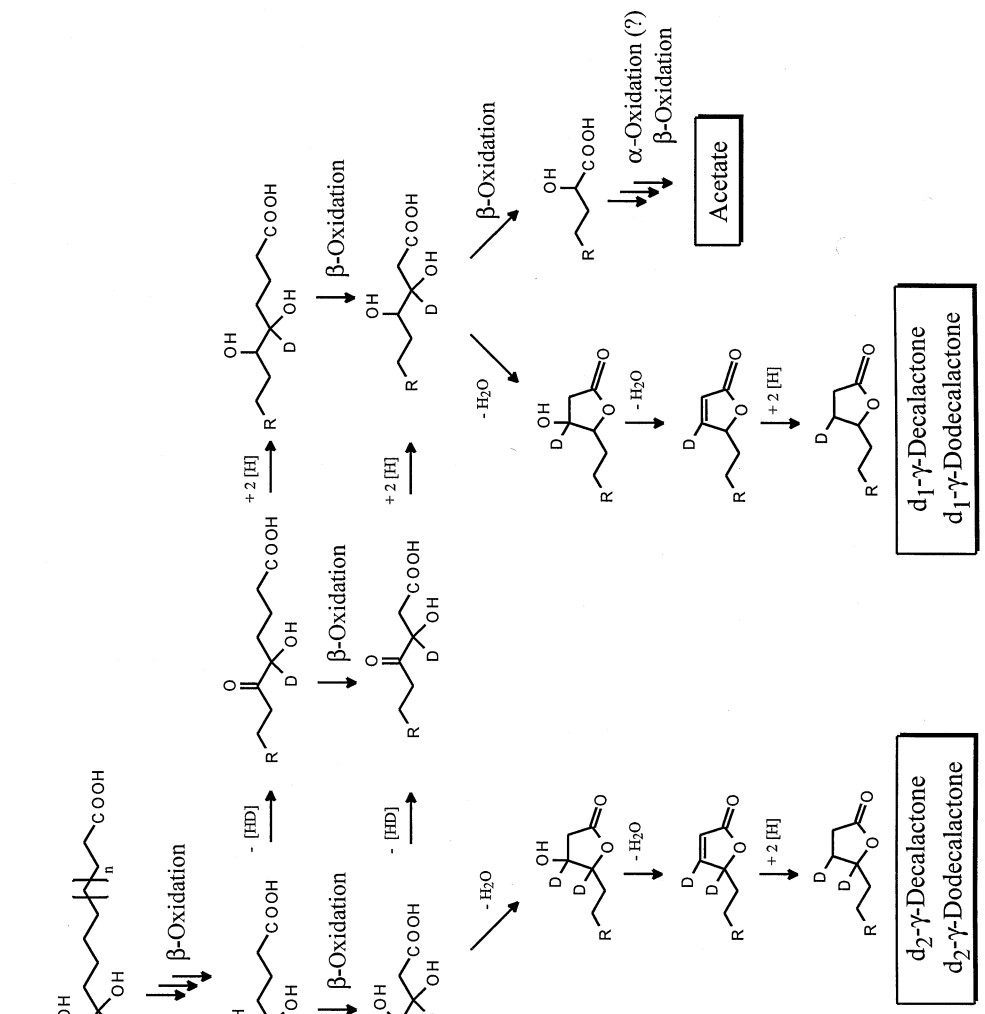
1. Epoxide Hydrolase
2. Esterase
β-Oxidation
- [HD]
+ 2 [H]
- H2O
α–Oxidation (?)
β-Oxidation
Acetate
d2-γ-Decalactone
d2-γ-Dodecalactone
d1-γ-Decalactone
d1-γ-Dodecalactone

SCHEME 1

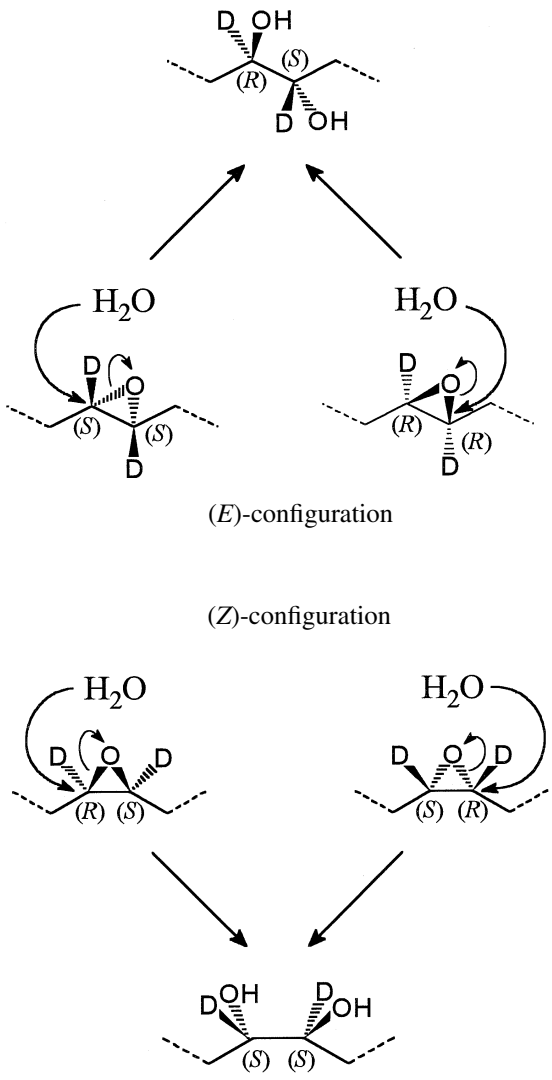

FIG. 5. Proposed stereoselectivity of the epoxide hydrolase involved in the metabolism of racemic epoxyfatty acids in *Sporidiobolus salmonicolor.*

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[Received August 1, 1997, and in final revised form December 3, 1997; revision accepted December 8, 1997]

Biosynthesis of Triacylglycerols Containing Ricinoleate in Castor Microsomes Using 1-Acyl-2-oleoyl-*sn*-glycero-3-phosphocholine as the Substrate of Oleoyl-12-hydroxylase

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ABSTRACT: We have examined the biosynthetic pathway of triacylglycerols containing ricinoleate to determine the steps in the pathway that lead to the high levels of ricinoleate incorporation in castor oil. The biosynthetic pathway was studied by analysis of products resulting from castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, the substrate of oleoyl-12-hydroxylase, using high-performance liquid chromatography, gas chromatography, mass spectrometry, and/or thin-layer chromatography. In addition to formation of the immediate and major metabolite, 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine, ^{14}C-labeled 2-linoleoyl-phosphatidylcholine (PC), and ^{14}C-labeled phosphatidylethanolamine were also identified as the metabolites. In addition, the four triacylglycerols that constitute castor oil, triricinolein, 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linolenoyl-*sn*-glycerol, were also identified as labeled metabolites in the incubation along with labeled fatty acids: ricinoleate, oleate, and linoleate. The conversion of PC to free fatty acids by phospholipase A_2 strongly favored ricinoleate among the fatty acids on the *sn*-2 position of PC. A major metabolite, 1-palmitoyl-2-oleoyl-*sn*-glycerol, was identified as the phospholipase C hydrolyte of the substrate; however, its conversion to triacylglycerols was blocked. In the separate incubations of 2-[^{14}C]ricinoleoyl-PC and [^{14}C]ricinoleate plus CoA, the metabolites were free ricinoleate and the same triacylglycerols that result from incubation with 2-oleoyl-PC. Our results demonstrate the proposed pathway: 2-oleoyl-PC → 2-ricinoleoyl-PC → ricinoleate → triacylglycerols. The first two steps as well as the step of diacylglycerol acyltransferase show preference for producing ricinoleate and incorporating it in triacylglycerols over oleate and linoleate. Thus, the productions of these triacylglycerols in this relatively short incubation (30 min), as well as the availability of 2-oleoyl-PC *in vivo*, reflect the *in vivo* drive to produce triricinolein in castor bean.

Lipids 33, 59–69 (1998).

Ricinoleate has many industrial uses; however, the only commercial source of ricinoleate, castor (*Ricinus communis* L.) bean, contains the toxin ricin and potent allergens that pose serious health hazards to growers and processors. Because of the potential danger of these components in harvest and processing, it is desirable to produce ricinoleate in an oilseed of a transgenic plant lacking these components. The cDNA for oleoyl-12-hydroxylase, the key enzyme in the biosynthesis of ricinoleate, has recently been cloned (1). Expression of this enzyme in transgenic plants resulted in low levels of hydroxy fatty acids (2) compared to castor oil (20% vs. 90% ricinoleate in castor). It is therefore important to maximize expression of the hydroxylase and optimize incorporation of ricinoleate into triacylglycerols to develop transgenic plants that produce higher levels of ricinoleate. To accomplish this, it is essential to determine which enzymatic steps drive ricinoleate into triacylglycerols. It is also crucial to determine how the oleate supply is maintained for oleoyl-12-hydroxylase, since the final oil contains 5% or less of oleate.

In order to optimize incorporation of ricinoleate into triacylglycerols in a transgenic plant, the biosynthetic pathway of triricinolein must be known. Bafor *et al.* (3) proposed the biosynthetic pathway of triricinolein from microsomal incubations of oleoyl-CoA which is rapidly incorporated into 2-oleoyl-phosphatidylcholine (PC), the proposed substrate of oleoyl-12-hydroxylase (4). We report here the identifications of some intact metabolites of 2-oleoyl-PC, including the immediate metabolite of oleoyl-12-hydroxylase, 2-ricinoleoyl-PC, free ricinoleate and triacylglycerols containing rici-

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Abbreviations: DGDG, digalactosyl diacylglycerol; FAB, fast atom bombardment; FAME, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; HPLC, high-performance liquid chromatography; 2-linolenoyl-PC, 1-acyl-2-linolenoyl-*sn*-glycero-3-phosphocholine; 2-linoleoyl-PC, 1-acyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; MGDG, monogalactosyl diacylglycerol; MS, mass spectrometry or mass spectrum; NAPE, *N*-acyl-phosphatidylethanolamine; 2-oleoyl-PC, 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; 2-ricinoleoyl-PC, 1-acyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

noleate, using high-performance liquid chromatography (HPLC) of intact lipids, demonstrating the major biosynthetic pathway to triricinolein. We also report the identification of enzymatic steps which drive ricinoleate into triacylglycerols. The key enzyme in the pathway, oleoyl-12-hydroxylase, has been characterized recently using both 2-oleoyl-PC (5) as immediate substrate and oleoyl-CoA (6) which is the acyl donor for biosynthesis of the substrate, 2-oleoyl-PC (3,4).

EXPERIMENTAL PROCEDURES

Microsomal incubation. Microsomes from castor bean were prepared as described previously (5). In order to keep the enzymes active after the microsomes were thawed for use in incubations, antipain-dihydrochloride (5 μg/mL; Boehringer Mannheim Corp., Indianapolis, IN) was added to the extraction buffer and microsome suspension buffer during microsomal preparation. The microsomal incubation mixture included in a total volume of 1 mL was: sodium phosphate (0.1 M, pH 6.3), NADH (0.5 mol), ATP (0.5 μmol), $MgCl_2$ (0.5 μmol), catalase (1000 units), and microsomal fraction of endosperm from immature castor bean (15 μL, 138 μg of protein). The radioactive substrate, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (0.125 μCi, 2.16 nmol, 58.0 Ci/mol; DuPont NEN, Boston, MA) in 20 μL of ethanol was added last into a screw- capped tube followed by immediate mixing. The mixture was then incubated in a shaking water bath for 30 min at 22°C. The incubation was stopped by addition of 3.75 mL of chloroform/methanol (1:2, vol/vol). The mixture was again mixed with 0.63 mL of chloroform and 0.63 mL of water. The lower chloroform layer containing the lipid extract was dried and fractionated on a silica HPLC system for the separation of lipid classes described here. The radioactive 2-ricinoleoyl-PC was prepared by a microsomal incubation of 2-[1-^{14}C]oleoyl-PC up to 48 times the incubation mixture given above (48 × volume). Then the PC fraction obtained was purified by the C_8 HPLC system described here to obtain radioactive 2-ricinoleoyl-PC. In the study of metabolism of free ricinoleate, radioactive ricinoleate was added to the incubation (1 h) in place of radioactive 2-oleoyl-PC as described here, and CoA (0.5 μmol) also was added to the incubation. Radioactive ricinoleate was prepared by the castor microsomal incubation of radioactive oleoyl-CoA (6), hydrolysis by sodium hydroxide, and purification by C_{18} HPLC system of free fatty acids (FFA) (7). For the purpose of identifying the fatty acid constituents of intact lipids, lipid extracts were hydrolyzed and methylated in 5% HCl/methanol (1 mL) at 80°C for 1 h. The fatty acid methyl esters (FAME) formed were extracted with 2 × 1 mL of hexane.

HPLC. Different HPLC systems were carried out as described previously (5). Radioactive lipids were separated by HPLC and identified by cochromatography with lipid standards. The flow rates of eluents of different HPLC systems were 1 mL/min. Different HPLC systems provided separations for various products as follows:

(i) Separation of lipid classes: Lipid classes were separated according to Singleton and Stikeleather (8) by a silica column (25 × 0.46 cm, 5 μm, Spherisorb S5W; Phase Separations, Norwalk, CT) with a linear gradient starting at isopropanol/hexane (4:3, vol/vol) to isopropanol/hexane/water (4:3:0.75, by vol) in 20 min, then isocratically for 15 min. A Pre-Sat silica saturation column (25 × 0.46 cm; Alltech Associates Inc., Deerfield, IL) was installed between the pump and injector to saturate the mobile phase with silica before it reached the analytical column. Free phosphatidylglycerol (PG), cardiolipin, phosphatidylinositol (PI), and phosphatidic acid standards were obtained by acidification of their salts (Sigma Chemical Co., St. Louis, MO) with HCl.

(ii) Separation of PC molecular species: PC molecular species were separated by a C_8 column (25 × 0.46 cm, 5 μm, Ultrasphere C8; Beckman Instruments Inc., Fullerton, CA) with a linear gradient of 90–100% methanol (containing 0.1% of conc. NH_4OH) in 40 min. A Pre-Sat silica saturation column was installed between the pump and injector to prevent the rapid deterioration of the C_8 column by the pH 9.5 mobile phase.

(iii) Separation of FAME: FAME were separated in the same manner as we previously described (5) using a short C_{18} column (5 × 0.46 cm, 3 μm, Microsorb MV; Rainin Instrument Co. Inc., Woburn, MA) to save time. A linear gradient of 90–100% methanol in 15 min was used for 10 min. For the purpose of identifying the FAME, a regular C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) was used (7) with a linear gradient of 90–100% methanol in 40 min.

(iv) Separation of FFA: FFA were separated as we reported previously (7) using a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) with a linear gradient of 85–100% methanol (containing 0.05% HAc as ion suppressor) in 40 min.

(v) Separation of acylglycerol molecular species: Acylglycerol molecular species (triacylglycerols and diacylglycerols) were separated by the use of a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) with a linear gradient starting at 100% methanol to 100% isopropanol in 40 min as we have reported (9). Another linear gradient (9) starting at 100% methanol to methanol/isopropanol (50:50) in 40 min was used for the identification of 1,2-dioleoyl-*sn*-glycerol.

Mass spectrometry (MS) by fast atom bombardment (FAB). Lipids were applied to the probe of a Jeol HX-110 double-focusing instrument (Tokyo, Japan). Glycerol was added to the sample to create optimal conditions for FAB ionization. FAB ionization was performed using xenon gas at 6 keV. Ions were accelerated out of the ion source at 10 kV. Both positive and negative ion acquisitions were obtained with the exception of only negative-ion acquisitions obtained for PI since this compound gave no signal in the positive mode. Mass ranges scanned were 400–1300 daltons for positive-ion and 200–1300 daltons in the negative-ion mode. Scan speed was 5 s per decade. Ion source temperature was maintained at 37°C.

Identification of FAME by gas chromatography (GC) and GC–MS. All FAME were tentatively identified and quantitated with an HP6890 gas chromatograph (Hewlett-Packard Co., San Fernando, CA) and identities were confirmed by GC–MS using an HP5970A quadrupole-based mass selective detector as previously described (10). Data were analyzed

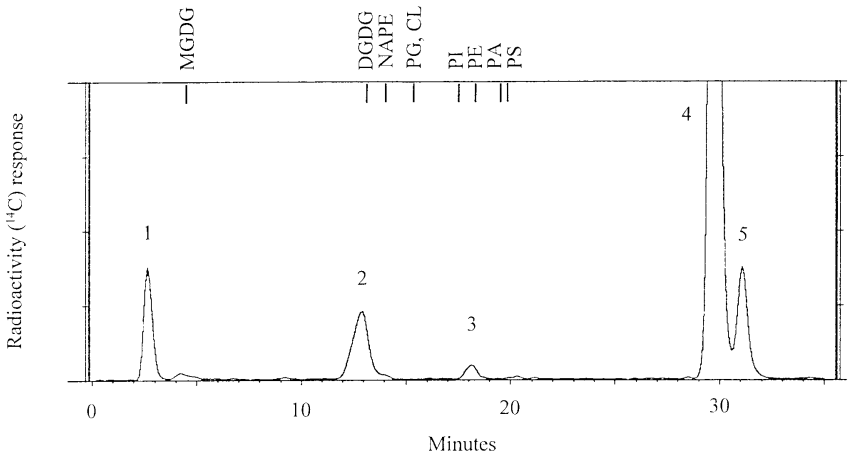


FIG. 1. Separation of lipid classes of total lipid extract from the castor microsomal incubation with 2-[^{14}C]oleoyl-phosphatidylcholine (PC) using a silica high-performance liquid chromatography (HPLC) system (see the Experimental Procedures section, HPLC, i). The radioactive peaks were: (1) acylglycerols and free fatty acids (FFA), retention time 2.7 min; (2) unknown, 13.0 min; (3) phosphatidylethanolamine (PE), 18.2 min; (4) PC, 29.8 min; (5) 2-ricinoleoyl-PC, 31.1 min. Retention times of other lipid classes in this HPLC system are also shown in the figure. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; NAPE, *N*-acyl-phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

using Hewlett-Packard MS ChemStation (DOS series) software (HP G1034C).

Thin-layer chromatography (TLC) of phospholipids and glycolipids. Phospholipids were separated by TLC on silica gel plates which were first immersed in 100 mM ammonium sulfate and dried for 1.5 h at 120°C. Samples were applied to the plates in a spotting chamber. Separation was with the solvent system 1-propanol/chloroform/propanoic acid/0.1%(wt/vol) KCl (3:2:2:1, by vol). Glycolipids were separated on a silica gel plate in the solvent system chloroform/methanol/conc. ammonia (13:5:1, by vol). Bands containing lipids to be analyzed were routinely localized by iodine staining. Lipids were identified by co-migration with standards and by specific spray reagents (11). The radioactivity was localized by autoradiography with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

RESULTS AND DISCUSSION

It is generally accepted that oleoyl-12-hydroxylase catalyzes the reaction from 2-oleoyl-PC to 2-ricinoleoyl-PC in castor bean. We have previously (5) used the putative substrate, 2-oleoyl-PC, to characterize oleoyl-12-hydroxylase in microsomes from the endosperm of immature castor bean. In the previous study (5), the enzyme activity of oleoyl-12-hydroxylase was determined by the radioactivity of total ricinoleate after methanolysis of total lipid in the incubation. In this report, the intact lipid metabolites of 2-[^{14}C]oleoyl-PC have been identified, and the incorporation of radiolabel in each metabolite has been quantified in order to follow the label through the biosynthetic pathway of triacylglycerols containing ricinoleate.

The castor microsomal incubation of 2-[^{14}C]oleoyl-PC was performed as previously described (5). Figure 1 shows the radiochromatogram of the separated lipid classes from the total lipid extract of the incubation. By using standards, the radioactive peaks in Figure 1 were identified as follows: peak 1: acylglycerols (neutral lipids) and FFA, 2.7 min (retention time), 6% of total radioactivity; peak 2: unknown, 13.0 min, 7%; peak 3: phosphatidylethanolamine (PE), 18.2 min, 1%; peak 4: PC, 29.8 min, 78%; peak 5: 2-ricinoleoyl-PC, 31.1 min, 8%. Many standards of lipid classes were used for identifications as shown in Figure 1. Radioactive peak 2 in Figure 1 corresponded to digalactosyl diacylglycerol (DGDG) or *N*-acyl-PE (NAPE) on this HPLC system; however, it did not correspond to DGDG or NAPE on TLC. DGDG is synthesized from diacylglycerols by galactosylacyltransferases located in the plastid envelope (12), and NAPE is synthesized from PE and FFA by microsomal NAPE synthase (13). We have not yet identified the radioactive peak 2 in Figure 1.

Radioactive peak 3 in Figure 1 was identified as PE (tentatively) by HPLC retention time and TLC. However, the MS of this fraction [17.0–19.0 min in Fig. 1 which included PE and phosphatidylinositol (PI)] indicated the presence of PI. The FAB(–) showed the molecular ions, M – H (834 – 1 and 836 – 1), with their relative abundances at *m/z* 833 (52%) and 835 (27%) which correspond to 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoinositol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol, respectively. Other ions shown were *m/z* 671 (11%, M – 163 inositol), 571 (18%, M – 263 linoleoyl), 299 (40%, M – palmitoyl – linoleoyl), 281 (35%, oleoyl), 279 (60%, linoleoyl), 255 (100%, palmitoyl). The MS of peak 3 did not indicate the presence of PE. PE and PI were well separated by TLC as ammonium salts [with their

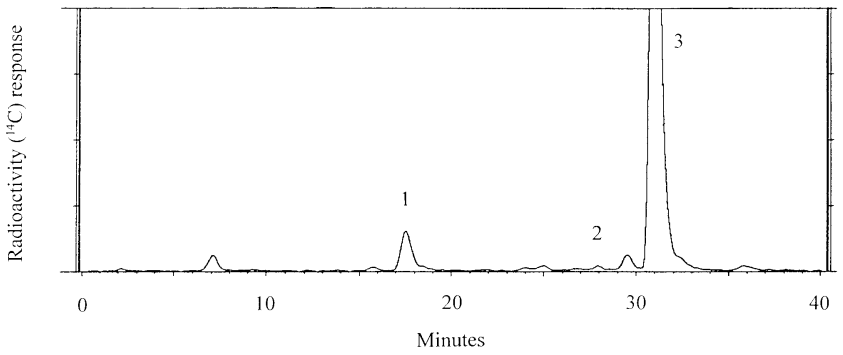


FIG. 2. Purification of 2-[^{14}C]ricinoleoyl-PC (peak 1), using a C_8 HPLC system (see the Experimental Procedures section, HPLC, ii). Radioactive peaks were: (1) 1-palmitoyl-2-ricinoleoyl-PC, 17.6 min; (2) 1-palmitoyl-2-linoleoyl-PC, 28.0 min; (3) 1-palmitoyl-2-oleoyl-PC, 31.0 min. See Figure 1 for abbreviations.

retention factor (R_f) values of 0.47 and 0.23, respectively] while HPLC did not resolve the free acids well. PE was radioactive on TLC, and its amount was not high enough to be detected by MS, while PI was not radioactive on TLC, and could be detected by MS. The radioactive peak 3 in Figure 1 thus contained radioactive PE together with unlabeled PI. Thus, the labeled 2-oleoyl-PC was metabolized in part to PE, but not to PI. The labeled PE could be derived from labeled diacylglycerols which were abundant in this incubation (as shown in Scheme 1) by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase in castor microsomes (14). The labeled PE also could be synthesized in castor microsomes by a base group exchange reaction (15). The biosynthesis of radioactive PI in this incubation was unlikely (16).

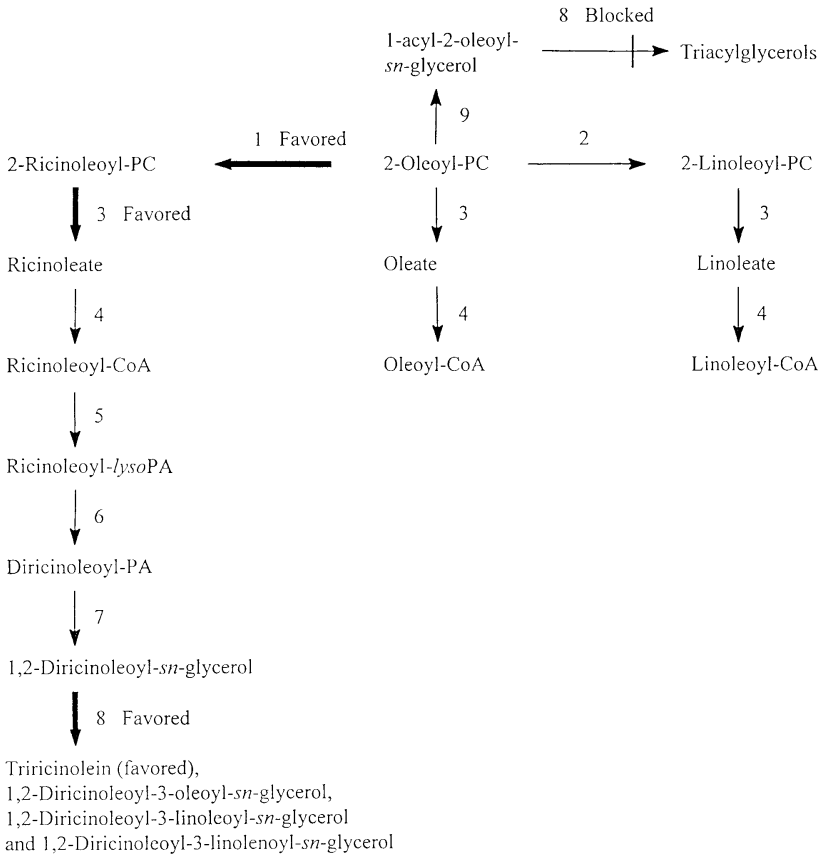
Since there was no 2-ricinoleoyl-PC standard available to us, peak 5 in Figure 1 was identified as follows. Half-minute fractions of radioactive peaks 4 and 5 in Figure 1 were collected and then hydrolyzed and methylated. HPLC of the radioactive FAME obtained showed that the radioactive peaks 4 and 5 on Figure 1 corresponded to 2-oleoyl-PC and 2-ricinoleoyl-PC, respectively. About 1% of peak 4 corresponded to radioactive 2-linoleoyl-PC. The radioactive peak 5, 2-ricinoleoyl-PC, was further purified by C_8 HPLC as shown in Figure 2. The radioactive peak 1 (19.8 min) in Figure 2 appeared to be 1-palmitoyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine by MS. A molecular weight of 776 was shown in MS of FAB(+). The MS of FAB(−) showed the characteristic negative ion of PC, 761 ($M - CH_3^-$). The hydrolysis of peak 1 in Figure 2 by phospholipase A_2 (from *Naja mocambique mocambique,* Sigma P-4034) showed the presence of free [^{14}C]ricinoleate by HPLC in the incubation product. We have thus identified 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine as the metabolite of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine by oleoyl-12-hydroxylase in castor microsomes. Since we have previously used 2-oleoyl-PC as the substrate of oleoyl-12-hydroxylase in the castor microsomal incubation (5) and we now identify its immediate metabolite, we have thus proved that 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine is the substrate of oleoyl-12-hydroxylase in castor bean. Ricinoleate was mainly or completely made at the *sn*-2 position (3).

The only metabolites (fatty acids) of oleate from the *sn*-2 position of 2-oleoyl-PC were identified previously as ricinoleate and linoleate (5). Radioactive lipid classes shown in Figure 1 were hydrolyzed and methylated, and the FAME formed were separated by HPLC on a 5-cm, C_{18} column which resolves the three labeled FAME (5). All lipid classes contained radioactive ricinoleate, linoleate, and oleate. The distribution of labeled fatty acids in each lipid class is shown in Table 1. The PC fraction shown in Table 1 was the combination of radioactive peaks 4 and 5 in Figure 1 which included 2-oleoyl-PC (the substrate), 2-ricinoleoyl-PC, and 2-linoleoyl-PC. 2-Ricinoleoyl-PC was the major immediate metabolite of 2-oleoyl-PC. In the microsomal incubation, the conversion of PC to acylglycerols (neutral lipids) and FFA strongly favored ricinoleate among the three fatty acids on the *sn*-2 position of PC as shown by others (17). The incorporation of radioactivity into PE also showed preference for ricinoleate. Thus, the biosynthesis of PE likely occurred from the radioactive 1,2-diacyl-*sn*-glycerols shown in Scheme 1 by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (14). Since 1,2-diacyl-*sn*-glycerols were enriched

TABLE 1
Radioactive Fatty Acid Composition of Lipid Classes and Acylglycerols from the Castor Microsomal Incubation of 2-[^{14}C]Oleoyl-PC[a]

Lipid classes and acylglycerols	Ricinoleate	Linoleate	Oleate
PC	6	3	91
PE	52	4	44
Unknown	6	4	90
Acylglycerols and FFA	63	7	30
FFA	73	5	22
Triricinolein	100	0	0
Diricinoleoyl-linolenoyl-glycerol	100	0	0
Diricinoleoyl-linoleoyl-glycerol	96	4	0
Diricinoleoyl-oleoyl-glycerol	93	0	7
1-Palmitoyl-2-oleoyl-*sn*-glycerol	0	0	100

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine; FFA, free fatty acid.



SCHEME 1

in ricinoleate, they were mostly formed after the enzymatic action of oleoyl-12-hydroxylase and phospholipase A_2 which provide ricinoleate for 1,2-diacyl-*sn*-glycerol. 1,2-Diacyl-*sn*-glycerols can also be formed by phospholipase C hydrolysis of the three labeled PC shown in Scheme 1, but this reaction is unlikely since 1-palmitoyl-2-oleoyl-*sn*-glycerol was identified as the major labeled metabolite (Fig. 5) while 1-palmitoyl-2-ricinoleoyl-*sn*-glycerol and 1-palmitoyl-2-linoleoyl-*sn*-glycerol were not identified. The biosynthesis of PE was unlikely from the base exchange reaction (15), incorporating free ethanolamine into PE, because the base exchange reaction would likely reflect the fatty acid composition of PC and thus not favor ricinoleate. The incorporation of label in the unknown peak 2 (Fig. 1) showed no fatty acid preference. The unknown peak 2 (Fig. 1) contained the unchanged diacylglycerol portion of PC which might be derived from PC by phospholipase C or D (18).

Radioactive FFA in the fraction of acylglycerols and FFA in Figure 1 (peak 1, 2–5 min) were identified by the C_{18} HPLC system for the separation of FFA as shown in Figure 3. Free ricinoleate, linoleate, and oleate were identified by HPLC cochromatography with the standards. The FFA represented about 23% of the radioactivity in this fraction, and the percentage radioactivity of each FFA shows that phospholipase A_2 favored release of ricinoleate (Table 1). Although the release of oleate was not favored in this study, it was much higher than that of previous studies (3,17) which showed that ricinoleate was specifically released from PC in castor microsomes.

The fraction of acylglycerols and FFA in Figure 1 (peak 1, 2–5 min) was cochromatographed with castor oil by the C_{18} HPLC system to separate molecular species of acylglycerols as shown in Figure 4. The radioactive triacylglycerols (peaks 1–4 in Fig. 4C) identified were the four major triacylglycerols in castor oil shown in Figure 4A, triricinolein (peak 1, the main triacylglycerols in castor oil, 8.5% of the total radioactivity in the fraction of acylglycerols and FFA), diricinoleoyl-linolenoyl-glycerol (peak 2, 0.8%), diricinoleoyl-linoleoyl-glycerol (peak 3, 4.0%), and diricinoleoyl-oleoyl-glycerol (peak 4, 11.9%). The four major triacylglycerols in castor oil (peaks 1–4, Fig. 4A) were identified by GC and GC–MS of the FAME after methanolysis of these peaks. The radioactive peak 5 in Figure 4C at the retention time of 2.9 min corresponded to free ricinoleate and was about 16% of the total radioactivity in the fraction of acylglycerols and FFA. The radioactive peaks of free linoleate (3.5 min) and oleate (4.5 min) were not detectable in Figure 4C, and were masked by other radioactive peaks as shown in Figure 4C.

Radioactive peak 6 in Figure 4C with retention time 17.3 min, the largest radioactive peak representing 23% of the total radioactivity in the fraction of acylglycerols and FFA (peak 1, Fig. 1), did not correspond to any acylglycerols and FFA detectable in castor oil (Fig. 4A) and castor microsomes (Fig.

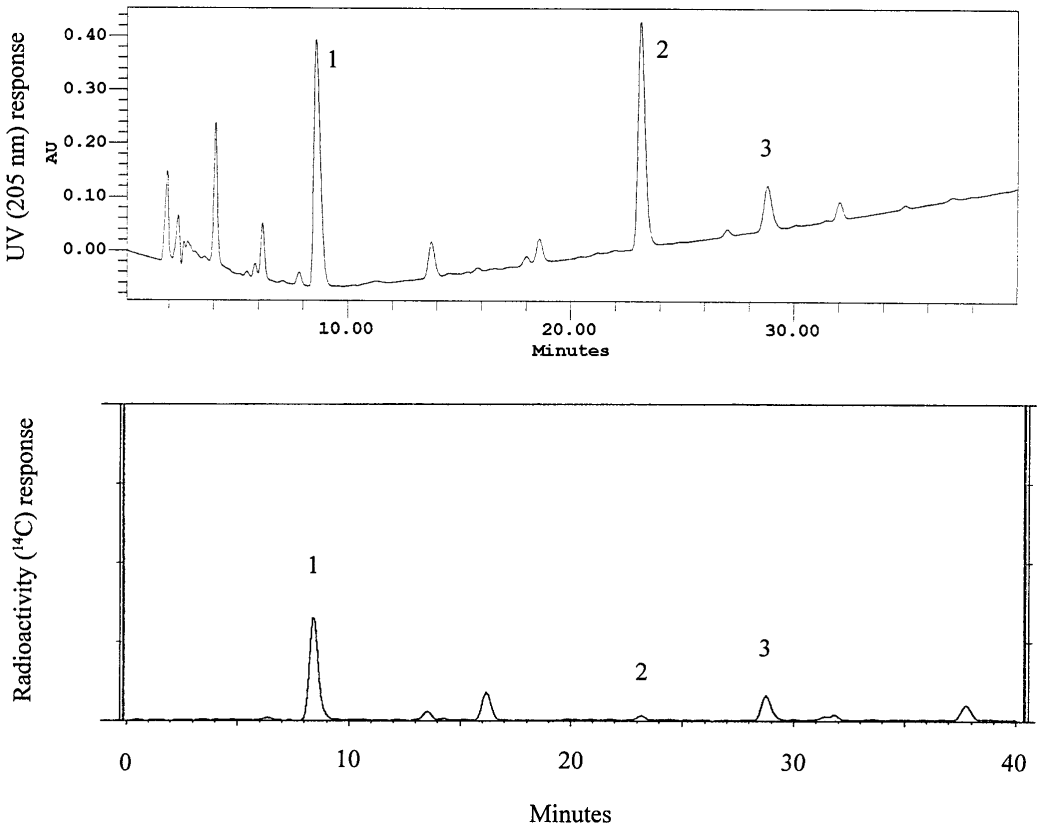


FIG. 3. HPLC identification of radioactive FFA in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, iv). The sample was cochromatographed with free ricinoleate (10 μg), linoleate (10 μg), and oleate (5 μg). The radioactive peaks were: (1) ricinoleate, 8.57 min ; (2) linoleate, 23.12 min; (3) oleate, 28.82 min. UV, ultraviolet. See Figure 1 for other abbreviations.

4B). The HPLC of the FAME of peak 6 in Figure 4C after methanolysis showed that the only radioactive fatty acid was oleate. However, the GC showed equal amounts of palmitate and oleate only. The radioactive peak 6 in Figure 4C was coeluted with 1,2-dioleoyl-*sn*-glycerol as shown in Figure 5 using the linear gradient of 100% methanol to methanol/isopropanol (50:50) in 40 min. We have recently reported the HPLC of 45 synthetic diacylglycerols and triacylglycerols (9). The relative retention times of 1,3-dioleoyl-glycerol and 1,2-dioleoyl-*sn*-glycerol were 18.67 and 18.95 min, respectively, and they can be resolved by this HPLC system. The relative retention time of 1,2-dipalmitoyl-*sn*-glycerol was 18.97 min and was almost exactly the same as that of 1,2-dioleoyl-*sn*-glycerol. Even though we do not have the standard of 1-palmitoyl-2-oleoyl-*sn*-glycerol, it should be coeluted with 1,2-dioleoyl-*sn*-glycerol; and the radioactive peak 6 of Figure 4 was identified as 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol, the product of substrate hydrolysis by phospholipase C in castor microsomes. This labeled product also coeluted with 1,2-dioleoyl-*sn*-glycerol in the HPLC system of Figure 5. We can thus infer that phospholipase C activity toward PC is present in castor bean. The neutral metabolites (triacylglycerol) of 1-palmitoyl-2-oleoyl-*sn*-glycerol which eluted after 1-palmitoyl-2-oleoyl-*sn*-glycerol were minor, if any, as shown in Figure 4C. The minor radioactive peaks in Figure 4C were not identified. The unidentified radioactive peaks in Figure 4C might be triacylglycerols and diacylglycerols containing labeled ricinoleate, oleate, and/or linoleate.

Increasingly longer microsomal incubations with 2-[1-^{14}C]oleoyl-PC, up to 2 h, showed as in Figure 6 continuous increases in the labeling of acylglycerols and FFA (peak 1 of Fig. 1, including triricinolein), of the unknown (peak 2 of Fig. 1), and of PE (peak 3 of Fig. 1). However, radioactive 2-ricinoleoyl-PC (peak 5 of Fig. 1) was maximal at 30 min. Clearly, 2-ricinoleoyl-PC is an intermediate in the metabolic pathway. To further corroborate this, microsomal incubation of the radioactive 2-ricinoleoyl-PC showed the labeled metabolites, free ricinoleate, triricinolein, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol as shown in Figure 7. Radioactive peak 6 in Figure 7 corresponded to 1-palmitoyl-2-oleoyl-*sn*-glycerol which was the same as peak 6 in Figure 4. Its origin needs to be investigated. 1-Palmitoyl-2-oleoyl-*sn*-glycerol was the hydrolyte of 2-oleoyl-PC by phospholipase C in the incubation.

Incubation with radioactive free ricinoleate also showed the incorporation of ricinoleate into triacylglycerols. The HPLC

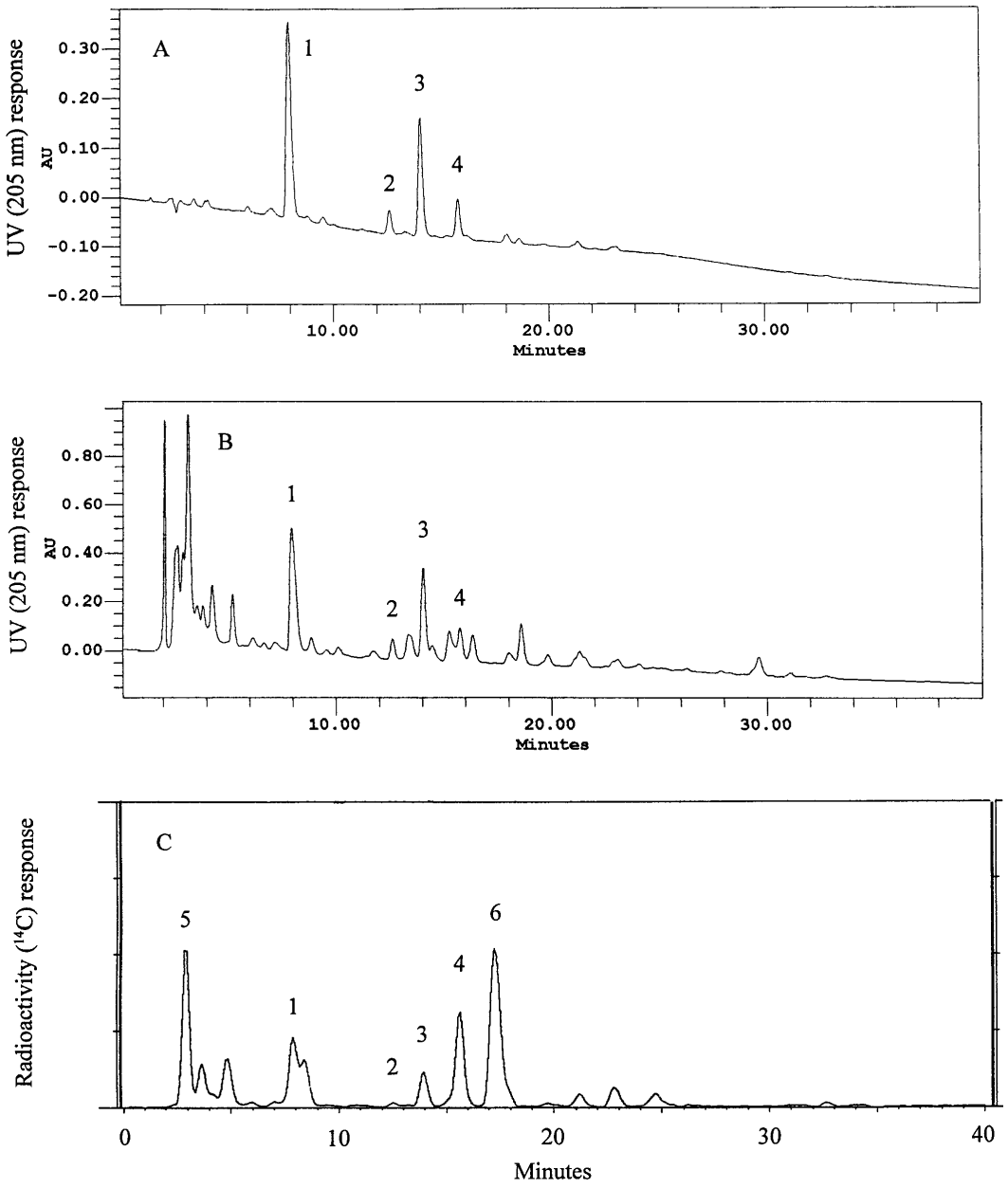


FIG. 4. HPLC identification of radioactive triacylglycerols in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. Peak 1, triricinolein; peak 2, diricinoleoyl-linolenoyl-glycerol; peak 3, diricinoleoyl-linoleoyl-glycerol; peak 4, diricinoleoyl-oleoyl-glycerol. Figure 4A was the HPLC profile of castor oil (80 µg) which shows the retention times of triacylglycerols as: (peak 1) 7.94 min, (2) 12.62 min, (3) 14.05 min, (4) 15.79 min. Figure 4B was the HPLC profile of the fraction of acylglycerols and FFA of the lipid extract from the incubation (peak 1, Fig. 1) which was cochromatographed with castor oil (80 µg). The retention times were: (peak 1) 7.95 min, (2) 12.61 min, (3) 14.04 min, (4) 15.75 min. Figure 4C was the radiochromatogram of the same HPLC run as Figure 4B. The retention times obtained from the radioactive flow detector were: (peak 1) 7.9 min, (2) 12.6 min, (3) 14.0 min, (4) 15.7 min. Peak 5, free ricinoleate (2.9 min); peak 6, 1-palmitoyl-2-oleoyl-sn-glycerol (17.3 min). See Figures 1 and 3 for abbreviations.

of the fraction of acylglycerols and FFA cochromatographed with castor oil (Fig. 8) showed the identifications of radioactive triricinolein, 1,2-diricinoleoyl-3-linolenoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol. However, radioactive peak 6 of Figure 4C (1-palmitoyl-2-oleoyl-*sn*-glycerol, hydrolyte of the substrate 2-oleoyl-PC by phospholipase C) was not present in Figure 8 as expected. No incorporation of free ricinoleate into triacylglycerols was observed in the incubation without the addition of CoA. It is interesting that the incorporation of exoge-

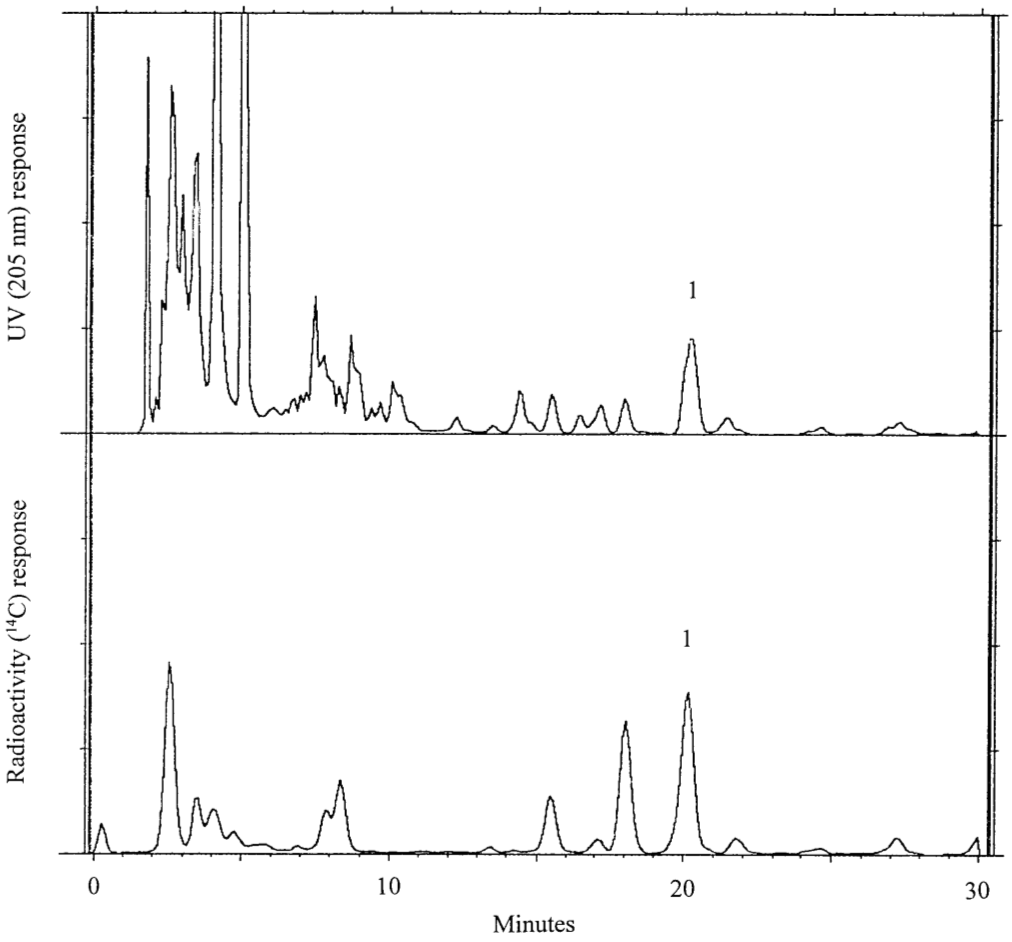


FIG. 5. HPLC identification of radioactive 1-palmitoyl-2-oleoyl-*sn*-glycerol in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). The linear gradient was 100% methanol to methanol/isopropanol (50:50) in 40 min. The sample was cochromatographed with 1,2-dioleoyl-*sn*-glycerol (50 μg), and its retention time (20.2 min, peak 1 of UV chromatogram) was the same (20.2 min) as that of peak 1 of the radiochromatogram. Both the UV chromatogram and radiochromatogram were from the printer of radioactivity flow detector. See Figures 1 and 3 for abbreviations.

nous free ricinoleate into triacylglycerols in the incubation required CoA while the incorporation of ricinoleate from 2-ricinoleoyl-PC or 2-oleoyl-PC into triacylglycerols does not. It is possible that lipids derived from these substrates participate in acyl exchange reactions as recently described (19). Endogenous CoA was available for the activation of ricinoleate formed from 2-ricinoleoyl-PC (Scheme 1) while it was not available for exogenous ricinoleate. We have previously added CoA to the incubation (5), and it has not made a difference in the total ricinoleate-containing lipids produced. The radioactivity ratio of peaks 2, 3, and 4 in Figure 8 was similar to the ratio of peaks 2, 3, and 4 of castor oil of Figure 4A. The ratio probably reflected the substrate (acyl CoA) specificity of the diacylglycerol acyltransferase (step 8, Scheme 1) and/or the availabilities of acyl CoA in castor endosperm *in vivo*. The radioactivity of triricinolein (peak 1, Fig. 8) was lower than those of peaks 3 and 4 in Figure 8 and was probably due to the depletion of ricinoleoyl CoA used in the biosynthesis of 1,2-diricinoleoyl-*sn*-glycerol in the incubation. The radioactive peak 6 in Figure 8 has not been identified yet and might be 1,2-diricinoleoyl-*sn*-glycerol, the intermediate in the pathway (Scheme 1), according to the elution characteristic of acylglycerols (9). The possible labeled 2-ricinoleoyl-PC (identical relative retention time) was also shown in the total lipid extract in the radiochromatogram (not shown) using the HPLC system of Figure 1. The incorporation of [^{14}C]ricinoleoyl-CoA into triacylglycerols in castor microsomes previously has been reported (3,20). The incorporation of the ammonium salt of [^{14}C]ricinoleate into triricinolein, diricinoleoyl-acyl-glycerols, ricinoleoyl-diacyl-glycerols, and ricinoleoyl-acyl-glycerols has also been recently reported in castor endosperm *in vivo* (20). We have shown the phospholipase A_2 hydrolysis of 2-ricinoleoyl-PC and the incorporation of ricinoleate from 2-ricinoleoyl-PC into triacylglycerols in castor microsomes, thus demonstrating the pathway *in vitro:* 2-oleoyl-PC → 2-ricinoleoyl-PC → ricinoleate → triricinolein.

The major biosynthetic pathway from 2-oleoyl-PC to triacylglycerols containing ricinoleate is shown in Scheme 1.

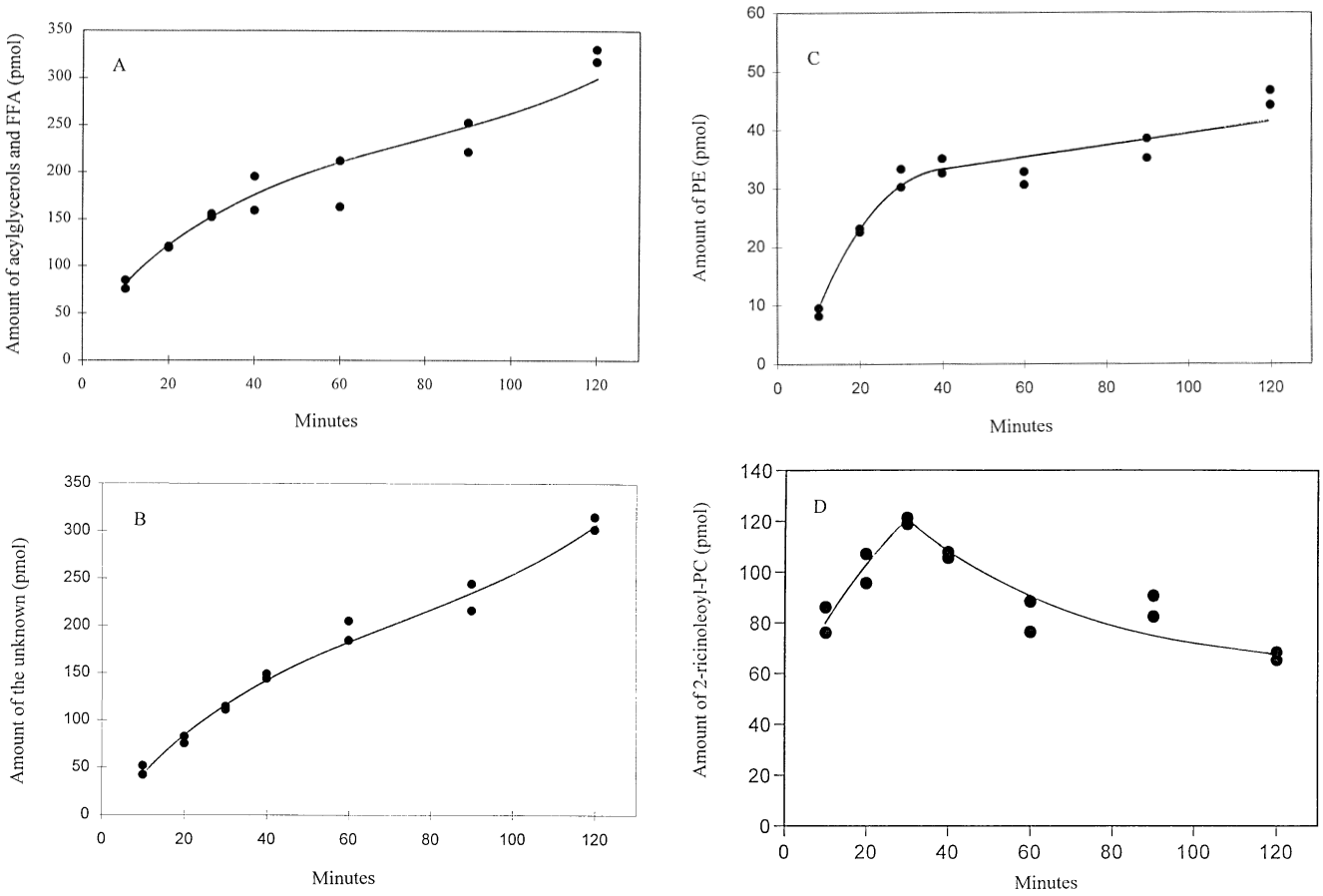


FIG. 6. Amounts of lipid classes shown in Figure 1 formed at various times of castor microsomal incubations with 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine. Units for y-axes are the pmol of labeled fatty acids in each lipid class derived from [^{14}C]oleate of the substrate. Figure 6A, acylglycerols and FFA (including triricinolein); Figure 6B, the unknown; Figure 6C, PE; Figure 6D, 2-ricinoleoyl-PC. See Figure 1 for abbreviations.

Step 1: oleoyl-12-hydroxylase, by cytochrome b_5, NADH, O_2, ATP (21). This step is favored between steps 1 and 2. Step 2: oleoyl-12-desaturase, by cytochrome b_5, NADH, O_2, ATP (21). Step 3: phospholipase A_2. Step 4: acyl-CoA synthetase, by ATP, CoA. Step 5: glycerol-3-phosphate acyltransferase, by acyl-CoA. Step 6: lyso-phosphatidic acid acyltransferase. Step 7: phosphatidic acid phosphatase. Step 8: diacylglycerol acyltransferase, by acyl CoA, blocked (completely or partially) when the substrate contains no ricinoleate, in favor of the formation of triricinolein. Because of the block, 1-acyl-2-oleoyl-*sn*-glycerol was accumulated. The conversion of 1,2-diricinoleoyl-*sn*-glycerol to PC by CDP-choline:diacylglycerol cholinephosphotransferase was blocked owing to the presence of membrane-incompatible fatty acid (22). Step 9: phospholipase C. The hydrolysis of PC at the *sn*-2 position by phospholipase A_2 favors ricinoleate as shown in Table 1. Removal of ricinoleate from PC has been explained in part by the action of phospholipid acyl hydrolases in castor microsomes that display strong preference for releasing ricinoleate from the *sn*-1 and *sn*-2 positions of PC (17). The accumulation of 1,2- diricinoleoyl-*sn*-glycerol was not significant as shown in Figure 4C, because it would elute before triricinolein (radioactive peak 1 in Fig. 4C, 7.9 min) and after ricinoleate (peak 5, 2.9 min), and the radioactive peaks at retention times of 3.7 and 4.9 min contained mostly radioactive oleate after the hydrolysis of these two peaks. The conversion from 1-palmitoyl-2-oleoyl-*sn*-glycerol to 1-palmitoyl-2-oleoyl-3-acyl-*sn*-glycerols was blocked (completely or partially) and the enzyme, diacylglycerol acyltransferase, favored the conversion of 1,2-diricinoleoyl-*sn*-glycerol to 1,2-diricinoleoyl-3-acyl-*sn*-glycerols. The diacylglycerol acyltransferase favored the formation of triricinolein and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol as shown in Figure 4C. The castor endosperm diacylglycerol acyltransferase displays a 3- to 4-fold preference for acylating 1,2-diricinoleoyl-*sn*-glycerol over 1,2-dioleoyl-*sn*-glycerol and exhibits low activity toward diacylglycerols with only one ricinoleate residue (23). In a separate study, the diacylglycerol acyltransferase showed selectivities for substrates containing ricinoleate on both 1,2-diricinoleoyl-*sn*-glycerol and ricinoleoyl-CoA (24). The accumulation of a large quantity of radioactive 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol in this incubation (Fig. 4C), in contrast to castor oil constituents (Fig. 4A), might be due to the presence of a large quantity of oleoyl-CoA in the incubation which came from the large quantity of 2-oleoyl-PC as the substrate. In this study, we have produced metabolic evidence that three enzymatic steps, oleoyl-12-hydroxylase, phospholipase A_2 and diacylglycerol acyltransferase, drive ricinoleate into triricinolein. The prolonged and

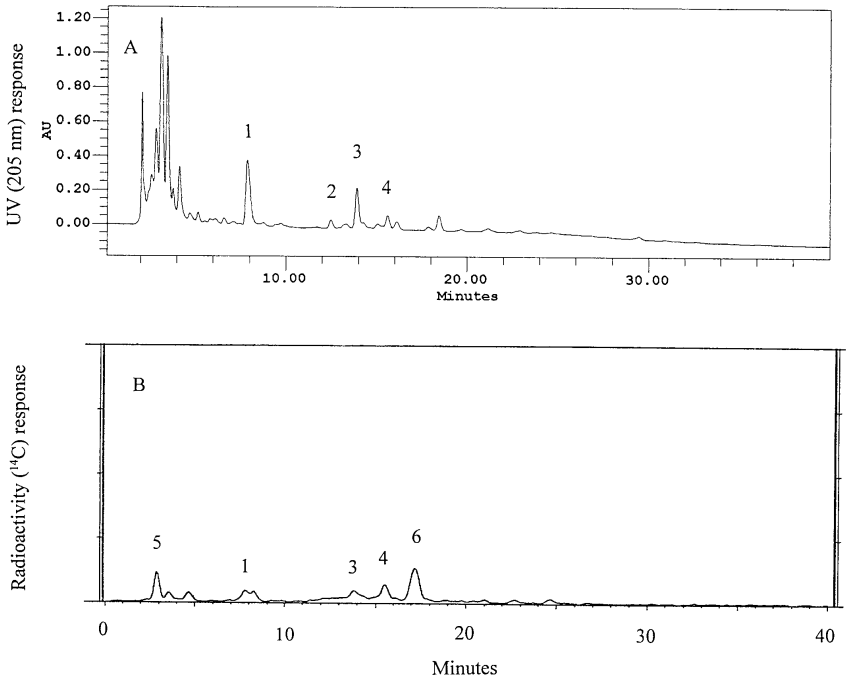


FIG. 7. HPLC identification of acylglycerols in the total lipid extract from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. The sample was cochromatographed with castor oil (80 μg). Peak 1, triricinolein (7.8 min); peak 2, diricinoleoyl-linolenoyl-glycerol (12.4 min); peak 3, diricinoleoyl-linoleoyl-glycerol (13.8 min); peak 4, diricinoleoyl-oleoyl-glycerol (15.6 min); peak 5, ricinoleate (2.9 min); peak 6, 1-palmitoyl-2-oleoyl-*sn*-glycerol (17.2 min). See Figures 1 and 3 for abbreviations.

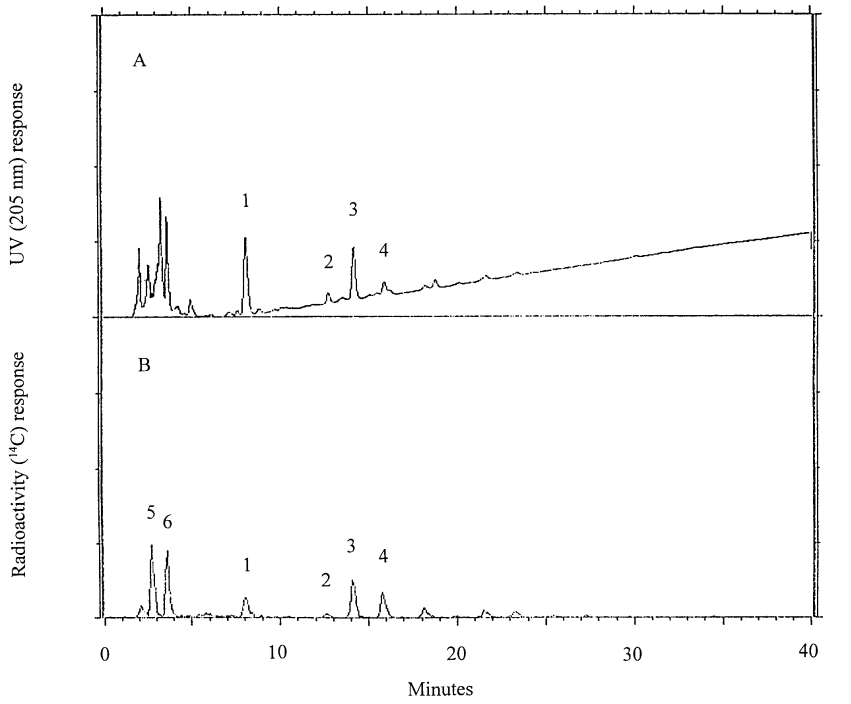


FIG. 8. HPLC identification of acylglycerols in the fraction of acylglycerols and FFA from the castor microsomal incubation of free [^{14}C]ricinoleate using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. The sample was cochromatographed with castor oil (80 μg). Peak 1, triricinolein (8.1 min); peak 2, diricinoleoyl-linolenoyl-glycerol (12.6 min); peak 3, diricinoleoyl-linoleoyl-glycerol (14.1 min); peak 4, diricinoleoyl-oleoyl-glycerol (15.8 min); peak 5, ricinoleate (2.7 min); peak 6, may be 1,2-diricinoleoyl-*sn*-glycerol (3.6 min). See Figures 1 and 3 for abbreviations.

increasing activity of oleoyl-12-hydroxylase and diminishing activity of oleoyl-12-desaturase throughout development (5) are also important factors in the production of triacylglycerols containing ricinoleate.

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[Received July 7, 1997, and in final revised form November 8, 1997; revision accepted November 26, 1997]

Studies of Lipase-Catalyzed Esterification Reactions of Some Acetylenic Fatty Acids

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ABSTRACT: Esterification of five positional isomers of acetylenic fatty acids [*viz.* 9:1(2a), 11:1(10a), 18:1(6a), 18:1(9a) and 22:1(13a)] of different chain lengths with *n*-butanol in *n*-hexane in the presence of eight different lipases [Lipozyme IM 20 (*Rhizomucor miehei*), Lipolase 100T (*R. miehei*), Novozyme 435 (*Candida antarctica*), PPL (porcine pancreatic lipase), CCL (*C. cylindracea*), PS-D (*Pseudomonas cepacia*), Lipase A-12 (*Aspergillus niger*) and Lipase AY-30 (*C. rugosa*)] was studied. 2-Nonynoic acid was not esterified except when catalyzed by the lipase from *C. antarctica* (Novozyme 435) to give 42% butyl ester after 48 h. The lipases from *A. niger* (Lipase A-12) and *C. rugosa* (Lipase AY-30) showed poor biocatalytic behavior in the esterification of the acetylenic fatty acids studied. 10-Undecynoic acid gave the highest conversion rate of esterification with each kind of lipase used. 6-Octadecynoic acid showed a marked degree of resistance to esterification carried out in the presence of *C. cylindracea* (CCL), *P. cepacia* (PS-D), or porcine pancreatic (PPL) lipase but not significantly in the presence of the lipases of *R. miehei* (Lipozyme IM 20), *R. miehei* (Lipolase 100T), or Novozyme 435. 9-Octadecynoic acid and 13-docosynoic acid were not discriminated and were readily esterified by the remaining six lipases, but when compared to oleic acid the acetylenic fatty acids were comparatively much slower in conversion to the esters.

Lipids 33, 71–75 (1998).

Lipase-catalyzed esterification of long-chain fatty acids has become common practice in laboratories for its prominent advantage over chemical methods owing to the mild conditions used and the high specificity in product formation. Numerous reports on this topic describe a large variety of enzymes, substrates and conditions employed (1–6). Knowledge of the specificity of lipases toward different types of fatty acids is essential, especially in the metabolic studies carried out *in vivo* or *in vitro*. For example, triglycerides containing short-chain fatty acids are more readily hydrolyzed by lipases than triglycerides composed of long-chain fatty acid moieties (7–9). In the hydrolysis of positional isomers of unsaturated fatty esters, substrates containing the double bond nearer to the carboxylate function (between Δ^2 and Δ^7) were found resistant to hydrolysis in the presence of porcine pancreatic lipase (PPL) with the highest discrimination found for the isomer with the double bond in the Δ^5 position of the alkyl chain (10). More recent results showed that lipases from papaya (*Carica papaya*) latex discriminate fatty esters containing a *cis*-double bond at Δ^4, Δ^6 and Δ^8, but not fatty esters with a Δ^9 double bond (11). However, the lipase from *Chromobacterium viscosum* showed preference for the Δ^6 olefinic isomer (12).

Fatty acid or ester substrates containing a Δ^9 olefin are most easily hydrolyzed or esterified by a variety of lipases (13–16). However, polyunsaturated long-chain fatty acids are often discriminated depending on their chain length and positions of the double bonds. This specificity allows particular fatty acids to be enriched using specific lipases for such processes (17–19). Most lipase studies have concentrated on saturated and olefinic fatty acids or esters. There is yet another family of unsaturated fatty acids, namely the acetylenic fatty acids, which exist in various plants, such as tariric acid (6-octadecynoic acid) (20) and stearolic acid (9-octadecynoic acid) (21), which deserve attention. The presence of stearolic acid in seed oils prompted Bu'lock and Smith (22) to postulate that C_{18} polyacetylenic fatty acids might be formed by successive dehydrogenation reactions of the fatty acid precursors (22). Acetylenic fatty acids were found to inactivate lipoxygenase (23) and stearolic acid was observed to act as a strong DNA-binding agent (24).

In view of the importance of acetylenic fatty acids in plants, this paper presents the results of enzymatic esterification reactions of some acetylenic fatty acid isomers and homologues with *n*-butanol in the presence of different lipases.

MATERIALS AND METHODS

Immobilized lipases from *Rhizomucor miehei* (Lipozyme IM 20 and Lipolase 100T) and *Candida antarctica* (Novozyme 435) were gifts from Novo Nordisk A/S (Hong Kong). Lipases from *C. cylindracea* (CCL, type VII, 905 U/mg) and PPL (type II, 179 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Lipases from *Pseudomonas cepacia* (PS-D, type I), *Aspergillus niger* (Lipase A-12), and *C. ru-*

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Abbreviations: CCL, *Candida cylindracea* lipase; NMR, nuclear magnetic resonance; PPL, porcine pancreatic lipase; PS-D, *Pseudomonas cepacia* lipase; TMS, tetramethylsilane.

gosa (Lipase AY-30) were gifts from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Methyl 2-nonynoate, oleic acid, and 10-undecenoic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Carrot seeds and high erucic acid rape seed oil were obtained from local sources.

Gas–liquid chromatographic analyses were performed on a Hewlett-Packard (Hewlett-Packard Inc., Palo Alto, CA) model 5890 instrument equipped with a flame-ionization detector and fitted with a fused-silica capillary column coated with Nukol™ (15 m, 0.53-mm diameter, 0.50-μm film thickness) or with Omegawax™ (30 m, 0.32-mm diameter, 0.25-μm film thickness) as stationary phase at a column temperature of 200°C. Silica gel (type 60, 70–230 mesh, Merck, Darmstadt, Germany) was used for column chromatography. ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained using a Brucker Avance DPX_{300} NMR spectrometer (Brucker, Fallanden, Switzerland) with the sample dissolved in deuteriochloroform and with tetramethylsilane as internal reference standard.

Preparation of acetylenic fatty acids. 2-Nonynoic acid was obtained by alkali hydrolysis of methyl 2-nonynoate. 10-Undecynoic acid was prepared from 10-undecenoic acid by the method described by Khan (25). 6-Octadecynoic acid, 9-octadecynoic acid, and 13-docosynoic acid were obtained by similar bromination and dehydrobromination reactions involving 6-octadecenoic acid from carrot seed oil and oleic acid and 13-docosenoic acid from high erucic acid rape seed oil, respectively, as described elsewhere (26).

General method for the enzymatic esterification of acetylenic acid as exemplified by the reaction of 9-octadecynoic acid with n-*butanol in the presence of* C. cylindracea *lipase.* A mixture of 9-octadecynoic acid (25 mg), *n*-butanol (0.10 mL), *n*-hexane (1.2 mL) and *C. cylindracea* lipase (25 mg) was stirred (800 rpm) at 40°C for definite periods in a glass culture tube. The reaction mixture was centrifuged, and the *n*-hexane solution was loaded on a silica gel (5.0 g) column (12-mm internal diameter). The mixture was eluted with a mixture of *n*-hexane/diethyl ether (95:5, vol/vol, 40 mL) to remove the butyl ester. The remaining fraction on the column was eluted with a mixture of *n*-hexane/diethyl ether (2:3, vol/vol, 120 mL) to isolate the unreacted acetylenic fatty acid. The solvent of each fraction was evaporated under reduced pressure and the weights of the butyl ester and free acetylenic acid were determined. The results (Fig. 1–6) represent the average of at least two determinations.

Competitive enzymatic esterification reaction of a mixture of acetylenic acids as exemplified by the reaction with a mixture of 10-undecynoic acid and 9-octadecynoic acid. A mixture of 10-undecynoic acid (30 mg) and 9-octadecynoic acid (30 mg), *n*-butanol (0.12 mL), *n*-hexane (1.6 mL) and PS-D lipase (18 mg) was stirred at 40°C for 6 or 12 h. The *n*-butyl esters and free acetylenic acids were isolated by column chromatography as described above. Yields were calculated from the weight of the butyl esters and the results of the gas–liquid chromatographic analysis of each isolated fraction. In the case where the mixture of acetylenic acids is composed of 6-octadecynoic acid and 9-octadecynoic acid, the relative amount

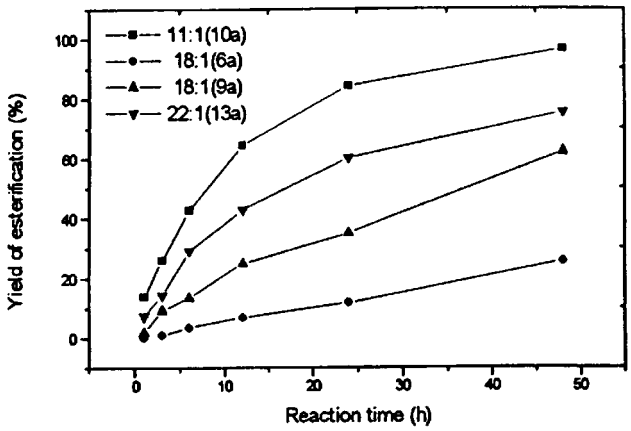

FIG. 1. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by *Candida cylindracea* lipase (25 mg) at 40°C.

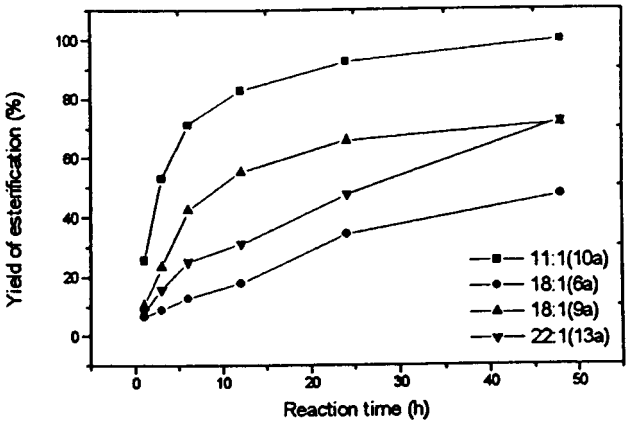

FIG. 2. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by porcine pancreatic lipase (25 mg) at 40°C.

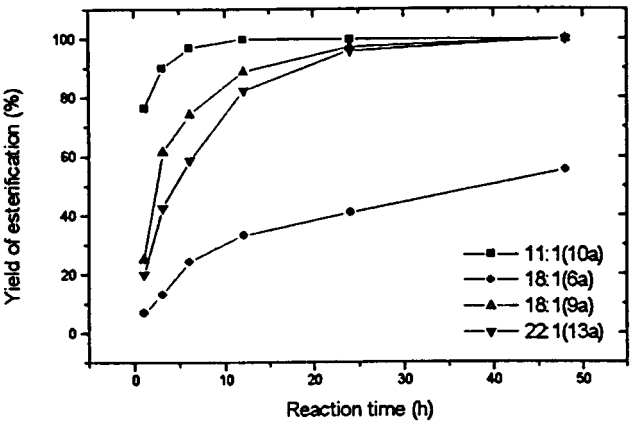

FIG. 3. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by *Pseudomonas cepacia* lipase (25 mg) at 40°C.

of each isomer in the resulting mixture of *n*-butyl esters was determined from the intensities of the pair of signals (*viz.* 34.72/34.26, C-2; 64.47/64.54, -COO*C*H_2-; 25.34/24.55, -COOCH_2*C*H_2-; of butyl esters of Δ^9/Δ^6 esters, respectively) in the ^{13}C NMR spectrum to furnish an average ratio of the

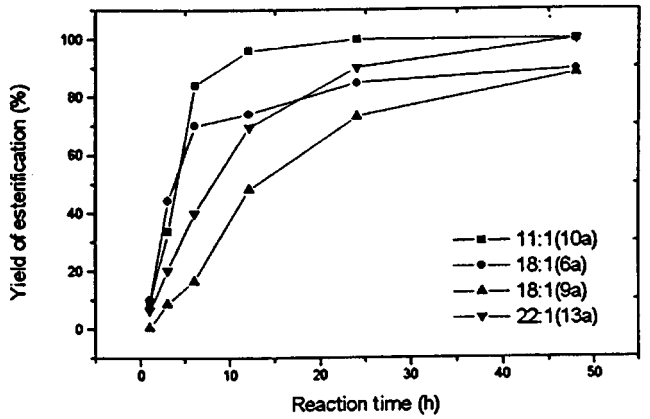

FIG. 4. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by *Rhizomucor miehei* lipase (Lipolase 100T; Novo Nordisk, Hong Kong; 25 mg) at 40°C.

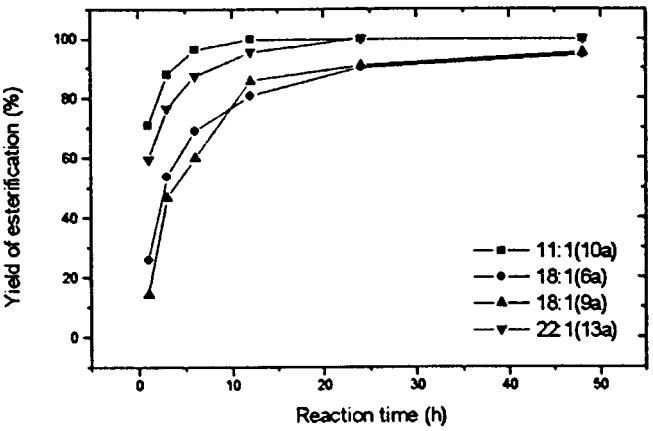

FIG. 5. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by *Rhizomucor miehei* lipase (Lipozyme IM 20; Novo Nordisk, Hong Kong; 3.6 mg) at 40°C.

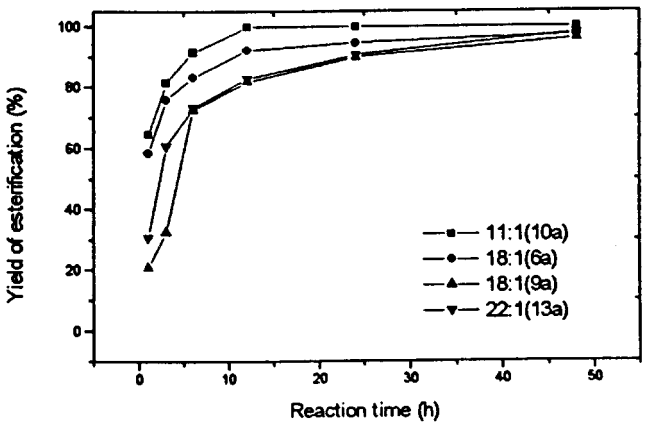

FIG. 6. Time course of esterification of acetylenic acids (25 mg) with *n*-butanol catalyzed by *Candida antarctica* lipase (Novozyme 435; Novo Nordisk, Hong Kong; 3.6 mg) at 60°C.

two positional isomers contained in the isolated butyl ester mixture.

RESULTS AND DISCUSSION

A total of eight lipases [Lipozyme IM 20 (*R. miehei*), Lipolase 100T (*R. miehei*), Novozyme 435 (*C. antarctica*), CCL (*C. cylindracea*), PPL, PS-D (*P. cepacia*), Lipase A-12 (*A. niger*), Lipase AY-30 (*C. rugosa*)] were used as biocatalysts to study the rate of esterification between *n*-butanol and five acetylenic fatty acids [*viz.* 9:1(2a), 11:1(10a), 18:1(6a), 18:1(9a), and 22:1(13a)]. (The first number denotes the chain length of the fatty acid. The number after the colon denotes the number of unsaturation, the number within the brackets indicates the position of the unsaturation, and the letter "a" stands for acetylene.)

When 2-nonynoic acid [9:1(2a)] was used as the substrate, none of the lipases used as biocatalysts furnished any butyl ester even after 48 h of contact time, except with the lipase of *C. antarctica* (Novozyme 435), which gave a 42% yield of the corresponding butyl ester. The poor performance or inactivity of this type of positional isomer with unsaturation at the Δ^2 position was similar to that previously observed in the case of the Δ^2 olefinic fatty acids (10), and also for that of 2-thia fatty acids (27). A plausible reason for such poor conversion rates was suggested to be the conjugative effect of the unsaturation center adjacent to the carboxylic acid group or, in the case of the 2-thia fatty acids, the electronic effect of the lone pair of electrons of the sulfur atom (27).

The results of enzymatic esterification of 11:1(10a), 18:1(6a), 18:1(9a), and 22:1(13a) with six different lipases for different reaction periods are graphically presented in Figures 1–6. In all six cases studied, 10-undecynoic acid [11:1(10a)] was most readily esterified. The lipases used appeared not to discriminate or have any adverse effect on the terminal acetylenic bond. It seemed that the position of the acetylenic bond from the carboxylic acid group was of greater importance than the nature of this unsaturated system in affecting the efficiency of the lipase in these reactions. 9-Octadecynoic acid [18:1(9a)] and 6-octadecynoic acid [18:1(6a)] were readily discriminated by CCL (Fig. 1), PPL (Fig. 2) and PS-D (Fig. 3), which showed that only about 40% of the 18:1(6a) was esterified after 48 h. By using Lipolase, Lipozyme or Novozyme, all four acetylenic substrates showed relatively efficient esterification rates irrespective of the position of acetylenic bond in the alkyl chain or difference in chain lengths (Fig. 4–6). Lipozyme and Novozyme were considered the most efficient enzymes for esterification where all substrates were converted to the corresponding butyl ester (>80%) within a period of 12 h. It must also be stressed that in the case of the reactions involving Lipozyme and Novozyme, the amount of catalyst used was only about 15% of the weight of the substrate, while in the other reactions the amount of lipase used was in a 1:1 ratio by weight of catalyst to substrate. The chain length of 13-docosynoic acid [22:1(13a)] did not cause much discrimination when compared to 18:1(9a), as the acetylenic bond is remotely positioned from the carboxylic function.

To compare the relative rates of esterification between oleic acid [18:1(9c)] and its acetylenic analogue [18:1(9a)], four experiments were conducted using the following lipases:

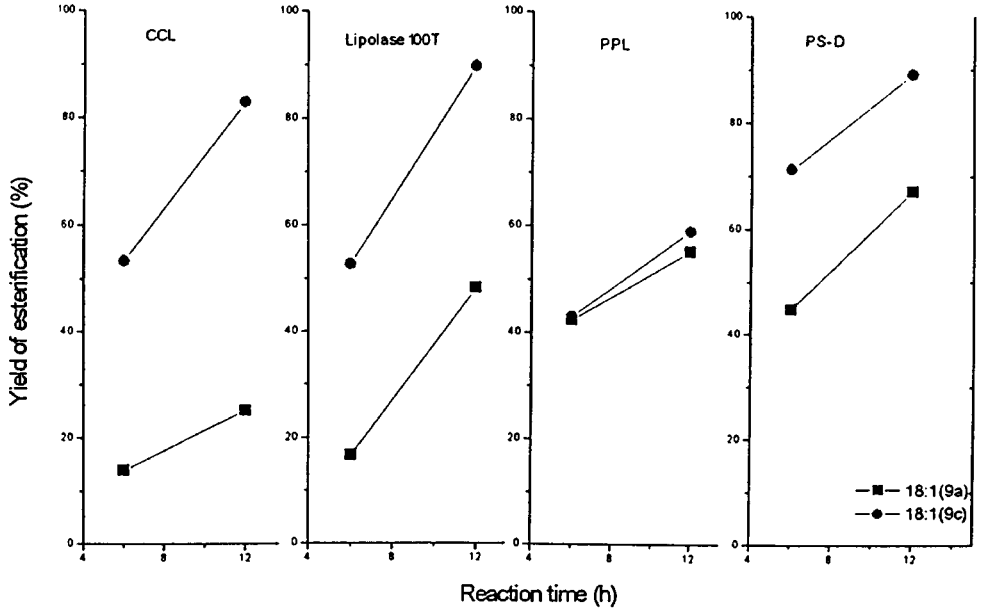

FIG. 7. Comparison of the yield of esterification of 9-octadecenoic acid and 9-octadecynoic acid catalyzed by lipases [*Candida cylindracea* (CCL), *Rhizomucor miehei* (Lipolase 100T), porcine pancreatic lipase (PPL), and *Pseudomonas cepacia* (PS-D)] at 40°C.

CCL, Lipolase 100T, PPL, and PS-D. The results are shown in Figure 7. In all instances, except for PPL, the rate of esterification of oleic acid was much faster than for the corresponding acetylenic analogue. When PPL was used, the rates of esterification for these substrates appeared to be very similar.

In view of the strong discriminating effects exhibited by PS-D, three mixtures of fatty acids (mixtures A–C) were used to test the case. Mixture A consisted of 30 mg each of 11:1(10a) and 18:1(6a). After 6 h of reaction time, the amounts of butyl esters of 11:1(10a) and 18:1(6a) were 94.4 and 11.7%; and after 12 h of reaction the yields of the butyl esters were 96.6 and 18.6%, respectively. Mixture B consisted of 30 mg each of 11:1(10a) and 18:1(9a). After 6 h of reaction time, the amounts of butyl esters of 11:1(10a) and 18:1(9a) were 97.6 and 62.3%; and after 12 h of reaction the yields of the butyl esters were 99.3 and 78.9%, respectively. These results were in good agreement with results described above for the individual isomer. Mixture C consisted of 30 mg each of 18:1(6a) and 18:1(9a). After 6 h of reaction time, the amounts of butyl ester of 18:1(6a) to that of 18:1(9a) as deduced from the relative intensities of signals from the ^{13}C NMR spectrum were in a ratio of 1:6 after 6 h and 1:4 after 12 h of reaction time. This experiment clearly showed that the PS-D was able to discriminate the fatty acid with the acetylenic bond at different positions albeit of the same chain length.

Lipase A-12 and Lipase AY-30 performed very poorly as biocatalysts. Lipase A-12 could esterify 11:1(10a), 18:1(9a) and 22:1(13a) in yields of only 5, 5, and 24%, respectively, after 48 h. Lipase AY-30 esterified the same set of fatty acids in yields of 33, 5, and 20%, respectively, after 48 h. Neither of these two lipases could esterify 9:1(2a) and 18:1(6a).

It can be concluded from these results that fatty acids containing an acetylenic bond at the Δ^2 position of the alkyl chain are very resistant to esterification by most lipases. Lipases of *A. niger* (Lipase A-12) and *C. rugosa* (Lipase AY-30) are also poor biocatalysts for esterification. Lipozyme (*R. miehei*) and Novozyme (*C. antarctica*) appeared to be the most efficient enzymes for esterification, while CCL, PPL, and especially PS-D showed a significant degree of discrimination toward positional isomers, such as 18:1(6a) and 18:1(9a).

ACKNOWLEDGMENTS

The authors thank the Lipid Research Fund, the Research Grant Council and the Research Grants Committee of the University of Hong Kong for financial assistance, and Novo Nordisk A/S (Hong Kong) and Amano Pharmaceutical Co. Ltd. (Nagoya, Japan) for samples of lipases.

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[Received June 4, 1997, and in final revised form October 27, 1997; revision accepted November 3, 1997]

Reaction of α-Tocopherol in Heated Bulk Phase in the Presence of Methyl Linoleate (13*S*)-Hydroperoxide or Methyl Linoleate

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ABSTRACT: α-Tocopherol and methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MeLOOH) were allowed to stand at 100°C in bulk phase. The products were isolated and identified as methyl 13-hydroxyoctadecadienoate (**1**), stereoisomers of methyl 9,11,13-octadecatrienoate (**2**), methyl 13-oxo-9,11-octadecadienoate (**3**), epoxy dimers of methyl linoleate with an ether bond (**4**), a mixture of methyl (*E*)-12,13-epoxy-9-(α-tocopheroxy)-10-octadecenoates and methyl (*E*)-12,13-epoxy-11-(α-tocopheroxy)-9-octadecenoates (**5**), a mixture of methyl 9-(α-tocopheroxy)-10,12-octadecadienoates and methyl 13-(α-tocopheroxy)-9,11-octadecadienoates (**6**), α-tocopherol spirodiene dimer (**7**), and α-tocopherol trimer (**8**). α-Tocopherol and 13-MeLOOH were dissolved in methyl myristate, and the thermal decomposition rate and the distributions of reaction products formed from α-tocopherol and 13-MeLOOH were analyzed. α-Tocopherol disappeared during the first 20 min, and the main products of α-tocopherol were **5** and **6** with the accumulation of **1–4** which were the products of 13-MeLOOH. The results indicate that the alkyl and alkoxyl radicals from the thermal decomposition of 13-MeLOOH could be trapped by α-tocopherol to produce **5** and **6**. The reaction products of α-tocopherol during the thermal oxidation of methyl linoleate were compounds **6** and **7**. Since the radical flux during the autoxidation might be low, the excess α-tocopheroxyl radical reacted with each other to form **7**.

Lipids 33, 77–85 (1998).

The primary products of lipid peroxidation are hydroperoxides, which can dissociate into free radicals. Lipid hydroperoxides are fairly stable at physiological temperatures, but they become susceptible to chemical change by transition metals, heat, and light (1). α-Tocopherol, an antioxidant in foods and living cells, can suppress further reactions of lipid hydroperoxides by donating hydrogen atoms to free radicals (2,3). Free radical reactions of α-tocopherol take place *via* the α-tocopheroxyl radical as intermediate (4,5). If a suitable free radical is present, a nonradical product may be formed from the coupling of the free radical with α-tocopheroxyl radical. The reaction products of α-tocopherol with lipid-peroxyl radicals have been reported to be 8a-(lipid-dioxy)-α-tocopherones (6–10). α-Tocopherol also traps alkyl and alkoxyl radicals to give alkylated products (11–16). In our previous study (16), we characterized 8a-alkyldioxy-α-tocopherones, 8a-epoxyalkyldioxy-α-tocopherones, 6-*O*-alkyl-α-tocopherols, and 6-*O*-epoxyalkyl-α-tocopherols as the reaction products of α-tocopherol during iron-catalyzed decomposition of methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MeLOOH) under aerobic and anaerobic conditions. The thermal decomposition of hydroperoxides is believed to occur *via* homolytic cleavage of the oxygen-oxygen bond to yield a hydroxyl radical and an alkoxyl radical (1). Thus, α-tocopherol can react with these radicals to stop the thermal decomposition of lipid hydroperoxides. It has been reported that α-tocopherol can suppress the thermal decomposition of methyl linoleate hydroperoxides (MeLOOH) and inhibited the formation of volatile and nonvolatile decomposition products (2,3). However, the reaction mechanisms of α-tocopherol on thermal decomposition of hydroperoxide are still unclear.

In this study, we have characterized the reaction products of α-tocopherol during the decomposition of 13-MeLOOH at 100°C under air. A reaction mechanism of α-tocopherol during the decomposition of 13-MeLOOH and the autoxidation of methyl linoleate is discussed based on the reaction products observed in bulk phase of methyl myristate.

MATERIALS AND METHODS

Materials. (2*R*,4′*R*,8′*R*)-α-Tocopherol (Type V) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by reversed-phase high-performance liquid chromatography (HPLC) before use (16). γ-Tocopheryl acetate, used as an internal standard, was prepared by the acetylation of γ-tocopherol with acetic anhydride in pyridine. Authentic samples of the oxidation products of α-tocopherol were prepared as described previously (16–18). The spirodiene dimer of α-tocopherol was synthesized following the procedure of Nelan and Robe-

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Abbreviations: CIMS, chemical ionization mass spectrometry; EIMS, electron impact mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; 13-LOOH, (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate; MeLOH, methyl linoleate hydroxy compounds; MeLOOH, methyl linoleate hydroperoxides; 13-MeLOH, methyl (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoate; 13-MeLOOH, methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate; NMR, nuclear magnetic resonance; UV, ultraviolet.

son (19). (9*Z*,11*E*)-(*S*)-13-Hydroperoxy-9,11-octadecdienoate (13-LOOH) was prepared by oxidation of linoleic acid (99% purity; Wako Pure Chemical Industries, Osaka, Japan) with soybean lipoxygenase-1 (Sigma) (20). The 13-LOOH was converted to its methyl ester with diazomethane in diethyl ether and purified by silica gel column chromatography using a mixture of hexane/diethyl ether. The purity of 13-MeLOOH was checked by normal-phase HPLC after reduction (21). The purity of 13-MeLOOH was about 90% of the total MeLOOH, the remainder being the (*E,E*)-13- and 9-isomers. Methyl myristate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and used as received. Methyl linoleate (Tokyo Chemical Industry Co.) was purified and made peroxide-free by silica gel column chromatography just before use (18).

Apparatus. HPLC was performed with a Jasco Trirotar pump (Japan Spectroscopic Co., Tokyo, Japan) equipped with a Model GP-A40 gradient programmer. A Jasco Model 875 ultraviolet (UV) detector or a Model 820-FP spectrofluorometer was used as the detector. ^{1}H (200 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Gemini 2000 FT NMR spectrometer (Varian Japan Ltd., Tokyo, Japan) using $CDCl_3$ as the solvent and tetramethylsilane as the internal standard. Mass spectra (MS) were obtained on a QP-1000 instrument (Shimadzu Co., Kyoto, Japan) operated in the electron impact (EI) mode with a 70-eV electron beam or in the chemical ionization (CI) mode with isobutane as the reagent gas. UV spectra were measured with a Jasco Ubest-30 spectrophotometer. Infrared (IR) spectra of samples in liquid film were measured on a Perkin-Elmer 2000 Fourier transform infrared spectrometer (Yokohama, Japan).

Reaction of α-tocopherol with 13-MeLOOH. A mixture of α-tocopherol (0.10 g, 0.23 mmol) and 13-MeLOOH (0.40 g, 1.2 mmol) in a test tube (1.6 cm in diameter) was incubated at 100°C for 20 min under air. The products were analyzed by normal-phase and reverse-phase HPLC. Normal-phase HPLC was done with a μBondasphere 5 μ Si-100 Å column (3.9 × 150 mm; Nihon Waters, Ltd., Tokyo, Japan) developed with hexane/diethyl ether (85:15, vol/vol) at a flow rate of 1.0 mL/min. Compounds were detected by an absorbance at 235 or 268 nm. Reversed-phase HPLC was done with a μBondasphere 5 μ C_{18} 100 Å column (3.9 × 150 mm) and a 15-min linear gradient of acetonitrile/methanol/water (10:88:2, by vol) to acetonitrile/2-propanol/hexane (30:40:30, by vol) at a flow rate of 1.0 mL/min. The eluent was monitored by an absorbance at 240 nm. The several reaction products detected by HPLC were isolated by preparative HPLC using an Inertsil Prep ODS column (10 × 250 mm; GL Sciences Inc., Tokyo, Japan) developed with methanol or methanol/ethanol (1:1, vol/vol) at 5.0 mL/min.

Time-course experiment. α-Tocopherol (2 or 10 mM) and 13-MeLOOH (40 mM) in methyl myristate solution (2 mL) were placed in a test tube (1.4 cm in diameter) and incubated at 100°C. Periodically, aliquots of the sample were withdrawn, dissolved in hexane or ethanol, and injected into HPLC. The amount of MeLOOH and its reaction products were determined by normal-phase HPLC with UV detection (3). α-Tocopherol was determined by reversed-phase HPLC with fluorescence detection (13). The reaction products of α-tocopherol were determined by reversed-phase HPLC with UV detection (18). γ-Tocopheryl acetate was used as the internal standard. Methyl linoleate (40 mM) with or without α-tocopherol in methyl myristate was also incubated in the same condition as described above.

RESULTS

Reaction products of α-tocopherol and 13-MeLOOH. A mixture of α-tocopherol and 13-MeLOOH was reacted at 100°C for 20 min, and the reaction mixture was analyzed by normal-phase and reversed-phase HPLC (Fig. 1). In normal-phase HPLC, the eluent was monitored at 235 or 268 nm. Peaks corresponding to 13-MeLOOH and its isomers, **a**–**c**, and a prod-

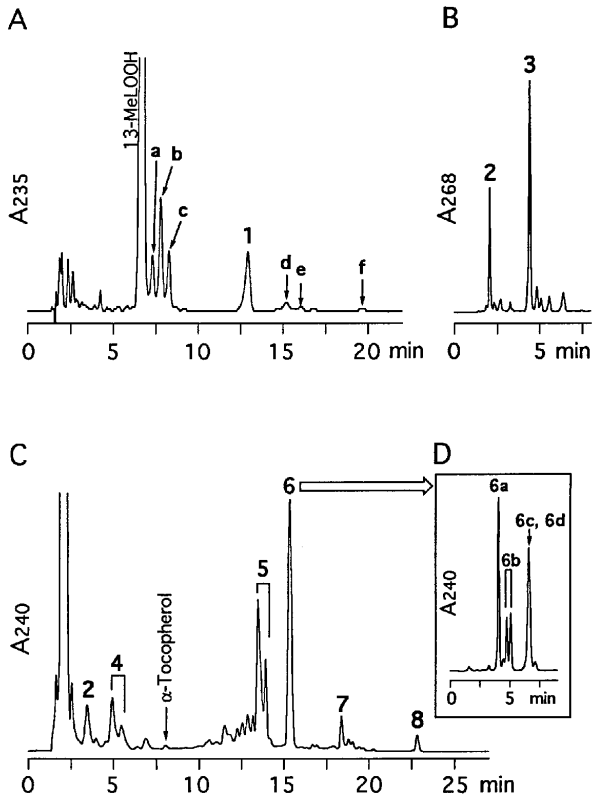


FIG. 1. High-performance liquid chromatography (HPLC) of the reaction products of α-tocopherol with methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MeLOOH) at 100°C for 20 min. Normal-phase HPLC was done with a μBondasphere 5 μ Si-100 column (3.9 × 150 mm) developed with hexane/diethyl ether (85:15, vol/vol) at a flow rate of 1.0 mL/min. The eluent was monitored at 235 (A) and 268 nm (B). Reversed-phase HPLC was done with a μBondasphere 5 μ C_{18} column (3.9 × 150 mm) which was developed with a 15-min linear gradient of acetonitrile/methanol/water (10:88:2, by vol) to acetonitrile/2-propanol/hexane (30:40:30, by vol) at 1.0 mL/min (C). The eluent was monitored at 240 nm. Peak **6** was collected and analyzed by normal-phase HPLC, which was developed with hexane/2-propanol (1000:1, vol/vol) at 1.0 mL/min (D).

uct peak **1** appeared at 235 nm (Fig. 1A); and two product peaks, **2** and **3**, appeared at 268 nm (Fig. 1B). Peaks **a**–**c** were assigned to the MeLOOH isomers by comparison of the HPLC behavior with that of the authentic samples (3,21); **a**, the (*E,E*)-13-isomer; **b**, the (*Z,E*)-9-isomer; and **c**, the (*E,E*)-9-isomer. Peak **1** was identified as methyl (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoate (13-MeLOH) based on the reference sample prepared by reducing 13-MeLOOH with $NaBH_4$ (3). In addition, other hydroxy isomers were also detected as peaks **d**, the (*E,E*)-13-isomer; **e**, the (*Z,E*)-9-isomer; and **f**, the (*E,E*)-9-isomer.

Peaks **2** and **3** were isolated by preparative reversed-phase HPLC using methanol as the solvent. Compound **2** showed absorption maxima at 258, 267, and 279 nm in ethanol, indicating the presence of conjugated triene in the molecule. The structure of compound **2** was identified as a mixture of methyl (9*Z*,11*E*,13*E*)-octadecatrienoate (**2a**) and methyl (9*E*,11*E*,13*E*)-octadecatrienoate (**2b**) from the spectral data (Table 1). Compound **3** was identified as methyl (9*Z*,11*E*)-13-oxo-9,11-octadecadienoate (3,22,23) (Table 1).

The reversed-phase HPLC gave peaks corresponding to the new reaction products, **4**–**8**, and some unknown peaks in addition to a peak corresponding to compound **2** (Fig. 1C). Peaks **4**, **5** and **6** were isolated by preparative HPLC using methanol/ethanol (1:1, vol/vol) as the solvent. Compound **4** showed absorption maximum at 234 nm in ethanol. The ^1H NMR spectrum has signals at δ 2.86 and 3.10 due to methin protons on an epoxy ring and at δ 4.35–4.43 due to two methin protons on an ether bond (Table 1). Thus, the structure of **4** was assumed to be epoxy dimers of methyl linoleate with an ether bond. Compound **4** might be produced from the reaction between the 12,13-epoxyalkyl radicals of methyl linoleate and the 13-alkoxyl radical. However, the isomeric structures of this compound were not further investigated.

Compound **5** contained two enantiomers of methyl (*E*)-(12*S*,13*S*)-9-(α-tocopheroxy)-12,13-epoxy-10-octadecenoate (**5a**) and two enantiomers of methyl (*E*)-(12*S*,13*S*)-11-(α-tocopheroxy)-12,13-epoxy-9-octadecadienoate (**5b**) by UV, IR, MS, and ^1H NMR data and coelution with the authentic samples (16). Compound **6** was identified as a mixture of methyl 9-(α-tocopheroxy)-10,12-octadecadienoate and methyl 13-(α-tocopheroxy)-9,11-octadecadienoate by UV, IR, MS, and ^1H NMR data (13,18). Compound **6** gave four peaks by normal-phase HPLC using hexane/2-propanol (1000:1, vol/vol)

TABLE 1
Spectral Data of Compounds 2–4

2	UV (ethanol) λ_{max} nm (ε)	258 (30500), 267 (40600), 279 (31700)
	IR (film) ν_{max} cm^{-1}	2926, 1742, 1464, 1440, 1371, 1254, 1200, 1171, 996, 960
	EIMS (70 eV) *m/z*	292 (M^+, 75%), 261 (10), 163 (12), 149 (34), 135 (27), 121 (25), 107 (40), 93 (100), 79 (100), 67 (62), 55 (57)
	CIMS (isobutane) *m/z*	293 ($[M + 1]^+$, 100%)
	^1H NMR[a] ($CDCl_3$) δ	0.88 (*m*, 3H, H-18), 1.30 (*br. m*, 8H, H-4,-5,-6,-17), 1.35 (*m*, 4H, H-7, H-16), 1.62 (*m*, 2H, H-3), 2.07 (*m*, 2H, H-8, H-15, **2b**), 2.09 (*m*, 1H, H-15, **2a**), 2.17 (*m*, 1H, H-8, **2a**), 2.30 (*t*, *J* = 7.5 Hz, 2H, H-2), 3.67 (*s*, 3H, OCH_3), 5.40 (*dt*, *J* = 7.5, 17.2 Hz, 1/2H, H-9, **2a**), 5.65 (*m*, 1H, H-9,H-14, **2b**), 5.69 (*dt*, *J* = 7.0, 13.7 Hz, 1/2H, H-14, **2a**), 6.07 (*m*, 3/2H, H-11, H-12, H-13, **2b**), 6.09 (*dd*, *J* = 9.1, 14.3 Hz, 1/2H, H-13, **2a**), 6.16 (*dd*, *J* = 10.6, 15.4 Hz, 1/2H, H-12, **2a**), 6.40 (*m*, 1/2H, H-11, **2a**)
3	UV (ethanol) λ_{max} nm (ε)	278 (22800)
	IR (film) ν_{max} cm^{-1}	2926, 1742, 1459, 1366, 1260, 996, 962, 870
	EIMS (70 eV) *m/z*	308 (M^+, 40%), 278 (10), 237 (7), 209 (7), 177 (27), 151 (88), 135 (28), 99 (55), 95 (58), 81 (100), 67 (55), 55 (56)
	CIMS (isobutane) *m/z*	309 ($[M + 1]^+$, 100%)
	^1H NMR ($CDCl_3$) δ	0.90 (*m*, 3H, H-18), 1.31 (*m*, 14H, H-4, H-5, H-6, H-7, H-16, H-17), 1.63 (*m*, 4H, H-3, H-15), 2.31 (*m*, 4H, H-2, H-8), 2.55 (*t*, *J* = 7.4 Hz, 2H, H-14), 3.67 (*s*, 3H, OCH_3), 5.89 (*dt*, *J* = 7.7, 10.6 Hz, 1H, H-9), 6.13 (*t*, *J* = 11.1 Hz, 1H, H-10), 6.17 (*d*, *J* = 15.4 Hz, H-12), 7.49 (*dd*, *J* = 11.4,15.3 Hz, 1H, H-11)
4	UV (ethanol) λ_{max} nm (ε)	234 (18800)
	IR (film) ν_{max} cm^{-1}	2936, 1742, 1454, 1186, 986, 894
	EIMS (70 eV) *m/z*	489 (4%), 431 (3), 309 ($[OC_{17}H_{30}COOCH_3]^+$, 24), 293 ($[C_{17}H_{30}COOCH_3]^+$, 65), 277 (20), 151 (29), 149 (36), 123 (22), 109 (32), 99 (71), 95 (67), 81 (95), 67 (66), 55 (100)
	CIMS (isobutane) *m/z*	447 (25%), 325 ($[O_2C_{17}H_{30}COOCH_3]^+$, 28), 309 ($[OC_{17}H_{30}COOCH_3]^+$, 100), 293 ($[C_{17}H_{30}COOCH_3]^+$, 85), 277 (16), 239 (20)
	^1H NMR ($CDCl_3$) δ	0.87 (*m*, 6H), 1.30 (*m*, 28H), 1.63 (*m*, 6H), 1.75–2.19 (*m*, 6H), 2.30 (*t*, *J* = 7.5 Hz, 4H), 2.86 (*m*, 1H), 3.10 (*m*, 1H), 3.67 (*s*, 6H, OCH_3), 4.35–4.43 (*m*, 2H), 5.32–6.50 (*m*, 6H)

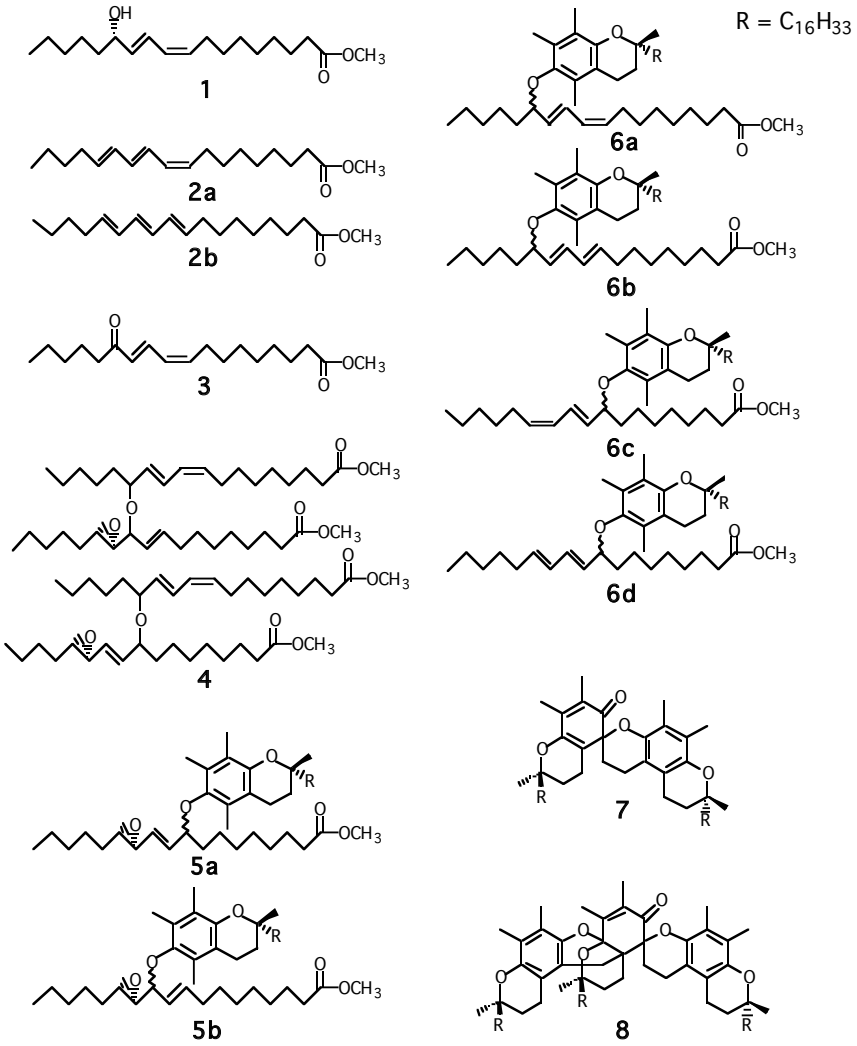
[a]Shifts in parts per million downfield relative to tetramethylsilane. Multiplicities are designated by *s*, singlet; *d*, doublet; *t*, triplet; *m*, multiplet; UV, ultraviolet; IR, infrared; EIMS, electron impact mass spectrometry; CIMS, chemical ionization mass spectrometry; NMR, nuclear magnetic resonance.

as the mobile phase (Fig. 1D), corresponding to an enantiomeric mixture of the (*Z,E*)-13-isomer (**6a**), the (*R,E,E*)- and (*S,E,E*)-13-isomers (**6b**), and a mixture of the (*E,Z*)- (**6c**) and (*E,E*)-9-isomers (**6d**) (18). Compound **7** was identified as α-tocopherol spirodiene dimer, and **8** was α-tocopherol trimer by comparison of the HPLC behavior with those of the authentic samples (17,19). Scheme 1 shows the structures of compounds **1**–**8**.

Stability of α-tocopherol. α-Tocopherol (10 mM) was dissolved in methyl myristate without or with 13-MeLOOH (40 mM) or methyl linoleate (40 mM), and incubated at 100°C (Fig. 2). Most of the α-tocopherol disappeared during the first 10-min incubation in the presence of 13-MeLOOH, and during the 6-h incubation in the presence of methyl linoleate, respectively. On the other hand, 80% of the α-tocopherol remained during the 6-h incubation without 13-MeLOOH or methyl linoleate. Thus, the free radical reaction of α-tocopherol occurred in the reaction mixture containing 13-MeLOOH or methyl linoleate.

Reaction of α-tocopherol during the decomposition of 13-MeLOOH. 13-MeLOOH (40 mM) in methyl myristate was heated at 100°C in the absence or presence of two different concentrations of α-tocopherol (2 and 10 mM). Figure 3 shows the results of the fate of α-tocopherol and 13-MeLOOH and the formation of their reaction products. α-Tocopherol at 2 mM inhibited the decomposition of 13-MeLOOH, but α-tocopherol at 10 mM accelerated the decomposition. The α-tocopherol at two concentrations disappeared during the first 20 min. The main product of α-tocopherol at 2 mM was **6**, and those at 10 mM were **5** and **6**, respectively. In addition to these products, compounds **1**–**4** accumulated in the 2 mM α-tocopherol and compounds **1**–**3** in the 10 mM α-tocopherol, respectively. Table 2 compares the relative product yields of compounds **1**–**6** from 13-MeLOOH. The reaction products from 13-MeLOOH in the 10-min reaction mixture containing 10 mM α-tocopherol could account for 96% of the consumed 13-MeLOOH.

Reaction of α-tocopherol during the autoxidation of methyl linoleate. Methyl linoleate (40 mM) in methyl myristate was incubated at 100°C in the absence or presence of α-tocopherol (2 and 10 mM). Figure 4 shows the fate of α-tocopherol and



SCHEME 1

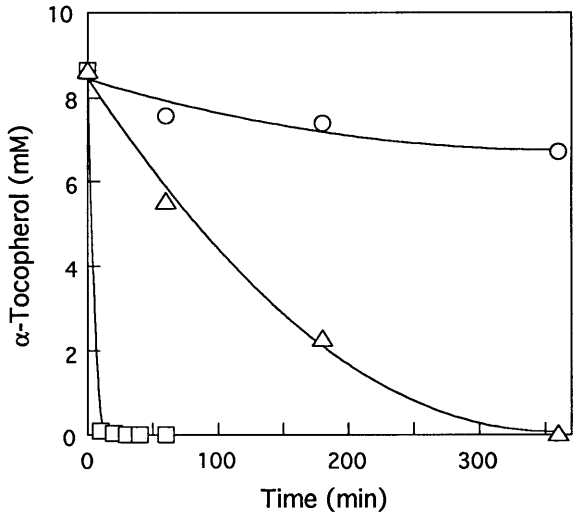


FIG. 2. Decomposition of α-tocopherol in bulk phase of methyl myristate. α-Tocopherol (10 mM) in methyl myristate without (○) or with 40 mM methyl linoleate (△) or 40 mM methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (□) was incubated at 100°C under air.

the formation of the reaction products: MeLOOH, MeLOH including **1**, conjugated triene compounds including **2**, ketodiene compounds including **3**, epoxy dimers of methyl linoleate with an ether bond including **4**, 6-*O*-alkyl-α-tocopherol (**6**), and spirodiene dimer of α-tocopherol (**7**), during the autoxidation of methyl linoleate. α-Tocopherol suppressed the formation of MeLOOH until it was consumed. The reaction products of α-tocopherol were compounds **6** and **7**. Compound **7** was the major product in the reaction mixture containing 10 mM α-tocopherol, and the ketodiene compounds also accumulated. The thermal oxidation of unsaturated lipids could result in carbon-carbon linked dimers (13,24–26) and epoxy dimers (18). However, we did not analyze the dimeric products of methyl linoleate other than the epoxy dimers of methyl linoleate.

Table 3 compares the relative product yields of α-tocopherol during the decomposition of 13-MeLOOH and the autoxidation of methyl linoleate. The reaction products from the 10 mM α-tocopherol during the decomposition of 13-MeLOOH accounted for 48% of the consumed α-tocopherol, but the reaction products during the autoxidation of methyl linoleate accounted only for 5–16% of the consumed α-tocopherol.

DISCUSSION

α-Tocopherol reacts with a variety of free radicals and stops free-radical chain reactions to form various reaction products. In the present study, the reaction products of α-tocopherol during the thermal decomposition of 13-MeLOOH were al-

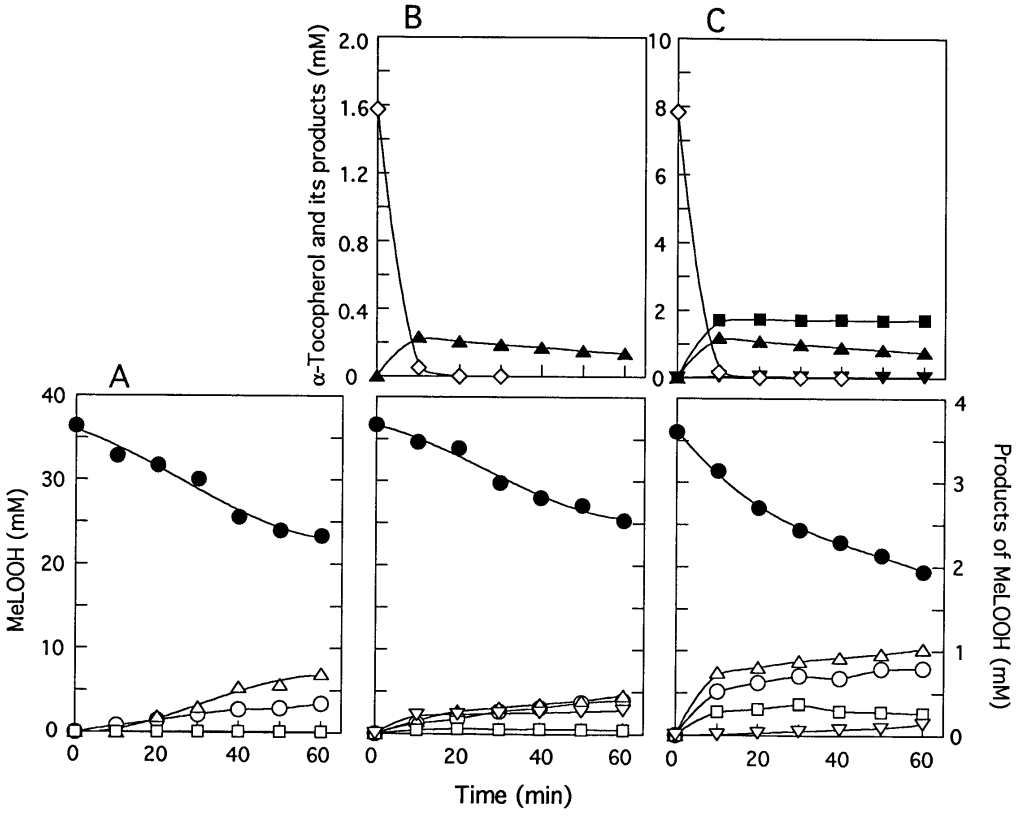


FIG. 3. Reaction of α-tocopherol during the decomposition of methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MeLOOH) in methyl myristate. Methyl myristate containing 40 mM 13-MeLOOH was incubated at 100°C in the absence (A) or presence of 2 mM (B) or 10 mM (C) α-tocopherol. Residual amounts of 13-MeLOOH (●) and α-tocopherol (◇), and the reaction products, compound **1** (○), compound **2** (□), compound **3** (△), compound **4** (▽), compound **5** (■), compound **6** (▲), and compound **7** (▼), in the reaction mixture are shown. Compounds **1–7** are identified in Scheme 1.

TABLE 2
Product Distributions from the Decomposition of 13-MeLOOH[a] After 10-min Incubation[b]

Sample	Yield (%)[c]						
	MeLOOH	**1**	**2**	**3**	**4**	**5**	**6**
13-MeLOOH (40 mM)	90.3	0.2	trace[d]	n.d.	n.d.	n.d.	n.d.
+ 2 mM α-tocopherol	94.5	0.3	0.1	0.4	0.6	n.d.	0.6
+ 10 mM α-tocopherol	87.1	1.4	0.8	2.1	trace	4.8	3.3

[a]MeLOOH, methyl linoleate hydroperoxide.
[b]These data are from the experiments described in Figure 3.
[c]Mol% to each theoretical yield based on the starting material.
[d]Trace amount (less than 0.1 mol%); n.d., not detectable.

most the same as those of α-tocopherol during the iron-catalyzed reactions between α-tocopherol and MeLOOH reported by Gardner *et al.* (14), Kaneko and Matsuo (15), and ourselves (16). The main products of α-tocopherol were 6-*O*-epoxyalkyl-α-tocopherols (**5**) and 6-*O*-alkyl-α-tocopherols (**6**) with small amounts of the dimer (**7**) and the trimer (**8**). Besides these products, we found 13-MeLOH (**1**), conjugated triene compounds (**2**), a ketodiene compound (**3**), and epoxy dimers of methyl linoleate with an ether bond (**4**) as the reaction products of 13-MeLOOH (Fig. 1 and Scheme 1).

Decomposition pathways of lipid hydroperoxides depend markedly on the reaction conditions, and several mechanisms for lipid hydroperoxide decomposition have been suggested (1,27,28). According to the bond dissociation energies, the thermal decomposition of MeLOOH may occur mainly through the alkoxyl radical (44 kcal/mol) rather than through the peroxyl radical (90 kcal/mol). Therefore, decomposition through the alkoxyl radical is considered to be an important pathway (1). Hopia *et al.* (3) have shown that the phenolic antioxidants, α-tocopherol and Trolox, at high concentrations efficiently inhibit the decomposition of MeLOOH at 60°C. They suggested that the phenolic antioxidants might markedly affect the reaction pathway involving the alkoxyl radical; they inhibit β-scission of the alkoxyl radical by hydrogen donation, and the hydrogen donation of antioxidants to the alkoxyl radical would lead to the formation of MeLOH. On the con-

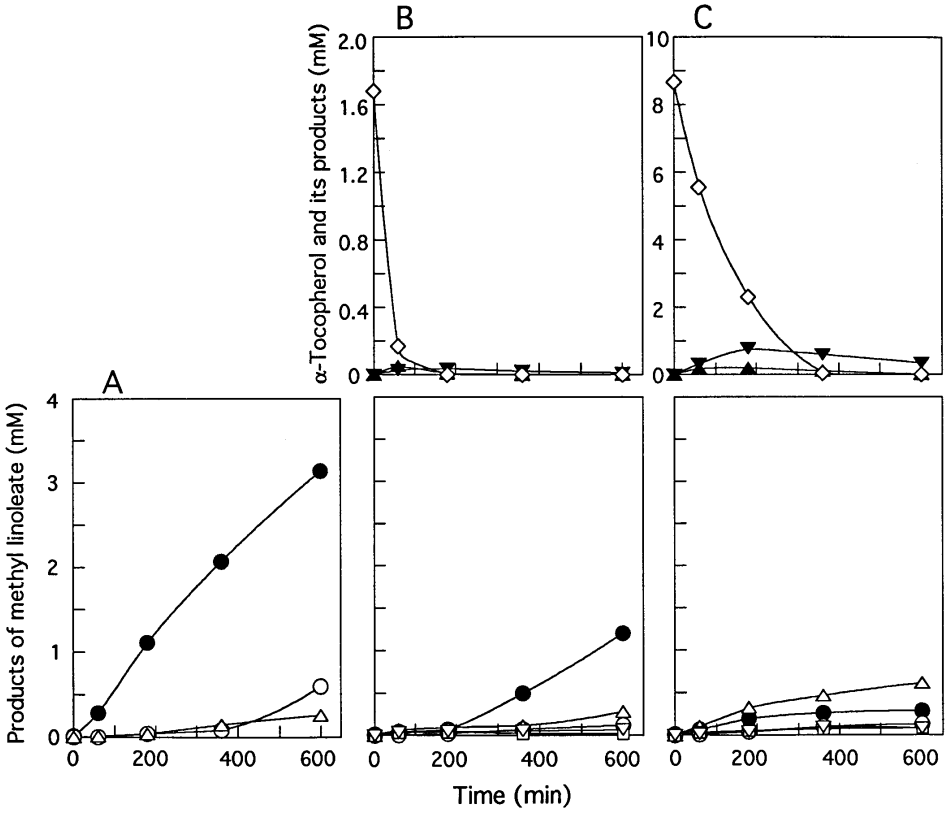


FIG. 4. Reaction of α-tocopherol during the autoxidation of methyl linoleate in methyl myristate. Methyl myristate containing 40 mM methyl linoleate was incubated at 100°C in the absence (A) or presence of 2 mM (B) or 10 mM (C) α-tocopherol. α-Tocopherol (◇), methyl linoleate hydroperoxides (●), methyl linoleate hydroxy compounds including **1** (○), conjugated triene compounds including **2** (□), ketodiene compounds including **3** (▲), methyl linoleate dimers containing two extra oxygen atoms including **4** (▽), compound **6** (▲), and compound **7** (▼) in the reaction mixture are shown. Compounds **1–7** are identified in Scheme 1.

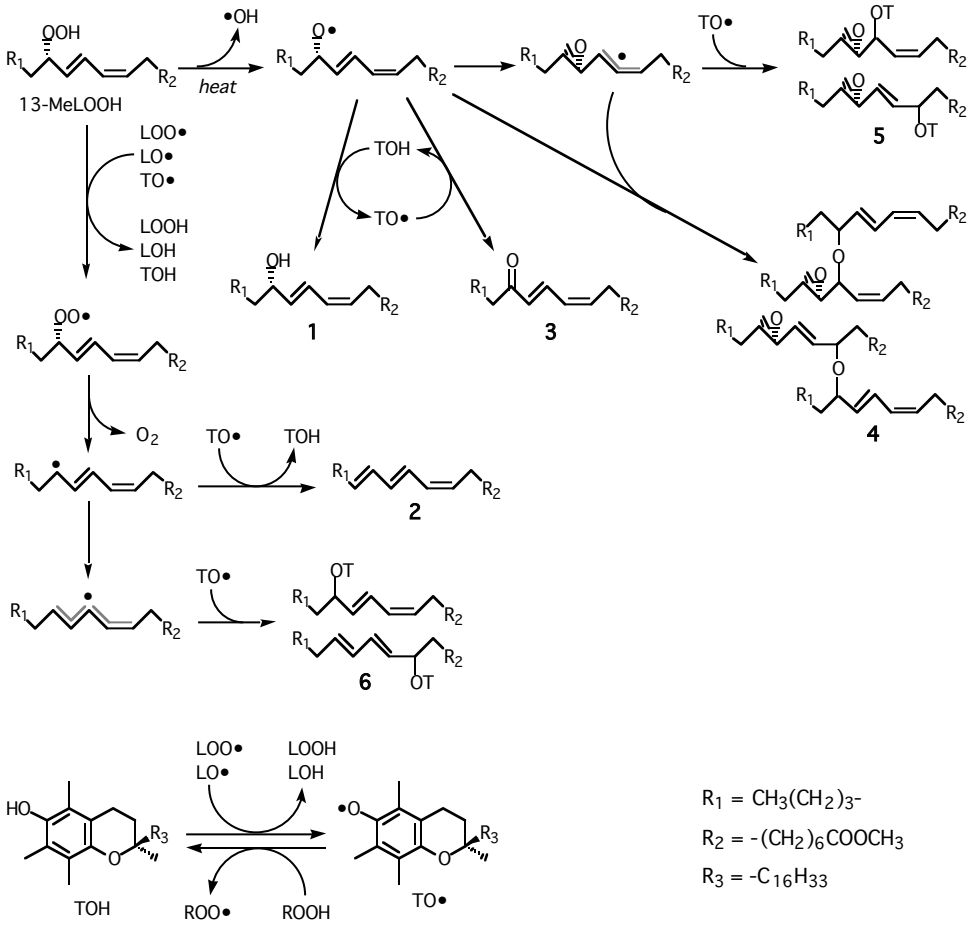
TABLE 3
Product Distributions from the Reaction of α-Tocopherol[a]

Sample	Incubation time (min)	Yield (%)[b] α-Tocopherol	**5**	**6**	**7**
In 13-MeLOOH (40 mM)					
+ 2 mM α-tocopherol	10	3.2	n.d.[c]	14.8	n.d.
+ 10 mM α-tocopherol	10	20.9	21.9	15.3	0.6
In methyl linoleate (40 mM)					
+ 2 mM α-tocopherol	60	10.1	n.d.	3.1	1.7
+ 10 mM α-tocopherol	60	64.0	n.d.	2.1	3.7
	180	26.6	n.d.	2.3	9.0

[a]These data are from the experiments described in Figures 3 and 4.
[b]Mol% to each theoretical yield based on the starting material.
[c]n.d., not detectable.

trary, the 10 mM α-tocopherol in our study accelerated the decomposition of 13-MeLOOH and produced reaction products, **1**–**6** (Fig. 3 and Table 2). Therefore, incubations at higher temperature result in the immediate loss of α-tocopherol, and the resulting α-tocopheroxyl radical may accelerate the decomposition of 13-MeLOOH.

The thermal decomposition of 13-MeLOOH and the reaction with α-tocopherol in methyl myristate are illustrated in Scheme 2 [proposed mechanism of the thermal decomposition of methyl (9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MeLOOH) and the reaction with α-tocopherol (TOH). LOOH, hydroperoxide; LOO·, peroxyl radical; LOH, hydroxy compound; LO·, alkoxyl radical; and TO·, the 8a-carbon radical of TOH]. The first reaction would be the formation of alkoxyl and peroxyl radicals of MeLOOH (1,23,27). The alkoxyl radicals generally are known for their



SCHEME 2

ability to abstract hydrogens. α-Tocopherol in the reaction mixture trapped the alkoxyl radicals by hydrogen-atom transfer, giving the α-tocopheroxyl radical and MeLOH (**1**). Because of the presence of unsaturation in the hydroperoxides, other reactions are evidently more competitive than hydrogen abstraction (1). Alkoxyl radicals of methyl linoleate tend to rearrange into epoxyallylic radicals, even in the presence of compounds with a readily abstractable hydrogen, like α-tocopherol (14–16). In the absence of oxygen, the resulting epoxyallylic radical is susceptible to a variety of radical combination reactions. In the present study, the epoxyallylic radicals reacted with the α-tocopheroxyl radical to form 6-*O*-epoxyalkyl-α-tocopherols (**5**). If the production of the tocopheroxyl radical is insufficient, the epoxyallylic radicals can react with the alkoxyl radical of 13-MeLOOH to form dimeric products. The epoxy dimers of methyl linoleate with an ether bond (**4**) appeared in the reaction mixture containing 2 mM α-tocopherol instead of the formation of 6-*O*-epoxyalkyl-α-tocopherols (Fig. 3 and Table 2). The results indicate that the alkoxyl radical rearrangement to epoxide is an important pathway in the homolysis of 13-MeLOOH by heat. The conversion of lipid hydroperoxides to the corresponding lipid ketones usually accounts for a relatively large portion of end products and they can be derived from hydroperoxides *via* alkoxyl radicals (22,23). From 13-MeLOOH, the corresponding oxodiene, methyl 13-oxo-9,11-octadecadienoate (**3**), was obtained, and high concentrations of α-tocopherol accelerated the formation of **1** and **3** (Fig. 3). Therefore, we assumed that the catalytic reactions of α-tocopherol to the alkoxyl radical might produce MeLOH and ketodiene compounds.

The formation of the peroxyl radical is the other important pathway of the decomposition of lipid hydroperoxides. Oxidation by higher oxidation states of transition metal ions is a pathway to form peroxyl radicals (27), but no metal catalyst is added in this study. The most likely process is that the peroxyl radicals might be produced from hydroperoxides by hydrogen abstraction; the free radicals, such as peroxyl, alkoxyl, and α-tocopheroxyl radicals could abstract the hydrogen atom from hydroperoxides (1). The peroxyl radicals thus formed should be trapped by the α-tocopheroxyl radical to form 8a-substituted α-tocopherones and epoxy-α-tocopherones (9,10,18). However, we could not detect such products in the present system. Since the 8a-alkyldioxy-α-tocopherones have been reported to be thermolabile (18), the produced tocopherones might immediately be decomposed at 100°C. The decomposition of these products to unidentified secondary products could account for the low recovery of α-tocopherol in terms of the products identified (Table 3). Alternatively, radical elimination of the peroxyl radicals results in the release of O_2 as a leaving group to form carbon-centered radicals (29). The formation of the carbon-centered radicals is substantiated by the finding that the isomerization of MeLOOH is accompanied by the exchange of the oxygen atoms of the hydroperoxy group with atmospheric oxygen (30). The alkyl radicals produced then react with the α-tocopheroxyl radical to form 6-*O*-alkyl-α-tocopherols (**6**) (18). In another pathway, the hydrogen atom of alkyl radical might be abstracted by the α-tocopheroxyl radical to produce the conjugated triene compounds (**2**). These results suggest that the elimination reaction of peroxyl radicals might occur during the thermal decomposition of 13-MeLOOH.

α-Tocopherol inhibited the autoxidation of methyl linoleate at 37 and 60°C and produced some reaction products including adducts of the α-tocopheroxyl radical with methyl linoleate-peroxyl radicals and methyl linoleate-alkyl radicals (18). When the autoxidation was performed at 60°C under air-insufficient conditions, the adducts of the α-tocopheroxyl radical with alkyl radicals accumulated, owing to the radical elimination of the peroxyl radicals followed by the addition of the α-tocopheroxyl radical. In the present study, α-tocopherol inhibited the formation of MeLOOH. α-Tocopherol traps the peroxyl radicals to produce hydroperoxide and α-tocopheroxyl radical (4,5). The resulting hydroperoxide is decomposed to alkoxyl radical at 100°C, and reacts with α-tocopheroxyl radical to form the ketodiene. Thus the ketodiene products accumulated in the reaction mixture at 10 mM α-tocopherol (Fig. 4). The adducts of α-tocopherol with methyl linoleate-alkyl radicals and spirodiene dimer of α-tocopherol were the other reaction products (Fig. 4 and Table 3). Because the radical flux of the autoxidation of methyl linoleate might be very low compared with that of the thermal decomposition of 13-MeLOOH, the produced α-tocopheroxyl radical reacted with each other to form the dimer as the main product.

In the present study, the main products of α-tocopherol with 13-MeLOOH were 6-*0*-epoxyalkyl-α-tocopherol (**5**) and 6-*O*-alkyl-α-tocopherol (**6**), whereas the product at 2 mM of α-tocopherol was only **6** and the epoxyalkyl radical reacted with the alkoxyl radical of 13-MeLOOH to form dimeric products (**4**). Therefore, the α-tocopheroxyl radical might prefer to react with alkyl radicals rather than epoxyalkyl radicals of polyunsaturated lipids.

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[Received August 19, 1997, and in final revised form December 21, 1997; revision accepted December 11, 1997]

Penetration and Distribution of α-Tocopherol, α- or γ-Tocotrienols Applied Individually onto Murine Skin

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ABSTRACT: To evaluate skin penetration of various vitamin E homologs, a 5% solution of either α-tocopherol, α-tocotrienol, or γ-tocotrienol in polyethylene glycol was topically applied to SKH-1 hairless mice. After 0.5, 1, 2, or 4 h (*n* = four per time point and four per vitamin E homolog), the skin was washed, the animals killed, the skin rapidly removed, frozen on dry ice, and a biopsy taken and sectioned: stratum corneum (two uppermost, 5- μm sections—SC1 and SC2), epidermis (next two 10-μm sections—E1 and E2), papillary dermis (next 100 μm, PD), dermis (next 400 μm, D), and subcutaneous fat (next 100 μm, SF). SC1 contained the highest vitamin E concentrations per μ thickness. To compare the distribution of the various vitamin E forms into the skin layers, the percentage of each form was expressed per its respective total. Most surprising was that the largest fraction of skin vitamin E following topical application was found in the deeper subcutaneous layers—the lowest layers, PD (40 ± 15%) and D (36 ± 15%), contained the major portion of the applied vitamin E forms. Although PD only represents about 16% of the total skin thickness, it contains sebaceous glands—lipid secretory organs, and, thus, may account for the vitamin E affinity for this layer. Hence, applied vitamin E penetrates rapidly through the skin, but the highest concentrations are found in the uppermost 5 microns.

Lipids 33, 87–91 (1998).

Skin is protected from oxidative stress by a variety of enzymatic and nonenzymatic antioxidants (1–5). Among these, vitamin E is generally regarded as the most important lipid-soluble antioxidant and an obvious choice for enhancement of the antioxidant protection by topical application.

Vitamin E is a generic description for all tocol and tocotrienol derivatives that qualitatively exhibit the biological activity of α-tocopherol, and is the collective name for the eight major, naturally occurring molecules, four tocopherols and four tocotrienols. Tocotrienols differ from tocopherols in that they have an isoprenoid, instead of a phytyl side chain; the four forms of tocopherols and tocotrienols differ in the number of methyl groups on the chromanol nucleus (α- has three, β- and γ- have two, while δ- has one).

α-Tocopherol is generally regarded as the most important lipid-soluble antioxidant in plasma, circulating lipoproteins, and tissues (6–10), while γ-tocopherol and the tocotrienols are present in most tissues at much lower concentrations than α-tocopherol (11–14). Remarkably, however, we found that tocotrienols represent about 15% of the vitamin E in murine skin from hairless mice fed a chow diet (15). Earlier reports suggested that *in vitro* α-tocotrienol has higher antioxidant activity than α-tocopherol against Fe^{2+}/ascorbate and Fe^{2+}/NADPH induced lipid peroxidation in rat liver microsomes (16). Therefore, we hypothesized that tocotrienols might be more effective than tocopherols against oxidative damage to skin. Our studies demonstrated that a 5% solution containing TRF (tocotrienol-rich fraction) to the skin of hairless mice protected skin from oxidative stress produced by ultraviolet light (17) or ozone (18).

Our studies using ozone suggested that the surface of skin is particularly susceptible to oxidation. When hairless mice were exposed to 10 ppm ozone for 2 h, it increased skin malondialdehyde (MDA), depleted topically applied vitamin E, but not the vitamin E inherent in the skin (18). Next we localized the ozone-caused damage to the various murine skin layers. Ozone (10 ppm for 2 h) damaged the upper epidermis, decreasing both vitamins C and E and increasing MDA concentrations (19). Notably, antioxidants in the lower layers of skin were unaffected. Therefore, we undertook assessment of the antioxidant content of the uppermost skin layer, the stratum corneum (20). Sequential tape strippings of mouse stratum corneum demonstrated that ozone exposures as low as 1 ppm for 2 h on six consecutive days depleted vitamin E, and 5 ppm increased MDA formation in stratum corneum.

Our studies suggest that skin is quite susceptible to oxidative stress and that the stratum corneum serves as a barrier to oxidative insults. Since applied vitamin E was readily de-

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Abbreviations: D, dermis; E, epidermis; MDA, malondialdehyde; PD, papillary dermis; SC, stratum corneum; SF, subcutaneous fat; TRF, tocotrienol-rich fraction.

pleted by ozone exposure, it was of interest to evaluate how well various vitamin E homologs penetrate skin. Knowledge about the topical bioavailabilities and the kinetics of absorption of a substance is essential for determination of its pharmacologic potency. The purpose of the present study was to measure the bioavailability of topically applied α-tocopherol in comparison with α-tocotrienol and γ-tocotrienol by determining the localization of each in murine skin at various time points after topical application.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest grade available. Tocopherol standards were provided by Henkel Corporation (LaGrange, IL). Dr. Asaf A. Qureshi, University of Wisconsin (Madison, WI), purified the tocotrienols from TRF that was kindly provided by PORIM (Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia).

Animals. The animal care, handling, and experimental procedures were carried out as described in the animal use protocol approved by Animal Care and Use Committee of the University of California, Berkeley. SKH-1 hairless mice (females, between 8 and 10 wk old; Charles River Laboratories, Wilmington, MA were kept under standard light and temperature conditions. Food (Harlan Teklad Rodent Diet #1846, Madison, WI) and water were provided *ad libitum*. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight, Nembutal^{R}; Abbott Laboratories, North Chicago, IL) and remained anesthetized during the entire experimental period.

Vitamin E application. A 5% wt/vol solution of each of the vitamin E homologs was prepared in polyethylene glycol-400 (PEG; Sigma, St. Louis, MO). Mice were anesthetized, then a polypropylene plastic ring (2 cm^2) was glued onto the animals' backs, and the vitamin E solution (40 µL) was applied (n = four per time point and four per vitamin E form). After 0.5, 1, 2 or 4 h, the treated area was washed as described (21). Briefly, the skin was rinsed three times rapidly with 300 µL ethanol/water (95:05) using a pipettor to flush the solution in and out of the ring surrounding the application site. Based on our previous experience with solvent extraction of skin (22), it is unlikely that the ethanol washes changed the barrier permeability characteristics. Then, the site was rinsed twice in a similar manner with water alone and dried with a cotton-tipped swab. Subsequently, the location of the application site was marked and the plastic ring removed. The animal was killed, the skin removed, immediately flattened on dry ice, and a 6-mm punch biopsy was taken. The frozen biopsy was sliced parallel to the skin surface with a cryomicrotome (Kryostat 1720; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) as described (23). Previously, cryosections of skin were hematoxylin/eosin stained, following tape stripping, and examined by microscopy to identify cell morphology of the individual sections. On the basis of this examination, the epidermis was localized to the first four slices of 5-µm thickness, and the subsequent 10 sections (10 µm) corresponded to papillary dermis (PD) (24). In the present study, two 5-µm sections are labeled stratum corneum (SC1 and SC2), the next two 10-µm sections are labeled epidermis (E1 and E2), the next 100 µm are labeled PD, the next 400 µm are labeled dermis (D), the remainder (approximately 100 µm) is labeled subcutaneous fat (SF).

Skin extraction and antioxidant analysis. Tocopherols and tocotrienols were extracted from skin, as described (25,26). Briefly, the skin sections were transferred to a tube containing 2 mL buffer (10 mM phosphate, 130 mM NaCl, 1 mM EDTA, pH 7.0) and 50 µL butylated hydroxytoluene (1 mg/mL) followed by mixing and the addition of 1 mL of 0.1 M sodium dodecylsulfate, 2 mL ethanol and extraction with 2 mL hexane. An appropriate aliquot was used for high-performance liquid chromatography (HPLC) analysis, as described (15). The electrochemical detector was operated with 0.5 V potential, full recorder scale at 50 nA for quantitation of α-tocopherol, α-, and γ-tocotrienols. The amounts of applied vitamin E (even the tocotrienols in lower layers) far exceeded the amounts present in controls tissues (data not shown). Thus, these inherent vitamin E concentrations were not subtracted from the data presented here.

Statistical analyses. All statistical analyses were carried out using SuperAnova (Abacus Concepts, Inc., Berkeley, CA) for the Macintosh. Analyses included three-factor analysis of variance with least square means comparisons. A P-value <0.05 was considered statistically significant. Values are given as means ± standard deviation of the concentrations in layers of skin (pmol/cm^2/µ) or as the percentage found in a given layer of total vitamin E recovered (pmol/cm^2).

RESULTS

The concentrations (pmol/cm^2/µ) of γ-tocotrienol, α-tocotrienol, or α-tocopherol after topical application of a 5% solution for 0.5, 1, 2, or 4 h were measured in murine skin layers SC, E, PD, D, and SF. Although there was a significant increase in the concentrations in the layers with time (main effect, $P < 0.001$), the relationship between the various vitamin E forms did not change differently with time, nor did the vitamin E contents of the layers change differently with time (no significant interactions). Therefore, for simplicity, the concentrations of each of the vitamin E forms in each of the layers were averaged over all time points as shown in Figure 1; the effects of time are shown in Figure 2. The uppermost layer of the skin (5 µm, SC1) contained the highest γ-tocotrienol, α-tocotrienol, and α-tocopherol concentrations of any of the layers ($P < 0.0001$ for each of the vitamin E forms). Furthermore, SC1 α-tocopherol concentrations were significantly greater than those of either SC1 γ-tocotrienol ($P < 0.0001$) or SC1 α-tocotrienol ($P < 0.0001$). None of the vitamin E concentrations in the other layers per µ were significantly greater than any other layers, possibly because of the large standard deviations observed.

The lengths of time that the vitamin E homologs penetrated the skin in relationship to the cutaneous layer vitamin

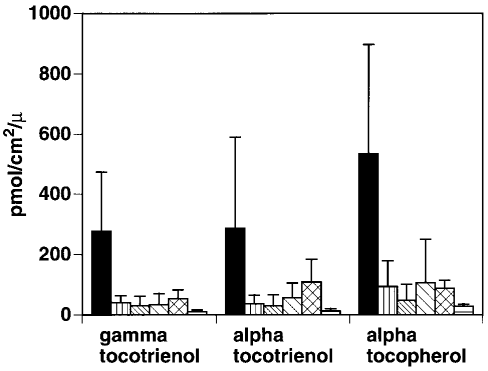

FIG. 1. Vitamin E content of murine skin after topical application of either γ-tocotrienol, α-tocotrienol, or α-tocopherol. The concentrations (pmol/cm^2/μ, mean ± SD, n = 16) of γ-tocotrienol, α-tocotrienol, or α-tocopherol after topical application of a 5% solution for 0.5, 1, 2, or 4 h were measured in skin layers (α-tocopherol, n = 16 for each layer): stratum corneum [SC1 (solid bar) γ-tocotrienol, n = 14; α-tocotrienol, n = 14], SC2 (vertical striped bar) γ-tocotrienol, n = 14; α-tocotrienol, n = 15], epidermis [E1(narrow, slanted, striped bar) γ-tocotrienol, n = 15; α-tocotrienol, n = 14], E2 (wide, slanted) (γ-tocotrienol, n = 16; α-tocotrienol, n =14) papillary dermis [PD cross-hatched bar (γ-tocotrienol, n = 15; α-tocotrienol, n = 16)], dermis [D (horizontal line bar) (γ-tocotrienol, n =15; α-tocotrienol, n = 13)]. Although subcutaneous fat vitamin E was measured, the thickness varied, so is not reported per μ. The average concentrations for all time points are shown. By least square means comparisons: γ-tocotrienol, α-tocotrienol, α-tocopherol in SC1 were significantly greater than their respective concentrations in any other layer ($P < 0.0001$); SC1 α-tocopherol concentrations were significantly greater ($P < 0.0001$) than those of either γ-tocotrienol or α-tocotrienol. There were no other significant differences between layers or between vitamin E forms.

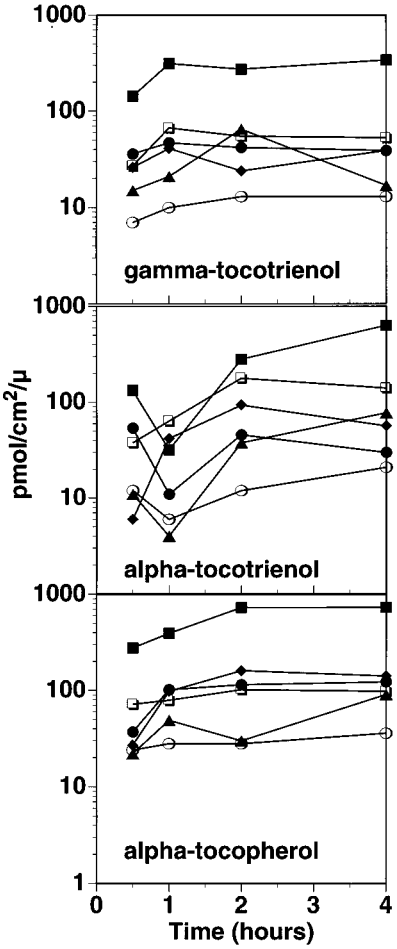

FIG. 2. Vitamin E content of murine skin at various times after topical application. The vitamin E concentrations from the same samples described in Figure 1 are shown for each of the time points (0.5, 1, 2, 4 h). By three-factor analysis of variance, there was a significant interaction between time and layer ($P < 0.0001$), but not with vitamin E form. The SC 1 vitamin E at 0.5 h was significantly less than for 2 h ($P < 0.0001$) or 4 h ($P < 0.0001$). In none of the other layers did the vitamin E concentrations significantly increase with time, although for each of the layers there was a trend toward increasing concentrations with time. See Figure 1 for abbreviations. SC1, ■; SC2, ●; ▲, E1; ◆, E2; □, PD; ○,D.

E are shown in Figure 2. It is clear that at all time points, SC1 contained the highest vitamin E concentrations, except for the comparison between SC1 at 0.5 and 1 h for α-tocotrienol.

SC1 contained the highest vitamin E concentrations when it was expressed per μ. If the thickness of the various skin layers is taken into account, it is apparent that the lower skin layers contain appreciable amounts of vitamin E. In order to compare the distribution of the various vitamin E forms into the skin layers, the total amount of each form in each of the layers was summed, then the percentage of each form was expressed per its respective total. When expressed as a percentage, the lowest layers (PD and D) contained the major portion of the applied vitamin E forms (Fig. 3). Although the percentage γ-tocotrienol in SC1 (10 ± 5%) was significantly greater ($P < 0.01$) than in SC2 (2 ± 1%), E1 (2 ± 2%), or E2 (2 ± 2%), it was significantly less than in PD (40 ± 15%) or D (36 ± 15%) ($P < 0.0001$). The SC1 percentage α-tocotrienol or percentage α-tocopherol was also significantly less than in PD or D ($P < 0.0001$).

The distribution into the PD was different for α-tocopherol compared with the tocotrienols. The percentage PD γ-tocotrienol (40 ± 15%) was similar to the fraction found in D (35 ± 15%); the percentage PD α-tocotrienol (53 ± 19%) was greater than that found in D (29 ± 16%) ($P < 0.0001$), while the percentage PD α-tocopherol (31 ± 6%) was less than that found in the D (41 ± 8%) ($P < 0.0005$). In all cases the percentage PD vitamin E homolog was greater than that found in the subcutaneous fat ($P < 0.0001$), which contained from 11 to 14% of the applied vitamin E. Thus, the penetration of the various vitamin E forms was rapid through the entire skin.

DISCUSSION

This study demonstrates that vitamin E applied to the skin is found in the highest concentrations in the uppermost layers of skin, if the data is expressed per μ of skin thickness (Fig. 1). It is not unsurprising that this is true whether measured at 0.5 h or up to 4 h (Fig. 2). What is surprising is that the largest fraction of skin vitamin E following topical application is not

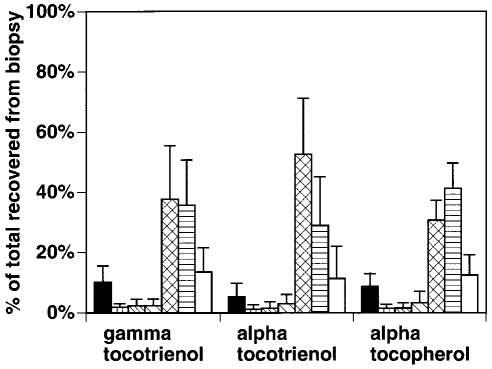

FIG. 3. Skin Vitamin E distribution following topical application. The percentage of each of the vitamin E forms found in each of the biopsies from γ-, α-, or α-tocopherol from the skin treated as described above is shown. The percentage γ-tocotrienol in SC1 was significantly greater ($P < 0.01$) than in SC2, E1, or E2, and significantly less than in PD or D ($P < 0.0001$). SC1 percentage α-tocotrienol or percentage α-tocopherol were also significantly less than their respective percentage in PD or D ($P < 0.0001$). The percentage α-tocotrienol was greater in PD than in D ($P < 0.0001$), while percentage α-tocopherol was less in PD than in D ($P < 0.0005$). In all cases the percentage PD vitamin E homolog was greater than its respective percentage in subcutaneous fat (SF) ($P < 0.0001$). SF (shaded bar). See Figure 1 for other abbreviations and symbols' explanations.

at the surface, but is found in the deeper subcutaneous layers (Fig. 3).

It should be noted that topical vitamin E application markedly increased skin vitamin E content. For example, α-tocopherol contents of untreated mice ($n = 8$) were: SC1 0.25 ± 0.09 pmol/cm^2/μ (Traber, M.G., Rallis, M., and Packer, L., unpublished observations) compared with 534 ± 363 pmol/cm^2/μ reported here; PD were 0.089 ± 0.037 pmol/cm^2/μ compared with 88 ± 27 pmol/cm^2/μ reported here, and D were 0.159 ± 0.059 pmol/cm^2/μ compared with 29 ± 7 pmol/cm^2/μ reported here. Thus, the application of a 5% α-tocopherol solution resulted in a 200- to 2000-fold increase in the skin α-tocopherol content. Trivedi *et al.* (27) proposed that topically applied vitamin E function as a penetration enhancer. Based on the large increases of vitamin E content, its rapid penetration and its well-known properties of increasing fluidity (28), it is not surprising that vitamin E has this effect.

We have calculated, based the thickness of each layer, that SC1 or SC2 each represents 0.8% of the skin thickness, E1 and E2 represent 1.6%, PD represents 16%, D represents 64%, and subcutaneous fat 15%. Comparing these percentages with those of the distribution of vitamin E homologs, about 10% of the skin vitamin E was found in SC1, far exceeding the percentage expected based on its thickness, while SC2, E1, or E2 contained a fraction of total equivalent to that expected from its fraction of the total. Most remarkably, PD represents about 16% of the total skin thickness but contained 40 to 50% of the various vitamin E homologs. This layer contains the sebaceous glands, which are lipid secretory organs, and, thus, may account for the affinity of this layer for vitamin E. The D represents about 64% of the thickness of the skin, but was comparatively unenriched in vitamin E (30–40% of total). The subcutaneous fat contained about 11–14% of the skin vitamin E and represents about 15% of the skin thickness. Remarkably, at the earliest time point 0.5 h, significant increases in vitamin E were found in the subcutaneous fat (data not shown), suggesting rapid penetration of the applied vitamin E through the skin.

In part, this study was carried out to evaluate whether tocotrienols, which have a higher fluidity (29,30), might more readily penetrate murine skin. In fact, we found that the distribution of tocotrienols was somewhat different than that of α-tocopherol. α-Tocotrienol appeared to accumulate more in the PD, while a greater fraction of α-tocopherol was present in the D. It is unknown what regulates tissue concentrations and whether this represents a real preference of the PD for tocotrienols and the D for α-tocopherol.

The high concentration of the applied vitamin E forms in SC1 is consistent with our observations of the high susceptibility of applied TRF to ozone exposure (18). These data also emphasize that topically applied vitamin E largely protects the first few microns of skin. Since oxidative stress depletes skin oxidants, it is now important to determine whether damage caused by oxidation by ozone at the surface, or ultraviolet light in deeper layers can be prevented by applied vitamin E and whether it protects other skin antioxidants and proteins, lipids, and DNA.

In conclusion, application of either γ-tocotrienol, α-tocotrienol, or α-tocopherol to murine skin results in extraordinary increases in stratum corneum vitamin E. If oxidative stress is applied to the surface of the skin, this level of vitamin E is sufficient to provide protection, as we have described in our studies with ozone exposure (18). Vitamin E homologs penetrated through the entire skin to the subcutaneous fat layer within the first 0.5 h. It is unclear whether this rapid penetration is into skin cells (keratinocytes or fibroblasts), around the cells in the skin lipids, or perhaps down the hair follicles into the deepest layers. It is clear from these studies that PD, the location of sebaceous glands, has a special affinity for vitamin E; the mechanism for this affinity remains to be determined.

ACKNOWLEDGMENTS

Beth Koh provided excellent technical assistance. We gratefully acknowledge the efforts of Dr. Asaf A. Qureshi, University of Wisconsin (Madison, WI), who isolated tocotrienols for use as standards for this study. This study was supported in part by the Palm Oil Research Institute of Malaysia.

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[Received September 12, 1997; accepted December 10, 1997]

Bile Acid Metabolism in Analbuminemic Rats

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ABSTRACT: The bile acid concentrations in the serum, liver, bile, intestines, and feces of 3- and 19-mon-old male and female Nagase analbuminemic (NA) rats were compared with those in Sprague-Dawley (SD) rats. There was no significant difference in the bile acid levels between NA and SD rats. However, increased biosynthesis and pool size of cholic acid (CD) derivatives and decreased levels of chenodeoxycholic acid (CDCA) derivatives (increased CA/CDCA ratio) were detected in male NA rats as compared to SD rats. The CA/CDCA ratio in female NA rats was not different from that in their SD counterparts. There were no significant differences between NA and SD rats in the biliary bile flow, bile acid levels in the small and large intestines, fecal bile acid excretion, bile acid concentration in the portal and systemic circulation, and in the pool size of bile acids. The blood lipid concentrations were significantly higher in the NA rats than in the SD rats. The hepatic levels of lipids were not significantly different between the two rat strains. In conclusion, this study showed that metabolism of bile acids in NA rats is not significantly affected, and that the hypercholesterolemia observed in these strains is not related to abnormalities of bile acid metabolism.

Lipids 33, 93–99 (1998)

Analbuminemic rats established from the normal Sprague-Dawley (SD) rats (Nihon Clea, Tokyo, Japan) by Nagase *et al.* (1,2) (Nagase albuminemic [NA] rats) exhibit several features resembling human analbuminemia. Analbuminemia of NA rats is inherited as an autosomal recessive trait. Deficiency or defect in the albumin mRNA has been reported in hepatocytes of NA rats (3). This mutant strain is characterized by a marked hypoalbuminemia, hypercholesterolemia, mild anemia, hypocalcemia, and hyperfibrinogenemia.

Albumin plays a crucial role in the blood transport of bile acids (BA). Hypoalbuminemic states are frequently associated with increased plasma level of cholesterol, which is converted to BA in the liver. In the present study, to investigate the alterations of the BA metabolism during hypercholesterolemia associated with analbuminemic states, we determined the BA concentrations in the serum, bile, intestines, and feces in NA and SD rats.

MATERIALS AND METHODS

Animals. Three-month-old and 19-mon-old male and female SD and NA rats (Sasaki Kenkyusho, Tokyo, Japan) were used in the experiments. The animals were raised in cages at room temperature (25 ± 1°C), with a humidity of 50–60% and illumination 12 h/day (from 7:00 to 19:00). The rats were kept in separate cages 2 wk before collection of feces. Food (CE-2; Nihon Clea) and tap water were given *ad libitum.*

Sample collections. Rats were laparotomized under pentobarbital anesthesia (50 mg/kg, i.p.); a polyethylene tube (PE-10; Clay Adams, Parsipanny, NJ) was inserted into the bile duct, and bile was collected for 30 min. Blood samples were then drawn from the portal vein and descending aorta, and serum was separated by centrifugation at 1,800 × *g* for 15 min. Thereafter, resection of the liver, small and large intestines was performed. Feces were collected continuously during the last 2 d before sacrificing the animals.

Sample preparations. After adding 25 μg of 7-α, 12-α-dihydroxy-5β-cholan-24-oic acid to 500 μL of bile samples, lipid extraction was performed using 40 vol of 99.5% ethanol for 10 min. The extract was pooled, adjusted to an appropriate volume, and filtered. The filtrates were used to measure the concentrations of BA, cholesterol, and phospholipids (PL).

For the determination of sterol and BA concentrations in feces, samples were lyophilized and pulverized using a commercial coffee mill. Absolute ethanol was then added to 500 mg of pulverized feces, and the filtrates were pooled and evaporated to dryness. Small and large intestines were separately homogenized with distilled water, and a fixed amount of each intestinal homogenate (1/30 of the total volume of the small intestine and 1/5 of the large intestine) was collected and lyophilized. The small and large intestine extracts were then

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Abbreviations: BA, bile acids; C/P ratio, cholesterol/phospholipid ratio; CA, cholic acid; CDCA, chenodeoxycholic acid; EC, esterified cholesterol; FC, free cholesterol; MCA, muricholic acid; NA, Nagase analbuminemic; PL, phospholipids; SD, Sprague-Dawley; TC, total cholesterol; TG, triglyceride.

prepared as described above for fecal samples. Lyophilized samples were then dissolved in 70% ethanol, and triglycerides (TG) were partition-extracted with *n*-hexane. After removing the solvent by evaporation, the residue was used for assaying BA (4).

Serum samples were also added to 25 μg of 7-α, 12-α-dihydroxy-5β-cholan-24-oic acid, and extracts were prepared using 10–20 vol of 99.5% ethanol for 1 h. The extracts were then cooled, filtered, and used for assaying BA. The liver was resected, weighed, and 1 g of the left lateral lobe was homogenized. One-tenth volume of the homogenate was lyophilized, and extraction of hepatic lipids was performed using 95.5% ethanol. The extract of the remaining homogenate was cooled, filtered, and used for the assay of lipids.

Assays of BA, sterols, PL, and TG. BA were determined following methods previously described (4,5,8).

Conjugated BA were hydrolyzed enzymatically and then analyzed by gas chromatography. Sterols in bile and feces were assayed by gas chromatography (column DB-17; J&W Scientific, Folsom, CA; temperature 240°C) after dissolving the samples in methyl-ethyl ketone/carbon disulfide (1:1, vol/vol). Measurements of total cholesterol (TC), free cholesterol (FC), TG, and PL were carried out as previously described (6,7,9).

Statistical analysis. Data are expressed as the mean ± standard error. The difference between the means of variables was calculated by the Student's *t*-test. A $P < 0.05$ was considered as statistically significant.

RESULTS

Body and liver weights and the blood and hepatic concentrations of lipids (*Table 1*). The body weight of NA rats was 30–35% lower than that of SD rats of the same age. Little difference was found in the liver weight between the two strains, but the liver weight per unit of body weight (g/100 g body weight) was significantly higher in NA rats than in SD rats. Serum cholesterol levels were significantly increased in both female and male NA rats as compared to their SD counterparts. The degree of hypercholesterolemia was particularly high in the 19-mon-old female NA rats. The serum concentrations of FC and esterified cholesterol (EC) were significantly higher in NA rats than in the SD strains. On the other hand, the percentage of EC included in the TC was decreased in NA rats as compared to that observed in SD strains. No consistent difference in the serum levels of TG was observed between the two animal strains, except for increased serum TG levels in the 19-mon-old female NA rats as compared to their SD counterparts. NA rats presented higher serum PL levels than SD rats. The increase rate in cholesterol levels was proportionally greater than that of PL, resulting in a high cholesterol/phospholipid ratio (C/P ratio).

The lipid concentration in liver homogenates was not significantly different between the two strains in most experimental animal groups, except for higher levels of EC in the 19-mon-old male SD rats than in NA rats of the same age and gender. The hepatic level of EC was significantly lower in female than in male rats of both strains. This increased proportionally with age in female rats of both strains. The hepatic concentration of PL was not correlated with the strain, sex, or age of rats.

Bile flow and biliary lipid concentration (Table 2). Bile flow was decreased in 3-mon-old male NA rats as compared to their SD counterparts. However, it did not change markedly in all other groups when expressed per unit weight of the liver. There was no difference between SD and NA rats in the secretory levels of biliary cholesterol, PL, or BA, except for a

TABLE 1
Plasma and Liver Lipid Levels in SD and NA Rats

	Male				Female			
	3 Mon		19 Mon		3 Mon		19 Mon	
	SD	NA	SD	NA	SD	NA	SD	NA
Number of rats	5	5	5	4	5	5	5	3
Body weight (g)	486 ± 13.1[a]	340 ± 19.0*	864 ± 14.7	577 ± 22.8*	298 ± 2.6	213 ± 21.6*	509 ± 34.1	338 ± 14.4*
Serum TC (mg/100 mL)	54 ± 2.7	137 ± 7.8*	93 ± 12.3	139 ± 7.1*	70 ± 2.6	184 ± 5.9	87 ± 13.9	288 ± 2.8*
FC (mg/100 mL)	11 ± 0.9	35 ± 2.3*	21 ± 4.2	38 ± 0.8*	15 ± 0.4	46 ± 1.4*	20 ± 2.6	77 ± 2.9*
EC (mg/100 mL)	43 ± 2.3	101 ± 5.6*	72 ± 8.3	100 ± 6.4*	54 ± 2.3	137 ± 4.5*	68 ± 11.4	2.12 ± 4.6*
EC (%)	80 ± 1.3	74 ± 0.4*	79 ± 1.9	72 ± 1.1*	78 ± 0.5	75 ± 0.2*	77 ± 1.1	73 ± 1.1*
TG (mg/100 mL)	100 ± 23.0	147 ± 15.4	117 ± 28.1	94 ± 1..6	57 ± 7.0	110 ± 5.3*	168 ± 50.0	485 ± 116.6
PL (mg/100 mL)	103 ± 5.5	202 ± 12.7*	139 ± 17.1	173 ± 3.7*	136 ± 5.0	256 ± 5.2*	178 ± 25.7	470 ± 22.1*
C/P ratio	0.53 ± 0.02	0.68 ± 0.01*	0.67 ± 0.02	0.80 ± 0.03*	0.52 ± 0.01	0.72 ± 0.02*	0.48 ± 0.02	0.62± 0.03*
Liver weight (g)	17.5 ± 0.93	15.3 ± 1.17	20.6 ± 0.88	19.4 ± 0.48	11.5 ± 0.17	10.6 ± 1.16	14.7 ± 0.94	14.9 ± 0.31
Weight (g/100 g BW)	3.6 ± 0.10	4.5 ± 0.11*	2.4 ± 0.09	3.4 ± 0.11*	3.8 ± 0.03	5.0 ± 0.08*	2.9 ± 0.15	4.4 ± 0.10*
TC (mg/g)	2.56 ± 0.14	2.45 ± 0.08	4.96 ± 0.82	2.64 ± 0.03*	2.08 ± 0.04	2.18 ± 0.05	3.07 ± 0.54	2.70 ± 0.06
FC (mg/g)	1.94 ± 0.08	2.05 ± 0.04	2.67 ± 0.17	2.15 ± 0.06	2.03 ± 0.06	2.02 ± 0.05	2.7 ± 0.37	2.30 ± 0.03
EC (mg/g)	0.61 ± 0.09	0.41 ± 0.05	2.29 ± 0.75	0.50 ± 0.07*	0.04 ± 0.03	0.16 ± 0.01*	0.37 ± 0.20	0.40 ± 0.04
TG (mg/g)	10.6 ± 0.73	12.9 ± 1.00	34.9 ± 10.37	13.4 ± 0.95	7.0 ± 0.60	9.4 ± 0.45*	29.5 ± 5.72	12.8 ± 0.40
PL (mg/g)	34.6 ± 0.46	37.6 ± 1.02*	34.8 ± 0.70	37.8 ± 1.43	35.8 ± 0.95	38.6 ± 0.77	35.2 ± 1.24	37.0 ± 1.24

[a]Mean ± SE. *$P < 0.05$, compared to corresponding SD rats. SD, Sprague-Dawley rat; NA, Nagase analbuminemic rat; TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol; TG, triglycerides; PL, phospholipids; C/P ratio, cholesterol (total)/phospholipid ratio; BW, body weight.

TABLE 2
Bile Flow and Biliary Lipid Secretion in SD and NA Rats

	Male				Female			
	3 Mon		19 Mon		3 Mon		19 Mon	
	SD	NA	SD	NA	SD	NA	SD	NA
Number of rats	5	5	5	4	4	5	5	3
Bile flow (mL/h/rat)	1.58 ± 0.10[a]	1.27 ± 0.07*	1.60 ± 0.10	1.67 ± 0.10	1.05 ± 0.09	0.84 ± 0.06	0.94 ± 0.03	1.03 ± 0.11
(mL/h/10 g liver)	0.91 ± 0.08	0.86 ± 0.09	0.73 ± 0.01	0.85 ± 0.03	0.91 ± 0.07	0.82 ± 0.07	0.64 ± 0.03	0.69 ± 0.06
TC (mg/h)	0.28 ± 0.02	0.12 ± 0.01*	0.20 ± 0.02	0.21 ± 0.02	0.10 ± 0.03	0.08 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
PL (mg/h)	4.68 ± 0.42	2.39 ± 0.19*	7.1 ± 0.85	5.85 ± 0.68*	2.86 ± 0.51	1.72 ± 0.14	4.11 ± 0.82	3.11 ± 0.25
BA (mg/h)	10.49 ± 1.44	11.30 ± 0.92	14.72 ± 2.54	15.70 ± 1.42	6.76 ± 0.87	7.68 ± 0.56	9.40 ± 0.70	8.70 ± 0.66
BA composition								
CA group (%)	53 ± 1.7	71 ± 1.7*	65 ± 0.9	72 ± 1.7**	58 ± 1.8	56 ± 1.9	55 ± 2.8	51 ± 2.9
3-α,7-α,12-α (CA)	48 ± 2.0	63 ± 1.7*	60 ± 0.8	67 ± 1.8**	52 ± 1.7	51 ± 2.0	50 ± 2.8	47 ± 3.1
3-α,12-α[1]	3 ± 0.4	6 ± 0.5*	4 ± 0.5	4 ± 0.3	4 ± 0.3	3 ± 0.2	3 ± 0.8	3 ± 0.6
3-β,12-α	<1	<1	<1	<1	<1	<1	<1	<1
3-α,12-α,7=O	1 ± 0.1	1 ± 0.4	<1	1 ± 0.1	1 ± 0.2	1 ± 0.2	1 ± 0.2	1 ± 0.3
3-α,7-α,12=O	<1	<1	<1	<1	1 ± 0.4	1 ± 0.1	1 ± 0.2	1 ± 0.1
3-α,12=O	<1	<1	<1	<1	<1	<1	<1	<1
CDCA group (%)	48 ± 1.7	29 ± 1.7*	35 ± 0.9	28 ± 1.7**	42 ± 1.8	45 ± 1.9	45 ± 2.8	49 ± 2.9
3-α,7-α (CDCA)	7 ± 0.9	1 ± 0.2*	4 ± 0.5	3 ± 0.2	13 ± 0.6	10 ± 1.1	13 ± 2.9	8 ± 1.7
3-α,6-β,7-α[2]	6 ± 0.8	2 ± 0.2*	3 ± 0.3	3 ± 0.2	9 ± 0.7	10 ± 0.5	7 ± 0.9	8 ± 1.3
3-α,6-β,7-β[3]	16 ± 3.0	15 ± 1.7	16 ± 2.8	9 ± 0.5	7 ± 1.4	12 ± 0.7	11 ± 3.2	12 ± 1.4
3-α,6-α,7-β[4]	1 ± 0.2		1 ± 0.1	<1	1 ± 0.2	1 ± 0.3	1 ± 0.1	1 ± 0.2
Δ^{22}-3-α,6-β,7-β	9 ± 2.9	5 ± 0.5	6 ± 3.0	9 ± 0.8*	4 ± 0.9	4 ± 0.7	3 ± 1.5	12 ± 2.7
3-α,6-α[5]	5 ± 0.8	4 ± 0.6	3 ± 0.6	3 ± 0.5	4 ± 1.5	4 ± 1.7	5 ± 1.2	3 ± 0.3
3-α,7-β	2 ± 0.1	1 ± 0.1	1 ± 0.2	1 ± 0.1	1 ± 0.1	1 ± 0.1	1 ± 0.3	1 ± 0.1
3-α	1 ± 0.1	1 ± 0.1	<1	1 ± 0.1	1 ± 0.1	1 ± 0.1	1 ± 0.1	1 ± 0.1
3-α,7=O	1 ± 0.1	<1	<1	<1	3 ± 0.4	2 ± 0.2	3 ± 0.6	3 ± 0.2
3-α,6=O	<1	<1	<1	<1	1 ± 0.1	1 ± 0.01	1 ± 0.3	1 ± 0.1
CA/CDCA ratio	1.11 ± 0.08	2.45 ± 0.18*	1.85 ± 0.17	2.61 ± 0.23**	1.38 ± 0.10	1.26 ± 0.09	1.26 ± 0.15	1.06 ± 0.13

[a]Mean ± SE. *$P < 0.05$, compared to corresponding SD rats. **$P < 0.01$, compared to corresponding SD rats. BA, bile acids; CA, cholic acid; CDCA, chenodeoxycholic acid α or β indicates presence and position of OH residue. Superscripted numbers: 1, DCA; 2, α-muricholic acid (MCA); 3, β-MCA; 4, ω-MCA; 5, hyodeoxycholic acid. See Table 1 for other abbreviations.

significant decrease in the secretion of biliary cholesterol and PL in the 3-mon-old male NA rats and of biliary PL in 19-mon-old male NA rats as compared to those in the corresponding SD rats. Although the secretory level of each biliary lipid per animal was significantly lower in female than in male rats, it was nearly equal in both groups when the secretory activity was expressed per unit weight of the liver. With regard to the BA composition, CA was the most abundant component, followed by β-muricholic acid (β-MCA) in males and CDCA and α- and β-MCA in females. The BA composition was not significantly different between NA and SD rats, but the ratio of total CA and CDCA derivatives (CA/CDCA ratio) was significantly higher in male NA rats than in SD counterparts.

Fecal concentrations of cholesterol and BA (*Table 3*). The dietary intake and fecal excretion of cholesterol were lower in both male and female NA rats than in the corresponding SD rat groups at the age of 3 mon. However, little difference was observed between the same groups in 19-mon-old rats. The cholesterol/coprosterol ratio was almost the same in both animal strains.

No difference was observed in the rate of fecal BA excretion between SD and NA rats. However, the fecal BA excretion per gram liver weight was reduced in 19-mon-old male rats of both strains. This age-associated decrease in the fecal excretion per gram liver weight was not significant in female rats.

Deoxycholic acid, ω-MCA, and hyodeoxycholic acid were the major fecal BA components. The BA composition was not different between NA and SD rats. The difference in the CA/CDCA ratio between female and male rats was significant in the NA (higher in males) but not in the SD strain. This CA/CDCA ratio was higher in the aged rat groups than in their young counterparts.

Blood concentration of BA (Table 4). Decreased portal BA concentration and increased portal and systemic CA/CDCA ratios were observed in 3-mon-old male NA rats as compared to male SD rats of the same age. No difference was detected in other groups. The BA concentration in the systemic circulation was lower (male rats, 7%; female rats, 5%) than in the portal circulation in both NA and SD rats.

Size and composition of BA pool (Table 5). The BA pool size was calculated as the sum of the total amount of BA in bile collected during a 30-min period and the total amount of BA detected in the small and large intestines. The BA pool size was smaller in female than in male rats, but it was not significantly different between NA and SD rats. The CA/CDCA ratio was higher in male NA rats than in SD counterparts. No other substantial differences were observed.

The distribution of BA in the body was as follows: about

TABLE 3
Fecal Sterol and BA Excretion in SD and NA Rats

	Male				Female			
	3 Mon		19 Mon		3 Mon		19 Mon	
	SD	NA	SD	NA	SD	NA	SD	NA
Number of rats	5	5	5	4	5	5	4	3
Diet intake (g/day/rat)	26.5 ± 2.26[a]	18.7 ± 1.05*	22.4 ± 0.99	22.8 ± 0.75	19.4 ± 0.97	14.1 ± 1.25*	18.3 ± 1.36	20.3 ± 0.67
Feces dry wt (g/day/rat)	6.4 ± 0.43	5.0 ± 0.34*	5.6 ± 0.48	6.3 ± 0.23	4.4 ± 0.11	4.4 ± 0.37	4.5 ± 0.39	5.1 ± 0.09
Fecal sterols (mg/day/rat)								
Total	21.6 ± 1.70	10.1 ± 0.69*	12.4 ± 0.62	10.6 ± 0.58	12.3 ± 0.29	9.4 ± 0.74*	8.4 ± 0.70	10.7 ± 0.46
Cholesterol	9.2 ± 0.90	4.9 ± 0.49*	5.4 ± 0.46	5.7 ± 0.27	6.3 ± 0.44	4.7 ± 0.27*	4.4 ± 0.41	6.2 ± 0.10*
Coprostanol	2.5 ± 1.07	5.2 ± 0.25*	7.0 ± 0.63	4.9 ± 0.33*	5.9 ± 0.50	4.7 ± 0.48	4.0 ± 0.31	4.4 ± 0.41
Fecal BA								
(mg/day/rat)	10.8 ± 0.84	9.9 ± 0.44	8.3 ± 1.13	7.6 ± 0.95	8.4 ± 0.62	9.0 ± 0.29	9.6 ± 0.83	8.6 ± 1.21
(mg/10 g liver weight)	6.24 ± 0.53	6.58 ± 0.40	4.07 ± 0.69	3.92 ± 0.45	7.35 ± 0.48	7.74 ± 0.45	6.86 ± 1.18	5.70 ± 0.71
BA composition								
CA group (%)	32 ± 2.3	38 ± 1.6*	43 ± 2.0	51 ± 3.1	36 ± 1.4	29 ± 1.2*	46 ± 1.8	32 ± 0.8*
3-α,7-α,12-α (CA)	1 ± 0.3	3 ± 0.9	1 ± 0.2	3 ± 0.8	2 ± 0.2	3 ± 0.4	5 ± 0.8	3 ± 0.2
3-α,12-α[1]	18 ± 1.4	26 ± 0.8*	24 ± 1.3	22 ± 2.9	22 ± 2.0	20 ± 0.5	25 ± 2.3	16 ± 0.8*
3-β,12-α	1 ± 0.2	1 ± 0.3	2 ± 0.4	1 ± 0.4	2 ± 0.2	<1	2 ± 0.5	1 ± 0.3
3-α,12-α,7=O	1 ± 0.3	1 ± 0.3	1 ± 0.3	1 ± 0.7	2 ± 0.4	1 ± 0.4	2 ± 0.2	2 ± 0.5
3-α,7-α,12=O	3 ± 0.3	<1	3 ± 0.2	4 ± 0.5	2 ± 0.2	<1	2 ± 0.3	3 ± 0.3
3-α,12=O	7 ± 1.0	8 ± 0.8	12 ± 1.6	19 ± 0.6*	7 ± 0.5	5 ± 0.5	10 ± 1.4	8 ± 0.6
CDCA group	68 ± 2.3	62 ± 1.6	57 ± 2.0	49 ± 3.1	64 ± 1.4	71 ± 1.2*	54 ± 1.8	68 ± 0.8*
3-α,7-α (CDCA)	1 ± 0.2	1 ± 0.1	<1	1 ± 0.1	1 ± 0.2	2 ± 0.2	2 ± 0.8	<1
3-α,6-β,7-α[2]	1 ± 0.3	1 ± 0.2	1 ± 0.3	1 ± 0.1	5 ± 0.9	5 ± 0.6	5 ± 0.8	5 ± 0.9
3-α,6-β,7-β[3]	6 ± 0.6	9 ± 1.4	5 ± 1.2	9 ± 0.8*	4 ± 0.5	9 ± 0.4*	4 ± 0.1	9 ± 0.3*
3-α,6-α,7-β[4]	23 ± 3.2	16 ± 3.3	16 ± 2.1	16 ± 1.4	15 ± 3.6	13 ± 1.4	12 ± 4.0	15 ± 1.9
3-α,6-α[5]	30 ± 3.0	27 ± 4.0	27 ± 1.8	14 ± 1.0*	19 ± 4.3	25 ± 2.7	17 ± 3.7	16 ± 3.5
3-α,7-β	<1	<1	<1	<1	<1	<1	<1	<1
3-α	6 ± 0.8	3 ± 0.2*	5 ± 0.4	4 ± 0.7	17 ± 0.5	13 ± 0.5*	10 ± 1.5	19 ± 1.4*
3-α,7=O	1 ± 0.1	<1	1 ± 0.1	1 ± 0.2	1 ± 0.1	1 ± 0.1	2 ± 0.1	2 ± 0.1
3-α,6=O	2 ± 0.3	4 ± 0.9	2 ± 0.3	3 ± 0.6	2 ± 0.2	3 ± 0.3	2 ± 0.3	2 ± 0.5
CA/CDCA ratio	0.47 ± 0.05	0.63 ± 0.04*	0.77 ± 0.06	1.05 ± 0.13*	0.57 ± 0.03	0.41 ± 0.02*	0.86 ± 0.06	0.47 ± 0.02*

[a]Mean ± SE. *$P < 0.05$, compared to corresponding SD rats. See Tables 1 and 2 for abbreviations and Table 2, for further footnotes/explanations.

10% was present in bile, about 80% in the small intestine, and about 10% in the large intestine. Interestingly, the CA/CDCA ratio was significantly lower in large intestine and feces than in bile and small intestine. These findings suggest that absorption of CA group in the small intestine is predominant in relation to that of CDCA group. No difference was observed in the distribution of BA between NA and SD rats.

DISCUSSION

BA are bound primarily to albumin in biological fluids (10–12) and are eluted from rat liver only when the liver is perfused with a buffer containing albumin (13). Thus, we hypothesized that the metabolism of BA would be affected in NA rats. To demonstrate this, in the present study, we compared the BA metabolism between NA and SD rats (the parental strain of NA rats).

The results of our present study showed that there are insignificant differences between the two rat strains in the rate of biliary BA secretion, BA concentration in the small and large intestines, fecal BA excretion, BA concentrations in the portal and systemic circulations, BA pool size, and in the BA composition. Increases in the synthesis and in the pool size of BA of the CA group and decreases in the synthesis and pool size of BA of the CDCA group (increased CA/CDCA ratio) were the only abnormalities observed in male NA rats. Thyroid hormones previously have been reported to enhance the synthesis of BA of the CDCA group, and this hormonal activity may explain the reduced CDCA levels observed in hypothyroid states (14,15). The blood levels of thyroid hormones were reported to be significantly lower in male NA rats than in male SD strains (16), and low levels of thyroid hormones may explain the increased CA/CDCA ratio observed in male NA rats.

There were no statistically significant differences between NA and SD rats in the rate of BA secretion and in the blood BA levels. The fecal BA excretion indicates the daily amount of BA synthesis in the liver (4,5). Although we found no difference in the BA excretion between SD and NA rats, a previous study suggested that it is high in NA rats (17). The mechanism of this discrepancy remains to be clarified. On the other hand, little difference was observed between NA and

TABLE 4
Blood BA Levels in SD and NA Rats

	Male				Female			
	3 Mon		19 Mon		3 Mon		19 Mon	
	SD	NA	SD	NA	SD	NA	SD	NA
Number of rats	5	4	5	4	5	5	5	3
Portal vein blood								
Serum BA (μ/mL)	46.5 ± 4.42[a]	32.3 ± 1.75*	36.6 ± 2.07	31.1 ± 3.71	30.8 ± 3.01	29.6 ± 2.82	45.1 ± 7.83	32.1 ± 3.30
BA composition								
CA group (%)	53 ± 2.8	63 ± 1.5	60 ± 2.0	67 ± 3.5	50 ± 2.4	46 ± 3.0	57 ± 4.1	47 ± 2.4
3-α,7-α,12-α (CA)	47 ± 3.0	51 ± 2.0	51 ± 1.3	60 ± 3.8	44 ± 2.3	34 ± 3.2	47 ± 3.7	42 ± 2.4
3-α,12-α[1]	5 ± 1.6	6 ± 0.5	8 ± 1.8	5 ± 0.3	4 ± 0.8	3 ± 0.6	6 ± 1.2	4 ± 0.4
3-β,12-α	<1	<1	<1	<1	<1	<1	<1	<1
3-α,12-α,7=O	1 ± 0.2	3 ± 0.6	1 ± 0.1	1 ± 0.4	1 ± 0.2	3 ± 1.3	3 ± 1.7	1 ± 0.3
3-α,7-α,12=O	<1	2 ± 0.4*	<1	1 ± 0.2	1 ± 0.2	6 ± 0.8	1 ± 0.1	1 ± 0.1
3-α,12=O	<1	1 ± 0.2	1 ± 0.2	<1	<1	<1	<1	<1
CDCA group	47 ± 2.8	37 ± 1.5*	40 ± 2.0	33 ± 3.5	50 ± 2.4	54 ± 3.0	44 ± 4.1	53 ± 2.4
3-α,7-α (CDCA)	5 ± 0.8	2 ± 0.3	3 ± 0.7	3 ± 0.8	13 ± 1.5	17 ± 4.9	11 ± 3.2	8 ± 2.1
3-α,6-β,7-α[2]	7 ± 1.0	3 ± 0.5*	5 ± 0.9	5 ± 0.3	9 ± 0.4	10 ± 1.2	8 ± 0.9	11 ± 1.2
3-α,6-β,7-β[3]	14 ± 2.1	11 ± 1.3	14 ± 2.4	8 ± 0.5	8 ± 0.3	8 ± 0.8	11 ± 2.2	10 ± 1.5
3-α,6-α,7-β[4]	2 ± 0.2	7 ± 0.4*	2 ± 0.4	1 ± 0.2	1 ± 0.3	5 ± 1.0	2 ± 0.4	2 ± 0.2
Δ^{22}-3-α,6-β,7-β	10 ± 2.7	7 ± 1.1	5 ± 1.3	10 ± 1.7	7 ± 2.0	4 ± 0.5	4 ± 2.0	15 ± 3.2
3-α,6-α[5]	5 ± 0.8	6 ± 0.5	7 ± 1.2	2 ± 0.4	6 ± 1.4	4 ± 1.5	3 ± 1.2	2 ± 0.1
3-α,7-β	2 ± 0.2	1 ± 0.2	2 ± 0.3	1 ± 0.1	<1	2 ± 1.2	1 ± 0.3	1 ± 0.3
3-α	1 ± 0.1	1 ± 0.2	1 ± 0.2	1 ± 0.1	2 ± 0.1	2 ± 0.2	1 ± 0.2	2 ± 0.2
3-α,7=O	1 ± 0.2	<1	<1	<1	3 ± 0.3	3 ± 1.0	1 ± 0.3	2 ± 0.5
3-α,6=O	1 ± 0.2	<1	1 ± 0.2	<1	1 ± 0.2	<1	1 ± 0.2	<1
CA/CDCA ratio	1.15 ± 0.13	1.74 ± 0.11*	1.56 ± 0.13	2.13 ± 0.34	1.03 ± 0.09	0.86 ± 0.10	1.38 ± 0.22	0.91 ± 0.09
Systemic blood								
Serum BA (μg/mL)	3.55 ± 1.42	2.29 ± 0.32	1.89 ± 0.7	2.20 ± 0.4	1.86 ± 0.2	1.48 ± 0.2	2.40 ± 0.4	1.78 ± 0.1
CA/CDCA ratio	1.15 ± 0.10	1.94 ± 0.24*	1.88 ± 0.36	1.50 ± 0.15	1.22 ± 0.08	1.70 ± 0.25	1.11 ± 0.12	0.96 ± 0.07

[a]Mean ± SE. *$P < 0.05$, compared to corresponding SD rats. See Tables 1 and 2 for abbreviations and Table 2 footnote for further explanations.

SD rats in the BA concentration and composition in the portal blood, suggesting that BA are probably bound to other proteins such as lipoproteins in the plasma of NA rats (18).

The hypercholesterolemia found in NA rats is consistent with the results reported in previous studies. The age-associated increase in the serum levels of cholesterol, which were particularly of esterified form, was observed in both male and female SD rats, but not in male NA rats (7,19). The serum PL level was also particularly high in NA rats. This was associated with an increase in the C/P ratio due to a more significant increase in the serum level of cholesterol than that of PL. Serum concentrations of TG were significantly higher in female NA rats than in the SD counterparts. Serum TG also tended to be higher in male NA rats, but their increase was not significant.

The mechanism of hypercholesterolemia associated with analbuminemia is not clear, but experimental and clinical studies carried out in the nephrotic syndrome, a kidney disease that is also associated with decreased plasma levels of proteins and hypercholesterolemia, suggested that it occurs due to increased synthesis and/or reduced catabolism of lipoproteins (20–22). The increased level of cholesterol is downregulated by its conversion to BA in the liver through the neutral and acid pathways. The ratio of the two major BA, CA and CDCA, produced in the liver varies according to the activity of either of these two pathways. In the present study, neither the production of the major BA nor their ratio (CA/CDCA ratio) was changed in NA rats, suggesting that analbuminemia induces no major changes in BA metabolism. In addition, previous studies have shown that the activities of the hepatic enzymes (i.e., HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis; cholesterol 7 α-hydroxylase, the rate-limiting enzyme in BA synthesis), responsible for increasing BA production in the liver, are not altered in the hypoalbuminemic states associated with the puromycin-induced nephrotic syndrome (23,24). Overall, the results of our present study and those reported in previous investigations suggest that the increased levels of cholesterol observed in analbuminemic conditions do not affect the metabolism of BA.

In contrast to the marked increase in the serum levels of lipids, little changes were observed in the hepatic lipid concentrations of NA rats. This finding differs from that observed during increased dietary cholesterol intake. In this situation, the lipid levels in the liver increase significantly as compared to the blood lipid concentrations (4,25).

In conclusion, this study showed that the metabolism of BA is not affected in NA rats. This result suggests that the hypercholesterolemia observed in these mutant strains is not related to abnormalities of the BA metabolism.

TABLE 5
Pool Size of BA and CA/CDCA Ratio in SD and NA Rats (3 mon)

	Male		Female	
	SD	NA	SD	NA
Number of rats	5	5	4	5
Pool size (mg/rat)	57.04 ± 4.90[a]	51.38 ± 2.49	38.95 ± 2.86	34.30 ± 2.55
Bile (mg/rat/30 min)	5.25 ± 0.72 (9.2%)	5.65 ± 0.46 (11.0%)	3.38 ± 0.44 (8.7%)	3.84 ± 0.28 (11.2%)
Small intestine (mg/rat)	47.92 ± 3.91 (84.0%)	40.24 ± 2.32 (78.3%)	29.48 ± 2.66 (75.7%)	27.85 ± 2.44 (81.2%)
Large intestine (mg/rat)	3.87 ± 0.41 (6.8%)	5.48 ± 0.80 (10.7%)	6.09 ± 0.29 (15.6%)	2.61 ± 0.77 (7.6%)
Bile acid composition (%)				
CA group (%)	54 ± 1.2	63 ± 1.4*	59 ± 1.3	53 ± 2.3
3-α,7-α,12-α (CA)	49 ± 1.4	55 ± 1.9*	50 ± 0.9	47 ± 2.4
3-α,12-α[1]	3 ± 0.5	6 ± 0.6*	5 ± 0.4	4 ± 0.2
3-β,12-α	<1	<1	1 ± 0.1	<1
3-α,12-α,7=O	1 ± 0.1	1 ± 0.4	1 ± 0.2	1 ± 0.2
3-α,7-α,12=O	<1	<1	1 ± 0.1	1 ± 0.1
3-α,12=O	1 ± 0.1	<1	2 ± 0.2	<1
CDCA group	47 ± 1.2	37 ± 1.4*	42 ± 1.3	47 ± 2.3
3-α,7-α (CDCA)	4 ± 0.4	1 ± 0.1*	8 ± 0.5	7 ± 0.7
3-α,6-β,7-α[2]	5 ± 0.7	2 ± 0.2*	9 ± 0.6	9 ± 0.8
3-α,6-β,7-β[3]	15 ± 2.9	14 ± 1.4	6 ± 1.2	11 ± 0.7*
3-α,6-α,7-β[4]	3 ± 0.4	3 ± 0.5	4 ± 1.1	2 ± 0.2
Δ22-3-α,6-β,7-β	9 ± 3.5	6 ± 0.7	4 ± 0.7	5 ± 1.0
3-α,6-α[5]	7 ± 1.4	9 ± 1.5	8 ± 2.1	7 ± 1.8
3-α,7-β	<1	1 ± 0.1	1 ± 0.1	1 ± 0.1
3-α	1 ± 0.3	1 ± 0.1	2 ± 0.4	2 ± 0.1
3-α,7=O	<1	<1	<1	2 ± 0.1
3-α,6=O	<1	<1	<1	<1
CA/CDCA ratio	1.15 ± 0.06	1.75 ± 0.10*	1.41 ± 0.08	1.15 ± 0.10
Bile	1.11 ± 0.17	2.45 ± 0.17*	1.38 ± 0.10	1.26 ± 0.09
Small intestine	1.24 ± 0.06	2.05 ± 0.12*	1.77 ± 0.10	1.25 ± 0.11*
Large intestine	0.48 ± 0.02	0.41 ± 0.02*	0.51 ± 0.07	0.41 ± 0.08
Feces	0.47 ± 0.05	0.63 ± 0.04*	0.57 ± 0.03	0.41 ± 0.02*

[a]Mean + SE. *$P < 0.05$, compared to corresponding SD rats. See Tables 1 and 2 for abbreviations and Table 2 footnote for further explanations.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Esteban C. Gabazza for critical reading and preparation of the manuscript and to Dr. Nobuo Yamaga, Tottori University School of Medicine, Yonago, for the gift of 7-α, 12-α-dihydroxy-5β-cholan-24-oic acid.

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[Received July 23, 1997, and in final revised form December 1, 1997; revision accepted December 2, 1997]

Structure–Retention Correlation of Isomeric Bile Acids in Inclusion High-Performance Liquid Chromatography with Methyl β-Cyclodextrin

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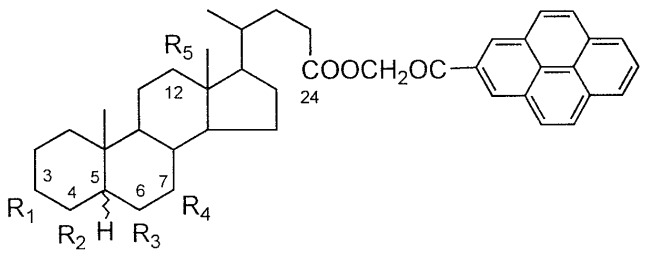
ABSTRACT: The structure–retention correlation of various C_{24} bile acid isomers was studied by the addition of methyl β-cyclodextrin (Me-β-CD) to mobile phases in reversed-phase high-performance liquid chromatography (HPLC). The compounds examined include a series of monosubstituted bile acids related to cholanoic acids differing from one another in the position and configuration of an oxygen-containing function (hydroxyl or oxo group) at the position C-3, C-6, C-7, or C-12 and the stereochemistry of the A/B-ring fusion (*trans* 5α-H and *cis* 5β-H) in the steroid nucleus. The inclusion HPLC with Me-β-CD was also applied to biologically important 4β- and 6-hydroxylated bile acids substituted by three to four hydroxyl groups in the 5β-steroid nucleus. These bile acid samples were converted into their fluorescence prelabeled 24-pyrenacyl ester derivatives and chromatographed on a Capcell Pak C_{18} column eluted with methanol–water mixtures in the presence or absence of 5 mM Me-β-CD. The effects of Me-β-CD on the retentions of each compound were correlated quantitatively to the decreasing rate of capacity factors and the relative strength of host–guest interactions. On the basis of the retention data, specific and nonspecific hydrogen-bonding interactions between the bile acids and the Me-β-CD were discussed.
Lipids 33, 101–108 (1998).

Cyclodextrins (CD) are toroidal-shaped cyclic oligosaccharides consisting of six to eight D(+)-glucopyranose units connected by α-(1,4) bonds and are able to include specific guest molecules into their hydrophobic cavity to form inclusion complexes (1). The stability of the inclusion complexes, i.e., strength of host–guest interaction, is influenced by the hydrophobic and/or hydrogen-bonding interactions between the cavity of CD and the guest molecules, including the shape and the size of the guests (2–5). By utilizing the properties of inclusion complex formation of CD, Armstrong *et al.* (1,3–5) have successfully separated many recalcitrant pairs of the positional, geometrical, and optical isomers of bioactive compounds using chromatographic techniques as they fit differently into the cavity of CD (1).

In reversed-phase high-performance liquid chromatography (HPLC), CD have been used in two different manners, either by adding a CD to the mobile phase (6) or by covalently bonding a CD to a silica gel (4). Of the two methods proposed in inclusion HPLC, Shimada *et al.* (6) have reported apparent superiorities of CD, particularly for β-CD or its methylated derivative (Me-β-CD), as a mobile phase additive in steroid analysis. By using the method, they have selectively resolved many difficult-to-separate pairs of cardiac steroids related to cardenolides (7,8) and bufadienolides (8,9), estrogens (6,10), and bile acids (11–14), which overlap one another in conventional HPLC without CD in the mobile phases.

The above findings in inclusion HPLC prompted us to explore in more detail the relationship between the retentions and the structures of various bile acid isomers and to compare the results with that observed in conventional HPLC. In addition, the retention data are expected to estimate what factors are responsible for the host–guest interactions of the bile acids with Me-β-CD and to elucidate the role of oxygen-containing functions in the complex formation. In this study, inclusion HPLC behavior induced by adding Me-β-CD in the mobile phases was examined for a series of monosubstituted bile acids related to 5α- and 5β-cholanoic acids having an oxygen-containing function (hydroxyl or oxo group) at the position C-3, C-6, C-7, or C-12 as their fluorescence-labeled 24-pyrenacyl ester derivatives (Scheme 1).



R_1, R_2, R_3, R_4, R_5 = H, α-OH, β-OH or =O

SCHEME 1

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Abbreviations: CD, cyclodextrin; HPLC, high-performance liquid chromatography; Me-β-CD, methyl β-cyclodextrin; *rk′*, relative capacity factor; *rΔk′*, the ratio of the difference in the capacity factors between the k'_1 and k'_2 values of the same compound to that of a reference compound.

The inclusion HPLC of biologically important 4β- and 6-hydroxylated bile acids substituted by three to four hydroxyl groups in the 5β-steroid nucleus was also studied.

EXPERIMENTAL PROCEDURES

Materials and reagents. Almost all of the C_{24} bile acids related to 5α- and 5β-cholanoic acids having one to four oxygen-containing functions at the positions C-3, C-4, C-6, C-7, and/or C-12 in the steroid nucleus were taken from our laboratory collection. The fluorescence prelabeling reagent, 1-bromoacetylpyrene, was obtained from Wako Pure Industries Ltd. (Tokyo, Japan). Me-β-CD (heptakis-(2,6-di-D-methyl)-β-cyclodextrin; mean degree of substitution, 10.5–14.7) and dicyclohexyl 18-crown-6 ether were available from Sigma Chemical Co. (St. Louis, MO) and Nakarai Tesque (Kyoto, Japan), respectively. Prepacked silica cartridges, Sep-Pak Light (sorbent weight, 120 mg), for solid-phase extraction were purchased from Waters Associates (Milford, MA).

HPLC. The apparatus used for this work was Hitachi (Tokyo, Japan) L-6200 and L-6000 pumps equipped with an L-7610 degasser and an L-1050 fluorescence spectrophotometer. A Capcell Pak C_{18} UG120 column (250 mm × 4.6 mm i.d; particle size, 5 μm; Shiseido; Tokyo, Japan) was used at ambient temperature.

Methanol–water mixtures, ratios from 92:8 to 80:20 (vol/vol), were used as mobile phases at a flow rate of 1.0 mL/min under an isocratic condition. The effluents from the column were monitored with the fluorophotometer by using an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Sample preparation. Each of the C_{24} free bile acids (*ca.* 200 μg) was converted into the 24-pyrenacyl ester derivative by a procedure reported by Kamada *et al.* (15), using 1-bromoacetylpyrene as a prelabeling reagent and dicyclohexyl 18-crown-6 ether as a catalyst. After the reaction, sample solution was diluted with benzene (5 mL), applied to a preconditioned Sep-Pak silica cartridge, and washed successively with benzene (5 mL) and benzene/EtOAc (4:1, vol/vol; 5 mL). The desired pyrenacyl ester derivative of a bile acid was then eluted with methanol/CH_3CN (1:9, vol/vol; 10 mL), and an aliquot of the solution was injected into the HPLC system with an internal reference standard. The esters of 3α-hydroxy-5β-cholanoic acid and 3α, 6α, 7α-trihydroxy-5β-cholanoic acid were chosen as internal reference standards for the analysis of monosubstituted and polyhydroxylated bile acids, respectively.

RESULTS AND DISCUSSION

Preliminary study. Previously, optimal conditions for inclusion HPLC using CD as mobile phase additives have been studied by Shimada *et al.* (11–14) for the unconjugates of five common bile acids (i.e., lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, and cholic acids) and their glycine- and taurine-amidated, sulfated, and glucuronidated conjugates. On the basis of these studies, methanol and Me-β-CD were employed in this study as an organic mobile phase modifier and as a mobile phase additive, respectively. In addition, the carboxyl groups of free bile acid samples were converted into their 24-pyrenacyl ester derivatives using dicyclohexyl 18-crown-6 ether and 1-bromoacetylpyrene as precolumn labeling reagents, because of the high-sensitivity with fluorescence detector and of the excellent chromatographic properties in both conventional and inclusion HPLC (13–15).

When the eluent is low in water percentage (*ca.* 10%), excess fluorescence prelabeling reagent and reaction by-products in the 24-pyrenacyl esterification of monosubstituted bile acids with 1-bromoacetylpyrene do not generally interfere with the desired bile acid ester peaks of interest, so a preliminary purification step is avoided. However, with the esters of more polar polyhydroxylated bile acids, which require a higher percentage of water in the eluent for good resolution, the contaminating peaks can often interfere with early-emerging ester peaks. For this reason, a crude esterification product was purified by a preconditioned Sep-Pak silica cartridge for solid-phase extraction prior to HPLC analysis. After washing the product successively with benzene and benzene–ethyl acetate, the desired bile acid ester was recovered with eluting methanol/CH_3CN (1:9, vol/vol).

The pyrenacyl ester derivatives of bile acids were first chromatographed on a Capcell Pak C_{18} column eluting with methanol/water mixtures (92:8–80:20, vol/vol) and subsequently measured with the same eluent system containing 5 mM Me-β-CD. As expected, the presence of 5 mM Me-β-CD in the eluents did not affect the peak shape and symmetry. Under the HPLC conditions used, the retentions (i.e., capacity factors) observed for each compound in the absence and presence of Me-β-CD were expressed as k_1' and k_2' values, respectively. In order to facilitate a comparison of the effects of Me-β-CD on the k_1' and k_2' values of each substituent and to estimate the relative strength of host–guest interactions from bile acids and Me-β-CD, rk' and $r\Delta k'$ values were proposed in this study.

Thus, the rk' value is the relative capacity factor (k_2'/k_1') for the same compound, representing the decreasing rate of retentions induced by adding Me-β-CD (14); the effect of Me-β-CD on the retention is larger for compounds with a small rk' value than for compounds with a large one. On the other hand, the $r\Delta k'$ value is defined as the ratio of the difference in the capacity factors between the k_1' and k_2' values of the same compound to that of a reference compound, which reflects a relative time to pass through the Me-β-CD cavity; alternatively, compounds with a large $r\Delta k'$ value are more tightly complexed with Me-β-CD than compounds with a small one, indicating the relative strength of the host–guest interactions. Since the rk' and $r\Delta k'$ values are specific ones for individual compounds and are not being related to each other, the combined use of the two relative values would be useful for characterizing the structures of unknown bile acids (see below).

Chromatographic behavior of monosubstituted bile acids. At first, chromatographic behavior of the pyrenacyl ester derivatives of 24 monosubstituted bile acids was examined.

TABLE 1
Effect of Me-β-CD on the Retention of Monosubstituted Bile Acids[a]

5β-Series	k'_1[b]	k'_2[c]	rk'[d]	$r\Delta k'$[e]	$(\Delta k')$[f]
3α	3.61	1.75	0.48	1.00	(1.86)
3β	3.61	1.38	0.38	1.20	(2.23)
6α	3.71	1.81	0.49	1.02	(1.90)
6β	4.83	2.05	0.42	1.49	(2.78)
7α	5.79	3.59	0.62	1.18	(2.20)
7β	4.79	2.06	0.43	1.47	(2.73)
12α	6.06	5.43	0.90	0.34	(0.63)
12β	5.93	4.57	0.77	0.73	(1.36)
3-Oxo	3.96	1.43	0.36	1.36	(2.53)
6-Oxo	3.79	1.90	0.50	1.11	(2.07)
7-Oxo	4.62	2.82	0.61	0.97	(1.80)
12-Oxo	5.69	4.86	0.85	0.45	(0.83)
5α-Series					
3α	4.07	2.25	0.55	0.98	(1.82)
3β	3.61	1.75	0.48	1.00	(1.86)
6α	4.13	2.46	0.60	0.90	(1.67)
6β	6.28	3.29	0.52	1.61	(2.99)
7α	5.47	3.59	0.66	1.01	(1.88)
7β	5.47	2.71	0.50	1.48	(2.76)
12α	5.93	4.95	0.83	0.53	(0.98)
12β	6.50	4.95	0.76	0.83	(1.55)
3-Oxo	3.96	2.17	0.55	0.96	(1.79)
6-Oxo	4.62	2.82	0.61	0.97	(1.80)
7-Oxo	5.47	3.68	0.67	0.96	(1.79)
12-Oxo	6.24	5.28	0.85	0.52	(0.96)

[a]Column, Capcell Pak C_{18} (Shiseido, Tokyo, Japan), mobile phase, methanol/water (92:8, vol/vol); detection, fluorescence (excitation wavelength, 370 nm; emission wavelength, 440 nm); the designations 5β- and 5α-series refer to 5β- and 5α-cholanoates, respectively; measured as the 24-pyrenacyl ester derivatives.
[b]Capacity factor obtained without methyl-β-cyclodextrin (Me-β-CD) in the mobile phase.
[c]Capacity factor obtained with 5 mM Me-β-CD in the mobile phase.
[d]Relative capacity factor; the k'_1 value for the same compound was taken as 1.0.
[e]Relative $\Delta k'$ value; the $\Delta k'$ value of 3α-hydroxy-5β-cholanoic acid was taken as 1.0 for the calculation of the $r\Delta k'$ values.
[f]Difference in the k'_1 and k'_2 values for the same compound.

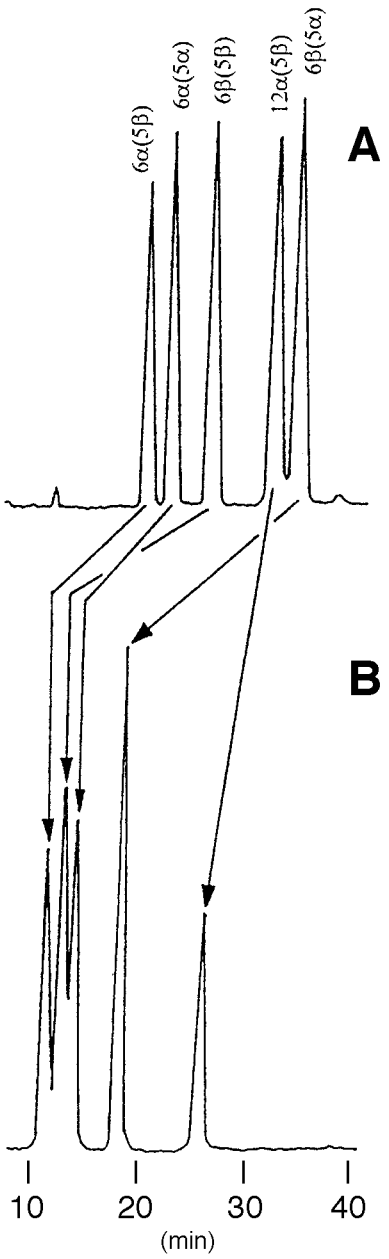

FIG. 1. High-performance liquid chromatography (HPLC) chromatograms of a mixture of the 24-pyrenacyl ester derivatives of monohydroxylated bile acid isomers eluted by methanol/water (92:8, vol/vol) (A) without or (B) with 5 mM methyl-β-cyclodextrin (Me-β-CD) in the mobile phase.

These compounds differ from one another in the position and configuration of an oxygen-containing function (hydroxyl or oxo group) at the position C-3, C-6, C-7, or C-12 and the stereochemistry of the A/B-ring fusion (5β-H normal and 5α-H "*allo*" series) in the steroid nucleus. A methanol/water mixture of 92:8 (vol/vol) was employed as the eluent without or with 5 mM Me-β-CD. The compounds examined and their retention data are compiled in Table 1. It is noticed here that since the capacity factors are dependent on both the Me-β-CD concentration (14) and mobile phase composition (unpublished results; Momose, T., and Iida, T.), the data are essential for the HPLC conditions used. Typical chromatograms are also shown in Figure 1.

In conventional HPLC without Me-β-CD in the mobile phase, the elution orders of the pyrenacyl esters of each group of monohydroxy and monooxo compounds in the 5α- and 5β-series on a Capcell Pak C_{18} column were essentially identical with those observed previously for the corresponding 4-nitrophthalimide methyl esters on a Nova-Pak C_{18} column (16). However, slight differences observed in the separation efficiency and the elution order of some of the isomeric pairs are probably attributable to the differences in the nature of the C_{18} reversed-phase columns used and of the ester derivatives at C-24.

By adding 5 mM Me-β-CD to the eluent, the retention of these compounds responded dramatically to the minor structural differences in the steroid nucleus, and the observed k_2' values were decreased to a large extent, compared to the corresponding k_1' values. The decrease in the retentions observed for the same compound indicates the formation of host–guest interactions between the Me-β-CD and the bile acid. Two different mechanisms have accounted for the host–guest interac-

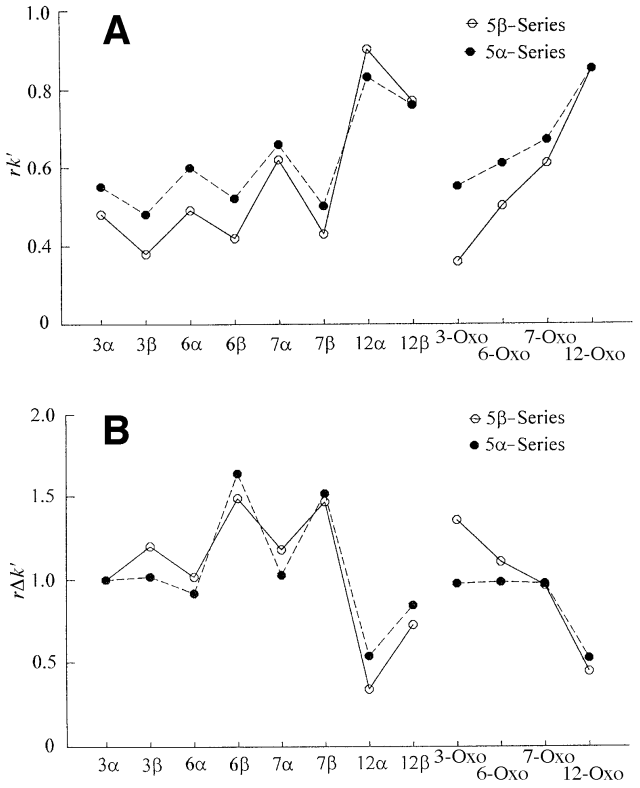

FIG. 2. Relationship of the (A) relative capacity factor (*rk′*) values and (B) the ratio of the difference in the capacity factors between the k'_1 and k'_2 values of the same compound to that of a reference compound (*rΔk′*) for monosubstituted bile acids.

tions (17): a hydrophobic interaction between the inside of the hydrophobic cavity of a host CD and the hydrophobic face of a guest molecule, and a hydrogen-bonding interaction between hydroxyl groups around the rims of the CD and polar functional groups of the guest molecule. Of the two interactions, the hydrogen-bonding interaction seems to be essential for the observed changes in the retention of the bile acids, because the polar and hydrophilic functional groups in the nucleus and the carbonyl group in the side-chain of the solutes accelerate the formation of strong hydrogen bonds with Me-β-CD (12,17).

As shown in Figure 2, the following general trends were observed for the *rk′* values in the monosubstituted bile acids. The effects of Me-β-CD on the *rk′* values of analogous compounds are usually similar in both the 5β- and 5α-series, and the following order of the increasing *rk′* values was observed for each series: 12α- >> 12β- > 7α- > 3α-, 6α- > 6β-, 7β- > 3β-ols; 12- > 7- > 6- > 3-ketones. Retentions of compounds having a β-oriented hydroxyl group are much more strongly influenced by Me-β-CD than those of the corresponding α-epimers. The largest decrease (*ca.* 50–60%) in the retentions was observed for 3β-hydroxy compounds (*rk′*, 0.38 and 0.48), whereas 12α-hydroxy and 12-oxo compounds (*rk′*, 0.83–0.90) were slightly affected by Me-β-CD (*ca.* 10–15%). In addition, the decrease in retentions was found to be more prominent in the 5β-series of compounds than in the corresponding 5α-analogs, except for 12α- and 12β-hydroxy compounds. These results imply that in bile acids, β-configuration of hydroxyl groups and shape of the *cis*-A/B (5β-H) ring fusion of the steroid nucleus, as well as the position of an oxygen-containing function, are preferentially oriented to fit well into the Me-β-CD cavity.

On the other hand, inspection of the *rΔk′* values revealed that the relative strength of the host–guest interactions of Me-β-CD with monohydroxylated bile acids in each of the 5β- and 5α-series increases in the following order: 12α- << 12β- < 3α-, 6α- < 3β-, 7α- < 6β-, 7β-ols (Fig. 2). This order of the *rΔk′* values is inconsistent with that observed for the *rk′* values, suggesting that the decreasing rate of retentions is not necessarily proportional to the relative strength of host–guest interactions. For example, both 6α-hydroxy-5β-cholanoate and 7β-hydroxy-5α-cholanoate exhibited very similar *rk′* values of 0.49 and 0.50, respectively, but their *rΔk′* values differed greatly from each other and showed 1.02 and 1.48, respectively. The combined use of the *rk′* and *rΔk′* values is therefore useful for characterizing the structures of the types of bile acids. In each of the 5β- and 5α-series, compounds having a β-hydroxyl group form more stable inclusion complexes than the corresponding α-isomers, in accord with their decreasing rate of the retentions as mentioned above. In particular, 6β- and 7β-hydroxy compounds (*rΔk′*, 1.47–1.61) exhibit the strongest affinity with Me-β-CD, whereas 12α-hydroxy compounds (*rΔk′*, 0.34–0.53) show the weakest affinity to give the least-stable complexes. In the case of oxo-bile acids, the complex stability increased in the order of 12- << 7- < 6- < 3-ketones in the 5β-series and 12- << 3-, 6- and 7-ketones in the 5α-series. Again, the order of the *rΔk′* values in the 5α-series also differed from that of the *rk′* values.

The above finding that compounds having a hydroxyl group at the A- or B-ring of the steroid nucleus form much more stable inclusion complexes with Me-β-CD than compounds having an oxygen-containing function at C-12 in the C-ring can be reasonably explained by suitable spatial distances between hydroxyl groups and carbonyl groups of the former, which allow for specific hydrogen bondings with the Me-β-CD. As illustrated in Figure 3A, assuming that if a bile acid pyrenacyl ester enters from the less bulky side of the A/B-ring of the solute into the wider rim of Me-β-CD, hydrogen bonds may occur at the two sites: firstly, a hydrogen bond between a hydroxyl of the steroid nucleus in the solute and one or two of seven primary hydroxyls ($-CH_2OH$) at the 6′-position of each glucopyranose moiety, which are situated in the narrower rim of the Me-β-CD cone; secondly, a hydrogen bond between a carbonyl group in the side-chain in the solute and one of secondary hydroxyls (>CHOH) at the 2′- or 3′-position of each glucopyranose unit, which is located at the wider rim of the Me-β-CD cone.

Examination of a molecular model revealed that hydroxyl groups at the position C-3, C-6, or C-7 in the steroid nucleus and a carbonyl group in the side-chain of bile acid pyrenacyl esters are situated in preferred positions, which form easily the specific hydrogen bonds with a primary 6′-hydroxyl group

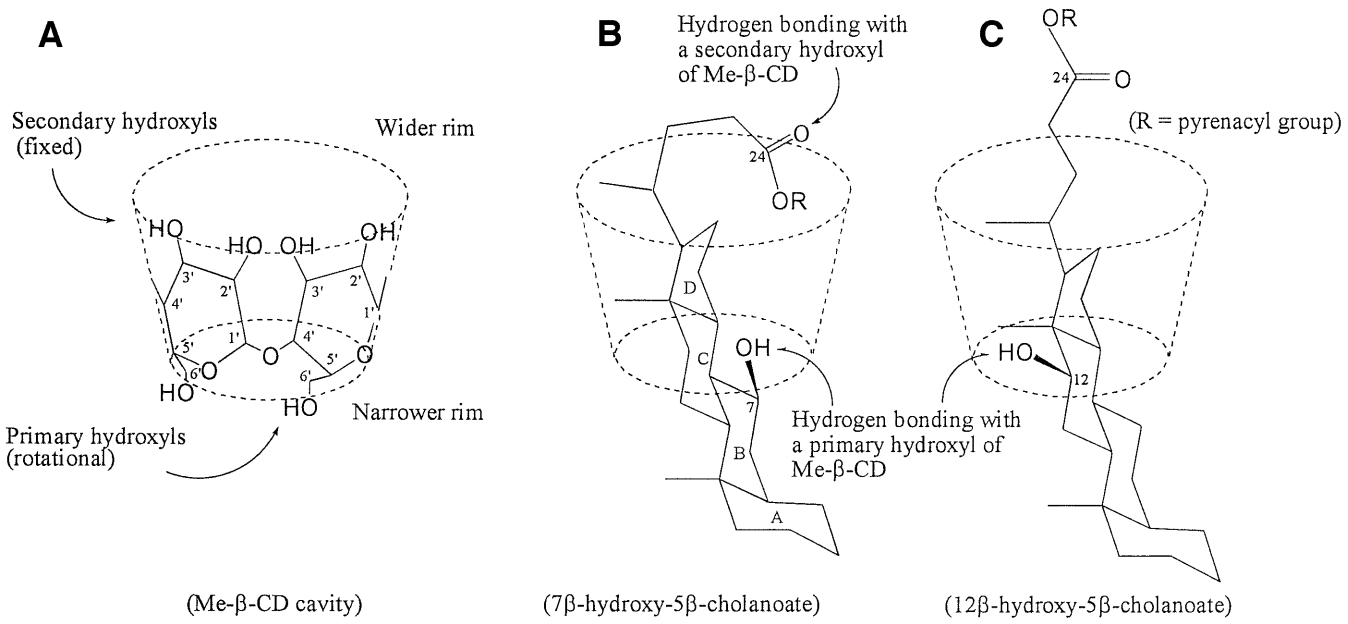

FIG. 3. Conceptual illustration of inclusion complexes between Me-β-CD and monohydroxylated bile acids. See Scheme 1 for abbreviation.

(free rotational) at the narrower rim and a secondary 2′- or 3′-hydroxyl group (fixed) at the wider rim of the Me-β-CD cone, respectively (Fig. 3B). These compounds would therefore be held by the two specific hydrogen bonds at both rims of the Me-β-CD, forming stable complexes. The validity of the above assumptions is supported by the previous observations in similar inclusion HPLC studies of polyhydroxylated sterols having an alkyl side-chain (17) and C_{24} unconjugated bile acids and their glycine- and taurine-amidated conjugates (12,14).

In a similar manner, the HPLC behavior of 12-oxygenated compounds which show the least-stable hydrogen bonds with Me-β-CD can be interpreted as follows. Concerning the anomaly of analogous 12α-hydroxy bile acids such as deoxycholic and cholic acid derivatives, previous workers have reported that a steric interaction between the 12α-hydroxyl group and the carbonyl group of the solutes interferes with the hydrogen bonding between the carbonyl group of the solutes and hydroxyl groups of Me-β-CD (12). However, the restriction of the free rotation of the side-chain by a steric hindrance between the functional group at C-12 and the side-chain appears to be more well-explained than the anomalous behavior of these compounds. According to our previous study of bile acids by nuclear magnetic resonance spectroscopy (18), they should have a preferential conformation about the 17(20) bond such that C-21 and C-22 lie pseudo-equatorially to the rear of the 17(20) bond with the third substituent (H) on C-20 pseudo-axially to the front, opposing C-18 (C-22, *trans*-oriented to C-13). If the presence of a 12-substituent in the bile acids restricts the free rotation of the side-chain, the most preferred conformer at the 17(20) bond is fixed as mentioned above. Such conformation of the side-chain may interfere with the two hydrogen bonds of the solutes with Me-β-CD. In fact, a molecular model exhibited that a hydrogen bonding of the solutes with Me-β-CD is possible at the one site, either at the wider or narrower rim of the Me-β-CD cone (Fig. 3C). These compounds would therefore be held only by one hydrogen bond with Me-β-CD, forming less-stable complexes. It is, however, noticed here that further study is necessary to obtain more precise information on the hydrogen-bonding mechanism.

Chromatographic behavior of polyhydroxylated bile acids. In recent years, considerable attention has been focused on the metabolism of unusual bile acids such as 4β- and 6-hydroxylated bile acids (19). These unusual bile acids, most of which possess a vicinal 3,4- or 6,7-glycol structure, are present in significant amounts in biological materials excreted by patients with hepatobiliary diseases and by newborn infants and in the fetus. The concurrent occurrence of 4β- and 6-hydroxylated bile acids is of keen current interest in biological and metabolic studies in connection with liver diseases. Although conventional HPLC of the bile acids on a reversed-phase column has been reported by Batta *et al.* (20) and by us, Iida *et al.* (21), their inclusion HPLC behavior has not yet been studied. We performed here additional experiments using various unusual C_{24} bile acids substituted by three to four hydroxyls in the 5β-steroid nucleus, in order to elucidate further what factors are responsible for changing the retentions of the compounds.

Analogous with the monosubstituted bile acids mentioned above, the polyhydroxylated bile acids were measured as the 24-pyrenacyl ester derivatives on a Capcell Pak C_{18} column, eluting with a methanol–water mixture (80:20, vol/vol) in the presence or absence of 5 mM Me-β-CD as a mobile-phase additive. The retention data for the compounds examined and typical chromatograms obtained are shown in Table 2 and Figure 4, respectively.

TABLE 2
Effect of Me-β-CD on the Retention of Polyhydroxylated Bile Acids[a]

Position and configuration of hydroxyls									
C-3	C-4	C-6	C-7		k'_1	k'_2	rk'	$r\Delta k'$	($\Delta k'$)
α	β		α		9.38	4.41	0.47	1.38	(4.97)
β	β		α		8.30	3.40	0.41	1.36	(4.90)
α		α	α		6.54	2.94	0.45	1.00	(3.60)
α		α	β		3.94	1.35	0.34	0.72	(2.59)
α		β	α		4.28	1.73	0.40	0.70	(2.55)
α		β	β		4.67	1.54	0.33	0.87	(3.13)
β		α	α		6.31	2.50	0.40	1.06	(3.81)
β		α	β		5.12	1.35	0.26	1.05	(3.77)
β		β	α		4.46	1.73	0.39	0.76	(2.73)
β		β	β		5.96	1.54	0.26	1.23	(4.42)
C-3	C-4	C-6	C-7	C-12					
α		α	β	α	1.26	0.93	0.74	0.09	(0.33)
α		β	α	α	1.44	1.05	0.73	0.11	(0.39)
β		β		α	1.52	1.12	0.74	0.11	(0.40)
α		β	β	α	1.54	1.14	0.74	0.11	(0.40)
α		β		α	1.64	1.23	0.75	0.11	(0.41)
α		α	α	α	2.58	1.95	0.76	0.18	(0.63)
β		α		α	2.52	1.86	0.74	0.18	(0.66)
β	β		α	α	3.48	2.74	0.79	0.20	(0.74)
α		α		α	3.02	2.26	0.75	0.21	(0.76)
α	β		α	α	4.35	3.44	0.79	0.25	(0.91)
β	α			α	5.90	4.61	0.78	0.36	(1.29)
β	β			α	5.75	4.41	0.77	0.37	(1.34)
α	β			α	6.92	5.39	0.78	0.42	(1.53)

[a]All the compounds examined are of 5β-series; measured as the 24-pyrenacyl ester derivatives; column, Capcell Pak C_{18}; mobile phase, methanol/water (80:20 vol/vol); detection, fluorescence (excitation wavelength 370 nm; emission wavelength 440 nm); the $\Delta k'$ value of 3α,6α,7α-trihydroxy-5β-cholanoic acid was taken as 1.0 for the calculation of the $r\Delta k'$ values; see footnotes to Table 1 for other explanations, abbreviations, and company source.

In the absence of Me-β-CD in the mobile phase, four stereoisomeric 3α,6,7-trihydroxy acids afforded well-resolved peaks, emerging from the column in the elution order of 3α 6α 7β < 3α 6β 7α < 3α 6β 7β << 3α 6α 7α. Analogously, 3α,6,7,12α-tetrahydroxy stereoisomers follow the same elution order with that observed for the 3α,6,7-triols, while 3β,6,7-trihydroxy stereoisomers were eluted in the order of 3β6β7α < 3β6α7β < 3β6β7β < 3β6α7α. Four stereoisomeric 3,6,12α-trihydroxy acids were less strongly adsorbed on the column than any other 3,6,7-triols, and the following elution order was observed: 3β6β12α < 3α6β12α < 3β6α12α < 3α6α12α. These elution orders observed on a Capcell Pak C_{18} column are at slight variances with those reported previously by Batta *et al.* (20) and by us, Iida *et al.* (21), in which we used different columns, mobile phases, and/or C-24 ester derivatives. In each of the stereoisomeric groups, compounds having all α-oriented hydroxyls had definitely the lowest mobility, because they do not have a β-substituent which reduces the hydrophobic β-surface area of steroid molecules and increases the polarity of the molecules, resulting in a decrease in the retentions (22).

Addition of Me-β-CD to the mobile phase resulted in a considerable reduction of the retentions of 3,4β,7- and 3,6,7-trihydroxy compounds and caused a significant change in the elution order of several isomeric pairs due to a combined effect of Me-β-CD on the retentions of each hydroxyl. However, the retention data for the polyhydroxylated compounds did not demonstrate an additive property of each hydroxyl contribution. Hence, the result probably suggests that nonspecific hydrogen bondings occur between the solutes and the Me-β-CD (17).

In the inclusion HPLC, the retention (k_2') of the 3,6,7-trihydroxy acids was shortened greatly by *ca.* 50–70%, compared with those (k_1') observed in the conventional HPLC, and the observed rk' and $r\Delta k'$ values for these compounds were 0.26–0.47 and 0.70–1.38, respectively. A comparison of the rk' values between two epimeric pairs at the 3-, 6-, or 7-position in the eight stereoisomeric 3,6,7-triols (12 epimeric combinations are possible) showed that the β-hydroxy isomers in each combination always have smaller rk' values than the corresponding α-epimers and that the difference in the rk' values between the two epimers is usually larger for the 3β- and 7β-hydroxy isomers than for the 6β-epimers: e.g., 3α6α7β vs. 3β6α7β, 0.08; 3α6α7β vs. 3α6β7β, 0.01; 3α6α7α vs. 3α6α7β, 0.11. A similar comparison of the difference in the $r\Delta k'$ values between the two epimeric pairs also provided additional information for characterizing each of the eight stereoisomers: e.g., 3α6α7α vs. 3β6α7α, 0.06; 3α6α7α vs. 3α6β7α, –0.30; 3α6α7α vs. 3α6α7β, 0.28.

Particular interest should be paid for the inclusion HPLC behavior of 3,4,12α- and 3,6,12α-trihydroxy and 3,4,7α,12α- and 3α,6,7,12α-tetrahydroxy acids, all of which possess a 12α-hydroxyl group. The retention of all the compounds was

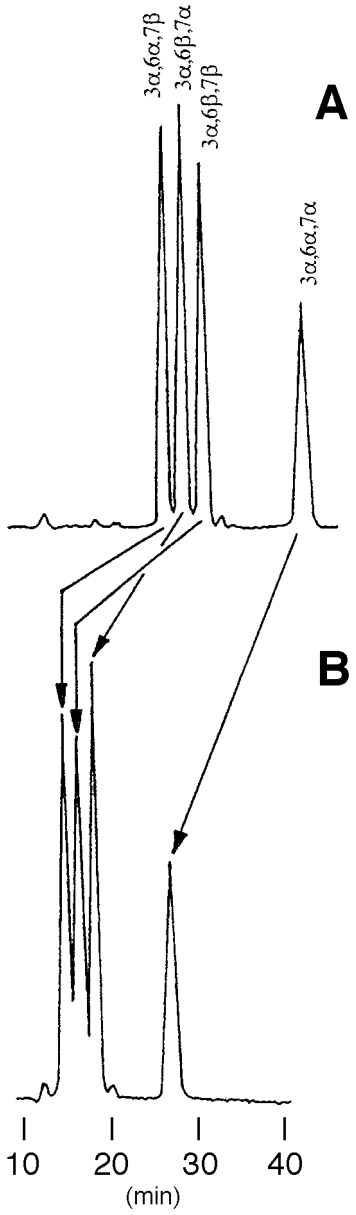

FIG. 4. HPLC chromatograms of a mixture of the 24-pyrenacyl ester derivatives of stereoisomeric 3α,6,7-trihydroxylated bile acids eluted by methanol/water (80:20, vol/vol) (A) without or (B) with 5 mM Me-β-CD in the mobile phase. See Scheme 1 for abbreviations.

decreased to a certain extent by Me-β-CD, but the elution order of each stereoisomeric group was unchanged. Such anomaly of the 12α-hydroxylated bile acids in the inclusion HPLC is compatible with those observed for 12-monosubstituted compounds mentioned above and for deoxycholic and cholic acid derivatives reported previously (12). Furthermore, all the 12α-hydroxylated compounds had a nearly constant rk' value of 0.76 (n = 13; standard deviation, 0.02; coefficient of variation, 2.69%), irrespective of the other structural features. On the contrary, the $r\Delta k'$ values of the 12α-hydroxylated compounds were somewhat influenced by the presence or absence and the configuration of additional hydroxyls at the positions C-6 and/or C-7 in the 5β-nucleus and classified roughly into the three classes of compounds. Those are compounds ($r\Delta k'$, 0.09–0.11) having a β-hydroxyl either at the 6- or 7-position, compounds ($r\Delta k'$, 0.18–0.25) having an α-hydroxyl either at the 6- or 7-position, and compounds ($r\Delta k'$, 0.36–0.42) without both the hydroxyls. The order of the $r\Delta k'$ values for the compounds reflects their structural features and nearly corresponds to that of the k_1' values. The above results may suggest that essentially there is either no or little effect of the addition of Me-β-CD on the retention of the 12α-hydroxylated compounds.

The reason why the retention of all the 12α-hydroxylated compounds is decreased by *ca.* 24% by adding 5 mM Me-β-CD in the mobile phase (methanol/water, 80:20, vol/vol) may be attributed to reduction of the retention ability (i.e., partition coefficient) of the C_{18}-bonded stationary phase in a Capcell Pak column employed. It is known that in inclusion HPLC, an organic mobile phase modifier such as methanol forms a weak inclusion complex with a CD (14,23). Analogously, if the stationary phase causes hydrophobic interactions not only with bile acid molecules but also with Me-β-CD, the solubility of the bile acids into the stationary phase is reduced. The decrease (*ca.* 24%) of the capacity factors of the bile acids in the mobile phase containing 5 mM Me-β-CD would therefore correspond to that of the retention ability of the column. Although the usefulness of the inclusion HPLC in the analysis of naturally occurring bile acids has been reported previously (14), a more detailed study is now being conducted in these laboratories.

In conclusion, the relationship between retentions and structures of a variety of monosubstituted and polyhydroxylated bile acid isomers was examined as their 24-pyrenacyl ester derivatives on a C_{18} reversed-phase column, eluting with methanol–water mixtures in the presence or absence of Me-β-CD. Retentions of all the compounds were decreased by the addition of Me-β-CD, and the magnitude of the decreases depended significantly on the position and configuration of oxygen-containing functions and the stereochemistry of the A/B-ring fusion in the solutes. The observed changes in the retentions were found to be essentially ascribed to hydrogen- bonding interactions between the functional groups of both the nucleus and the side-chain in the solutes and hydroxyl groups of both the narrower and wider rims in the Me-β-CD. The retention characteristics reported here provide much useful information in the understanding of the analysis of bile acid molecules.

ACKNOWLEDGMENT

We are indebted to Natsumi Suzuki for her technical assistance and typing the manuscript.

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[Received August 8, 1997, and in final revised form November 12, 1997; revision accepted November 17, 1997]

COMMUNICATION

Seasonal Changes in Leaf Lipid and Fatty Acid Composition of Nine Plants Consumed by Two African Herbivores

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ABSTRACT: In the wild, nutrients from plants provide for the requirements of all herbivores and ultimately all animals, yet little work has been published on how such nutrients change with environmental conditions. Plant lipids are nutritionally important for browsing mammals, initially with respect to taste and palatability, but ultimately how they fulfill a variety of functions. This study reports on seasonal changes in fatty acid (FA) profiles of nine plants commonly consumed by two herbivore species (kudu and impala) in South Africa. The FA composition of the plants varied with both season and plant species but not with geographic location. The overall profile of FA provided a maximum of n-3-essential FA during the transition and wet seasons, but there was no parallel increase in n-6-essential FA.
Lipids 33, 109–113 (1998).

Browsing mammals in the wild systematically vary the selection of their food sources with season (1,2), yet little work has been published on variations in plant nutrient profiles. Seasonal, geographic, or inter/intraspecific variation of plant composition, in particular those components of nutritional significance to mammals, has received minimal attention. An exception to this is that seasonal differences in the mineral composition of plants have been shown to be significant in determining the spatial distribution (3) and migratory patterns (4) of East African herbivores.

Plants react to mammalian predation by production of a variety of unpalatable compounds (5) and fatty acid (FA)-derived intra-individual plant pheromones (6–10). Similarly, plants exhibit varying tolerance to insect-induced damage (11). Lipids contribute greatly to the flavor of a food, thus the FA composition of leaf lipids may be significant in the consumption patterns adopted by herbivorous mammals. General mammalian nutritional requirements also vary seasonally (12–13), with more of the FA and their derivative postdesaturation FA needed during growth, reproduction, and lactation (14–18). In southern Africa, these periods are usually coincident with the wet season, which is also the period of most active plant growth.

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Abbreviation: FA, fatty acid.

Variation with time of food-plant selection has been noted for many herbivores but only quantitated for two of the more common South African browsing herbivore species, the kudu (*Tragelaphus strepsiceros*) and the impala (*Aepyceros melampus*) (1,2). Nine plant species constitute >95% of their food intake, but the amount of each plant species consumed varies with both season and mammal species (1,2). The present work was carried out in a small but geologically diverse reserve (Nylsvley, Fig. 1) situated in the subtropical region of the South African Northern Province highveld at an elevation of about l000 m.

An initial survey at Nylsvley of the FA profiles of these plant species showed minimal variation in FA between soil types, but significant differences dependent on both season and plant species (19,20). However, the distance between individual sampling sites was small (<5 km) and this may have limited any variation reflecting site or soil type.

The purpose of the study reported here was to extend observations obtained at Nylsvley to a wider geographical area. Samples were collected from the nine plant species every year for a 4-yr period, during the same months, but from eight geologically different regions of the Kruger National Park (Fig. 1, left insert) covering about 250 km^2. The Kruger Park itself spans the subtropical and tropical areas of Mpumalanga Province lowveld. The average elevation of the park is about 200 m, and it covers an area of more than 300,000 km^2.

MATERIALS AND METHODS

The eight sampling sites within the Kruger National Park were situated within the following geographical/geological areas, as categorized by the National Parks Board: Mopane Shrub Veld, Mixed Red Bushwillow–Mopane Veld, Lebombo Mountain, Knobthorn–Maroela Veld on Basalt and Dolomite, Mixed Combretum Veld, Mixed Combretum-Acacia Veld, Silver Clusterleaf-Sicklebush Veld, and Delagoa Thron Veld.

Roughly 50-g samples of leaves were collected at between l and 2 m above ground level, which approximated the average browsing heights of the impala and kudu. Samples were weighed, chopped, and extracted with a mixture of chloroform/methanol (2:1,vol/vol) (21). The lipid extracts were stored at −20°C under nitrogen, and the residual leaf material

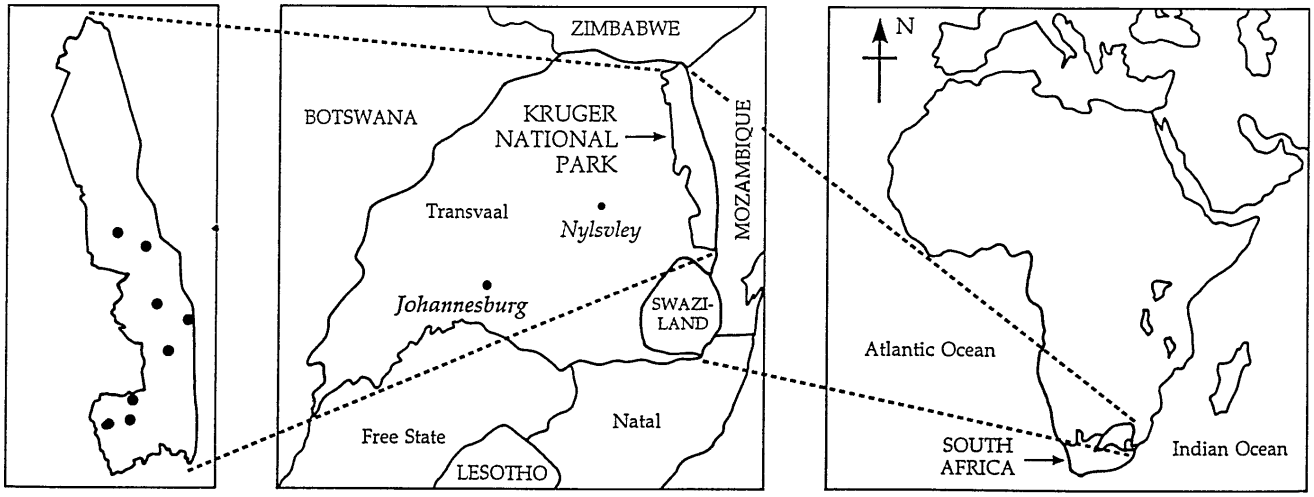

FIG. 1. Location of Nylsvley Reserve and Kruger National Park within South Africa.

was dried and reweighed. Aliquots of the extracts were used for lipid weight determination. FA methyl esters were prepared from 20 mg of each lipid sample using BF_3 in methanol (22). Analyses were performed using a Varian 3400 gas chromatograph (Varian-Aerograph, Walnut Creek, CA) with 4270 integrator and a 30-m SP2330 capillary column (SMM Instruments, Johannesburg, South Africa) run isothermally at 210°C.

In the Kruger National Park, materials were collected from the same individual specimens of each tree species at each sampling time in the different seasons. Wet season was defined as the period from November to April, dry season from May to August, and transition season from September to November, inclusive. The exact months of sampling were the same as those used for the Nylsvley study (19,20), i.e., January, June, and September. The nine plant species were also the same: *Acacia karroo*, *A. nilotica*, *A. tortilis*, *Dichrostachys cinerea*, *Burkea africana*, *Rhus leptodictya*, *Grewia flavescens*, *Euclea natalensis*, and *Terminalia sericea*. Individual trees were selected by arbitrary choice of compass direction from each start point and fell within approximately 5 m either side of a straight line from that point. Where more than one individual fell within the collection area, the first specimen was marked and used. Each species was sampled once only per site per season per year. No sampling trees were lost to fire, animal damage or other causes during the years of the study.

Statistical comparisons were carried out using the Student's *t*-test, as recommended by the Statistical/Research Advisory Service of the South African Medical Research Council.

RESULTS

Table 1 presents *A. karroo* dry-season data as a typical example of all the species, and shows the variation between the individual sampling sites on a yearly basis. There were no significant differences at $P > 0.25$ for all species.

Table 2 compares the FA profiles for *A. karroo* at the different sampling sites within the Kruger Park. There were no significant differences between the sampling sites, as in all cases $P > 0.25$. For the sake of brevity, only data for *A. karroo* in the dry seasons are shown; however, the same held true for all the species analyzed irrespective of season or FA class.

TABLE 1
Effect of Site and Year on the Percentage of Saturated Fatty Acids in *Acacia karroo* Across Four Years

Site	Year 1[a]	Year 2[a]	Year 3[a]	Year 4[a]	Mean
1	37.2	39.7	39.8	36.1	38.2 ± 1.6
2	37.9	34.1	37.4	34.3	35.9 ± 1.7
3	35.2	38.7	35.1	38.1	36.8 ± 1.6
4	36.9	35.5	39.3	39.9	37.9 ± 1.8
5	37.8	35.6	37.5	35.1	36.5 ± 1.2
6	35.5	36.5	39.1	38.8	37.5 ± 1.5
7	36.4	39.7	39.9	35.1	37.8 ± 2.1
8	34.9	35.7	37.3	38.1	36.5 ± 1.3
Mean	36.7 ± 1.1	37.1 ± 2.0	38.4 ± 1.5	37.1 ± 1.9	37.8 ± 1.5

[a]Each data point represents $n = 1$ sample/yr/sampling site, Σ:4. Means shown ± SEM. The *P* values in all cases were at least >0.25. The data for *A. karroo* in dry season are shown as an example. The data for Kruger National Park showed no significant differences from those of Nyslvley (Refs. 19,20), $P > 0.25$ in all cases. Units are saturated fatty acids as percentage of total fatty acids.

TABLE 2
Effect of Sampling Site on the Fatty Acid Types as a Percentage of Total Fatty Acids of *Acacia karroo* in Kruger National Park

Reserve	Site no.	Total saturated[a]	Total mono-unsaturated[a]	18:2n-6[a]	18:3n-3[a]
Kruger NP	1	38.2 ± 1.6	14.8 ± 3.1	17.1 ± 3.1	41.2 ± 4.3
	2	35.9 ± 1.7	11.8 ± 2.6	14.3 ± 3.0	32.4 ± 4.5
	3	36.8 ± 1.6	11.7 ± 3.3	12.5 ± 2.7	37.3 ± 3.7
	4	37.9 ± 1.8	12.4 ± 4.1	13.5 ± 3.3	32.4 ± 3.8
	5	36.5 ± 1.2	10.2 ± 1.7	15.9 ± 2.6	30.6 ± 4.1
	6	37.5 ± 1.5	12.8 ± 2.3	13.1 ± 2.8	37.2 ± 4.6
	7	37.8 ± 2.1	15.4 ± 2.4	17.6 ± 2.5	31.1 ± 3.7
	8	36.5 ± 1.3	15.9 ± 2.6	16.0 ± 2.7	39.4 ± 3.8
	Mean	37.8 ± 1.5	13.1 ± 1.9	15.0 ± 1.8	35.2 ± 3.8
Nylsvley	Mean	37.2 ± 1.4	11.6 ± 0.8	15.6 ± 1.3	35.8 ± 2.5

[a]Each data point based on $n = 4$. Means shown ±SEM. The *P* values in all cases were at least >0.25. The mean data for *A. karroo* for the four dry seasons are shown as an example. The data for Kruger National Park (NP) showed no significant differences from those of Nyslvley (Refs. 19,20), $P > 0.25$ in all cases.

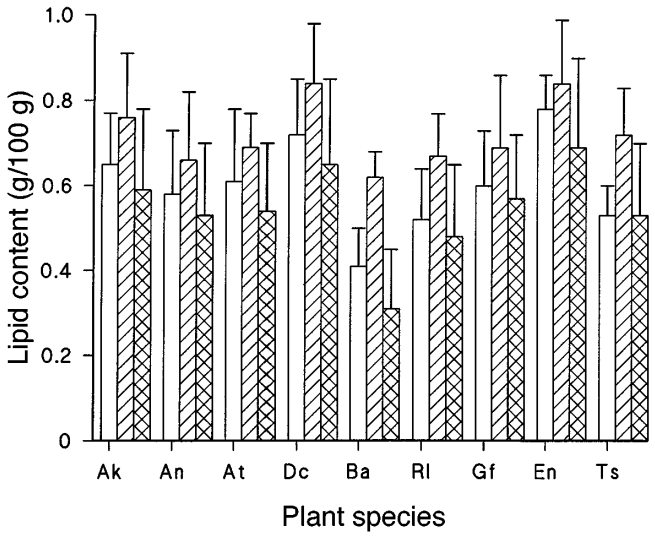

FIG. 2. Seasonal variation in lipid content (g/100 g) among the following nine plant species: *Acacia karroo* (Ak), *A. nilotica* (An), *A. tortilis* (At), *Dichrostachys cinerea* (Dc), *Burkea africana* (Ba), *Rhus leptodictya* (Rl), *Grewia flavescens* (Gf), *Euclea natalensis* (En), and *Terminalia sericea* (Ts). Data are shown as mean ± SEM, $n = 32$ (eight sampling sites over four years). Open bars are wet season, diagonal lines are dry season, and crosshatch are transition season.

Figure 2 shows the lipid content (g/100 g leaf matter). There were changes in total lipid between wet and dry season, with the amounts increasing across all the species, but only the changes for *B. africana* and *T. sericea* were significant at $P < 0.10$. Generally, the transition-season samples showed levels of total lipid slightly and nonsignificantly decreased compared to wet season.

Figure 3 shows the profiles of the FA for each species across the three seasons. In general, the saturated fatty acids (Fig. 3A) increased during dry season (only *Acacia karroo* and *B. africana* decreased) but showed a slight drop during transition season. The greatest increase between wet and dry season was shown by *T. sericea* (29.8 to 56.2 g FA/100 g dry matter), while the greatest decrease was that of *B. africana* (52.4 to 28.1 g FA/100 g dry matter). All differences were significant ($P < 0.05$ at least) except for *R. leptodictya,* where there was no significant change. There were fewer significant differences between wet and transition seasons with *A. nilotica* and *tortilis*, *D. cinerea*, *G. flavescens* and *T. sericea* showing no significant change, and the other species showing signficant decreases.

The monoenoic FA (Fig. 3B) also showed increases between wet and dry seasons, with the greatest increase occurring for *A. tortilis* (6.4 to 23.8 g FA/100 g). The only species not showing an increase was *R. leptodictya*; however, the decrease was not significant. In comparing wet and transition seasons, four species (*A. tortilis*, *D. cinerea*, *E. natalensis,* and *T. sericea*) showed no significant changes, whereas *A. karroo*, *A. nilotica,* and *B. africana* showed increases significant at $P < 0.10$. *Rhus leptodictya* monoenoic FA increased, but P was only <0.10, whereas *G. flavescens* showed an increase with $P < 0.05$.

Apart from *A. nilotica*, *A. tortilis*, and *T. sericea*, 18:2n-6 increased markedly in the dry season compared to the wet season (Fig. 3C). Both *A. nilotica* (15.7 to 11.3 g FA/100 g) and *T. sericea* (16.2 to 13.3 g FA/100 g) showed decreases significant at $P < 0.10$, whereas the differences for *A. tortilis* and *D. cinerea* were not significant. The other four species showed highly significant increases with $P < 0.05$, and the greatest change was shown by *A. karroo* (7.5 to 15.0) g FA/100 g. When wet and transition seasons were compared, all the species demonstrated increased levels of 18:2n-6 during transition. Only that of *A. nilotica* was not significant. In contrast *A. karroo, G. flavescens,* and *E. natalensis* showed changes significant at the $P < 0.05$ level.

The species showing the greatest decrease in 18:3n-3 in comparing wet and dry seasons were *G. flavescens* (44.3 to 11.6 g FA/100 g) and *T. sericea* (43.6 to 17.0 g FA/100 g), and these were significant at $P < 0.01$. Only *B. africana* showed no significant change, whereas the other species exhibited decreases significant at either $P < 0.10$ or $P < 0.05$ (Fig. 3D). When comparing wet and transition seasons, there were few significant differences. Only *A. karroo* and *E. natalensis* showed increased 18:3n-3 levels, increased with $P < 0.05$, while *G. flavescens* showed a decrease significant only at $P < 0.10$.

DISCUSSION

The site variation at Nylsvley reflected differences between soil types, mainly sandy vs. clay, while the variation at Kruger Park reflected much greater geological and botanical differences (Fig. 1). Figure 2 shows the variation between seasons in total lipid across the nine plant species. The dry-season samples showed higher levels of total lipid, but this may reflect the reduced availability of water, for the samples appeared less turgid than those from other seasons. There were no significant differences at $P < 0.05$ between the seasons; however *B. africana* and *T. sericea* showed differences between wet and dry seasons, but only at $P < 0.10$.

The FA profiles of the nine plant species showed no significant variation within a species or within a season when site, soil type, or location were compared (Tables 1 and 2). The profiles were thus independent of these parameters even between reserves over 300 km apart and at markedly different altitudes. There were, however, very significant differences both between plant species and between seasons (Fig. 3). The wet season is the period when metabolic activity is high but stable, whereas during dry season metabolic activity is relatively low. The transition period is the period of greatest change in metabolic activity. The adaptation of the species to the change from dry to transition appears to have been very fast, as the data for 18:3n-3 during the transition were very close to that for the wet season. However, *A. karroo* and *E. natalensis* both showed levels higher than wet season, whereas *G. flavescens* had lower levels of 18:3n-3 during transition.

The most significant FA was *cis*-C18:3n3 (α-linolenic acid), the polyunsaturate (polyenoic) precursor of the n-3 series. This FA is the major FA found ln photosynthetic plant

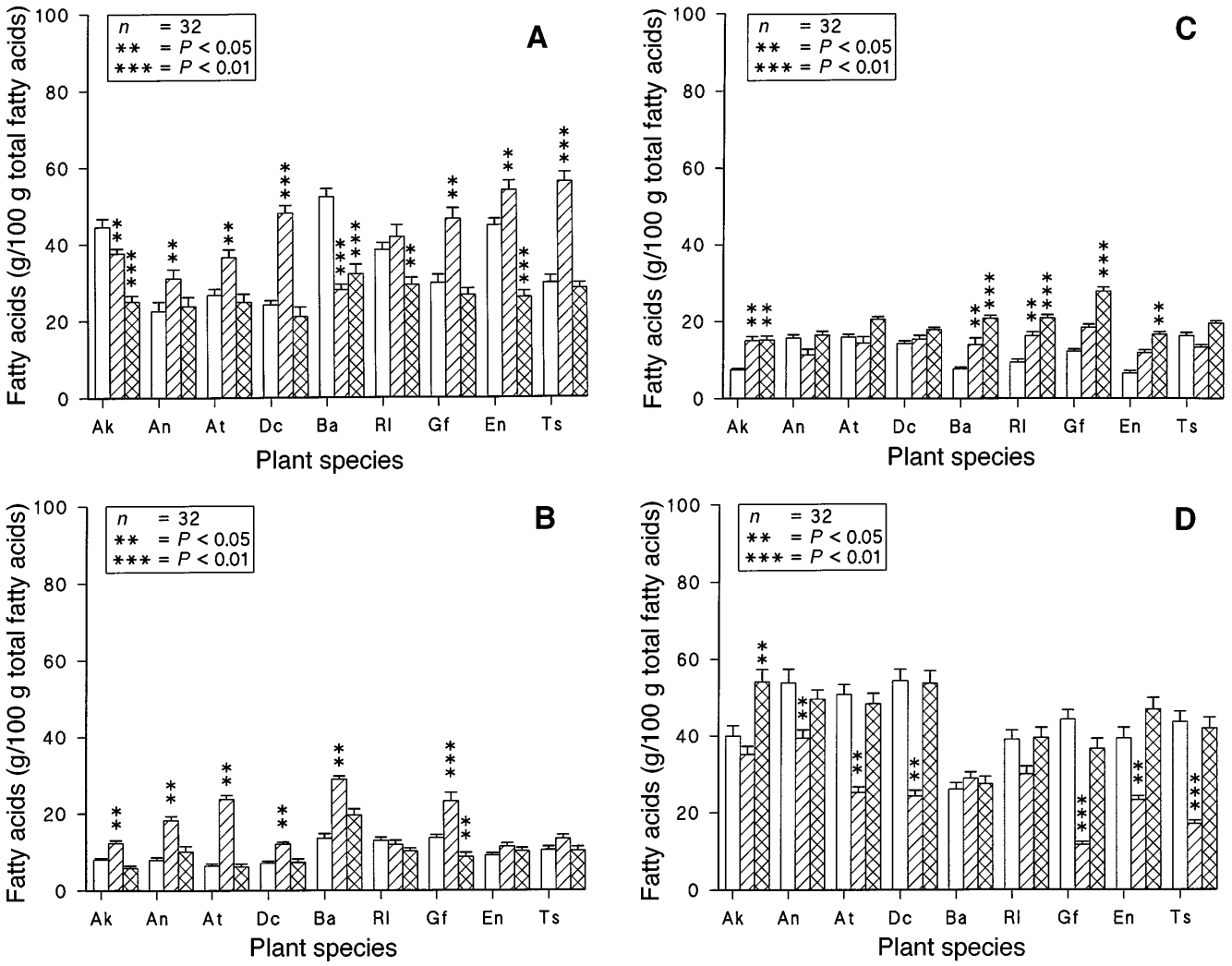

FIG. 3. Seasonal variations in fatty acid profiles among the nine plant species. Open bars are wet season, diagonal lines are dry season, and cross-hatch are transition season. Species identification is the same as used in Figure 2. Data are shown as mean ± SEM. The *P* values are relative to wet season data in all cases. (A) Total saturated fatty acids; (B) total monoenoic fatty acids; (C) *cis*-C18:2n-6 (linoleic acid); (D) *cis*-C18:3n-3 (α-linolenic acid).

matter (23), and it is interesting that it was this FA that most rapidly returned to wet-season levels during transition. This may correlate with a need to produce active photosynthetic organelles early to maximize energy production capability.

Conversely, the levels of *cis*-C18:2n-6 (linoleic acid), the n-6 series precursor, increased during dry season and remained elevated during transition. This may reflect that during dry season there may be a relatively greater removal of 18:3n-3 with the decrease in photosynthetic potential, thus leaving an apparently higher level of 18:2n-6 which is fulfilling a structural role. The continued higher levels of 18:2n-6 during transition may also reflect the need for more structural lipid during rapid leaf growth.

The pattern of species/seasonal differences did not vary greatly from year to year and appeared to be independent of variations in actual annual rainfall, at least within the time scale of these studies. During the period of this study, the actual rainfall did vary quite considerably, from a low of 13 mm to a high of 99 mm in January, 2 to 38 mm in June and 0 to 81 mm in September (wet, dry, and transition season collection months, respectively).

The intraspecies and seasonal differences among the three *Acacia* species emphasize that even relatively closely related species such as the acacias may show distinctly different patterns of FA content and FA change.

Both the kudu and the impala are diurnal browsing feeders and consume the same nine plant species, but the time spent browsing varied considerably with season and herbivore species (1,2). Quantitatively, *G. flavescens* and *D. cinerea* contributed the most to the intakes of these two herbivores; however, the plants contributing less may be providing nutrients not present in adequate levels in these two main species.

No quantitative data were available as to the contribution that the seeds of these plant species made to the herbivores' diets, except that it was sporadic and highly seasonal, being largely confined to the months from March to May. Given that the levels of linoleic acid (*cis*-18:2n-6) are commonly high in seeds (24), it is possible that they may have provided a source of this FA, but not on a regular year-round basis.

ACKNOWLEDGMENTS

The author thanks the Foundation for Research Development of South Africa and the National Parks Board of South Africa for their wholehearted cooperation during the Nylsvley and Kruger National Park phases of this project, respectively. They also thank Efamed (Pty.) Ltd., South Africa; Scotia Pharmaceuticals, United Kingdom; Medical Research Council, South Africa; and the University of the Witwatersrand, South Africa, for their financial and logistical support during this study. The editorial comments of Dr. Andrew Sinclair were invaluable in finalizing this manuscript.

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[Received February 12, 1996, and in final revised form December 8, 1997; revision accepted December 10, 1997]

ARTICLE

Arachidonic Acid Supplementation Enhances Synthesis of Eicosanoids Without Suppressing Immune Functions in Young Healthy Men

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ABSTRACT: This study was conducted to determine the effects of arachidonic acid (AA) supplementation on human immune response (IR) and on the secretion of prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). Ten healthy men (20–38 yr) participated in the study and lived at the Metabolic Suite of the Western Human Nutrition Research Center. They were fed a basal diet (57, 27, and 16 energy percentage from carbohydrate, fat, and protein, respectively, and AA 200 mg/d) for the first 15 d of the study. Additional AA (1.5 g/d) was added to the diet of six men from day 16 to 65, while the remaining four subjects remained on the basal diet. The diets of the two groups were crossed-over from day 66 to 115. *In vitro* indices of IR were examined using blood drawn on days 15, 58, 65, 108, and 115. Influenza antibody titers were determined in the sera prepared from blood drawn on days 92 and 115 (23 d postimmunization). AA supplementation caused significant increases in the *in vitro* secretion of LTB4, and PGE2, but it did not alter the *in vitro* secretion of tumor necrosis factor α; interleukins 1β, 2, 6; and the receptor for interleukin 2. Nor did it change the number of circulating lymphocytes bearing markers for specific subsets (B, T, helper, suppressor, natural killer) and the serum antibody titers against influenza vaccine. The opposing effects of PGE2 and LTB4 may have led to the lack of change in immune functions tested.
Lipids 33, 125–130 (1998).

Studies conducted in cultured cells and animal models show that n-6 polyunsaturated fatty acids (PUFA) inhibit immune cell functions (1–10). The results from studies in humans are mixed. A moderate increase in dietary linoleic acid (18:2n-6, LA) did not affect human immune response (IR) when dietary fat was held constant (11–13). But a negative association between plasma LA concentration and the number of circulating natural killer (NK) cells has been reported in a group of elderly individuals (14).

LA is the most abundant dietary n-6 PUFA for humans. It can be converted to arachidonic acid (20:4n-6, AA) by humans and several other animal species. AA is the major n-6 fatty acid of the membranes and it is a precursor of the eicosanoids. It is metabolized by two distinct oxygenase pathways. The cyclooxygenase pathway converts AA to prostaglandins, thromboxanes, and prostacyclins; the lipoygenase pathway converts it to leukotrienes.

In vitro studies show that AA and its metabolic products can alter a number of lymphocyte and monocyte functions, including the maturation of immature T cells into helper and suppressor cells, lymphocyte proliferation, cytokine and antibody secretion (15–21). Prostaglandin E2 (PGE2) inhibited a number of lymphocyte functions in a dose-dependent manner, whereas the leukotriene B4 (LTB4) was stimulatory at low concentrations and inhibitory at higher concentrations (22). When eicosanoid synthesis was blocked with inhibitors of cyclooxygenase and lipooxygenase pathways, addition of AA still inhibited immune cell functions *in vitro* (23–27). Thus, it is not clear if n-6 PUFA inhibit immune cell functions through eicosanoids or through other mechanisms.

The effects of AA supplementation on both immune cell functions and eicosanoid synthesis have not been examined previously in humans, because of concerns regarding its safety and the nonavailability of natural triglycerides of AA. An algal source of natural AA triglycerides recently became available, making human studies of AA supplementation feasible. Use of natural triglycerides of AA permits one to study its effects on human IR while the total amounts of other dietary fatty acids are held constant.

The purpose of this study therefore was to measure eicosanoid secretion and immune functions in humans given a supplement of AA while consuming a constant diet. We examined *in vivo* antibody production in response to influenza vaccine, lymphocyte subsets, and *in vitro* secretion of PGE2, LTB4, interleukin (IL) 1β, IL 2, IL 2R, IL 6, and tumor necrosis factor α using peripheral blood mononuclear cells (PBMNC).

SUBJECTS AND METHODS

Subjects, protocol, and diets. Ten healthy men (20–38 yr) were selected to participate in the study. All subjects were nonsmokers, had no history of alcohol or drug abuse, had moderate exercise levels, had body weights within −10 to

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Abbreviations: AA, arachidonic acid; IL, interleukin; IR, immune response; LA, linoleic acid; LPS, lipopolysaccharide; LTB4, leukotriene B4; NK, natural killer; PBMNC, peripheral blood mononuclear cells; PGE, prostaglandin E; PHA, phytohemagglutinin; PUFA, polyunsaturated fatty acids.

+20% of ideal body weight, and had chemical and hematological measurements within normal ranges. The participants lived and ate all meals at the Metabolic Unit of the Western Human Nutrition Research Center for 130 d (August 14–December 22, 1994). A crossover design was used. All subjects consumed the basal, low-AA, diet during the first 15 d. For the next 50 d six subjects (group 1) consumed the diet supplemented with AA (1.5 g/d) and the other four (group 2) continued to consume the basal diet. Diets of the two groups were switched on day 66, and the new diets were fed for 50 d (days 66–115). Both groups were fed the basal diet for the last 15 d (days 116–130). Body weights of the subjects were maintained constant throughout the study by adjusting the energy intake and by maintaining a constant physical activity schedule. All subjects walked two miles twice every day. Blood samples were collected on days 16, 58, 65, 108, and 115.

Basal diet consisted of natural foods and was adequate in all nutrients. A 5-d rotating menu with three meals and two snacks every day was used. The proportion of energy from carbohydrate, fat, and protein was 57, 27, and 16%, respectively (28). The energy was equally distributed among the saturated, monounsaturated, and n-6 PUFA. The basal diet provided 200 mg AA and 250 mg cholesterol per day. The calculated amount of vitamin E in the basal diet was two times the Recommended Daily Allowance. Three grams ARASCO® oil (a gift from Martek Biosciences Corporation, Columbia, MD) was added to the diet to provide 1.5 g AA/d. The total fat content of the two diets was held constant by replacing an equivalent amount of monounsaturated fat with AA.

Laboratory procedures. Blood was collected by antecubital venipuncture into evacuated tubes containing heparin for cell culture experiments, or EDTA for lymphocyte phenotypic analysis, or without anticoagulants for preparation of sera. Blood samples for all subjects were collected after an overnight fast, between 0700 and 0800 h.

Secretion of cytokines and eicosanoids. PBMNC were isolated using Histopaque-1077 as previously reported (29). The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) containing 10% autologous serum and L-glutamine (2 mmoI/L), penicillin (100 KU/L), streptomycin (100 mg/L), and gentamicin (20 mg/L). Five hundred μL of the culture medium containing 5×10^5 PBMNC was inoculated in each well of a 24-well flat-bottom culture plate. An additional 500 μL of the culture medium with or without the mitogens was added to each well.

The production of IL 1β, IL 6, tumor necrosis factor, PGE2, and LTB4 was stimulated by the addition of lipopolysaccharide (LPS, 0.5 mg/L), and that of IL 2 and IL 2R was stimulated by adding phytohemagglutinin (PHA, 10 mg/L). Tissue culture media were collected by centrifugation 24 h after stimulation with LPS, and 48 h after PHA. The media were stored frozen at −70°C until the cytokine and eicosanoid concentrations were determined.

ELISA kits for cytokine assays were purchased from T Cell Diagnostics, Inc. (Cambridge, MA), and those for eicosanoids from Cayman Chemical Company (Ann Arbor, MI).

Influenza vaccination and serum antibody titers. All subjects were immunized on study day 92 with the trivalent influenza virus vaccine (Fluzone, 1993–1994 Formula) purchased from Connaught Laboratories Inc. (Swiftwater, PA). Preimmunization sera were prepared from the blood drawn on day 92, and the postimmunization sera from the blood drawn on day 115. The antibody titers were determined at the Centers for Disease Control using the hemagglutination inhibition assay (30) and the viral strains A/TEXAS/36/91, A/BEIJING/32/92, and B/PANAMA/45/90ET. Results for antibody titers are expressed as the geometric means of the antibody titer, the geometric mean transformations, and the percentage of subjects with an antibody titer ≥40 or 160.

Lymphocyte phenotypic analysis. Lymphocyte subsets, including B ($CD19^+$), T ($CD3^+$), helper ($CD3^+$, $CD4^+$), suppressor ($CD3^+$, $CD8^+$), and NK ($CD3^-$, $CD16^+$, $CD56^+$) cells, in peripheral blood were determined using the Becton-Dickinson Flowcytometer as previously reported (29).

Data analysis. The data were analyzed by an analysis of variance model using SAS/STAT PROC GLM (31). The cross-over design model included effects of order, subject (order), period, and diet, using subject (order) as an error term for order. The significance of the difference between with and without AA supplementation was assessed from the *P* values for the diet main effects. The data from the two groups were analyzed separately using a paired *t*-test when the treatment was given only during one period, such as immunization with influenza vaccine. Changes in the variables examined are considered significant for $P < 0.05$ or otherwise stated.

RESULTS

The mean ± SEM for age (yr), weight (kg), height (cm), and body mass index (kg/m^2) for subjects in groups 1 ($n = 6$) and 2 ($n = 4$) were 31.2 ± 3.2 and 32.2 ± 2.9, 73.8 ± 2.4 and 71.0 ± 5.4, 177.4 ± 3.6 and 175.5 ± 1.8, 23.8 ± 1.8 and 23.0 ± 1.3, respectively. The composition of the diets and the average daily intake of nutrients have been previously reported (28). None of these parameters was different between the two groups.

Eicosanoid secretion. PGE2 and LTB4 secretion into the tissue culture medium within 24 h of PBMNC stimulation with LPS is shown in Table 1. AA supplementation significantly ($P < 0.05$) increased the secretion of both of these eicosanoids, compared to the values at the end of stabilization period (day 16). The percentage stimulation of LTB4 secretion with AA supplementation was greater (200–400%) than that of PGE2 secretion (50–100%). In the group receiving AA supplementation first (days 16–65), eicosanoid secretions remained elevated 50 d after the discontinuation of the AA supplementation. The amount of PGE2 secreted into the medium in the absence of LPS (unstimulated cultures) was about 25% of that secreted in the presence of LPS and was not altered by AA supplementation (not shown). The amount of LTB4 secreted in unstimulated cultures was about 50% of the amount secreted in the presence of LPS, and it increased significantly ($P < 0.05$) with AA supplementation (not shown).

TABLE 1
Dietary Arachidonic Acid Increases *in vitro* Secretion of Eicosanoids by PBMNC Stimulated with LPS[a]

	Group 1 (n = 6)			Group 2 (n = 4)		
Eicosanoid	Day 15	Day 65	Day 115	Day 15	Day 65	Day 115
PGE2 (ng/mL)	0.81 ± 0.06[a]	1.25 ± 0.12[b]	1.11 ± 0.10[b]	0.53 ± 0.05[c]	0.47 ± 0.01[c]	0.87 ± 0.06[d]
LTB4 (ng/mL)	0.20 ± 0.02[a]	0.43 ± 0.04[b]	0.39 ± 0.04[b]	0.14 ± 0.01[c]	0.19 ± 0.02[c]	0.85 ± 0.14[d]

[a]Data shown are the mean ± SEM for the number of subjects shown. Diet of subjects in Group 1 was supplemented with arachidonic acid from days 16 to 65, and that of Group 2 from days 66 to 115. For each eicosanoid, comparisons were made within each group using a paired *t*-test, and the numbers bearing different superscripts within each group are significantly different ($P < 0.05$). PBMNC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; PGE2, prostaglandin E2; LTB4, leukotriene B4.

Cytokine secretion. The effects of AA supplementation on cytokine secretion are presented in Table 2. The concentrations of IL 1β, IL 6, and tumor necrosis factor α secreted into the tissue culture media by the PBMNC are in response to stimulation with LPS, and that of IL 2 and IL 2R are in response to stimulation with PHA. Mitogens caused a several-fold increase in the release of all cytokines tested, compared to the corresponding cytokine concentrations in the media from unstimulated cultures, which were below the detection limits of the assay systems (not shown). AA supplementation did not alter the secretion of all the cytokines at any time in the study.

Influenza antibody titers. The pre- and postimmunization antibody titers for the A/Texas, A/Beijing, and B/Panama strains of influenza are shown in Table 3. Immunization caused an approximately 10-fold increase in the serum titer for B/Panama and 30-fold increase in A/Texas in both the AA-supplemented and -nonsupplemented groups. The amount of AA in the diet did not influence the antibody titer for these two strains. Immunization caused a 19-fold increase in the antibody titer for A/Beijing in Group 1 (fed low-AA diet at the time of immunization) and a 9-fold increase in Group 2 (fed high-AA diet). The apparent difference in the antibody titers between the two groups for this viral strain was also evident if the results for antibody titers were expressed as percentage of subjects with titers ≥40 or 160. These results suggest an inhibition of A/Bejing antibody production by AA supplementation, however, the differences between the two groups did not attain statistical significance.

Lymphocyte subsets. The percentage and the absolute numbers for the B, T, helper, suppressor, and NK cells at study days 16, 65, and 115 are shown in Table 4. AA supplementation did not alter the absolute numbers or the percentage of these lymphocytes in circulation. Nor was there an effect of AA supplementation on the ratio between the helper and suppressor T lymphocytes.

DISCUSSION

AA feeding caused significant increase in the *in vitro* secretion of both PGE2 and LTB4, without any change in a number of indices of IR tested. These results suggest that either AA has no effect on IR or it may alter immune functions independently of the changes in eicosanoid production. The second interpretation is consistent with the results from previous studies with inhibitors of lipoxygenase and cyclooxygenase (23–27), in which AA inhibited cytokine production *in vitro*, even when there was no change in eicosanoid synthesis. This inhibition could be due to changes in membrane fluidity or receptor expression. The lack of inhibition of cytokine secretion by AA in our study may be a function of AA concentration, or some other differences in experimental conditions.

Results obtained from *in vitro* studies have demonstrated that PGE2 inhibited (17,20,25) and LTB4 stimulated cytokine production. These opposing effects of PGE2 and LTB4 on cytokine production can also explain our results regarding immune functions. Our study design cannot distinguish between this and the possibility discussed above. We are not aware of any studies where the effects on immune cell functions of adding PGE2 and LTB4 concurrently have been tested. Evaluation of such interaction between these two cytokines is important and needs to be monitored in future studies.

Our results showing increases in eicosanoid production *in vitro* are consistent with the results from *in vivo* studies show-

TABLE 2
Dietary Arachidonic Acid Does Not Alter *in vitro* Secretion of Cytokines by PBMNC[a]

	Group 1 (n = 6)			Group 2 (n = 4)		
Cytokine	Day 15	Day 65	Day 115	Day 15	Day 65	Day 115
TNF α (pg/mL)	309 ± 93	296 ± 79	309 ± 51	299 ± 45	335 ± 85	503 ± 101
IL 1β (ng/mL)	2.99 ± 1.03	4.10 ± 0.87	3.27 ± 0.63	2.99 ± 0.78	3.55 ± 0.98	3.65 ± 0.67
IL 6 (ng/mL)	1.85 ± 0.19	2.07 ± 0.19	2.17 ± 0.15	2.01 ± 0.15	2.19 ± 0.14	2.56 ± 0.25
IL 2 (pg/mL)	376 ± 45	349 ± 27	313 ± 74	272 ± 38	453 ± 14	472 ± 98
IL 2R (nM/mL)	1.34 ± 0.15	1.47 ± 0.01	1.60 ± 0.01	1.37 ± 0.18	1.51 ± 0.10	1.65 ± 0.27

[a]Data shown are the mean ± SEM for the number of subjects shown for each group. Abbreviations: TNF, tumor necrosis factor; IL, interleukin; R, receptor. Synthesis of IL 2 and IL 2R were stimulated with phytohemagglutinin, while that of the other three cytokines with lipopolysaccharide. Arachidonic acid supplementation did not alter the secretion of all cytokines tested.

TABLE 3
Dietary Arachidonic Acid Does Not Alter Serum Influenza Antibody Titers[a]

		Group 1 (n = 6)		Group 2 (n = 4)	
Strain	Index	Pre-immun.	Post-immun.	Pre-immun.	Post-immun.
A/Texas	GM	0.95 ± 0.20[a]	2.45 ± 0.09[b]	0.92 ± 0.15[c]	2.35 ± 0.31[d]
	GMT	9	285	8	226
	% ≥40	17	100	0	100
	% ≥160	0	100	0	75
A/Beijing	GM	0.80 ± 0.10	2.05 ± 0.32	0.70 ± 0.10	1.68 ± 0.56
	GMT	6	113	5	48
	% ≥40	0	83	0	50
	% ≥160	0	33	0	25
B/Panama	GM	1.40 ± 0.48	2.40 ± 0.22	1.08 ± 0.14	2.05 ± 0.15
	GMT	25	254	12	113
	% ≥40	17	100	0	100
	% ≥160	17	83	0	25

[a]Data shown are mean ± SEM for the number of subjects shown for each group. Abbreviations: GM, geometric mean; GMT, geometric mean transformation. Immunization significantly increased the antibody titers for all three viral strains in both groups. There was no effect of arachidonic acid supplementation on the antibody titers for all three strains.

ing an increase in AA metabolites in the urine of subjects supplementing their diets with n-6 PUFA (32–35). Results regarding immune functions are also consistent with our previous report from the same study in which we found no changes in lymphocyte proliferation in response to PHA and Concanavalin A, NK cell activity, and delayed hypersensitivity skin response (28). These results, however, differ from those of several *in vitro* studies, which showed inhibition of cytokine production with PGE2 (20,25) and stimulation with LTB4 (18). As stated earlier, the opposing effects of the two eicosanoids may have canceled each other.

The lack of an AA effect on cytokine secretion in our study could also result from the failure of the mitogens to increase the synthesis of the receptors for the cytokines. This is unlikely, because the mitogens and the experimental conditions used in this study have previously been shown (18,20,29) to increase the secretion of the cytokines examined in this report. Even in the present study the cytokine secretion was severalfold higher in the cultures treated with mitogens, compared to the parallel cultures without mitogens. Together these data indicate that cytokine secretion was increased by the mitogens, but it was not altered by AA feeding.

AA feeding did not alter the concentration of serum antibody production against the A/Texas and B/Panama strains. There was a trend toward reduction in antibody produced in response to A/Beijing strain by AA feeding, but it did not attain significance. This is perhaps due to the small number of subjects (six in Group 1, and four in Group 2), or the crossover study design. We believe a 50-d wash-out period should have removed most of the AA in Group 1; however, the amount of eicosanoids produced in this group at day 115 suggests that the wash-out was not complete. Our study design cannot distinguish whether the residual AA leads to the lack of difference in antibody production between the two groups, or AA has no effect on antibody production. There was no carryover effect for other immune functions tested in the six subjects in Group 1. Thus, it is safe to assume that AA feeding did not alter cytokine production and the number of phenotypically distinct lymphocytes in circulation.

Our data indicate that the amount, form, and duration of AA fed had no adverse health effects, as long as the total fat and vitamin E intakes were maintained constant. Further-

TABLE 4
Dietary Arachidonic Acid Does Not Alter Number of Phenotypically Distinct Lymphocytes in Circulation[a]

		Group 1 (n = 6)			Group 2 (n = 4)		
Cell type		Day 16	Day 65	Day 115	Day 16	Day 65	Day 115
B	10^9/L	218 ± 36	280 ± 38	260 ± 30	185 ± 93	198 ± 54	208 ± 51
	%	12.0 ± 1.3	14.6 ± 1.4	14.3 ± 1.4	10.0 ± 2.3	9.9 ± 2.2	11.5 ± 1.9
T	10^{12}/L	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
	%	73.1 ± 3.4	72.8 ± 3.3	69.3 ± 2.9	77.1 ± 4.1	79.1 ± 4.3	74.5 ± 3.9
Helper	10^9/L	840 ± 118	897 ± 126	788 ± 87	788 ± 65	801 ± 112	690 ± 36
	%	46.3 ± 2.7	45.9 ± 1.6	41.8 ± 1.6	42.5 ± 4.1	42.3 ± 3.8	40.3 ± 3.3
Suppressor	10^9/L	418 ± 73	497 ± 63	448 ± 71	596 ± 91	657 ± 82	555 ± 78
	%	24.5 ± 1.9	25.7 ± 1.3	25.7 ± 1.9	32.5 ± 5.3	36.0 ± 6.0	32.3 ± 4.3
NK	10^9/L	241 ± 44	223 ± 36	287 ± 51	239 ± 69	190 ± 39	240 ± 75
	%	14.7 ± 3.2	12.1 ± 2.0	14.7 ± 2.4	12.4 ± 2.9	10.0 ± 1.8	14.5 ± 3.9

[a]Data shown are the mean ± SEM for the number of subjects shown for each group. Arachidonic acid supplementation did not alter the number or the percentage of lymphocytes within each subset.

more, the concurrent increase in PGE2, and LTB4 secretion had no effect on immune cell functions. These results are consistent with the results from our LA studies, in which several indices of IR were not inhibited by increase in LA intake as long as the total fat intake was maintained constant (8,9). Together these studies show that moderate intake of n-6 PUFA do not inhibit human IR if the diet is adequate in antioxidant nutrients and the total dietary fat is held constant.

ACKNOWLEDGMENTS

Authors thank the volunteers for their participation in the study, the nursing and dietary staff of the Metabolic Unit, and the staff of the Bioanalytical Laboratory at the Western Human Nutrition Research Center for their help in conducting the study. We also thank Dr. Helen Regenary of the Centers for Disease Control for analysis of serum antibody titers, and Dr. Janet King of our Center for a critical review of the manuscript.

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[Received October 27, 1997, and in final revised form December 21,

Effects of Highly Purified Eicosapentaenoic Acid and Docosahexaenoic Acid on Fatty Acid Absorption, Incorporation into Serum Phospholipids and Postprandial Triglyceridemia

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ABSTRACT: Fourteen healthy volunteers were randomly allocated to receive 4 g highly purified ethyl esters of eicosapentaenoic acid (EPA) (95% pure, $n = 7$) or docosahexaenoic acid (DHA) (90% pure, $n = 7$) daily for 5 wk in supplement to their ordinary diet. The n-3 fatty acids were given with a standard high- fat meal at the beginning and the end of the supplementation period. EPA and DHA induced a similar incorporation into chylomicrons which peaked 6 h after the meal. The relative uptake of EPA and DHA from the meal was >90% compared with the uptake of oleic acid. During absorption, there was no significant elongation or retroconversion of EPA or DHA in total chylomicron fatty acids. The concentration of EPA decreased by 13% and DHA by 62% ($P < 0.001$) between 6 and 8 h after the meal. During the 5-wk supplementation period, EPA showed a more rapid and comprehensive increase in serum phospholipids than did DHA. DHA was retroconverted to EPA, whereas EPA was elongated to docosapentaenoic acid (DPA). The postprandial triglyceridemia was suppressed by 19 and 49% after prolonged intake of EPA and DHA, respectively, indicating that prolonged intake of DHA is equivalent to or even more efficient than that of EPA in lowering postprandial triglyceridemia. This study indicates that there are metabolic differences between EPA and DHA which may have implications for the use of n-3 fatty acids in preventive and clinical medicine.
Lipids 33, 131–138 (1998).

n-3 Polyunsaturated fatty acids (FA), especially long-chain eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have antithrombotic, antiinflammatory and hypolipemic properties (1,2), suggesting that they may be useful in the prevention and treatment of atherothrombotic diseases. Postprandial triglyceridemia is thought to play an important role in atherogenesis (3). Male survivors of myocardial infarction have higher postprandial lipid levels following fat tolerance tests than control subjects (4–6). Moreover, patients with angiographically verified coronary artery disease displayed larger triglyceride increase and magnitude of postprandial lipemia as compared to control subjects (6). Thus, any therapy that lowers postprandial lipemia may be expected to be antiatherogenic (3,6).

Daily ingestion of moderate (7) and large amounts (8) of fish oil for some weeks reduces postprandial lipemia significantly, independent of the type of fat present in the test meal (8). Similar results were obtained after long-term fish oil feeding when a 24-h oral fat-load was administered to mimic normal eating patterns (9). These studies used mixtures of EPA and DHA and can therefore not discriminate between possible separate actions of these FA.

There is growing evidence that EPA and DHA may have specific, and partly different, effects on lipid metabolism. In rats, EPA have been shown to decrease serum triglycerides, probably by induction of mitochondrial oxidation of FA, whereas DHA did not influence the triglyceride level (10). Recently, Grimsgaard *et al.* (11) reported that DHA had a more pronounced triglyceride-lowering effect than EPA. Concentrations of EPA and DHA in serum phospholipids have also been shown to be divergently associated with high density lipoproteins (HDL) in humans (12).

EPA and DHA have been given as triglycerides, free fatty acids, or esters of methanol and ethanol. Ethyl esters (EE) and triglycerides have been preferred in clinical studies. The absorption of a mixture of EPA and DHA as EE has been reported to be attenuated and delayed compared to triglyceride-formulas or free fatty acids (13,14). However, other studies indicate that mixed EE of both EPA and DHA are apparently well absorbed and incorporated into blood lipids (15–17). The purpose of the present study was to investigate the absorption, FA incorporation, and effect on postprandial triglyceridemia of highly purified EPA or DHA supplemented as EE in the diet of healthy males.

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Abbreviations: CM, chylomicrons; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EE, ethyl esters; EPA, eicosapentaenoic acid; FA, fatty acid; HDL, high density lipoproteins; LA, linoleic acid.

METHODS

Participants. Fourteen healthy normolipemic male employees at the University of Tromsø (mean age 36.2, range 28–49) on a traditional Western diet were recruited into the present study. They were healthy as determined by medical history, physical examination, and measurements of blood pressure, hematological variables, blood glucose, serum lipids, and routine biochemistry. One individual smoked cigarettes. The exclusion criteria were: regular use of drugs, history of peptic ulcers, gastrointestinal disturbances likely to influence absorption, alcoholism, drug abuse, and mental illness. The subjects were asked to maintain their regular physical activity and dietary habits throughout the trial. Informed written consent was obtained from each participant. The study was approved by the regional board of research ethics.

Experimental design. The study was carried out as a randomized double-blind, parallel trial. After a 2-wk run-in phase with weekly physical examinations and blood collections, the participants were randomly allocated at visit 3 to receive either 4 g/day of EPA (95% pure) or 4 g/day DHA (90% pure), as EE (Pronova Biocare AS, Oslo, Norway) for 5 wk. The EPA and DHA were given in soft gelatin capsules with a reddish-brown capsule shell, each containing 1.0 g of FA. The content of tocopherol was 4–6 IU/capsule, the peroxide value <0.020 meq/g and *p*-anisidine value <35.

Physical examination and blood collection were conducted weekly during the dietary intervention. Height was measured at baseline, and body weight was recorded at baseline and after 5 wk of supplementation using a digital weight with the subjects wearing light inner clothing. Compliance was assessed by capsule counts and by measurements of serum phospholipid FA.

Blood sampling. After 12 h of overnight fasting, blood was drawn at 8 a.m. from an antecubital vein by using a 19-gauge needle in a vacutainer system with minimal stasis. Serum was prepared by clotting whole blood in a glass tube at room temperature for 1 h and then centrifuged at 2000 × *g* for 15 min. Aliquots of 1 mL were transferred into sterile cryovials (Greiner Laboratechnik, Germany), flushed with nitrogen, and frozen at −70°C until further analysis. Blood for plasma preparation was collected into vacutainers (Becton Dickinson, Meylan Cedex, France) containing disodium EDTA as anticoagulant (EDTA k_3 0.12 mL, 0.34 M per tube). Plasma was prepared by centrifugation at 2000 × *g* for 15 min at 22°C, transferred into sterile cryovials in aliquots of 1 mL, flushed with nitrogen, and stored at −70°C until further analysis.

Fat-tolerance test. A fat-tolerance test was conducted before (visit 3) and at the end of the dietary supplementation period (visit 8). A standard 2.64 MJ (631 kcal) test meal was prepared as a 375-mL liquid formula containing 28.6 g carbohydrate, 22.6 g protein, and 47.0 g fat. The fat was mainly from cream and milk and comprised 65.4% saturated, 30.9% monounsaturated, and 3.8% polyunsaturated FA. The test meals were freshly prepared each morning, served at 8:15 a.m. and consumed over a 10-min period. Individuals allocated to the EPA and DHA groups received four capsules of EPA and DHA, respectively, with the test meal. The subjects were given 100 mL of orange juice with the meal, and were allowed to drink 350 mL calorie-free beverages and eat an apple during the following 8 h. Blood samples for chylomicron isolation and plasma preparation were collected before the meal and every second hour during the next 8 h.

Chylomicron (CM) isolation. CM were isolated by overlayering 8 mL plasma with 4 mL of buffer (150 mmol/L NaCl and 1 mmol/L EDTA) in a cellulose nitrate tube (18) and centrifuging in a Beckman SW40 Ti swinging bucket rotor at 20.000 rpm for 1 h at 4°C. The CM were carefully removed by aspiration, divided into three aliquots in cryovials, flushed with nitrogen, and frozen at −70°C until further analysis.

FA analysis. Total lipids were extracted from 500 µL serum according to Folch *et al.* (19) with phosphatidylcholine deheptadecanoyl added as internal standard (P-5014 Sigma Chemical Company, St. Louis, MO), chloroform/methanol (2:1, vol/vol) as the solvent, and butylated hydroxytoluene (75 mg/L) as the antioxidant. Total phospholipids were separated by solid-phase extraction using NH_2 columns (size 3 cc, Analytichem Bond Elut LRC; Varian, Harbor City, CA) (20), followed by transmethylation using boron trifluoride, extracted into hexane and evaporated to dryness. The fatty acid methyl esters were dissolved in hexane and analyzed by gas–liquid chromatography (Shimadzu GC-14 A; Shimadzu Corporation, Kyoto, Japan), fitted with a capillary column (CP-Sil 88, length 50 m, i.d. 0.25 mm) obtained from Chrompack Inc. (Raritan, NJ). Retention times and response factors for each FA were determined using standards obtained from Nu-Chek-Prep (Elysian, MN). The results were integrated on a Shimadzu C-R4A integrator, and the FA concentrations were reported as µmol/L serum. The intraprocedural coefficients of variation (n = 10) for 16:0, 18:2n-6, 20:4n-6, 20:5n-3, and 22:6n-3 were 1.4, 1.7, 2.3, 2.3, and 4.3%, respectively. The unsaturation index was calculated as the sum (in µmol/L) of all unsaturated FA within serum phospholipids, multiplied by the number of double bonds in each FA divided by 1000. Total FA were extracted from 500 µL CM with heptadecaenoic acid (17:0) as internal standard according to the above-mentioned procedure. The concentrations of FA in CM were reported as µmol/L plasma.

Serum lipids. Serum lipids were analyzed on a Hitachi 737 Automatic Analyzer (Boehringer Mannheim, Germany) with reagents from the manufacturer. Total cholesterol was measured with an enzymatic colorimetric method (CHOD-PAP) and HDL cholesterol was assayed by the same procedure after precipitation of lower density lipoproteins with heparin and manganese chloride. Triglyceride concentration was determined with an enzymatic colorimetric test (GPO-PAP). Apolipoprotein A-1 and apolipoprotein B were measured immunochemically by rate nephelometry, using the Array Protein System from Beckman Instruments Inc. (Brea, CA).

Statistics. Change was calculated as the value obtained after minus the normally distributed value obtained before in-

tervention. Paired *t*-test was applied for changes in normally distributed variables within a treatment group, whereas unpaired *t*-test was used for comparison of changes between treatment groups. Repeated measures analysis of variance was performed to assess changes in plasma phospholipid and postprandial CM FA over time. The postprandial increase in plasma triglycerides and CM triglycerides were assessed in two ways. First, we calculated the area under the curve for the triglyceride concentrations after intake of a standardized fat meal as the deviation from the basal value integrated over the sampling time (8 h). Next, the triglyceride response was calculated as the average of the two highest postprandial triglyceride concentrations minus the baseline value. Two-sided *P*-values <0.05 were regarded as significant. The SAS statistical software package was used (21).

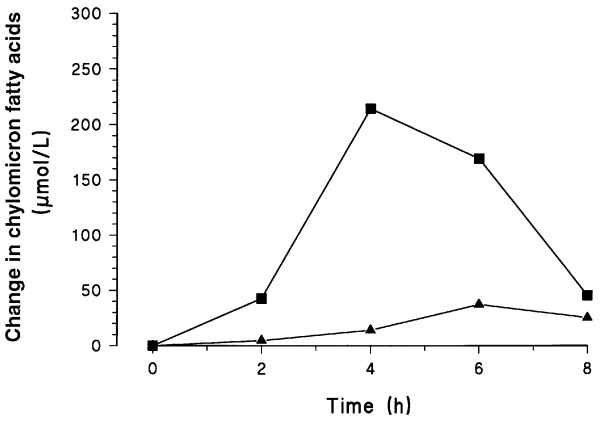

FIG. 1. Change in total saturated fatty acids (FA) (■) and total n-3 FA (▲) in chylomicrons after ingestion of a standardized high-fat meal with the addition of highly purified n-3 FA either as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Values are means of pooled data from the EPA and DHA groups ($n = 14$).

RESULTS

None of the participants experienced discomfort, nausea, or diarrhea after ingestion of the liquid test meal with addition of highly purified n-3 FA. The participants did not experience significant side effects while taking EPA or DHA. All participants completed the study per protocol. The percentage of capsules taken was 98 (range 96–100%) in the EPA group and 91 (69–100%) in the DHA group.

The EPA and DHA groups were well balanced with regard to age, body mass index, and blood lipids (Table 1). During 5 wk of supplementation with EPA or DHA, serum triglycerides decreased by 13.4 ± 17.6% ($P = 0.09$) and 15.8 ± 19.4% ($P = 0.11$), respectively. Only minor changes were observed for other lipids and apolipoproteins.

Absorption of EPA and DHA before prolonged dietary intervention. Figure 1 shows absorption curves for saturated FA and total n-3 FA in total lipids extracted from CM after a standard test meal supplemented with 4 g n-3 FA, either as EPA ($n = 7$) or DHA ($n = 7$) (pooled data $n = 14$ at baseline). While saturated FA peaked 4 h after the meal, the peak concentrations of polyunsaturated n-3 FA were observed 6 h after the meal. The increase in EPA and DHA after a standard high fat meal with the addition of EPA (A) or DHA (B) at baseline are shown in Figure 2. Ingestion of both EPA and DHA induced a similar maximal increase in CM FA (8.4-fold and 8.5-fold increase, respectively) which peaked 6 h after the meal. The relative uptake of EPA and DHA compared to oleic acid calculated from peak concentrations was 94 and 100% respectively, whereas calculations based on the area under the concentration curve resulted in an estimated relative uptake of 91 for EPA and 93% for DHA. In the EPA group, the CM concentration of DHA did not change after the meal, and there was no change in the EPA concentration in the DHA group (Fig. 2). The clearance pattern for EPA and DHA differed, since the concentration of EPA decreased by 13.0% ($P = 0.57$) from 6 to 8 h after the meal, whereas the corresponding decrease in DHA was 61.9% ($P < 0.001$). The ratio between EPA and triglycerides in CM in the EPA group increased from 0.12 6 h after the meal to 0.25 8 h after the meal, mainly due to decreased levels of CM triglycerides. The corresponding ratios between DHA and CM triglycerides in the DHA group were 0.19 and 0.11, respectively, indicating a selective decrease of DHA from CM particles.

FA in serum phospholipids after prolonged intervention. Table 2 shows the concentration of FA in serum phospholipids at baseline and changes from baseline after intervention. EPA supplementation caused an increase in n-3 FA at

TABLE 1
Age, Body Mass Index, and Serum Lipids at Baseline and Changes from Baseline After Dietary Intake (4 g/d for 5 wk) of Either EPA or DHA

	EPA ($n = 7$)		DHA ($n = 7$)		EPA vs. DHA
	Baseline	Change	Baseline	Change	
Age (yr)	32.4 (5.0)		40.0 (8.4)		
Body mass index (kg/m^2)	25.2 (1.8)	0.13 (0.41)	24.5 (2.7)	0.26 (0.38)	NS
Total cholesterol (mmol/L)	5.80 (1.16)	0.00 (0.47)	5.34 (0.53)	0.09 (0.34)	NS
HDL cholesterol (mmol/L)	1.28 (0.12)	0.05 (0.11)	1.38 (0.26)	0.05 (0.19)	NS
Triglycerides (mmol/L)	1.19 (0.53)	–0.22 (0.36)	1.04 (0.41)	–0.21 (0.31)	NS
Apo-A1 (g/L)	1.30 (0.16)	–0.02 (0.13)	1.33 (0.11)	–0.04 (0.07)	NS
Apo-B (g/L)	0.99 (0.21)	0.01 (0.18)	0.89 (0.18)	0.02 (0.07)	NS

[a]Values are means (SD). HDL, high density lipoproteins. Apo-A1, Apolipoprotein-A1. Apo-B, Apolipoprotein B. NS, no significant difference between groups. EPA, eicosapentaenoic acid. DHA, docosahexaenoic acid.

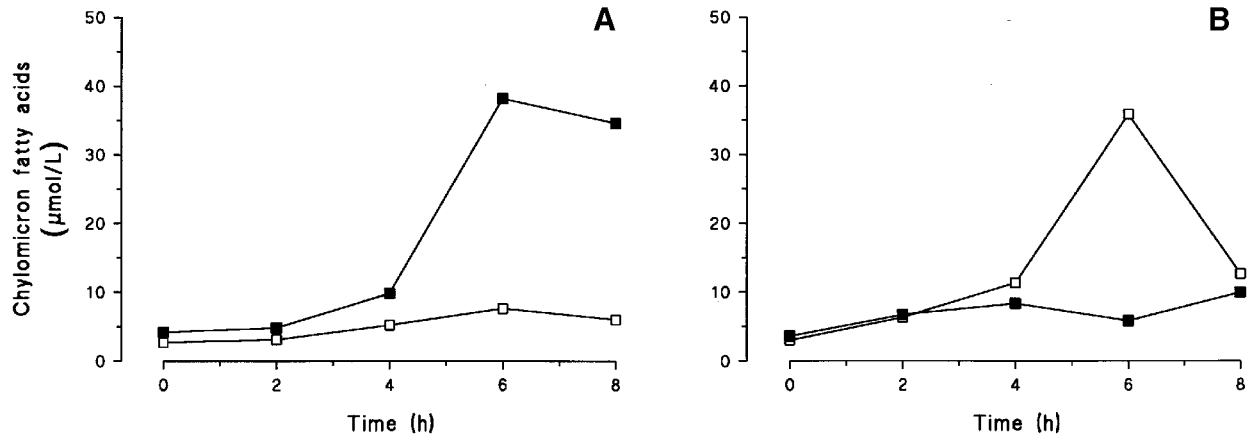

FIG. 2. Concentration of EPA (■) and DHA (□) in plasma chylomicrons after a standard high fat meal with the addition of 4 g of either EPA (A) or DHA (B). The values are means from seven subjects in each group. See Figure 1 for abbreviations.

the expense of n-6 FA. The decrease in linoleic acid (LA; 18:2n-6) was significantly greater after EPA than after DHA supplementation. The increase in n-3 FA after EPA was due to a 5.2-fold increase in EPA, and a 2.1-fold increase in docosapentaenoic acid (DPA), whereas the DHA concentration did not change. DHA supplementation caused a smaller increase in the total concentration of n-3 FA than did EPA (0.7- and 1.2-fold, respectively), but the difference between the two groups was not statistically significant. The increase in n-3 FA after DHA was due to 0.9-fold increase in DHA and 0.7-fold increase in EPA, whereas the DPA concentration did not change. A modest increase in the unsaturation index was seen both after EPA and DHA supplementation (0.36 ± 0.47 vs. 0.34 ± 0.53, $P = 0.94$). The incorporation of DHA in serum phospholipids after DHA supplementation was delayed compared to the incorporation of EPA after EPA supplementation (Fig. 3).

Influence of EPA and DHA on postprandial triglyceridemia. A standard test meal (2.64 MJ) with 4 g n-3 FA was given at the beginning and at the end of the 5-wk intervention period. Addition of EPA or DHA with the standard meal at baseline did not influence postprandial triglycerides or CM triglycerides differently as determined by the area under the curve or the peak response for the triglyceride curves (Table 3). Daily supplementation with EPA and DHA for 5 wk was associated with a 20 ($P = 0.11$) and 15% ($P = 0.13$) decrease in plasma triglycerides, respectively. After the intervention period, the peak response for plasma triglycerides decreased by 26 ($P = 0.04$) and 41% ($P = 0.02$) in the EPA and DHA groups, respectively, whereas the area under the curve decreased by 19 ($P = 0.20$) and 49% ($P = 0.05$),

TABLE 2
Concentrations of Fatty Acids (μmol/L) in Serum Phospholipids at Baseline and Changes from Baseline After Dietary Intake (4 g per day for 5 wk) of Either EPA or DHA

	EPA ($n = 7$)		DHA ($n = 7$)		EPA vs. DHA
	Baseline	Change	Baseline	Change	*P*-value
Saturated	2168.8 (410.3)	46.1 (230.9)	2098.4 (184.5)	36.5 (112.5)	NS
Monounsaturated	395.3 (112.5)	2.8 (71.9)	389.8 (77.5)	18.6 (43.8)	NS
Polyunsaturated	1410.0 (219.8)	–113.1 (126.3)	1388.0 (114.6)	–69.7 (121.4)	NS
n-6 Family	1203.3 (228.3)	–337.7 (129.3)[b]	1164.5 (95.4)	–214.9 (111.3)[c]	NS
LA	857.6 (150.0)	–275.7 (76.1)[b]	831.0 (90.8)	–168.5 (90.6)2	<0.05
AA	240.1 (79.7)	–36.7 (44.6)[d]	226.1 (23.4)	–17.3 (42.8)	NS
n-3 Family	190.7 (64.9)	229.6 (54.2)[b]	206.4 (43.9)	146.5 (80.4)[c]	NS
EPA	34.7 (16.9)	180.7 (49.2)[b]	40.2 (14.4)	29.7 (23.0)[d]	<0.001
DPA	24.8 (10.3)	51.5 (11.3)[b]	26.6 (4.8)	–5.0 (6.6)	<0.001
DHA	125.2 (42.8)	–0.6 (29.7)	133.1 (28.8)	123.6 (56.8)[c]	<0.001
Unsaturation index	4.49 (0.75)	0.36 (0.47)	4.47 (0.45)	0.34 (0.53)	NS

[a]The values are means (SD). LA, linoleic acid (18:2n-6). AA, arachidonic acid (20:4n-6). EPA, eicosapentaenoic acid (20:5n-3). DPA, docosapentaenoic acid (22:5n-3). DHA, docosahexaenoic acid (22:6n-3).
[b]$P < 0.001$.
[c]$P < 0.01$.
[d]$P < 0.05$.

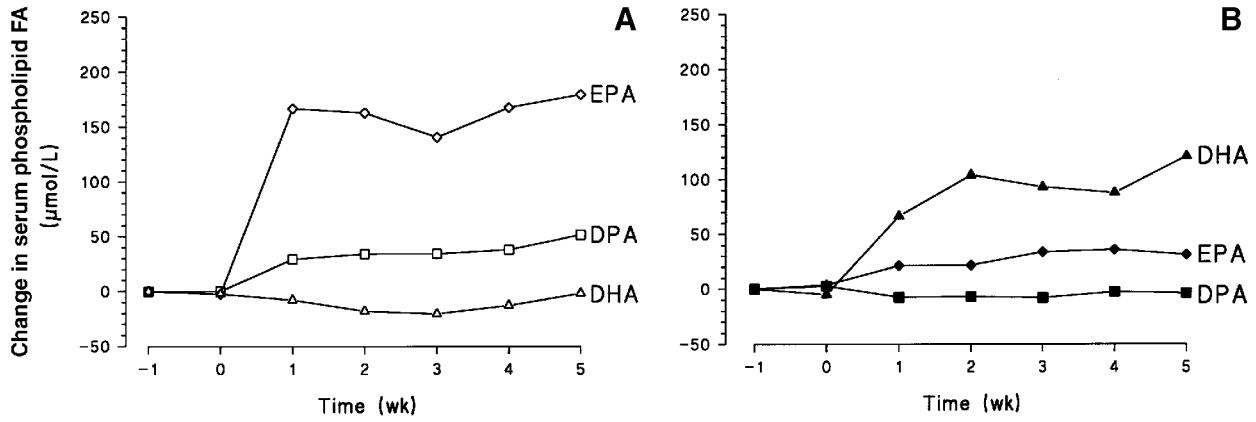

FIG. 3. Time-course changes in serum phospholipid n-3 fatty acid concentration after dietary intake of 4 g per day for 5 wk of EPA (A) or DHA (B). Values are means. DPA, docosapentaenoic acid. See Figure 1 for other abbreviations.

TABLE 3
The Effect of a Standard Fat Meal on Postprandial Triglyceridemia at Baseline and Changes After Dietary Intake (4 g EPA and DHA per day for 5 wk)

	EPA (n = 7)		DHA (n = 7)		EPA vs. DHA
	Before	Change	Before	Change	P-value[b]
P-triglycerides (mmol/L)	1.13 (0.44)	−0.24 (0.33)	0.94 (0.24)	−0.15 (0.24)	0.73
AUC-TG (mmol/L)	1.79 (0.71)	−0.34 (0.63)	1.39 (0.33)	−0.68 (0.74)	0.38
PR-TG (mmol/L)	0.78 (0.26)	−0.20 (0.20)[c]	0.63 (0.17)	−0.26 (0.23)[c]	0.63

[a]Values are means (SD). P-triglycerides, plasma concentration of triglycerides. See Table 2 for other abbreviations. AUC-TG, area under the plasma triglyceride curve during the fat tolerance test. PR-TG, peak response of plasma triglycerides after the fat tolerance test.
[b]P-values for between group comparisons of change.
[c]$P < 0.05$; P-value for changes within a group.

respectively. The differences between groups were not statistically significant. A similar pattern was seen for CM triglyceride concentrations (Fig. 4).

DISCUSSION

The present investigation examined the incorporation of EPA and DHA into CM after ingestion of purified EPA and DHA separately as EE together with a standard meal. Our data suggest that the enterocytic processing of the ingested fat and generation of CM do not involve significant elongation or retroconversion of EPA and DHA. This implies that ingested EPA and DHA are presented to their target organ(s) or cell(s) in their original form. Furthermore, DHA is apparently cleared from CM more efficiently than is EPA.

The relative uptakes from the standard meal of EPA and

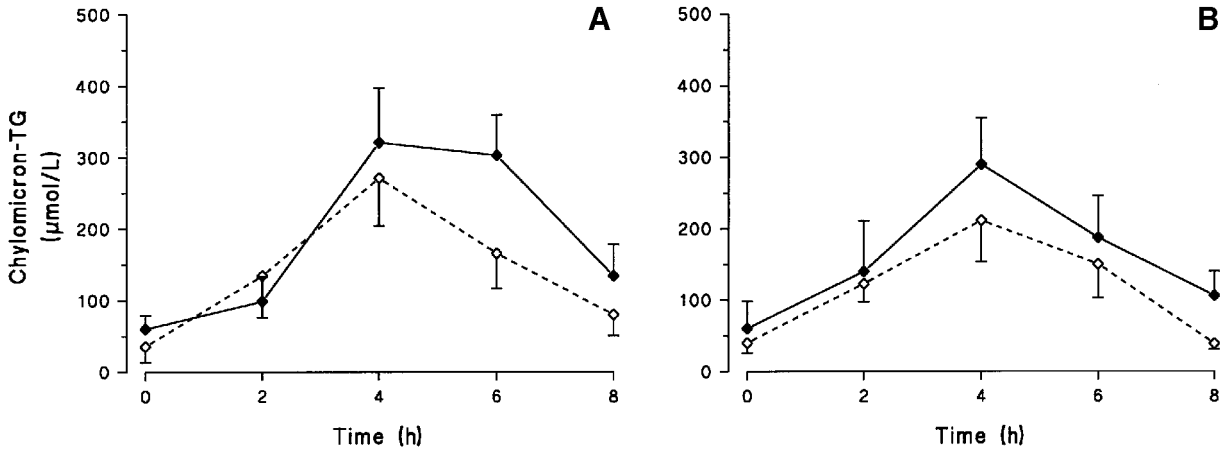

FIG. 4. Chylomicron triglyceride (TG) concentrations after a standard high-fat meal before (—) and after (– – –) a daily intake of 4 g EPA (A) or DHA (B) for 5 wk. Symbols are means ± SD.

DHA into CM were similar to that of oleic acid. Although the absorption of EPA and DHA was delayed compared with saturated FA, our data indicate that moderate and therapeutic amounts given as EE were well absorbed. These observations contrast with studies where equivalents of only 1.0 g EPA were given (13,14), and incomplete absorption of EE compared to triglycerides was observed. In a later study, Nordøy *et al.* (15) demonstrated that n-3 FA were equally well absorbed as EE or triglycerides when given in amounts of 28–40 g with the meal. Apart from differences in the amount of n-3 FA, the latter study measured absorption directly by analyzing CM triglycerides, rather than serum triglycerides, which is more precise. Equal absorption was further documented in a study showing similar incorporation of EPA and DHA into blood lipids after daily intake of 3.5 g EPA and DHA for 7 wk given either in a triglyceride or an EE formula (16).

It has been suggested, based on results obtained from *in vitro* studies, that the EE of n-3 FA are poor substrates for pancreatic lipase (4,7), and that this lipase has a reduced hydrolyzing activity toward trioctadecanoates containing double bonds in the third to sixth positions from the carboxyl end (22). The first double bonds of DHA and EPA are in positions 4 and 5, respectively. However, coingestion of EPA-EE with a meal normal for the American diet has been found to improve the uptake threefold (14). This may partly explain the substantial absorption of EPA and DHA in our study. A more recent study found that rates of release of oleic acid and DHA by lipase *in vitro* differ only by a factor of six (23). It was suggested that with the very high pancreatic lipase activity in the intestinal lumen, there should be little differences between the composition of FA ingested and that of FA released. Perhaps the delay in the maximal incorporation of EPA and DHA into CM compared to saturated FA reflected this difference.

Time-course incorporation of EPA and DHA into serum phospholipids had a different pattern after dietary supplementation with EPA and DHA. Daily ingestion of EPA caused a prominent and steep increase of EPA in serum phospholipids which reached a plateau after 1 wk. In contrast, ingestion of DHA caused a more gradual increase during the intervention period. In addition to different incorporation into and clearance from CM, this indicates different metabolism of EPA and DHA. The incorporation pattern for EPA indicates priority of the circulatory pool which seemed to be in equilibrium with other compartments after 1 wk.

Compared to EPA, more of the ingested DHA seemed to be channeled into extracirculatory compartments, since we observed a more rapid clearance from the circulation and a decreased incorporation of DHA into serum phospholipids. The mechanism(s) involved is unknown, but the selective decrease in DHA may suggest a more efficient cleavage of DHA than EPA by lipoprotein lipase. DHA is selectively enriched in the central nervous system (24) and is mandatory for normal central nervous system function. Recently, Anderson *et al.* (25) demonstrated incorporation of CM FA, and especially DHA, into the developing rat brain. They suggested that the brain derives a significant fraction of essential FA from CM. Whether this is a specific mechanism for the developing brain only is not known.

In agreement with previous studies (26,27), dietary EPA was not metabolized to DHA but caused a prominent increase in DPA (22:5n-3). The biological significance of the accumulated DPA after dietary EPA remains unknown. DHA supplementation caused a significant increase in EPA, confirming the retroconversion previously demonstrated both in rats (28) and humans (27). Thus, DHA may serve as a reservoir for EPA *via* partial β-oxidation (29) and saturation of the resulting *trans*-double bond (28).

Long-term intake of fish oil, rich in long-chain FA of the n-3 family (EPA and DHA) is reported to lower postprandial triglycerides by 30–55% in normolipemic subjects (7–9). The decrease in postprandial triglycerides by prolonged feeding of n-3 FA was seen regardless of whether saturated fat or fish oil was given with the meal (8). Harris and Muzio (9) also reported a similar decrease in postprandial triglycerides after a 24-h oral fat-load test, designed to mimic a normal eating pattern. Prolonged supplementation of n-3 FA may decrease postprandial triglyceridemia by diminished absorption, reduced synthesis, and release into the circulation by enterocytes, or by a more rapid removal of CM from the circulation. There is no evidence of diminished absorption (30) or enhanced removal of CM assessed by clearance rates of intravenous administered fat emulsions (9). However, fish oils have been reported to reduce triglyceride synthesis in and secretion from CaCO-2 cells, a human intestinal cell line (31). A similar inhibitory effect on the triglyceride synthesis pathway of n-3 fatty acids has also been observed in hepatocytes (32,33).

The present study indicates that prolonged intake of DHA is equivalent to or even more efficient than EPA in reducing postprandial triglyceridemia and fasting serum triglycerides. The postprandial triglyceridemia (area under the curve) was suppressed by 19 and 49% after ingestion of 3.8 g EPA or 3.6 g DHA daily for 5 wk. However, the present study was underpowered to detect statistically significant differences between groups. A recent study among 234 healthy subjects found that DHA tended to decrease serum triglycerides more efficiently than EPA (11). Experimental studies in perfused rat livers (34), $HepG_2$ cells (35), and hepatocytes (32,36) support our observation that both EPA and DHA inhibit triglyceride synthesis and secretion.

Patients with coronary artery disease have been found to have high plasma increased plasma concentrations of CM remnants (37) and postprandial lipemia (6). Fish oils containing a mixture of EPA and DHA lower postprandial triglyceridemia and thereby CM remnants. To our knowledge, the present study is the first to examine the separate actions of EPA and DHA on postprandial triglyceridemia. Our observations indicate that DHA may be more efficient than EPA in reducing postprandial CM remnants, but further studies are needed to establish the possible separate effects on lipid metabolism of the two major n-3 FA.

ACKNOWLEDGMENTS

This study was supported in part by grants from Pronova Biocare a.s. (Oslo, Norway), the Norwegian Council on Cardiovascular Dis-

eases, and the Norwegian Research Council. The skillful technical assistance of Vegard Lyngmo and Birgit Svensson at the Department of Medicine, Institute of Clinical Medicine, University of Tromsø is appreciated.

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[Received March 31, 1997, and in final revised form and accepted December 15, 1997]

Effect of Apolipoprotein E Polymorphism on Serum Lipoprotein Response to Saturated Fatty Acids

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ABSTRACT: This report summarizes two studies which investigated the effects of apolipoprotein E (apoE) polymorphism on the serum total cholesterol (TC) and lipoprotein cholesterol responses to 8:0 + 10:0 and 12:0 diets (Study I) and 14:0, 16:0, and 18:0 diets (Study II). Eighteen healthy premenopausal women (3 apoE 3/2, 12 apoE 3/3, 3 apoE 4/3) in study I and another 18 healthy premenopausal women (4 apoE 3/2, 10 apoE 3/3, 3 apoE 4/3, 1 apoE 4/2) in study II consumed a baseline diet providing 40 en% total fat, 11 en% 18:2, 15 en% 18:1, 11.5 en% saturated fat for the first week of each 5-wk period. The experimental diets for both studies provided 40 en% total fat, 13–14 en% as one of five test saturated fatty acids (SFA), 14–16 en% 18:1, and 3–4 en% 18:2. Analysis by apoE phenotypes showed that both the 8:0 + 10:0 diet and the 12:0 diet in Study I induced significant increases in serum TC in subjects with different apoE phenotypes with the exception of apoE 3/2 in the medium-chain triglyceride group. In contrast, in Study II, individuals with apoE 4/3 consuming the 14:0 diet showed significant increases in serum TC, high density lipoprotein-cholesterol (HDL-C), and HDL2-C, but the same subjects consuming the 16:0 diet showed significant increases in serum TC and low density lipoprotein-cholesterol. The findings from both studies indicated serum lipoprotein responses to SFA were different and the variation of responsiveness may be regulated, at least in part, by apoE polymorphism, especially when 14:0, 16:0, or 18:0 was consumed.

Lipids 33, 139–148 (1998).

Apolipoprotein E (ApoE) is a major protein constituent of chylomicrons, very low density lipoprotein (VLDL) and their remnants and, to a lesser extent, of high density lipoprotein (HDL) (1). It is also found to function as a ligand for the binding of lipoproteins to the LDL receptor and apoE receptor (1). There are three homozygous phenotypes (E 4/4, E 3/3, and E 2/2) and three heterozygous phenotypes (E 4/3, E 3/2, and E 4/2) (2). The different apoE forms have different affinities for lipoproteins. For example, apoE 2 compared to apoE 3 is associated less with VLDL, intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) and more with HDL, while apoE 4 compared to apoE 3 is associated more with VLDL, IDL, and LDL and less with HDL (2). In addition, apoE phenotype is associated with changes in plasma total cholesterol (TC) and LDL-cholesterol (LDL-C). Among apoE 2, apoE 3, and apoE 4 phenotypes, individuals with apoE 2 phenotype tend to have the lowest plasma cholesterol concentrations while individuals with apoE 4 phenotype have the highest plasma levels of TC, LDL-C and apoB (3). With respect to the effect of dietary saturated fatty acids (SFA) on plasma cholesterol levels, SFA may act differently (4–8). For example, stearic acid (18:0) seems to be neutral compared to myristic acid (14:0) and palmitic acid (16:0) because of its partial conversion to oleic acid (18:1) or poor digestibility (6). In the general population, apoE polymorphism may account for some degree of the interindividual variation in plasma TC and LDL-C (2). In some studies individuals with apoE 4 phenotype have a greater LDL-C response to dietary changes in fat and/or cholesterol intakes than individuals with other apoE phenotypes (9–14). However, there are few reports elucidating the effect of varied apoE phenotypes on the response to dietary SFA changes. The purpose of the present study was to investigate the effect of apoE polymorphism on the serum TC and lipoprotein cholesterol responses to different SFA ranging in chain length from 8 to 18 carbons.

MATERIALS AND METHODS

Subjects. The apoE phenotype was determined in a total of 18 healthy premenopausal women recruited from among The Ohio State University female students in a two-period crossover study (Study I) and in a total of another 18 healthy premenopausal women in a three period-crossover study (Study II), the minimal number needed to detect a 10% mean difference in serum lipoprotein concentrations between treatments with a standard deviation of 8% by a statistical power of 0.9. Before starting the study, all subjects in both Study I and Study II signed a consent form approved by The Ohio State University Biomedical Human Studies Committee. All subjects were screened on the basis of information from a health questionnaire and a physical examination. The subjects' characteristics including age, weight, ethnic back-

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Abbreviations: apo, apolipoprotein; ANOVA, analysis of variance; CETP, cholesteryl ester transfer protein; HDL-C, high density lipoprotein-cholesterol; IDL, intermediate density lipoprotein; IEF, isoelectric focusing; LDL-C, low density lipoprotein-cholesterol; LCAT, lecithin cholesterol acyl transferase; MCT, medium-chain triglyceride; MNC, mononuclear cell; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TC, total cholesterol; TG, triglyceride; VLDL-C, very low density lipoprotein-cholesterol.

TABLE 1
Characteristics of Subjects in Study I and Study II[a]

Variables	Study I (n = 18)	Study II (n = 18)
Age (year)	24 ± 4 (19–33)	28 ± 2 (19–43)
Weight (kg)	62.2 ± 11.7 (40.5–91.8)	61.3 ± 7.4 (50–77)
Serum cholesterol level at screening (mg/dL)	167 ± 22 (143–237)	178 ± 9 (122–252)
Ethnic background	Caucasian (n = 10) Asian (n = 7) African-American (n = 1)	Caucasian (n = 12) Asian (n = 4) African-American (n = 2)
Experimental diet	MCT diet Lauric acid diet	Myristic acid diet Palmitic acid diet Stearic acid diet
ApoE phenotype	E3/2 (n = 3) E3/3 (n = 12) E4/3 (n = 3)	E3/2 (n = 4 E4/2 (n = 1) E3/3 (n = 10) E4/3 (n = 3)

[a]MCT, medium-chain triglyceride; ApoE, apolipoprotein E.

ground, apoE phenotype, and serum TC concentration at screening are shown in Table 1.

Diets. The diets in both Study I and Study II were prepared in a metabolic kitchen in the Department of Human Nutrition and Food Management at The Ohio State University. Subjects ate breakfast and dinner in the metabolic dining room and took out a sack lunch. Weekend meals were also taken out except for Saturday night dinner. The baseline diet for both studies provided 40 en% total fat, 11 en% 18:2, 15 en% 18:1, 11.5 en% saturated fat, and about 323 mg cholesterol. The experimental diets for both studies were formulated to provide 40 en% total fat, 13–14 en% as one of five test SFA (8:0 + 10:0, 12:0, 14:0, 16:0, 18:0), 14–16 en% 18:1, 3–4 en% 18:2, and about 308–328 mg cholesterol. A basal menu providing 8.3 MJ (about 2000 Kcal) was given initially. Subjects requiring more food energy to maintain a stable weight were given extra cookies and cakes made up of fat blends with the same proportion of fatty acids as the overall diet. Dietary records of the subjects' food intake were kept and analyzed using Food Processor II software (ESHA Research, Salem, OR) with added data on fatty acid composition of the foods in the menu. Composites of the rotating 4-d menus were made and analyzed for specific fatty acid composition by gas–liquid chromatography (Table 2).

Research design. The protocols for both Study I and Study II were similar with the exception of participating subjects and experimental diets. All subjects were randomized into different groups using either a two-period (Study I) or three-period (Study II) crossover design. The baseline diet was fed for 1 wk and then each dietary treatment lasted 4 wk with a 7-wk washout period between experimental periods. Blood samples were obtained by venipuncture after an overnight fast twice during the last 3 d of the baseline period and two times during the last 3 d of each feeding period. Depending on the requirements of the procedure, either analyses commenced immediately or serum (or plasma), obtained by centrifugation at 2500 rpm for 20 min at 4°C, was frozen at −80°C for later analysis.

EXPERIMENTAL PROCEDURES

Plasma lipids. The concentrations of TC and triglyceride (TG) were measured by an enzymatic assay using a commercial kit (Sigma Chemical Company, St. Louis, MO; procedure#352-50 and #339, respectively) (15). HDL-C including HDL2-C and HDL3-C were measured by the same enzymatic procedure as TC. This involved precipitation of and removal by centrifugation of VLDL- and LDL-C by a precipitation reagent composed of 10 g/L of dextran sulfate and 0.5 M of Mg^{2+} with buffer (Sigma Chemical Company; procedure#352-2) (16). LDL-C was calculated by the Friedewald equation (17).

ApoE phenotyping. ApoE phenotype was determined by isoelectric focusing (IEF) according to a modification of the method of Kamboh *et al.* (18) and Kataoka *et al.* (19). Plasma

TABLE 2
Daily Energy and Nutrient Intake on Baseline Diet and Five Saturated Fat Diets[a]

Dietary variable	Baseline (all subjects)	MCT (n = 17)	Lauric acid (n = 18)	Myristic acid (n = 17)	Palmitic acid (n = 16)	Stearic acid (n = 18)
Energy (MJ)[b]	8.1 ± 0.1	8.3 ± 0.1	8.4 ± 0.1	8.5 ± 0.2	8.2 ± 0.1	8.3 ± 0.1
Protein (g)[b]	72 ± 0 (15%)	73 ± 0 (15%)	73 ± 0 (15%)	74 ± 1 (15%)	73 ± 1 (15%)	74 ± 1 (15%)
Carbohydrate (g)[b]	225 ± 2 (46%)	230 ± 3 (46%)	231 ± 3 (46%)	229 ± 4 (45%)	225 ± 3 (46%)	227 ± 3 (46%)
Total fat (g)[b]	86 ± 1 (40%)	89 ± 1 (40%)	89 ± 1 (40%)	91 ± 3 (40%)	87 ± 2 (40%)	89 ± 2 (40%)
Fatty acids (g)[c]						
8:0 + 10:0 (MCT)	1.8 ± 0 (1%)	30.1 ± 0.5 (14%)	1.0 ± 0 (1%)	1.3 ± 0 (1%)	1.5 ± 0 (1%)	0.8 ± 0 (1%)
12:0	3.6 ± 0 (1.7%)	2.1 ± 0 (1%)	31.4 ± 0.5 (14%)	2.7 ± 0.1 (1%)	3.2 ± 0.1 (2%)	2.4 ± 0 (1%)
14:0	2.3 ± 0 (1%)	1.4 ± 0 (1%)	1.4 ± 0 (1%)	31.1 ± 1.3 (14%)	2.1 ± 0 (1%)	1.1 ± 0 (1%)
16:0	8.2 ± 0.1 (3.8%)	5.9 ± 0.1 (3%)	5.9 ± 0.1 (3%)	6.3 ± 0.2 (3%)	27.8 ± 0.7 (13%)	5.7 ± 0.1 (3%)
18:0	7.9 ± 0.1 (4%)	2.9 ± 0.1 (1%)	2.9 ± 0.1 (1%)	3.5 ± 0.4 (2%)	4.0 ± 0.1 (2%)	28.2 ± 0.9 (13%)
MUFA	32.0 ± 0.4 (15%)	32.8 ± 0.4 (15%)	32.9 ± 0.5 (15%)	31.0 ± 0.9 (14%)	32.8 ± 0.7 (15%)	35.3 ± 0.7 (16%)
PUFA	22.6 ± 0.3 (11%)	7.5 ± 0.1 (3%)	7.5 ± 0.1 (3%)	7.6 ± 0.2 (3%)	7.9 ± 0.2 (4%)	7.5 ± 0.1 (3%)
Cholesterol (mg)[b]	323 ± 1	316 ± 1	316 ± 1	319 ± 2	328 ± 2	308 ± 2

[a]Mean ± SEM: mean ± standard error of mean. Values in parentheses are calculated values for percentage of food energy provided by the nutrient.
[b]Analyzed for daily dietary records using Food Processor II Analyses Software (ESHA Research, Salem, OR).
[c]As determined by gas–liquid chromatography. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See Table 1 for other abbreviation.

samples were delipidated with 100 µL of dithiothreitol solution containing 0.771% of dithiothreitol and 250 µl of Tween 20 at 4°C for overnight. The IEF was carried out by 7.5% polyacrylamide gel electrophoresis (PAGE)-3 M urea gel. The gel was freshly prepared as follows: 7.2 g urea was dissolved in 10 mL polyacrylamide solution containing 30 g of monomer acrylamide and 0.8 g of bis acrylamide per 100 mL and the final volume was adjusted to 40 mL with deionized water. The pH gradient was created by adding 0.6 mL Ampholine pH 4–6 (Amersham Pharmacia Biotech, Piscataway, NJ) and 1.2 mL Pharmalyte pH 5–8 (Amersham Pharmacia Biotech). Before casting, 25 µL of *N*,*N*,*N*′,*N*′-tetramethylenediamine and 500 µL of 10% ammonium persulfate solution were added to the gel solution. After polymerization, the gel was placed on an IEF machine (Bio-Phoresis Horizontal Electrophoresis Cell; Bio-Rad Laboratories, Hercules, CA) connected with a Lauda RM6 cooling unit (Brinkmann Instruments, Inc., Westbury, NY) operating at 6°C and thus prefocused for 15 min at voltage of 1500 V, 60 mA, 15 W. The cathode strip and anode strip were saturated with cathode solution (1 M NaOH) and anode solution (1 M H_3PO_4), respectively, and strips were laid across the length of the gel as close to the edge of the gel as possible. After prefocusing, the sample wicks saturated with a 30 µL sample were located on the gel near the cathode strip. Then the gel was focused at the voltage of 1500 V, 60 mA, 15 W for 30 min. After that, the sample wicks were removed and the IEF was continued for an additional 1.5 h. Then, immunoblotting was conducted according to a modification of the method of Kamboh *et al.* (18).

LDL receptor activity. Degradation and binding of ^{125}I-LDL by mononuclear cell (MNC) were determined by a modification of methods of Ho *et al.* (20) as described previously by Park and Snook (21). An aliquot of freshly isolated MNC in Hank's buffer (21) was incubated with ^{125}I-LDL (20 µg/mL) in the presence and absence of a 20-fold excess of unlabeled LDL at 37°C for 5 h. Degradation values were expressed as specific degradation which was the difference in degradation in the presence and absence of a 20-fold excess unlabeled LDL. Expression of specific degradation was as nanograms of ^{125}I-LDL protein degraded/10^6 cells/h. Specific and nonspecific binding activity of LDL receptors was determined by incubation of MNC at 4°C with ^{125}I-LDL in the presence and absence of excess unlabeled LDL for 1 h. Bound counts were expressed as micrograms of LDL protein bound per 10^6 cells.

CETP and LCAT activity. The rates of cholesterol ester transfer (cholesterol ester transfer protein, CETP) and endogenous cholesterol esterification (lecithin-cholesterol acyl transferase, LCAT) were assessed by the isotopic method of Channon *et al.* (22). Briefly, for determination of esterification rate, fresh serum was preincubated at 4°C for 1 h with ^{3}H-cholesterol-albumin emulsion; after equilibration of the added cholesterol and endogenous cholesterol, the mixture was incubated at 37°C for 3 h. Lipids were extracted, separated by thin-layer chromatography, and radioactivity of cholesterol ester and free cholesterol spots was counted by liquid scintillation. For cholesterol transfer activity, transfer of radiolabeled cholesterol ester into VLDL and LDL was measured first by precipitating the two apoB-containing lipoproteins from the incubate with dextran sulfate. Then, total radioactivity in the incubate, in the HDL-containing supernatant, and in the apoB-containing lipids was measured by liquid scintillation counting. These data were used to calculate the rate of transfer of the cholesterol ester.

Statistical analysis. MINITAB statistical software (Minitab Inc., State College, PA) and the Statistical Analysis System (SAS; SAS Institute, Cary, NC) were used in the present study for analysis of serum lipid and lipoprotein responses to dietary manipulation. The significance of the differences between the beginning and the end of each period (mean ± SEM) was calculated using the paired *t*-test in the Minitab statistical software. Least squares means, adjusted for unequal subclasses, of changes in serum TC, TG, and lipoprotein cholesterol levels from baseline analyzed by apoE phenotypes and experimental diets were analyzed using the general linear models procedure of SAS. Two-way analysis of variance (ANOVA) was used to determine interactions between experimental diets and apoE phenotypes, as well as their independent effects. In addition, stepwise regression analysis was conducted to quantify the contribution of race, diet, and apoE isoforms to the variation in serum lipid and lipoprotein responses to experimental diets. The probability of significant difference was defined as $P < 0.05$ for all statistical analysis in both Study I and Study II.

RESULTS

Daily energy and nutrient intake on the baseline diet and five saturated fat diets is shown in Table 2. The experimental diets provided similar amounts of poly- and monounsaturated fat as well as dietary cholesterol, and the baseline diet provided three times as much 18:2 as the experimental diets and only half as much saturated fat (Table 2). However, there was no effect of apoE phenotype on any of the variables when subjects consumed the baseline diet (Table 3). Changes from

TABLE 3
Variables Measured in Subjects with Different ApoE Phenotypes Consuming Baseline Diet in Both Study I and Study II (mean ± SEM)[a]

Variable	ApoE 3/2 (n = 7)	ApoE 3/3 (n = 19)	ApoE 4/3 (n = 6)
Total cholesterol (mg/dL)	151 ± 11	158 ± 5	153 ± 8
Triglyceride (mg/dL)	65 ± 7	69 ± 8	68 ± 7
LDL-cholesterol (mg/dL)	85 ± 9	92 ± 5	84 ± 7
HDL-cholesterol (mg/dL)	53 ± 4	52 ± 2	56 ± 4
HDL_2-cholesterol (mg/dL)	19 ± 3	17 ± 1	20 ± 3
HDL_3-cholesterol (mg/dL)	33 ± 1	35 ± 1	35 ± 2
CETP (nmol/mL/h)	22 ± 4	23 ± 1	21 ± 3
LCAT (nmol/mL/h)	50 ± 4	49 ± 2	51 ± 4
Receptor-mediated ^{125}I-LDL degradation in MNC (ng/h/10^6 MNC)	12 ± 1	15 ± 1	11 ± 1
Binding of ^{125}I-LDL by MNC LDL receptor (ng/10^6 MNC)	15 ± 1	18 ± 1	18 ± 3

[a]ApoE, apolipoprotein E; LDL, low density lipoprotein; HDL, high density lipoprotein; CETP, cholesteryl ester transfer protein; LCAT, lecithin cholesterol acyl transferase; MNC, mononuclear cell.

TABLE 4
Changes from Baseline[a] in Serum TC, TG, and Lipoprotein Cholesterol Levels

	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	HDL_2-C (mg/dL)	HDL_3-C (mg/dL)
Study I						
MCT diet						
E3/2 ($n = 3$)	–3.5 ± 16.2	5.0 ± 5.7	–5.8 ± 5.6	1.2 ± 2.7	–3.3 ± 0.9	4.6 ± 2.8
E3/3 ($n = 11$)	10.9 ± 4.9 ($P = 0.049$)	1.1 ± 4.8	10.2 ± 4.8	0.5 ± 1.3	–3.4 ± 1.6	3.9 ± 1.0 ($P = 0.0043$)
E4/3 ($n = 3$)	22.1 ± 4.6 ($P = 0.041$)	5.7 ± 9.4	22.6 ± 8.0	–1.6 ± 1.6	–5.2 ± 0.9 ($P = 0.031$)	3.6 ± 1.6
Lauric acid diet						
E3/2 ($n = 3$)	34.6 ± 7.2 ($P = 0.041$)	–11.2 ± 1.1 ($P = 0.0098$)	29.1 ± 6.0 ($P = 0.041$)	7.7 ± 4.1	3.0 ± 2.7	4.6 ± 1.4
E3/3 ($n = 12$)	15.0 ± 5.0 ($P = 0.012$)	–5.3 ± 6.2	11.2 ± 5.0 ($P = 0.046$)	4.9 ± 1.6 ($P = 0.013$)	2.3 ± 1.1 ($P = 0.050$)	2.5 ± 1.0 ($P = 0.040$)
E4/3 ($n = 3$)	21.7 ± 5.1 ($P = 0.050$)	17.2 ± 3.2 ($P = 0.033$)	14.6 ± 8.7	3.6 ± 3.9	0.7 ± 2.7	2.9 ± 1.7
Study II						
Myristic acid diet						
E3/2 ($n = 4$)	13.9 ± 6.4	8.2 ± 12.5	11.1 ± 5.9	0.2 ± 2.5	2.4 ± 3.5	–2.2 ± 3.2
E3/3 ($n = 9$)	5.3 ± 7.1	–18.4 ± 7.1 ($P = 0.032$)	2.4 ± 7.7	–0.6 ± 2.8	–1.5 ± 3.4	0.9 ± 2.1
E4/3 ($n = 3$)	22.6 ± 3.6 ($P = 0.025$)	14.4 ± 12.1	4.8 ± 5.5	14.9 ± 1.5 ($P = 0.010$)	10.1 ± 1.5 ($P = 0.022$)	4.7 ± 2.1
Palmitic acid diet						
E3/2 ($n = 4$)	5.2 ± 3.4	–4.6 ± 8.8	–2.5 ± 3.6	6.5 ± 3.8	4.7 ± 4.9	1.8 ± 2.1
E3/3 ($n = 8$)	11.5 ± 5.6	3.5 ± 11.3	7.2 ± 3.3	0.3 ± 4.1	3.5 ± 3.3	–1.4 ± 1.2
E4/3 ($n = 3$)	46.9 ± 10.9 ($P = 0.050$)	–7.2 ± 16.9	44.4 ± 8.9 ($P = 0.038$)	0.6 ± 3.2	–0.8 ± 4.6	1.4 ± 5.2
Stearic acid diet						
E3/2 ($n = 4$)	–18.1 ± 13.7	5.2 ± 16.1	–17.8 ± 10.3	–2.9 ± 2.9	–0.2 ± 2.9	–2.6 ± 2.6
E3/3 ($n = 10$)	–9.2 ± 5.4	–4.7 ± 8.0	–7.4 ± 5.0	0.2 ± 4.5	2.2 ± 2.3	–3.8 ± 1.5 ($P = 0.037$)
E4/3 ($n = 3$)	6.1 ± 5.6	4.4 ± 13.9	5.8 ± 3.8	–0.5 ± 1.0	1.9 ± 1.9	–2.4 ± 2.4

[a]Mean ± SEM = mean ± standard error of mean. Significant difference between baseline diet and experimental diet (*P* value) according to paired *t*-test. TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol. See Table 1 for other abbreviation.

baseline in lipid and lipoprotein concentrations, variables associated with reverse cholesterol transport, and LDL receptor activity in MNC in subjects with different apoE phenotypes consuming both Study I diets (including 12:0 diet and 8:0 + 10:0 diet) and Study II diets (including 14:0, 16:0, and 18:0 diets) are shown in Table 4 and Table 5, respectively.

Serum changes in lipid and lipoprotein concentrations. In Study I (Table 4), both the 12:0 diet and 8:0 + 10:0 diet induced significant increases in serum TC in subjects with different apoE phenotypes (with the exception of apoE 3/2 on 8:0 + 10:0 diet). Specifically, the 12:0 diet significantly elevated serum TC, LDL-C, HDL-C, HDL_2-C, and HDL_3-C in subjects with apoE 3/3, while the same subjects consuming the 8:0 + 10:0 diet showed significant increases only in serum TC and HDL_3-C. Serum LDL-C increased and TG decreased when individuals with apoE 3/2 consumed the 12:0 diet. There was a trend toward elevated and reduced serum HDL_2-C on the 12:0 diet and 8:0 + 10:0 diet, respectively, in all subjects with different apoE phenotypes. In Study II (Table 4), individuals with apoE 4/3 consuming the 14:0 diet showed significant increases in serum TC, HDL-C and HDL_2-C, but the same subjects consuming the 16:0 diet showed significant increases in serum TC and LDL-C. There were no significant changes from baseline in lipid and lipoprotein levels when subjects with different apoE phenotypes consumed the 18:0 diet (with the exception of apoE 3/3).

Changes in variables associated with reverse cholesterol transport. In comparison to values measured on the baseline diet, the rate of CETP from HDL to VLDL + LDL tended to increase on all of the Study II diets (14:0, 16:0, 18:0), but only the mean increase from baseline in subjects with apoE 3/2 consuming the 14:0 diet was statistically significant (Table 5). The 12:0 diet raised the rate of cholesterol ester transfer whereas the 8:0 + 10:0 diet tended to decrease the rate of cholesterol ester transfer in all subjects among all apoE phenotypes, but none of the changes was significant (with the exception of apoE 3/3 consuming the 12:0 diet). The rate of cholesterol esterification (LCAT) increased in subjects with apoE 3/3 and 3/2 consuming the 12:0 diet but tended to decline nonsignificantly on the 18:0 diet for all apoE phenotypes (Table 5).

Changes in LDL receptor activity in MNC. In Study I (Table 5), in subjects with apoE 3/3, the rate of receptor-mediated ^{125}I-LDL degradation significantly declined on 12:0 diet and increased on 8:0 + 10:0 diet. On the other hand, data shown in Table 5 illustrate that the 12:0 diet tended to reduce LDL binding while the 8:0 + 10:0 diet enhanced LDL binding significantly in subjects with apoE 3/3. In Study II (Table 5), only subjects with apoE 3/3, the most common apoE phenotype, consuming the 16:0 diet showed a significant decrease in the rate of receptor-mediated ^{125}I-LDL degradation.

Effect of interactions between experimental diets and apoE phenotypes. In both Study I and Study II, two-way ANOVA was used to determine interactions between experimental diets and apoE phenotypes, as well as their independent effects (Table 6 and Table 7). There were no statistically ($P < 0.05$) significant interactions between experimental diets and apoE phenotypes for variables analyzed in both studies although the effect of interactions on LDL-C ($P = 0.0669$) approached significance in Study II. However, an analysis of the independent effects of experimental diets and apoE pheno-

TABLE 5
Changes from Baseline[a] in Variables Associated with Reverse Cholesterol Transport and LDL Receptor Activity in Mononuclear Cell (MNC)

	CETP (nmol/mL/h)	LCAT (nmol/mL/h)	Receptor-mediated ^{125}I-LDL degradation in MNC (ng/h/10^6 MNC)	Binding of ^{125}I-LDL by MNC LDL receptors (ng/10^6 MNC)
Study I				
MCT diet				
E3/2 (n = 3)	−1.7 ± 5.9	−1.3 ± 0.3	2.6 ± 2.3	3.7 ± 5.2
E3/3 (n = 11)	0.8 ± 1.2	5.2 ± 5.3	4.9 ± 1.6 (P = 0.014)	4.4 ± 1.8 (P = 0.038)
E4/3 (n = 3)	−3.8 ± 4.3	−1.5 ± 9.6	6.7 ± 3.9	5.3 ± 1.8
Lauric acid diet				
E3/2 (n = 3)	2.4 ± 1.5	7.3 ± 0.4 (P = 0.0029)	−3.3 ± 4.4	−4.1 ± 3.7
E3/3 (n = 12)	5.3 ± 1.4 (P = 0.0033)	7.2 ± 2.1 (P = 0.0057)	−2.8 ± 0.8 (P = 0.0052)	−2.0 ± 1.4
E4/3 (n = 3)	6.8 ± 2.6	13.8 ± 7.8	−0.5 ± 0.5	0.0 ± 5.2
Study II				
Myristic acid diet				
E3/2 (n = 4)	6.2 ± 1.5 (P = 0.029)	−2.7 ± 5.4	4.8 ± 2.9	−4.5 ± 8.3
E3/3 (n = 9)	8.1 ± 7.1	1.8 ± 9.5	−0.1 ± 2.9	−8.9 ± 4.6
E4/3 (n = 3)	3.8 ± 2.0	12.5 ± 6.7	−0.2 ± 3.2	−4.8 ± 15.2
Palmitic acid diet				
E3/2 (n = 4)	4.0 ± 2.5	−1.4 ± 4.5	−0.5 ± 3.9	1.8 ± 0.7
E3/3 (n = 8)	4.6 ± 3.1	−0.4 ± 2.4	−9.7 ± 2.2 (P = 0.0034)	−4.3 ± 4.7
E4/3 (n = 3)	22.6 ± 11.7	11.2 ± 15.5	−1.4 ± 5.4	−3.9 ± 2.7
Stearic acid diet				
E3/2 (n = 4)	6.9 ± 3.0	−1.6 ± 4.4	5.2 ± 3.3	−2.5 ± 4.6
E3/3 (n = 10)	2.2 ± 2.6	−5.2 ± 3.2	−3.5 ± 1.6	8.1 ± 7.1
E4/3 (n = 3)	3.9 ± 1.8	−7.4 ± 6.6	2.7 ± 5.0	0.9 ± 7.1

[a]Mean ± SEM = mean ± standard error of mean. Significant difference between baseline diet and experimental diet (P value) according to paired t-test. See Tables 1 and 3 for abbreviations.

types showed that the 8:0 + 10:0 and 12:0 diets in Study I had significant influences on HDL-C, HDL_2-C, CETP, receptor-mediated ^{125}I-LDL degradation and binding, while the 14:0, 16:0, and 18:0 diets in Study II showed significant effects on TC, LDL-C, and receptor-mediated ^{125}I-LDL degradation. With respect to the independent effect of apoE phenotype, the serum TC and LDL-C responses to the 14:0, 16:0, and 18:0 diets in Study II were regulated by apoE phenotypes.

Stepwise regression analysis. Race (Asian, African-American, and Caucasian), diet (8:0 + 10:0, 12:0, 14:0, 16:0, and 18:0), and apoE phenotype (E2, E3, and E4) were used in a stepwise regression analysis as dummy variables. Diet and apoE phenotype entered the regression model as significant factors explaining the variation in serum TC, LDL-C, and ^{125}I-LDL degradation responses to experimental diets. The regression equations were described as follows: (i) change of TC = 8.65 − 23.28 (18:0) + 23.98 (E4), the partial R^2 = 0.2579, 0.1420, and the model R^2 = 0.3999; (ii) change of LDL-C = 3.36 − 16.05 (18:0) + 22.85 (E4), the partial R^2 = 0.1826, 0.1663, and the model R^2 = 0.3489; (iii) change of ^{125}I-LDL degradation = −1.60 − 6.73 (16:0) + 7.05 (E2), the partial R^2 = 0.1639, 0.1666, and the model R^2 = 0.3305.

DISCUSSION

This was a controlled dietary intervention study in which the effects of apoE phenotypes on serum lipid and lipoprotein responses to five different SFA diets were investigated. The five SFA diets were medium-chain triglycerides (MCT) (8:0 + 10:0), lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0). In order to maximize their individual effects, these SFA were administered in amounts 2 to 10 times higher than those typically selected in an American diet.

Recently, considerable evidence has accumulated that suggests that apoE polymorphism may affect the serum lipid and lipoprotein responses to dietary intervention (5,9–14,23–33). A summary of a variety of studies of dietary-induced changes in serum lipids and lipoproteins in relation to apoE phenotype is shown in Table 8. Some studies demonstrated greater serum lipid and lipoprotein responses in individuals carrying the apoE 4 allele (apoE 4/3 or apoE 4/4) (9–14) while others failed to have the same conclusion (5,23–33). These various findings can be attributed, at least in part, to differences between these studies in the subjects' age and gender, composition of the baseline diet and experimental diet, content of fat and/or cholesterol, and the length of the period of dietary intervention. In addition, such characteristics as individual apoE phenotypes, subject's individual variation, and/or other genetic factors may also play a role in the serum lipid and lipoprotein responses to dietary manipulation. Among those studies (Table 8), most either modified the amounts of dietary fat and cholesterol or adjusted the ratio of polyunsaturated fat to saturated fat. There have been few studies (5) elucidating the effect of varied apoE phenotypes on the response to manipulation of dietary SFA. Like most studies (Table 8), however, the present report also found that subjects with apoE 4/3 showed the greatest lipid and lipoprotein responsiveness to dietary manipulation but only to certain SFA diets. This re-

TABLE 6
Least Squares Means[a] of Changes from Baseline in Serum TC, TG, and Lipoprotein Cholesterol Levels Analyzed by ApoE Phenotypes and Experimental Diets

	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	HDL_2-C (mg/dL)	HDL_3-C (mg/dL)
Study I						
ApoE phenotypes						
E3/2	15.5 ± 7.0	–3.1 ± 6.6	11.7 ± 6.4	4.5 ± 2.0	–0.1 ± 2.0	4.6 ± 1.3
E3/3	13.5 ± 3.6	–2.6 ± 3.4	11.5 ± 3.3	2.5 ± 1.7	–0.8 ± 1.0	3.3 ± 0.6
E4/3	21.9 ± 7.0	11.4 ± 6.6	18.6 ± 6.4	1.0 ± 2.0	–2.2 ± 2.0	3.2 ± 1.3
Experimental diets						
8:0 + 10:0	10.2 ± 4.7	3.6 ± 5.3	9.5 ± 5.3	–0.0 ± 1.6[a]	–4.2 ± 0.9[a]	4.1 ± 1.1
12:0	23.8 ± 4.7	0.2 ± 5.2	18.3 ± 5.3	5.4 ± 1.6[b]	2.0 ± 0.9[b]	3.3 ± 1.1
Main effects						
Phenotype	NS[c]	NS	NS	NS	NS	NS
Diet	NS ($P = 0.0608$)	NS	NS	$P = 0.0305$	$P = 0.0004$	NS
Interaction[b]	NS ($P = 0.0822$)	NS	NS	NS	NS	NS
Study II						
ApoE Phenotypes						
E3/2	0.3 ± 4.3[a]	2.9 ± 10.0	–3.0 ± 4.4[a]	1.2 ± 2.9	2.3 ± 2.5	–1.0 ± 1.4
E3/3	2.8 ± 2.8[a]	–7.0 ± 6.6	1.3 ± 3.0[a]	0.9 ± 1.9	1.4 ± 1.7	–1.5 ± 0.9
E4/3	25.2 ± 5.0[b]	3.8 ± 11.6	18.3 ± 5.1[b]	4.9 ± 3.3	3.7 ± 2.9	1.2 ± 1.7
Experimental diets						
14:0	13.4 ± 5.2[a]	0.9 ± 5.8	5.5 ± 4.4[a,b]	4.9 ± 2.7	3.6 ± 2.2	1.3 ± 1.5
16:0	22.0 ± 5.2[a]	–2.8 ± 5.8	17.5 ± 4.5[a]	3.4 ± 2.7	2.5 ± 2.2	0.3 ± 1.5
18:0	–7.0 ± 5.2[b]	1.6 ± 5.7	–6.4 ± 4.4[b]	–1.0 ± 2.6	1.2 ± 2.2	–3.0 ± 1.5
Main effects						
Phenotype	$P = 0.0033$	NS	$P = 0.0176$	NS	NS	NS
Diet	$P = 0.0017$	NS	$P = 0.0035$	NS	NS	NS
Interaction[b]	NS	NS	NS ($P = 0.0669$)	NS	NS	NS

[a]LSMEAN ± SEM = least squares means ± standard error of LSMEAN.
[b]Main effects of diet and phenotype and interaction were determined by two-way analysis of variance of General Linear Model (GLM) procedure.
[c]NS = not significant. Values with different superscripts (a,b) within a data set are significantly different ($P < 0.05$). See Table 4 for other abbreviations.

sult may be due to the characteristics of the individual SFA, since SFA may not have the same influence on lipoprotein metabolism. Interestingly, in this report, there were no effects of apoE phenotype on any of the variables measured in both Study I and Study II when subjects consumed a baseline diet providing higher polyunsaturated fat (Table 2 and Table 3). It may be possible to hypothesize that the high polyunsaturated fat diet has its own hypocholesterolemic effect regardless of apoE phenotypes. In addition, Cobb and Risch (30, Table 8) reported that the degree of change in dietary saturated fat content was one of the most significant predictors of responses of plasma LDL-C to dietary intervention. According to results from stepwise regression analyses, 14% of the variance in change of serum TC and 16% of the variance in change of serum LDL-C were explained by apoE 4/3 phenotype. Although there were Asians and African-Americans in both Study I and Study II, ethnic difference did not contribute to serum lipid and lipoprotein (except for TG in Study II) responses to experimental diets. Therefore, the present study was a well-controlled feeding study designed to investigate whether the effects of different SFA on serum TC and lipoprotein cholesterol depend on the apoE phenotype in healthy premenopausal women.

Does apoE polymorphism influence SFA-induced serum lipoprotein responses? It is known that SFA have different cholesterolemic effects on the serum lipids and lipoproteins (4–8). The MCT (8:0 + 10:0) are thought not to raise cholesterol concentrations (4), while myristic acid (14:0) and palmitic acid (16:0) raise both serum total and LDL-C (4,5). Stearic acid (18:0) is considered neutral owing to its failure to increase either serum TC or LDL-C levels because of poor absorption and possible conversion to oleic acid (18:1) (6,34). With respect to effects of the apoE phenotypes on lipoprotein metabolism, it has been known that chylomicron remnants with apoE 2 on the surface are poorly recognized by receptors which results in accumulation of these particles in plasma (3), while clearance of chylomicron remnants is more rapid and cholesterol absorption is more enhanced in individuals with the apoE 4 phenotype (35,36). Therefore, when taking apoE polymorphism into account, the magnitude of cholesterolemic effects described above may not stay the same. In the present report, changes from baseline showed that both the 12:0 diet and 8:0 + 10:0 diet induced significant increases in serum TC in subjects with different apoE phenotypes (with the exception of apoE 3/2 in the 8:0 + 10:0 group) in contrast to previous reports suggesting 8:0 + 10:0 was neutral (4). The 16:0 diet significantly raised both serum TC and LDL-C in subjects with apoE 4/3. It is not clear that the cause of increased atherogenic factors in subjects with apoE 4/3 consuming the 16:0 diet is due to the hypercholesterolemic prop-

TABLE 7
Least Squares Means[a] of Changes from Baseline in Variables Associated with Reverse Cholesterol Transport and LDL Receptor Activity in Mononuclear Cell (MNC) Analyzed by ApoE Phenotypes and Experimental Diets

	CETP (nmol/mL/h)	LCAT (nmol/mL/h)	Receptor-mediated ^{125}I-LDL degradation in MNC (ng/h/10^{6}MNC)	Binding of ^{125}I-LDL by MNC LDL receptors (ng/10^{6}MNC)
Study I				
ApoE phenotypes				
E3/2	0.3 ± 2.2	3.0 ± 5.4	−0.3 ± 1.9	−0.2 ± 1.8
E3/3	2.8 ± 1.1	6.0 ± 2.8	0.9 ± 1.0	1.2 ± 0.9
E4/3	1.5 ± 2.2	6.1 ± 5.4	3.1 ± 1.9	2.6 ± 1.8
Experimental diets				
8:0 + 10:0	−1.7 ± 1.5[a]	0.6 ± 3.4	4.6 ± 1.3[a]	4.5 ± 2.1[a]
12:0	4.8 ± 1.4[b]	9.4 ± 3.4	−2.2 ± 1.3[b]	−2.0 ± 2.1[b]
Main effects				
Phenotype	NS[c]	NS	NS	NS
Diet	$P = 0.0075$	NS ($P = 0.0928$)	$P = 0.0025$	$P = 0.0460$
Interaction[b]	NS	NS	NS	NS
Study II				
ApoE phenotypes				
E3/2	5.7 ± 3.0	−1.9 ± 4.0	3.2 ± 1.7[a]	−1.7 ± 5.4
E3/3	4.6 ± 2.0	−1.6 ± 2.7	−4.3 ± 1.2[b]	−1.7 ± 3.6
E4/3	10.1 ± 3.4	5.4 ± 4.6	0.3 ± 2.0[ab]	−2.6 ± 6.2
Experimental diets				
14:0 10.0 ± 3.7	2.6 ± 5.0 −3.6 ± 2.1	−1.0 ± 4.1		
16:0	6.1 ± 3.6	3.9 ± 4.9	1.3 ± 2.1	−7.2 ± 4.0
18:0	4.4 ± 3.6	−4.7 ± 4.8	1.4 ± 2.1	2.1 ± 4.0
Main effects				
Phenotype	NS	NS	$P = 0.0093$	NS
Diet	NS	NS	NS	NS
Interaction[b]	NS	NS	NS	NS

[a]LSMEAN ± SEM = least squares means ± standard error of LSMEAN.
[b]Main effects of diet and phenotype and interaction were determined by two-way analysis of variance of General Linear Model (GLM) procedure.
[c]NS = not significant. Values with different superscripts (a,b) within a data set are significantly different ($P < 0.05$).

erty of the palmitic acid (4,5) or the greater responsiveness of apoE 4 (9–14) or interaction of both. The 18:0 diet tended to decrease LDL-C concentrations. Taken together, all variations regarding the cholesterolemic responses to dietary intervention providing enhanced individual SFA may be associated with the different metabolism of lipoproteins regulated by the apoE polymorphism. For example, Kesaniemi *et al.* (36) reported that subjects with apoE 2 showed lower intestinal cholesterol absorption efficiency than those with the apoE 4. Miettinen *et al.* (28) also showed that subjects with apoE 2 have more effective bile acid and cholesterol syntheses, fecal elimination of cholesterol, removal of LDL apoB, and low cholesterol absorption. In the present study, results from statistical analysis by two-way ANOVA showed that there were no significant interactions between experimental diets and apoE phenotypes although the interaction between the saturated fats in Study II and apoE phenotypes for LDL-C responsiveness ($P = 0.0669$) was close to a significant level (Table 6 and Table 7). However, with respect to the independent effects of experimental diets and apoE phenotypes, we found that serum TC and LDL-C level were independently regulated by apoE polymorphism: apoE 4/3 increased levels of TC and LDL-C the most, when subjects consumed the experimental diets in Study II (14:0, 16:0, and 18:0 diets).

Does apoE phenotype influence parameters related to reverse cholesterol transport? In a previous report (37), in which apoE polymorphism was not taken into account, we demonstrated that SFA had different effects on HDL-C in healthy women. For example, the 12:0 diet significantly raised HDL-C fractions (HDL_2-C and HDL_3-C), and the concentration of HDL_2-C declined and HDL_3-C increased in serum when subjects consumed 8:0 + 10:0, while 14:0 and 16:0 diets had no significant effect on the HDL-C fractions compared to the baseline diet (37). With respect to the influences of apoE phenotype on this aspect, Martin *et al.* (31) found that there was a significant effect of apoE phenotype on both the HDL-C and CETP responses to a high cholesterol diet. In their study, subjects with apoE 4/3 had a small increase in CETP and a large increase in plasma HDL-C whereas the apoE 3/2 subjects showed the opposite association (31). In the present study, the 12:0 diet significantly raised both HDL-C-including HDL fractions and CETP in the apoE 3/3 group, the most common apoE phenotype. In addition, no consistencies existed in the experimental diet-induced changes of the rate of LCAT among apoE phenotypes. Overall, statistical analysis of the independent effects of experimental diets and apoE phenotypes showed that apoE polymorphism had no significant influence on parameters re-

TABLE 8
Summary of a Variety of Studies of Dietary-Induced Changes in Plasma Lipids and Lipoproteins in Relation to ApoE Phenotype

Investigator, date (Ref. no.)	Subject	Diet	Period	ApoE phenotype showing a significant dietary-induced change in:			
				TC	TG	LDL-C	HDL-C
Fisher *et al.*, 1983 (23)	E3/2 (n = 3) E3/3 (n = 5)	Corn oil diet vs. coconut oil diet	18 d	NS	NS	NS	NS
Miettinen *et al.*, 1988 (9)	E2 (n = 6) E4 (n = 10)	Low-fat/low-cholesterol (150–200 mg/d) vs. low-fat/high-cholesterol (900 mg/d)	5–6 wk	E4[a]	NA	E4[a]	E4[a], E2
Gylling *et al.*, 1989 (10)	E2 (n = 8) E3 (n = 9) E4 (n = 12)	Low-fat/low-cholesterol (24%, 210 mg/d) vs. low-fat/high cholesterol (24%, 880 mg/d)	6 wk	E3, E4[a] E3, E4[a]	NA	E3, E4[a]	E2
Tikkanen *et al.*, 1990 (11)	E3/2 (n = 12) E3/3 (n = 48) E4/3 (n = 42) E4/4 (n = 8)	Low-fat/high P/S diet vs. usual diet	6 or 12 wk	E4/4[a]	NS	E4/4[a]	NS
Savolainen *et al.*, 1991 (24)	E3/3 (n = 23) E4 (n = 21)	Low-fat/low-cholesterol (25% fat, 240 mg/12.6 MJ) vs. high-fat/high-cholesterol (38% fat, 420 mg/12.6 MJ)	4 wk	E3/3, E4	E3/3	E3/3, E4	E3/3, E4
Boerwinkle *et al.*, 1991 (25)	E3/2 (n = 13) E3/3 (n = 48) E4/3 (n = 10)	Low-cholesterol diet (300 mg/d) vs. high-cholesterol diet (1700 mg/d)	3 wk	NS	NS	NS	NS
Gaddi *et al.*, 1991 (26)	E3/2 (n = 7) E3/3 (n = 9) E4/3 (n = 4)	Low-fat/low cholesterol (25% fat, 120–250 mg/d) vs. soy protein diet (25% fat, <10 mg/d, 15% soy protein)	4 wk	E3/3, E4/3	NA	E3/3, E4/3	NA
Uusitupa *et al.*, 1992 (27)	E3/3 (n = 12) E4 (n = 7)	High-fiber diet (29.8 g oat bran or 20.5 g wheat bran)	8 wk	E3/3[a]	NA	E3/3[a]	NA
Miettinen *et al.*, 1992 (28)	E2 (n = 8) E3 (n = 9) E4 (n = 12)	Low-fat/low-cholesterol (24% fat, 208 mg/d) vs. basal diet (38% fat, 574 mg/d)	5 wk	E2, E3, E4	NS	E2, E3, E4	E4
Lehtimaki *et al.*, 1992 (12)	E3/2 (n = 9) E3/3 (n = 11) E4/3 (n = 13) E4/4 (n = 3)	High-cholesterol diet (750 mg/d) vs. usual diet	3 wk	E3/2 E3/3 E4/3 E4/4	E3/2 E3/3 E4/3 E4/4	E3/2 E3/3 E4/3 E4/4[a]	E3/2 E3/3 E4/3 E4/4
Jenkins *et al.*, 1993 (29)	E2 (n = 13) E3 (n = 38) E4 (n = 16)	High-fiber diet (6.8 g/1000 kcal oat bran or wheat bran) vs. usual diet	2 wk	E2[a]	NS	E2[a]	NS
Cobb and Risch, 1993 (30)	E3/2 (n = 13) E3/3 (n = 44) E4/3 (n = 8) E4/4 (n = 2)	Low P/S vs. high P/S	NA	NA	NS	NA	
Martin *et al.*, 1993 (31)	E3/2 (n = 5) E3/3 (n = 11) E4/3 (n = 14)	Low-cholesterol diet (80 mg/1000 kcal) vs. high-cholesterol diet (320 mg/1000 kcal)	35 d	NS E4/3	NS	NS	E3/3
Lopez-Miranda *et al.*, 1994 (13)	E3/2 (n = 17) E3/3 (n = 94) E4/3 (n = 17)	Cholesterol-lowering diet (26% fat, 201 mg/d) vs. American diet (39% fat, 435 mg/d)	4–24 wk	E3/2 E3/3 E4/3	E4/3 E3/3 E4/3[a]	E3/2 E3/3 E4/3[a]	E3/2
Sarkkinen *et al.*, 1994 (32)	E3/2 or E4/2 (n = 3) E3/3 (n = 27) E4/3 (n = 10)	Monoene-enriched diet (38% fat, S/M/P = 14:18:6) vs. control diet (38% fat, S/M/P = 18:15:5)	6 mon	NS	NA	E3/3[a]	NA
Dreon *et al.*, 1995 (14)	E3/2 (n = 10) E3/3 (n = 65) E4 (E4/3, 4/4) (n = 28)	High-fat diet (46%) vs. low-fat diet (24%)	6 wk	E3/3 E4[a]	E3/3 E4	E3/2, E3/3 E4	E3/3 E4
Zambon *et al.*, 1995 (33)	E3/2 (n = 27) E3/3 (n = 48) E4/3 (n = 47)	Hypolipidemic diet (31% fat, S/M/P = 7:19:5) vs. basal diet (40% fat, S/M/P = 12:22:6)	12 wk	NS	NS	NS	NS
Park *et al.*, 1996 (5)	E3/2 (n = 3) E3/3 (n = 14)	12:0 + 14:0, 14:0 + 16:0, 16:0 + 18:0 (39% fat, S/M/P = 15:17:7)	4 wk	NS	NS	NS	NS

[a]ApoE phenotype showing the significantly greatest response. NS = no significant effect of apoE phenotype on the response to dietary manipulation. NA = not applicable. See Table 4 for other abbreviations. P/S = polyunsaturated/saturated; S/M/P = saturated/monounsaturated/polyunsaturated.

lated to reverse cholesterol transport regardless of experimental diets, whereas the experimental diets in Study I had significant effects on HDL-C and HDL_2-C, the 12:0 diet increased their serum levels, and the 8:0 + 10:0 diet lowered them. We may hypothesize that variations in reverse cholesterol transport system induced by the dietary manipulation are regulated not by apoE polymorphism but by certain SFA.

Is experimental diet-induced LDL-receptor mediated LDL metabolism regulated by apoE polymorphism? Since the steady-state concentrations of LDL-C in blood are predominantly a function of the rate of LDL-C production and the activity of the LDL-receptor, the SFA fed in cholesterol-containing diets have been found to enhance LDL-C production and reduce LDL-receptor activity (34). In addition, LDL-receptor activity in MNC has been shown to reflect the LDL-receptor activity in liver (38). In both Study I and Study II, MNC were isolated from fresh fasting blood to detect the LDL-receptor activity. In Study I, all apoE phenotypes consuming the 12:0 diet tended to decrease the receptor-mediated degradation of ^{125}I-LDL in MNC. This reduction may help explain why the 12:0 diet significantly raised both serum TC and LDL-C among all apoE phenotypes in the present study. Surprisingly, the 8:0 + 10:0 diet significantly induced the elevation of both receptor-mediated degradation and binding of ^{125}I-LDL in subjects with apoE 3/3 phenotypes. It has been known that apoE 4 may increase the intrahepatic pool of cholesterol, downregulate hepatic cholesterol synthesis and LDL receptor activity, and eventually elevate serum LDL-C levels, while the reduced binding affinity seen in subjects with apoE 2 may upregulate LDL receptor activity and increase LDL-C clearance (3,39). In the present study, however, the independent effect of apoE polymorphism on receptor-mediated degradation of ^{125}I-LDL in MNC was significant (P = 0.0093) only when subjects consumed the experimental diets in Study II (14:0, 16:0, and 18:0 diets) (Table 7). Compared to experimental diets in Study II, the 8:0 + 10:0 and 12:0 diets in Study I seemed to be more responsible for variation of LDL receptor-mediated degradation (P = 0.0025) and binding (P = 0.046) of ^{125}I-LDL in MNC (Table 7). It seems that the effect of apoE polymorphism on the response of receptor-mediated mechanisms to experimental diets may depend on the characteristics of individual saturated fats.

In summary, serum TC and LDL-C responses to the 14:0, 16:0, and 18:0 diets were regulated, in part, by apoE polymorphism, because subjects with apoE 4/3 responded well to the dietary manipulation providing certain SFA enhanced at levels above usual intake (Table 6). According to findings of the present report, it is possible to hypothesize that variations in the serum lipid and lipoprotein responses to dietary intervention providing enhanced individual SFA may be associated with differences in lipoprotein metabolism regulated in part by apoE polymorphism. However, since there were only three or four subjects in either the apoE 3/2 or apoE 4/3 groups participating in the present dietary intervention studies (both Study I and Study II), more controlled studies with larger subject sample sizes are needed. It is also important to study the less common phenotypes such as apoE 2/2, apoE 4/4, and apoE 4/2.

ACKNOWLEDGMENT

This work was supported by the USDA Grant 93-37200-8978.

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[Received June 19, 1997, and in final revised form November 19, 1997; revision accepted December 30, 1997]

Low-Fat, Monounsaturate-Rich Diets Reduce Susceptibility of Low Density Lipoproteins to Peroxidation *ex vivo*[1]

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ABSTRACT: Oxidative modification of low density lipoprotein (LDL) plays an important role in the process of atherosclerosis. The susceptibility of LDL to oxidation and the amount of peroxidation products formed are influenced by the lipoprotein content of 18:1n-9, 18:2n-6, and the 18:2n-6/18:1n-9 ratio, which is dependent in part on dietary fatty acids. The purpose of this study was to determine if changing from a typical American diet to a low-fat, monounsaturate-rich diet (LFMR) would result in favorable alterations in the fatty acid composition and oxidative profile of LDL in hypercholesterolemic individuals. Free-living postmenopausal hypercholesterolemic women who routinely consumed a diet moderately high in total fat and total saturates (34 and 11%, respectively) followed an LFMR diet (26% fat, 6% saturated fat, and 14% monounsaturated fat) for 6 mon. Sixteen postmenopausal hypercholesterolemic women already following standard low-fat (LF) diets acted as a control for seasonal variations in serum lipids. LDL from randomly selected subjects (LF $n = 6$, LFMR $n = 5$) was evaluated. LFMR diets resulted in LDL with increased concentrations and percentages of 18:1n-9, reduced 18:2n-6/18:1n-9 ratio, and lower percentages of 18:2n-6. No significant changes in LDL fatty acids occurred in the LF group. Conjugated diene lag time increased in both groups during copper-induced *in vitro* oxidation. Only the LFMR group experienced an increase in lipid peroxide lag time and a decrease in lipid peroxide formation. The LFMR diet was well tolerated and may be of therapeutic value in the treatment of hypercholesterolemia.

Lipids 33, 149–157 (1998).

Oxidative modification of low density lipoprotein (LDL) is thought to play an important initiating role in the process of atherosclerosis (1–6). Diets high in monounsaturated fatty acids (MUFA) have been shown to enrich LDL with oleic acid and result in lipoprotein particles that are more resistant to oxidation and produce less conjugated dienes (CD), lipid peroxides (LP), or malondialdehyde (MDA), relative to LDL rich in polyunsaturated fatty acids (PUFA) (7–12). The rate and extent of LDL oxidation during *in vitro* studies have been shown to be influenced by the fatty acid content of the lipoprotein. The amount of LDL oxidative products generated, and to a lesser extent the oxidative rate, have been positively associated with the ratio of 18:2n-6 to 18:1n-9, %18:2n-6, and %PUFA, and inversely correlated with %18:1n-9 and %MUFA (7,9,11,12). The influence of LDL fatty acid composition on lag time has been less clear (7,11,13–15).

To date, experimental diets used to alter the fatty acid composition and oxidative profile of human LDL have been relatively high in fat (34–45% energy) and consumed for only a few weeks. High MUFA diets often have been compared to high PUFA diets to determine efficacy, so it is not certain if high MUFA diets actually improve baseline oxidative resistance of LDL. In general, most of the experimental diets employed in previous studies do not reflect standard therapeutic practices for treating hypercholesterolemia. Current dietary guidelines for the prevention of coronary heart disease recommend that individuals consume <30% energy from fat and <10% from saturated fat (SFA), with MUFA making up the majority of dietary fat. The impact of such dietary modifications on the lipid composition and oxidative susceptibility of LDL has not been studied. It was the purpose of this investigation to determine if the fatty acid profile and oxidative potential of LDL can be altered when hypercholesterolemic individuals change from typical American diets to low-fat, high-MUFA diets.

SUBJECTS AND METHODS

Study design. This study was approved by the Institutional Review Board of the University of Florida. Thirty-six free-living healthy postmenopausal hypercholesterolemic women participated in this study. Twenty subjects habitually consuming a typical American diet (34% fat, 11% saturated fat) were placed on an experimental low-fat monounsaturate-rich diet (LFMR) for 6 mon. Sixteen subjects already following a low-

[1]A portion of this material was presented earlier at the annual meeting of the American Oil Chemists' Society and in abstract form [O'Byrne, D.J., Shireman, R.B., Knauft, D. (1993) The effects of a low-fat/high oleic acid diet on lipoproteins in postmenopausal hypercholesterolemic women, *INFORM 4*:553, #SS7].

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Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CD, conjugated diene; LDL, low density lipoprotein; LF, low-fat diet; LFMR, low-fat monounsaturate-rich; LP, lipid peroxide; MDA, malondialdehyde; MUFA, monounsaturated fatty acid; NCEP, National Cholesterol Education Program; PBS, phosphate buffered solution; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; WHHL, Watanabe heritable hyperlipidemic.

fat diet (LF) similar to the National Cholesterol Education Program (NCEP) Step 1 guidelines were asked to continue following their typical diets. LF subjects were used as cohorts to monitor seasonal variation in lipids which might confound the results of the study. LDL was isolated before and after the experimental diet period from randomly selected subjects in both groups (LFMR $n = 8$, LF $n = 6$). LDL fatty acid composition and *in vitro* formation of CD, LP, and MDA were determined.

Subjects. Subjects were unpaid volunteers who gave informed consent to participate in the study. These women ranged in age from 50 to 65 yr, had fasting serum cholesterol levels between 220 and 300 mg/dL (5.68–7.76 mmol/L), did not smoke, were free of endocrine disorders, were not on hormone replacement therapy, were not taking medications that alter blood lipids, and discontinued all vitamin/mineral supplements 3 mon prior to initiation of the study.

The data from 12 of the participants were not used because the women were unable to successfully complete the study due to injury, illness, schedule conflicts, or noncompliance with the study protocol. Three of these subjects were part of the randomly selected LFMR subgroup used for the LDL studies. Therefore, only the results of the subjects who successfully completed the study are reported here (LFMR $n = 5$, LF $n = 6$).

Diet. The participants were taught how to weigh, measure, and record all foods and beverages consumed. Subjects completed 7-d diet records prior to the initiation of the study and on a monthly basis during the 6-mon experimental diet phase. The nutrient composition of the initial and 6-mon diet records were analyzed by computer (Professional Dietitian, Wellsource, Clackamas, OR). The database of the nutritional analysis program was expanded to include specific processed foods and information on the dietary fiber and vitamin E content of foods frequently eaten (16–27).

A registered dietitian instructed the free-living participants on their diet protocol, designed individualized daily eating plans, and provided monthly nutrition classes and counseling throughout the study. The LF and LFMR daily eating plans were based on the NCEP Step 1 guidelines as outlined by the American Heart Association (28). The energy distributions of the LF and LFMR diets were <30% fat, 50–60% carbohydrate, and 15–20% protein. Dietary SFA was limited to <10% energy, and dietary cholesterol was restricted to <300 mg/d. Monounsaturated fat comprised 50–60% of the fat in the LFMR diet. For convenience and to enhance compliance, a majority of the monounsaturated fat in the LFMR diet was supplied by a high-oleic acid peanut cultivar developed at the University of Florida (Gainesville, FL) (29). This peanut contains approximately 76–80% of the lipid as MUFA. Prepackaged daily rations of the dry roasted high-oleic peanuts were given to the participants. Depending upon energy requirements, subjects consumed 35–68 g of peanuts per day. Small amounts of olive oil and canola oil were allowed for cooking purposes.

Subjects were asked to maintain weight and activity level throughout the study. Dietary compliance was determined through monthly telephone conversations and evaluation of subject's body weight and diet records.

Isolation of LDL. Approximately 60 mL blood was drawn from randomly selected subjects after a 12-h fast. Subjects refrained from vigorous exercise and alcohol consumption for 24 h prior to donating blood. Serum was isolated, and 1 mL proteolysis inhibitor (10 mg EDTA, 10 mg merthiolate, and 20 mg sodium azide per 100 mL) was added per 100 mL serum. A Sorvall OTD-2 ultracentrifuge equipped with a T865 rotor (Sorvall, Newton, CT) was used to isolate LDL by sequential density gradient ultracentrifugation of serum (30) at 4°C under N_2. The KBr buffers used during ultracentrifugation contained 0.01% EDTA to prevent oxidation during the procedure (31). Isolated samples of LDL were dialyzed at 4°C with five changes of N_2-flushed phosphate buffered saline (PBS, pH 7.2) containing 0.01% EDTA.

LDL protein concentration. The protein concentration of freshly dialyzed LDL was determined in triplicate by the method of Lowry *et al*. (32) using bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) for the standard curve.

LDL fatty acid profile. The lipid from freshly dialyzed LDL was extracted by a modified method of Bligh and Dyer (33). During each step of the extraction and methylation process, samples were flushed with N_2 to prevent oxidation of the lipids. The extract residue was dissolved in chloroform with 0.05% butylated hydroxytoluene (BHT) and transferred to 2 mL Pyrex® sample containers with Teflon-lined screw caps. Samples were flushed with N_2 and stored at −70°C until the fatty acid analysis was performed at the end of the study. The initial and final LDL samples were methylated (34) and analyzed at the same time to minimize procedure variability. Fatty acid methyl esters were analyzed using a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and C-R5A Chromatopac integrator (Shimadzu, Kyoto, Japan). A DB-225 capillary column, 30 m × 0.25 mm internal diameter (Alltech Associates, Deerfield, IL), was used at a constant temperature of 200°C. Duplicate injections were made for each LDL sample. Fatty acid methyl esters were identified by comparison of peak retention times with a standard containing a mixture of fatty acid methyl esters (GLC-68A; Nu-Chek-Prep, Inc., Elysian, MN). The fatty acid profile was calculated by dividing the area of each peak by the total area of the fatty acid peaks. The concentrations of individual fatty acids were calculated by comparing the mean area of each peak to the mean peak area of the 17:0 internal standard (Nu-Chek-Prep). The results were expressed as μg fatty acid/mg protein.

LDL oxidation studies. An aliquot of each freshly isolated LDL sample was dispensed into 2 mL polystyrene cryovials containing sufficient sucrose and EDTA for a final concentration of 20 and 0.01%, respectively. The samples were flushed with N_2 and stored at −20°C for oxidation experiments to be conducted at the end of the study. Prior to the oxidation experiments, each subject's initial and final LDL samples were

thawed and dialyzed together at 4°C in five changes of N_2-saturated PBS (pH 7.2) without EDTA.

Each LDL sample was diluted with PBS to a concentration of 50 µg protein/mL. *In vitro* oxidation of LDL was induced by adding 5 µM $CuSO_4$ to each sample and incubating at 37°C for 4 h (35). Initial and final LDL samples were analyzed at the same time. The production of CD was continuously monitored at 234 nm for 4 h in a DU-7 spectrophotometer (Beckman, Palo Alto, CA), and measurements were recorded every 5 min. CD concentration was calculated using the extinction coefficient 29,500 L mole^{-1} cm^{-1} (7). Lipid peroxides were measured every 30 min by an iodometric method (36). Absorbance was measured at 365 nm using a Beckman DU-40 spectrophotometer and the LP concentration was calculated from the extinction coefficient 24,600 L mole^{-1} cm^{-1}. MDA was determined every 30 min by a modification of the method of Tatum *et al*. (37). Thiobarbituric acid-MDA adduct was measured using a Perkin Elmer 204-A fluorimeter (Norwalk, CT) set at an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

Statistical analysis. Statistical analyses were performed on data from the subgroups (LFMR $n = 5$, LF $n = 6$). Results are expressed as the mean ± standard deviation. The initial and final group mean values were compared by paired *t*-test. Because the LF group was already following a low-fat diet and served the purpose as a cohort for determining potential seasonal lipid variations, comparisons between the two groups were not made. Correlations were performed between variables selected "a priori" to assess the impact of specific variables on LDL fatty acid composition and oxidation. Statistical analyses were performed using the Microsoft Excel™ 4.0 (Microsoft Corporation, Redmond, WA) statistical program.

RESULTS

Diet. The LFMR diet was well-tolerated by the subjects. Table 1 summarizes the nutrient composition of the diets consumed by the subjects. Both LF and LFMR subjects reduced their reported energy intake during the study. The LF group continued to follow the NCEP guidelines for a low fat diet. LFMR subjects successfully modified their eating habits to meet the dietary guidelines by significantly reducing the amount of fat, saturated fat, and polyunsaturated fat consumed, and maintaining dietary cholesterol intake at <300 mg/d.

Overall, both diets provided at least 75% of the RDA for major vitamins and minerals (data not shown). Dietary fiber increased in the LFMR group, and Vitamin A and β-carotene increased in the LF group. The standard deviation was high for vitamin A, vitamin C, and β-carotene in both groups, which probably reflects the varying consumption of foods rich in these nutrients in this free-living population. High-oleic peanuts contain approximately the same amount of vitamin E as regular peanuts (7.8 mg α-tocopherol per 100 g edible portion) (23), and may have contributed to the slight increase in vitamin E in the LFMR diet ($P = 0.07$).

The fatty acid composition of the diet consumed by the LFMR group was significantly altered (Table 2). The ratios of SFA/UFA, PUFA/MUFA, SFA/fat, and PUFA/fat decreased and the ratio of MUFA/fat increased. A significant reduction in PUFA/fat was observed only in the LF group.

LDL fatty acid analysis. The fatty acid composition of LDL was significantly altered in the LFMR group, but did not change in the LF group (Table 3). The %18:0, %18:2n-6, and %total PUFA decreased while %18:1n-9 and %total MUFA increased in the LFMR LDL. The change in the proportion of LDL MUFA was due to an increase in the concentration of

TABLE 1
Daily Nutrient Intake of Postmenopausal Hypercholesterolemic Women on Low-Fat (LF) and Low-Fat, Monounsaturate-Rich (LFMR) Diets[a]

Nutrient	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
Energy (KJ)	6146 ± 1261	5694 ± 1364*	8970 ± 1615	7816 ± 1372*
Protein (g)	72 ± 16	81 ± 16	85 ± 20	94 ± 11
Carbohydrate (g)	228 ± 53	212 ± 58	279 ± 84	259 ± 60
Alcohol (g)	0.4 ± 0.9	0.3 ± 0.8	5 ± 12	3 ± 7
Total fat (g)	33 ± 13	24 ± 8	76 ± 16	54 ± 7*
Saturated fat (g)	9 ± 5	6 ± 1	22 ± 4	12 ± 2**
Monounsaturated fat (g)	11 ± 4	9 ± 5	26 ± 7	29 ± 4
Polyunsaturated fat (g)	6 ± 2	4 ± 2*	14 ± 5	5 ± 1*
Cholesterol (mg)	145 ± 61	122 ± 27	238 ± 93	144 ± 51
Dietary fiber (g)	16 ± 4	16 ± 2	11 ± 4	24 ± 5**
Vitamin A (µg)	1174 ± 359	2123 ± 951*	1992 ± 967	1307 ± 369
Vitamin C (mg)	136 ± 69	177 ± 70	220 ± 106	266 ± 60
Vitamin E (mg)	3 ± 2	3 ± 2	5 ± 1	8 ± 3
β-carotene (µg)	3412 ± 1949	9668 ± 4955*	8538 ± 4195	4741 ± 1669

[a]Values represent the mean ± standard deviation. The Seven Day Diet Records from each subject (LF $n = 6$, LFMR $n = 5$) were analyzed prior to the initiation and after 6 mon of the diet phase. *$P \leq 0.05$, **$P \leq 0.01$; significantly different from the initial group mean.

TABLE 2
Ratio of Dietary Fatty Acids in Postmenopausal Hypercholesterolemic Women on LF and LFMR Diets[a]

Dietary fatty acid ratio	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
SFA/PFA	1.45 ± 0.34	2.04 ± 0.34*	1.82 ± 0.72	2.34 ± 0.42
SFA/UFA	0.53 ± 0.15	0.54 ± 0.18	0.57 ± 0.09	0.34 ± 0.03**
PFA/MFA	0.60 ± 0.12	0.43 ± 0.16	0.55 ± 0.25	0.18 ± 0.04*
SFA/fat	0.27 ± 0.06	0.27 ± 0.06	0.29 ± 0.03	0.21 ± 0.01**
MFA/fat	0.32 ± 0.04	0.38 ± 0.12	0.33 ± 0.05	0.54 ± 0.03**
PUFA/fat	0.19 ± 0.05	0.15 ± 0.02*	0.18 ± 0.6	0.09 ± 0.02*

[a]Values represent the mean ± standard deviation. The Seven Day Diet Records from each subject (LF $n = 6$, LFMR $n = 5$) were analyzed prior to the initiation and after 6 mon of the diet phase. *$P \leq 0.05$, **$P \leq 0.01$; significantly different from the initial group mean. SFA, saturated fatty acid; UFA, unsaturated fatty acid; PFA, polysaturated fatty acid; MFA, monosaturated fatty acid; PUFA, polyunsaturated fatty acid. See Table 1 for other abbreviations.

16:1n-7 and 18:1n-9, rather than a decrease in 18:2n-6 or total PUFA (Table 4). As a consequence of the oleic-enrichment of LFMR LDL, the ratio of 18:2n-6/18:1n-9 was significantly lower. A nonsignificant trend toward increased fatty acid content was also observed in the LDL of LFMR subjects.

LDL oxidative profile. After subjects followed the LF and LFMR diets for 6 mon, several significant changes in the oxidative profile of LDL were noted. Lag time before the onset of CD formation increased by approximately 10–16 min for both groups (Table 5). The LFMR group also had a significant increase in LP lag time. Figure 1 illustrates the time course of LDL oxidation and depicts the mean change in absorbance at 234 nm during CD formation. No change in oxidative rate was seen in either group (Table 6). A decrease in LP production occurred after oleic enrichment of the LFMR LDL (Table 7). The LFMR diet was also associated with a trend ($P = 0.12$) toward lower CD production when the amount of oxidative product was expressed per mg LDL fatty acid. No changes in the concentration of oxidation products were seen in the LDL samples from the LF group.

DISCUSSION

The LFMR diet effectively increased the percentage and concentration of 18:1n-9 in LDL and lowered the proportion of 18:2n-6 and the 18:2n-6/18:1n-9 ratio. The change in the 18:2n-6/18:1n-9 ratio was primarily due to the enrichment of LDL with oleic acid. Although the LFMR diet did not result in the high proportions of LDL oleic acid associated with diets containing 30–34% MUFA (9,11,12), the %18:1n-9 was similar to that elicited by diets supplemented with olive oil or low-erucic acid rapeseed oil (13,38). The percentage decrease in the proportion of LDL linoleic acid and the resulting 18:2n-6/18:1n-9 ratio was also in line with that reported for diets high in fat and MUFA. Our study shows that significant modifications in the proportion of oleic and

TABLE 3
LDL Fatty Acid Composition[a] of Postmenopausal Hypercholesterolemic Women on LF and LFMR Diets

Fatty acids	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
14:0	0.7 ± 0.3	0.8 ± 0.3	0.5 ± 0.1	0.5 ± 0.2
16:0	24.6 ± 0.9	25.4 ± 2.1	23.7 ± 1.1	23.6 ± 1.2
16:1n-7	1.6 ± 0.4	1.7 ± 0.3	1.0 ± 0.1	1.3 ± 0.3
18:0	11.3 ± 0.6	10.9 ± 1.1	11.9 ± 1.1	10.4 ± 0.8**
18:1n-9	17.4 ± 1.1	17.8 ± 2.2	15.7 ± 1.8	20.5 ± 1.3**
18:2n-6	21.6 ± 3.0	19.9 ± 1.7	25.7 ± 1.9	21.7 ± 3.1**
18:3n-3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:3n-6	2.7 ± 0.3	2.8 ± 0.4	2.4 ± 0.7	2.4 ± 0.6
20:4n-6	8.7 ± 1.3	8.7 ± 1.4	9.1 ± 1.8	9.2 ± 1.8
20:5n-3	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.5
22:5n-3	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
22:6n-3	2.3 ± 0.5	2.2 ± 0.7	2.0 ± 0.5	2.4 ± 1.8
Total SFA	36.6 ± 1.1	37.1 ± 3.1	36.1 ± 1.5	34.5 ± 0.9
Total MUFA	21.8 ± 1.7	22.3 ± 2.5	19.2 ± 1.9	24.0 ± 1.6*
Total PUFA	36.9 ± 2.9	35.3 ± 1.7	40.5 ± 0.9	37.2 ± 2.1*

[a]Low density lipoprotein (LDL) fatty acid composition is expressed as percentage of total fatty acid. Values represent the mean ± standard deviation; LF $n = 6$, LFMR $n = 5$. *$P \leq 0.05$, **$P \leq 0.01$; significantly different from the initial group mean. MUFA = monounsaturated fatty acid. See Tables 1 and 2 for other abbreviations.

TABLE 4
LDL Fatty Acid Concentration[a] in Postmenopausal Hypercholesterolemic Women on LF and LFMR Diets

Fatty acid	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
14:0	19.8 ± 11.9	21.7 ± 11.6	10.2 ± 3.0	12.4 ± 5.8
16:0	634.2 ± 197.3	677.3 ± 140.0	480.0 ± 66.5	594.6 ± 115.2
16:1n-7	42.2 ± 20.4	46.8 ± 15.0	20.2 ± 1.9	32.4 ± 10.0*
18:0	289.5 ± 86.4	289.2 ± 64.1	243.0 ± 52.6	258.2 ± 37.2
18:1n-9	448.3 ± 132.7	478.8 ± 112.7	316.0 ± 41.1	513.4 ± 85.7**
18:2n-6	556.3 ± 199.2	532.0 ± 117.2	517.6 ± 54.5	552.0 ± 163.2
18:3n-3	11.8 ± 6.2	11.3 ± 4.0	7.2 ± 0.8	10.4 ± 4.3
20:3n-6	68.8 ± 21.1	74.8 ± 19.2	49.4 ± 21.7	59.2 ± 14.1
20:4n-6	218.0 ± 55.4	233.0 ± 66.2	186.0 ± 55.9	226.0 ± 29.0
20:5n-3	13.5 ± 5.0	16.3 ± 6.0	9.4 ± 3.4	15.8 ± 9.1
22:5n-3	18.8 ± 7.3	20.0 ± 3.0	12.0 ± 2.0	13.2 ± 2.8
22:6n-3	58.5 ± 22.2	61.3 ± 30.9	39.0 ± 7.6	55.4 ± 32.3
Total SFA	943.7 ± 294.0	988.7 ± 209.5	733.0 ± 119.3	864.8 ± 151.8
Total MUFA	561.8 ± 169.0	598.0 ± 130.6	387.2 ± 45.9	600.4 ± 102.0*
Total PUFA	946.2 ± 291.8	948.8 ± 214.4	821.0 ± 114.5	931.6 ± 157.7
Total fatty acid	2578.2 ± 789.6	2676.0 ± 548.0	2027.8 ± 271.3	2507.4 ± 413.2
18:2n-6/18:1n-9	1.25 ± 0.24	1.13 ± 0.14	1.65 ± 0.19	1.06 ± 0.17**

[a]Concentration expressed as μg fatty acid/mg protein. Values represent the mean ± standard deviation. LF $n = 6$, LFMR $n = 5$. *$P \leq 0.05$, **$P \leq 0.01$; significantly different from the initial group mean. See Tables 1–3 for abbreviations.

linoleic acid in LDL can be elicited by an LFMR diet. Strong correlations were observed between LDL %18:1n-9 and the following: dietary %PUFA ($r = -0.825$, $P \leq 0.01$), dietary PUFA/MUFA ($r = -0.899$, $P \leq 0.001$), and MUFA/fat ($r = 0.943$, $P \leq 0.001$). Similarly, LDL %18:2n-6 was associated with the ratio of dietary PUFA and MUFA. Thus, the proportion of dietary PUFA and MUFA may be important factors influencing the 18:1n-9 and 18:2n-6 content of LDL, independent of the actual quantity of dietary fat.

An unexpected result of the LFMR diet was a trend ($P = 0.09$) toward lipid enrichment of LDL. The LDL particles tended to increase in total fatty acid content despite the fact that LFMR subjects were consuming less dietary fat compared to baseline. Similarly, the LDL-cholesterol/apolipoprotein B ratio increased slightly in the LFMR subgroup (1.63 ± 0.14 vs. 1.75 ± 0.46). Although serum LDL-cholesterol decreased significantly after subjects followed the LFMR diet, apolipoprotein B concentrations also tended to decrease (39). The resulting increase in the ratio of LDL-cholesterol/apolipoprotein B indicates that the diet resulted in slightly fewer circulating LDL particles, but the lipoprotein tended to contain more cholesterol. It is thought that LDL which is more lipid-rich and buoyant is less atherogenic while small dense LDL have been associated with increased oxidative susceptibility (40,41) and atherosclerosis (42,43). Increased oxidative susceptibility of small dense LDL may be due to the greater content of linoleic acid, cholesterol ester, and PUFA/vitamin E in this subfraction (41,44). In other studies, supplementation with dietary MUFA has resulted in LDL with greater 18:1n-9 concentration and a tendency toward increased fatty acid content (13). Oleate-enriched diets have also increased the 18:1n-9 content of small dense LDL, rendering them more resistant to oxidation (45). In contrast, low-fat diets have reduced the lipid content of LDL (46,47) and promoted the conversion of LDL from pattern A to the more dense pattern B phenotype in some individuals (46). Because LDL was not subfractionated in this study and the major lipid classes were not analyzed, it is unclear exactly how the LFMR diet may

TABLE 5
Lag Time Before Formation of Conjugated Diene (CD), Lipid Peroxide (LP), and Malondialdehyde (MDA) in LDL from Postmenopausal Hypercholesterolemic Women Following LF and LFMR Diets[a]

Lag time	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
CD (min)	39.6 ± 8.3	50.0 ± 10.8*	36.9 ± 8.8	52.7 ± 16.4*
LP (min)	45.9 ± 33.2	62.5 ± 36.7	48.7 ± 26.6	64.3 ± 30.6*
MDA (min)	44.4 ± 39.6	61.1 ± 45.9	63.7 ± 63.7	73.1 ± 60.5

[a]Values represent the mean ± standard deviation, LF $n = 6$, LFMR $n = 5$. LDL samples isolated prior to initiation of the diet phase and after 6 mon of the diet were diluted to a concentration of 50 μg protein/mL and incubated with 5 μM $CuSO_4$ at 37°C. Measurements were taken every 5 min for CD and every 30 min for LP and MDA during the 4-h incubation period. *$P \leq 0.05$, **$P \leq 0.01$; significantly different from the initial group mean. See Tables 1 and 3 for other abbreviations.

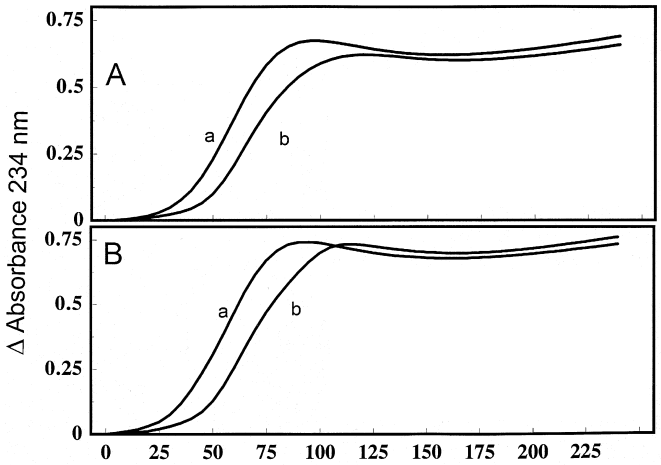

FIG. 1. Formation of conjugated dienes (CD) in LDL from postmenopausal hypercholesterolemic women before and after 6 month diet period. LDL samples were diluted to 50 μg protein/mL and incubated with 5 μM $CuSO_4$ at 37°C. The absorbance at 234 nm was measured every 5 min during a 4 h incubation period, and the results are expressed as the mean change in absorbance relative to baseline. (A) Oxidative profile of LDL from LF subjects (n = 6) before (a) and after (b) the diet period. (B) Oxidative profile of LDL from LFMR subjects (n = 5) before (a) and after (b) the diet period.

have affected the compositional pattern of LDL. However, it is possible that the relatively high MUFA composition and moderate dietary fat restriction of the LFMR diet may have prevented a potentially detrimental shift in LDL lipid composition from pattern A to pattern B. Additionally, already existing small dense LDL may have become enriched with oleic acid, potentially protecting against lipid peroxidation.

Changes in the fatty acid composition of LDL after the LFMR diet were accompanied by improved oxidative resistance as evidenced by increased CD and LP lag time. Two studies have also shown that, compared to baseline, lag time increased after humans (13) or Watanabe heritable hyperlipidemic rabbits (WHHL) (48) consumed high-MUFA diets. It has been proposed that lag time is affected by the ratio of LDL oleic acid and linoleic acid (15). The reduction in LDL 18:2n-6/18:1n-9 and %18:2n-6 may account for the prolonged lag time in the LFMR group, but does not fully explain the increased lag time in the LF group. Even though subjects in the LF group were instructed to maintain a constant diet throughout the study, subjects did reduce dietary PUFA and PUFA/fat, and tended to decrease the ratio of PUFA/MUFA in their diet. As a consequence LDL %18:2n-6, %PUFA, and 18:2n-6/18:1n-9 exhibited a downward trend in the LF group. Pooled data from both groups showed significant correlations between lag time and dietary MUFA/fat (r = 0.489) and PUFA/MUFA (r = –0.454). This correlation may represent the influence of the more oxidatively stable dietary MUFA or the potential impact of phytochemicals and antioxidants associated with sources of dietary MUFA (38,49). Surprisingly, no significant correlations were found between LDL fatty acid composition and lag time, although there was a slight inverse relationship between LDL %PUFA and CD lag time (r = –0.341). No single fatty acid appeared to have a significant relationship with lag time, which may indicate that lag time is a product of the overall LDL fatty acid and lipid composition, and endogenous and dietary antioxidants.

Antioxidant vitamins are known to influence oxidative resistance. The LFMR group had nonsignificant increases in vitamin E intake, both groups tended to consume more vitamin C, and the LF group had significant increases in dietary vitamin A and β-carotene. Vitamin E has been shown to inhibit LDL oxidation when given in relatively high doses (50–53), but most researchers have found little relationship between lag time and the content of endogenous vitamin E (14,15,54–56). Vitamin C is thought to enhance the antioxidant action of α-tocopherol (57), but it is doubtful that the small dietary increase in this study would have afforded protection when similar dietary levels have failed to prolong lag time (58). Abbey *et al.* (58) demonstrated a reduction in oxidative products when subjects were given diets containing approximately 19 mg of β-carotene, but lag time was not changed. Since the dietary antioxidant levels in the LF and LFMR diets were less than in supplementation studies, and no significant correlations were found between dietary antiox-

TABLE 6
Rate of Formation of CD, LP, and MDA in LDL from Postmenopausal Hypercholesterolemic Women on LF and LFMR Diets[a]

Oxidative rate	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
CD/protein/min[a]	12.8 ±1.6	11.1 ± 1.7	14.7 ± 2.1	15.0 ± 2.1
CD/fatty acid/min[b]	5.7 ± 3.0	4.3 ± 1.3	7.3 ± 0.9	6.1 ± 1.2
LP/protein/min[a]	1.7 ± 0.6	1.6 ± 0.5	1.6 ± 0.3	1.4 ± 0.2
LP/fatty acid/min[b]	0.8 ± 0.7	0.6 ± 0.2	0.8 ± 0.2	0.6 ± 0.2
MDA/protein/min[a]	1.7 ± 0.6	1.8 ± 0.4	2.1 ± 0.8	1.7 ± 0.9
MDA/fatty acid/min[b]	0.7 ± 0.4	0.7 ± 0.2	1.1 ± 0.5	0.7 ± 0.5

[a]Values represent the mean ± standard deviation, LF n = 6, LFMR n = 5. LDL samples isolated prior to initiation of the diet phase and after 6 mon of the diet were diluted to a concentration of 50 μg protein/mL and incubated with 5 μM $CuSO_4$ at 37°C. Measurements were taken every 5 min for CD and every 30 min for LP and MDA during the 4-h incubation period. Oxidative rate is expressed as: [a]nmol oxidative product/mg LDL protein/min, [b]nmol oxidative product/mg LDL total fatty acid/min. See Tables 1, 3, and 5 for abbreviations.

TABLE 7
Maximal Amount of CD, LP, and MDA Formed in LDL Samples from Postmenopausal Hypercholesterolemic Women on LF and LFMR Diets[a]

Oxidative products	LF baseline value	LF 6-mon value	LFMR baseline value	LFMR 6-mon value
CD/protein[a]	465 ± 54	440 ± 54	511 ± 43	503 ± 65
CD/fatty acid[b]	206 ± 110	175 ± 66	258 ± 53	205 ± 47
LP/protein[a]	83 ± 12	83 ± 10	95 ± 6	82 ± 8**
LP/fatty acid[b]	37 ± 20	33 ± 10	48 ± 7	33 ± 7*
MDA/protein[a]	106 ± 21	109 ± 16	104 ± 30	107 ± 71
MDA/fatty acid[b]	45 ± 19	43 ± 17	53 ± 19	46 ± 32

[a]Values represent the mean ± standard deviation, LF $n = 6$, LFMR $n = 5$. LDL samples isolated prior to initiation of the diet phase and after 6 mon of the diet were diluted to a concentration of 50 μg protein/mL and incubated with 5 μM $CuSO_4$ at 37°C. Measurements were taken every 5 min for CD and every 30 min for LP and MDA during the 4-h incubation period. Maximal oxidative product is expressed as: [a]nmol oxidative product/mg LDL protein, [b]nmol oxidative product/mg LDL total fatty acid, [c]nmol oxidative product/mg LDL PUFA. *$P \leq 0.05$; **$P \leq 0.01$; significantly different from the initial group mean. See Tables 1–3 and 5 for abbreviations.

idants and lag time, it is doubtful that vitamins A, C, E, or β-carotene were the primary cause of increased lag time. However, because subjects in both groups consumed more plant-based foods in accordance with the NCEP Step 1 guidelines, it is possible that an increased consumption of various dietary antioxidants in combination with alterations in LDL fatty acid composition may have had a synergistic effect on oxidative resistance. Abbey *et al.* (7) observed a significant variation in lag time after subjects consumed fixed and free diets and hypothesized that this difference may be due to dietary antioxidants. An alternate explanation for the increased lag time in both groups may be that the endogenous content of LDL antioxidants decreased during storage, even though LDL samples were stored under N_2 at –20°C with 0.01% EDTA.

It has been suggested that a change in LDL fatty acid composition may influence oxidative rate, but this was not confirmed by this study. Kleinveld *et al*. (48) reported a significant reduction in the rate of oxidation when lipoprotein fatty acid composition was altered by a high MUFA diet. These investigators dramatically lowered the proportion of LDL 18:2n-6 and increased 18:1n-9 in WHHL rabbits fed high amounts of MUFA. The LFMR diet did not alter LDL fatty acid composition to a similar degree. Even very high MUFA diets have failed to alter the fatty acid composition of human LDL to the same extent as seen in WHHL rabbits (9,12,38), and LDL oxidation rates have not changed (9). Therefore, it is doubtful that changing from a typical American diet to one high in MUFA would have sufficient impact on the lipid composition of LDL to reduce the rate of lipoprotein oxidation.

The LFMR diet did result in LDL particles which produced less LP during *in vitro* oxidation. Significant correlations were found between LP/protein and the following: LDL %18:1n-9 ($r = –0.815$, $P \leq 0.01$), LDL %MUFA ($r = –0.832$, $P \leq 0.01$), and LDL %PUFA ($r = 0.877$, $P \leq 0.001$). These correlations are in line with the results of other studies (7,9,11,12). However, MDA and CD production were not significantly altered by the LFMR diet. CD formation is a good marker for the oxidation of PUFA, and is generally a function of the linoleic and arachidonic acid content of LDL (7,35). The LFMR diet lowered the proportion of LDL fatty acid as 18:2n-6, but the actual concentration of linoleic acid, arachidonic acid, and total PUFA did not change. This may explain the lack of significant reduction in the amount of CD or MDA formed during oxidation.

In conclusion, this study demonstrated that changing from a typical American diet to a low-fat diet rich in MUFA (26% fat, 14% MUFA) led to reductions in the 18:2n-6/18:1n-9 ratio of LDL and improvements in the LDL oxidative potential of hypercholesterolemic postmenopausal women. Alterations in LDL fatty acid composition were correlated with reduced LP production during Cu^{2+}-induced oxidation of LDL, but not to changes in oxidative rate. The LFMR diet was also associated with prolonged CD and LP lag time, but it was unclear if the change in LDL fatty acid composition was solely responsible for increased oxidative resistance. It is important to note that these free-living hypercholesterolemic women did not have to consume excessive amounts of MUFA in order to change LDL fatty acid composition or improve the oxidative profile of LDL. Switching from a typical American diet to the LFMR diet required only a reduction in the intake of SFA and PUFA and a modest increase in MUFA consumption. These dietary changes were easily incorporated into each individual's eating habits, were well tolerated, and provided a degree of protection against oxidative modification of LDL.

ACKNOWLEDGMENTS

We gratefully acknowledge the volunteers who participated in the study. The high-oleic peanuts were generously supplied by the Department of Agronomy, University of Florida (Gainesville, FL). The study was supported in part by a grant from the American Dietetic Association's Sports, Cardiovascular, and Wellness Nutritionists Practice Group and by special funding from the Institute of Food and Agricultural Sciences, University of Florida. University of Florida Agricultural Experiment Station Journal Series #R-05504.

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[Received January 2, 1997, and in final revised form and accepted December 17, 1997]

A Comparison of Lycopene and Canthaxanthin Absorption: Using the Rat to Study the Absorption of Non-Provitamin A Carotenoids

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ABSTRACT: The purpose of this study was to validate the use of the mesenteric lymph duct cannulated rat to study the absorption of carotenoids which do not have provitamin A activity. The absorption of two carotenoids, a hydrocarbon carotenoid (lycopene) and a xanthophyll carotenoid (canthaxanthin), were investigated. In the first experiment, lipid emulsions containing lycopene (LYC) or canthaxanthin (CTX) were continuously infused into the duodenum, and lymph was collected for analysis at 2-h intervals. The time course for absorption of carotenoids and triacylglycerol (TAG) was similar. Carotenoids and TAG reached steady-state concentrations in the lymph by 6 h. There was no evidence for a delayed release of either carotenoid from the intestine relative to TAG. During a second experiment, emulsions containing increasing concentrations of LYC or CTX (5, 10, 15, 20 μmol/L) were infused. The LYC and CTX in the lymph increased in a dose-dependent manner. The average efficiency of CTX absorption was 16% while the efficiency of LYC absorption averaged only 6%. Efficiency of carotenoid absorption was not related to concentration infused. Finally, to test whether LYC and CTX interact during absorption both were added to a lipid emulsion at equal concentrations (20 μmol/L) and infused. The carotenoids did not significantly affect each other's absorption. These results demonstrate the usefulness of the rat as an animal model to study the absorption of non-provitamin A carotenoids.

Lipids 33, 159–163 (1998).

Recently much attention has been given to the possible role of carotenoids in the prevention and treatment of a wide variety of illnesses such as heart disease, cancer, and those of aging such as macular degeneration and cataract formation (1–3). Because there are differences in the metabolism, transport, and tissue distribution of individual carotenoids, specific carotenoids may be associated with different diseases. For example, lutein and zeaxanthin are found in the macula of the eye and may be important in prevention of age-related macular degeneration, while lycopene accumulates in the prostate and has been associated with reduced risk of prostate cancer (1). Because of the potential health effects of specific carotenoids, many without provitamin A activity, we were interested in the use of the mesenteric lymph duct cannulated rat as an animal model to study the absorption of non-provitamin A carotenoids. The lymph duct cannulated rat has been a useful animal model for studies of lipid absorption and in the past has been used in studies of β-carotene absorption and carotenoid conversion to vitamin A (4,5). Because the rat more efficiently cleaves β-carotene during absorption than does the human, it may not be an appropriate animal model for studies of carotenoids with provitamin A activity; however, it should be a useful model to study carotenoids that are absorbed intact.

The present work contains three experiments utilizing lycopene (LYC) and canthaxanthin (CTX), two carotenoids without provitamin A activity. The objective of the first experiment was to establish the time course for absorption of carotenoids to determine if the release of carotenoid and triacylglycerol (TAG) from the enterocyte is similar. In studies with humans, the time to maximum plasma concentration of carotenoid is slower than for TAG when both are consumed together, suggesting possible discrimination during absorption (6–8).

The objective of the second experiment was to compare the efficiency of absorption of a hydrocarbon carotenoid (LYC) and a xanthophyll (CTX). Chemically, the carotenoids can be classified as carotenes (containing carbon and hydrogen only) or xanthophylls (containing oxygen in addition to carbon and hydrogen). From limited data, it appears that the more polar xanthophylls may be handled differently during absorption than the hydrocarbon carotenoids (9,10).

The objective of the final experiment was to determine if LYC and CTX affect each other's absorption. Because both carotenoids are lipid-soluble, the potential to interact in the lumen of the digestive tract or during absorption is great. There are reports that suggest carotenoids interact during absorption (11–14).

MATERIALS AND METHODS

Animals and surgical procedure. Male Holtzman albino rats obtained from Harlan Sprague Dawley (Indianapolis, IN)

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Abbreviations: CTX, canthaxanthin; HPLC, high-pressure liquid chromatography; LYC, lycopene; TAG; triacylglycerol.

were used in this study. At the time of surgery the rats weighed 350–400 g and were consuming a standard laboratory diet: Purina Rodent Chow 5001 (Purina Mills, St. Louis, MO) and water *ad libitum*. The animal protocol was approved by The University of Connecticut Institutional Animal Care and Use Committee.

A feeding tube was placed into the duodenum, and the major mesenteric lymph duct was cannulated as previously described (15). Immediately following surgery, the rats were placed in a warm dark environment and allowed to recover approximately 36 h. The rats had access to water and received a glucose/electrolyte solution (Pedialyte, Ross Laboratories, Columbus, OH) at 2.5 mL/h during the recovery period *via* the duodenal feeding tube.

Preparation of carotenoid stock solutions. LYC was purified from tomato paste on a 5% water-weakened alumina column eluted with hexane/ethyl acetate (95:5, vol/vol). Solvent was removed under reduced pressure and a stock solution prepared in dichloromethane. A CTX stock solution (Sigma Chemical, St. Louis, MO) also was prepared with dichloromethane. The concentration of carotenoid in each stock solution was determined by absorption spectroscopy. CTX concentration was estimated using the extinction coefficient E at 466 nm ($E^{1\%}_{1\ cm}$ = 2200), and LYC concentration was estimated at 472 nm ($E^{1\%}_{1\ cm}$ = 3450) (16). Purity of CTX and LYC was verified by spectral characteristics and high-pressure liquid chromatography (HPLC).

Treatment emulsions. The basic emulsion consisted of a buffer solution (115 mmol/L NaCl, 5.0 mmol/L KCl, 6.8 mmol/L Na_2HPO_4, and 16.5 mmol/L NaH_2PO_4), 10 mmol/L sodium taurocholate (Sigma Chemical), and 1.5% (wt/vol) olive oil with different concentrations of CTX or LYC.

To prepare treatment emulsions, olive oil was placed in a round-bottomed flask, and an appropriate amount of carotenoid stock solution was added to the oil. The carotenoid, either CTX or LYC, and olive oil were mixed and the solvent removed with a stream of nitrogen. Sodium taurocholate and buffer were added to the lipid–carotenoid mixture. The contents of the flask then were emulsified using a probe sonicator producing approximately 40 watts output for 15 s, repeated four or five times until no lipid droplets were observed (Branson Sonifier, Model 185; Branson Sonic Power, Danbury, CT). An aliquot of the emulsion was extracted three times with hexane and the final concentration of carotenoid in the emulsion determined by HPLC as described below for lymph analysis.

Experiment 1. The time course for absorption of each carotenoid was determined using three rats per carotenoid. After a recovery period of 36 h, rats were infused through the feeding tube (2.5 mL/h) with an emulsion containing 20 μmol/L of either CTX or LYC. Lymph samples were collected at 2, 4, 6, 8, 10, and 12 h after the start of the infusion.

Experiment 2. To compare the absorption of a hydrocarbon carotenoid and a xanthophyll, emulsions with four differing concentrations of LYC or CTX (5, 10, 15, 20 μmol/L) were prepared. A total of 24 animals, three rats per treatment, were intraduodenally infused at 2.5 mL/h for 12 h. Based on the results of Experiment 1, a sample of lymph collected from 6 to 12 h of infusion was used for analysis. The carotenoid in these samples therefore represents absorption under steady-state conditions.

The results of Experiment 2 were statistically analyzed as a 2×4 factorial experiment, the first factor being carotenoid type and the second factor being carotenoid concentration. Because the four concentrations of carotenoid infused were equally spaced, the linear, quadratic, and cubic relationships between amount of carotenoid infused and absorption into the lymph were statistically tested (see Reference 17 for description).

The efficiency of absorption for each carotenoid was calculated by dividing the concentration of carotenoid recovered in the lymph per hour by the concentration of carotenoid infused into the duodenum per hour.

Experiment 3. To investigate the possible interaction of LYC and CTX during absorption, the recovery of carotenoid in the lymph from three treatment emulsions was compared. Data from LYC (20 μmol/L) and CTX (20 μmol/L) treatments collected in Experiment 2 plus one additional animal per treatment were used as a measure of absorption of each carotenoid by itself. Four additional animals were infused with an emulsion containing both LYC (20 μmol/L) and CTX (20 μmol/L). The infusion of treatment emulsions and collection of lymph were the same as described for Experiment 2. The results for each carotenoid when infused alone were compared to those when they were infused together. Differences were tested for significance using the Student's *t*-test.

Carotenoid and TAG analysis of lymph. After collection, lymph was stored at −70°C until analyzed. On the day of analysis, lymph samples were thawed to room temperature and total lipid was extracted from the lymph by the method of Folch *et al.* (18). To correct for recoveries of carotenoids during extraction, ethyl β-apo-8′-carotenoate (Fluka, Ronkonkoma, NY) was added to the lymph as an internal standard. Carotenoids were analyzed by HPLC using a Waters C18 Resolve column (15 cm × 3.9 mm; Millipore, Milford, MA) protected with an Upchurch C18 guard column (Upchurch Scientific, Oak Harbor, WA) and an isocratic mobile phase consisting of acetonitrile/dichloromethane/methanol/*n*-butanol/ammonium acetate (90:15:10:0.1:0.1, by vol) (19). Carotenoids and ethyl β-apo-8′-carotenate were identified and quantified at a wavelength of 450 nm.

Lymph samples were diluted 1:10 with deionized water and TAG determined by an enzymatic assay (Sigma Chemical Co.).

RESULTS AND DISCUSSION

Carotenoid absorption involves a series of interdependent steps that include release of carotenoid from the food matrix, solubilization into mixed bile salt micelles, transfer into the intestinal mucosal cell, and incorporation into lymphatic lipoproteins (20–23). While *in vitro* methods may be used to

study individual steps in this process, investigation of the overall process of carotenoid absorption requires the use of a whole animal system. Unfortunately, animal models that perfectly mimic human carotenoid metabolism are not available. Nevertheless, animal models must be used because only a few carotenoids have been approved for formulation into products for ingestion by humans, and methods for direct measurement of carotenoid absorption in humans are not available. Data presented here utilize the mesenteric lymph duct cannulated rat, an animal model used extensively to study the digestion and absorption of lipids and a potential animal model for investigation of factors that influence carotenoid absorption.

In the current study, we chose to investigate LYC and CTX absorption because previous work has shown these carotenoids to be absorbed intact and stored in various tissues by the rat (24). The dose of carotenoid provided to the rats in the present study was approximately 0.2–1 mg/kg body weight. This is in the same range as human intervention studies with β-carotene which provided doses of 0.2–4.3 mg/kg body weight (25).

The results of Experiment 1 are shown in Figure 1. The appearance of LYC and CTX in the lymph coincided with that of TAG. The concentrations of carotenoids and TAG reached a plateau or steady-state transport in the lymph by 6 h of continuous intraduodenal infusion of the carotenoid emulsions.

The time course for appearance of carotenoids in the lymph does not support the notion that there is a delayed release of carotenoids from the intestine relative to other dietary lipids. In human studies a delayed release of carotenoid by the enterocyte has been suggested because the time to maximum plasma concentration after an oral dose of a carotenoid is slower than the time needed to reach maximum plasma concentration of TAG or retinyl esters (6,7). This effect in humans can be explained by clearance of chylomicra by the liver and reintroduction of carotenoids into very low density lipoproteins during normal lipoprotein metabolism and is not the result of discrimination between carotenoids and other dietary lipids during absorption. This conclusion is supported by time-course studies in which individual lipoprotein classes have been separated. In these studies, carotenoid in the chylomicron fraction was reported to reach a maximum concentration about the same time, by 6 h, as maximum concentration of chylomicron TAG, while carotenoids associated with low density lipoproteins and high density lipoproteins reach a maximum later at 16 to 24 h (8,26).

In the second experiment, the recoveries of CTX and LYC in the lymph were compared (Fig. 2). CTX was significantly ($P < 0.01$) better absorbed than LYC. Both carotenoids were absorbed in a concentration-dependent manner. There was a significant ($P < 0.05$) linear relationship, for both carotenoids, between the amount of carotenoid infused and the amount recovered in the lymph. The LYC response, as seen in Figure 2, appears to have some curvature, but this was not statistically significant and was probably due to normal variation.

The average efficiency of absorption for CTX was approximately 16% with a range from individual samples of 10 to 20%. The recovery of CTX in the lymph for the lower concentration intraduodenally infused averaged 18.5% and for the highest concentration was 14.4%; however, these values were not statistically different.

The efficiency of CTX absorption in the current study is approximately the same as reported for β-carotene absorption

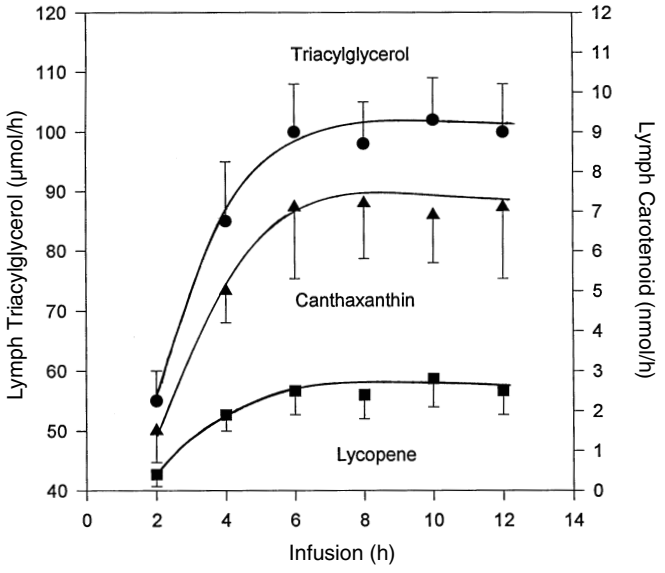

FIG. 1. Time course for absorption of canthaxanthin, lycopene, and triacylglycerol in the mesenteric lymph. Lipid emulsions containing 20 μmol/L of carotenoid were continuously infused (2.5 mL/h) into the duodenum. Shown are the mean ± SD of data collected from three rats per carotenoid.

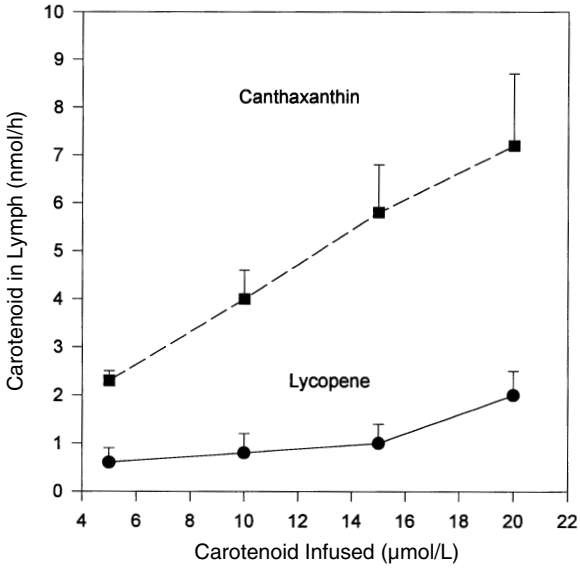

FIG. 2. Relationship between concentration of carotenoid continuously intraduodenally infused at the rate of 2.5 mL/h and concentration of carotenoid recovered per hour in the mesenteric lymph. The lymph samples were collected from 6 to 12 h after the initiation of infusion. These samples represent absorption under steady-state conditions. Each point is the mean ± SD from three individual rats. For both carotenoids, there was a significant ($P < 0.05$) linear relationship between the amount of carotenoid infused and the amount absorbed.

in the human. Using hospitalized patients with cannulated thoracic ducts, Goodman *et al.* (27) presented data suggesting that 10% of an oral dose of radioactive β-carotene was absorbed into lymph, while Blomstrand and Werner (28) presented data from a similar study suggesting that 23% of a similar dose was absorbed into lymph. Recently using stable isotope β-carotene-d_8, Novotny *et al.* (29) suggest that 22% of an oral dose of β-carotene in olive oil was absorbed in humans.

Under the conditions of the current study, LYC was absorbed poorly compared to CTX. The efficiency of LYC absorption ranged from 2 to 8% with an average recovery of LYC in the lymph of only 6%. The efficiency of absorption was not significantly affected by concentrations of LYC infused. A study comparing absorption of five carotenoids in preruminant calves noted poor LYC absorption compared to other carotenoids (30). Supplementation studies with humans would also suggest LYC is not efficiently absorbed (31,32).

In the final study, equal amounts of CTX and LYC were infused into the duodenum to determine if LYC and CTX affect each other's absorption. Based on indirect measures of absorption, there are several studies that suggest carotenoids interact during absorption. Almost 50 yr ago, by using liver vitamin A stores as a measure of β-carotene absorption, a xanthophyll (lutein) was reported to interfere with the absorption of β-carotene (33). In a more recent study with domestic ferrets, a negative interactive effect of β-carotene and either CTX or LYC on serum and tissue accumulation of β-carotene was observed (11). On the basis of kinetic analysis of serum carotenoids, two human studies also provide data that suggest carotenoids interact during absorption (12,13). White *et al.* (12) report that β-carotene appeared to inhibit intestinal absorption of CTX. In a similar human study, when a combined oral dose of β-carotene and lutein was administered, β-carotene reduced plasma lutein response by 40% (13).

As seen in Figure 3, LYC and CTX did not significantly affect each other's absorption. Lycopene in the lymph when infused alone was 2.0 ± 0.5 nmol/h compared to 1.6 ± 0.6 nmol/h when infused in equal molar amounts with CTX. When CTX was provided by itself, lymphatic CTX was 7.4 ± 1.6 nmol/h compared to 8.8 ± 1.9 nmol/h when infused simultaneously with LYC. The lack of significant interaction during absorption between these carotenoids may be a result of our choice of carotenoids. Because LYC was so poorly absorbed, its potential interaction with CTX may be limited. Also the relative concentrations of CTX and LYC used in the current study may not have been optimal for interactions. Using liver vitamin A stores as an index of β-carotene absorption, High and Day (34) reported that large amounts of lutein decrease β-carotene absorption, but small amounts had the opposite effect. Further studies are needed to investigate potential interactions during absorption using a wider range of concentrations and variety of carotenoids.

In conclusion, LYC and CTX were absorbed intact by the rat and appear in the lymph following a similar time course as other dietary lipids. Both carotenoids were absorbed in a dose-dependent manner with the xanthophyll (CTX) more efficiently absorbed than the nonpolar hydrocarbon carotenoid (LYC). When both carotenoids were infused together, LYC and CTX did not significantly affect each other's absorption. The results of this study demonstrate the usefulness of the rat as an animal model to study the absorption of non-provitamin A carotenoids. To our knowledge this is the first report using the mesenteric lymph duct cannulated rat to study the absorption of carotenoids other than β-carotene.

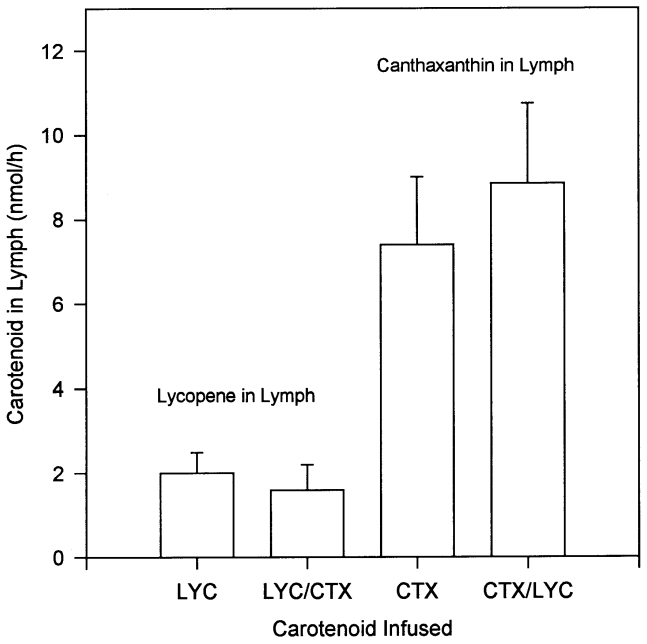

FIG. 3. A comparison of lymphatic lycopene (LYC) and canthaxanthin (CTX) when intraduodenally infused (2.5 mL/h) individually or together. LYC treatment contained 20 μmol/L lycopene; CTX treatment contained 20 μmol/L canthaxanthin; LYC/CTX and CTC/LYC treatments contained 20 μmol/L lycopene plus 20 μmol/L canthaxanthin. Shown are the mean ± SD (n = 4). CTX and LYC did not significantly affect each other's absorption.

ACKNOWLEDGMENTS

Supported in part by federal funds available through provisions of the Hatch Act and USDA Competitive Grant 92-37200-7703. This work constitutes Scientific Contribution No. 1762 of the Storrs Agricultural Experiment Station, The University of Connecticut (Storrs, CT).

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[Received August 1, 1997, and in final revised form December 19, 1997; revision accepted December 29, 1997]

Hepatic Cholesterol Metabolism in Experimental Nephrotic Syndrome

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ABSTRACT: Hypercholesterolemia is a consistent feature of the nephrotic syndrome. However, the mechanisms underlying this perturbation are unclear. In the present work, we have investigated different factors that influence hepatic cholesterol metabolism using the nephrotic rat as a model. The induction of nephrosis resulted in a severe and sustained hypercholesterolemia. However, no effect on the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl CoA reductase, could be detected. Further, plasma lathosterol/cholesterol ratio, a measure of cholesterol synthesis, was not altered. Also, plasma levels of mevalonate, both a substrate for cholesterogenesis beyond the rate-limiting step and a marker for cholesterol synthesis, did not differ between control rats and those with established hypercholesterolemia. There was no detectable change in the expression of low density lipoprotein (LDL) receptor between the two experimental groups. We conclude that the early increase in cholesterol synthesis reported after the induction of nephrosis is not necessary for the maintenance of hypercholesterolemia. Established hypercholesterolemia of the nephrotic syndrome seems to represent a steady state in which neither enhanced hepatic cholesterol synthesis nor retarded LDL cholesterol clearance is of major importance.
Lipids 33, 165–169 (1998).

Hypercholesterolemia with increased plasma and LDL cholesterol levels is a major lipid abnormality in the nephrotic syndrome (1–3). As a consequence, this condition carries a risk for accelerated atherosclerosis and thereby coronary heart disease and progressive renal damage (4–7). The mechanisms underlying hypercholesterolemia of the nephrotic syndrome are complex, which explains the large and partially conflicting body of data reported. While early studies demonstrated a decrease in the incorporation of ^{14}C-labeled acetate into hepatic cholesterol in nephrotic rats suggesting a curtailment of cholesterogenesis (8), cholesterol synthesis as determined by the incorporation of ^3H_2O into cholesterol was later found to be increased in liver slices from nephrotic rats compared to controls (9). Also, *in vivo* studies in nephrotic rats have shown an increased incorporation of ^3H_2O into hepatic lipids (10). The increase in cholesterol biosynthesis in the nephrotic state has been ascribed to an increase in the activity of the rate-limiting enzyme in this pathway, namely 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, and also to a disturbed mevalonate metabolism leading to increased substrate availability for cholesterol synthesis (11,12). However, in a study by Thabet and co-workers (13), neither HMG-CoA reductase activity nor the activity of the rate-limiting enzyme in cholesterol degradation, cholesterol 7α-hydroxylase, was altered in nephrotic rats. These findings argued against a marked alteration of hepatic cholesterol synthesis or degradation as a cause of hypercholesterolemia of the nephrotic syndrome. Alternatively, decreased removal of plasma cholesterol was suggested (13). However, apo-B kinetic measurements in puromycin aminonucleoside (PAN)-treated rats demonstrated a higher low density lipoprotein (LDL) synthetic rate in nephrotic rats relative to controls, while the fractional catabolic rate was only marginally affected (14).

In view of these conflicting data, we have in the present study examined different markers of cholesterol synthesis and catabolism in PAN-induced nephrotic syndrome, including the activity of HMG-CoA reductase; plasma levels of lathosterol, an indicator of cholesterol synthesis (15); plasma levels of mevalonate, which is both an indicator of (16) and a substrate for cholesterogenesis; and the expression of hepatic LDL receptor, which is responsible for the catabolism of cholesterol-rich plasma lipoproteins (17).

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats weighing 200–250 g were housed in metal cages and maintained on a 12-h light–dark cycle. The animals were allowed to move freely in the cages and had free access to standard chow and water throughout the study.

Nephrosis was induced by a single intraperitoneal injection of PAN (Sigma Chemical Co., St. Louis, MO) in a dose of 100 mg/kg body weight. One week after the injection, rats

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Abbreviations: GFR, glomerular filtration rate; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LDL, low density lipoprotein; PAN, puromycin aminonucleoside.

were transferred to individual metabolic cages, and urine was collected for 24 h. Plasma and urine albumin concentrations were measured by an immunonephelometric method using rat albumin antibodies (Nordic Immunology, Tilburg, The Netherlands). Nephrosis was confirmed by the presence of hyperlipidemia, low plasma albumin, and urine albumin loss exceeding 250 mg albumin/24 h. The rats were killed 11–12 d after PAN injection. On the day of sacrifice, blood sampling was performed by open heart puncture under neuroleptic anesthesia (Hypnorm®; Janssen Pharmaceutica, Beerse, Belgium) aided by a muscle relaxant (Stesolid®; Dumex Ltd., Copenhagen, Denmark).

Analytical procedures. Cholesterol levels were determined in plasma and liver using a commercially available enzymatic kit (Boehringer Mannheim, Mannheim, Germany). Liver samples were homogenized with 4 vol of 10 mmol/L Tris/HCl, pH 7.4 containing 0.25 mol/L sucrose, 1 mmol/L EDTA and 0.2 mmol/L dithiothreitol (Buffer A). The homogenate was then treated with 20 vol of chloroform/methanol (2:1, vol/vol) and cholesterol measured in the resulting lipid extract.

Lathosterol was measured by isotope dilution–mass spectrometry. Plasma samples were treated with chloroform/methanol (2:1) and the extract was subjected to C_{18} column chromatography (Isolute MF C_{18}; International Sorbent Technology, United Kingdom). Unesterified lathosterol was eluted with methanol, derivatized into trimethylsilyl ether and analyzed by gas chromatography/mass spectrometry using $^2H_3$ lathosterol as internal standard (18).

Mevalonate levels were determined in plasma and urine by isotope dilution–mass spectrometry, essentially as described (19). In brief, samples were acidified to pH 3.5 using phosphoric acid, proteins were precipitated with acetone, and lipids removed by an extraction with cyclohexane. Mevalonolactone was extracted into ethylacetate/acetone (2:1, vol/vol) using a salt-out effect by saturing the aqueous phase with NaCl. Mevalonolactone was converted to mevalonic acid by adding 0.5 mol/L NaOH. After derivatization with methyl-tertiary-butyl-dimethylsilyl-trifluoracetamide, the derivative was extracted into *n*-decane and measured by gas–liquid chromatography/mass spectrometry using $^2H_3$-MVA as internal standard.

For the assay of the HMG-CoA reductase activity, liver homogenates were centrifuged at 20,000 × *g* for 15 min and supernatants recentrifuged at 100,000 × *g* for 60 min. Prior to use, the microsomal pellets obtained were suspended in Buffer A to a protein concentration of about 40 mg/mL and stored at −70°C. Microsomal fractions were thawed and diluted in 20 mmol/L imidazol/HCl (pH 7.4) containing 10 mmol/L DTT, and HMG-CoA reductase activity was measured as the conversion of [3-^{14}C] labeled HMG-CoA (NEN DuPont, Boston, MA) to mevalonate, essentially as described by Brown *et al.* (20). Tritium-labeled mevalonate was used as internal standard. The reaction product was purified by thin-layer chromatography and analyzed for radioactivity.

LDL receptor expression was measured by a ligand blot assay. Hepatic membranes were prepared as described in detail elsewhere (21). In brief, frozen liver samples were homogenized with a polytron in a buffer (50 mmol/L Tris-HCl, pH 7.5, 2 mmol/L $CaCl_2$, 0.5% Triton x-100 supplemented with 1 mmol/L leupeptin, 1 mmol/L phenanthroline, and 1 mmol/L phenylmethyl sulfonyl fluoride). After quick sonication, the homogenates were ultracentrifuged for 10 min and the supernatants collected and frozen in multiple aliquots at −70°C. Membrane proteins and molecular weight standards were separated by SDS polyacrylamide gels (6%) and electrotransferred to nitrocellulose filters. Filters were incubated with ^{125}I-labeled rabbit β-very low density lipoprotein (VLDL) and subjected to autoradiography on x-ray film for 3 h at −70°C after aligning molecular weight standard lanes which had been cut out and visualized by protein staining. The 120 kD bands corresponding to the receptor protein were quantified in arbitrary units after background subtraction using a Bio-imaging analyzer (Fujix BAS 2000; Fuji Photo Film Co., Tokyo, Japan).

Glomerular filtration rate (GFR) was determined as the endogenous creatinine clearance (mL/min) using the formula: $GFR = C_u \times V_u/C_p$ where C_u is urine creatinine concentration, C_p is plasma creatinine concentration, and V_u is urine flow (mL/min) (22).

Statistical analysis. Data were evaluated by Student's *t*-test with the level of statistical significance set at $P < 0.05$.

RESULTS

Treatment of rats with PAN resulted in albuminuria (>250 mg/24 h), hypoalbuminemia, and a mixed hyperlipidemia with elevations of plasma levels of cholesterol (6-fold) and triglycerides (20-fold) (Table 1).

Nephrotic rats displayed a 5-fold increase in plasma levels of lathosterol, a steroid precursor to cholesterol that has been suggested to reflect the rate of hepatic cholesterol synthesis (15,23,24). However, when expressed relative to plasma cholesterol levels, no difference between normal and nephrotic rats could be disclosed (Table 2). Further, no difference in the activity of the rate-limiting enzyme in cholesterol biosynthesis (HMG-CoA reductase) could be detected between nephrotic animals and normal controls (Table 2).

As shown in Table 3, neither plasma nor urine levels of mevalonate were altered in nephrotic rats. Also, the induction

TABLE 1
The Effect of Nephrosis on Plasma Levels of Albumin, Cholesterol and Triglycerides as Well as on Urinary Albumin Excretion[a]

	Control rats	Nephrotic rats	*P*-value
Plasma albumin (g/L)	21.3 ± 0.3[a]	4.7 ± 0.5[b]	<0.001
Urinary albumin excretion (mg/24 h)	1.8 ± 0.4[c]	322.2 ± 9.2[d]	<0.001
Plasma cholesterol (mmol/L)	2.2 ± 0.1[e]	13.1 ± 0.8[a]	<0.001
Plasma triglycerides (mmol/L)	1.0 ± 0.04[e]	20.2 ± 3.2[a]	<0.001

[a]Values are means ± SEM. [a](n = 13), [b](n = 18), [c](n = 5), [d](n = 15), [e](n = 11). Samples were collected on day 11–12 after puromycin aminonucleoside injection.

TABLE 2
The Effect of Nephrosis on Metabolic Markers of Cholesterol Metabolism[a]

	Control rats	Nephrotic rats	*P*-value
HMG-CoA reductase activity (pmol/min·mg protein)	414 ± 49[a]	329 ± 45[b]	N.S
Plasma lathosterol (μmol/L)	1.93 ± 0.33[c]	10.18 ± 0.95[d]	<0.0001
Plasma lathosterol: cholesterol ratio (μmol/mmol)	0.81 ± 0.11[c]	0.84 ± 0.23[d]	N.S
Liver cholesterol (μmol/g liver)	7.9 ± 0.3[e]	8.6 ± 0.3[f]	N.S

[a]Values are means ± SEM . N.S, not significant, $P > 0.05$. [a]($n = 12$), [b]($n = 14$), [c]($n = 11$), [d]($n = 13$), [e]($n = 16$), [f]($n = 20$). Samples were collected on day 11–12 after puromycin aminonucleoside injection.

of nephrosis did not affect the GFR, as reflected by essentially unchanged plasma creatinine levels (Table 3).

In order to examine whether retarded plasma cholesterol clearance might contribute to the hypercholesterolemia of nephrotic syndrome, a major determinant of plasma cholesterol clearance, namely the LDL receptor expression, was measured. As shown in Figure 1, no significant difference between nephrotics and normals was observed. This is in accordance with the finding that hepatic cholesterol levels were similar in the two experimental groups (Table 2).

DISCUSSION

In the present study, a number of key metabolic markers for hepatic cholesterol metabolism were examined and found to be unaltered in rats with PAN-induced nephrotic syndrome, despite a pronounced hypercholesterolemia. Hypercholesterolemia can arise from increased hepatic production and secretion of cholesterol-rich lipoproteins, decreased removal of cholesterol from the circulation, or a combination of both factors. An overproduction of lipoproteins has been demonstrated in perfused livers from nephrotic rats (25). Furthermore, these lipoproteins had a higher content of cholesterol (26), implying enhanced cholesterol synthesis. These findings are in accord with earlier *in vivo* and *in vitro* studies that showed an increased incorporation of ^3H_2O into hepatic cholesterol (9,10). However, the mechanisms underlying this effect have been a matter of debate.

It is well-established that the HMG-CoA reductase is rate-limiting in cholesterol biosynthesis under most experimental conditions (20). The activity of this enzyme has therefore been considered a good index of the absolute rate of hepatic cholesterol synthesis (27). As shown in Table 2, HMG-CoA reductase activity was not significantly different between nephrotic rats and normal controls, well in line with the results of Thabet *et al*. (13). The plasma concentration of the steroid cholesterol precursor lathosterol has been reported to be a good indicator of cholesterol synthesis (15,23,24). As shown in Table 2, plasma lathosterol levels were 5-fold higher in nephrotic rats compared to controls. However, since lathosterol is carried with plasma lipoproteins, changes in the absolute concentrations of lathosterol might merely reflect changes in the number of lipoprotein particles. In order to cor-

	Control rats			Nephrotic rats		
Lane	1	2	3	4	5	6
Protein loaded (μg)	200	150	100	200	150	100
Receptor expression (arb. units)	253	189	128	229	183	123

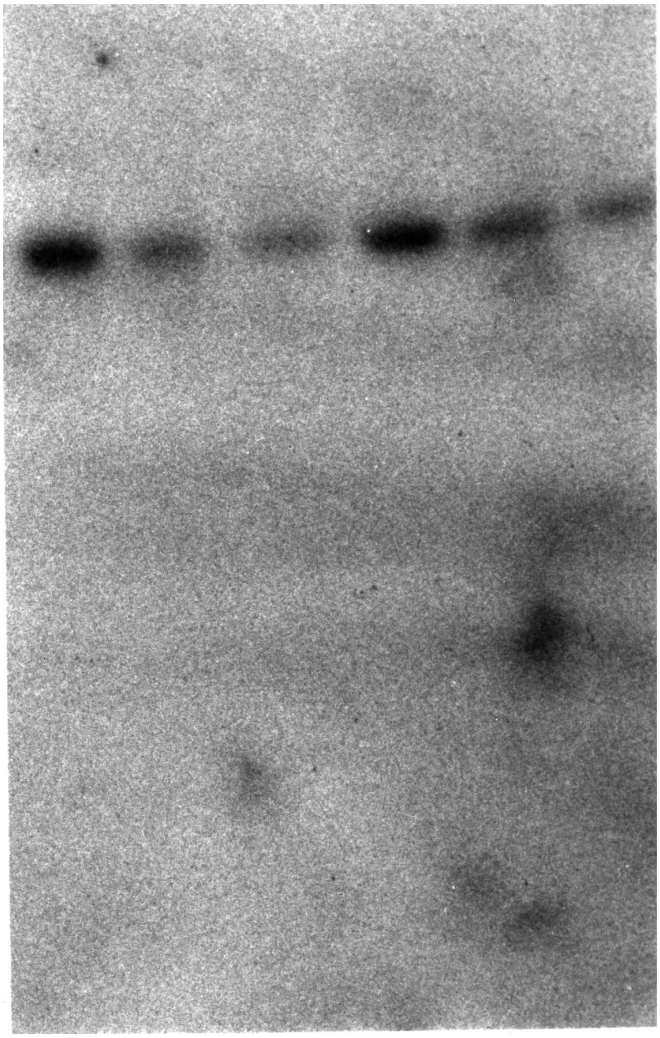

FIG. 1. Ligand blot of hepatic low density lipoprotein receptor from nephrotic rats and normal controls, prepared as described in detail in the Experimental Procedures section. The 120 kD bands corresponding to the receptor protein were quantified in arbitrary units after background subtraction using a bio-imaging analyzer.

TABLE 3
The Effect of Nephrosis on Plasma Mevalonate Levels, Urinary Mevalonate Excretion and Glomerular Filtration[a]

	Control rats $n = 6$	Nephrotic rats $n = 6$
Plasma mevalonate levels (ng/mL)	11.9 ± 1.7	12.2 ± 1.1
Urinary mevalonate excretion (μg/24 h)	16.5 ± 3.0	16.8 ± 3.4
Glomerular filtration rate (mL/min)	1.1 ± 0.1	0.9 ± 0.1
Plasma creatinine (μmol/L)	48 ± 1	55 ± 2

[a]Values are means ± SEM. None of the parameters given was significantly different between the two experimental groups, $P > 0.05$.

rect for this, plasma lathosterol levels were expressed relative to cholesterol. Indeed, the lathosterol/cholesterol ratio has been shown to correlate better with the overall rate of cholesterol biosynthesis than the absolute levels of lathosterol in plasma (15,24). This ratio was not significantly different between normal and nephrotic rats (Table 2). Interestingly, similar results were obtained in patients with glomerular proteinuria (28).

Of interest is the report that hepatic HMG-CoA reductase mRNA and HMG-CoA reductase activity showed a transient increase after the induction of nephrosis in rats by PAN (29). However, both subsequently reverted to normal despite a persistent hypercholesterolemia. These data suggest that an effect on the reductase might play a role in initiating, though not in maintaining, a hypercholesterolemic state.

It has been demonstrated that oxidation, excretion, and removal of mevalonate are decreased in isolated perfused kidneys from nephrotic rats compared to controls (12). Also, livers from nephrotic rats have been shown to synthesize increased amounts of cholesterol from exogenous ^{14}C-labeled mevalonate (11). These data suggested that an impaired mevalonate metabolism in nephrosis would increase the delivery of this substrate to the liver and thereby stimulate cholesterogenesis (11). However, as shown in Table 3, plasma mevalonate levels were unaltered in nephrotic rats. Further, the renal excretion of mevalonate was not different between the two experimental groups. These data do not support the hypothesis that the availability of mevalonate for cholesterol synthesis in the liver is increased in the nephrotic state. The increase of cholesterol synthesis from exogenous mevalonate (11) might reflect a general increase in the liver capacity for lipid synthesis (30). Our findings on mevalonate in nephrotic rats are in contrast with those in mice with chronic renal failure where hypercholesterolemia was associated with reduced urinary excretion of mevalonate and elevated plasma levels of this precursor (31). It is noteworthy that in our study the nephrotic rats had normal GFR measured as creatinine clearance (Table 3). The absence of renal failure in these animals may explain why mevalonate retention did not occur. Altogether, our findings on plasma lathosterol/cholesterol ratio along with those on mevalonate do not indicate enhanced cholesterol biosynthesis in established nephrotic hypercholesterolemia.

As shown in Figure 1, the expression of LDL receptor binding activity was largely unaffected in nephrotic rats compared to controls. Further, the activity of HMG-CoA reductase was not affected by the nephrotic state (Table 2). It has been shown that hepatic mRNA levels for LDL receptor and HMG-CoA reductase seem to be coordinately regulated (32). Thus, the findings on HMG-CoA reductase activity and LDL receptor expression were in accord. Further, the lack of effect of nephrosis on the LDL receptor binding activity is well in line with the observations of Joven *et al*. (14), who found no significant effect of nephrosis on the fractional catabolic rate of LDL in PAN-treated rats. In a recent study, LDL receptor expression was found to be decreased in nephrotic rats 30 d after the induction of nephrosis (33). However, no significant changes in the receptor expression were seen at earlier time points despite a pronounced hypercholesterolemia. These findings, together with our results, suggest that down-regulation of the LDL receptor does not occur early after the induction of nephrosis but may be a secondary phenomenon in the course of PAN-induced nephrotic syndrome.

The data obtained in our study, together with those reported earlier, suggest that the mechanisms that mediate the initiation of hyperlipidemia of the nephrotic syndrome are different from those operative when the hyperlipidemia becomes established and reaches a steady state. It is plausible that the hypoalbuminemia characteristic of the nephrotic syndrome or some other related factor initially increases the availability of apolipoproteins with concomitant increases in hepatic synthesis of cholesterol and triglycerides leading to hyperlipoproteinemia (25,26,34,35). When a new steady state is reached, there will be an increased influx of cholesterol into the liver, largely determined by a concentration-dependent uptake that does not necessarily involve the LDL receptor. An increased uptake would down-regulate hepatic cholesterol synthesis (17) whereby the synthetic rate would revert to normal. However, since the lipoprotein output is increased (25,26), the flux of cholesterol from the liver is maintained at a high level. This would explain the findings on hepatic cholesterol levels, and hepatic cholesterol synthesis, as well as hepatic LDL receptor expression (Table 2, Fig. 1).

To summarize, using several independent metabolic parameters, we found no evidence for increased hepatic cholesterol synthesis in established nephrotic hypercholesterolemia. Further, a major determinant of LDL cholesterol clearance, namely the LDL receptor expression, did not appear to be altered in this condition. Taking into account previously published data that support enhanced cholesterol synthesis early in the course of the disease, our results suggest that a sustained increase in cholesterogenesis is not a prerequisite for hypercholesterolemia which is maintained by other mechanisms. Rather, it appears that the mechanisms that mediate the initiation of hypercholesterolemia are not necessary for the maintenance of this condition. Altogether, the results obtained suggest that established hyperlipidemia of the nephrotic syndrome constitutes a steady state in which neither enhanced hepatic cholesterol synthesis nor retarded LDL cholesterol clearance is of major importance.

ACKNOWLEDGMENTS

This work was supported by The Swedish Medical Research Council (Project 10349) and Karolinska Institutet. Dr. Berglund is an Established Scientist of the American Heart Association, New York Affiliate, and a Florence Irving Associate Professor of Medicine. Dr Lindenthal is supported by a grant from Deutsche Forschungsgemeinschaft.

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[Received June 25, 1997, and in final revised form December 2, 1997; revision accepted December 29, 1997]

Eicosapentaenoic and Docosahexaenoic Acids Alter Rat Spleen Leukocyte Fatty Acid Composition and Prostaglandin E_2 Production But Have Different Effects on Lymphocyte Functions and Cell-Mediated Immunity

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ABSTRACT: Weanling rats were fed on high-fat (178 g/kg) diets which contained 4.4 g α-linolenic (ALA), γ-linolenic, arachidonic (ARA), eicosapentaenoic (EPA), or docosahexaenoic acid (DHA)/100 g total fatty acids. The proportions of all other fatty acids, apart from linoleic acid, and the proportion of total polyunsaturated fatty acids (PUFA) (approximately 35 g/100 g total fatty acids) were constant, and the n-6 to n-3 PUFA ratio was maintained as close to 7 as possible. The fatty acid compositions of the serum and of spleen leukocytes were markedly influenced by that of the diet. Prostaglandin E_2 production was enhanced from leukocytes from rats fed the ARA-rich diet and was decreased from leukocytes from the EPA- or DHA-fed rats. Replacing dietary ALA with EPA resulted in diminished *ex vivo* lymphocyte proliferation and natural killer (NK) cell activity and a reduced cell-mediated immune response *in vivo*. In contrast, replacing ALA with DHA reduced *ex vivo* lymphocyte proliferation but did not affect *ex vivo* NK cell activity or the cell-mediated immune response *in vivo*. Replacement of a proportion of linoleic acid with either γ-linolenic acid or ARA did not affect lymphocyte proliferation, NK cell activity, or the cell-mediated immune response. Thus, this study shows that different n-3 PUFA exert different immunomodulatory actions, that EPA exerts more widespread and/or stronger immunomodulatory effects than DHA, that a low level of EPA is sufficient to influence the immune response, and that the immunomodulatory effects of fish oil may be mainly due to EPA.

Lipids 33, 171–180 (1998).

In recent years there has been great interest in the effects of different types of dietary fatty acids upon the immune system. Much of this interest has focused on polyunsaturated fatty acids (PUFA), partly because the n-6 PUFA arachidonic acid (ARA; 20:4n-6) is the precursor of prostaglandins and leukotrienes which have potent pro-inflammatory and immunoregulatory properties (see Refs. 1–3 for reviews). ARA is formed from linoleic acid (18:2n-6), a major component of vegetable oils such as corn, sunflower, soybean, and safflower oils; an intermediate in the synthesis of ARA is γ-linolenic acid (GLA; 18:3n-6). The second reason for the interest in the immunomodulatory effects of fatty acids is that epidemiological studies have found that populations such as Greenland Eskimos, who consume large quantities of fish oil, which is rich in the n-3 PUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), have a very low incidence of inflammatory and autoimmune disorders (4). Furthermore, a number of clinical studies have reported that fish oil supplementation of the human diet has some beneficial effects in acute and chronic inflammatory conditions (e.g., 5–11) and prolongs the survival of grafts (12). The precursor of EPA and DHA is α-linolenic acid (ALA; 18:3n-3), a major component of green plant tissues and of linseed oil. Interest in the potential clinical effects of oils rich in n-6 or n-3 PUFA has given rise to a number of investigations of the effects of fatty acids and dietary oils upon immune cell functions (see 13–19 for reviews).

Feeding laboratory animals (rats, mice, rabbits, chickens) diets rich in n-3 PUFA (canola, linseed, fish oils) results in suppressed *ex vivo* lymphocyte proliferation (20–27), cytotoxic T lymphocyte activity (28–32), and natural killer (NK) cell activity (26,27,30,33–35). Investigations which have directly compared the effects of these oils indicate that fish oil is more suppressive than linseed oil which is, in turn, more suppressive than canola oil (see 14–18 for references). Diets rich in n-6 PUFA appear to be either less suppressive than those containing n-3 PUFA or without effect (see 14–18 for references). Taken together, these *ex vivo* observations indicate that consumption of diets containing n-3 PUFA, particularly diets rich in fish oil, may result in immunosuppression *in vivo*. Indeed, three studies have reported that feeding laboratory rodents fish oil results in diminished *in vivo* graft vs. host and/or host vs. graft responses (36–38); feeding diets rich in n-6 PUFA [safflower oil (SO) or evening primrose oil] did

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Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; Con A, concanavalin A; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; NK, natural killer; PBS, phosphate-buffered saline; PG, prostaglandin; PLN, popliteal lymph node; PUFA, polyunsaturated fatty acid; SO, safflower oil.

not suppress these responses (38). In addition, one study has reported that, compared with sunflower oil feeding, linseed oil reduces the graft vs. host response (27).

Thus, the consumption of diets which contain large amounts of n-3 PUFA clearly causes immunosuppression whereas those rich in n-6 PUFA are less suppressive. However, the oils used in such experiments (e.g., sunflower, safflower, linseed, and fish oils) differ greatly in background fatty acid composition, in total PUFA content, and in n-6/n-3 PUFA ratio. Because both the level of PUFA and the n-6/n-3 PUFA ratio of the diet have significant effects on immune cell function (39), the influences of these two characteristics of the diet on immune cell functions have been difficult to distinguish in previous studies. Furthermore, most previous studies did not strictly control the proportions of dietary fatty acids other than those under study. Palmitic and oleic acids are now known to influence immune cell function (23,35, 40–42), and the proportions of these fatty acids differ greatly among diets previously used [e.g., fish oil contains twice as much palmitic acid as SO (23)]. By using single oils or mixtures of two oils, most previous studies investigated the effects of large amounts of n-3 or n-6 PUFA; the amounts used often could not be achieved in the human diet. As a result, there is little information about how much n-3 PUFA needs to be present to bring about the effects of fish oil. Finally, whether the immunomodulatory effects of fish oil are due to EPA or DHA or to both of these fatty acids is not yet clear. The current study was designed to investigate the immunomodulatory effects of specific PUFA (GLA, ARA, EPA, DHA) fed at the level of 4.4 g/100 g total fatty acids and as components of diets with very similar background fatty acid composition and with a near-constant n-6/n-3 PUFA ratio. A control diet containing approximately 31 g linoleic acid and 4.4 g ALA/100 g total fatty acids was used; the effects of GLA and ARA were examined by replacing a portion of the linoleic acid, whereas the effects of EPA and DHA were examined by replacing ALA. In this way the total PUFA content and the n-6/n-3 PUFA ratio of the diets were kept as constant as possible.

TABLE 1
Proportions of Different Oils Used

	Diet[a]				
Oil	Control	GLA	ARA	EPA	DHA
Canola	28.7	31.0	31.5		
High-oleic acid sunflower seed				12.3	16.3
Sunflower seed	16.2		9.8	43.4	43.8
Soybean	23.4	19.9	19.7		
Fractionated palm	31.8	29.8	28.0	31.1	29.2
Borage		19.3			
ARASCO®[b]			11.0		
DHASCO®[b]					10.8
Enzymically modified fish				13.2	

[a]g/100 g oil blend; abbreviations: GLA, γ-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
[b]A gift from Martek Biosciences Corporation (Columbia, MD).

MATERIALS AND METHODS

Animals and diets. Weanling male Lewis or DA/Lewis rats (aged 4 wk, weighing between 65 and 80 g) were obtained from Harlan-Olac, Bicester, Oxfordshire, United Kingdom. The rats were housed in the Department of Biochemistry, University of Oxford for a period of 5 (DA/Lewis) or 6 (Lewis) wk prior to sacrifice, during which time they were given free access to water and to one of five experimental diets provided by Unilever Research Colworth Laboratory, Sharnbrook, Bedfordshire, United Kingdom. Each diet contained 178 g/kg lipid. The diets differed according to the proportions of different n-6 and n-3 PUFA they contained; these proportions were varied by using mixtures of oils containing triacylglycerols rich in different PUFA (see Table 1). All diets contained 182 g/kg high-nitrogen casein, 520 g/kg corn starch, 60 g/kg fiber (Solkafloc), 42 g/kg AIN-76 mineral mix, 12 g/kg AIN-76 vitamin mix, 4 g/kg DL-methionine, 2 g/kg choline bitartrate, and 170 mg/kg α-tocopherol; the α-tocopherol content of the oil blends was measured and normalized by the addition of commercial α-tocopherol (Sigma Type V; Sigma Chemical Co., Poole, Dorset, United Kingdom) to give an α-tocopherol content equivalent to 80 mg/kg of the final diet (the diets also contained 90 mg/kg α-tocopherol as a component of the AIN-76 vitamin mix). The fatty acid composition of each diet is shown in Table 2. The diets contained very similar levels of palmitic (approx. 20 g/100 g total fatty acids), stearic (approx. 4.0 g/100 g fatty acids), total saturated (approx. 27 g/100 g fatty acids), oleic (approx. 36 g/100 g fatty acids), and total PUFA (approx. 35 g/100 g fatty acids). Furthermore, the ratio of n-6/n-3 PUFA was kept fairly constant (Table 2); thus, in the diets containing GLA or ARA, these fatty acids partly replaced linoleic acid, whereas in the

TABLE 2
Fatty Acid Composition of the Diets Used

	Diet[a]				
Fatty acid	Control	GLA	ARA	EPA	DHA
12:0	n.d.	n.d.	n.d.	n.d.	0.4
14:0	0.3	0.3	0.3	0.4	2.0
16:0	22.5	22.2	20.9	20.5	20.3
16:1n-7	0.1	0.2	0.2	2.1	0.2
18:0	4.2	4.0	4.9	4.3	4.4
18:1n-9	36.9	37.0	37.0	32.3	36.4
18:2n-6	30.8	26.0	26.0	31.2	30.8
18:3n-3	4.4	4.4	4.4	0.3	0.1
18:3n-6	n.d.	4.4	n.d.	n.d.	n.d.
20:4n-6	n.d.	n.d.	4.4	n.d.	0.2
20:5n-3	n.d.	n.d.	n.d.	4.1	n.d.
22:5n-3	n.d.	n.d.	n.d.	0.3	0.1
22:6n-3	n.d.	0.4	n.d.	0.7	4.4
Total PUFA	35.2	35.2	34.8	36.6	35.6
n-6/n-3	7.0	6.3	6.9	5.8	6.7

[a]g/100 g total fatty acids; n.d. indicates not detected; PUFA, polyunsaturated fatty acids. For other abbreviations see Table 1.

diets containing EPA or DHA, these fatty acids replaced ALA (Table 2). Diets were stored at 4°C and were provided fresh to the rats every 2 d. Rats were killed in the fed state by an overdose of CO_2. Blood was collected by cardiac puncture and allowed to clot at room temperature for 1 h; serum was then collected by centrifugation at $1000 \times g$. Serum was stored at −20°C until analysis. Spleen leukocytes were prepared as described elsewhere (23). All procedures involving animals were approved under the Animals (Scientific Procedures) Act 1986 by the Home Office.

Chemicals. Chemicals and cell culture media and supplements were obtained from the sources described elsewhere (23,27,35,38). Prostaglandin (PG) E_2 concentrations were determined using ELISA kits purchased from Cayman Chemical Co. (Ann Arbor, MI).

Lipid extraction and fatty acid composition analysis. Lipid extraction and fatty acid composition analysis were performed as described elsewhere (27), except that fatty acid methyl esters were prepared by incubation with 14% boron trifluoride at 80°C for 15 min.

Analysis of lymphocyte subpopulations. Flow cytometry was used to measure the presence of various markers on the surface of freshly-prepared spleen leukocytes. The cells were stained with monoclonal antibodies to the T-cell receptor (a T lymphocyte marker), κ-light chain of immunoglobulins (a B lymphocyte marker), or CD11b/c (a macrophage marker) and analyzed as described elsewhere (23,27).

Lymphocyte proliferation and NK cell activity. Spleen lymphocyte proliferation in response to 5 μg/mL concanavalin A (Con A) was determined as described elsewhere (23–27); cultures contained 2.5% (vol/vol) autologous serum, and data are expressed as stimulation index (see 23–27). Spleen leukocyte NK cell activity toward YAC-1 cells was determined as described elsewhere (27).

Measurement of PGE_2 concentrations. Spleen leukocytes were cultured at 37°C in an air/CO_2 (19:1) atmosphere in 24-well culture plates at a density of 5×10^6 cells/well and a total culture volume of 2 mL in HEPES-buffered Roswell Park Memorial Institute medium supplemented with 2 mM glutamine, 2.5% (vol/vol) autologous serum, antibiotics (streptomycin and penicillin), and 5 μg/mL Con A. After 48 h of culture, the medium was collected and PGE_2 concentrations were determined by ELISA, performed according the instructions of the manufacturers of the kits; samples were diluted by 1 in 4 before analysis. This assay can measure a minimum of 29 pg/mL PGE_2, and the level of serum present in samples from cell culture does not interfere with the assay.

Popliteal lymph node (PLN) assay. This *in vivo* measure of cell-mediated immunity was performed as described elsewhere (27,38). Briefly, a graft vs. host response was elicited by injecting 6×10^6 lymph node lymphocytes (prepared from adult male Lewis rats maintained on standard laboratory chow) subcutaneously into the footpad of one leg of weanling male DA/Lewis rats which had been fed for 4 wk on the diets described above. The total injection volume was 0.1 mL and the cells were resuspended in phosphate-buffered saline (PBS); the footpad of the control leg was injected with 0.1 mL PBS. The animals were sacrificed 7 d after injection; during this time they were fed the same diet that they had been previously fed. The PLN were dissected out and weighed. Preliminary experiments indicated that injection of 6×10^6 cells and sacrifice 7 d postinjection resulted in maximal PLN size (38). Injection of PBS or of 6×10^6 DA/Lewis lymph node lymphocytes did not cause any increase in PLN weight (38).

Data presentation and statistical analysis. Data are means ± SEM of five rats fed each diet. Statistical comparions between groups were made using one-way analysis of variance and a *post hoc* least significant difference test. Spearman linear rank correlation coefficients, one-way analysis of variance and *post hoc* least significant difference tests were determined using SPSS Version 6.0 (SPSS Inc., Chicago, IL); in all cases a value for P of less than 0.05 was taken to indicate statistical significance.

RESULTS

Rat growth. The diets used in this study were isocaloric, and there was no difference in food intake among rats fed the different diets (data not shown). Final body weights, and therefore the total growth, were not different among rats fed the different diets (data not shown). Liver and spleen weight and weights of the epididymal and dorsal wall of abdomen adipose depots did not differ among animals fed the different diets (data not shown).

Serum fatty acid composition. The fatty acid composition of the serum was markedly influenced by that of the diet (Table 3). There were significant positive linear relationships between the proportions of each of the major fatty acids in the diet, except palmitic acid, and the proportions of those fatty acids in the serum (Table 4). The proportion of linoleic acid in the serum of rats fed diets containing n-3 PUFA was significantly higher than in the serum of rats fed the GLA- or ARA-rich diets (Table 3); there was a significant ($r = 0.651$; $P < 0.001$) linear relationship between the proportion of DHA in the diet and the proportion of linoleic acid in the serum. This is in agreement with previous observations that feeding rats diets containing fish oil leads to increased linoleic acid levels in plasma and liver lipids (43). These observations suggest that n-3 PUFA might inhibit the further metabolism of dietary linoleic acid and/or that n-3 PUFA might increase the intestinal absorption of linoleic acid. There is some evidence in support of both these suggestions. For example, DHA is known to inhibit Δ-6 desaturase, the enzyme responsible for further metabolism of linoleic acid (44). On the other hand, feeding rats a fish oil-rich diet has been shown to result in enhanced absorption of linoleic acid, at least from some dietary oils (45). Only the serum of GLA-fed rats contained appreciable proportions of GLA (Table 3); serum from these animals contained more dihomo-γ-linolenic acid than that from animals fed the control or ARA diets (Table 3). The proportion of ARA was high in the serum of ARA-fed rats and low in the serum of rats fed the EPA or DHA diets (Table 3). This

TABLE 3
Effect of Feeding Diets Containing Different PUFA upon the Fatty Acid Composition of Rat Serum[a]

	Diet[b]				
Fatty acid	Control	GLA	ARA	EPA	DHA
16:0	22.9 ± 1.3[a,b]	24.6 ± 1.3[a]	20.8 ± 1.2[b]	20.9 ± 0.5[b]	22.4 ± 0.8[a,b]
18:0	11.0 ± 0.7	10.4 ± 0.2	11.8 ± 0.8	11.0 ± 0.4	10.2 ± 0.2
18:1n-9	16.7 ± 1.8[a,b]	18.7 ± 0.9[a]	14.9 ± 1.4[b,d]	12.1 ± 0.4[c,d]	16.8 ± 0.7[a,b]
18:2n-6	27.2 ± 0.5[a]	22.6 ± 0.7[b]	16.7 ± 0.6[c]	28.4 ± 0.7[a]	31.5 ± 0.4[d]
18:3n-3	1.1 ± 0.1[a,b]	1.3 ± 0.1[a]	1.0 ± 0.1[b]	n.d.	0.17 ± 0.01[c]
18:3n-6	0.13 ± 0.02[a]	1.9 ± 0.1[b]	0.18 ± 0.01[a]	n.d.	n.d.
20:3n-6	0.05 ± 0.01[a]	0.5 ± 0.1[b]	n.d.	0.3 ± 0.1[a]	0.4 ± 0.1[a]
20:4n-6	13.3 ± 2.3[a,c]	13.2 ± 1.3[a]	26.1 ± 2.5[b]	9.3 ± 1.0[c,d]	7.9 ± 1.1[d]
20:5n-3	0.9 ± 0.4[a]	0.6 ± 0.2[a]	0.8 ± 0.2[a]	6.3 ± 0.5[b]	0.9 ± 0.2[a]
22:6n-3	1.2 ± 0.2[a]	0.6 ± 0.1[b]	0.5 ± 0.1[b]	1.8 ± 0.2[a]	4.5 ± 0.6[c]

[a]Data are mean ± SEM of five animals fed each diet. Values which do not share a superscript letter are significantly different from one another.
[b]g/100 g total fatty acids; for abbreviations see Tables 1 and 2.

is in accordance with observations made following the feeding of an ARA-rich diet to hamsters where ARA levels in liver, heart, lung, kidney, testes, and platelets were significantly elevated compared with feeding a control diet or a diet enriched in EPA (46). Thus, when a significant level of ARA is available from the diet, blood and tissue ARA levels exceed those observed in the absence of significant levels of ARA in the diet; in the latter situation dietary linoleic acid is used as a substrate for the synthesis of blood and tissue ARA. The reduced proportion of linoleic acid in the plasma of ARA-fed rats (Table 3) is in accordance with the reduced proportion of linoleic acid observed in the tissues of ARA-fed hamsters (46). The serum of EPA-fed rats contained a high proportion of EPA and DHA, whereas the serum of DHA-fed rats contained a high proportion of DHA (Table 3).

Spleen leukocyte fatty acid composition. The fatty acid composition of spleen leukocytes was markedly influenced by that of the diet (Table 5). There were significant positive linear relationships between the proportions of each of the PUFA in the diet, except ALA, and the proportions of those fatty acids in spleen leukocytes (Table 4). The proportion of linoleic acid in the leukocytes of rats fed diets containing EPA or DHA was significantly higher than in the leukocytes of rats fed the GLA- or ARA-rich diets (Table 5); there was a significant ($r = 0.639$; $P < 0.001$) linear relationship between the proportion of DHA in the diet and the proportion of linoleic acid in leukocytes. Only leukocytes from GLA-fed rats contained appreciable proportions of GLA (Table 5). These cells also contained a significantly greater proportion of dihomo-γ-linolenic acid than cells from rats fed each of the other diets (Table 5). This observation reflects the availability of GLA, which is rapidly metabolized to dihomo-γ-linolenic acid. The proportion of ARA was high in the leukocytes of ARA-fed rats and low in the leukocytes of rats fed the EPA or DHA diets (Table 5). This agrees with the study of Whelan *et al.* (46) in which feeding an ARA-rich diet to hamsters increased spleen lipid ARA levels (and reduced spleen lipid linoleic acid levels, as found in the current study). Leukocytes from EPA-fed rats contained a relatively high proportion of EPA, whereas those from DHA-fed rats contained a high proportion of DHA (Table 5).

TABLE 4
Spearman Linear Rank Correlation Coefficients for the Relationships Between the Proportions of Particular Fatty Acids in the Diet and the Proportions of Those Fatty Acids in the Serum or Spleen Leukocytes[a]

	Proportion of fatty acid in serum		Proportion of fatty acid in leukocytes	
Dietary fatty acid	*r*	*P*	*r*	*P*
Palmitic acid	0.393	NS[b]	–0.151	NS
Oleic acid	0.457	<0.05	–0.402	<0.05
Linoleic acid	0.859	<0.001	0.701	<0.001
ALA	0.819	<0.001	0.364	NS
GLA	0.987	<0.001	0.571	<0.01
ARA	0.822	<0.001	0.764	<0.001
EPA	0.962	<0.001	0.934	<0.001
DHA	0.886	<0.001	0.928	<0.001

[a]Data are for 25 animals.
[b]NS, not significant; ALA, α-linolenic acid. For other abbreviations see Tables 1 and 2.

Analysis of spleen leukocyte subpopulations. Diet did not affect the proportions of T lymphocytes (approximately 60%), B lymphocytes (approximately 20%), or macrophages (approximately 20%) in spleen leukocyte preparations (data not shown).

Proliferation of spleen lymphocytes. The proliferation of spleen lymphocytes from rats fed the EPA- and DHA-rich diets was significantly lower than that of lymphocytes from rats fed the control diet (Fig. 1). Maximal proliferation of lymphocytes from rats fed the DHA-rich diet was 65% that of lymphocytes from rats fed the control diet (Fig. 1).

Spleen NK cell activity. The NK cell activity of spleen lymphocytes from rats fed the EPA-rich diet was significantly lower than that of lymphocytes from rats fed the control or the ARA- or DHA-rich diets (Fig. 2).

TABLE 5
Effect of Feeding Diets Containing Different PUFA upon the Fatty Acid Composition of Rat Spleen Leukocytes

	Diet[a]				
Fatty acid	Control	GLA	ARA	EPA	DHA
16:0	24.8 ± 1.7	29.6 ± 1.1	26.6 ± 1.6	29.4 ± 0.5	27.1 ± 1.8
18:0	15.8 ± 1.4	18.3 ± 0.9	18.5 ± 1.0	18.1 ± 0.5	18.2 ± 1.0
18:1n-9	10.9 ± 0.3^{a}	12.1 ± 0.2^{b}	11.1 ± 0.7^{a,b}	12.5 ± 0.3^{b}	12.2 ± 0.7^{a,b}
18:2n-6	11.9 ± 0.8^{a,c}	10.7 ± 0.1^{a}	7.9 ± 0.2^{b}	14.6 ± 1.7^{c,d}	16.4 ± 1.2^{d}
18:3n-3	0.4 ± 0.2	n.d.	0.2 ± 0.1	n.d.	n.d.
18:3n-6	n.d.	1.2 ± 0.6	n.d.	n.d.	n.d.
20:3n-6	0.8 ± 0.1^{a}	2.3 ± 0.1^{b}	0.7 ± 0.1^{a,c}	0.9 ± 0.1^{a}	0.4 ± 0.1^{c}
20:4n-6	16.2 ± 0.5^{a}	18.7 ± 0.7^{b}	23.5 ± 1.0^{c}	11.6 ± 0.5^{d}	12.8 ± 0.4^{d}
20:5n-3	0.20 ± 0.06^{a}	0.8 ± 0.5^{a}	0.26 ± 0.01^{a}	2.9 ± 0.2^{b}	0.32 ± 0.04^{a}
22:6n-3	0.7 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	1.3 ± 0.4^{a}	3.8 ± 0.3^{b}

[a]g/100 g total fatty acids; data are mean ± SEM of five animals fed each diet. Values which do not share a superscript letter are significantly different from one another.
[b]For abbreviations see Tables 1 and 2.

PGE_2 production by cultured leukocytes. It is likely that the bulk of the PGE_2 produced in spleen leukocyte cultures is of macrophage origin (47). The highest level of PGE_2 production was from spleen leukocytes from rats fed the diet rich in ARA; this was greater than production by cells from rats fed the n-3 PUFA-containing diets (Fig. 3). PGE_2 production was lowest in leukocytes from rats fed the EPA- or DHA-rich diets; this was lower than production by cells from rats fed each of the other three diets (Fig. 3). There was a significant correlation between spleen leukocyte ARA levels and *ex vivo* PGE_2 production ($r = 0.738$; $P < 0.001$). This suggests that the main cause of the differences in PGE_2 production among leukocytes from rats fed different diets is the availability of substrate.

Graft vs. host response. The PLN assay was used as an *in vivo* measure of cell-mediated immunity. PLN weights were lowest in rats fed the EPA-rich diet (Fig. 4); these were significantly lower than those in rats fed the control diet.

DISCUSSION

In this study we have compared the effects of feeding rats high-fat (178 g/kg) diets with a near-constant PUFA content (35 g/100 g fatty acids) and a near-constant ratio of n-6/n-3 PUFA (approximately 7), but with different long-chain n-6 or n-3 PUFA. The characteristics of the diets used were selected so that this study would be of relevance to humans. For example, the level of fat in the diet was selected so that fat would provide approximately 35% of dietary energy, the current recommended level for the United Kingdom population (48). The levels of total saturated fatty acids, monounsaturated fatty acids, and total PUFA were selected so that they

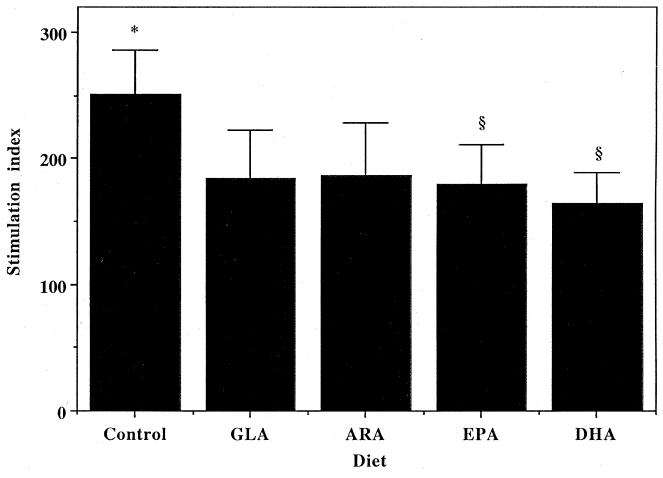

FIG. 1. Effect of feeding diets containing different polyunsaturated fatty acids (PUFA) upon the proliferation of rat spleen lymphocytes. Values are mean ± SEM of five animals fed each diet. Values indicated by different symbols are significantly different from one another. GLA, γ-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

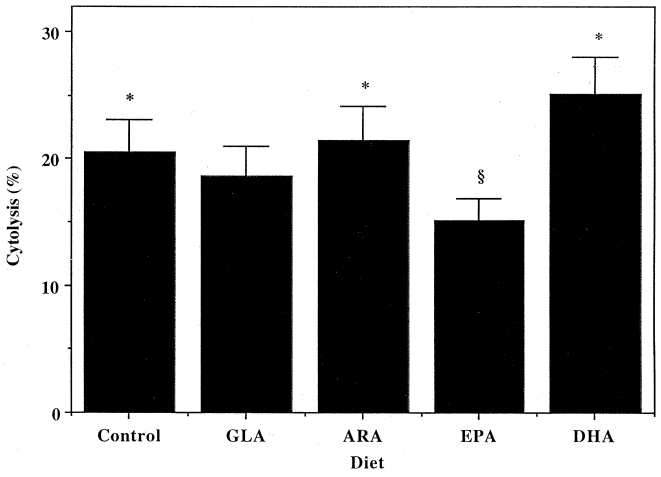

FIG. 2. Effect of feeding diets containing different PUFA upon the natural killer cell activity of rat spleen lymphocytes. Values are mean ± SEM of five animals fed each diet. Values indicated by different symbols are significantly different from one another. For abbreviations see Figure 1.

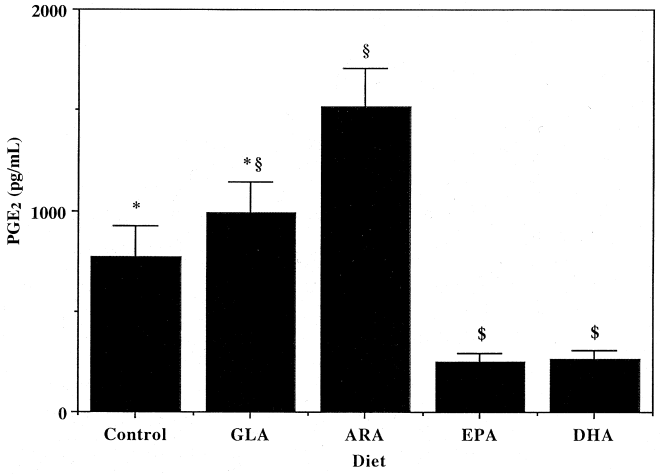

FIG. 3. Effect of feeding diets containing different PUFA upon *ex vivo* prostaglandin E_2 (PGE_2) production by rat spleen leukocytes. Values are mean ± SEM of five animals fed each diet. Values not sharing a common symbol are significantly different from one another. For abbreviations see Figure 1.

approximated those currently recommended for the United Kingdom diet (11, 14, and 7% of dietary energy, respectively; 48); however, the level of total PUFA actually used was higher than this. The n-6/n-3 PUFA ratio of 7 was selected so that it approximated that of the current United Kingdom diet (approximately 6.5; 49). The control diet contained 31 g linoleic acid and 4.4 g ALA/100 g total fatty acids. In all diets the content of long-chain PUFA was maintained at approximately 4.4 g/100 g fatty acids; therefore, in the experimental diets GLA and ARA partially replaced linoleic acid, and EPA and DHA replaced ALA. Importantly, the levels of all fatty acids other than PUFA were almost identical in all diets. Therefore, these diets investigate the effect of replacing a portion of dietary linoleic acid with a relatively low level of another n-6 PUFA (either GLA or ARA) without introducing any other changes in dietary fatty acid composition, total PUFA content, or n-6/n-3 PUFA ratio. Similarly, these diets investigate the effect of replacing dietary ALA with another n-3 PUFA (either EPA or DHA) without introducing any other changes in dietary fatty acid composition, total PUFA content, or n-6/n-3 PUFA ratio. Thus, feeding these diets represents a more strictly controlled situation than does feeding diets rich in particular oils (e.g., sunflower, safflower, evening primrose, linseed, fish), since these differ significantly in the proportions of fatty acids other than those being compared (e.g., palmitic and oleic acids). In addition, the characteristics of such oils differ widely. For example, linseed oil contains 50 g ALA/100 g total fatty acids, fish oil contains 25 g EPA plus DHA/100 g total fatty acids, and n-6 PUFA-rich oils such as sunflower or SO contain 60 to 85 g linoleic acid/100 g total fatty acids. Thus, experiments which compare the effects of feeding these oils are comparing diets with widely differing n-6/n-3 PUFA ratios and levels of PUFA. For instance, the n-6/n-3 PUFA ratio in sunflower and SO is greater than 100, whereas this ratio in linseed and fish oils is approximately 0.3.

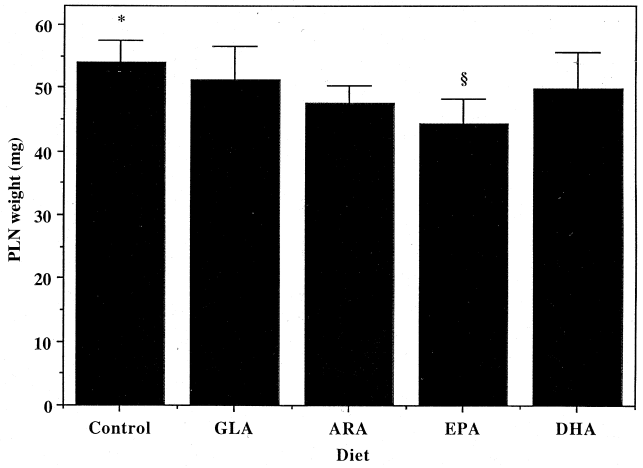

FIG. 4. Effect of feeding diets containing different PUFA upon the graft vs. host response in rats. Values are mean ± SEM of five animals fed each diet. Values indicated by different symbols are significantly different from one another. PLN, popliteal lymph node. For other abbreviations see Figure 1.

Feeding rats a diet containing 200 g/kg fish oil results in suppressed *ex vivo* lymphocyte proliferation (23,26) and NK cell activity (26,35), and diminishes the graft vs. host response *in vivo* (38). Similar effects of fish oil feeding have been reported in other species, including mice (25,30,33, 34,37), rabbits (21), chickens (22), and humans (50–52). Feeding linseed oil to laboratory animals also suppresses *ex vivo* lymphocyte proliferation (20–22,27) and NK cell activity (27) and diminishes the *in vivo* graft vs. host response (27). However, as described above, diets containing fish or linseed oils as the sole source of fat include very high levels of n-3 PUFA; the current study investigates the effects of lower levels of these PUFA. Differential effects of dietary n-3 PUFA were observed. Replacing dietary ALA with either EPA or DHA decreased lymphocyte proliferation. In contrast, replacing dietary ALA with EPA, but not with DHA, reduced NK cell activity and the graft vs. host reponse. These observations suggest that the suppressive effect of dietary fish oil upon lymphocyte proliferation might be due to the presence of both EPA and DHA, whereas the inhibitory effect of fish oil upon NK cell activity might be due to EPA alone. This study does not rule out the possibility that higher levels of DHA in the diet might exert effects upon NK cell activity. Furthermore, we have not investigated the effect of altering the ratio of EPA to DHA in the diet; this might be important in determining the precise effect of long-chain n-3 PUFA and of different fish oils upon lymphocyte function and cell-mediated immunity.

It is unlikely that the differences in lymphocyte function relate to differences in the populations of cells cultured: diet did not affect the proportions of T lymphocytes, B lymphocytes, or macrophages present in the spleen. This is in accordance with the lack of effect of diets rich in fish oil, evening primrose oil, or linseed oil upon spleen leukocyte subpopulations (23,27).

This study also reveals that replacing a proportion of dietary linoleic acid with another n-6 PUFA, either GLA or ARA, does not influence spleen lymphocyte proliferation or NK cell activity and does not affect the graft vs. host response.

The observations made in the current study can be compared with those of some other recent studies (53,54). Jolly *et al.* (53) fed mice for 10 d on diets containing 30 g SO/kg, 20 g SO plus 10 g ARA-rich triacylglycerol/kg, 20 g SO plus 10 g EPA-ethyl ester/kg or 20 g SO plus 10 g DHA-ethyl ester/kg. They found that compared with the SO diet, the ARA-rich diet did not affect spleen lymphocyte proliferation in response to Con A. In contrast, both EPA- and DHA-rich diets suppressed lymphocyte proliferation (by 80%); the two n-3 PUFA were equipotent. Qualitatively this agrees with the current study which found no effect of ARA on lymphocyte proliferation, but a suppressive effect of diets containing either EPA or DHA, which were equipotent in their effects (Fig. 1). However, lymphocyte proliferation was suppressed by about 30 to 35% in the current study (compared with the control diet). This quantitative difference in the effects of EPA and DHA between the two studies could be due to at least five key differences between the studies. First, the studies used different levels of n-3 PUFA in the diets; in the current study, EPA or DHA constituted approximately 4.4% of total fatty acids and approximately 1.5% of dietary energy, whereas in the study of Jolly *et al*. (53) EPA or DHA made up approximately 30% of total fatty acids and approximately 2.3% of dietary energy. Second, the total level of fat in the diets used was different; in the current study the diets contained 178 g fat/kg diet, whereas in Jolly *et al*. (53) the diets contained 30 g fat/kg diet. The level of dietary fat has been shown to influence lymphocyte proliferation (18), and this makes direct quantitative comparison between the two studies difficult. Third, the diets used in the two studies differed greatly in their PUFA content; in the current study PUFA constituted approximately 35% of total fatty acids whereas in the the study of Jolly *et al*. (53) PUFA made up 90% of total fatty acids. Lymphocyte proliferation has been shown to be markedly influenced by the level of PUFA in the diet (39); furthermore, the total PUFA content of the diet influences the effect of ALA (39), and perhaps other n-3 PUFA, on lymphocyte proliferation. Fourth, the diets used in the two studies differ greatly in the ratio of n-6/n-3 PUFA; in the current study the n-6/n-3 PUFA ratio was kept as constant as possible at approximately 7, whereas in the study of Jolly *et al*. (53) the n-6/n-3 PUFA ratio varied greatly between diets. Although the exact fatty acid compositions of the diets used were not given by Jolly *et al*. (53), sufficient information is available to indicate that the n-6:n-3 PUFA ratio of the control SO diet was at least 100, and perhaps as much as 800, while the n-6/n-3 PUFA ratios of the EPA and DHA diets were approximately 1.7. It is known that lymphocyte proliferation is markedly influenced by the n-6/n-3 PUFA ratio of the diet (27,39). Fifth, the exact comparisons being made are different between the two studies: in the current study the effect of replacing an exact amount of one n-3 PUFA (ALA) with the same amount of another n-3 PUFA (EPA or DHA) without altering any other dietary variable was examined, whereas in Jolly *et al*. (53) the effect of replacing a significant amount (30%) of linoleic acid in a diet almost devoid of n-3 PUFA with EPA or DHA was examined. Thus, the control diets are different between the two studies; in the current study the control diet contained 30 g linoleic acid, 4.4 g ALA, and 35 g PUFA/100 g total fatty acids and had an n-6/n-3 PUFA ratio of 7, whereas in the study of Jolly *et al.* (53) the control diet contained at least 84 g linoleic acid and PUFA/100 g total fatty acids and a negligible amount of ALA and had an n-6/n-3 PUFA ratio of at least 100. These profound differences most likely explain the quantitative differences in the effects of EPA and DHA upon lymphocyte proliferation between the two studies. It seems logical that, since adding ALA to an n-6 PUFA-rich diet reduces lymphocyte proliferation (27,39), adding EPA or DHA to an n-6 PUFA-rich diet (53) will have a greater effect upon lymphocyte proliferation than replacing ALA with either EPA or DHA (this study).

The lack of an immunological effect of feeding laboratory rodents diets enriched in ARA (this study; 53) agrees with a recent study in man, the first study in animals or man to have examined the effects of dietary ARA on components of the immune response, which showed no effect of 1.5 g ARA/day for 50 d on the proliferative response of peripheral blood mononuclear cells to Con A, phytohemagglutinin, or pokeweed mitogen (54). Human peripheral blood NK cell activity was unaffected by the consumption of ARA (54); again this observation is in agreement with that of the current study (Fig. 2). The current study observed a lack of effect of ARA upon the graft vs. host response, an *in vivo* measure of cell-mediated immunity (Fig. 4). This also agrees with the human study (54) which found no effect of the ARA diet on the delayed-type hypersensitivity response to seven recall antigens applied intradermally.

An n-3 PUFA-induced reduction in lymphocyte reactivity and cell-mediated immunity, although potentially useful in chronic inflammatory diseases (5–11) and following transplantation (12), could make individuals increasingly susceptible to bacterial and viral infections and to the development of cancers. Certainly inclusion of high levels of fish oil in the diet of experimental animals results in increased mortality following a challenge with *Salmonella typhimurium* (55), *Staphylococcus aureus* (56), or *Listeria monocytogenes* (57). However, the current study indicates that n-3 PUFA at the level of 4.4 g/100 g total dietary fatty acids cause rather minimal immunosuppressive effects. To achieve this level of n-3 PUFA intake, individuals would need to consume between 3.5 and 6 g long-chain n-3 PUFA per day. Since the current average United Kingdom intake of long-chain n-3 PUFA is less than 0.2 g/day (49), this would represent a vast increase in intake, achievable only by supplementation with encapsulated n-3 PUFA. We have not identified the lowest level of EPA or DHA required to suppress lymphocyte proliferation, but DHA at the level of 4.4 g/10 g total fatty acids did not af-

fect NK cell activity or a measure of the *in vivo* cell-mediated immune response. The data suggest that a modest increase in dietary long-chain n-3 PUFA or supplementation of the diet with relatively low levels of encapsulated fish oil will not compromise immunity. In agreement with this conclusion, Meydani *et al*. (51) reported that supplementation of the diet of women aged 23 to 33 yr with 2.4 g EPA plus DHA per day for up to 3 mon did not affect the response of peripheral blood lymphocytes to Con A or phytohemagglutinin. Similarly, supplementation of the diet with 6 g long-chain n-3 PUFA in the form of ethyl esters per day for 4 mon did not affect the response of peripheral blood lymophocytes to phytohemagglutinin (58). Interestingly, these levels of supplementation with n-3 PUFA significantly reduce the *ex vivo* production of proinflammatory cytokines (50–52; see 19 for a review), suggesting that proinflammatory cytokine production is more sensitive than lymphocyte function to n-3 PUFA intervention.

In the past it has been speculated that effects of dietary n-3 PUFA on immune cell function relate to changes in production of eicosanoids, particularly PGE_2. PGE_2 levels were elevated in the medium of leukocytes from rats fed the ARA-rich diet and were reduced in that of leukocytes from rats fed the EPA- or DHA-rich diets (Fig. 3). Thus, differences in PGE_2 production cannot be responsible for the differences in lymphocyte proliferation observed. This agrees with the outcomes of a number of *in vitro* studies which have concluded that the inhibitory effects of EPA and DHA upon lymphocyte proliferation are independent of their effects upon PGE_2 production (59–62). There is also agreement with studies in humans in which supplementation of the diet with fish oil has been shown to reduce both lymphocyte proliferation and PGE_2 production (50,51); these studies concluded that the effect of n-3 fatty acid supplementation on lymphocyte proliferation seems independent of, and is unlikely due to, decreases in PGE_2 production (50,51). The recent study of Wu *et al*. (63) in part also supports this conclusion: these workers found that feeding monkeys diets containing increased amounts of ALA resulted in significantly reduced PGE_2 production but did not affect lymphocyte proliferation. Where the study of Wu *et al*. (63) differs from this study and from that of Jolly *et al.* (53), as well as from earlier studies in rats (23,26), mice (25,30,33,34,37), rabbits (21), chickens (22), and humans (50–52), is in the effect of enrichment of the diet in EPA plus DHA upon lymphocyte proliferation. Adding EPA plus DHA to the diet of monkeys significantly reduced PGE_2 production but increased the response of lymphocytes to mitogens (63). The authors' explanation for the latter observation was that the monkeys had also been fed variable levels of α-tocopherol and so were better able to maintain antioxidant defenses in the face of differing dietary PUFA levels. In the current study, as well as that of Jolly *et al*. (53), the levels of antioxidants were constant in all diets.

If long-chain n-3 PUFA do not inhibit lymphocyte proliferation *via* changes in eicosanoid production, other mechanisms must be identified. The current study did not examine the possible mechanisms beyond measuring PGE_2 concentrations. However, the study by Jolly *et al*. (53) provides some insight into how EPA and DHA might act to suppress lymphocyte proliferation. These workers observed a marked reduction in generation of the intracellular second messengers diacylglycerol and ceramide in Con A-stimulated cells from EPA- or DHA-fed mice. Both diacylglycerol and ceramide are key second messengers in lymphocytes, ultimately giving rise to transcription factor activation and so regulating gene expression. The data are suggestive of effects of fish oil-derived n-3 PUFA on intracellular signaling pathways which control the functional activities of the cells. In agreement with Jolly *et al.* (53), we have observed diminished generation of inositol-1,4,5-trisphosphate in stimulated lymphocytes taken from rats fed fish oil; this appeared to be due to reduced activity of phospholipase C-γ (Sanderson, P., and Calder, P.C., unpublished observations). The mechanism(s) of action of EPA and DHA warrant further examination; in light of the current study it would seem important to identify the reasons why these fatty acids exert similar effects upon lymphocyte proliferation but different effects upon NK cell activity.

ACKNOWLEDGMENTS

This research is supported by Unilever Research Colworth Laboratory and the Ministry of Agriculture, Fisheries and Food under the Agro-Food Quality LINK Programme (AFQ51); NMJ held a Graduate Studentship and FT holds a Postdoctoral Research Fellowship under this support. LMP was supported by Rotary International and the Leopold Schepp Foundation, and PS held a Medical Research Council Graduate Studentship when this work was performed.

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[Received July 23, 1997, and in final revised form January 12, 1998; revision accepted January 14, 1998]

Changes in Cultured Arterial Smooth Muscle Cells Isolated from Chicks upon Cholesterol Feeding

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ABSTRACT: We have developed cultures of smooth muscle cells (SMC) isolated from arterial hypercholesterolemic chicks (cholesterol-SMC). These cultures are suitable for the study at the molecular level of the changes in arterial SMC induced by a cholesterol diet. By using a strong dose of cholesterol (5%) for 10 d, we obtained very proliferative SMC which became foam cells after 30 d in culture. On the other hand, SMC cultures isolated from control-fed chicks had a lower growth rate than the SMC ones under the same culture conditions. DNA synthesis was fourfold greater in cholesterol-SMC than in control-SMC cultures. Intracellular cholesterol concentrations were the same in both cholesterol and control SMC during the first 14 d of culture but afterward increased in differing ways: after 20 d of culture the cholesterol-SMC increased their cholesterol content to double the control. We give here the results obtained from transmission electron microscopy, lipid analysis, proliferation studies, DNA, RNA and protein synthesis, and then discuss their implications.

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Hypercholesterolemia is a known risk factor in arteriosclerosis, a complex disease involving prolonged processes in its evolution (1–4). The contractile-state smooth muscle cell (SMC) is the only cell type in the arterial media (5,6) and undergoes migration into the intima, proliferation, and abundant extracellular matrix production during the early stages of atherosclerosis (7,8).

In cell culture and in atherogenic processes, SMC changes from a contractile to a synthetic state (9–14). This modulation has been associated with phenotypic changes (15) characterized through cytoskeletal and cytoenzymatic studies (16,17) and makes the SMC responsive to chemotactic or mitogenic stimuli (2,18,19).

SMC is thought to be crucial in the development of arteriosclerotic plaques (20,21). In a rat aortal SMC culture model—including contractile, synthetic, and transformed cells—it has been shown that an increase in cholesterol biosynthesis associated with phenotypic changes (22) plus dedifferentiation in arterial SMC *in vitro* are associated with a decrease in intracellular cholesterol efflux and also a decrease in high-affinity binding sites for native high density lipoprotein (HDL) (23). Other cell-culture models use different additional elements in the culture to obtain foam cells *in vitro,* such as an elastin substrate (24) or lipid droplets isolated from cultured macrophages (25). Nevertheless, the molecular event that triggers the transformation of SMC in dedifferentiated and proliferative cells by cholestrol feeding is not yet completely understood.

Avian models were used in trials of atherosclerosis, and, in fact, cholesterol feeding has been described elsewhere as producing atherosclerotic lesions in fowl species (26–29). We have also used chicks previously to study lipid metabolism after cholesterol administration (30–33). The aim of this study was to develop a cell-culture avian model to look into the effect of a cholesterol diet on the transformation of SMC *in vivo*. We show a comparative study between cultures of SMC isolated from two chick groups: control- and high-cholesterol-feeding chicks. We have examined the morphological, molecular, and proliferative changes in arterial SMC in culture under conditions mimicking such a cholesterol diet and the reproducibility of our methods.

MATERIALS AND METHODS

Animals. Newborn white leghorn male chicks *(Gallus domesticus)* were bought from a commercial hatchery and fed *ad libitum* in a chamber with a daily light cycle from 0900 to 2100 and a controlled temperature of 29–31°C.

Diet and treatment. Different groups of newborn white leghorn male chicks were used. The control groups were 10-day-old chicks (C_{10}) and 20-day-old chicks (C_{20}) kept on a standard diet (Sanders A-00). The treated groups were 10-day-old chicks fed on the same diet supplemented with 2% w/w powdered cholesterol mixed homogeneously (Panreac reagent Barcelona, pure grade; Montplet & Esteban SA, Barcelona, Spain) (T_{10}-2%) or 5% w/w (T_{10}-5%). This treatment was also assayed with 20-day-old chicks (T_{20}-2% and

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Abbreviations: DMEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum; HDL, high density lipoprotein; LDL, low density lipoprotein; PBS, phosphate-buffered saline; SMC, smooth muscle cell.

T_{20}-5%). The diets were started at hatching and kept on until the chicks were killed. Water was available at all times. None of the chicks died a natural death during the treatment nor developed any illness.

Lipids analysis. Lipids were extracted by the Bligh–Dyer method (34). Serum total cholesterol content and intracellular cholesterol were determined by enzymatic colorimetry method CHOD-PAD, Cat. No. 290319 ("Test–Combination cholesterol," Boehringer Mannheim Chemicals GmbH, Mannheim, Germany).

Serum triglyceride and intracellular triglyceride contents were determined by enzymatic colorimetry Test Peridochrom ("Triglycerides GPO-PAP," Cat. No. 701904, Boehringer Mannheim GmbH).

Isolation and culturing of cells. Unless otherwise specified, at 10 d of age both the control and cholesterol-fed animals were decapitated and the aortic arch was removed. SMC explants from the medial layer of the arterial wall were prepared as described before (35,36) with slight modifications as described below.

Under meticulously sterile conditions, the arteries were placed in phosphate-buffered saline (PBS) (Flow) pH 7.4 at 37°C containing an antibiotic cocktail composed of penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotricin (0.25 µg/mL) (Sigma-Aldrich, Madrid, Spain). The blood vessels were cleaned of all adipose and connective tissues and were then cut open longitudinally and incubated in PBS containing type II (1 mg/mL) collagenase and type IIA (0.5 mg/mL) elastase (Sigma) at 37°C for 30 min. The adventitious veins were cleanly stripped and discarded. The endothelium was removed using a paint brush. The artery was then washed extensively in PBS.

The aortas (100 mg) were cut into uniform-sized pieces of 1 mm^2 and then put into 75-cm^3 flasks with a small amount (1 mL) of normal culture medium, consisting of Dulbecco's modification of Eagle's medium (DMEM) supplemented with D-glucose (4.5 g/L), L-glutamate (0.584 g/L) (Flow), antibiotic cocktail, and 20% (vol/vol) fetal calf serum (FCS). The flasks were allowed to stand with the cap end up, to drain most of the medium in order to promote tissue adherence. The explants were maintained in a humidified incubator (5% CO_2) at 37ºC for 24 h. More medium (2 mL) was then added to cover the explants, and these were checked every day under an inverted light microscope. Half of the medium was replaced every 2–3 d. The cells that grew out from the explants were detached with 0.05%/0.02% Trypsin/EDTA solution (Flow), centrifuged at 200 × *g* for 10 min, resuspended in complete medium, counted, and seeded into new flasks (first passage). While in first passage, the cells were fed every day and maintained prior to subsequent trypsinization and repassage. The medium used for feeding after the first passage was DMEM with 10% FCS.

Unless otherwise specified, all the experiments were conducted using third- or fourth-passage cells plated at a density of 2.5×10^6 cells/P-75 tissue culture dish. Cells were determined to be vascular smooth muscle by their hill-and-valley configuration at confluence (36) and positive fluorescence staining for smooth muscle actin and myosin (10).

Examination of cell viability. Trypan blue (Sigma) was added to the cells with the help of a hemocytometer, and the flasks were examined with an inverted light microscope (Olympus Opticals Co. Ltd., Tokyo, Japan) to count live and dead cells. Cell viability in our cultures was 90%.

Electron microscopy. After the chicks were killed, their aortic arches were immediately removed and samples were taken for electron microscope examination. A small segment was taken from the aortic arch and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1.5% osmium tetroxide in the same buffer, and embedded in epoxy resin. Ultrathin sections were cut and double-stained with uranyl acetate and lead citrate and observed under a Zeiss 902 transmission electron microscope (Carl Zeiss, Obercochen, Germany.

SMC cultures were also prepared for electron microscope examination. Aortic SMC growing in plastic dishes were fixed in cacodylate-buffered 2.5% glutaraldehyde (pH 7.2), postfixed in 1.5% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were viewed in a Zeiss 902 Microscope.

Cell proliferation studies. For cell proliferation studies confluent cultures were trypsinized, and the cells were seeded into 24-well plates (cel-Cult, Sterilin; Biby Sterilin, Staffordshire Stone, England) at a density of 1×10^5 cells/cm^3 with no precoating of the dishes with adhesion factors. The medium was changed every 24 h after seeding. For all cell proliferation studies, DMEM with glucose, glutamine, and antibiotic cocktail (Flow) supplemented with 10% FCS was used. At different times during the 10-d culture, the cells of three wells were washed with PBS and trypsinized to yield single cells. The cells were then pooled and transferred to a cell counter (Neubauer chamber; Rudolph Brand GmbH Co., Wertheim, Germany) for counting. Cell viability was checked by using Trypan blue.

Cell cycle arrest. To arrest cells in G_1, we deprived them of serum for 12 to 48 h. Serum deprivation was carried out by incubation in DMEM with 0.2% FCS added 12, 24, or 48 h after the cells were seeded (37).

Measurement of the rates of cell DNA, RNA, and protein synthesis. To measure the rate of cell DNA synthesis, we incubated the cells (5×10^5 per 25-cm^2 flask) for 24 h after inoculation to permit their recovery and then synchronized (0.2% FCS for 48 h) and pulse-labeled for 15 min at various times with 1 µCi/mL of [^{3}H]-*d*-thymidine (Amersham International, Little Chalfont, Buckinghamshire, England). The plates were washed five times with ice-cold PBS, and then the cells were harvested by scraping them with a cell-lifter (Costar; Corning Coster Corporation, Cambridge, MA) into 3 mL of ice-cold PBS and were centrifuged for 5 min at 220 × *g*; the pellet was resuspended in 0.5 mL of ice-cold PBS, sonicated, and mixed with a final volume of 10% ice-cold CCl_3COOH. Insoluble material was collected on a Whatman

GF/C filter (Maidstone, England) and washed extensively with cold 5% CCl_3COOH. The filter was then dried under a heat lamp, and the radioactivity incorporated was quantitated in Ready-Safe by liquid scintillation counting (Beckman LS 6000; Beckman Instruments, Inc., Fullerton, CA). The protein was determined according to Lowry *et al.* (38).

To measure the rate of RNA synthesis, the cells were pulsed as described above with 1 μCi/mL of [^{3}H]uridine (Amersham International) in DMEM medium. The CCl_3COOH-insoluble material was then determined as described for DNA.

To measure the rate of cellular protein synthesis [4,5-^{3}H]L-leucine (1 μCi/mL) incorporation into CCl_3COOH-insoluble material was performed as described for DNA synthesis except that a 30-min rather than a 15-min pulse was used.

Statistical analyses. The significance of differences between the SMC from cholsterol-fed and control chicks was computed using the unpaired Student's *t*-test. Results are presented as means ± SEM.

RESULTS

Outgrowth of the explants. As previously described in the Materials and Methods section, a simple, fast, and reproducible routine must be established between the removal of the artery and the outgrowth of the explants.

Smaller explants showed a reduced lag phase prior to outgrowth. When the tissues were cut into uniform 1-mm^2 pieces with a scalpel blade, 80–90% of the explants showed outgrowth after 10 d. Various concentrations of FCS were tested (5, 10, 20%); 20% FCS gave the best outgrowth from explants and was used routinely. The outgrowth from explants was better in 10- than in 20-day-old chick tissue.

Explants prepared using hypercholesterolemic chick arteries gave the shortest lag phase. The migration of SMC from explants of atherosclerotic tissues occurred 6–7 d after planting. The lag phase from control explants was 10 d or less. The migration out of the cells from the explants was much faster for cholesterol-fed chicks than for control chicks.

Maintenance of the SMC cultures' life. At the beginning of the study, tissue samples for preparing the explants were collected from different vessels (aortic arch and aorto-iliac bifurcation) to find the best arterial specimen to culture the SMC. This study was made by comparing the number of days that the different cultures remained viable. Aorto-iliac bifurcation and aortic arch SMC from different groups of control and treated animals were used. It can be seen in Table 1 that the SMC cultures from the aortic arches of younger chicks were kept in culture for longer times than the older ones. Moreover, Table 1 shows the effect of the proportion of cholesterol fed on the SMC culture life, which was longer with SMC from 5 than from 2% cholesterol-fed chicks. Results using SMC cultures from aorto-iliac bifurcation gave the same results (results not shown).

Transmission electron microscope analysis. Transmission electron microscope analysis of the aortas was made to choose the period of diet treatment because our first aim was to isolate the SMC prior to intimal thickening, considering that the effect of a high-cholesterol diet on SMC was a very early atherogenic process.

TABLE 1
Effect of Cholesterol Feeding on the Life of Smooth Muscle Cell (SMC) Cultures[a]

Chicks age (d)	Days that SMC cultures were maintained		
	C	T-2%	T-5%
10	38	40	80
20	15	14	28

[a]SMC obtained from control chicks group (C). SMC obtained from chicks fed on the same diet with 2 (T-2%) and 5% (T-5%) cholesterol. Results are expressed as the means of five experiments. Variations did not exceed 10%.

The effect of short-term (10 d) and long-term (20 d) cholesterol feeding on the morphological aspects in aorta tissues of chicks has been studied. No histological alterations were observed in aortas after 10 or 20 d of 2% cholesterol feeding (T_{10}-2% and T_{20}-2%) compared to the control chicks of the same age (C_{10} and C_{20}) (results not shown). Nevertheless, the histology of aortas from chicks fed long term (T_{20}-5%) on 5% cholesterol was different (Fig. 1A), with a considerable number of lipid droplets and a focal aggregation of foam cells, resulting in intimal thickening. When 5% cholesterol treatment was short-term (T_{10}-5%), no lipid droplets accumulated within 10 d in the aortas (Fig. 1B), similar to what happened in controls (Fig. 1C). However, we observed two morphological changes in T_{10}-5% cells compared to control: first, the relaxed aspect of the nuclear membrane (Fig. 1B) comparative to that of the control (Fig. 1C), and second, the increase of collagen fiber in the intracellular space (Fig. 1B). All of this suggests a preliminary indication of a change to a synthetic state from a contractile state and therefore to a more dedifferentiated SMC. Further studies using differentiation markers will be necessary in order to prove this.

On the basis of no short-term accumulation of lipid droplets in the aorta, the short-term treatment chicks (T_{10}-5%) were chosen for isolating and culturing SMC. Electron microscopy of the cultured cells was made to compare possible ultrastructural alterations. After 3- or 4-d culture the cells did not show any appreciable difference (result not shown), but after 30 d, whereas the control cells remained normal (Fig. 2A), the cells from treated chicks (T_{10}-5%) became more rounded and contained numerous lipid inclusions, like foam cells (Fig. 2B). Most of the lipid inclusions in the lipid-loaded SMC were membrane-delimited, and nonmembrane-bound intracellular lipid inclusions were also present. No plasma membrane-associated lipid was observed; extracellular matrix of collagen fibrils and extracellular lipid droplets were present (Fig. 2B).

Serum lipid analysis. The high-cholesterol diet caused a significant increase in circulating total cholesterol and triglycerides. Figure 3 shows the profile of serum cholesterol concentration of the chicks used in the experiments. As can be seen, the levels were more than twice as high in chicks after

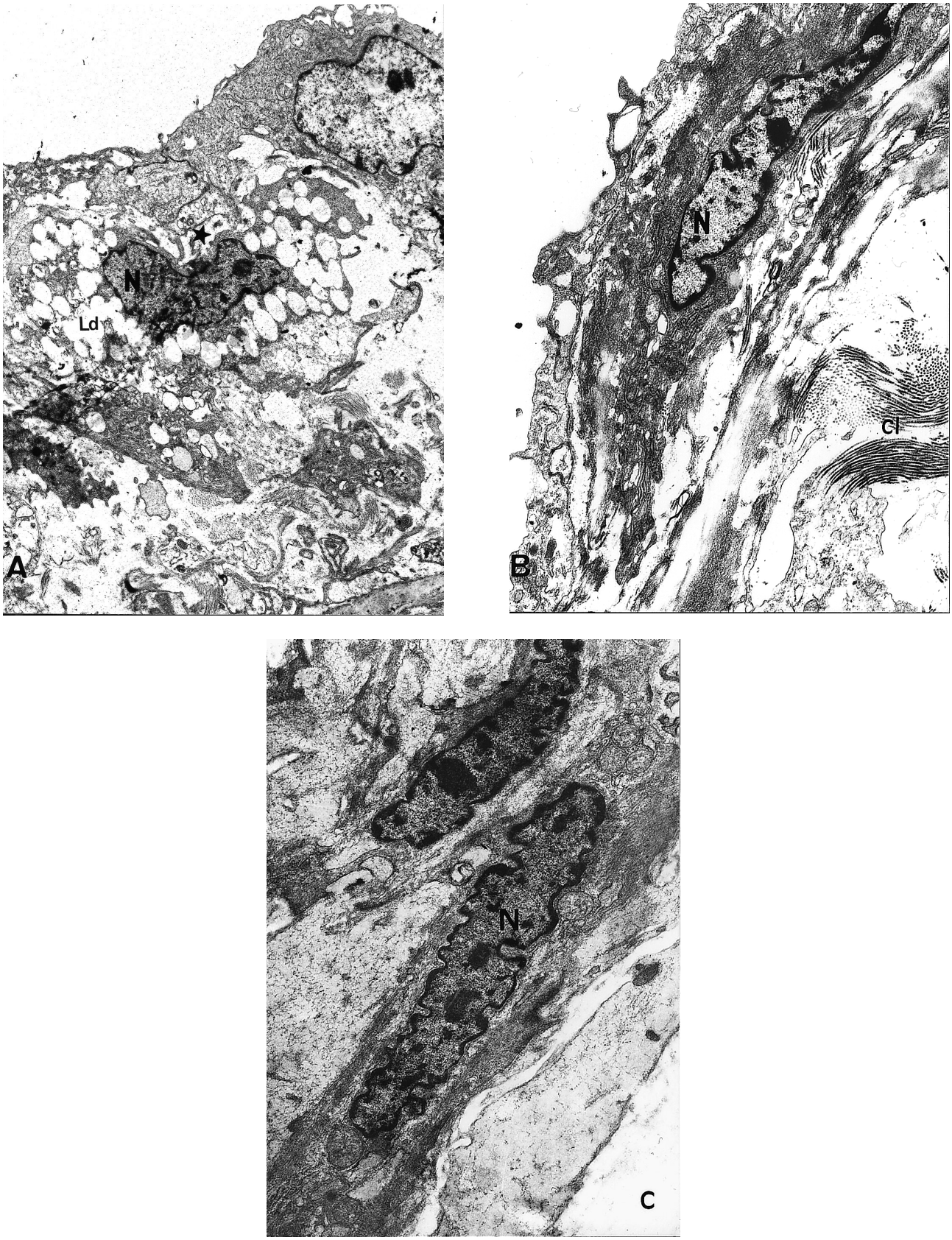

FIG. 1. Electron micrographs of aorta. (A) from T_{20}-5% chicks. Note the presence of smooth muscle cell (SMC)-like foam cells and focal aggregation in the intima (★), Ld = lipid droplets; (B) from T_{10}-5% chicks, cl = collagen fibers; N = nucleus. Original magnification, ×5.625, scale bar represents 183 nm; (C) from control chicks; N = nucleus. Original magnification, ×10.500, scale bar represents 350 nm.

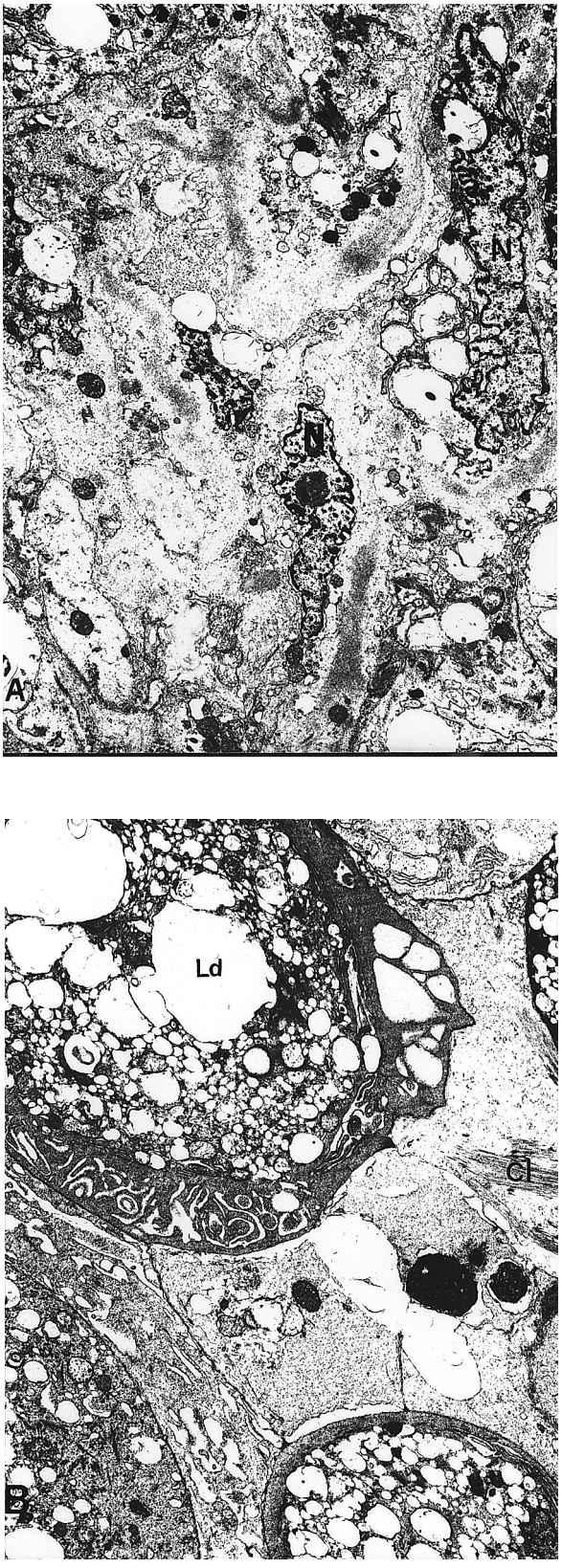

FIG. 2. Electron micrographs of SMC after 30 d in culture. (A) SMC isolated from control chicks. (B) SMC isolated from T_{10}-5% chicks. Ultrastructural morphology of SMC-like foam cells. Ld = lipid droplets; N = nucleus; cl = collagen fibrils. Original magnification, ×4.500, scale bar represents 150 nm. For other abbreviation see Figure 1.

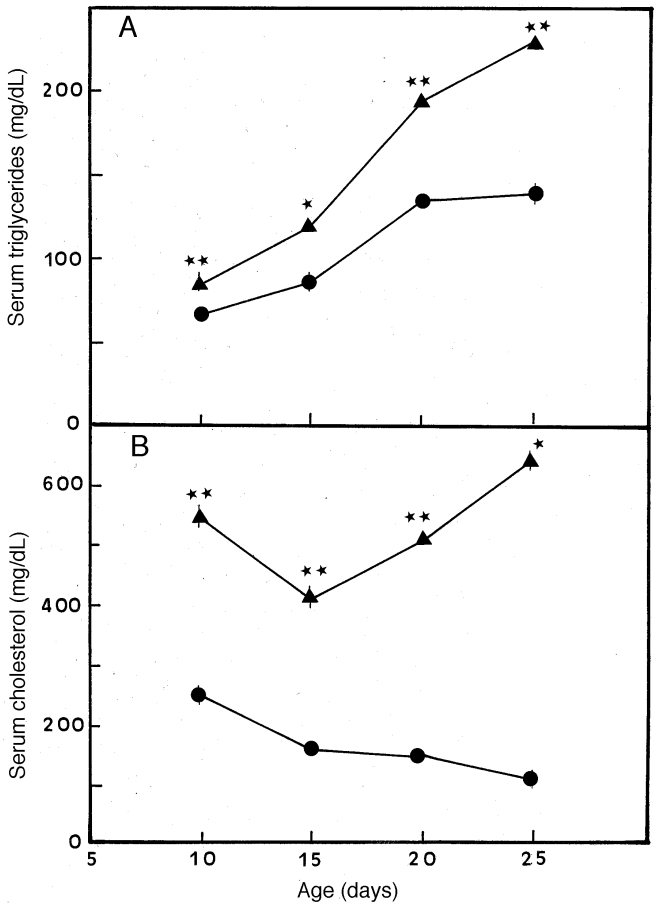

FIG. 3. Profiles of concentrations of serum triglycerides (A) and cholesterol (B) during the postnatal developments of two chick groups: control (●) and 5% cholesterol feeding (▲). Results are expressed as means ± SEM of five chicks. $*P < 0.001$, $**P < 0.0001$.

10 d of 5% cholesterol feeding compared with those on control diets. They decreased slightly 5 d afterward but increased significantly again (fivefold) until day 25 of cholesterol feeding. Figure 3 shows the evolution of triglyceride serum levels in the controls and those of 5% cholesterol feeding during 25 d after hatching. The levels in cholesterol-fed chickens were higher than the controls throughout the whole experimental period.

Intracellular lipid contents in SMC in culture. SMC were isolated and cultured from C_{10} and T_{10}-5% chick groups to obtain two lines of cultures, control-SMC, and cholesterol-SMC. The maintenance of the two lines of SMC cultures was identical, as described in the Materials and Methods section. DMEM and 10% FCS were used. The changes caused in the SMC triglyceride contents are shown in Figure 4. In the cholesterol-SMC cultures, there was an increase in triglycerides compared to the control-SMC. The triglyceride concentration of both the control-SMC and the cholesterol-SMC cultures increased during the first 18 d but decreased slightly afterward.

Although the SMC cholesterol content (Fig. 4) was the same and constant in both lines of cultures during the first

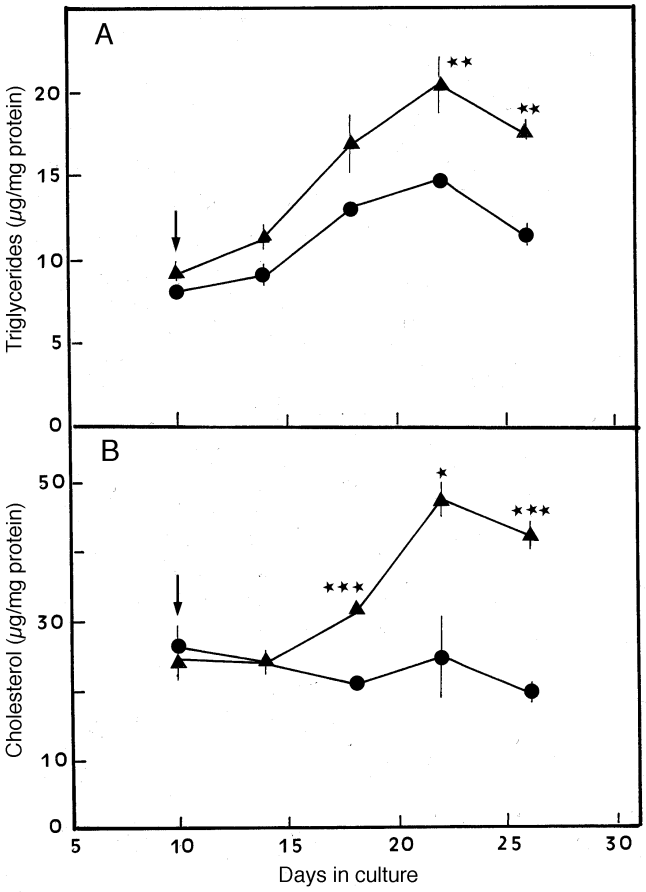

FIG. 4. Evolution of intracellular concentration of triglycerides (A) and cholesterol (B) during the period of culture. Control-SMC (●) and cholesterol-SMC (▲). After 10 d of explants, culture cells were seeded out in 150-cm^2 cell culture flasks (approximately 2.5×10^6 cells/flask), in 10 mL of Dulbecco's modification of Eagle's medium and 10% fetal calf serum (FCS). The arrows indicate the 0 d of culture of SMC (first passage). Results are expressed as means ± SEM of three determinations. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$. For abbreviation see Figure 1.

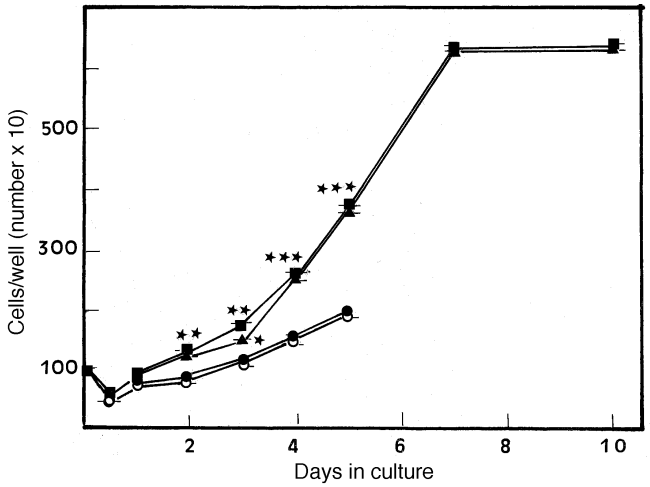

FIG. 5. Growth curves of SMC cultures derived from arteries: aortic arch from C_{10} (●); aortic arch from T_{10}-5% (▲); aorto-iliac bifurcation from C_{10} (○); aorto-iliac bifurcation from T_{10}-5% (■). Results are expressed as means ± SEM of three determinations. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$. For abbreviation see Figure 1.

14 d, the cholesterol-SMC increased afterward, rising to more than double the control-SMC after 22 d of culture.

Cell number and proliferation. We tested growth curves of four different SMC cultures obtained from the aortic arch and aorto-iliac bifurcation of control chicks and cholesterol-fed chicks. The growth curves were made by counting the number of cells. Analysis of growth curves (Fig. 5) showed that after a lag phase of 12 h the number of cells began to increase in the same way in the four SMC cultures. Both cholesterol-SMC from the aortic arches and those from the aorto-iliac bifurcation arteries began to increase at approximately double the rate of the control-SMC cultures.

Macromolecule synthesis in SMC cultures. Macromolecule synthesis in synchronized SMC cultures is shown in Figure 6, where the differences in DNA (A), RNA (B), and protein (C) synthesis can be seen between control-SMC and cholesterol-SMC cultures. As the maintenance of the two lines of cultures was the same, the differences in macromolecule synthesis must be due to the transformation of the SMC *in vivo* caused by cholesterol feeding. There was a minor effect on RNA synthesis compared to both DNA and protein synthesis, both of which increased substantially in the cholesterol-SMC cultures.

The DNA and protein biosynthesis peak occurred 8 h after the supplementation with 10% serum, the synthesis of DNA in cholesterol-SMC becoming fourfold more than that in the control-SMC cultures.

DISCUSSION

Arterial SMC proliferation is an important early event in atherosclerosis (2). Hypercholesterolemia develops atherosclerotic plaques in roosters fed upon a high-cholesterol diet (26–29). Thus, in this report we have focused our attention on developing a model of avian SMC cultures, isolated from control and cholesterol-fed chicks. These two lines of SMC were kept in culture under identical conditions to compare the molecular changes in SMC in the arterial wall *in vivo* induced by cholesterol. We have concluded that cholesterol feeding triggers proliferation of SMC. High-cholesterol blood concentration is a known cardiovascular risk factor, and for this reason a study of the effects of hypercholesterolemia on the behavior of vascular SMC in culture has been the main aim of our study.

The aortic arches of male chicks were chosen for this study for two reasons: first, for the ability of chicks to absorb high amounts of dietary cholesterol (39,40); and second, because intimal thickening in the aortas from these hypercholesterolemic chicks can be detected after only 20 d (41,42). This short diet period is very convenient and contrasts with those reported for other experimental animal models, mainly non-human primates (43) and rabbits (21), which must be fed a cholesterol-containing diet for between 1 and 18 mon to ob-

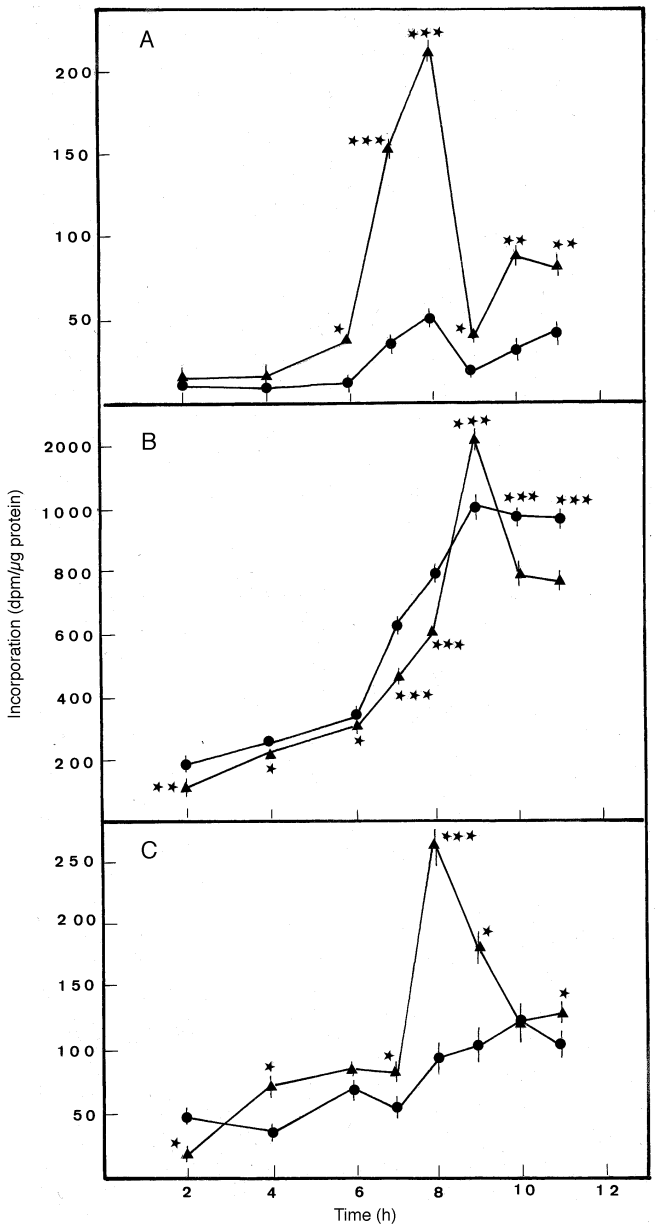

FIG. 6. SMC macromolecule synthesis. SMC were seeded out in 25-cm^2 cell culture flasks (approximately 0.5×10^6 cells/flask). Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (vol/vol) FCS. In log. linear (50% confluence) cultured cells were synchronized by serum deprivation (0.2% FCS during 48 h). For details see the Materials and Methods section. After the FCS stimulation, the cells were collected every hour and the [^{3}H]-*d*-thymidine incorporation into DNA for 15 min (A); [^{3}H]uridine incorporation into RNA for 15 min (B); and [4,5-^{3}H]leucine incorporation into proteins for 30 min (C) were determined in control-SMC (●) and cholesterol-SMC (▲). Results are expressed as means ± SEM of three experiments. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$. For abbreviations see Figures 1 and 4.

tain an atherosclerotic lesion. The short time of the chicken model is comparable with that necessary to induce mitotic activity in aortas of white carneau pigeons or swine (44,45). The high serum cholesterol levels observed in 10-day-old control chicks is normal because newly hatched chickens reabsorb the yolk during the first week of life (46), thus developing hypercholesterolemia, which becomes almost three times higher in cholesterol-fed chicks. The significantly elevated cholesterol and triglyceride plasma levels are in accordance with previously reported plasma lipids levels (39,47).

Our research has shown that medial SMC from chick aorta undergoes changes in primary culture when it is obtained from 10-day-old chicks fed on a 5% cholesterol diet compared to SMC cultures from control chicks. Although morphological and ultrastructural studies of the aortas before the explants for the cultures were performed did not show any apparent differences, the behavior of the cells in the cultures was very different. The migration of the cells from the explants was much faster in the cholesterol-SMC than in the control-SMC cultures. In addition, after 4 d in culture, the proliferation rate of the cholesterol-SMC in culture was twofold compared to the control-SMC, as measured by the number of cells. This difference in the proliferation is comparable with that described elsewhere between human SMC from nonatherosclerotic arteries and atheromatous plaques (48).

Furthermore, DNA synthesis was four times greater in the cholesterol-SMC than in the control-SMC in the S phase at 8 h of culture. The change in the DNA synthesis profile observed between the two lines of SMC cultures agrees with the differences presented in the growth rates of the cultures expressed by the growth curves and population doubling per day. These observations are in close accord with the data from SMC cultures obtained directly from human atheromatous plaque (49) and with another arteriosclerosis experimental model made by cultures of SMC derived from atherosclerosis-susceptible pigeons (50). Synchronization procedures frequently introduce abnormalities into the cell cycle, particularly with chemical agents such as thymidine and hydroxyurea (37), and so we used serum deprivation to arrest the cells at the start of DNA replication (S phase). Only deprivation for 48 h resulted in satisfactorily synchronized cell cultures. Serum deprivation for 12 or 24 h resulted in randomly growing cells (nonsynchronized).

The proliferation and DNA synthesis profiles of cholesterol-SMC are comparable with the tumorogenic properties found in transformed phenotype SMC of rats in long-term cultures (51,52) and the involvement of mutagen/carcinogen exposure in atherogenesis (53); to establish the effect of cholesterol feeding we have shown the differences vs. control-SMC.

Progressive thickening of the intima and development of atherosclerotic changes can be observed by electron microscopy after 20 d of 5% cholesterol-feeding chicks (T_{20}-5%), similar to that which has been found in a previous accelerated arteriosclerosis study (54), which showed a proliferation of spindle-shaped cells and an increase in the amount of fibrous connective tissue and lipid vacuoles in the cells. Nevertheless, electron microscopy of the arteries after 10 d of 5% cholesterol feeding (T_{10}-5%) did not yet show any lipid droplet accumulation. Thus, we report cholesterol treatment of a period as short as 10 d in the belief that this chick SMC cultures model is suitable for study at a molecular level for a very early atherogenesis process.

While we did not find any differences in the concentration of intracellular cholesterol between control-SMC and cholesterol-SMC during the first 14 d of culture, we have found clear differences after 15 d, this amount was doubled in cholesterol-SMC after 20 d. These results are consistent with our aim of isolating SMC from the arteries prior to the transformation into foam cells, and they agree well with the electron microscopy analysis, which showed a very similar morphology between arterial control and cholesterol-fed chicks after 10 d of treatment (Fig. 1B,C). Nevertheless, cholesterol-SMC developed the characteristics of macrophage-like foam cells after 30 d in culture under the same culture conditions as the control-SMC (Fig. 2). All of these results demonstrate a higher interiorization of cholesterol in the cholesterol-SMC than in the control-SMC cultures. Further studies on cholesterol metabolism will be necessary to look into this. SMC *in vivo* were shown to increase cholesterol biosynthesis significantly and decrease the intracellular cholesterol efflux associated with phenotype changes in SMC in culture (22,23). In this work, however, we are making a comparison with a control-SMC which underwent the same phenotype modulation in culture as the cholesterol-SMC. Thus, the changes found between the two lines of cultures must be due to cholesterol feeding of the chicks, and this suggests that the high-cholesterol diet might induce changes in the expression of the genes related to lipid metabolism, or greater expression of the low density lipoprotein-receptor gene, or perhaps the expression of a scavenger receptor similar to the ones that convert macrophages into foam cells (55). In addition, the great amount of collagen secreted into the cholesterol-SMC cultures (Fig. 1B) may also explain the formation of SMC foam cells. Collagen binds many molecules (56,57), including oxidized low density lipoprotein (58), which is deposited in collagen-rich tissues such as atherosclerotic plaques. The scavenger receptor might compete with collagen for binding oxidized low density lipoprotein (59). In fact, the formation of characteristic foam cells in arterial smooth muscle cells growing in cultures was very difficult to achieve except in cells growing on elastin membranes (24).

Despite all these molecular changes, it is possible that in feeding the animals with a cholesterol-rich diet we are selecting one of two different phenotypes that exist in the normal chicken aorta (26). This possibility could agree with the monoclonal hypothesis as to the origin of atherosclerotic plaques (60). In any case, SMC cultures from cholesterol-fed chicks show a "transformed" SMC and are a good model to study this and other questions concerning the alterations of the differentiated state of SMC caused by a cholesterol-rich diet in vascular disease. Further studies will be necessary to characterize these SMC cultures and to determine how the cholesterol-rich diet might cause molecular and genetic changes that trigger the transformation of the SMC in the atherogenic process induced by cholesterol.

ACKNOWLEDGMENTS

This work was supported partly by a grant from DGICYT (PB93-1160) and also from the Research grant from the Junta de Andalucía (Group code 3229). Electron microscopy was done at the Centro de Instrumentación Científica at the University of Granada. We thank our colleague Dr. J. Trout for revising the English text.

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[Received July 23, 1997, and in final revised form December 12, 1997; revision accepted December 15, 1997]

Catalytic Properties of Allene Oxide Synthase from Flaxseed (*Linum usitatissimum* L.)

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ABSTRACT: We investigated the catalytic and kinetic properties of allene oxide synthase (AOS; E.C. 4.2.1.92) from flaxseed (*Linum usitatissimum* L.). Both Michaelis constant and maximal initial velocity for the conversion of 9(*S*)- and 13(*S*)-hydroperoxides of linoleic and linolenic acid were determined by a photometric assay. 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD] as the most effective substrate was converted at 116.9 ± 5.8 nkat/mg protein by the flax enzyme extract. The enzyme was also incubated with a series of variable conjugated hydroperoxy dienyladipates. Substrates with a shape similar to the natural hydroperoxides showed the best reactivity. Monoenoic substrates as oleic acid hydroperoxides were not converted by the enzyme. In contrast, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid was a strong competitive inhibitor for AOS catalyzed degradation of 13(*S*)-HPOD. The inhibitor constant was determined to be 0.09 μM. Based on these results, we concluded that allene oxide synthase requires conjugated diene hydroperoxides for successful catalysis. Studying the enantiomeric preference of the enzyme, we found that AOS was also able to metabolize (*R*)-configurated fatty acid hydroperoxides. Conversion of these substrates into labile allene oxides was confirmed by steric analysis of the stable α-ketol hydrolysis products.

Lipids 33, 191–196 (1998).

Allene oxide synthase (AOS; hydroperoxide dehydratase, E.C. 4.2.1.92) is one of the key enzymes involved in the plant lipoxygenase pathway. This fatty acid metabolic pathway is assumed to be present in all plants (1). Formerly, allene oxide synthase was named "hydroperoxide dehydrase" (2) and, even before, "hydroperoxide isomerase" when it was first discovered in 1966 (3). Indeed, all three names offer aspects of the enzyme's catalytic properties. In plants, AOS is the second enzyme in the biosynthetic route leading to the plant hormone jasmonic acid. The starting substrate, linolenic acid, is oxygenated by lipoxygenase (LOX; E.C. 1.13.11.12) using molecular oxygen to yield 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*)-HPOT] (4). The resulting fatty acid hydroperoxide is dehydrated by AOS to form an intermediate, extremely labile allene oxide [12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid] (2,5). This intermediate compound, in turn, is cyclized enantioselectively into 9(*S*),13(*S*)-12-oxo-phytodienoic acid by allene oxide cyclase (6). Consecutive reduction and threefold β-oxidation yield the plant hormone jasmonic acid (4). Due to its instability, the allene oxide is also hydrolyzed by nucleophils such as water or methanol (7). The resulting products exhibit mainly an α-ketol structure [12-oxo-13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid], which initially was thought to result from direct enzymatic isomerization of the hydroperoxide (8). To a lesser extent, and depending on pH (9), there occurs also a γ-ketol structure [9-hydroxy-12-oxo-10(*E*),15(*Z*)-octadecadienoic acid] during hydrolysis of the allene oxide.

The first AOS described was the enzyme from flaxseed (*Linum usitatissimum* L.) (3). The purified enzyme was proved to be a specialized type of cytochrome P450 (*CYP74A*) by ultraviolet (UV) spectroscopy (10) and its amino acid sequence (11). Veldink and coworkers (12) have described flaxseed AOS to be specific for the 13-regioisomers of linoleic and linolenic acid hydroperoxides, whereas Feng and Zimmerman (13) have reported also the conversion of the 9-regioisomers, certainly by about 36-fold decreased maximal velocity. Baertschi *et al.* (14) reported that flaxseed AOS preferentially converted the (*S*)-enantiomer of a racemic mixture of 13-linolenic acid hydroperoxides. Based on these findings, we attempted to reinvestigate some catalytic properties of AOS from flaxseed using a broad series of different hydroperoxide substrates. In addition, we checked the enantiomeric preference of the enzyme against racemic mixtures of linoleic and linolenic acid hydroperoxides.

MATERIALS AND METHODS

Chemicals. Linoleic acid (99% pure) and (−)-menthoxychloroformate were purchased from Fluka (Neu-Ulm, Germany). Linolenic acid (99% pure) and soybean lipoxygenase (type I-

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Abbreviations: AOS, allene oxide synthase; ee, enantiomeric excess; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; 12-HPOD, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid; 9(*S*)-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 9-HPOE, 9-hydroperoxy-10(*E*)-octadecenoic acid; 10-HPOE, 10-hydroperoxy-8(*E*)-octadecenoic acid; 9(*S*)-HPOT, 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 13(*S*)-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; K_m, Michaelis constant; LOX, lipoxygenase; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet; V_{max}, maximum initial velocity.

S, L-8383) were from Sigma (Steinheim, Germany). High-performance liquid chromatography (HPLC) grade solvents, hexane, methanol, ethanol, *iso*-propanol, tetrahydrofuran (THF), and water were obtained from Merck (Darmstadt, Germany), and acetic acid from Baker Chemicals (Deventer, The Netherlands). All other chemicals were of analytical grade quality; solvents were redistilled before use.

Enzyme preparations. For preparation of AOS flaxseed (*L. usitatissimum* L.) (obtained from local market) was homogenized and the fine powder defatted with cold hexane. After evaporating the solvent *in vacuo*, the powder (80 g) was extracted with 800 mL of 0.1 M phosphate buffer (pH 7.0) at 0°C for 1 h. After centrifugation (15000 × *g*, 20 min), the supernatant was saturated up to 50% with solid ammonium sulfate and stirred for 1 h at 0°C. The precipitate was collected by centrifugation, dissolved in 50 mL of 0.1 M phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer. As shown by gas chromatography–mass spectrometry (GC–MS) analysis of products formed upon incubation, the hydroperoxide degradation activity was caused by AOS in the enzyme preparation. We did not detect the aldehydes as formed by the hydroperoxide lyase-catalyzed cleavage of substrates.

LOX isoenzymes L-2 and L-3b were purified from soybean seeds (*Glycine max* L. cv. Mapple Arrow) as described before (15) and freeze-dried after addition of 0.1 M sodium chloride for storage. Tomato (*Lycopersicon esculentum* Mill.) LOX was prepared according to the procedure described (16).

Protein was measured by the bicinchoninic acid method (17), using bovine serum albumin as standard protein.

Enzyme assay. AOS activity was monitored in a double-beam recording UV spectrophotometer (DMR21; Carl Zeiss, Oberkochen, Germany) at 234 nm (18). The assays were conducted at constant ambient temperature (22°C) in 2 mL of 0.1 M Tris-HCl buffer pH 7.4 in a 1-cm path-length quartz cuvette. Substrates were taken from 15-mM stock solutions in ethanol. Additional ethanol was added up to a constant concentration of 20 μL per cuvette. Absorbance was recorded for 2 min after addition of 20 μL [for 13(*S*)-hydroperoxides] or 50 μL [for 9(*S*)-hydroperoxides], respectively, of the dialyzed and diluted (1:80) enzyme solution. AOS activity was quantified as the initial rate of decrease in 234 nm absorbance. One unit (kat) is defined as conversion of 1 nmol of hydroperoxide substrate per second as measured in the photometric assay using the molar extinction coefficient of 25,000 $M^{-1}cm^{-1}$ for all conjugated hydroperoxides. Kinetic data were obtained from assays that used 0–80 μM substrate concentrations.

For inhibitor assays, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD] (0–60 μM) and 12-HPOD (0, 0.13, and 0.26 μM; taken from 0.13 mM stock solution in ethanol) were given individually into the cuvette. Again, the ethanol concentration was kept constant at 20 μL in the cuvette by adding additional ethanol when appropriate. Reaction was started by adding 40 μL of 1:80 diluted enzyme solution and was measured photometrically.

Preparation of hydroperoxide substrates. Linoleic {9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPOD] and 13(*S*)-HPOD} and linolenic {9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid [9(*S*)-HPOT] and 13(*S*)-HPOT} acid hydroperoxides were prepared as described previously using tomato and soybean LOX (16).

Enantiomeric 9- and 13-HPOD and -HPOT were obtained by enzymatic oxygenation of the fatty acids with soybean LOX isoenzymes L-2 and L-3b, respectively. One hundred mg of fatty acid was dissolved in 5 mL of ethanol and poured into 300 mL of 0.1 M phosphate buffer (pH 6.5). After the solution was saturated with oxygen, 10 mg of freeze-dried LOX (L-2 and L-3b, respectively) was added. The oxygenation was allowed to proceed for 2 h and was then terminated by lowering the pH to 3. After saturation with sodium chloride, the products were extracted with three portions of diethyl ether. Products were purified by column chromatography on silica gel (1 × 20 cm) using hexane/EtOAc/HOAc (85:15:1, by vol) as eluent. Regioisomers were separated by normal-phase HPLC on a preparative column (Lichrospher Si 60, 250 × 16 mm, 5 μm; Knauer, Berlin, Germany) with hexane/EtOH/HOAc (100:2:0.5, by vol) at a flow rate of 7.5 mL/min. Chromatography was monitored at 234 nm.

12-Hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid (12-HPOD) was prepared by singlet oxidation of linoleic acid (19). A 50 mL solution of 50 mg linoleic acid and 20 mg methylene blue in methanol was placed in a Pyrex photolysis well equipped with a side arm that allowed bubbling of oxygen gas through the solution during photolysis (300 W). The reaction mixture was cooled at −10°C. After photolysis was completed (24 h), the solvent was removed *in vacuo*, the residue was diluted in diethyl ether and purified by column chromatography on silica gel (1 × 20 cm) using hexane/EtOAc/HOAc (85:15:1, by vol) as eluent. The pooled hydroperoxide fraction was further purified by analytical scale normal-phase HPLC. Separation was performed on a 250 × 4 mm Eurospher Si 100 column (5 μm, Knauer, Berlin, Germany) with hexane/*iso*-PrOH/HOAc (100:1:0.1, by vol) as mobile phase at 1 mL/min flow rate. The UV detector was set at 205 nm.

For the synthesis of 9-hydroperoxy-10(*E*)-octadecenoic acid (9-HPOE) and 10-hydroperoxy-8(*E*)-octadecenoic acid (10-HPOE), oleic acid (50 mg) was diluted in 100 mL dichloromethane and poured into the photolysis well. After the addition of 2 mg of tetraphenylporphin, the solution was cooled to −10°C and bubbled through with oxygen. After 24 h of photolysis (300 W), the solvent was removed *in vacuo*; product work-up was carried out as described above.

The variable conjugated 3′(*Z*),5′(*E*)-7′-hydroperoxides **1b**–**5b** were obtained by soybean LOX-1 oxygenation of the corresponding 3′(*Z*),6′(*Z*)-pentadienes **1a**–**5a** (20). Work-up procedure of the products was performed as described above. The substrates for kinetic analyses (0–60 μM in 2 mL of 0.1 M Tris-HCl pH 7.4) were taken from 10 mM ethanolic stock solutions without further separation of regioisomers (or enantiomers). In the photometric assays, 50 μL of a 1:10 dilution of the dialyzed flax extract was used.

Chiral-phase HPLC of fatty acid hydroperoxides was carried out after reduction ($NaBH_4$) and methylation (etheral diazomethane) of the purified positional isomers. Enantiomers were separated by chiral-phase HPLC using a slightly modified procedure (21) for separation of methylated hydroxy-octadecadienoates. We used a Chiralcel OB-H column (250 × 4 mm, 5 µm; Daicel, Baker Chemicals) eluted with hexane/*iso*-PrOH 100+7 (by vol) at 0.5 mL/min flow rate.

Conversion of fatty acid hydroperoxides with AOS. Hydroperoxide samples in 5 mg amounts were incubated at ambient temperature with 7 mL of dialyzed flax extract in 100 mL of 0.1 M Tris-HCl buffer pH 7.4 constantly flushed with nitrogen. The following hydroperoxides were incubated individually: 13(*S*)- and 9(*S*)-HPOD, 13(*S*)- and 9(*S*)-HPOT, and enantiomeric 9- and 13-HPOD and -HPOT from L-2 and L-3b oxygenation of linoleic and linolenic acids, respectively. All products were extracted with diethyl ether after a reaction time of 15 min and termination with HCl up to pH 3.

For reversed-phase HPLC analysis of substrates and products a Spherisorb ODS2 column (250 × 4 mm, 5 µm; Knauer) was eluted with THF/methanol/water/HOAc (18:48:33.9:0.1, by vol) at 0.8 mL/min flow rate. Chromatography was monitored at UV 205 nm.

Steric analysis of ketol products. Steric analysis of the ketol products was carried out according to the procedure described by Hamberg (2). Briefly, 500 µg of the reaction products was taken up in 100 µL dry toluene, 20 µL dry pyridine and 100 µL (−)-menthoxychloroformate (1 µmol/µL in dry toluene), and incubated for 3 h at 60°C. The solvents were removed under a stream of nitrogen, and the material was dissolved in 300 µL acetic acid and treated with 15 mg $KMnO_4$ at 37°C for 1 h. The solution was diluted with brine and extracted with diethyl ether; the residue obtained after drying and evaporation was treated with etheral diazomethane. Authentic (*S*)-standards of methyl-2-hydroxy-heptanoate and dimethyl-2-hydroxy-sebacate were prepared by incubation of linoleic acid with soybean (Sigma) or tomato LOX, respectively. Resulting 13(*S*)- and 9(*S*)-HPOD were reduced with sodium borohydride, derivatized with (−)-menthoxychloroformate, cleaved by oxidative ozonolysis and finally methylated. Racemic and (*S*)-malic acid were also derivatized with (−)-menthoxychloroformate and methylated. GC analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a J&W DB-Wax column (15 m × 0.25 mm) (Folsom, CA) programmed from 50°C (3 min) to 240°C (15 min) at 4°C/min. GC–MS (EI, 70 eV) was performed on a Finnigan MAT44S (San Jose, CA) directly connected to a Varian (Palo Alto, CA) Aerograph 1440 gas chromatograph using identical GC conditions.

RESULTS

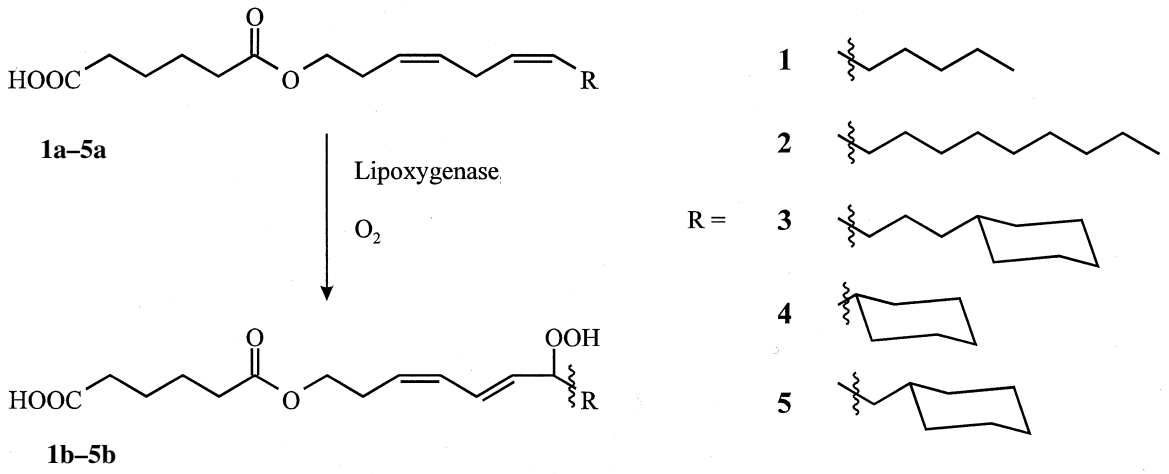
AOS kinetics. The spectrophotometric assay as described by Brash and Song (18) was used. The kinetic data for enzymatic dehydration of the four fatty acid hydroperoxides under study are summarized in Table 1. With a maximal initial velocity V_{max} of 116.9 ± 5.8 nkat/mg protein and a Michaelis constant K_m of 8.4 ± 1.2 µM, 13(*S*)-HPOD was the most effective substrate for flaxseed AOS. The 9-isomeric hydroperoxides reacted at comparable K_m but reduced V_{max}. We did not observe first-order kinetics for the substrate 13(*S*)-HPOT, but rather a sharp maximum of activity around 5 µM substrate that declined rapidly at concentrations higher than 10 µM, suggesting a mixture of overlapping enzymatic activities. This was probably caused by allene oxide cyclase which is also present in the flaxseed enzyme preparation (6). Allene oxide cyclase from corn (*Zea mays* L.) kernels was most effective at concentrations of 3–10 µM 13(*S*)-HPOT (6) and therefore would contribute noticeably—in addition to allene oxide hydrolysis—to the decline in absorbance at 234 nm. The enzymatic product, 9(*S*),13(*S*)-12-oxo-phytodienoic acid, contributes absorbance at 234 nm at a lesser extent than 13(*S*)-HPOT.

TABLE 1
Maximal Initial Velocity (V_{max}) and Michaelis Constant (K_m) for the Allene Oxide Synthase Catalyzed Dehydration of Fatty Acid Hydroperoxides[a,b]

Substrate	Maximal initial velocity (nkat/mg protein)	Michaelis constant (µM)
13(*S*)-HPOD	116.9 ± 5.8	8.4 ± 1.2
13(*S*)-HPOT	n.d.	n.d.
9(*S*)-HPOD	3.3 ± 0.05	3.4 ± 0.4
9(*S*)-HPOT	3.3 ± 0.17	8.6 ± 1.4

[a]The kinetic data were obtained by the photometric assay as described in detail in the Materials and Methods section. Substrates were used in 0–60 µM concentration in 2 mL 0.1 M Tris-HCl pH 7.4; the dialyzed flaxseed extract (2 mg/mL protein) was diluted 1:80 and 20 µL (for 13-isomers) or 50 µL (for 9-isomers) was used. All values are averages of a triplicate.
[b]Abbreviations: 9(*S*)-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 9(*S*)-HPOT, 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 13(*S*)-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; n.d., could not be determined.

Enzymatic conversion of variable conjugated 3′(Z),5′(E)-7′-hydroperoxides. Conjugated hydroperoxydienes **1b**–**5b** were obtained by enzymatic oxidation of the corresponding 3′(*Z*),6′(*Z*)-dienes **1a**–**5a** with soybean LOX-1 (Scheme 1) (20). Steric analysis of the hydroperoxy products (see Ref. 20) revealed a 4:1 ratio of 7′- to 3′-hydroperoxides for all substrates. The ratio of (*S*)- to (*R*)-enantiomer was about 80:20. The hydroperoxides were used in the photometric assay without further purification. The kinetic data of AOS catalyzed dehydration are summarized in Table 2. In this series, hydroperoxide **1b** is an analogon to 13(*S*)-HPOD, having a similar distal and proximal carbon chain length. Also, it was the substrate out of this series with lowest K_m (6.8 ± 1.0 µM) which was comparable to 13(*S*)-HPOD and highest V_{max} (26.7 ± 2.2 nkat/mg protein). Elongation of the distal carbon chain length (**2b**) led to depletion of the enzymatic reaction. When a bulky residue (substrates **4b** and **5b**) was connected directly to the conjugated hydroperoxy diene, there was no conversion of the substrates at all. Only when the cyclohexyl ring was moved in the distal direction (**3b**) was the enzymatic activity restored.



SCHEME 1

Inhibition of AOS activity. Photosensitized oxygenation of linoleic acid yielded a mixture of racemic 9-, 10-, 12-, and 13-hydroperoxy-octadecadienoic acids. Attempts to convert this crude substrate mixture with the flaxseed extract failed due to complete inhibition of enzymatic activity. We supposed one of the positional isomers to be responsible for the inhibition. Consequently, the substrate mixture was separated by normal-phase HPLC into the four hydroperoxy isomers by multiple injection. Purified 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid was tested for its ability to inhibit AOS. K_m and V_{max} for the dehydration of 13(*S*)-HPOD were determined at different fixed initial concentrations (0, 0.13, and 0.26 mM) of 12-HPOD in the photometric assay. As outlined in Table 3, increasing amounts of 12-HPOD led to increased values for K_m', whereas V_{max} was not or was only slightly affected. K_m' is indicated as apparent K_m in the presence of an initial concentration [I] of the inhibitor 12-HPOD. A Lineweaver-Burk plot of the obtained data (Fig. 1) showed typical criteria of a competitive inhibition: point of intersection of all three straight lines (0, 0.13, and 0.26 mM 12-HPOD) at $1/V_{max}$ with the y-axis and intersection at different $-1/K_m'$ with the abscissa. The inhibitor constant K_i was determined by a graphical method using secondary plots. Both types of secondary plots revealed a K_i of 0.09 μM 12-HPOD for the inhibition of AOS activity against 13(*S*)-HPOD.

Conversion of enantiomeric fatty acid hydroperoxides. Enantiomeric preference of AOS was studied using racemic fatty acid hydroperoxides derived from linoleic and linolenic acid. Enzymatic synthesis with LOX isoenzymes from soybean resulted in different enantiomeric compositions of the hydroperoxide products (Table 4). Only L-3b oxygenation yielded a genuine racemate for both 9- and 13-hydroperoxides, while L-2 produced predominantly 13(*S*)-hydroperoxides. Nevertheless, all synthesized substrates were used for conversion with the flax enzyme extract. The applied enantiomers [9(*R*,*S*)- and 13(*R*,*S*)-HPOD and -HPOT] were completely converted by the enzyme within 15 min. Enzymatic utilization of the substrates was proved by reversed-phase HPLC and thin-layer chromatography (TLC) analysis after extraction of the reaction mixture.

In order to prove dehydration of the (*R*)-hydroperoxides into the corresponding allene oxides, we studied the stereochemistry of the resulting α-ketol hydrolysis products. It is known from literature (2) that pure (*S*)-hydroperoxides are

TABLE 2
Maximal Initial Velocity (V_{max}) and Michaelis Constant (K_m) for the Allene Oxide Synthase Catalyzed Dehydration of Variable Conjugated 3′(*Z*),5′(*E*)-7′-Hydroperoxides[a]

Substrate	Maximal initial velocity (nkat/mg protein)	Michaelis constant (μM)
1b	26.7 ± 2.2	6.8 ± 1.0
2b	<1	n.d.[b]
3b	21.7 ± 7.3	21.3 ± 1.1
4b	—[c]	—
5b	—	—

[a]The substrate numbers **1b–5b** refer to Scheme 1. Details of the photometric assay are described in the Materials and Methods section. The assay contained 2 mL of 0.1 M Tris-HCl pH 7.4 and 50 μL of the dialyzed flaxseed extract in a 1:10 dilution, i.e., 10 μg protein per 2 mL cuvette. All values are averages of a triplicate.
[b]n.d., could not be determined.
[c]—, no conversion observed.

TABLE 3
Inhibition of Allene Oxide Synthase Catalyzed Dehydration of 13(*S*)-HPOD by 12-HPOD[a,b]

Concentration 12-HPOD (μM)	Maximal initial velocity (nkat/mg protein)	Michaelis constant (μM)
0	118.6 ± 4.5	9.4 ± 1.2
0.13	118.6 ± 4.4	24.6 ± 2.0
0.26	108.6 ± 6.7	32.6 ± 3.8

[a]V_{max} and K_m were obtained in the photometric assay at fixed initial concentrations of 12-HPOD (see the Materials and Methods section). All values are averages of a triplicate.
[b]Abbreviations: 12-HPOD, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid. For other abbreviations see Table 1.

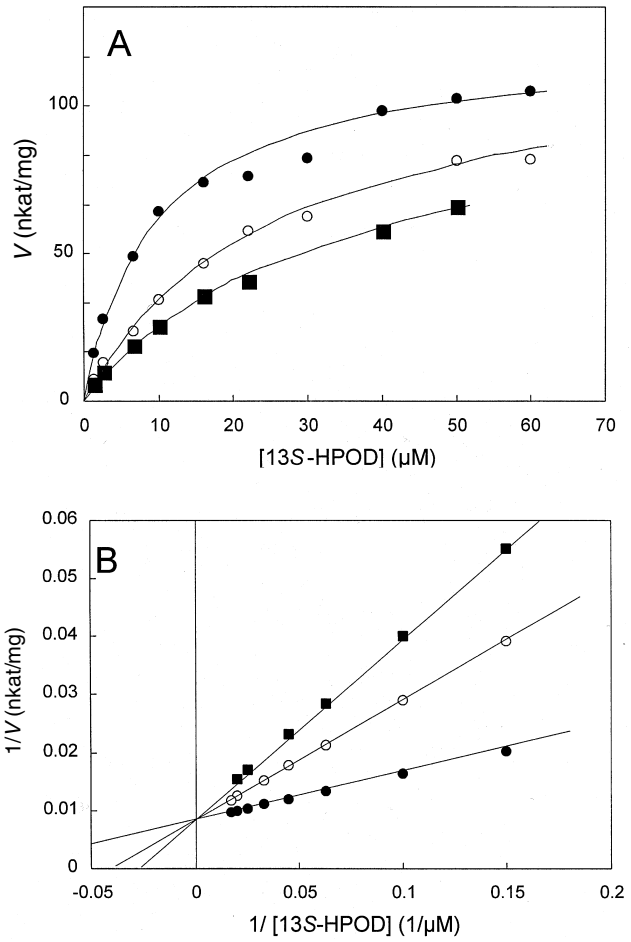


FIG. 1. Inhibition of allene oxide synthase catalyzed dehydration of 13(*S*)-HPOD by 12-HPOD. (A) Michaelis-Menten graph of *V* against [13(*S*)-HPOD] and (B) detail of the Lineweaver-Burk plot 1/*V* against 1/[13(*S*)-HPOD] at fixed concentrations of 12-HPOD (●, 0 μM, ○, 0.13 μM, and ■, 0.26 μM 12-HPOD). Abbreviations: 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 12-HPOD, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid; V, reaction velocity; kat, one unit, defined as conversion of 1 nmol hydroperoxide molar extinction coefficient of 25,000 $M^{-1}cm^{-1}$ for all conjugated hydroperoxides.

converted *via* nonenzymatic allene oxide hydrolysis into α-ketols exhibiting 70:30 (*R*) to (*S*) configuration. Based on this knowledge, one may expect a 1:1 ratio for α-ketols when applying racemic substrates. The results from these studies are summarized in Table 4. As demonstrated for 13-HPOD, the pure (*S*)-enantiomer was dehydrated and hydrolyzed into an α-ketol exhibiting 72:28 (*R*) to (*S*) configuration. Increasing the amount of (*R*)-hydroperoxides in the substrate mixture up to 50% of total hydroperoxides increased, in parallel, the amount of (*S*)-ketol products from 28 to 51%. A similar tendency was observed with the 13-HPOT substrates (see Table 4).

When oleic acid hydroperoxides (9- and 10-HPOE) were incubated with the flaxseed extract, no conversion of the substrates was observed. This was proved by TLC and reversed-phase HPLC after various incubation procedures (results not illustrated).

TABLE 4
Enantiomeric Composition of Substrates and α-Ketol Products in Allene Oxide Synthase Catalyzed Dehydration of Hydroperoxides from Linoleic (13-HPOD) and Linolenic Acid (13-HPOT)[a,b]

	Substrate[c]			α-Ketol[d]		
	13(*S*) (%)	13(*R*) (%)	ee (%)	13(*S*) (%)	13(*R*) (%)	ee (%)
13(*S*)-HPOD	98	2	96	28	72	44
13(*R,S*)-HPOD[e]	72	28	44	33	67	34
13(*R,S*)-HPOD[f]	50	50	0	51	49	2
13(*S*)-HPOT	94	6	88	34	66	32
13(*R,S*)-HPOT[e]	70	30	40	37	63	26
13(*R,S*)-HPOT[f]	50	50	0	43	57	14

[a]Five mg of each hydroperoxide was incubated with 14 mg of a dialyzed flaxseed protein extract in 100 mL of 0.1 M Tris-HCl pH 7.4 for 15 min. 13(*S*)-HPOD and -HPOT substrates contained about 5% of 9-hydroperoxides as estimated by analytical normal-phase high-performance liquid chromatography (HPLC). All (*R,S*)-hydroperoxides had been purified by preparative scale normal-phase HPLC and contained no 9-hydroperoxides.
[b]Abbreviations: ee, enantiomeric excess. For other abbreviations see Table 1.
[c]Enantiomeric composition determined by chiral-phase HPLC (see the Materials and Methods section).
[d]Enantiomeric composition determined by the gas chromatographic method as described in the Materials and Methods section.
[e]Obtained by L-2 oxygenation of fatty acid.
[f]Obtained by L-3b oxygenation of fatty acid.

DISCUSSION

We started our substrate studies by reexamining earlier work by Feng and Zimmerman (13) as well as by Veldink and coworkers (12). These authors have reported different results about the efficiency of 9-positional linoleic and linolenic acid hydroperoxides as substrates for flaxseed AOS. Our data confirm utilization of both 9- and 13-isomers by the enzyme, with the 9-isomer, however, being converted much more slowly. In fact, AOS reacted about 35 times faster with 13(*S*)-HPOD than with 9(*S*)-HPOD. The affinity of the substrates to the enzyme as shown by K_m was in the same range for the 13(*S*)- and 9(*S*)-hydroperoxides. The photometric assay we used was not suitable to determine the kinetic data of 13(*S*)-HPOT dehydration. We suppose this phenomenon to be caused by interference with allene oxide cyclase activity, which was also present in the enzyme preparation (22).

We extended the substrate investigations to a series of variable conjugated 3′(*Z*),5′(*E*)-7′-hydroperoxides. The chosen substrate feature allowed variations in the distal residue of the conjugated hydroperoxy diene moiety. Only those substrates with a shape similar to linoleic acid were converted at considerable rates by flax AOS.

The two oleic acid hydroperoxides, 9- and 10-HPOE, which show a monoene hydroperoxy moiety, were not converted into ketol fatty acids by the enzyme extract at all. This fact demonstrated the requirement of a 2,4-diene system conjugated to the hydroperoxy moiety for successful catalysis. Monoenoic fatty acid hydroperoxides do not serve as substrates for flaxseed AOS.

Interestingly, 12-HPOD was also no substrate for flax AOS. In fact, there is only one report in the literature about

the enzymatic conversion of 12-regioisomeric fatty acid hydroperoxides. Gardner and coworkers (23) have reported the cleavage of 12-HPOT by another fatty acid hydroperoxide degrading enzyme, hydroperoxide lyase, although the products of this reaction were not specifically defined. This substrate, however, also contains a conjugated hydroperoxy-diene moiety. When we incubated substrate mixtures containing 12-HPOD, we found no utilization of the substrates by the enzyme, but rather complete depletion of activity. Kinetic analyses revealed 12-HPOD as a strong competitive inhibitor of AOS. Like the oleic acid hydroperoxides, 12-HPOD does not exhibit a conjugated diene moiety. This also indicates the necessity of a conjugated hydroperoxy diene system for successful enzymatic conversion.

In order to prove dehydration of (*R*)-configurated hydroperoxides, mixtures of racemic regioisomeric HPOD and HPOT were incubated with AOS. By stereochemical analysis of the ketol hydrolysis products, we demonstrated the formation of (*R*)-configurated allene oxides by AOS. Using an excess of enzyme activity led to complete utilization of the substrates. This observation is in agreement with previous findings (14) in which enantiomeric selectivity for the (*S*)-enantiomers with sufficiently small amounts of enzyme has been described. Interestingly, a similar selectivity was reported recently for an enzyme named divinyl ether synthase from garlic bulbs (24). This activity dehydrated fatty acid hydroperoxides into a divinyl ether moiety. The enzyme reacted much more quickly with 13(*S*)-HPOD than with 13(*R*)-HPOD.

ACKNOWLEDGMENT

This work was funded by Deutsche Forschungsgemeinschaft, Bonn, Germany (Schr 211/17-1).

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[Received November 10, 1997; and in final revised form December 29, 1997; revision accepted December 29, 1997]

Positional Analysis of Triacylglycerols from Bovine Adipose Tissue Lipids Varying in Degree of Unsaturation

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ABSTRACT: The objective of this study was to demonstrate that changing the fatty acid composition of bovine adipose tissue concurrently changed (i) proportions of triacylglycerol species, (ii) fatty acid composition of triacylglycerol species, and (iii) positional distribution of the component fatty acids of the triacylglycerol species. To achieve this, we took advantage of adipose tissue lipids, from cattle fed in Australia and Japan, that varied widely in fatty acid composition and melting points. Treatment groups produced in Australia were cattle fed: a corn-based diet (MUFA1); a grain-based diet containing whole cottonseed (SFA); a grain-based diet containing protected cottonseed oil (PUFA); and a grain-based diet that resulted in high contents of *trans* fatty acids (TFA). Treatment groups produced in Japan (MUFA2 and MUFA3) were diets of unknown composition fed for over 300 d. The MUFA1, MUFA2, and MUFA3 samples all were rich in monounsaturated fatty acids, varying only in the proportions of the individual monounsaturates. The SFA, PUFA, and TFA samples had relatively high concentrations of stearic acid (18:0), PUFA, and TFA, respectively. Slip points (indicative of melting points) were 45.1, 41.5, 38.5, 30.7, 28.4, and 22.8°C, for the SFA, TFA, PUFA, MUFA1, MUFA2, and MUFA3 groups, respectively ($P < 0.05$). Triacylglycerols were separated by high-performance liquid chromatography on a silver nitrate-impregnated column into *sn*-1,2,3-saturated fatty acid triacylglycerol (SSS); [triacylglycerols containing two saturated acids and one *trans*-monounsaturated fatty acid (SSM*t* *sn*-positions unknown)]; *sn*-1-saturated, 2-monounsaturated, 3-saturated triacylglycerol (SMS); *sn*-1-saturated, 2-monounsaturated, 3-*trans*-monounsaturated triacylglycerol (SMM*t*); *sn*-1-saturated, 2,3-monounsaturated fatty acid triacylglycerol (SMM); *sn*-1-saturated, 2-polyunsaturated, 3-*trans*-monounsaturated triacylglycerol; *sn*-1,2,3-monounsaturated fatty acid triacylglycerol (MMM); and *sn*-1-saturated, 2-polyunsaturated, 3-monounsaturated triacylglycerol. Fatty acid methyl esters of each triacylglycerol species also were determined, and further analysis indicated *sn*-2, and *sn*-1/3 positions. As the percentage oleic acid increased in the total lipid extract, the proportions of SMM and MMM increased (e.g., from 31.4 and 2.4% in the SFA group to 55.4 and 17.8% in the MUFA3 group). The elevated 18:0 in the SFA group (26%) was reflected in increased percentages of SSS and SSM, and caused an increase in the proportion of 18:0 in all triacylglycerol species relative to the other treatment groups. The percentage of 18:0 in the *sn*-1/3 positions was elevated markedly in the SMS fraction of the SFA group (to 44%); this would account for the high melting point of the fat of these animals. We conclude that long-term feeding of cattle is sufficient to produce significant alterations in fatty acid composition in bovine adipose tissue. Alterations in the fatty acid composition of bovine adipose tissue changed both the distribution and the composition of the triacylglycerol species, which, in turn, accounted for marked differences in melting points among treatment groups.

Lipids 33, 197–207 (1998).

There is a renewed interest in the positional distribution of fatty acids within the triacylglycerol structure, as this appears to influence the metabolic effects of dietary fatty acids. For example, stearic acid (18:0) located in the *sn*-1/3 positions of triacylglycerols is absorbed poorly (1), which may explain its hypocholesterolemic nature. Also, there is a greater proportion of palmitic acid (16:0) in the *sn*-2 position of postprandial plasma, liver, and adipose tissue triacylglycerols in young piglets fed sow's milk (enriched with *sn*-2 16:0) than in piglets fed formula with 16:0 in the *sn*-1,3 position (2). Finally, the absorption of long-chain, polyenoic fatty acids apparently is not affected by positional distribution in triacylglycerols (3). Rather, the positional distribution of long-chain polyenoic fatty acids influences the rate of chylomicron clearance (4). These results suggest that positional distribution of fatty acids, in combination with their degree of saturation, could have a significant impact on the cholesterolemic nature of dietary fatty acids.

Cattle raised in Australia for the Japanese market are fed for long periods of time, and exhibit a high incidence of carcasses that are unacceptable for hand boning owing to excessively hard fat (5). Certainly, an increase in the adipose tissue concentration of 18:0 contributes to this problem (5,6). Re-

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Abbreviations: HPLC, high-performance liquid chromatography; MMM, *sn*-1,2,3-monounsaturated fatty acid triacylglycerol; MUFA1, MUFA2, and MUFA3, samples containing elevated monounsaturated fatty acids; PUFA, samples containing elevated polyunsaturated fatty acids; SFA, samples containing elevated saturated fatty acids; SMM; *sn*-1-saturated, 2,3-monounsaturated fatty acid triacylglycerol; SMM*t*, *sn*-1-saturated, 2-monounsaturated, 3-*trans*-monounsaturated triacylglycerol; SMS, *sn*-1-saturated, 2-monounsaturated, 3-saturated triacylglycerol; SPM, *sn*-1-saturated, 2-polyunsaturated, 3-monounsaturated triacylglycerol; SPMt, *sn*-1-saturated, 2-polyunsaturated, 3-*trans*-monounsaturated triacylglycerol; SSM*t*, triacylglycerols containing 2 saturated and 1 *trans*-monounsaturated fatty acid (*sn*-positions unknown); SSS, *sn*-1,2,3-saturated fatty acid triacylglycerol; TFA, samples containing elevated *trans* fatty acids.

cently, it has become apparent that positional distribution of saturated fatty acids (SFA) also may be important in determining fat hardness. Placement of 18:0 in the outer position of triacylglycerols can increase the melting point by as much as 10°C relative to triacylglycerols with 18:0 solely in the *sn*-2 position (7). This may exacerbate the fat hardness problem in Australian beef production. To address this, lipid samples, obtained from cattle raised in Australia and Japan under widely varying production conditions (and differing by more than 20°C in melting point), were analyzed for proportions of individual triacylglycerol species and the fatty acid positional distribution of each of these species.

MATERIALS AND METHODS

Animals. Treatment groups were classified as: three groups whose fat contained relatively high levels of monounsaturated fatty acids, MUFA1 ($n = 4$), MUFA2 ($n = 6$), and MUFA3 ($n = 6$); a group whose fat contained relatively high levels of 18:0 (SFA, $n = 6$); a group whose fat contained relatively high levels of linoleic acid (18:2n-6) [polyunsaturated fatty acid (PUFA), $n = 4$]; and a group whose fat contained relatively high levels of 18:1*t*11 [*trans* fatty acid (TFA), $n = 4$]. The cattle were fed in either Australia (MUFA1, SFA, TFA, and PUFA) or Japan (MUFA2 and MUFA3). The MUFA1 cattle were fed a corn-based diet in southern Australia for approximately 300 d, whereas the SFA group was fed a grain-based diet containing approximately 10% whole cottonseed for >400 d. The PUFA group was fed casein-formaldehyde-protected cottonseed oil for 100 d, and the TFA cattle were fed a grain-based diet for 150 d. The diets of the cattle fed in Japan are unknown, but the cattle were fed these diets for as long as 2 yr postweaning. A typical finishing diet in Japan contains flaked corn, barley, wheat and/or rice bran, and soybean meal (8). The MUFA2 cattle were Murray Grey, whereas the MUFA3 cattle were purebred Japanese Black steers produced in the Osaka region. The MUFA1, SFA, TFA, and PUFA animals were typical crossbred Murray Grey, Angus, and Grey Brahman steers.

Assignment to treatment groups was based on previous measurements of total fatty acids on stored adipose tissue samples (Tume, R.K., unpublished observations). Sample groups were chosen to give as wide a range of fatty acid compositions as possible, although the MUFA groups also were separated based upon differences in breed type and production location. Each group represented a distinct location in Japan and/or a distinct production diet.

Slip points. Subcutaneous adipose tissue was collected from the loin region of carcasses after overnight chilling. Lipids were extracted into chloroform/methanol (9). Slip points were measured to verify that lipid extracts from each treatment group differed in hardness. Solvents were removed exhaustively by heating the lipid samples to 60°C under a nitrogen jet. The lipids were drawn 1 cm into capillary tubes while still warm. Triplicate capillary tubes were collected for each sample. The samples in the capillary tubes were stored overnight at 4°C, and then placed vertically in a chilled water bath. The temperature was increased gradually in the water bath (1–2°C/min), and the temperature at which the lipid began to move up the capillary tube (slip point) was recorded. Within-sample variation was less than 1%.

Separation of triacylglycerol species. Triacylglycerols were separated from the total lipid extracts into molecular species by silver ion high-performance liquid chromatography (HPLC) using the method of Christie (10). Triacylglycerol species were separated on a Nucleosil SA 5-micron silica column (Waters Corp., Milford, MA) (4.6 × 250 mm) impregnated with 5% silver nitrate using a Waters HPLC gradient system (Waters Corp.) coupled to an evaporative light-scattering detector (Varex Mark III; Alltech, Deerfield, IL). Approximately 30% of the column eluant passed through the detector, the remainder being split off for collection of individual fractions. The proportions of each triacylglycerol species were quantified using Waters Millennium software, and the mass of each fraction collected was calculated. Owing to the hardness of the SFA samples, the carousel chamber of the sample injector (Waters 717 Sampler Injector) was warmed to the keep the samples in solution prior to injection. For each animal, the triacylglycerol fractions were collected from 3–5 separate runs to provide enough lipid in each fraction (approximately 20 µg) to allow for analysis of fatty acid positional distribution.

Lipase digestion. Lipase digestions were as described (11,12) with modifications. Whereas Williams *et al.* (12) digested approximately 2 mg (2 µmol) lipid with 100–120 units lipase from *Rhizopus arrhizus* (Boehringer Mannheim, Indianapolis, IN), we digested at most 20 µg lipid with 200–250 units of lipase. The triacylglycerol fractions were dried and emulsified in 1 mL of buffer containing 1 mg/mL Triton X-100, 0.04 M Tris (pH 7.2). Borate (0.05 M) was included to reduce positional migration (12). The fractions were sonicated for 1–2 min to ensure complete emulsification. One-half of each fraction was transferred to a separate tube, to which was added approximately 200 units of lipase from *R. arrhizus*, which removes only the fatty acids in the *sn*-1/3 positions (11,12). The fractions were digested with lipase for 60 min at 37°C (increased from 30 min at 22°C; 12). That portion of the sample which did not receive lipase was incubated under the same conditions. Reactions were terminated by the addition of 0.5 mL 1 N acetic acid and 3 mL chloroform/methanol (2:1, vol/vol). The lipids were extracted three times with chloroform/methanol. Reaction conditions were tested with 5 to 200 µg total lipid; reducing the amount of lipid or digesting for longer periods of time did not increase enrichment at the *sn*-2 position.

Trans-*methylation.* The solvent portion, containing the lipids, was dried exhaustively under a nitrogen gas jet at 60°C to eliminate all traces of solvent and acetic acid, and the remaining glycerides were *trans*-methylated by incubating for 30 min at 65°C in 1 mL 0.1 N NaOH in methanol. Lipids were extracted with 3 × 3-mL vol of hexane. The hexane was evaporated, and the samples were redissolved in 200 µL

hexane. A total of 6 to 8 μL of each sample was used for gas–liquid chromatography for the analysis of fatty acid methyl esters. Fatty acid methyl esters were separated by gas chromatography [Shimadzu GC17 (Kyoto, Japan) with AOC17 autoinjector] using a 50 m × 0.25 mm glass capillary column (CP-Sil-88, ChromPak, Middleburg, The Netherlands) with nitrogen as carrier gas. The initial temperature was set at 150°C, incrementing at 5°C/min to 235°C and held at that temperature for 8 min. Fatty acids were identified by comparison of retention times with standards (Alltech).

The NaOH/methanol methylation procedure does not methylate nonesterified fatty acids. Therefore, the fatty acid composition of the lipase-digested and undigested (total) fractions was used to calculate the average composition of fatty acids in the *sn*-1/3 positions (12):

$$\text{average } sn\text{-}1/3\ \% = (3 \times \%\ \text{fatty acid in total lipids}) - (2 \times \%\ \text{fatty acid in } sn\text{-}2\ \text{position}) \quad [1]$$

Statistics. Data were analyzed by single-factor analysis of variance by the Super Anova program (Abacus Concepts, Inc., Berkeley, CA), with treatment group as the main effect. Means were separated by the Fisher's Protected LSD method contained in the same software program. Differences between means were considered significant at $P < 0.05$.

RESULTS

Total fatty acid compositions. The SFA group exhibited an unusually high percentage of 18:0 (26%), whereas the TFA group had a high concentration of 18:1*t*11 (11%; Table 1). Lipids from the MUFA1, MUFA2, and MUFA3 groups contained 49 to 53% oleic acid (18:1), and those of the MUFA1 and MUFA3 groups were especially high in palmitoleic acid (16:1; >5%). The PUFA group was remarkable for its high concentration of 18:2 (over 4%), which is unusual for bovine adipose tissue (13–15).

In general, lipase digestion resulted in an enrichment of 18:1 and 18:2 at the *sn*-2 position (Table 1). Conversely, 16:0 was enriched primarily at the *sn*-1/3 positions. As the percentage of 18:0 was increased by dietary regimen, the proportion of 18:0 observed in the *sn*-1/3 positions increased. The MUFA2 group was unique in that the *sn*-2 position was more enriched in 18:0 than the *sn*-1/3 positions.

Slip points. As anticipated, slip point temperatures were highest for the SFA and TFA groups (45.1 and 41.5°C, respectively; Table 1). The PUFA group displayed a relatively

TABLE 1
Total, *sn*-2, and Average *sn*-1/3 Adipose Tissue Fatty Acid Compositions (wt%) and Slip Points (°C) for Each Treatment Group

	Fatty acid[a]												Slip
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3	points (°C)
Total[b]													
MUFA1	1.6	0.6^{b}	23.5	5.2^{a}	1.0^{c}	1.0^{a,b}	10.5^{c,d}	2.5^{b,c}	49.3^{b,c}	2.5^{b}	1.8^{b,c}	0.4	30.7^{d}
MUFA2	1.4	0.6^{b}	22.3	3.9^{a,b}	1.0^{c}	0.7^{b,c}	12.2^{c}	3.4^{b,c}	49.4^{a,b}	1.9^{c}	2.6^{b}	0.5	28.4^{d}
MUFA3	1.3	1.3^{a}	24.2	5.2^{a}	0.4^{d}	1.1^{a}	7.6^{d}	0.7^{d}	52.9^{a}	3.0^{a}	2.0^{b,c}	0.2	22.8^{e}
SFA	1.5	0.1^{c}	24.2	1.6^{c}	1.3^{b}	0.1^{d}	26.1^{a}	2.3^{c}	39.8^{d}	1.0^{d}	1.6^{c}	0.5	45.1^{a}
PUFA	1.8	0.2^{b,c}	23.8	2.8^{b,c}	1.1^{b,c}	0.4^{c,d}	17.7^{b}	3.7^{b}	42.4^{c,d}	1.3^{d}	4.5^{a}	0.2	38.5^{c}
TFA	2.0	0.5^{b,c}	26.3	2.2^{b,c}	1.7^{a}	0.5^{c}	20.0^{b}	11.1^{a}	32.4^{e}	1.0^{d}	2.2^{b,c}	0.1	41.5^{b}
Pooled SD	1.1	0.5	3.7	2.0	0.4	0.4	7.0	3.3	7.7	0.8	1.1	0.5	8.2
sn-2													
MUFA1	1.6	0.6^{a,b}	21.5	5.3^{a,b}	0.6	0.7^{a}	10.2^{c,d}	3.0^{b}	51.6^{c}	3.0^{a}	1.5^{c}	0.1	
MUFA2	0.6	0.1^{b}	17.4	2.1^{c}	0.1	0.1^{b}	14.4^{b,c}	1.6^{b,c}	59.7^{a,b}	1.0^{b}	2.4^{b,c}	0.1	
MUFA3	2.0	1.5^{a}	15.5	5.6^{a}	0.1	0.2^{a,b}	7.6^{d}	0.4^{c}	61.8^{a}	1.4^{b}	3.3^{b,c}	0.2	
SFA	2.0	0^{b}	19.9	2.2^{c}	0.3	0^{b}	21.2^{a}	1.9^{b,c}	49.5^{c,d}	0.5^{b}	2.1^{c}	0	
PUFA	0.7	0^{b}	17.3	2.3^{c}	0.6	0^{b}	17.2^{a,b}	1.9^{b,c}	53.0^{b,c}	0.4^{b}	6.2^{a}	0	
TFA	3.9	0.4^{b}	20.1	2.8^{b,c}	0.5	0.2^{a,b}	15.7^{b}	7.4^{a}	43.3^{d}	0.5^{b}	3.9^{b}	0.6	
Pooled SD	2.0	0.8	3.8	2.3	0.5	0.4	5.4	2.5	8.0	1.2	1.9	0.5	
Average *sn*-1/3													
MUFA1	1.6^{a,b}	0.1	32.8	4.0^{a}	1.7^{c,d}	1.2^{a,b,c}	12.9^{c,d}	4.5^{b,c}	34.6^{a}	3.6^{b,c}	1.9	0.5	
MUFA2	2.9^{a}	1.4	31.8	7.5^{a}	2.6^{a,b,c}	1.7^{a,b}	7.7^{d}	6.9^{b}	28.7^{a,b}	3.8^{b}	2.9	1.4	
MUFA3	–0.1^{a,b}	0.8	41.5	4.4^{a}	1.0^{d}	2.5^{a}	7.5^{d}	1.1^{d}	35.0^{a}	6.1^{a}	–0.5	0.3	
SFA	0.4^{a,b}	0.2	32.6	0.4^{b}	3.2^{a,b}	0.2^{c}	35.5^{a}	3.1^{c,d}	20.1^{a,b}	1.7^{c}	0.6	1.5	
PUFA	3.8^{a}	0.7	36.7	3.8^{a}	2.0^{b,c,d}	1.1^{b,c}	18.7^{b,c}	7.2^{b}	21.0^{a,b}	2.9^{b,c}	1.0	0.7	
TFA	–2.1^{b}	0.6	38.6	0.7^{b}	3.9^{a}	1.0^{b,c}	28.4^{a,b}	18.2^{a}	10.5^{b}	2.0^{b,c}	–1.3	–0.9	
Pooled SD	3.2	1.2	10.3	3.9	1.3	1.2	12.8	5.7	15.6	2.1	2.6	1.9	

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a particular fatty acid.
[b]MUFA1, MUFA2, MUFA3, samples containing elevated monounsaturated fatty acids; SFA, samples containing elevated saturated fatty acids; PUFA, samples containing elevated polyunsaturated fatty acids; TFA, samples containing elevated *trans* fatty acids; SD, standard deviation.

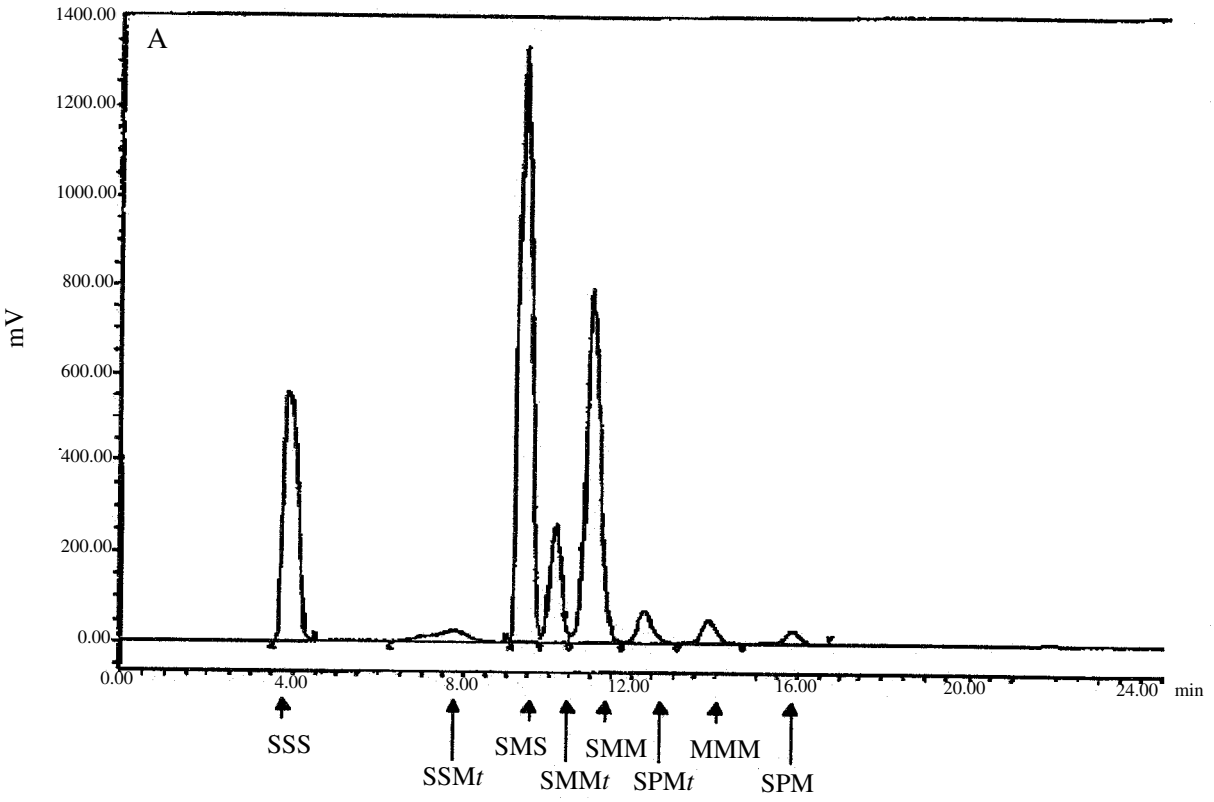

FIG. 1. Chromatograms from high-performance liquid chromatographic separation of triacylglycerol species from lipids extracted from SFA (A) and MUFA3 (B) adipose tissue tissue samples. Triacylglycerol species were separated on a Nucleosil SA 5-micron silica column (4.6 × 250 mm) impregnated with 5% silver nitrate. Abbreviations: SFA, samples containing elevated saturated fatty acids from long-fed Australian cattle; MUFA3, samples containing elevated monounsaturated fatty acids from long-fed Japanese Black cattle. Major positional species identified: SSS, *sn*-1,2,3-saturated fatty acid triacylglycerol; SSM*t*, triacylglycerols containing two saturated and one *trans*-monounsaturated fatty acid; SMS, *sn*-1-saturated, 2-monounsaturated, 3-saturated triacylglycerol; SMM*t*, *sn*-1-saturated, 2-monounsaturated, 3-*trans*-monounsaturated triacylglycerol; SMM, *sn*-1-saturated, 2,3-monounsaturated fatty acid triacylglycerol; SPM*t*, *sn*-1-saturated, 2-polyunsaturated, 3-*trans*-monounsaturated triacylglycerol; MMM, *sn*-1,2,3-monounsaturated fatty acid triacylglycerol; SPM, *sn*-1-saturated, 2-polyunsaturated, 3-monounsaturated triacylglycerol. The identities of the peaks are indicated below each chromatogram. The SSM*t* peak was undetectable in this MUFA3 sample. The MUFA3 sample was eluted several days after the SFA sample, and elution times had changed somewhat.

high slip point (38.5°C). The lowest slip point was exhibited by the MUFA3 group (22.8°C). The MUFA1 and MUFA2 samples were nearly identical in slip point temperatures.

Proportions of triacylglycerol species. The order of elution of triacylglycerols from the HPLC column was those containing: three saturated fatty acids (SSS); two saturated plus a *trans*-monounsaturated fatty acid, 18:1*t*11 (SSM*t*); two saturated plus a *cis*-monounsaturated fatty acid (SMS); one saturated plus a *cis*-monounsaturated fatty acid plus a *trans*-monounsaturated fatty acid (SMM*t*) (composition somewhat variable); one saturated plus two *cis*-monounsaturated fatty acids (SMM); one saturated plus a combination of polyunsaturated and *trans*- and *cis*-monounsaturated fatty acids (SPM*t*); three *cis*-monounsaturated fatty acids (MMM); and one saturated, one polyunsaturated, and one *cis*-monounsaturated fatty acid (SPM) (Fig. 1).

The SMM fraction constituted 47 to 48% of the triacylglycerols for the MUFA1 and MUFA2 groups, and was greatest ($P < 0.05$) for the MUFA3 group (55%) (Fig. 2). The MUFA3 group had the greatest percentage MMM triacylglycerols (nearly 18%). The PUFA group exhibited the highest proportions of SPM and SPM*t*. The SFA and TFA groups had the greatest proportion of SSS triacylglycerols, and the TFA was especially high in SSM*t* and SMM*t* (Fig. 2). The SSM*t* fraction eluted prior to the SMS fraction at variable retention times and was not analyzed other than for total fatty acid composition.

Fatty acid composition of triacylglycerol species. The MUFA3 and PUFA groups contained a small percentage of 18:1*c*9 and vaccenic acid (18:1*c*11) in their SSS fraction (Table 2), which should not have been observed and represents possible contamination within the column. The predominant fatty acid in the SSS fraction was 18:0 for the MUFA3, SFA, and TFA groups, but was 16:0 for the MUFA1, MUFA2, and PUFA groups. The PUFA group had a relatively small percentage of 18:0 in the *sn*-1/3 positions (15%), but a high percentage of 16:0 (68%). The SMS triacylglycerol fraction represented the predominant triacylglycerol species for the SFA and TFA groups (Fig. 2). This fraction contained the greatest percentages of 18:0 for the SFA and TFA treatment

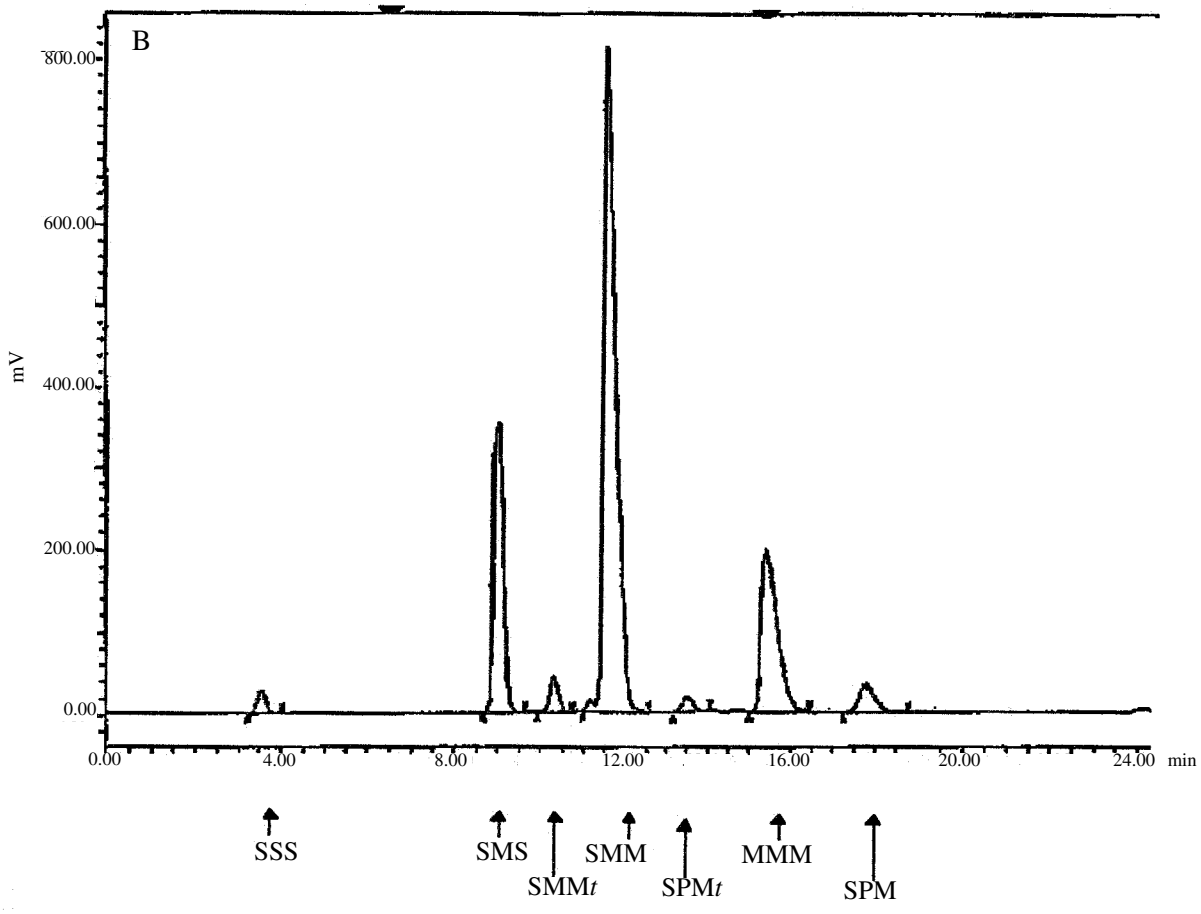

FIG. 1. (continued)

groups (Table 3). There was no difference ($P > 0.05$) in the percentage 18:0 in the *sn*-2 position of SMS triacylglycerols across treatment groups.

The composition of the SMM*t* fraction of the six sample types was rather variable in that the 18:1*t*11 content ranged from 10 to 23% (Table 4). This HPLC fraction may comprise a mixture of triacylglycerols; however, they always eluted in a sharp, discrete peak having a constant retention time. The MUFA3 group had the lowest percentage TFA in the SMM*t* fraction in addition to the lowest abundance of SMMt triacylglycerols (Fig. 2). There was no enrichment of 18:0 in the *sn*-1/3 positions in the SMM*t* fraction. The MUFA3 had an unusually high percentage myristic acid (14:0; over 9%) in the SMM*t* triacylglycerol fraction.

The SMM triacylglycerols were relatively enriched with myristoleic acid (14:1), 16:1, and 18:1 (Table 5). There was an enrichment of 18:1*c*9 in particular in the *sn*-2 position, whereas 16:0 was enriched at the *sn*-1/3 positions. The SPM*t* triacylglycerols (Table 6) were similar in composition to the SMM*t* triacylglycerols (Table 4) except for a relatively high abundance of 18:2 (up to 20%). For most treatment groups, the 18:2 was located primarily at the *sn*-2 position. However, in the PUFA group, there was a uniform distribution of 18:2 between the *sn*-2 and the *sn*-1/3 positions.

Monounsaturated *cis* fatty acids generally were abundant in the MMM triacylglycerols (Table 7) with the exception of the TFA group. Although the TFA MMM triacylglycerol peak eluted with the same retention time as for the other treatment groups, it contained only 47% 18:1, 1.5% 16:1, and no detectable 14:1. The MMM triacylglycerols from both the PUFA and TFA groups had relatively high concentrations of 18:1*t*11 and 18:2.

The composition of the SPM triacylglycerols was analyzed only for the MUFA1, SFA, and PUFA groups (Table 8). The SPM triacylglycerols contained approximately one-third SFA (primarily 16:0), one-third MUFA (18:1*c*9), and one-third 18:2. The 16:0 was concentrated primarily at the *sn*-1/3 positions, whereas the 18:0 was located primarily in the *sn*-2 position. There was a uniform distribution of 18:1*c*9 in the SFA and PUFA groups, but the *sn*-1/3 positions were enriched with 18:1*c*9 in the MUFA1 group. The 18:1*c*11 was located primarily in the outer *sn*-positions. The *sn*-2 position of the SPM triacylglycerols was particularly enriched with 18:2; and for the PUFA group, the *sn*-1/3 positions also contained a high percentage of 18:2. There was no detectable α-linolenic acid (18:3) in the SPM fraction. Rather, 18:3 was distributed thoughout the SMS, SMM*t*, SMM, and SPM*t* fractions, where it was concentrated in the *sn*-1/3 positions.

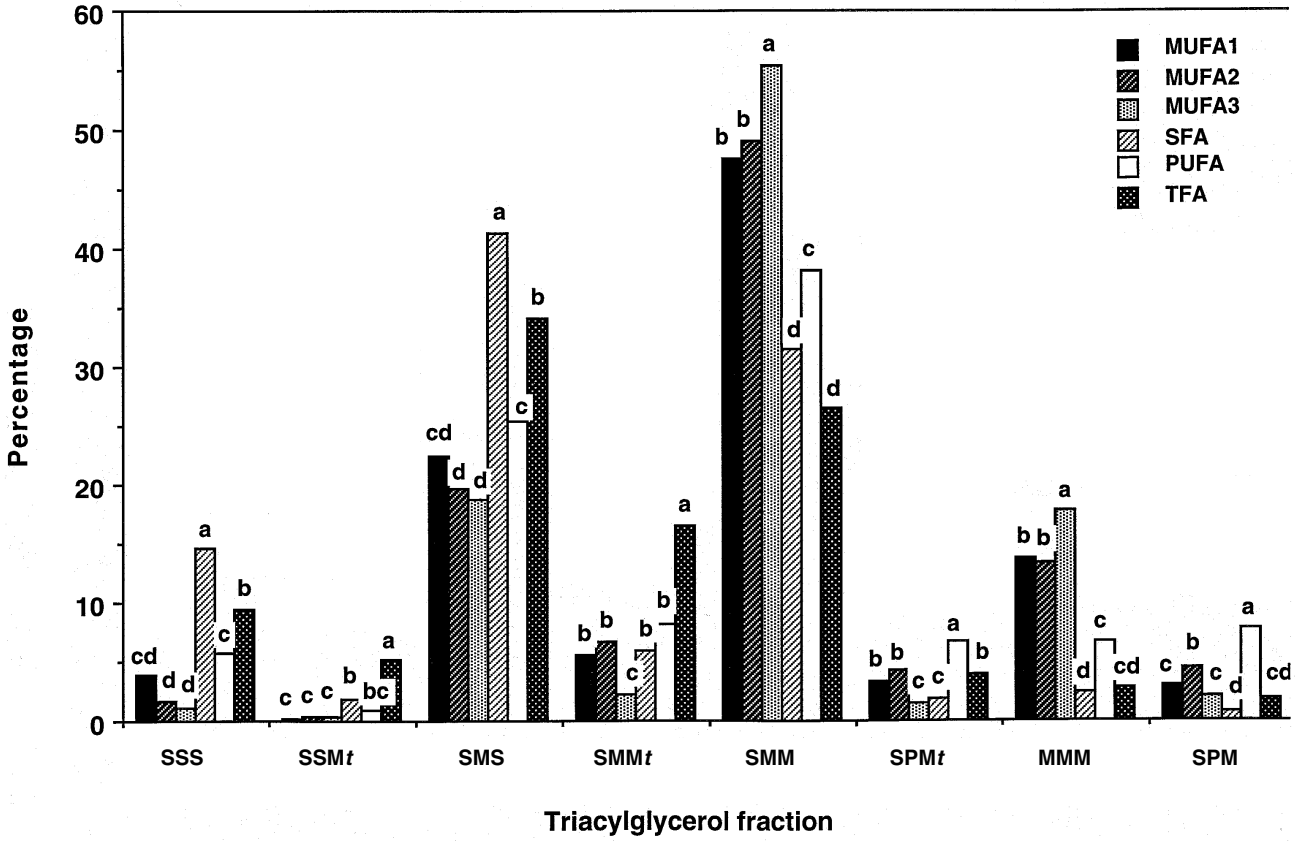

FIG. 2. Distribution of triacylglycerol molecular species in bovine subcutaneous adipose tissue lipids from cattle raised under widely varying conditions. Abbreviations: MUFA1, MUFA2, and MUFA3, samples containing elevated monounsaturated fatty acids; PUFA, samples containing elevated polyunsaturated fatty acids; TFA, samples containing elevated *trans* fatty acids. For other abbreviations see Figure 1. Means within a triacylglycerol fraction with different superscripts (a,b,c,d) are different ($P < 0.05$). Pooled standard deviations for the SSS, SSM*t*, SMS, SMM*t*, SMM, SPM*t*, MMM, and SPM fractions are 5.4, 1.8, 9.3, 4.5, 11.4, 2.0, 6.7, and 2.4, respectively.

Recovery of individual fatty acids after HPLC separation and subsequent methylation and extraction was estimated by summing the product of the proportions of each triacylglycerol fraction times the percentages of the individual fatty acids in all fractions. By this calculation, the sum of the 16:0 in the triacylglycerol fractions consistently was approximately 2 percentage units less than the percentage 16:0 observed in the lipid extracts that had not undergone HPLC separation (Table 1), and 18:0 was proportionately greater. The sums of the 18:1*c*9 proportions in the triacylglycerol fractions were nearly identical to the percentages listed in Table 1. This same pattern was observed in the total, *sn*-2, and *sn*-1/3 positions. None of these discrepancies alters the general finding that 18:0 was enriched in the *sn*-1/3 positions as its percentage increased in the total lipid extract.

DISCUSSION

The mechanism by which changes in fatty acid positional distribution are accomplished is unknown. Our results indicate that 16:0 and 18:0 composed up to 20 and 15%, respectively, of the fatty acids in the *sn*-2 position, and 18:1*c*9 constituted as much as 35% of the *sn*-1/3 positions. In these samples, 18:2 was preferentially incorporated into the *sn*-2 position, but became progressively enriched in the *sn*-1/3 positions as the total concentration of 18:2 increased. Thus, the acyltransferases responsible for triacylglycerol biosynthesis in bovine adipose tissue exhibit only partial specificity.

In those samples in which 18:0 accumulated, the proportion of 18:0 at the *sn*-2 and *sn*-1/3 positions increased perhaps merely owing to its greater concentration within the cell. When the concentration of total 18:0 approached 20%, glycerolphosphate acyltransferase and diacylglycerol acyltransferase placed more 18:0 in the outer positions. Because the concentration of 16:0 in the *sn*-1/3 positions generally was unaffected by the concentration of 18:0 in the samples, and because 16:0 generally is esterified to the *sn*-1 position prior to esterification of fatty acids in the other positions, we can conclude that enrichment of 18:0 occurred primarily at the *sn*-3 location. Jurriens (16) described the triacylglycerol composition of beef tallow as having a large percentage of triacylglycerols containing one *cis* double bond (35%, analogous to the SMS triacylglycerols in this study). Triacylglycerols with 18:0 in the *sn*-1/3 positions constituted over 40% of their "SMS" triacylglycerols, and nearly 30% of this fraction contained 18:0 solely in the *sn*-3 position (16). The samples de-

TABLE 2
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SSS Triacylglycerols[a]

	Fatty acid							
Fraction/group	14:0	14:1	16:0	16:1	17:0	18:0	18:1*c*9	18:1*c*11
Total								
MUFA1	16.0^{a}	0	50.1^{a,b}	0.1	1.4^{a,b}	32.1^{c}	0.03	0
MUFA2	4.0^{b}	0	53.8^{a}	0.3	0.4^{b,c}	41.3^{b,c}	0	0
MUFA3	5.9^{b}	0	23.6^{c,d}	0	0^{c}	63.1^{a,b}	5.54	1.72
SFA	6.1^{b}	0.2	30.6^{b,c,d}	0.3	1.1^{b}	61.5^{a,b}	0	0
PUFA	5.8^{b}	0.2	48.3^{a,b}	0.6	2.4^{a}	40.6^{b,c}	1.97	0
TFA	1.8^{b}	0	18.4^{d}	0	0^{c}	79.7^{a}	0	0
Pooled SD	6.9	0.2	20.9	0.4	1.1	23.9	5.22	1.62
sn-2								
MUFA1	11.7^{a}	0.2	52.7^{a}	0.3	2.0^{a}	31.6^{c}	0.7	0
MUFA2	1.6^{b}	0	54.2^{a}	0	0^{b}	44.0^{b}	0	0
MUFA3	1.9^{b}	0	23.0^{b}	0	0^{b}	70.2^{a,b}	4.8	0
SFA	3.5^{b}	0.2	29.4^{b}	0.4	1.1^{a}	65.2^{a,b}	0	0
PUFA	2.8^{b}	0.2	38.5^{a,b}	0.3	1.5^{a}	53.3^{a,b,c}	2.7	0
TFA	3.3^{b}	0	14.4^{b}	0	0^{b}	82.2^{a}	0	0
Pooled SD	5.0	0.2	21.1	0.4	1.1	24.7	4.8	0
Average *sn*-1/3								
MUFA1	24.6	–0.3	45.0^{a,b}	–0.1	0.3^{b}	33.0^{a,b}	–1.3	0
MUFA2	8.7	0	53.1^{a,b}	0.9	1.3^{b}	35.8^{a,b}	0	0
MUFA3	13.8	0	25.1^{b}	0	0^{b}	49.0^{a,b}	6.8	5.1
SFA	11.3	0.2	33.1^{b}	0.4	1.0^{b}	53.9^{a}	0	0
PUFA	12.0	0.1	67.9^{a}	1.1	4.2^{a}	15.3^{b}	0.3	0
TFA	–1.1	0	26.3^{b}	0	0^{b}	74.7^{a}	0	0
Pooled SD	16.9	0.3	25.8	1.2	2.3	31.5	6.4	4.9

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. There was no detectable 17:1, 18:1*t*11, 18:2 or 18:3 in the SSS fraction. SSS, *sn*-1,2,3-saturated fatty acid triacylglycerol. For other abbreviations see Table 1.

TABLE 3
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SMS Triacylglycerols[a]

	Fatty acid											
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3
Total												
MUFA1	6.1	0.7^{a}	37.8^{a}	3.6^{a}	1.4	0.6^{a}	17.9^{c}	1.4^{b,c}	29.1^{c}	1.1^{b}	0.1	0.1
MUFA2	3.3	0.2^{b}	34.8^{a,b}	1.9^{b,c}	1.1	0.2^{b}	22.6^{b,c}	1.8^{b,c}	32.0^{b,c}	1.0^{b,c}	0.7	0.1
MUFA3	2.8	0.1^{b}	28.8^{a,b,c}	2.1^{a,b}	1.2	0.1^{b}	24.6^{a,b,c}	0^{c}	38.2^{a}	1.8^{a}	0	0
SFA	2.9	0.1^{b}	23.3^{b,c}	1.3^{b,c}	0.9	0.1^{b}	34.5^{a,b}	0.6^{b,c}	35.4^{a,b}	0.5^{c}	0	0
PUFA	2.5	0.2^{b}	31.0^{a,b,c}	2.0^{a,b,c}	1.5	0.3^{a,b}	25.1^{a,b,c}	2.3^{b}	33.6^{a,b,c}	0.7^{b,c}	0.1	0.2
TFA	2.1	0.1^{b}	18.1^{c}	0.6^{c}	1.7	0.2^{b}	36.8^{a}	5.9^{a}	33.1^{a,b,c}	1.0^{b,c}	0	0
Pooled SD	2.4	0.3	11.5	1.3	0.6	0.3	11.2	2.3	4.8	0.5	0.8	0.2
sn-2												
MUFA1	4.3	0.6^{a}	29.9^{a,b}	4.0^{a}	1.2^{a}	0.6^{a}	18.9	0.8^{b}	38.7^{b}	0.9^{a}	0	0^{b}
MUFA2	2.6	0^{b}	31.4^{a}	1.4^{b}	0.4^{a,b}	0^{b}	24.5	0.9^{b}	37.5^{b}	0.6^{a,b}	0.4	0^{b}
MUFA3	3.3	0^{b}	18.5^{b}	1.9^{b}	0^{b}	0^{b}	27.5	0^{b}	47.8^{a}	0^{c}	0.7	0^{b}
SFA	1.7	0.1^{b}	18.1^{b}	1.1^{b}	0.6^{a,b}	0.2^{b}	29.8	0.3^{b}	47.3^{a}	0.3^{b,c}	0.1	0.1^{a}
PUFA	1.5	0.1^{b}	26.5^{a,b}	1.5^{b}	1.2^{a}	0.2^{b}	26.6	3.3^{a}	38.4^{b}	0.4^{a,b,c}	0	0^{b}
TFA	2.0	0^{b}	15.2^{b}	1.0^{b}	0.5^{a,b}	0.1^{b}	30.5	4.5^{a}	45.6^{a,b}	0.1^{b,c}	0	0^{b}
Pooled SD	2.4	0.2	11.3	1.6	0.7	0.3	9.5	2.3	7.4	0.4	0.6	0.1
Average *sn*-1/3												
MUFA1	9.5	1.0	53.4^{a}	2.8	1.7	0.4	16.8^{b}	2.5^{b,c}	9.9^{b}	1.3^{b,c}	0.1	0.1
MUFA2	4.7	0.7	41.6^{a}	2.9	2.6	0.6	18.6^{b}	3.7^{b}	20.9^{a,b}	1.8^{b,c}	1.3	0.2
MUFA3	1.8	0.3	49.4^{a,b}	2.5	3.5	0.2	19.0^{b}	0^{c}	18.8^{a,b}	5.5^{a}	–1.4	0
SFA	5.3	0.2	33.5^{a,b}	1.5	1.6	0.1	44.0^{a}	1.2^{b,c}	11.6^{b}	0.9^{c}	–0.2	–0.2
PUFA	4.6	0.4	40.1^{a,b}	3.1	2.1	0.7	22.2^{b}	0.3^{b,c}	24.2^{a}	1.4^{b,c}	0.1	0.6
TFA	2.3	0.2	23.7^{b}	–0.1	4.1	0.4	49.5^{a}	8.5^{a}	8.1^{b}	2.7^{a,b}	0	0
Pooled SD	7.2	0.6	18.6	2.5	2.0	0.6	19.9	3.9	10.6	2.0	2.7	0.5

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. SMS, *sn*-1-saturated, 2-monounsaturated, 3-saturated triacylglycerol. For other abbreviations see Table 1.

TABLE 4
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SMM*t* Triacylglycerols[a]

	Fatty acid											
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3
Total												
MUFA1	1.5[b]	0.3	20.7	2.1[a]	0.6	0.4[a]	12.9[b,c]	21.6[a]	38.4	1.2[a,b]	0	0[b]
MUFA2	0.8[b]	0.1	24.5	2.0[a]	0.5	0.1[a,b]	10.7[c]	15.8[a,b]	40.6	1.3[a]	0.1	3.1[a]
MUFA3	9.2[a]	0	20.8	0.5[b]	0	0[b]	22.1[a]	9.8[b]	37.3	0[c]	0	0[a,b]
SFA	1.3[b]	0.1	18.1	0.6[b]	0.3	0.2[a,b]	18.3[a,b]	16.2[a,b]	43.4	0.4[b,c]	0.1	2.2[a,b]
PUFA	0.2[b]	0	19.4	1.4[a,b]	0.6	0.1[a,b]	13.4[b,c]	23.4[a]	36.7	0.8[a,b,c]	0.3	3.3[a]
TFA	3.7[a,b]	0.1	17.8	0.9[a,b]	0.6	0.4[a]	19.0[a,b]	23.2[a]	32.6	0.6[a,c]	0.9	0[b]
Pooled SD	5.2	0.2	9.0	1.0	0.4	0.3	6.4	7.2	8.2	0.7	0.7	1.9
sn-2												
MUFA1	1.9	0.1	15.6	2.6[a]	0[b]	0.5[a]	14.2[a,b]	14.4[a,b]	49.1	1.2	0	0[b]
MUFA2	0.3	0.1	21.7	2.5[a]	0[b]	0[b]	11.9[b]	11.8[a,b]	47.7	0.3	0.1	3.3[a]
MUFA3	7.4	0	16.2	0[c]	0[b]	0[b]	22.4[a]	7.8[b]	45.9	0	0	0[b]
SFA	0.8	0.2	14.9	0.6[b,c]	0.2[a,b]	0[b]	20.1[a]	10.1[b]	51.2	0.7	0.5	1.1[a,b]
PUFA	1.2	0	17.4	1.4[a,b]	0.2[a,b]	0[b]	15.9[a,b]	17.0[a,b]	44.6	0.2	0	1.8[a,b]
TFA	3.3	0	16.0	1.0[b,c]	0.5[a]	0.3[a]	17.5[a,b]	20.7[a]	38.6	0.7	1.0	0[b]
Pooled SD	5.1	0.2	9.7	1.2	0.3	0.2	6.5	7.9	10.2	0.7	0.8	2.0
Average *sn*-1/3												
MUFA1	0.7	0.6[a]	31.1	1.0	1.8[a]	0.1	10.3	35.8	17.1	1.0[b]	0	0[c]
MUFA2	1.8	0.1[a,b]	30.0	1.0	1.7[a]	0.2	8.3	23.9	26.6	3.3[a]	0.1	2.6[b,c]
MUFA3	12.9	0[b]	29.8	1.5	0[b]	0	21.6	13.8	20.1	0[b]	0	0[c]
SFA	2.3	–0.2[b]	24.7	0.5	0.4[a,b]	0.5	14.8	28.4	27.7	–0.2[b]	–0.6	4.3[a,b]
PUFA	–1.8	0[b]	23.3	1.4	1.7[a]	0.2	8.5	36.2	21.1	1.9[a,b]	0.7	6.4[a]
TFA	4.7	0.2[a,b]	21.3	0.7	0.7[a,b]	0.4	21.9	28.2	20.6	0.3[b]	0.5	0[c]
Pooled SD	9.9	0.4	11.5	1.7	1.2	0.5	13.5	15.1	16.2	1.7	0.9	3.1

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. SMM*t*, *sn*-1-saturated, 2-monounsaturated, 3-*trans*-monounsaturated triacylglycerol. For other abbreviations see Table 1.

TABLE 5
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SMM Triacylglycerols[a]

	Fatty acid											
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3
Total												
MUFA1	1.4	1.2[a]	22.4	6.4[a]	0.7	1.1[a]	6.0[b]	0.1[b]	57.4	2.7[b,c]	0.1	0.1
MUFA2	1.0	1.0[a]	21.1	4.8[a,b,c]	0.5	1.1[a]	5.8[b]	1.0[b]	60.0	3.1[a,b]	0.1	0.2
MUFA3	0.6	0.8[a,b]	18.6	5.0[a,b]	0.4	0.9[a]	5.9[b]	0[b]	63.5	3.7[a]	0.2	0
SFA	0.5	0.2[b]	15.9	2.3c	0.4	0.4[b]	11.6[a]	0.1[b]	67.1	1.2[c]	0.2	0
PUFA	0.8	0.5[a,b]	20.1	3.7[a,b,c]	0.8	1.0[a]	7.9[a,b]	0.3[b]	62.3	1.9[c]	0.1	0.1
TFA	1.0	0.2[b]	18.1	3.1[b,c]	0.5	0.7[a,b]	12.6[a]	3.5[a]	58.4	1.6[c]	0	0
Pooled SD	0.6	0.6	6.2	2.4	0.3	0.4	4.1	1.4	7.2	1.1	0.2	0.2
sn-2												
MUFA1	2.1	1.9[a]	12.4	7.7[a]	0.4	1.3[a]	4.4[c]	0.1[b]	67.4	2.0[b]	0.1	0
MUFA2	0.7	0.9[b]	14.1	4.5[a,b]	0.2	0.9[a,b]	6.5[c]	0.8[b]	68.8	2.1[b]	0	0.1
MUFA3	0.9	0.9[b]	12.8	5.1[a,b]	0.1	0.5[b,c]	6.9[b,c]	0[b]	68.4	3.5[a]	0.5	0
SFA	1.4	0.1[b]	7.9	2.5[b]	0.1	0.2[c]	10.7[a,b]	0.1[b]	76.1	0.4[c]	0.1	0.1
PUFA	0.5	0.3[b]	13.6	2.9[b]	0.3	0.9[a,b,c]	7.5[b,c]	0.1[b]	71.8	1.5[b,c]	0.1	0.1
TFA	0.3	0.2[b]	11.9	2.9[b]	0.2	0.3[b,c]	13.2[a]	2.6[a]	66.9	1.1[b,c]	0	0
Pooled SD	1.2	0.8	6.4	3.0	0.3	0.5	4.1	1.3	8.3	1.3	0.4	0.2
Average *sn*-1/3												
MUFA1	0.1[a,b]	–0.2c	42.4	4.1[a,b]	1.3	0.7	9.3[a,b]	0.1[b]	37.4	4.1[a,b]	0.3	0.1
MUFA2	1.6[a]	1.2[a]	35.2	5.4[a]	1.2	1.4	4.4[b]	1.4[b]	42.3	5.3[a]	0.1	0.2
MUFA3	0.1[a,b]	0.6[a,b,c]	30.0	4.9[a]	1.1	1.8	3.9[b]	0[b]	53.6	4.1[a,b]	–0.5	0
SFA	–1.2[b]	0.2[b,c]	31.8	2.0[b]	1.0	0.6	13.3[a]	–0.1[b]	49.0	2.7[b]	0.4	–0.2
PUFA	1.5[a,b]	0.9[a,b]	33.2	5.4[a]	1.6	1.2	8.7[a,b]	0.7[b]	43.1	2.6[b]	0.3	0.3
TFA	2.6[a]	0.2[b,c]	30.5	3.3[a,b]	0.9	1.4	11.5[a,b]	5.2[a]	41.5	2.4[b]	0	0
Pooled SD	7.2	0.6	18.6	2.5	2.0	0.6	19.9	3.9	10.6	2.0	2.7	0.5

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. SMM, *sn*-1-saturated, 2,3-monounsaturated fatty acid triacylglycerol. For other abbreviations see Table 1.

TABLE 6
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SPM*t* Triacylglycerols[a]

	Fatty acid											
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3
Total												
MUFA1	4.3[a]	0.5[a]	14.2	4.1[a]	0.1[a,b]	0.6[a]	9.8[b]	13.7[a]	42.0[a]	2.0[a,b]	7.9[b]	0.3
MUFA2	1.2[b]	0.0[b]	18.2	3.8[a]	0.1[b]	0.4[a,b]	10.0[b]	12.6[a]	44.2[a]	2.5[a]	6.0[b]	0.6
MUFA3	1.8[a,b]	0[b]	14.7	2.6[a,b]	0[b]	0[b]	28.5[a,b]	2.1[b]	44.7[a]	1.3[a,b]	3.9[b]	0
SFA	0.6[b]	0[b]	11.3	0.7[b]	0.4[a,b]	0[b]	33.1[a]	3.7[b]	40.3[a,b]	0.9[b]	8.6[b]	0
PUFA	0.9[b]	0[b]	20.2	1.0[b]	0.7[a]	0[b]	19.9[a,b]	6.6[a,b]	28.4[b]	0.7[b]	20.8[a]	0.3
TFA	0.7[b]	0[b]	8.9	0.7[b]	0[b]	0[b]	27.0[a,b]	8.2[a,b]	48.3[a]	0.5[b]	5.3[b]	0
Pooled SD	2.0	0.3	8.0	1.8	0.4	0.4	14.8	6.1	9.6	1.0	7.5	0.6
sn-2												
MUFA1	5.7[a]	0.4	12.6	4.1[a]	0	0	16.9[b]	4.2[a]	44.0[a]	0.3[a]	11.4[a,b]	0
MUFA2	0.4[b]	0	19.5	3.8[a]	0	0.2	12.7[b]	4.3[a]	49.3[a]	0.9[a]	8.6[b]	0
MUFA3	2.0[b]	0	17.5	1.2[b]	0	0	30.7[a,b]	0[b]	44.9[a]	0[b]	3.4[b]	0
SFA	0.7[b]	0	8.8	0[b]	0	0	46.5[a]	0[b]	30.5[b]	0[b]	13.2[a,b]	0
PUFA	1.6[b]	0	15.7	0.7[b]	0	0	17.9[b]	4.9[a]	37.0[a,b]	1.3[a]	20.5[a]	0
TFA	0[b]	0	8.8	0[b]	0	0	28.1[a,b]	4.3[a]	50.9[a]	0[b]	7.7[b]	0
Pooled SD	2.6	0.3	7.8	2.0	0	0.2	16.8	2.8	10.4	0.9	8.5	0
Average *sn*-1/3												
MUFA1	1.4	0.9	17.4	4.2	0.5[a,b]	1.9[a]	–4.3	32.6[a]	38.0[a,b]	5.3[a]	0.8[b]	0.9
MUFA2	2.8	0.1	15.6	3.9	0.2[b]	0.8[a,b]	4.5	29.2[a]	33.9[a,b]	5.7[a]	0.9[b]	1.2
MUFA3	1.3	0	9.2	5.5	0[b]	0[b]	24.0	6.4[b]	44.4[a]	4.1[a]	4.8[b]	0
SFA	0.3	0	16.4	2.3	1.3[a,b]	0[b]	6.3	11.1[b]	59.7[a]	2.7[a,b]	–0.4[b]	0
PUFA	–0.4	0	29.3	1.8	2.1[a]	0[b]	24.0	10.1[b]	11.0[b]	–0.4[b]	21.5[a]	0.9
TFA	2.1	0	9.2	2.2	0[b]	0[b]	24.9	16.0[a,b]	43.1[a,b]	1.6[a,b]	0.4[b]	0
Pooled SD	4.1	1.2	11.9	3.1	1.2	1.0	22.3	15.1	22.9	3.1	10.0	1.9

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. SPM*t*, *sn*-1-saturated, 2-polyunsaturated, 3-monounsaturated triacylglycerol. For other abbreviations see Table 1.

TABLE 7
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the MMM Triacylglycerols[a]

	Fatty acid											
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2	18:3
Total												
MUFA1	0.5	1.5[a]	0.4[b]	10.3[a]	0.3[a,b,c]	1.8[a]	0.3[b]	0.5[b,c]	77.1[a]	6.1[a,b]	0.8[c]	0
MUFA2	0.2	0.9[a,b]	4.4[a,b]	7.3[a,b,c]	0.7[a]	1.3[a,b]	6.3[b]	0.8[b,c]	71.4[a]	4.5[b]	1.8[b,c]	0
MUFA3	0.3	0.4[b,c]	2.7[a,b]	7.9[a,b]	0.6[a,b]	0.9[a,b,c]	3.4[b]	0[c]	76.2[a]	6.9[a]	0.2[c]	0
SFA	0	0[c]	2.8[a,b]	2.3[c]	0[c]	0.5[b,c]	13.9[a,b]	0.8[b,c]	76.0[a]	1.5[c]	1.8[b,c]	0
PUFA	0.1	0.1[c]	6.0[a]	4.3[b,c]	0.1[b,c]	0.9[a,b,c]	4.9[b]	3.1[a,b]	70.5[a]	2.6[c]	7.0[a]	0
TFA	0	0[c]	7.9[a]	1.5[c]	0[c]	0[c]	31.4[a]	5.5[a]	46.8[b]	1.0[c]	5.7[a,b]	0
Pooled SD	0.5	0.7	3.5	4.4	0.5	0.7	13.4	2.4	11.7	2.5	3.3	0
sn-2												
MUFA1	1.2[a]	2.6[a]	1.4[b]	10.0[a]	0	1.9[a]	1.6[b]	0.9[a,b]	74.9[a]	3.0[a,b]	1.4[c]	0.5[a]
MUFA2	0.1[b]	0.4[b]	6.7[a,b]	6.1[a,b]	0	0.6[b]	7.4[b]	0.1[b]	72.3[a]	2.8[a,b]	3.0[b,c]	0[b]
MUFA3	0.2[b]	0.2[b]	3.4[a,b]	7.7[a,b]	0	0.2[b,c]	6.2[b]	0[b]	77.5[a]	4.0[a]	0.1[c]	0[b]
SFA	0[b]	0[b]	2.4[b]	1.7[c]	0	0[c]	14.1[a,b]	0.5[a,b]	76.1[a]	0.7[c]	4.1[a,b,c]	0[b]
PUFA	0.1[b]	0[b]	7.7[a,b]	3.3[b,c]	0	0.1[b,c]	7.0[b]	1.8[a]	68.5[a,b]	1.6[b,c]	9.5[a]	0[b]
TFA	0[b]	0[b]	9.9[a]	0[c]	0	0[c]	28.6[a]	0[b]	52.7[b]	0[c]	8.6[a,b]	0[b]
Pooled SD	0.7	1.0	4.5	4.5	0	0.7	11.6	1.0	11.8	1.8	4.8	0.3
Average *sn*-1/3												
MUFA1	–0.9	–0.7[b]	–1.5[b]	10.9	0.9[a,b]	1.5	–2.4[b]	–0.3[b]	81.5[a]	12.7[a]	–0.3[a,b]	0.4[a]
MUFA2	0.6	1.8[a]	–0.1[a,b]	9.6	2.2[a]	2.6	4.0[b]	2.1[b]	69.6[a]	7.8[b,c]	–0.6[a,b]	0[b]
MUFA3	0.5	0.8	1.3[a,b]	8.2	1.9[a,b]	2.5	–2.2[b]	0[b]	73.6[a]	12.6[a,b]	0.3[a]	0[b]
SFA	0	0[a,b]	3.7[a]	3.5	0[b]	1.6	13.5[a,b]	1.4[b]	75.6[a]	3.0[c]	–2.6[b]	0[b]
PUFA	1.7	0.3[a,b]	2.6[a,b]	6.2	0.2[b]	2.5	0.9[b]	5.6[a,b]	74.5[a]	4.6[c]	1.9[a]	0[b]
TFA	0	0[a,b]	3.8[a]	4.5	0[b]	0	37.0[a]	16.5[a]	34.9[b]	3.1[c]	–0.0[a,b]	0[b]
Pooled SD	1.6	1.5	3.3	5.5	1.5	1.7	19.2	6.8	15.3	5.2	2.2	0.5

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. MMM, *sn*-1,2,3-monounsaturated fatty acid triacylglycerol. For other abbreviations see Table 1.

TABLE 8
Total, *sn*-2, and Average *sn*-1/3 Fatty Acid Compositions (wt%) of the SPM Triacylglycerols for the Control, SFA, and PUFA Treatment Groups[a]

	Fatty acid										
Fraction/group	14:0	14:1	16:0	16:1	17:0	17:1	18:0	18:1*t*11	18:1*c*9	18:1*c*11	18:2
Total											
MUFA1	4.3	0.4	20.3	2.4[a]	0[b]	0	10.3	0	33.5	1.3	27.1[b]
SFA	0	0	22.7	1.1[b]	0.8[a]	0	9.7	0	33.8	1.4	30.2[a,b]
PUFA	0.5	0	20.2	1.1[b]	0.5[a]	0.2	10.4	0	33.6	0.9	32.2[a]
Pooled SD	3.0	0.4	2.3	0.8	0.4	0.2	2.6	0	1.9	0.6	3.0
sn-2											
MUFA1	4.2[a]	0.3	11.6	2.9[a]	0	0	13.0	0.3	29.6	0.4	37.1
SFA	0[b]	0	12.2	0[b]	0	0	12.6	0	34.0	0	40.9
PUFA	0[b]	0	13.6	0.4[b]	0	0	12.3	0	35.2	0.2	37.9
Pooled SD	2.3	0.4	6.0	1.6	0	0	2.9	0.5	3.9	0.5	6.0
Average *sn*-1/3											
MUFA1	4.4	0.8	37.8	1.4	0[b]	0	4.9	–0.7	41.1	3.1	6.9
SFA	0	0	43.6	3.4	2.5[a]	0	4.0	0	33.7	4.2	8.6
PUFA	1.6	0	31.8	2.8	1.5[a]	0.5	8.0	0	30.2	2.4	20.7
Pooled SD	6.2	1.6	12.9	2.4	1.2	0.5	7.6	0.9	8.4	2.0	10.6

[a]Means within a column for each fraction without a common superscript are different. Data are means for four (MUFA1, TFA, and PUFA) or six (MUFA2, MUFA3, and SFA) animals per treatment group. Some negative values occurred for the average *sn*-1/3 means when there was a low abundance of a fatty acid. There was no detectable 18:3 in the SPM fraction. SPM, *sn*-1-saturated, 2- polyunsaturated, 3-monounsaturated triacylglycerol. For other abbreviations see Table 1.

scribed by Jurriens (16) most closely resemble the SFA samples in their large proportions of triacylglycerols containing zero or one *cis* double bond. The data from the current study provide the first detailed description of bovine lipid samples with substantially more MUFA and consequently lower melting points, and substantiate an earlier, preliminary report from this laboratory (17).

The samples obtained from these cattle provided an unusual opportunity to investigate fatty acid positional distribution in bovine lipids. Under typical production conditions in the United States, cattle are slaughtered at 18 to 20 mon of age, and dietary treatments are restricted to less than 180 d. Feeding whole, high-oleic acid, canola rapeseed to cattle for 100 d did not increase the percentage of 18:1 in subcutaneous adipose tissue, although the MUFA/SFA ratio was increased significantly (from 0.85 to 0.93; 13). Even after feeding cattle high-oleic acid sunflower seed for 180 d, the percentage of 18:1 subcutaneous adipose tissue increased only marginally (from 44.7% in controls to 46.7%) (calculated from 18).

The cattle produced in Australia in the current study either were fed for very long times (300 d, MUFA1, and >400 d, SFA) or were fed casein-formaldehyde-protected lipids (PUFA). The latter have been shown to alter bovine adipose tissue fatty acid composition markedly (19–21), as the casein-formaldehyde protection prevents ruminal hydrogenation of ingested fatty acids. Compared to cattle of similar genotype raised in the United States and fed corn-based diets for 100 d (13), the MUFA1 group was enriched with 18:1 (49 vs. 40%) and 16:1 (5 vs. 3%), and was notably lower in 18:0 (10 vs. 15%). The cattle fed for 180 d in the study of Chang *et al.* (18) were intermediate in fatty acid composition; apparently, long-term feeding of cattle is sufficient to produce significant alterations in subcutaneous adipose tissue fatty acid composition. The TFA group was unusual in that substantial changes in adipose tissue fatty acid composition were achieved (especially for 18:0 and 18:1*t*11) after a relatively short feeding period (150 d).

The fatty acid composition of the SFA, PUFA, and TFA groups suggested that their dietary treatments inhibited the activity of adipose tissue stearoyl-CoA desaturase. Samples from these groups were especially low in 14:1, 16:1, 18:1*c*9 and 18:1*c*11, all products of the desaturase. The diet of the SFA group included approximately 10% whole cottonseed, which contains the cyclopropene fatty acid, sterculic acid. Sterculic acid inhibits stearoyl-CoA desaturase activity *in vitro* by irreversibly binding sulfhydryl groups (22,23). It has not been possible to demonstrate an effect of dietary whole cottonseed on adipose tissue fatty acid composition or desaturase activity in cattle fed whole cottonseed for only 54 (14) or 150 d (24), as compared to >400 d for the SFA group. Although hydrogenation of sterculic acid by rumen microflora should have inactivated the inhibitor (25), sufficient sterculic acid may have been absorbed postruminally to reduce desaturase activity in the SFA group. In support of this, we have demonstrated a marked reduction in microsomal stearoyl-CoA desaturase activity from adipose tissues of cattle fed casein-formaldehyde-protected cottonseed oil (similar to that fed the PUFA group; Tume, R.K., and Yang, A., unpublished observations).

The high concentration of MUFA in the MUFA3 group suggests elevated stearoyl-CoA desaturase activity in adipose tissue of those animals. We previously sampled animals from

the same geographical region in Japan (26) and noted extraordinarily elevated levels of 14:1 (2.5%), 16:1 (11%), and 18:1 (55%). Other studies of American Wagyu (27) and Japanese Black cattle raised in Japan (28) have supported a genetic basis for the elevated MUFA in this breed type.

In summary, these groups of cattle provided a unique opportunity to investigate fatty acid positional distribution in ruminant adipose tissue displaying a wide range of fatty acid compositions. Enrichment with 18:0, particularly in the *sn*-1/3 positions, contributed to the high melting points of the SFA, TFA, and PUFA samples. This may play an important role in the production of meat products. In Australia, where beef carcasses are hand-boned, excessively hard fat, particularly in grain-fed cattle at chiller temperatures, can lead to a larger percentage of carcasses that are unacceptable for boning (5). Thus, the high melting point of lipids containing 18:0 in the *sn*-1/3 can have very practical implications.

ACKNOWLEDGMENTS

This research was funded in part by a grant from the Australian Meat Research Corporation and by the Texas Agricultural Experiment Station.

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[Received July 10, 1997, and in final revised form December 10, 1997; revision accepted January 6, 1998]

METHOD

Stereospecific Analysis of Soybean Triacylglycerols

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ABSTRACT: Thirty soybean germplasm lines representing a wide distribution of fatty acid compositions were analyzed stereospecifically by using a chiral column to resolve the *sn*-1 and *sn*-3 positions of glycerol. The amounts of each acyl group on each of the *sn* positions were plotted vs. the amount of that acyl group in the triacylglycerols (TAG), and the plots were fitted by linear regression. The deviation of individual data points from the linear regressions was much greater than observed in previous studies. This could be attributed to the inclusion of a number of germplasm lines with elevated or reduced percentages of saturates. The stereospecific distributions could not be fit with previously suggested mathematical models because the plots had intercepts that were not allowed by the models. Statistical tests of the analytical procedure indicated that slight oxidation of or bias against the polyunsaturates had occurred and that the Grignard deacylation method gave slightly less representative analyses of the *sn*-2 position than pancreatic lipase deacylation on these TAG.
Lipids 33, 209–216 (1998).

Natural vegetable oils have nonrandom distributions of acyl groups on the *sn*-1, -2, and -3 positions of triacylglycerols (TAG) that can affect their physical properties and oxidative stability (1). Stereospecific plots of plant lines that differ in acyl group composition show linear relationships between the percentages of an acyl group on the *sn*-1, -2, and -3 positions and the percentage of that acyl group in the total TAG. This relation has been shown for several species (2–6). Fatemi and Hammond (4) suggested that these plots would be helpful in identifying TAG distribution mutants since such mutants would yield data points that departed from the linear plots typical of the species. Lee *et al.* (7) proposed two mathematical models to account for the stereospecific distribution of TAG produced in the oleaginous yeast *Apiotrichum curvatum* fed various fatty acid mixtures.

Previous studies of the stereospecific distribution of acyl groups in oilseed varieties have used the analytical method developed by Brockerhoff (8) and modified by Christie and Moore (9). More recently, methods based on separations on chiral columns (10–14) or by using chiral reagents (15) have been developed. The mutation breeding program at Iowa State University (Ames, IA) has generated soybean germplasm lines with a wider range of acyl compositions than those examined earlier (4,6), especially with respect to the concentrations of the saturated acyl groups. This study examined these mutants by using the chiral column method of Takagi and Ando (11).

MATERIALS AND METHODS

The solvents and reagents were A.C.S. certified grade. Diethyl ether was distilled for daily use in the presence of lithium aluminum hydride.

Soybean [*Glycine max* (L.) Merr.] seed from germplasm lines representing a wide range of fatty acid compositions was obtained from the Agronomy Department of Iowa State University and Pioneer Hi-Bred International (Johnston, IA). Fifty to 100 seeds from each germplasm line were split into two equal parts with a razor blade, and one-half of each seed was analyzed for fatty acid composition. The half seeds were crushed with a hydraulic press and extracted with hexane as described by Hammond (16) and analyzed by gas chromatography (GC). Ten to 16 seeds/line were selected for homogeneity of their fatty acid compositions.

The selected half seeds were ground in a Wiley mill (A.H. Thomas Co., Philadelphia, PA), and 500 mg of the meal was extracted with 4–6 mL of hexane overnight at ambient temperature. The hexane was adjusted by evaporation under nitrogen to 0.9 mL and applied to a 900-mg Maxi-Clean silica cartridge (Alltech Associates, Deerfield, IL) that had been preconditioned with 5 mL of hexane. The cartridge was treated with 4 mL of 7% diethyl ether in hexane to elute the TAG, and the solvent was evaporated under nitrogen. A sample of the TAG was analyzed for fatty acid composition.

The chiral analysis was based on the method of Takagi and Ando (11): 50 mg of the remaining TAG was dissolved in 3 mL of diethyl ether and treated with 0.15 mL of 3 M ethylmagnesium bromide in diethyl ether. After 1 min, 0.05 mL of acetic acid in 3.35 mL of 0.4 M boric acid was added, the reaction mixture was centrifuged, and the reaction products were extracted with several portions of diethyl ether. The extract was washed with 3 mL of 2% aqueous sodium bicarbonate solution followed by 3 mL of water, dried with sodium

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Abbreviations: diDNPU, dinitrophenylurethane derivatives; DNPU, dinitrophenylurethane derivatives; GC, gas chromatography; HPLC, high-performance liquid chromatography; TAG, triacylglycerols; TLC, thin-layer chromatography.

sulfate, and evaporated under nitrogen. The residue was dissolved in 0.5 mL of toluene and 0.05 mL of pyridine and reacted with 40 mg of 3,5-dinitrophenylisocyanate (Fluka Chemical Corp., Ronkonkoma, NY) overnight at ambient temperature to form dinitrophenylurethane derivatives (DNPU). The solvents were evaporated; the residue was then dissolved in 0.22 mL of chloroform and streaked on a 0.5-mm Adsorbisil®-Plus 1 silica thin-layer chromatographic (TLC) plate (Alltech). The plate was developed twice with chloroform/acetone (96:4, vol/vol), and the bands were visualized by spraying with 0.01% Rhodamine 6G in ethanol. The bands representing the *sn*-1, *sn*-3-diDNPU enantiomers and the *sn*-2-diDNPU were scraped from the plates separately and eluted from the silica with diethyl ether. Because the TLC separation was not as complete as desired, the two bands were rechromatographed on separate TLC plates as before. The band representing *sn*-2-diDNPU was converted to methyl esters and analyzed by GC. The *sn*-1, *sn*-3-diDNPU enantiomers were prepared for chiral high-performance liquid chromatography (HPLC) by dissolving them in 180 μL of HPLC-grade chloroform and filtering through 0.2-μm filters (Gelman Acrodisc, Ann Arbor, MI). HPLC separation of the *sn*-1 and *sn*-3-diDNPU enantiomers was accomplished on a 25-cm × 4-mm i.d. Sumichiral OA-4100 column (Sumika Ltd., Osaka, Japan) in a Shimadzu instrument (Shimadzu, Kyoto, Japan). The developing solvent was hexane/1,2-dichloroethane/ethanol (73:22:5, by vol) at 1 mL/min. Peaks were monitored at 254 nm. The two fractions were freed of solvent under nitrogen, dissolved in hexane, and prepared for GC analysis.

Acyl groups of the DNPU derivatives were converted to methyl esters by reaction with 1 M sodium methoxide in methanol for 50–60 min while shaking at 45°C. Methyl esters from other lipids were prepared by reaction at room temperature. GC analyses were performed on a Hewlett-Packard 5890A (Avondale, PA) on a J&W (Deerfield, IL) DB-23 column (15 m × 0.25-mm i.d.). Oven temperature was 180°C, inlet and detector 250°C. The peak areas were corrected by factors calculated according to Ackman and Sipos (17), and the weight percentages were converted to mole percentages.

Pancreatic lipase hydrolysis of the TAG by the procedure of Luddy *et al.* (18) was applied to 15 of the samples.

All analyses were duplicated, and Microsoft Excel was used to obtain least squares linear regression, *t*-test, Z-test, and analysis of variance.

RESULTS AND DISCUSSION

Plots showing the percentage of a given acyl group on each of the three glycerol positions vs. the percentage of that acyl group in the total TAG are shown in Figures 1–5. The data points in these plots were obtained from the separated DNPU derivatives. The data were fitted by least-squares linear regression, and the results are given in Table 1. Using a statistical Z-test on the linear regression residuals and normalizing them so that the residual mean is zero can identify outliers from the regression lines. Outliers were defined as falling more than 1.96 standard deviations from the normalized mean. By chance, about 5% of the data points should be defined as outliers by this procedure, and for our sample size of

TABLE 1
Linear Regression Statistics of the Mole Percentage of Acyl Groups at the Three Glycerol Positions vs. the Amount in the Total Triacylglycerol for 30 Samples of Soybean Oil

Acyl group	Position	Slope	Prob. slope ≠ 0	y-intercept	Prob. intercept ≠ 0	R^2	No. outliers[d]
16:0	*sn*-1	1.22 ± 0.04[a]	<0.01	3.52 ± 0.66[a]	<0.01	0.97	2
	sn-2	0.04 ± 0.01	0.01	1.29 ± 0.24	<0.01	0.21	0
	sn-3	1.72 ± 0.04	<0.01	−4.07 ± 0.69	<0.01	0.98	0
	Sum	2.98		0.74			
18:0	*sn*-1	1.24 ± 0.02	<0.01	2.33 ± 0.35	<0.01	0.99	1
	sn-2	0.08 ± 0.01	<0.01	0.33 ± 0.08	<0.01	0.85	1
	sn-3	1.67 ± 0.02	<0.01	−1.57 ± 0.31	<0.01	1.00	0
	Sum	2.99		1.09			
18:1	*sn*-1	0.94 ± 0.04[b]	<0.01	−1.19 ± 0.94	0.22	0.95	1
	sn-2	0.97 ± 0.05[b]	<0.01	1.30 ± 1.20	0.29	0.93	2
	sn-3	1.12 ± 0.06	<0.01	−0.24 ± 1.44	0.87	0.92	1
	Sum	3.02		−0.12			
18:2	*sn*-1	0.96 ± 0.05	<0.01	−7.09 ± 2.30	<0.01	0.93	0
	sn-2	0.15 ± 0.13	0.26	58.62 ± 6.14	<0.01	0.05	2
	sn-3	1.82 ± 0.09	<0.01	−49.46 ± 4.15	<0.01	0.94	1
	Sum	2.94		2.07			
18:3	*sn*-1	1.06 ± 0.05[c]	<0.01	−0.28 ± 0.37	0.46	0.94	3
	sn-2	1.16 ± 0.15[c]	<0.01	0.59 ± 1.11	0.60	0.67	1
	sn-3	0.52 ± 0.11	<0.01	0.61 ± 0.81	0.48	0.44	1
	Sum	2.74		0.92			

[a]Standard deviation.
[b]Not significantly different at the 5% level.
[c]Not significantly different at the 5% level.
[d]Z-test statistics—the number of outliers found were within the expected number for the 95% probability level.

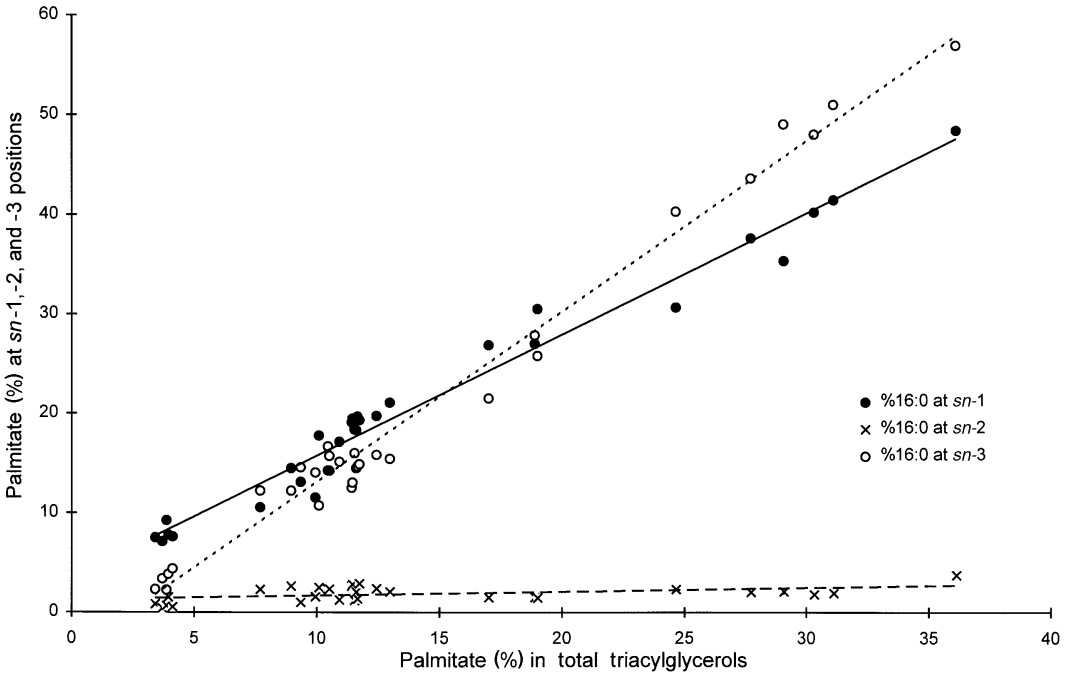

FIG. 1. The percentage of palmitate on the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol vs. the percentage of palmitate in the whole triacylglycerol for *Glycine max* germplasm lines.

30, one or two data points per regression would be expected to fall outside this limit. Thus, the number of outliers for the regressions in Table 1 is not greater than the number expected by chance.

The palmitate and stearate data in Figures 1 and 2 were fit well by the linear regression. In agreement with previous studies (4,6), there was very little saturated acyl group on the *sn*-2 positions. The slope of the regression line for palmitate on the *sn*-2 position was significantly different from zero, but this may be because palmitate migrated from the *sn*-1 and *sn*-3 positions in proportion to the amounts present in the TAG. In contrast to earlier studies, which had a much more limited

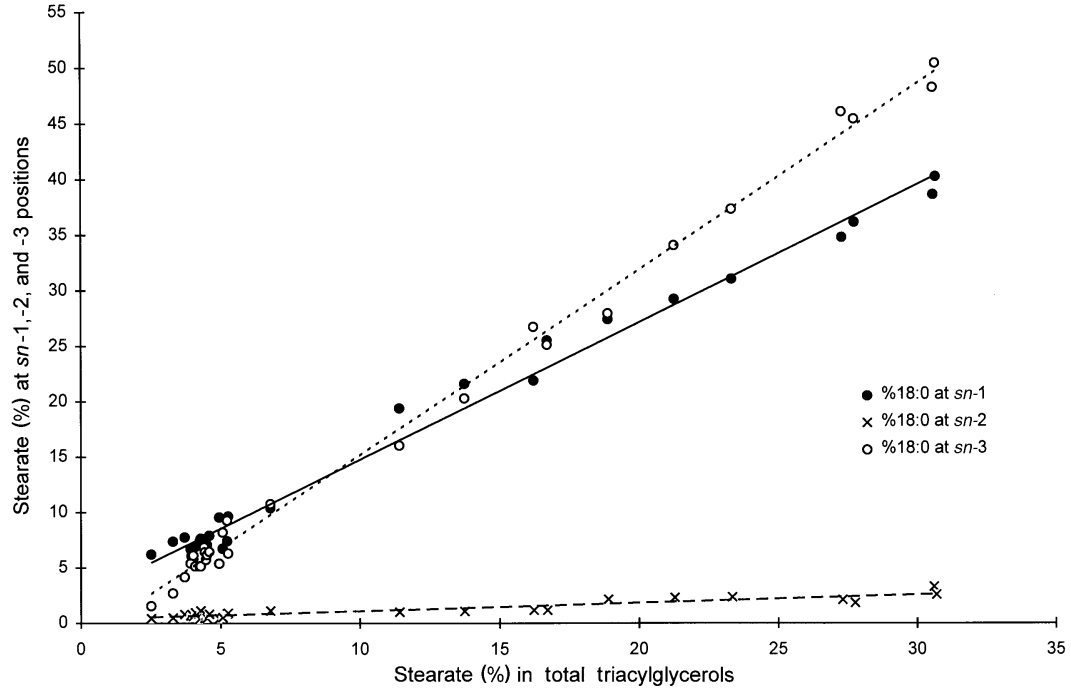

FIG. 2. The percentage of stearate on the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol vs. the percentage of stearate in the whole triacylglycerol for *Glycine max* germplasm lines.

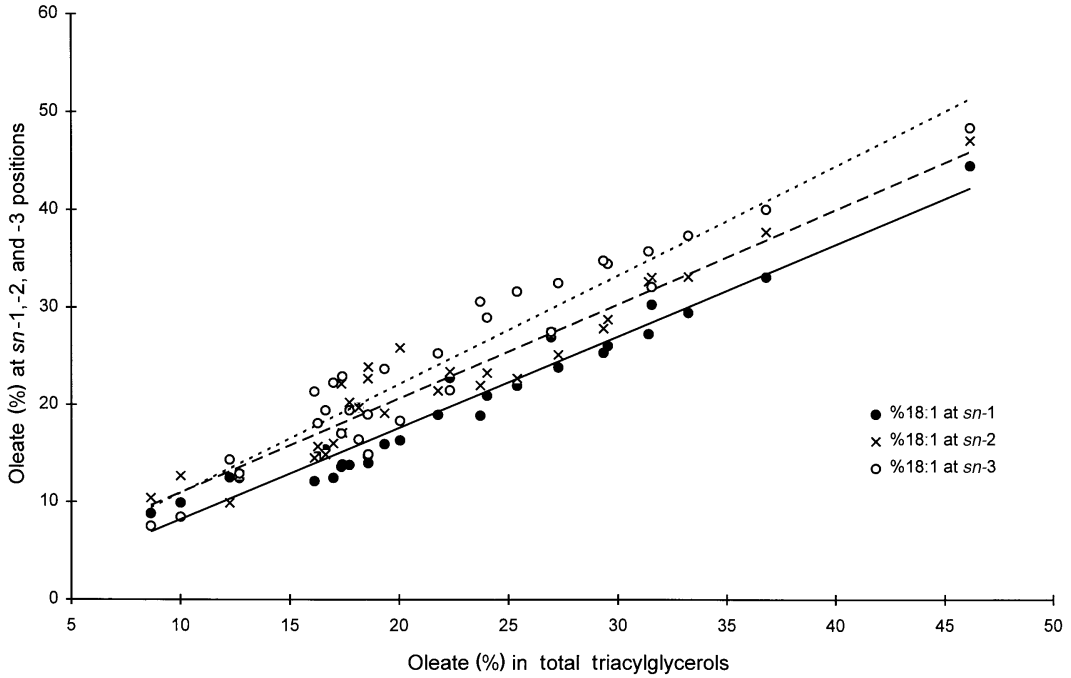

FIG. 3. The percentage of oleate on the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol vs. the percentage of oleate in the whole triacylglycerol for *Glycine max* germplasm lines.

range for saturated acyl groups than the present study, the regression lines for the *sn*-1 and *sn*-3 positions crossed at about 15% for palmitate in the TAG and 8% for stearate. In both instances the percentages for *sn*-1 were greater than those for *sn*-3 at TAG percentages below the crossover points, and the reverse was true above the crossover points.

Figures 3, 4, and 5 show the distribution of oleate, linoleate, and linolenate on the three *sn*-positions of glycerol. The data for oleate and the *sn*-1 and *sn*-3 positions of linoleate were fit reasonably well by the linear regressions, but the data for the *sn*-2 position of linoleate and all the positions for linolenate showed considerable scatter not seen in previous

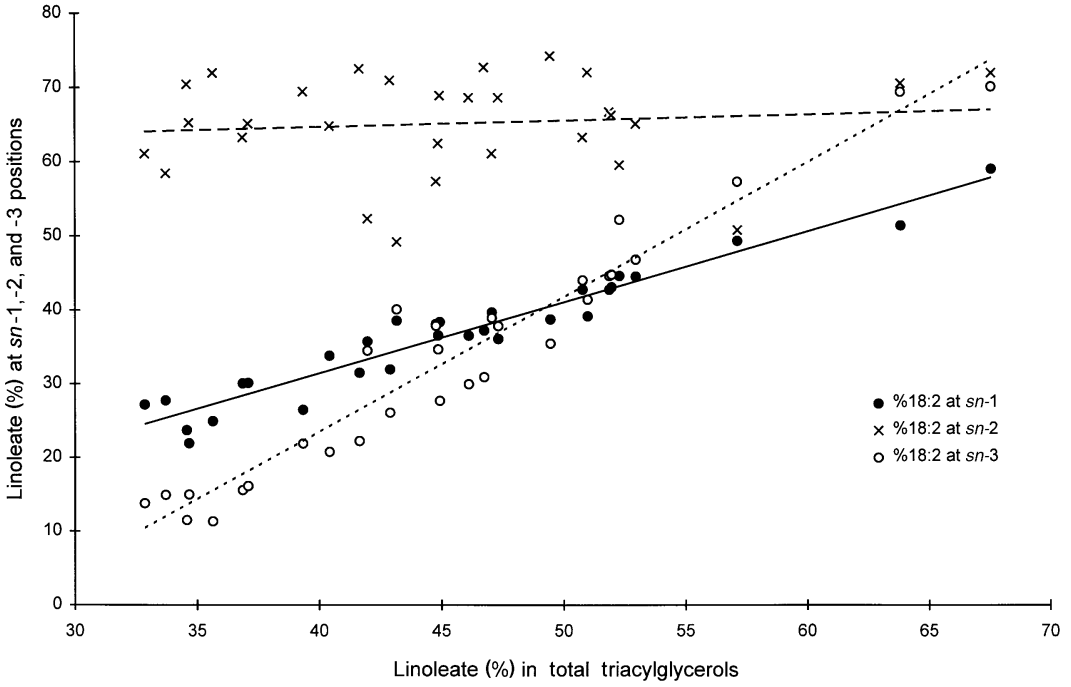

FIG. 4. The percentage of linoleate on the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol vs. the percentage of linoleate in the whole triacylglycerol for *Glycine max* germplasm lines.

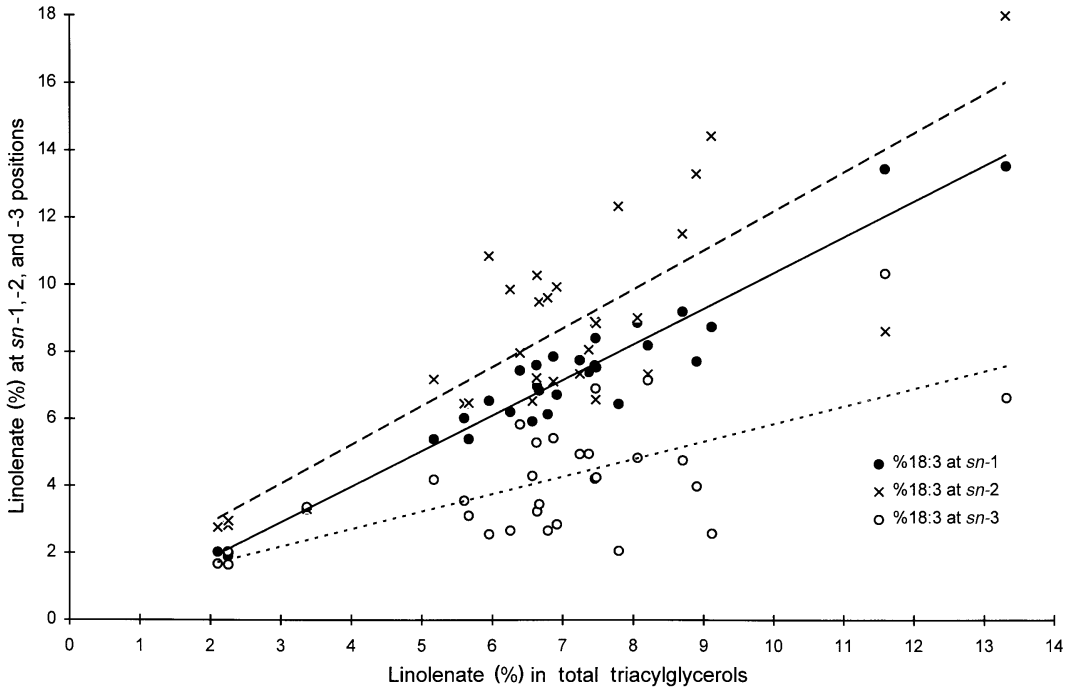

FIG. 5. The percentage of linolenate on the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol vs. the percentage of linolenate in the whole triacylglycerol for *Glycine max* germplasm lines.

studies. Much of the scatter in this study could be attributed to the presence of a number of samples with either elevated or reduced saturate concentrations. For example, samples with typical saturate concentrations (~10% palmitate and ~4% stearate) had an enrichment of oleate on the *sn*-3 position (data not shown), as reported in previous studies. But in germplasm lines with elevated stearate, the oleate often was more enriched on the *sn*-2 position. In germplasm lines elevated in both palmitate and stearate, the oleate distribution favored *sn*-2 > *sn*-1 > *sn*-3. In germplasm lines with reduced saturates, the oleate was distributed more or less equally among the three *sn*-positions.

For linoleate, placement on the *sn*-2 position was generally favored, but as reported previously by Pan and Hammond (6), the regression line for the *sn*-3 position had a greater slope, and the linoleate on the *sn*-3 position equaled that of the *sn*-2 position at high levels of TAG linoleate. Like the *sn*-1 and *sn*-3 regression lines for stearate and palmitate, those for linoleate crossed, in this instance, at about 49% linoleate in TAG.

Figure 6 shows the distribution of linoleate on the *sn*-2 position for samples having normal, elevated, and reduced levels of the saturated acyl groups. The samples with typical saturate levels were fairly well fit by a straight line, but the samples with elevated saturates deviated to greater ordinate values, and those with reduced saturates deviated to lower values. The extent of these deviations was correlated with the TAG saturate content and the saturate content on the *sn*-1 position.

Previous studies showed that linolenate placement was favored at the *sn*-1 over the *sn*-2 or *sn*-3 positions, but in this study this was true only for germplasm lines with low or normal saturate levels (data not shown). Figures 7 and 8 show how saturate content affected the distribution of linolenate on the *sn*-2 and *sn*-3 positions. In soybean germplasm lines with elevated saturate contents, the linolenate deviated to lower ordinate values on the *sn*-3 plot and to higher values on the *sn*-2 plot. In low and normal saturate samples, the linolenate deviated in the opposite directions from samples with elevated saturates. The *sn*-1 plot for linolenate showed deviations related to saturates that were similar to those of the *sn*-3 plot (data not shown), but as Figure 5 shows, the scatter for the *sn*-1 line was much less than those for *sn*-2 and *sn*-3. This may be because the elevated saturates tended to accumulate on the *sn*-3 position preferentially.

Possibly, these deviations from the stereospecific distributions previously reported resulted from glyceride structure mutations as predicted by Fatemi and Hammond (4), and germplasm lines that contained elevated saturates were accompanied by mutations in glyceride assembly. But the number of outliers for each of the regressions shown in Table 1 was not greater than the number expected by chance.

A more likely explanation of the deviations from the linear regressions was that the inclusion of germplasm lines with unusual amounts of saturates revealed normal effects of saturates on glyceride assembly that were not obvious previously. Linoleate and linolenate are formed on the *sn*-2 position of phosphatidylcholine (19). Possibly, linoleate and linolenate move from the *sn*-2 to the *sn*-1 and *sn*-3 positions by a coupled exchange with oleate on the *sn*-1 and *sn*-3 position. If the saturates are elevated on the *sn*-1 and *sn*-3 positions, there would be less oleate to effect the exchange. A lack of oleate with which to exchange would increase the linoleate and linolenate on the *sn*-2 position and decrease linoleate and linolenate on

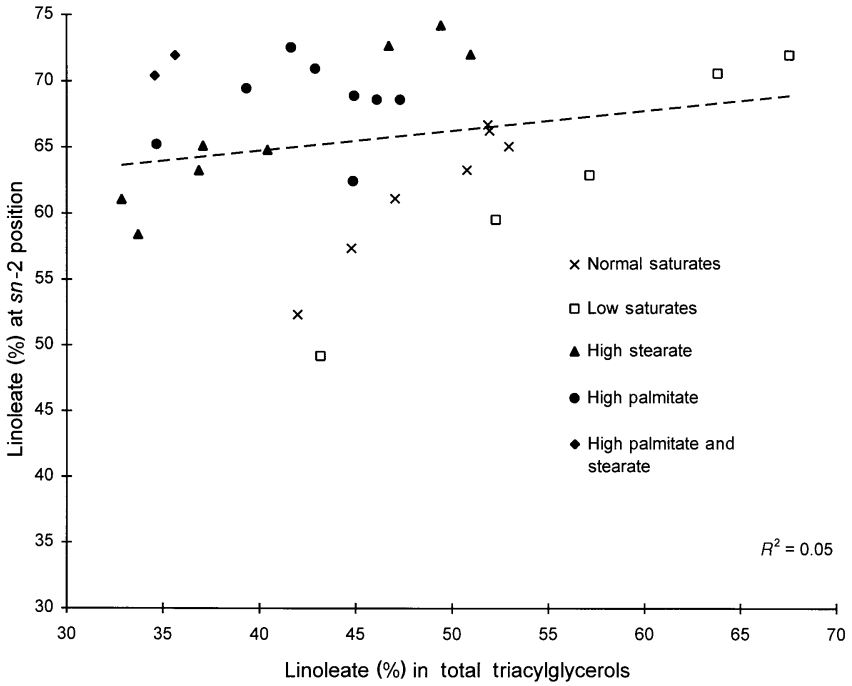

FIG. 6. The effect of germplasm lines with various saturate percentages on the percentage of linoleate on the *sn*-2 positions of glycerol vs. the percentage of linoleate in the whole triacylglycerol for *Glycine max* germplasm lines.

sn-3. Reduced saturate levels would have the opposite effect. Such mechanisms would explain the observed effects.

Samples rich in palmitate contained significant amounts (up to 2.4%) palmitoleate (16:1). Palmitoleate concentrations were approximately equal on the *sn*-1 and *sn*-3 positions, but the *sn*-2 position contained twice as much palmitoleate as *sn*-1 and *sn*-3.

Samples rich in stearate also contained significant amounts (up to 2%) arachidate (20:0). Arachidate was almost exclu-

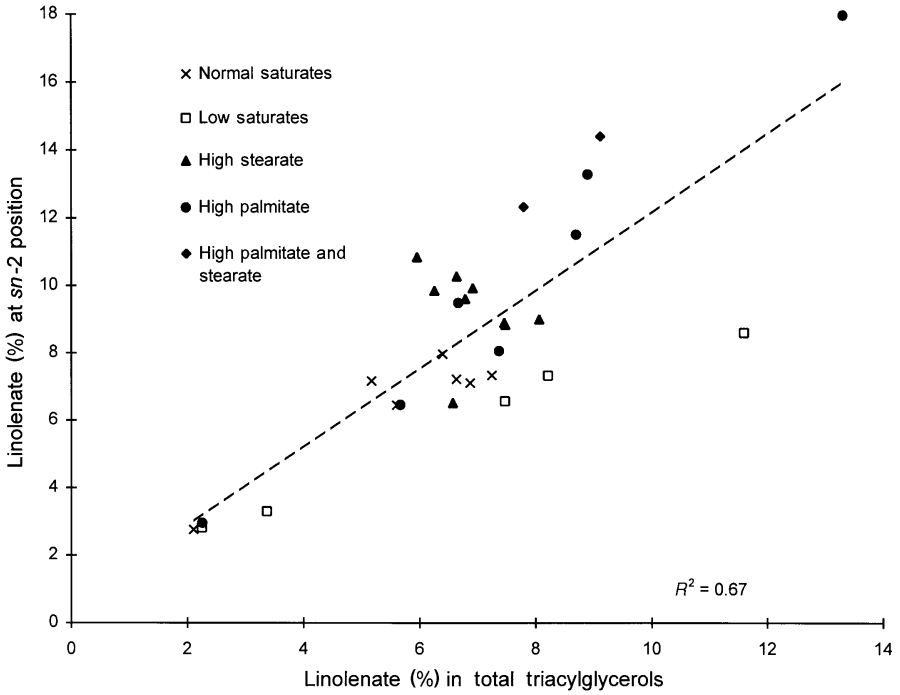

FIG. 7. The effect of germplasm lines with various saturate percentages on the percentage of linolenate on the *sn*-2 positions of glycerol vs. the percentage of linolenate in the whole triacylglycerol for *Glycine max* germplasm lines.

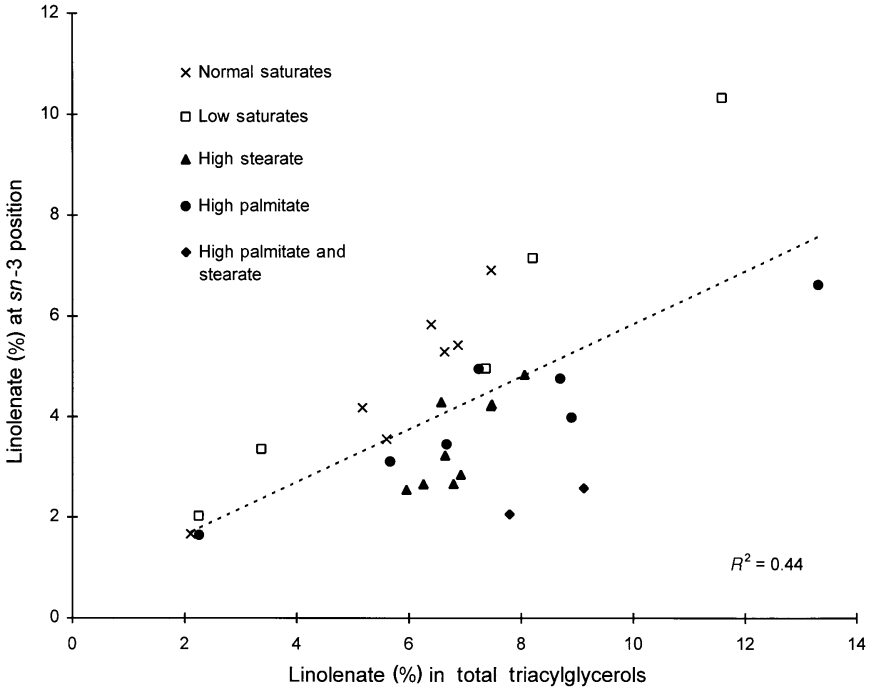

FIG. 8. The effect of germplasm lines with various saturate percentages on the percentage of linolenate on the *sn*-3 positions of glycerol vs. the percentage of linolenate in the whole triacylglycerol for *Glycine max* germplasm lines.

sively at the *sn*-3 position. A similar distribution of acyl groups longer than stearate has been reported for peanut (20).

Theoretically, if there were 0 or 100% of an acyl group present in a particular TAG sample then the amount of that acyl group on three *sn* positions would have to be either 0 or 100%. Thus, each of the stereospecific plots must go through 0,0 and 100,100. For a linear plot, only a line with a slope of 1 will pass through both of these points, so the seemingly linear stereospecific plots observed in some of the figures cannot be linear over their entire range. Lee *et al.* (7) proposed two mathematical models to account for the stereospecific distribution of acyl groups in TAG. Model 1 assumes that some fraction of the glycerol molecules contains fixed proportions of specific acyl groups. The acyl group distribution at the *sn*-1, -2, and -3 positions of this fraction of the glycerol is controlled by specific enzymes that place the acyl groups independently of the total amount available for esterification. The remainder of the glycerol molecules, not affected by this mechanism, is acylated according to the proportion of acyl groups available. This model gives linear plots over the span in which it can operate. The second model was based on chemical kinetics and assumed that the three glycerol positions were filled in proportion to substrate concentrations and the affinities of the enzymes filling the three *sn* positions. This model is approximately linear over much of its range but bends to go through 0,0 and 100,100. Neither of these models fits the present data, primarily because the data for the *sn*-3 plots of palmitate, stearate, and linoleate show intercepts on the abscissa that are not possible with these models.

Previous studies showed that for stereospecific plots the slopes summed to 3 and the intercepts to 0 (4,6). The models of Lee *et al.* (7) give a mathematical basis for these observations. Table 1 shows that although the slopes sum to approximately 3, the intercepts are considerably greater than zero except for oleate. This reflects the greater scatter of these data compared with previous studies and results from the inclusion of many samples with altered saturate content.

To check the reliability of the analyses, the average fatty acid composition of the TAG being analyzed and the average found for the three *sn* positions were calculated, and the results are shown in Table 2. If there were no analytical errors, these values should agree, but the differences were all statistically significant. The sample means for the saturates and oleate were greater for the stereospecific analyses than from the whole TAG, whereas the opposite was true for linoleate and linolenate. Takagi and Ando (12) reported a bias against the recovery of polyenoates during the formation of DNPU

TABLE 2
Sample Means of the Percentage of Fatty Acid Composition for the Original TAG Compared with the Average Found on the Three *sn* Positions

	Mean (mole%)[a]		
Acyl group	TAG	Avg. of *sn*-1, *sn*-2, and *sn*-3 positions	SEM[b]
16:0	14.20	14.45	0.05
18:0	10.73	11.10	0.04
18:1	22.22	22.35	0.04
18:2	45.53	45.10	0.06
18:3	6.83	6.53	0.06

[a]n = 60. All differences were significant at <2% level.
[b]Standard error of the mean. TAG, triacylglycerol.

TABLE 3
Comparison of the *sn*-2 Acyl Group Mole Percentages After Pancreatic Lipase Hydrolysis and Grignard Hydrolysis of Soybean Oil Triacylglycerols

	Composition (mole%)[a]	
	Pancreatic lipase[b]	Grignard[c]
Acyl group	*sn*-2	*sn*-2
16:0	0.50	1.79
18:0	0.31	1.21
18:1	18.06	17.89
18:2	70.62	68.69
18:3	10.26	10.14

[a]Average of 15 samples in duplicate.
[b]Modified method of Luddy *et al.* (18).
[c]Modified method of Takagi and Ando (11).

derivatives and their conversion to methyl esters. Our results show considerably better recovery than they reported, and the small losses of polyenoates that we observed may be caused by oxidation during TLC and handling.

An analysis of variance for each of the acyl groups in the 30 duplicate samples showed that the standard errors of the means for each acyl group percentage on each *sn*-position and for the whole TAG were small. In general, the hydrolysis and separation steps did not increase the standard errors over those for TAG.

The compositions of the *sn*-2 positions for 15 germplasm lines were determined by pancreatic lipase hydrolysis as well as by the analysis of the DNPU derivatives representing the *sn*-2 position, and the results are compared in Table 3. A paired *t*-test of the means for the two methods showed that the Grignard deacylation method gave more saturate and less unsaturate on the *sn*-2 positions than did the lipase method, and the differences were significant for palmitate, stearate and linoleate ($P < 0.01$). This suggests that slightly more acyl migration occurred with the Grignard method than with lipase.

ACKNOWLEDGMENT

This paper is Journal Paper No. J-1762 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011, Project No. 3414.

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[Received September 29, 1997, and in final revised form December 29, 1997; revision accepted January 6, 1998]

Silver-Ion High-Performance Liquid Chromatographic Separation and Identification of Conjugated Linoleic Acid Isomers

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ABSTRACT: This is the first report of the application of silver-ion impregnated high-performance liquid chromatography (Ag^+-HPLC) to the separation of complex mixtures of conjugated linolenic acid (CLA) isomers present in commercial CLA sources and foods and in biological specimens. This method showed a clear separation of CLA isomers into three groups related to their *trans,trans, cis,trans* or *trans,cis,* and *cis,cis* configuration of the conjugated double-bond system. In addition, this method separated individual positional isomers of the conjugated diene system within each geometrical isomeric group. Following Ag^+-HPLC isolation, gas chromatography (GC)–electron impact mass spectrometry, and GC–direct deposition-Fourier transformed infrared spectroscopy were used to confirm the identity of two major positional isomers in the *cis/trans* region, i.e., Δ8,10- and Δ11,13-octadecadienoic acids, which had not been chromatographically resolved previously. Furthermore, the potential of this method was demonstrated by showing different Ag^+-HPLC profiles exhibiting patterns of isomeric distributions for biological specimens from animals fed a diet containing a commercial CLA preparation, as well as for a commercial cheese product.

Lipids 33, 217–221 (1998).

"Conjugated linoleic acid (CLA)" is an imprecisely defined term describing a mixture of octadecadienoic acids (18:2) that contain a conjugated double-bond system. Interest in CLA has increased in the last decade with reports that dietary CLA reduced carcinogenesis (1–4), atherosclerosis (5), and body fat (6) and provided several other beneficial effects (7–9) in laboratory animals. However, commercial CLA products fed to experimental animals are complex mixtures of CLA isomers, many of which have not been identified. To date, there is no direct evidence as to which isomer or isomers within the complex CLA mixture are the potentially active components, other than an assumption that it may be the major isomer, *cis,trans*-octadeca-9,11-dienoic acid (*c,t*-9,11-18:2), found in milk and dairy products (10).

Techniques used previously to measure CLA have included high-performance liquid chromatography (HPLC) and gas chromatography (GC). HPLC procedures using C_{18} (10–13) or silica columns (14) produced inadequate separation of CLA isomeric mixtures, analyzed as either free fatty acids (10–12,14) or fatty acid methyl esters (FAME) (13). The CLA isomers were detected by ultraviolet (UV), which takes advantage of the conjugated diene chromophore that absorbs at 234 nm. In GC separations, long capillary columns with polar liquid phases (e.g., SP 2560, CP-Sil 88, or Supelcowax-10) were used (11,15–17). Although several peaks in the CLA isomeric region were resolved by GC depending on the column and conditions used, the separation was not complete as evidenced by GC–mass spectrometry (MS) of the 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives (11,16). In GC separations, CLA positional and geometrical isomers extensively overlap making their identification difficult, particularly of the minor components. Neither HPLC nor GC provided sufficient resolution for all the isomers in complex CLA mixtures.

We describe here an application of silver-ion HPLC (Ag^+-HPLC) that resolved CLA isomers on the basis of chain length, double-bond configuration, and position of the conjugated diene functional group in the fatty acid chain. For each CLA positional isomer, the corresponding *cis/trans* or *trans/cis* pair of geometric isomers were not separated by Ag^+-HPLC. The chromatographic resolution of complex mixtures of CLA isomers obtained by Ag^+-HPLC was substantially better than those reported previously by GC on polar phases, or by HPLC on C_{18} or silica columns.

MATERIALS AND METHODS

A mixture of CLA FAME was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Several isomers or isomeric mixtures of CLA were obtained as free fatty acids from Matreya Inc. (Pleasant Gap, PA) and from Dr. Michael W. Pariza of the University of Wisconsin (Madison, WI). Acetonitrile and

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Abbreviations: *cis/trans,* refers to the same positional isomers that have either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 2-alkenyl-4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC–DD–FTIR, gas chromatography–direct deposition–Fourier transform infrared; GC–EIMS, gas chromatography–electron impact mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet.

hexane were UV grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol, 95%, was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from TCI America (Portland, OR).

HPLC. HPLC analysis was performed with a Waters 510 solvent delivery system (Waters Associates, Milford, MA), a Waters 717 plus Autosampler with a 2-mL and 200-µL injection loop, a Waters 486 tunable absorbance UV detector (233 nm), a Waters fraction collector, and a Waters software program (Millennium™ version 2.15.01). A ChromSpher 5 Lipids semipreparative (10 mm i,d. × 250 mm stainless steel; 5 µm particle size) and an analytical (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size) silver-impregnated column were used (Chrompack, Bridgewater, NJ). The solvent flows were 4.0 and 1.0 mL/min, for the preparative and analytical columns, respectively. Best resolutions were obtained with a mobile phase of 0.1% acetonitrile in hexane and isocratic operation. The mobile phase mixture was prepared fresh each day because the retention volumes of the CLA isomers changed with time with little loss of resolution. The flow was commenced for 0.5 h prior to sample injection. Typical injection volumes were 150–200 µL for the semipreparative column and 5–15 µL for the analytical column.

Preparation of FAME. The free acids (CLA) were dissolved in a screw-cap test tube with 1 mL of 20% methanol/benzene and reacted with trimethylsilyldiazomethane as described (18). Unreacted trimethylsilyldiazomethane was removed by adding *ca.* five drops of acetic acid with gentle swirling until the yellow color disappeared. Then 5 mL H_2O and 1 mL isooctane were added, and the tube was capped and shaken for 10 s. The solution was dried over Na_2SO_4 and stored for future injection. Test portions of purchased FAME mixtures were dissolved in hexane prior to Ag^+-HPLC analysis.

DMOX derivatives. To a reaction tube, 10–20 mg of methyl heptadecanoate (17:0) and *ca.* 1–2 mg of CLA FAME (collected from semipreparative HPLC column) were added, and the mixture was hydrolyzed with 1 N KOH/95% ethanol (19), in order to obtain free fatty acid. The free fatty acids were added to a screw-cap reaction tube, and a threefold excess (w/w) of 2-amino-2-methyl-1-propanol was added. The tube was purged with argon, capped, and heated at 170°C for 0.5 h in an oven. DMOX derivatives were partitioned into petroleum ether as described previously (11).

GC–electron impact mass spectrometry (GC–EIMS). The GC–EIMS was performed by using a gas chromatograph (Hewlett-Packard 5890, series II, Palo Alto, CA) coupled to a mass spectrometer (Autospec Q mass spectrometer) and data system (OPUS 4000) (Micromass, Manchester, United Kingdom). The GC–MS system utilized version 2.1 BX software. This system was used with a 100-m CP-Sil 88 capillary column (Chrompack) described previously (9). The GC–MS conditions were: splitless injection with helium sweep restored 1 min after injection; injector and transfer lines' temperature 220°C; oven temperature 75°C for 1 min after injection, then ramped 20°C/min to 185°C, and held at 185°C for 15 min, 4°C/min to 220°C, and held there for 45 min.

GC–direct deposition Fourier transform infrared (GC–DD–FTIR) spectroscopy. A Bio-Rad (Cambridge, MA) Tracer™ GC–FTIR 60A spectrometer system was used. This system, which was used with a 50 m CP-Sil-88 capillary column, has been described earlier (20).

Quantitation. The response factors were calculated as follows. Each response was first determined by GC–DD–FTIR to have either a *cis,cis*, a *trans,trans*, or a *cis/trans* configuration (21). Quantitation of each *cis,cis* isomer was calculated by taking the percentage of that response $[R(c,c)_i]$ relative to the total of all *cis,cis* responses obtained by Ag^+–HPLC $\left[\sum_1^n R(c,c)_i\right]$, and then multiplying this fraction $\left[R(c,c)_i/\sum_1^n R(c,c)_i\right]$ by the total amount of *cis,cis* isomers measured by GC. Response factors were also determined in this manner for the *trans,trans* and the *cis/trans* isomers.

RESULTS AND DISCUSSION

Figure 1 shows a typical Ag^+-HPLC chromatographic separation obtained from a commercial CLA mixture. The resolution depended on the concentration of acetonitrile in hexane

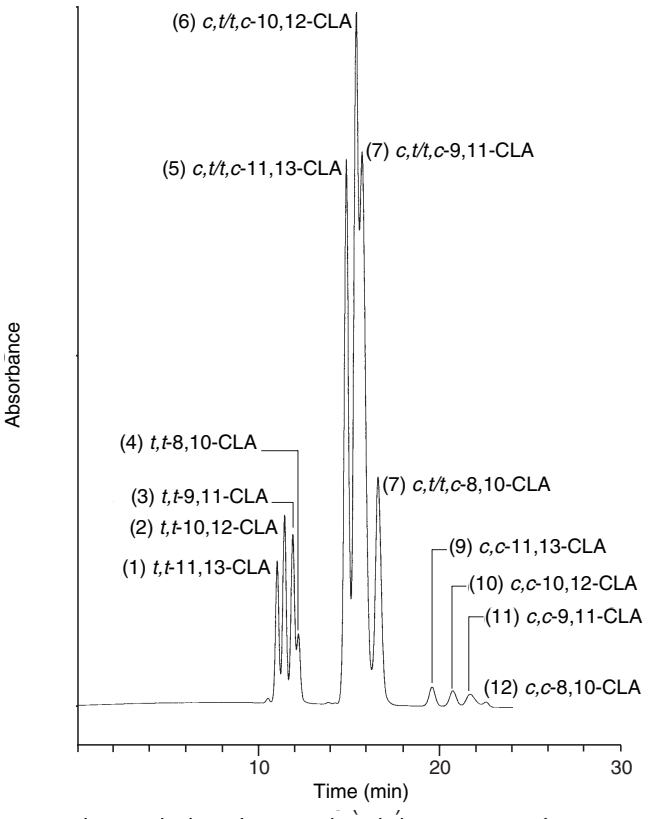

FIG. 1. Silver-ion high-performance liquid chromatogram of a commercial conjugated linoleic acid (CLA) standard (Nu-Chek-Prep, Elysian, MN), using a ChromSpher 5 Lipids analytical column (4.6 mm i.d. × 250 mm), and 0.1% acetonitrile in hexane as the mobile phase, at a flow rate of 1 mL/min. The slash between geometric isomers (i.e., *c,t/t,c*) means that either or both of the two isomers could be present.

of the mobile phase, first used by Adlof *et al*. (22) to separate isomeric *cis* and *trans* 18:1 FAME. At 1% acetonitrile, all the CLA isomers eluted as a single peak within 1 min. The resolution shown in Figure 1 was obtained with a concentration of 0.1% acetonitrile in hexane. Twelve peaks eluted, generally in three groups of four peaks each. The first group was shown to be *trans,trans* CLA isomers, while the second and third groups were *cis/trans* and *cis,cis* isomers, respectively. At 0.2 and 0.3% acetonitrile in hexane, all the peaks emerged earlier, and there was less resolution between the sets of geometric isomers. Evidence for the identification of the individual CLA isomers within each group was obtained by comparison with standard CLA isomers, from direct measurements of the isolated peaks by HPLC using both GC–DD–FTIR and GC–EIMS spectral techniques, and by simple comparison of the relative amounts of each isomer within each group.

Identification of the individual CLA isomers. Peaks 3, 7, and 11 had the same retention volumes as those of the standards, methyl *trans,trans*-octadeca-9,11-dienoate (*t,t*-9,11-18:2), *c,t*-9,11-18:2, and *c,c*-9,11-18:2, respectively. These standards were obtained from Matreya Inc. as the free fatty acids which we converted to their FAME. We confirmed the conjugated double-bond geometry and position, and purity of these three standards by GC–DD–FTIR and GC–EIMS (21).

The CLA mixture provided by Dr. Pariza was also methylated and analyzed by Ag^+-HPLC. This mixture showed peaks at retention volumes corresponding to peaks 2, 3, 6, 7, 10, and 11 (Fig. 1). Independent GC–EIMS analysis in our laboratory of Pariza's CLA mixture showed that this mixture contained CLA isomers with double bonds only at the Δ9,11 and Δ10,12 positions. These data, in conjunction with GC–DD–FTIR spectroscopy, GC–EIMS and the relative retention volumes on Ag^+-HPLC, indicated that peaks 2, 6, and 10 were due to *t,t*-10,12-18:2, *t/c*-10,12-18:2, and *c,c*-10,12-18:2, respectively.

Peaks 5 and 8 (Fig. 1) were collected after repeated injections using a semipreparative Ag^+-HPLC column. The DMOX derivatives of the isolated peaks 5 and 8 were shown by GC–EIMS to have a conjugated double-bond system at positions Δ11,13 (peak 5) and Δ8,10 (peak 8). The EIMS evidence for the double-bond position of these two conjugated octadecadienes with their characteristic fragmentation patterns is shown in Figure 2. The GC–DD–FTIR data were consistent with peaks 5 and 8 both containing isomers with a

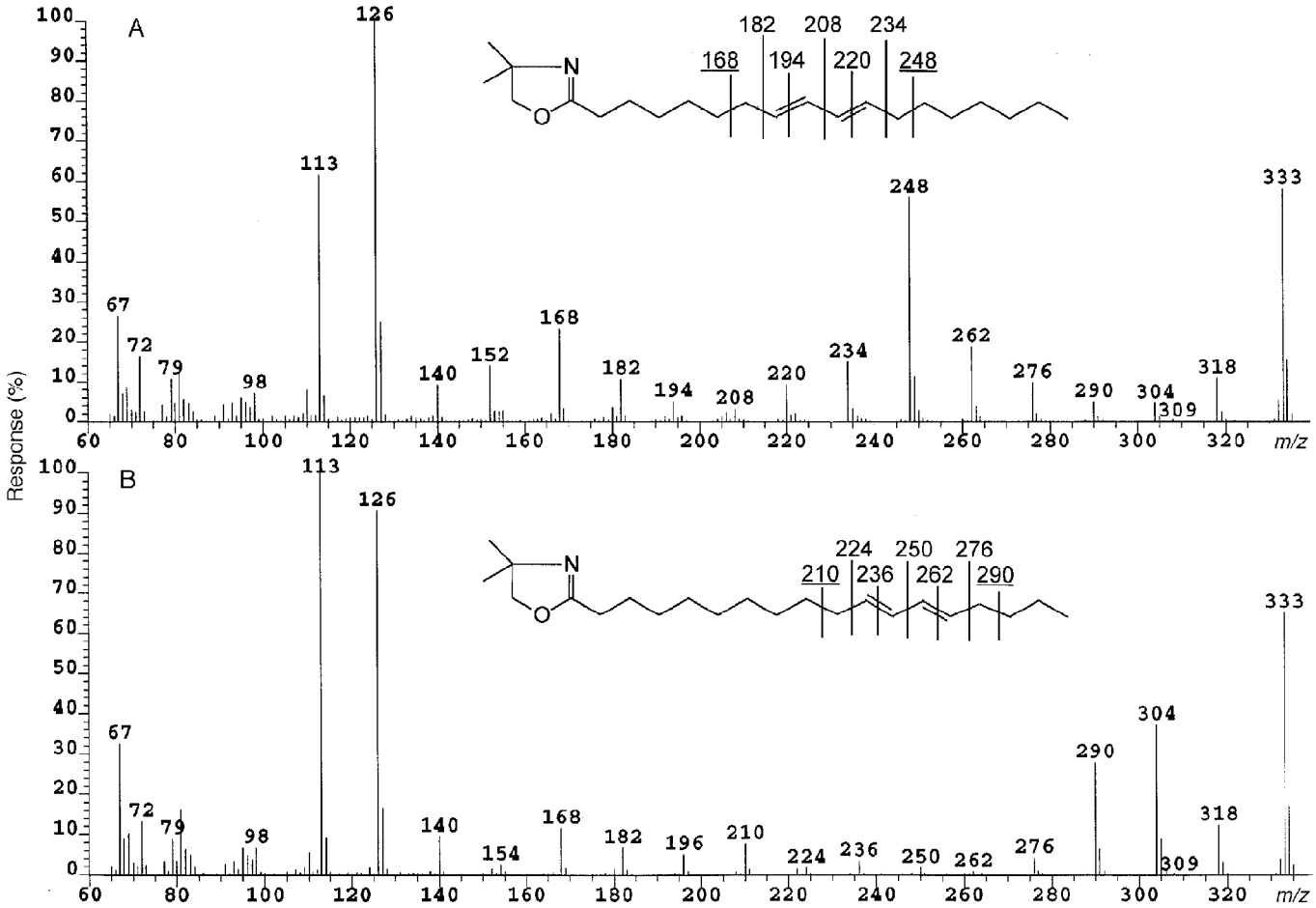

FIG. 2. Gas chromatography–electron impact mass spectrometry spectra of the 2-alkenyl-4,4-dimethyloxazoline derivatives of two CLA isomers isolated from a commercial CLA mixture which were identified as (A) *c,t/t,c*-8,10-18:2 and (B) *c,t/t,c*-11,13-18:2. The *trans,trans* structures shown in the figure are only meant to illustrate the *m/z* assignments. For abbreviations see Figure 1.

TABLE 1
Composition (% of total fatty acid methyl esters) of a Commercial Conjugated Linoleic Acid (CLA) Mixture[a]

CLA isomers	This study[b]	Nu-Chek-Prep assay[c]
t,t-11,13-18:2	0.74	—[d]
t,t-10,12-18:2	1.23	1.3[e]
t,t-9,11-18:2	1.18	[e]
t,t-8,10-18:2	0.46	—
c,t/t,c-11,13-18:2[f]	21.7	—
c,t/t,c-10,12-18:2	29.0	44.0
c,t/t,c-9,11-18:2	29.5	41.0
c,t/t,c-8,10-18:2	12.3	—
c,c-11,13-18:2	0.96	—
c,c-10,12-18:2	0.88	9.5
c,c-9,11-18:2	0.88	1.0
c,c-8,10-18:2	0.20	—

[a]Commercial CLA mixture was obtained from Nu-Chek-Prep, Inc. (Elysian, MN) (Ref. 24).
[b]For quantitative calculations see the Materials and Methods section.
[c]The composition was obtained from Nu-Chek-Prep, Inc. and reported previously (Ref. 24). It also included 0.7% 18:2n-6.
[d]—, Not reported.
[e]Represents content of both *t,t*-10,12-18:2 and *t,t*-9,11-18:2 (Ref. 24).
[f]The slash between geometric isomers (i.e., *c,t/t,c*) means that either or both of the two isomers could be present.

cis/trans and/or *trans/cis* double-bond configuration. This assignment was based on characteristic FTIR bands including those observed at 988 and 949 cm^{-1} (21,23). A tentative assignment of the minor peaks 1, 4, 9, and 12 (Fig. 1) was made based on a comparison of the retention volume order among the *cis/trans* group of isomers, and by the relative abundance of the CLA FAME present in the *cis/trans* region.

Application of the Ag^+-HPLC method for the quantitation of a commercial CLA standard. The Ag^+-HPLC method was used in conjunction with GC–EIMS and GC–DD–FTIR to analyze and quantitate the composition of the commercial standard obtained from Nu-Chek-Prep, Inc. (Fig. 1). The procedure is described in the Materials and Methods section. The revised composition of the Nu-Chek-Prep standard is shown in Table 1 and compared to the reported composition (24) of this material. The results from Table 1 showed that two major isomers of CLA [Δ8,10 (12.3%) and Δ11,13(21.7%)] were completely missed, and there was an incomplete analysis of the *trans,trans* (four isomers totaling 3.6%) and *cis,cis* isomers (four isomers totaling 2.9%). Erroneous analyses of commercial CLA mixtures used in feeding trials will result in misleading interpretations of observed biological response(s) in animals.

The revised assay (Table 1) reported for the CLA isomers was consistent with the finding of four similar sets of oxidation products, including four furan fatty acids from the Nu-Chek-Prep Inc. CLA mixture (9,24). This demonstrated that these four sets of oxidation products originated from four CLA positional isomers. It was just reported (25) that four positional isomers were found in a commercial CLA mixture using selected ion scanning of the 4-methyl-2,3,4-triazoline-3,5-dione derivative, but the CLA isomers were not separated chromatographically.

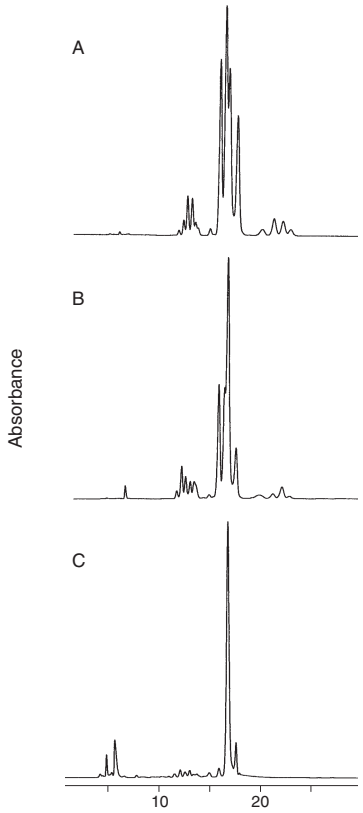

FIG. 3. Typical silver-ion high-performance liquid chromatographic separations. (A) A commercial CLA mixture used in a pig feeding study (Ref. 26); (B) the CLA composition of the total liver lipids of pigs fed this CLA mixture (Ref. 26); and (C) the CLA composition of a commercial cheese product. For abbreviation see Figure 1.

Application of the Ag^+-HPLC separation of CLA isomers in food and biological specimens. The potential of the Ag^+-HPLC method is illustrated with three examples. Figure 3 shows three additional Ag^+-HPLC separations of mixtures containing CLA isomers. Figure 3A represents the CLA composition of a commercial CLA mixture obtained from Natural Lipids Ltd. (Hovdebygda, Norway) fed to pigs in an experiment reported elsewhere (26), whereas Figure 3B shows the distribution of CLA isomers present in the total liver lipids of the pigs fed the above diet; pigs fed the control diet showed no observable CLA peaks. It is evident from Figures 3A and 3B that the commercial diet and biological matrix contained many geometric and positional isomers of CLA, some of which still remain to be identified. Some bioconcentration of the *c,t*-9,11-18:2 was evident compared to the dietary distribution of CLA isomers. A complete composition of CLA isomers in tissue lipids will be presented elsewhere. In addition, Figure 3C presents a typical CLA distribution found in a commercial cheese product. The only major CLA isomer in cheese is the *c,t*-9,11-18:2. Detailed analyses of food products are in progress.

In conclusion, Ag^+-HPLC proved to be an excellent method for the analysis and isolation of most geometric and positional isomers of CLA. Furthermore, this method allowed

the identification of CLA isomers not separable by other complementary techniques, such as GC. In combination with GC–DD–FTIR and GC–EIMS, a more accurate confirmation of the identity of CLA isomers in commercial and biological specimens is now possible.

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[Received November 26, 1997; accepted January 6, 1998]

Phospholipase D Hydrolyzes Short-Chain Analogs of Phosphatidylcholine in the Absence of Detergent

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ABSTRACT: Phospholipase D is an important enzyme in signal transduction in neuronal tissue. A variety of assays have been used to measure phospholipase D activity *in vitro*. The most typical measure of phospholipase D activity is the production of phosphatidylethanol in the presence of ethanol. Phosphatidylethanol is a product of transphosphatidylation activity that is considered a unique property of phospholipase D. To support transphosphatidylation activity, high concentrations of ethanol may be required. Furthermore, most assays in the literature utilize a detergent. These extreme conditions, detergent and ethanol, may alter phospholipase D and hinder the study of its regulation. In this manuscript we describe an assay that eliminates these potentially confounding conditions. It utilizes high specific activity [^{3}H]butanol as a nucleophilic receptor. This eliminates the need for high concentrations of alcohol. The substrate is an analog of phosphatidylcholine that contains short-chain fatty acids, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine. Phospholipase D readily hydrolyzes this substrate in the absence of detergent. This novel assay should be useful in the further characterization of phospholipase D.

Lipids 33, 223–227 (1998).

Phospholipase D is an important enzyme in neuronal signal transduction (1–3). It is activated by many neurotransmitters and hormones. In neuronal tissue, phospholipase D activity specifically hydrolyzes phosphatidylcholine (4–8). This hydrolysis leads to the production of phosphatidic acid and choline. Phosphatidic acid could act as a second messenger or be hydrolyzed by phosphatases to form diacylglycerol. Diacylglycerol activates protein kinase C, which can phosphorylate and activate other enzymes. Choline in cells that contain choline acetyltransferase can serve as a precursor to acetylcholine.

The most typical measure of phospholipase D activity is transphosphatidylation (6). Usually, this requires a high concentration of ethanol, ~400 mM. Furthermore, most *in vitro* assays for phospholipase D utilize a detergent, e.g., sodium oleate or Triton X-100 (5,6,9) . It is not clear whether the detergent is necessary to activate the enzyme or to make the substrate more accessible (10). These extreme conditions, detergent and ethanol, may alter phospholipase D and hinder the ability to study its regulation. In this paper we describe a novel assay for phospholipase D that attempts to eliminate these potentially confounding conditions. It utilizes high specific activity [^{3}H]butanol as a nucleophilic receptor. This eliminates the need for high concentrations of alcohol. The substrate is an analog of phosphatidylcholine that contains short-chain fatty acids, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (PC8). Phospholipase D readily hydrolyzes this substrate in the absence of detergent or other cofactors such as phosphatidylinositol-4,5-bisphosphate and GTPγS.

MATERIALS AND METHODS

Isolation of microsomal membranes. Crude microsomal membranes were isolated from whole rat brain. Brains were homogenized in 10 vol of cold 0.32 M sucrose containing 1 mM EGTA. The crude homogenate was centrifuged for 10 min at 1,000 *g*. The supernatant was then centrifuged for 30 min at 20,000 *g*. Finally, the microsomal membranes were pelleted by centrifugation at 100,000 *g* for 60 min. The pellet was then washed with 0.32 M sucrose. The membranes were resuspended at a concentration of 2 mg protein/mL and stored at −80°C until use.

Phospholipase D activity. PC8, stored in chloroform, was dried under a stream of nitrogen, resuspended in ether, and then dried again. The lipid was resuspended in 5 mM HEPES, pH 6.6 and allowed to sit at room temperature for 1 h. At the the end of this time, the lipids were sonicated for 30 s with a probe sonicator, at low power, using a 50% duty cycle (Branson Sonifier 250; Danbury, CT). The reaction was initiated by adding 20 μL of this lipid suspension. Unless otherwise indicated, the assay conditions were: 3.5 mM phospholipid; 50 mM HEPES, pH 6.6; 5 μCi [^{3}H]butanol (15 Ci/mmol; American Radiolabeled Chemical, St. Louis, MO) and 100 μg of crude membrane protein in a final volume of 120 μL. The activity of the enzyme with PC8 as a substrate is linear from 10 to 100 μg of protein. In some experiments the buffer was β-dimethylglutaric acid or Tris maleate, and in other experiments 10 mM EDTA or a divalent cation was added to the incubation. These different conditions did not affect the activity of the enzyme. Blanks contained 0.32 M sucrose rather than membranes. Incubations were carried out for 10 min at

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Abbreviations: CMC, critical micellar concentration; K_M, Michaelis constant; PC8, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine.

30°C unless otherwise stated. The reactions were stopped by adding 3 mL of ice-cold chloroform/methanol (1:2) and 0.7 mL of 1% perchloric acid. The mixture was vortexed for 1 min. The phases were separated by the addition of 1 mL of 1% perchloric acid and 1 mL of chloroform. After vortexing, the tubes were centrifuged and the upper phase was aspirated. The lower phase was then washed twice with 2 mL of 1% perchloric acid. An aliquot of 500 µL was removed and dried for thin-layer chromatography. When phosphatidylcholine from egg yolk was used as substrate, it was dried under a stream of nitrogen and then resuspended in 36 mM sodium oleate in 5 mM β-dimethylglutaric acid, pH 6.5. This suspension was allowed to sit for 60 min at room temperature. Vesicle formation was induced by sonicating in a small bath sonicator (Branson 1200) at 20-s intervals for 10 min. During the intervening 20-s interval, the tube was put on ice. In one experiment the substrate was L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]phosphatidylcholine (specific activity 2 mCi/mmol). It was prepared as above except that it was mixed with egg yolk phosphatidylcholine.

Thin-layer chromatography was routinely done according to Liscovitch (11). Whatman LK6 plates (Clifton, NJ) were dipped in 1.3% potassium oxalate made up in methanol/H_2O (2:3) and allowed to dry at room temperature. Just before use, the plates were activated by heating at 115°C for 1 h. The mobile phase was the upper layer of a mixture of: ethyl acetate/isooctane/acetic acid/H_2O (13:2:3:10). The thin-layer chromatography scrapings were counted by scintillation spectrometry. Dioctanoyl-phosphatidylbutanol from cold butanol had the same R_f as dioctanoyl-phosphatidyl[^{3}H]butanol generated from [^{3}H]butanol (see Fig. 2).

Materials. Male Sprague Dawley rats were obtained from ACE Animals (Boyertown, PA) at 150–200 g. [^{3}H]Butanol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals; and [^{3}H]dipalmitoyl-glycero-3-phosphocholine was from New England Nuclear (Boston, MA), sold as L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)] phosphatidylcholine. Phospholipids, sodium oleate, and dimethylglutaric acid were obtained from Sigma (St. Louis, MO). Phosphatidylethanol was obtained from Avanti Polar Lipids (Birmingham, AL).

RESULTS AND DISCUSSION

PC8 is a good substrate for phospholipase D. The production of phosphatidylalcohol is considered a reliable index of phospholipase D activity. The availability of high specific activity radiolabeled alcohol allows the use of novel unlabeled substrates that are not available in radiolabeled form. We have already used this approach to study the substrate specificity of neuronal phospholipase D (5). In this manuscript we further exploit this advantage to examine the use of short-chain analogs of phosphatidylcholine as substrates for phospholipase D. In the experiment shown in Figure 1, microsomal membranes prepared from whole rat brain were incubated with two different substrates: PC8 (3.5 mM) and phosphatidylcholine isolated from egg yolk (2 mM). The acceptor, [^{3}H]butanol, was added at a final concentration of 3 µM. We and many other investigators have shown that optimal activity of phospholipase D requires the presence of a detergent such as sodium oleate (6). Consistent with this finding, when egg yolk phosphatidylcholine was used as a substrate, detergent was absolutely required for activity. From the data in Figure 1 it is apparent that PC8 is a better substrate for phospholipase D even in the absence of detergent.

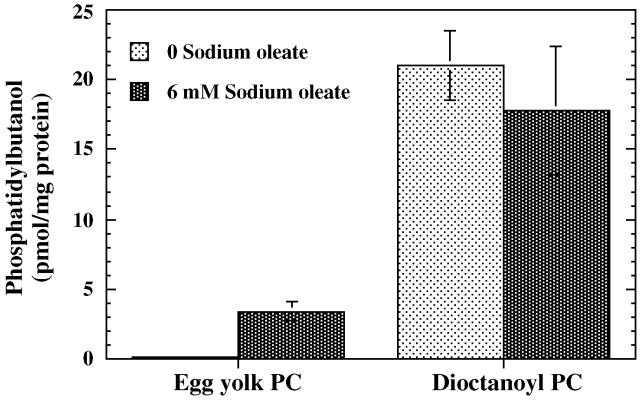

FIG. 1. The effect of sodium oleate on phospholipase D activity. Each reaction mixture contained 50 mM dimethylglutaric acid (pH 6.5), 10 mM EDTA, 5 µCi [^{3}H]butanol (15 Ci/mmol), and 100 µg of microsomal membrane protein. The final concentration of phosphatidylcholine (PC) from egg yolk was 2 mM, and the final concentration of dioctanoyl-PC was 3.5 mM. The substrate was prepared by sonicating the phospholipids in the presence and absence of 36 mM sodium oleate (final concentration = 6 mM) or buffer. Incubation time was 60 min. Data represent the mean ± SEM of three determinations.

PC8 is hydrolyzed to phosphatidic acid. The action of phospholipase D would be expected to lead to the production of phosphatidic acid. Figure 2 shows that there was an increase in phosphatidic acid in control incubations. This 1,2-dioctanoyl-*sn*-glycero-3-phosphate had the same R_f as a standard obtained from Avanti Polar Lipids (Birmingham, AL). Consistent with earlier findings, the greater the production of phosphatidylalcohol, the lower the production of phosphatidic acid (12). This was most apparent in the incubation with 100 mM butanol. Ethanol was added at a concentration of 400 mM because it is a less efficient acceptor than butanol (5). Phosphatidylethanol generated from the hydrolysis of PC8 has a lower R_f than phosphatidylethanol produced from egg yolk.

Phosphatidylbutanol is not produced via *a base exchange enzyme.* Phosphatidylbutanol could be formed by the action of a base exchange enzyme (13). This enzyme normally exchanges headgroups such as choline or serine. It is active at high pH and is stimulated by Ca^{2+}. If the base exchange enzyme is active, choline would be expected to compete with [^{3}H]butanol for this enzyme. However, choline at a concentration of 1 mM and in the presence of Ca^{2+} did not inhibit the production of phosphatidyl[^{3}H]butanol at pH 6 through 7.2 (data not shown). At pH 8.4 it caused a 34% inhibition of phosphatidyl[^{3}H]butanol production. Furthermore, when [^{3}H]choline was added to the incubation medium, there was only minimal incorporation into PC8. These data suggest that the production of phos-

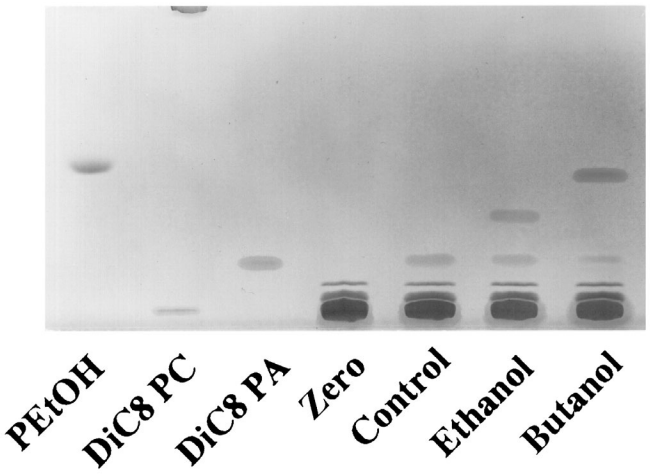

FIG. 2. Mass amount of phosphatidic acid and phosphatidylalcohol production. The first three lanes contain 4 mg of standards in the following order: egg yolk phosphatidylethanol (PEtOH), dioctanoyl-phosphatidylcholine (DiC8PC), and dioctanoyl-phosphatidic acid (DiC8PA). The last four lanes contain samples from standard reaction conditions without labeled [^{3}H]butanol but with unlabeled alcohol. Each reaction mixture contained 50 mM HEPES (pH 6.6), 1 mM $MgCl_2$, 25 mM NaF, and 100 µg of microsomal membrane protein. The final concentration of DiC8PC was 7 mM. The incubation time was 20 min. The lane labeled Zero contained sample that was not incubated. The lane labeled Control contained sample that was incubated without alcohol. The concentration of ethanol was 400 µM and the concentration of butanol was 100 µM. The phospholipids were visualized with molybdate phosphate spray and then photographed.

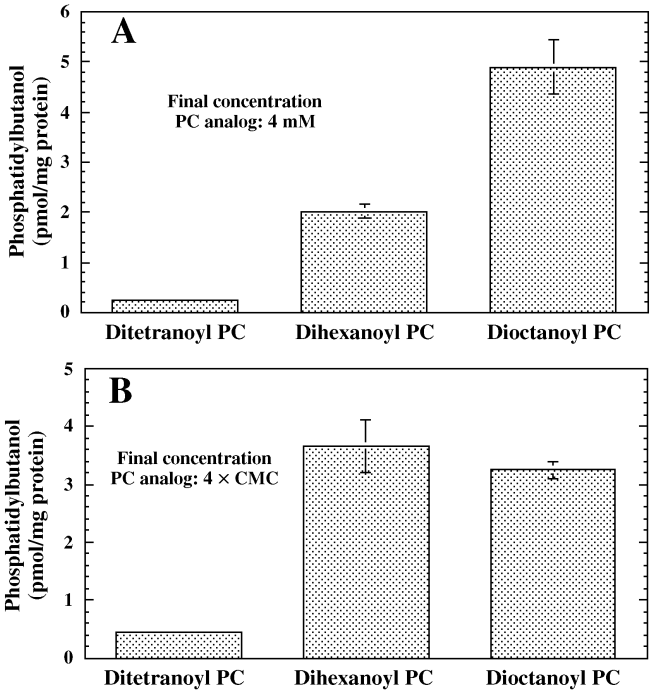

FIG. 3. Short-chain analogs of PC as substrates for phospholipase D. (A) Each reaction mixture contained 25 mM Tris maleate (pH 6.8), 1.25 mM EGTA, 6 mM $MgCl_2$, 5 µCi [^{3}H]butanol (15 Ci/mmol), and 90 µg of microsomal membrane protein. The final concentration of PC analog was 4 mM. Incubation time was 10 min. The substrate was prepared by sonicating the phospholipids for 30 s in water using a bath sonicator. (B) Each reaction mixture contained: 50 mM HEPES (pH 6.6), 5 µCi [^{3}H]butanol (15 Ci/mmol), and 70 µg of microsomal membrane protein. The final concentration of PC analog was 3.75 times the critical micellar concentration (CMC) for each phospholipid. 1,2-Ditetranoyl-*sn*-glycero-3-phosphocholine, 300 mM; 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, 56 mM; 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine, 1 mM. Incubation time was 20 min. The substrate was prepared by sonicating and vortexing in 5 mM HEPES, pH 6.6. Data represent the mean ± SEM of three or four determinations. For other abbreviation see Figure 1.

phatidylbutanol is not due to the base exchange enzyme.

Phospholipase D is affected by the chain length of the phosphatidylcholine analog. The activity of a phospholipase D is strongly influenced by the physical form of the substrate. The phospholipid may be present as a monomer, micelle, or bilayer. To address this issue, we measured the activity of phospholipase D against substrates containing fatty acids of different chain lengths. The data are shown in Figure 3A. All substrates were prepared such that the final concentration was 4 mM. 1,2-Ditetranoyl-*sn*-glycero-3-phosphocholine has a high critical micellar concentration (CMC). It does not form micelles up to 80 mM (14). Thus, at this concentration the 1,2-ditetranoyl-*sn*-glycero-3-phosphocholine would be present in the monomeric form. There is essentially no phospholipase D activity when this short-chain phosphatidylcholine is used as substrate. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine has a CMC of 14 mM but does aggregate to form small micelles at lower concentrations (15). There is a small but significant amount of activity when this analog is used as substrate. The CMC for 1,2-dioctanoyl-*sn*-glycero-phosphocholine is approximately 0.25 mM (16). Thus, this substrate is probably present entirely in the micellar form. The activity is highest when PC8 is the substrate.

The foregoing data suggest that micellar structure is important for the activity of phospholipase D. To investigate this possibility, we measured phosphospholipase D at a substrate concentration four times the CMC (Fig. 3B). Under these conditions a phospholipase D hydrolyzes 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine just as well as PC8. However, the activity against 1,2-ditetranoyl-*sn*-glycerophosphocholine is relatively low despite the fact that it is at the CMC. Thus, micellar structure is important; however, chain length of the fatty acid also influences the ability of the phospholipid to interact with phospholipase D. The longer-chain analogs may have the additional ability to act as emulsifiers to enhance activity (10). Since the activity was highest with PC8 at the lowest concentration, additional experiments to characterize phospholipase D activity were done with this substrate.

Kinetics of phospholipase D activity. At a substrate concentration of 8 mM, the production of phosphatidylbutanol is linear for at least 40 min (Fig. 4A). The production of phosphatidylbutanol began without any apparent lag after the addition of liposomes. Figure 4B shows production of phosphatidylbutanol as a function of substrate concentration using an incubation time of 10 min. Under these conditions the apparent K_m for the PC8 is 1 mM. Maximal activity was obtained at a PC8 concentration of 3.5 mM. The enzyme activ-

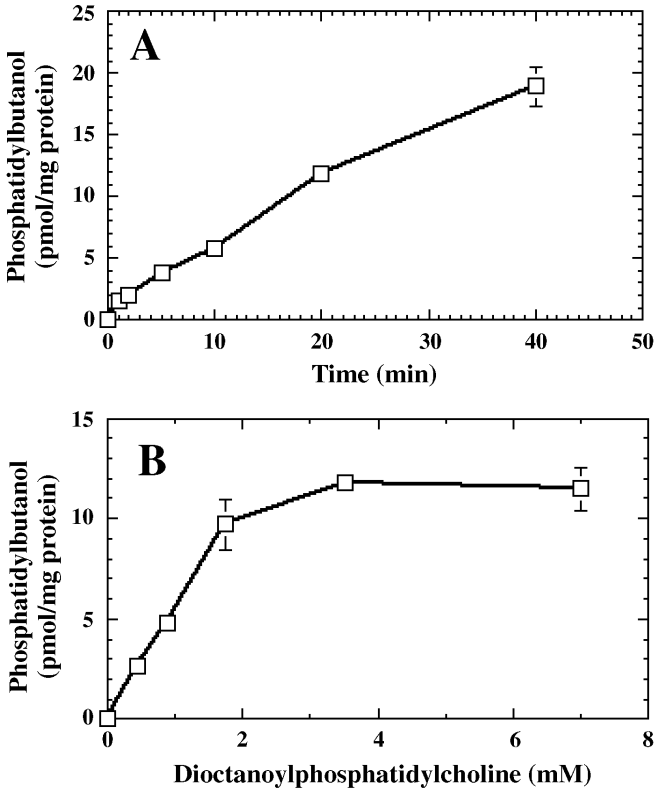

FIG. 4. Time course (A) and concentration dependence (B) of phospholipase D activity with dioctanoylphosphatidylcholine as substrate. Each reaction mixture contained 50 mM HEPES (pH 6.6), 1 mM $MgCl_2$, 5 μCi [^{3}H]butanol (15 Ci/mmol), and 100 μg of microsomal membrane protein. The incubation time for the determination of concentration dependence was 10 min. The substrate concentration in the time-course experiment was 8 mM. Data represent the mean ± SEM of four determinations.

ity appears to follow classic Michaelis-Menten kinetics.

The estimated specific activity of the radiolabeled alcohols obtained from American Radiolabeled Chemicals was 15 Ci/mmol. Each assay contained 5 μCi/120 μL. The actual concentration of cold alcohol therefore was approximately 3 μM. These data suggest that significant amounts of phosphatidylalcohol can be formed even at very low concentrations of alcohol. It is possible that the concentration of alcohol is too low to drive the reaction at an optimal rate. To investigate this issue, microsomal membranes were incubated with increasing concentrations of butanol while maintaining a constant specific activity (2 Ci/mmol). There was linear relationship between phosphatidylbutanol production and butanol concentration but only at concentrations higher than 10 μM (Fig. 5). Thus, at 5 μM the amount of phosphatidylbutanol formed in this particular experiment was 3.5 pmol/mg protein; at 10 μM the amount of production was 18 pmol/mg protein. Therefore, these data indicate that there is significant production of phosphatidylalcohol even at low concentrations; higher concentrations lead to a higher rate of reaction, proportional to the concentration of butanol.

Ionic and pH dependence of phospholipase D. Phospholipase D has been reported to have a pH optimum of 6.5 or 7.2.

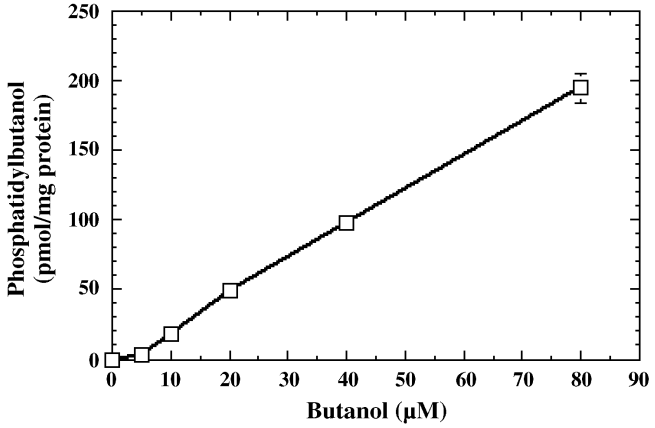

FIG. 5. Dependence of phospholipase D activity on butanol concentration. Each reaction mixture contained 50 mM HEPES (pH 6.6) and 100 μg of microsomal membrane protein. High specific activity [^{3}H]butanol was diluted with unlabeled butanol to make [^{3}H]butanol with a specific activity of 2 Ci/mmol. This labeled [^{3}H]butanol was added to the reaction mix to produce different concentrations of butanol. The final concentration of dioctanoylphosphatidylcholine was 8 mM. The incubation time was 10 min. Data represent the mean ± SEM. of three determinations.

Using the buffer dimethylglutaric acid and the detergent sodium oleate, Kobayashi and Kanfer (6) reported a pH optimum of 6.5 for microsomal membrane phospholipase D. In contrast, Chalifa *et al.* (17) using the same detergent but another buffer, HEPES, found that the pH optimum for phospholipase D in synaptic plasma membranes was 7.2. Figure 6 shows the pH dependence of this enzyme using PC8 as a substrate without any detergent and with HEPES as the buffer. There was activity in the entire pH range from 6 to 8.4 with a broad pH optimum between 7.2 and 7.8. Differences in reported pH optima may be due to different detergents used in the assay (9,18); alternatively these data may suggest the

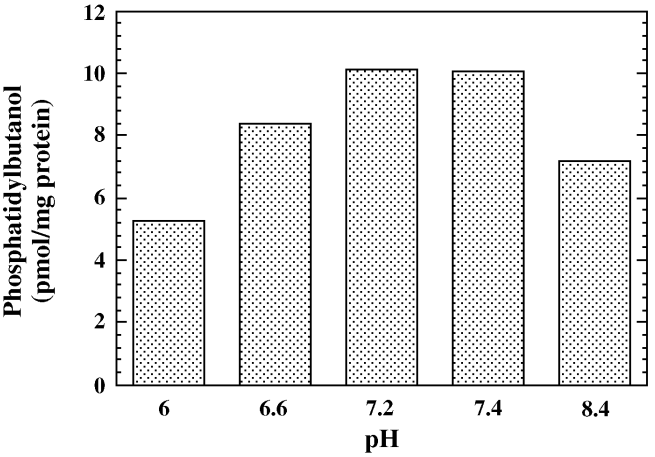

FIG. 6. The pH dependence of phospholipase D activity. Each reaction mixture contained 50 mM HEPES (pH 6.6 to 8.4), 1 mM $MgCl_2$, 5 μCi [^{3}H]butanol (15 Ci/mmol), and 100 μg of microsomal membrane protein. The final concentration of dioctanoylphosphatidylcholine was 7 mM. The substrate was prepared by sonicating the phospholipids in H_2O. Data represent the mean of two determinations.

presence of multiple isozymes of phospholipase D.

An investigation of the ionic dependence of phospholipase D was done. Mg^{2+} has been reported to stabilize the enzyme (17). Ca^{2+} has been reported to be both stimulatory and inhibitory (19–21). By using this assay, there was a high level of activity in the absence of any ions. Addition of Ca^{2+} and Mg^{2+} in concentrations ranging from 0.1 μM to 2 mM had only a mild stimulatory effect that was not significant and not reproducible (data not shown). Thus, there is no apparent ionic dependence under these conditions.

The novelty of this assay is that phospholipase D is active in the absence of phosphatidyinositol-4,5-bisphosphate. Liscovitch *et al.* (22) and Waksman *et al.* (23) have reported that phospholipase D hydrolyzes phosphatidylcholine analogs containing short-chain fatty acids in the 2-position in the absence of detergent. However, in the former case (22) this hydrolytic activity was dependent upon the presence of phosphatidylinositol-4,5-bisphosphate. In contrast, Vinggaard *et al.* (24) reported that phospholipase D hydrolyzes 1,2-didecanoyl-*sn*-glycero-3-phosphocholine in the absence of phosphatidylinositol-4,5-bisphosphate. Furthermore, the hydrolytic activity of phospholipase D against PC8 is not dependent on GTPγS or ADP ribosylation factor (data not shown). These studies represent a logical extension of the work of Vinggard *et al.* (24) and suggest that phospholipase D can hydrolyze short-chain analogs of phosphatidylcholine in the absence phosphatidylinositol-4,5-bisphosphate or other cofactors.

In conclusion, the use of [^{3}H]butanol in combination with the short-chain analog, PC8, represents a novel and potentially very useful method for assaying phospholipase D activity. PC8 is a very efficient substrate; therefore the assay is sensitive. The use of [^{3}H]butanol eliminates the need for high concentrations of alcohol which could affect enzymatic activity. Thus, this assay could be used to characterize phospholipase D under conditions that more closely reflect the situation *in vivo.*

ACKNOWLEDGMENTS

The authors thank Dr. Michael C. Phillips of the Biochemistry Department for reviewing the manuscript. This work was supported in part by the Alcoholic Beverage Medical Research Foundation, the Allegheny Health Education Research Foundation, and NIH grant AA10070.

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[Received October 22, 1996, and in final revised form December

Atherogenic Potential of Peanut Oil-Based Monounsaturated Fatty Acids Diets

Dear Editor,

In the dietary management of coronary heart disease (CHD), there is increasing recognition (1,2) that the traditionally recommended high carbohydrate, low-fat diet for hypercholesterolemia (3) may elicit undesirable blood lipid changes, including reductions in high-density lipoproteins and apolipoprotein A-1, while concurrently elevating triglycerides (TG) and very low density lipoproteins (4–6). Because of these untoward blood lipid changes induced by high-carbohydrate, low-fat diets, substitution of monounsaturated fatty acids (MUFA) for saturated fats may be a more effective strategy than substitution of carbohydrate for saturated fats in order to lower total and low density lipoprotein (LDL) serum cholesterol levels without adversely influencing high density lipoprotein, very low density lipoprotein, TG, and apolipoprotein A-1 (7–9). Most experimental diets have employed olive oil or canola oil as the MUFA source; however other MUFA-rich foods such as nuts (10) and avocados (11) have also been demonstrated to improve blood lipid profiles. In a recent report to *Lipids,* O'Byrne *et al.* (12) have shown that a low-fat, high-MUFA (14% energy) diet based upon high-MUFA (76–80%) peanuts improved total and LDL serum cholesterol levels in 12 postmenopausal women. Although diets based upon MUFA-rich peanuts (*Arachis hypogea*) should, in theory, be nonatherogenic because they reduce total and LDL cholesterol, there is substantial evidence to indicate that peanut oil, despite its hypolipidemic effects, is highly atherogenic to animals and possibly to humans as well.

Although the total MUFA content of peanut oil is high and can range from 36 to 59%, dependent upon the specific cultivar (13), it has been shown to be unexpectedly atherogenic when fed to laboratory animals (monkeys, rabbits, and rats) as part of a cholesterol-rich or a cholesterol-free diet (13–16). Because peanut oil can so rapidly produce atherosclerotic lesions which have similar biochemical and pathological characteristics to those in human atherosclerosis, it is routinely used in rabbit models to induce atherosclerosis (17,18). The reason for the high atherogenicity of peanut oil is unclear; however, it has been suggested that it may be due to residual lectins (glycoproteins with high affinity binding to cellular carbohydrate residues) found in the oil, since peanut oil induces fibromuscular arterial lesions in contrast to other vegetable oils which induce fatty lesions (19). Alternatively, the specific TG structure may also be responsible for its atherogenicity (20). In native peanut oil, all of the long-chain polyunsaturated fatty acids are found in the *sn*-3 position of glycerol; however, by utilizing a process to randomize the fatty acids within the TG, the atherogenicity of peanut oil has been shown to be reduced (21). It should be pointed out that the randomization process in some cases may also reduce peanut oil's lectin content (20,21).

When contrasting olive to peanut oil, rabbits which were fed peanut oil showed a higher frequency of arterial lesions, more intimal proliferation, and thicker intimas than did rabbits fed olive oil (15). Peanut oil-containing, atherogenic diets induce a preferential increase in intimal collagen and result in a characteristic fibromuscular lesion in intimal plaques that is attributable to the addition of peanut oil to the atherogenic diet (22,23). It has been suggested that residual peanut lectin (PNA) found in peanut oil, because of its specificity for D-galactose residues, may bind arterial smooth muscle cells expressing these sugar residues and thereby induce its characteristic fibromuscular lesions (19). In support of this concept are data which have shown that PNA stimulates *in vitro* vascular smooth muscle cell proliferation and that added lactose could inhibit the PNA-induced stimulation (24). A similar *in vivo* experiment would be able to distinguish if peanut oil's atherogenicity is more attributable to its PNA content or to its specific TG structure.

Although there are no direct epidemiological studies evaluating the atherogenic potential of peanut oil, there is suggestive information from India that implicates peanut oil with higher mortality rates from CHD. In India, wherein vegetable oils constitute 80% of the per capita fat consumption, there are regional preferences in the choice of oils, and peanut oil is preferred in southern states, whereas northern states use mustard oil (25). In southern India, the mortality rate from CHD 30 yr ago was reported to be seven times higher than that in northern India and similar to that in the United States and England (26,27). A more recent report showed the prevalence of CHD to be 61.6% higher in southern Indians compared to their more northerly counterparts (28). It is possible that one of the factors underlying these regional differences may be different levels of peanut oil consumption. In contrast to peanut oil, which is a fairly recent addition to the human diet, olive oil has been part of the traditional Mediterranean diet for thousands of years (29) and has been shown both epidemiologically (30) and clinically (31) to reduce the risk for

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CHD in subjects living in industrialized countries. These studies suggest that olive oil may be superior to peanut oil in terms of its cardiovascular health benefits in such populations. Until further trials are conducted, it would seem premature to recommend peanut oil as part of high-MUFA diets for the management of CHD in Western populations.

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ARTICLE

Dietary Methionine Level Affects Linoleic Acid Metabolism Through Phosphatidylethanolamine *N*-Methylation in Rats

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ABSTRACT: The effects of dietary methionine level on the profiles of fatty acids and phospholipids and on the plasma cholesterol concentration were investigated to confirm whether the methionine content of dietary proteins is one of the major factors that cause differential effects on lipid metabolism. The effect of dietary supplementation with eritadenine, which is shown to be a potent inhibitor of phosphatidylethanolamine (PE) *N*-methylation, was also investigated. Rats were fed six diets containing casein (100 g/kg) and amino acid mixture (86.4 g/kg) differing in methionine content (2.5, 4.5, and 7.5 g/kg) and without or with eritadenine supplementation (30 mg/kg) for 14 d. The ratio of arachidonic to linoleic acid of liver microsomal and plasma phosphatidylcholine (PC) was significantly increased as the methionine level of diet was elevated, indicating that dietary methionine stimulates the metabolism of linoleic acid. The PC/PE ratio of liver microsomes and the plasma cholesterol concentration were also increased by dietary methionine. These effects of methionine were completely abolished by eritadenine supplementation The *S*-adenosylmethionine concentration in the liver reflected the methionine level of diet. These results support the idea that the differential effects of dietary proteins on lipid metabolism might be ascribed, at least in part, to their different methionine contents, and that methionine might exert its effects through alteration of PE *N*-methylation.
Lipids 33, 235–242 (1998).

It is confirmed that lipid metabolism can be modified by the type of dietary proteins. For instance, plant proteins compared with animal proteins generally lead to lower plasma cholesterol concentrations (1–4). The metabolism of fatty acids, especially linoleic acid, also has been shown to be affected by dietary protein types (5,6). Additionally, the profile of liver microsomal phospholipid classes, as represented by the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE), could be altered by the type of dietary proteins (7). A number of studies so far reported have suggested that both the difference in the physicochemical properties and the difference in the amino acid composition of dietary proteins participate in the differential effects on the plasma cholesterol concentration. In contrast, the mechanism underlying the differential effects of dietary proteins on the metabolism of fatty acids appears to be less understood. Recently we demonstrated that methionine supplementation of a low-methionine diet containing soybean protein brought about a significant increase in the ratio of arachidonic to linoleic acid of liver microsomes and plasma in rats, suggesting that dietary methionine stimulates the metabolic conversion of linoleic acid into arachidonic acid (8). The PC/PE ratio of liver microsomes and the plasma cholesterol concentration also were increased by methionine supplementation (8). These results suggest that the methionine content of dietary proteins may have a wide range of effects on lipid metabolism, and that metabolism of linoleic acid, phospholipids, and cholesterol may not be independent of each other. Furthermore, previous studies have suggested that dietary methionine level may affect the PC/PE ratio of liver microsomes through alteration of PE *N*-methylation (7,8). If this is true, the effect of dietary methionine on lipid metabolism would be abolished or suppressed by the inhibitor of PE *N*-methylation.

In this study, the effects of dietary methionine level on the composition of fatty acids and molecular species of PC on the microsomal PC/PE ratio and on the plasma cholesterol concentration were investigated to confirm whether the methionine content of dietary proteins is one of the major factors that cause differential effects on lipid metabolism. The effect of supplementation with eritadenine, which strongly depresses PE *N*-methylation (9), also was investigated to see whether the effect of methionine can be abolished by eritadenine.

MATERIALS AND METHODS

Animals. Thirty-six male 5-wk-old rats of the Wistar strain were received at 90–100 g body weight from Japan SLC (Hamamatu, Japan). The rats were individually housed in hanging stainless-steel wire cages kept in an isolated room at

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Abbreviations: GLC, gas–liquid chromatography; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

a controlled temperature (23–25°C) and humidity (40–60%). Lights were maintained on a 12-h cycle (lights on from 0600 to 1800 h). For 5 or 6 d, rats were fed the powdered laboratory stock diet as described previously (10). Then, they were divided into six groups of six rats each with similar mean weights (~121 g) and allowed free access to the experimental diets and water for 14 d. The experimental plan was approved by the Laboratory Animal Care Committee of the Faculty of Agriculture, Shizuoka University.

Diets. Six diets differing in methionine content (2.5, 4.5, and 7.5 g/kg diet) and without or with eritadenine were utilized in this study. Table 1 shows the composition of experimental diets without eritadenine. Casein (100 g/kg) and casein-simulating amino acid mixture (86.4 g/kg) were used as protein sources. Therefore, the diets were comparable to 20% casein diet in the amino acid composition except for sulfur amino acids. The amount of total sulfur amino acids (methionine and cystine) in the diet was maintained to a constant level on a molar basis, which was equivalent to the amount of methionine of 8 g/kg diet. Eritadenine was added to the diet at a level of 30 mg/kg diet at the expense of lactose; this dose level was shown to be sufficient to elicit maximal effect (10). The eritadenine was isolated from dried *Lentinus edodes* mushroom as reported previously (10). The fatty acid composition of corn oil used was as follows (wt%): 16:0, 12.2; 18:0, 1.8; 18:1, 32.8; 18:2n-6, 51.4; and 18:3n-3, 1.6.

Tissue collection and fractionation. At the end of the feeding period, non-fasted rats were killed by decapitation under light anesthesia with diethyl ether between 1200 and 1300 h. Plasma was separated from heparinized whole blood by centrifugation at 2000 × *g* for 20 min at 4°C. An aliquot of the plasma was stored at 4°C until subsequent analyses for plasma lipid concentrations, and the residual plasma was stored at −80°C until analyses for phospholipids. After collection of blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, and weighed. The liver was homogenized in 4 vol (vol/wt) of an ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl, a modified solution of Nguyen *et al*. (11). An aliquot (2 mL) of the homogenate was stored at −30°C until subsequent analyses for liver lipid concentrations. Another aliquot (12 mL) of the homogenate was centrifuged at 10,000 × *g* for 12 min at 4°C, and the resultant supernatants were further centrifuged at 105,000 × *g* for 60 min at 4°C to obtain the microsomal fraction as a precipitate. The microsomes obtained were resuspended in the homogenizing buffer and stored at −80°C until analyses for phospholipids.

Lipid analysis. The plasma concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, free cholesterol, triglycerides, and phospholipids were measured enzymatically with kits (Cholesterol C-Test, HDL Cholesterol-Test, Free Cholesterol C-Test, Triglyceride G-Test, and Phospholipid B-Test, respectively; Wako Pure Chemical, Osaka, Japan). The difference between total cholesterol and HDL cholesterol or free cholesterol was assumed to be very low density lipoprotein (VLDL) + low density lipoprotein (LDL) cholesterol or esterified cholesterol, respectively. The total lipids of liver homogenate, liver microsomes, and plasma were extracted by the method of Folch *et al*. (12). The cholesterol, triglycerides, and phospholipids in the extract of liver homogenate were measured according to Zak (13), Fletcher (14), and Bartlett (15), respectively. For the determination of phospholipid class composition, the phospholipids in the extract of liver microsomes were separated into each class by thin-layer chromatography (TLC) on silica gel 60 (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by vol) as a developing solvent. The bands of each phospholipid class were visualized in iodine vapor, scraped off the plate, and analyzed directly for inorganic phosphorus (15). For the determinations of fatty acid and molecular species composition, PC was likewise separated by TLC from liver microsomes and plasma, visualized with dichlorofluorescein, scraped off the plate, and extracted with chloroform/methanol (1:2, vol/vol). An aliquot of PC was treated with 14% (w/w) BF_3/methanol reagent (Wako Pure Chemical), and fatty acid methyl esters formed were analyzed by gas–liquid chromatography (GLC) on a Model GC-17A (Shimadzu, Kyoto, Japan), equipped with a TC-FFAP capillary column (0.25 mm × 30 m; GL Sciences, Tokyo, Japan). Another aliquot of PC was converted to diacylglycerol benzoates according to the method of Blank *et al*. (16). The diacylglycerol benzoates were analyzed by high-performance liquid chromatography (HPLC) on a Model LC-6A system (Shimadzu), equipped with an ODS column (4.6 × 250 mm, Lichrosorb RP-18; E. Merck), essentially according to the method of Blank *et al*. (16). Since some peaks consisted of two molecular species, the ratio of two molecular species was determined by the analysis of fatty acids by GLC. A representative chromatogram of PC molecular species by HPLC was shown previously (17).

TABLE 1
The Composition of the Experimental Diets

	Diet		
	2.5M4.4C	4.5M2.8C	7.5M0.4C
Ingredient (g/kg)			
Casein	100	100	100
Amino acid mix[a]	86.4	86.4	86.4
Cornstarch	489.6	489.2	488.6
Sucrose	200	200	200
Corn oil	50	50	50
Mineral mix[b]	35	35	35
Vitamin mix[b]	10	10	10
Choline·Cl	4	4	4
Cellulose	20	20	20
Lactose	1	1	1
L-Methionine	0	2	5
L-Cystine	4	2.4	0
Concentration (g/kg)			
Methionine	2.5	4.5	7.5
Cystine	4.4	2.8	0.4

[a]The composition was similar to that of casein except that sulfur amino acids were omitted. The mixture contained the following amino acids (g/86.4 g): L-Ile, 4.51; L-Leu, 7.75; L-Lys·HCl, 8.27; L-Phe, 4.25; L-Tyr, 4.63; L-Thr, 3.38; L-Trp, 1.05; L-Val, 5.51; L-His, 2.51; L-Arg·HCl, 3.61; L-Ala, 2.51; L-Asp, 5.75; L-Glu, 17.51; Gly, 1.49; L-Pro, 9.38; L-Ser, 4.25.
[b]AIN-76 (Oriental Yeast, Tokyo, Japan).

TABLE 2
Growth, Food Intake, Liver Weight, and Liver Lipid Concentrations in Rats Fed Experimental Diets[a]

	Diet					
	–Eritadenine			+Eritadenine		
	2.5M4.4C	4.5M2.8C	7.5M0.4C	2.5M4.4C	4.5M2.8C	7.5M0.4C
Body weight gain (g/14 d)	68 ± 3	67 ± 2	64 ± 2	64 ± 2	70 ± 2	63 ± 3
Food intake (g/14 d)	192 ± 4	192 ± 2	186 ± 3	188 ± 3	193 ± 3	187 ± 4
Liver weight (g/100 g body weight)	4.6 ± 0.1	4.5 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.4 ± 0.1
Liver lipids (μmol/g liver)						
Cholesterol	6.8 ± 0.2^{c}	7.0 ± 0.1^{b,c}	7.3 ± 0.1^{b,c}	7.0 ± 0.3^{b,c}	7.1 ± 0.2^{b,c}	7.4 ± 0.2^{b}
Triglycerides	15.5 ± 1.3^{b}	17.0 ± 0.5^{b}	17.1 ± 1.0^{b}	11.4 ± 0.6^{c}	12.2 ± 0.7^{c}	14.3 ± 1.5^{b}
Phospholipids	28.7 ± 0.4^{c}	28.7 ± 0.6^{c}	29.8 ± 0.6^{c}	31.8 ± 0.7^{b}	30.5 ± 0.6^{b,c}	32.1 ± 0.8^{b}

[a]Values are mean ± SEM for six rats. Values in a row with different superscripts are significantly different at $P < 0.05$.

Assay of methionine metabolites and protein. The concentrations of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in the liver were measured with HPLC according to Cook *et al*. (18) with some modifications. Frozen livers were thawed and homogenized in 4 vol (vol/wt) of ice-cold 0.5 M perchloric acid, and the homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C. The resultant supernatants were filtered through a 0.45-μm Millipore filter (Nihon Millipore, Tokyo, Japan) and applied to an HPLC column (Shim-pak CLC-ODS, 6 × 150 mm; Shimadzu) . The mobile phase was a 100 mM KH_2PO_4 solution containing 10 mM sodium heptane sulfonate and 3% (vol/vol) methanol. The flow rate was 1.5 mL/min, and the elution was monitored at 254 nm. Liver microsomal protein was measured according to Lowry *et al*. (19).

Statistical analysis. Data were analyzed by one-way analysis of variance, and the differences between means were tested using Duncan's multiple range test (20) when the *F* value was significant. Simple correlation between variables was calculated by linear regression analysis using mean values. A *P* value of 0.05 or less was considered significant.

RESULTS

Growth, food intake, liver weight, and liver lipid concentrations. There was no significant difference in the body weight gain, food intake, and relative liver weights of animals among the six groups (Table 2). Dietary methionine level had little effect on liver lipid concentrations. Eritadenine supplementation tended to decrease the triglyceride concentration and inversely to increase the phospholipid concentration in the liver.

Plasma lipid concentrations. The plasma total cholesterol concentration was significantly increased by elevating dietary methionine level (Fig. 1). In the same manner, plasma concentrations of VLDL + LDL cholesterol, HDL cholesterol, and cholesteryl esters were significantly affected by dietary methionine level. The plasma concentrations of triglycerides and phospholipids were not affected by dietary methionine level. Eritadenine supplementation brought about marked decreases in the plasma concentrations of cholesterol and phospholipids, but not triglycerides. In those rats fed eritadenine-supplemented diets, the hypercholesterolemic effect of dietary methionine disappeared completely.

Methionine metabolites and microsomal phospholipids. The concentration of SAM in the liver was significantly increased by elevating dietary methionine level, whereas the concentration of SAH was unchanged (Fig. 2). Consequently, the SAM/SAH ratio was significantly increased by dietary methionine. Eritadenine supplementation significantly decreased the SAM/SAH ratio, which was mainly due to the increase in SAH concentration. The concentration of PC in liver microsomes was unaffected by dietary methionine level, whereas the concentration of PE was significantly decreased by elevating dietary methionine level. Eritadenine supplementation significantly increased the PE concentration and

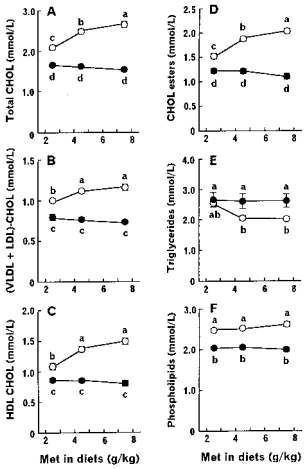

FIG. 1. Effects of dietary methionine (Met) level and eritadenine (Er) supplementation on plasma lipid concentrations in rats. The circle and its bar represent mean and SEM, respectively, for six rats. Values with different letters are significantly different at $P < 0.05$. CHOL, cholesterol; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein. ○, –Er; ●, + Er.

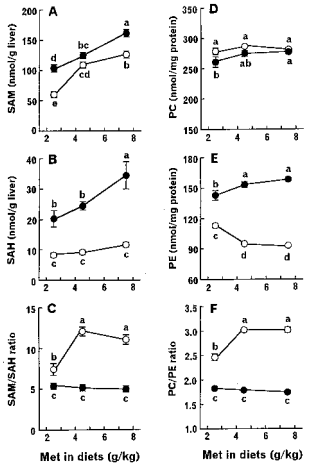

FIG. 2. Effects of dietary Met level and Er supplementation on the concentration of Met metabolites (A–C) and microsomal phospholipids (D–F) in the liver of rats. The circle and its bar represent mean and SEM, respectively, for six rats. Values with different letters are significantly different at $P < 0.05$. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine. See Figure 1 for other abbreviations and symbol legend.

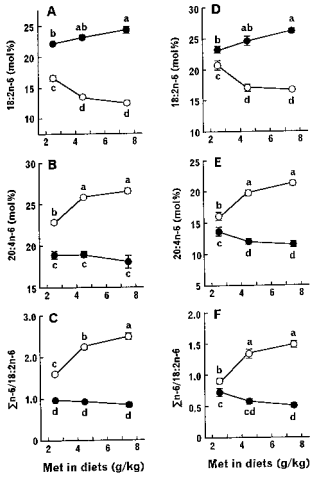

FIG. 3. Effects of dietary Met level and Er supplementation on the proportion of linoleic and arachidonic acids in liver microsomal (A–C) and plasma (D–F) PC in rats. The circle and its bar represent mean and SEM, respectively, for six rats. Values with different letters are significantly different at $P < 0.05$. Abbreviations: Σn-6, metabolites of 18:2n-6. See Figures 1 and 2 for other abbreviations. See Figure 1 for symbol legend.

consequently significantly decreased the PC/PE ratio. Eritadenine abolished the effect of dietary methionine on the PC/PE ratio.

Fatty acid and molecular species composition of PC. The proportion of linoleic acid in liver microsomal PC was significantly decreased, and conversely the proportion of arachidonic acid was significantly increased by elevating dietary methionine level (Fig. 3). Consequently, the ratio of linoleic acid metabolites to linoleic acid was significantly increased by dietary methionine. In the same manner, the proportion of linoleic and arachidonic acids in plasma PC was influenced by dietary methionine level. Table 3 shows the fatty acid composition of liver microsomal and plasma PC in rats fed diets without eritadenine. The diets higher in methionine brought about a decrease in the proportion of palmitic acid and increases in the proportion of 22:5n-6 and 22:6n-3, in addition to the suppression of linoleic acid metabolism. Table 4 shows the molecular species composition of plasma PC in rats fed diets without eritadenine. The diets higher in methionine caused a significant decrease in the proportion of 16:0-18:2 PC and a significant increase in the proportion of 18:0-20:4 PC. The effects of dietary methionine level on the other PC molecular species were relatively small. Figure 4 shows the effects of dietary methionine level and eritadenine supplementation on the proportion of plasma 16:0-18:2 and 18:0-20:4 PC molecular species. Eritadenine supplementation significantly increased the proportion of 16:0-18:2 PC and conversely significantly decreased the proportion of 18:0-20:4 PC. In the presence of dietary eritadenine, higher levels of dietary methionine potentiated the effect of eritadenine on plasma PC molecular species. There was a significantly negative or positive correlation between the concentration of plasma total cholesterol and the proportion of plasma 16:0-18:2 or 18:0-20:4 PC, respectively (Fig. 5).

DISCUSSION

Effects on fatty acid and phospholipid profiles. The conversion of linoleic acid into arachidonic acid is one of the most important metabolisms of fatty acids quantitatively and qualitatively in higher animals, since arachidonic acid exists in membrane phospholipids at relatively high levels, and since arachidonic acid is the predominant precursor for various types of eicosanoids. In rats, the metabolism of linoleic acid can be modified by dietary proteins types (5,6), in addition to the other dietary constituents, such as n-3 fatty acids (21) and cholesterol

TABLE 3
Effect of Dietary Methionine Level on the Fatty Acid Composition (mol%) of Phosphatidylcholine[a]

	Liver microsomes			Plasma		
	Diet (–eritadenine)			Diet (–eritadenine)		
Z						
14:0	0.6 ± 0.0[b]	0.5 ± 0.0[c]	0.4 ± 0.0[c]	0.9 ± 0.1[b]	0.9 ± 0.1[b]	0.6 ±0.0[c]
16:0	22.6 ± 0.6[b]	20.5 ± 0.2[c]	20.4 ± 0.2[c]	24.6 ± 0.3[b]	23.5 ± 0.3[c]	22.3 ± 0.4[d]
16:1n-7	2.8 ± 0.1[b]	2.3 ± 0.1[c]	2.2 ± 0.1[c]	3.1 ± 0.2[b]	2.1 ± 0.2[c]	2.7 ± 0.2[b]
18:0	16.5 ± 0.5[c]	18.4 ± 0.6[b]	19.0 ± 0.3[b]	14.6 ± 0.7[c]	17.8 ± 0.7[b]	16.4 ± 0.4[b,c]
18:1n-9	5.2 ± 0.1[b]	4.9 ± 0.1[c]	4.9 ± 0.1[c]	11.1 ± 0.8	9.1 ± 0.5	10.0 ± 0.8
18:1n-7	5.4 ± 0.2	5.7 ± 0.0	5.4 ± 0.1	3.6 ± 0.2	3.3 ± 0.0	3.4 ± 0.2
18:2n-6	16.6 ± 0.4[b]	13.5 ± 0.3[c]	12.4 ± 0.4[c]	20.7 ± 0.7[b]	17.0 ± 0.6[c]	16.7 ± 0.3[c]
18:3n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1n-7	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
20:2n-6	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:3n-9	0.3 ± 0.0[b]	0.2 ± 0.0[c]	0.2 ± 0.0[c]	0.3 ± 0.1[c]	0.5 ± 0.1[b]	0.2 ± 0.0[c]
20:3n-6	1.2 ± 0.1[b]	0.9 ± 0.0[c]	0.8 ± 0.0[c]	0.7 ± 0.0[b]	0.5 ± 0.0[c]	0.5 ± 0.0[c]
20:4n-6	22.8 ± 0.2[b]	25.8 ± 0.2[c]	26.5 ± 0.3[c]	16.0 ± 0.7[c]	19.7 ± 0.5[b]	21.3 ± 0.4[b]
22:4n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1[c]	0.5 ± 0.1[b,c]	0.7 ± 0.1[b]
22:5n-6	1.4 ± 0.1[c]	2.3 ± 0.1[b]	2.3 ± 0.1[b]	0.9 ± 0.2[c]	1.6 ± 0.1[b]	1.7 ± 0.1[b]
22:5n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
22:6n-3	2.6 ± 0.1[d]	3.1 ± 0.1[c]	3.4 ± 0.1[b]	1.6 ± 0.1	2.0 ± 0.2	2.1 ± 0.1
∑n-6/18:2n-6	1.6 ± 0.0[d]	2.2 ± 0.1[c]	2.5 ± 0.1[b]	0.9 ± 0.0[c]	1.3 ± 0.1[b]	1.5 ± 0.1[b]

[a]Values are mean ± SEM for six rats. Within a row of each tissue, values with different superscript letters (b–d) are significantly different at $P < 0.05$. ∑n-6, metabolites of 18:2n-6.

(22). It has been shown that dietary soybean protein as compared with casein suppresses the metabolism of linoleic acid and consequently decreases the ratio of arachidonic to linoleic acid of phospholipids (6). However, by what factor of dietary proteins linoleic acid metabolism is differentially affected has not yet been fully elucidated. The present study clearly demonstrated that linoleic acid metabolism is significantly affected by the dietary level of methionine; low methionine diet led to suppressed metabolism of linoleic acid and conversely high methionine diet stimulated the metabolic conversion of linoleic acid into arachidonic acid. It is well recognized that the methionine content of soybean protein is considerably lower than that of casein. Our previous study showed that methionine supplementation of a soybean protein diet caused a significant increase in the arachidonic/linoleic acid ratio of liver microsomal and plasma PC in rats (8). Thus, the results obtained here, together with previous results, strongly suggest the idea that the differential effect of different types of dietary proteins on linoleic acid metabolism might be attributable to the difference in their methionine contents.

TABLE 4
Effect of Dietary Methionine Level on the Molecular Species Composition (mol%) of Plasma Phosphatidylcholine[a]

Molecular species	Diet (–eritadenine)		
	2.5M4.4C	4.5M2.8C	7.5M0.4C
16:0-18:1[b]	10.8 ± 0.3[c]	9.1 ± 0.5[d]	8.4 ± 0.1[d]
18:0-18:1	1.4 ± 0.3	1.3 ± 0.2	1.3 ± 0.3
18:1-18:1	0.5 ± 0.1[d]	0.9 ± 0.1[c]	0.8 ± 0.1[c]
16:0-18:2	22.2 ± 0.4[c]	16.8 ± 0.3[d]	16.3 ± 0.4[d]
18:0-18:2	9.4 ± 0.1[c]	7.5 ± 0.3[e]	8.4 ± 0.1[d]
18:1-18:2	2.4 ± 0.1[c]	1.7 ± 0.3[d]	1.0 ± 0.2[e]
16:0-20:4	15.7 ± 0.3	16.1 ± 0.6	16.2 ± 0.2
18:0-20:4	18.2 ± 0.4[e]	21.5 ± 0.4[d]	24.2 ± 0.7[c]
18:1-20:4	3.6 ± 0.3	4.1 ± 0.3	4.4 ± 0.2
16:0-22:5	2.1 ± 0.3[e]	3.9 ± 0.2[c]	3.0 ± 0.4[d]
18:0-22:5	2.2 ± 0.2[d]	4.3 ± 0.4[c]	3.1 ± 0.3[c,d]
16:0-22:6	4.5 ± 0.1	4.5 ± 0.2	4.4 ± 0.1
18:0-22:6	3.5 ± 0.1[d]	4.3 ± 0.2[c]	4.8 ± 0.2[c]

[a]Values are mean ± SEM for six rats.
[b]Fatty acids in the *sn*-1 and *sn*-2 positions of phosphatidylcholine are indicated at the left and right sides, respectively. Values in a row with different superscript letters (c–e) are significantly different at $P < 0.05$.

The conversion of linoleic acid into arachidonic acid is thought to be regulated by the activity of Δ6-desaturase, the rate-limiting enzyme of linoleic acid metabolism. Actually, it was shown that the Δ6-desaturase activity was significantly lower in rats fed a soybean protein diet than in rats fed a casein diet (22). Although Δ6-desaturase activity was not measured in the present study, fatty acid profiles, as represented by the arachidonic/linoleic acid ratio, suggest that dietary methionine level might affect the enzyme activity. This may be further supported by the fact that 22:5n-6 and 22:6n-3 were increased by elevating dietary methionine level, despite 22:4n-6 and 22:5n-3 being unchanged. The enzyme Δ6-desaturase is also thought to participate in the formation of 22:5n-6 and 22:6n-3 from 22:4n-6 and 22:5n-3, respectively (23). It is interesting to know by what mechanism dietary methionine level affects the metabolism of linoleic acid. With regard to this, there are several reports to provide available information. For instance, Leikin and Brenner (24–26) have reported that the activity of Δ5 or Δ6 desaturase might be regulated by the PC/PE or cholesterol/PC ratio of liver microsomal membranes. Consistent with this, She *et al.* (27) have

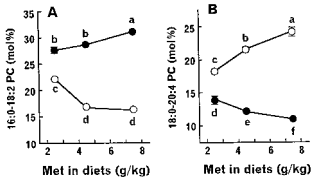

FIG. 4. Effects of dietary Met and Er supplementation on the proportion of 16:0-18:2 (A) and 18:0-20:4 (B) molecular species of plasma PC in rats. The circle and its bar represent mean and SEM, respectively, for six rats. Values with different letters are significantly different at $P < 0.05$. See Figures 1 and 2 for abbreviations. See Figure 1 for symbol legend.

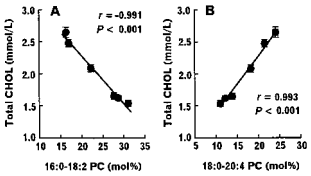

FIG. 5. Relationship between the plasma total CHOL concentration and the proportion of 16:0-18:2 (A) or 18:0-20:4 (B) molecular species of plasma PC in rats fed six experimental diets. The circle and its bar represent mean and SEM, respectively, for six rats. See Figures 1 and 2 for abbreviations.

shown that feeding a vitamin B_6-deficient diet caused decreases in both the PC/PE ratio and Δ6-desaturase activity of rat liver microsomes. Sugiyama *et al*. (10) also showed that there was a significantly positive correlation between the PC/PE ratio and the activity of Δ6 desaturase in rats fed graded levels of eritadenine. Furthermore, dietary supplementation with PE or ethanolamine (28,29) and diabetes caused by streptozotocin (30) have been shown to decrease both the PC/PE ratio and linoleic acid metabolism in rats. These results suggest that the activity of Δ6 desaturase can be affected by various types of dietary treatment through alteration of liver microsomal phospholipid profile, as represented by the PC/PE ratio. Previously we demonstrated that in rats fed different types of dietary proteins, the liver microsomal PC/PE ratio was significantly correlated with the methionine content of dietary proteins (7). In support of this, the present study showed that the PC/PE ratio of liver microsomes could be influenced by the level of dietary methionine. Taken together, it seems reasonable to consider that dietary methionine level might affect the metabolism of linoleic acid through alteration of the PC/PE ratio of liver microsomes in rats.

In the liver of rats, PC is synthesized either by the CDP-choline pathway or the PE *N*-methylation pathway, although the former is thought to be the main pathway (31). On the other hand, it appears that the PC/PE ratio of liver microsomes is regulated mainly by the extent of PE *N*-methylation (8). The decreased PC/PE ratio evoked by vitamin B_6-deficiency (27) or eritadenine supplementation (9) could be attributable to the increase in hepatic SAH concentration, since SAH is known to be a potent inhibitor of various types of methyltransferases, including PE *N*-methyltransferase. In contrast, the decreased PC/PE ratio caused by a low methionine diet cannot be ascribed to the change in SAH concentration (Fig. 2). It is confirmed that PE *N*-methylation is also influenced by the hepatic concentration of SAM, the substrate of the reaction. The K_m values for SAM of PE *N*-methyltransferase in rat liver microsomes for sequential methylation of PE were estimated to be 51, 58, and 79 μM, respectively (32). On the other hand, concentrations of SAM in the liver of rats fed the low- and high-methionine diets were found to be 60 and 126 μmol/g liver, respectively (Fig. 2). Taking these K_m values and hepatic SAM concentrations into consideration, it is reasonable to consider that the low-methionine diet might decrease the extent of PE *N*-methylation by decreasing hepatic SAM concentration, thereby leading to an increase in microsomal PE concentration and a resultant decrease in the PC/PE ratio. One of the most important findings of this study is that all of the effects tested of dietary methionine level on lipid metabolism disappeared completely under the condition of eritadenine supplementation. Since eritadenine strongly depresses the PE *N*-methylation (9), these results are taken to support the idea that dietary methionine level affects the metabolism of a variety of lipids (e.g., phospholipids, fatty acids, and cholesterol) through the depression of PE *N*-methylation.

Effect on plasma cholesterol concentration. Several reports have shown that methionine supplementation of a low-methionine diet, e.g., soybean protein diet, increases the plasma cholesterol concentration in rats (8,33–35). These results suggest that methionine has a hypercholesterolemic action at least under the experimental conditions used, although methionine also has been shown to have a hypocholesterolemic action under different dietary conditions. For instance, it was shown that methionine supplementation of a cholesterol-enriched low-protein diet, unlike standard or high-protein diet, resulted in a significant decrease in the plasma cholesterol concentration in rats (36). The hypercholesterolemic effect of methionine in rats fed a cholesterol-free diet was confirmed by the present study. However, the mechanism by which dietary methionine exerts its hypercholesterolemic action is not yet fully elucidated. The present study clearly demonstrated that the plasma cholesterol-elevating effect of methionine could be abolished by eritadenine supplementation, suggesting that the stimulation of PE *N*-methylation and a resultant increase in the PC/PE ratio of liver microsomes might participate in the enhancement of plasma cholesterol by methionine. In support of this, there was a significantly positive correlation between the PC/PE ratio and the plasma total cholesterol concentration in rats fed different

types of dietary proteins (7). However, it is possible that the stimulation of linoleic acid metabolism and a resultant decrease in the proportion of linoleic acid in phospholipids are more directly associated with the hypercholesterolemic action of methionine than the increase in the PC/PE ratio, since dietary linoleic acid is known to have a hypocholesterolemic action. In the present study, the depression of linoleic acid metabolism by the low-methionine diet was found to reflect mainly in the increase in the proportion of 16:0-18:2 molecular species of plasma PC (Table 4). This was also the case for the eritadenine-induced alteration of PC molecular species, although the extent of the effect of eritadenine was greater than that of dietary methionine. It was shown that the uptake rate of reconstituted HDL cholesterol by perfused rat livers was most stimulated by 16:0-18:2 PC of five PC molecular species used (16:0-18:2, 16:1-16:1, 18:0-18:2, 18:1-16:0, or 20:1-20:1) for the reconstitution of HDL (37). This PC molecular species-dependent differential uptake of lipoprotein cholesterol is thought to be mediated by hepatic lipase, since the hydrolysis of HDL phospholipids by the phospholipase A_1 activity of hepatic lipase is shown to be necessary for the subsequent uptake of HDL constituents (38), and since the 16:0-18:2 PC is the most preferred substrate for hepatic lipase (37). The uptake of the other lipoproteins, such as remnants of chylomicron (39) and VLDL (40), is also supposed to be mediated by hepatic lipase. Therefore, it is expected that the treatment to increase the proportion of 16:0-18:2 molecular species of plasma lipoprotein PC should enhance the cholesterol uptake of certain types of lipoproteins by the liver. Along this line, it seems reasonable to consider that dietary methionine level affects the phospholipid molecular species composition of plasma lipoproteins, thereby altering the uptake rate of lipoprotein cholesterol by the liver. This is also the case for the hypocholesterolemic action of eritadenine. In support of this, a significantly negative correlation was observed between the proportion of plasma 16:0-18:2 PC and the plasma total cholesterol concentration in rats fed six diets differing in methionine level and with or without eritadenine supplementation (Fig. 5).

Although 18:0-20:4 PC also had a significantly positive correlation with the plasma cholesterol concentration, it is currently unclear whether 18:0-20:4 PC exerts as a hypercholesterolemic factor. On the other hand, it has been shown that soybean protein increased the activity of lipoprotein receptor for β-VLDL in liver cells of rats fed a cholesterol-enriched diet (41). Hence, another mechanism is that a low-methionine diet may enhance the activity of lipoprotein receptors, thereby accelerating the uptake of plasma lipoprotein cholesterol by the liver. With regard to this, it is interesting that increased fluidity of LDL receptor membranes has been postulated to participate in the increase in LDL receptor activity in cynomolgus monkeys fed a diet rich in linoleic acid (42). Therefore, the possibility that dietary methionine level may affect the proportion of linoleic acid in lipoprotein receptor membrane phospholipids, in addition to plasma lipoprotein phospholipids, thereby possibly affecting lipoprotein receptor activities, cannot be ruled out. The effect of dietary methionine level on the activity of lipoprotein receptors in liver cell membranes remains to be further investigated.

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[Received September 22, 1997; accepted February 6, 1998]

Interaction of n-3 Long-Chain Polyunsaturated Fatty Acids with n-6 Fatty Acids in Suckled Rat Pups

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ABSTRACT: The addition of long-chain polyunsaturated fatty acids (LCP: C20, and C22) to infant formula may permit fatty acid accretion rates similar to breast-fed infants, and may have long-term outcome benefits, such as improved visual acuity and cognitive development. Although fish oil may provide a source of n-3 LCP, sources of n-6 LCP have been more difficult to identify. The present study evaluates the effects of n-3 and n-6 LCP derived from single-cell oils on liver, plasma, and brain fatty acid levels in a neonatal animal model. Newborn rat pups were suckled for 14 d by dams receiving diets containing n-3 LCP alone or combinations of n-3 LCP and increasing doses of linoleic acid (18:2n-6) or arachidonic acid (20:4n-6). Dietary groups received 2% n-3 LCP and 1, 2, or 5% of either 18:2n-6 or 20:4n-6. The 20:4n-6 source also contained modest levels of 18:2n-6. At the termination of the study, liver, plasma, and brain were obtained from the rat pups and the phospholipid fatty acid profiles determined. The results indicate complex interactions of n-3 and n-6 fatty acids. Groups receiving dietary 20:4n-6 incorporated higher levels of n-6 LCP into tissues than did the groups receiving 18:2n-6. The brain was relatively resistant to changes in fatty acid composition compared with the liver and plasma. As expected, tissue n-3 LCP levels were reciprocally related to n-6 levels. The present results document that single-cell LCP oils are bioavailable in a neonatal animal model. The use of 20:4n-6 is a more effective means of supporting n-6 status than the use of 18:2n-6. These results may have implications for the addition of LCP to infant formula.

Lipids 33, 243–250 (1998).

The ability of preterm and term infants to elongate and desaturate C18 essential fatty acids to long-chain polyunsaturated fatty acids (LCP) may not be optimal. Plasma and red blood cell levels of n-3 and n-6 LCP fall following birth in formula-fed infants, whereas the circulating LCP levels in human milk-fed infants remain relatively constant or fall to a lesser extent (1–4). To ensure an adequate supply of LCP, several published guidelines have indicated that preterm and/or term infant formula should contain both n-3 and n-6 LCP (5–7).

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Abbreviations: AA, arachidonic acid (20:4n-6); LA, linoleic acid (18:2n-6); LCP, long-chain polyunsaturated fatty acid(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine.

The incorporation of n-3 LCP into infant formula may be achieved by the addition of marine oil. However, this is an unbalanced addition, since most marine oils contain relatively high levels of n-3 LCP and much lower levels of n-6 LCP (8). *In vitro,* the presence of n-3 LCP down-regulates the chain elongation/desaturation of 18:2n-6 to n-6 LCP, primarily owing to inhibition of the Δ6 desaturase, resulting in reduced production of n-6 LCP (9). Therefore, addition of n-3 LCP, with the only source of n-6 fatty acids being 18:2n-6, may produce an undesirable reduction in tissue n-6 LCP and a concomitant elevation of n-3 LCP. Carlson *et al*. (10) observed a reduction in plasma and red blood cell arachidonic acid (AA: 20:4n-6) status in preterm infants receiving a marine oil containing infant formula compared with a nonsupplemented formula. Since 20:4n-6 status may exert a positive effect on growth in young infants (11), the optimal addition of LCP to infant formulas would provide a docosahexaenoic acid (22:6n-3)-rich source of n-3 LCP (to mimic the n-3 profile of human milk), as well as a source of 20:4n-6.

The present study examines the interaction of n-3 and n-6 fatty acids in suckling pups whose dams received dietary n-3 LCP (2% of total fat) and various levels of either 18:2n-6 or 20:4n-6 (1, 2, or 5%). The ratio of dietary 22:6n-3/20:5n-3 (eicosapentaenoic acid) was 2.3:1.0, similar to the ratio found in human milk (12). The source of 20:4n-6 and most of the 22:6n-3 was from single-cell oils. Lactating rats received diets for 14 d, and the suckled pup phospholipid fatty acid profiles of plasma, liver, and brain were determined [similar to a model described by Yeh *et al.* (13)].

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in a room controlled for temperature (21 ± 1°C), humidity (40–50%), and light (lights on 0600–1800 h). The rats were housed in suspended wire mesh cages before and during the mating period. They were fed a nonpurified diet (Purina Rat Chow #5001; Ralston Purina, St. Louis, MO) *ad libitum*; females continued the same diet during gestation and until postpartum day 2. The rats were mated (monogamous mating, one male/one female) for a 1-wk period. After the mating period, the pregnant females were divided into seven groups (six dams/group). The pregnant rats

were individually housed in plastic shoe box cages with ALPHA-dri™ bedding (Shepherd Specialty Papers, Inc., Kalamazoo, MI) and given free access to food and water. Body weights were recorded twice weekly.

Newborn pups were delivered naturally and weighed within 24 h of birth. Purified diets (stored at −70°C under argon and changed daily) were introduced to the dams on postpartum day 2. All diets contained 22% casein, 1% cellulose, 4% mineral mix (Bernhart-Tomarelli Salt Mixture), 1% AIN-76 vitamin mix (both mineral mix and vitamin mix from United States Biochemical Corp., Cleveland, OH), 0.1% choline chloride, 56.9% dextrose, and 15% fat. Seven fat blends were prepared to contain 2% of fat as n-3 LCP in all blends (22:6n-3/20:5n-3 = 2.3:1.0). This ratio was achieved by mixing a single-cell oil (Martek Biosciences Corp., Columbia, MD), rich in 22:6n-3, with menhaden oil. The blends contained n-3 LCP alone or n-3 LCP plus 1, 2, or 5% 18:2n-6 [linoleic acid (LA)] or 1, 2, or 5% 20:4n-6 (referred to as n-3 LCP, LA1, LA2, LA5, AA1, AA2, and AA5, respectively). The n-6 fatty acid sources for the diets were peanut oil (18:2n-6 source) and a 20:4n-6-rich single-cell oil (Martek Biosciences Corp.). The 20:4n-6 source contained modest levels of 18:2n-6; therefore, diets AA1, AA2, and AA5 also contained 18:2n-6. The remainder of the fat was hydrogenated coconut oil. Table 1 provides the fatty acid profiles of the diets. On postpartum day 0, the litter size (number live/number dead) was determined, and on day 7 the pups of each litter were weighed, sexed, and culled to four males and four females. On day 15 the pups were weighed, anesthetized with an intraperitoneal injection of Nembutal™ (Abbott Laboratories, North Chicago, IL) (5 mg/100 g body weight), and blood obtained by cardiac puncture with a 1-mL syringe containing sodium EDTA. Approximately 1.0 mL of blood was routinely obtained per pup; plasma was collected and pooled from each litter, and 20 μL of *d*-α-tocopherol (1.6 mg/mL EtOH) was added to each tube. The brain, liver, and stomach contents were obtained from one male pup per litter. The tissues were quickly removed and immediately rinsed in ice-cold saline. All samples were stored under argon at −70°C until lipid analysis.

Lipid extraction and analyses. Lipids were extracted from tissues according to the procedure of Folch *et al.* (14). The tissues were weighed, distilled water was added (10 mL for every gram of tissue), and tube contents were homogenized. For analyses of phospholipid fatty acids, an aliquot of the homogenate (1 mL) was added to 20 mL of a chloroform/methanol (2:1, vol/vol) mixture for lipid extraction. Phospholipid classes were separated from the lipid extracts by thin-layer chromatography using LK6 silica plates (Whatman Inc., Clifton, NJ) and a solvent system consisting of chloroform/methanol/petroleum ether/acetic acid/boric acid (40:20:30:10:1.8, vol/vol/vol/vol/wt) (15). Santoquin™ (Monsanto Co., St. Louis, MO) was added to the methanol to act as an antioxidant.

Lipids were methylated and extracted using the method of Morrison and Smith (16). Fatty acid methyl esters were analyzed by gas chromatography (Hewlett-Packard Model 5890 gas chromatograph interfaced with an HP 3365 Chemstation; Palo Alto, CA) using a capillary column (Omegawax, 30 m, 0.32 mm i.d.; Supelco, Bellefonte, PA) and flame-ionization detection. The following operating parameters were used: column temperature, 200°C; detector, 250°C; split ratio, 65:1; carrier gas, helium. Appropriate standards were analyzed to deter-

TABLE 1
Fatty Acid Profile of Dams' Diets[a]

Fatty acid	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
8:0	2.7	2.8	3.0	3.3	3.6	2.8	2.4
10:0	4.7	4.7	4.5	4.3	4.8	4.3	3.6
12:0	44.9	43.3	41.7	36.8	42.6	39.9	32.5
14:0	19.5	18.6	17.7	15.1	18.1	17.4	14.4
16:0	10.9	10.8	10.7	10.5	10.8	11.3	12.0
16:1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
18:0	11.8	11.3	10.6	9.1	11.4	11.7	11.8
18:1n-9	1.3	3.0	4.7	9.4	2.0	2.8	5.0
18:2n-6	0.1	1.3	2.4	5.6	0.7	1.3	3.1
18:3n-6	—[b]	—	—	—	0.3	0.5	1.3
18:3n-3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20:0	0.2	0.2	0.3	0.4	0.2	0.3	0.4
20:1n-9	0.1	0.1	0.2	0.4	0.1	0.2	0.3
20:2n-6	—	—	—	—	0.1	0.1	0.2
20:3n-6	—	—	—	—	0.3	0.5	1.3
20:4n-6	—	—	—	—	1.1	2.2	5.6
20:5n-3	0.7	0.7	0.7	0.6	0.7	0.7	0.7
22:0	0.03	0.2	0.3	0.7	0.2	0.4	0.8
22:5n-3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
22:6n-3	1.6	1.6	1.5	1.5	1.6	1.6	1.6
24:0	—	0.1	0.2	0.4	0.2	0.3	0.8

[a]LCP, long-chain polyunsaturated fatty acids; n-3 LCP plus 1, 2, or 5% linoleic acid (referred to as LA1, LA2, and LA5); n-3 LCP plus 1, 2, or 5% arachidonic acid (referred to as AA1, AA2, and AA5); values reported are weight percentage.

[b]—, Below detection limit.

mine retention times and response factors (Nu-Chek-Prep, Inc., Elysian, MN). Fatty acid composition was expressed as the weight percentage of the total fatty acids.

Statistics. The means of the seven diet groups were compared by the Student-Newman-Keuls test at the 1% level following a one-way analysis of variance (17). Multiple correlation coefficients (R^2) were calculated based on the most parsimonious multiple nonlinear regression model determined by stepwise regression. A single nonlinear coefficient, lambda, was fitted for the prediction model. A concentration additive model was employed to predict a fatty acid percentage in one tissue given the fatty acid composition of plasma. In this model a linear combination of all the independent variables was raised to lambda (18). The regression analysis and fatty acid Student-Newman-Keuls analyses used square root percentage as the response variable to induce normality and homogeneity of variance. The reported means and standard errors have been retransformed back to percentages.

RESULTS

Growth. All pups grew at similar rates with the exception of the high 18:2n-6 group (LA5). This group had a trend toward higher body weights at day 7, which reached statistical significance at day 15 vs. the other LA groups and the n-3 LCP group (Table 2). No differences in appearance or activity were noted in any group.

Stomach content (milk). Pup stomach contents at day 15 of life contained no particulates indicative of chow consumption, and therefore reflected the composition of their dams' milk. Stomach contents were generally related to the dams' diets (Table 3). The LA1, LA2, and LA5 diets of the dams contained no other n-6 fatty acids, although low levels of n-6 LCP were found in stomach contents. The diets containing n-6 LCP also contained modest amounts of 18:2n-6; as a result, the total n-6 level in diets AA1, AA2, and AA5 were higher than the corresponding LA diets. These differences were minimized in the pup stomach contents. The total n-6 levels in the stomach contents of the LA groups were 1.66, 2.32, and 5.93% vs. 2.06, 3.30, and 6.50% in the AA groups.

Liver phospholipids. The 18:2n-6 levels in both liver phosphatidylethanolamine (PE) and phosphatidylcholine (PC) increased progressively from the basal group through the groups receiving increasing dietary levels of 18:2n-6 (Table 4). Interestingly, 18:2n-6 levels in AA1–AA5 groups were lower (significant at $P < 0.05$, but not at $P < 0.01$) than the basal group, even though milk levels of 18:2n-6 were higher in the AA1–AA5 groups than in the basal group. Hepatic PE and PC 20:4n-6 levels increased progressively from the basal group to groups receiving high n-6 fatty acid levels in a dose-related manner as maternal diet and milk levels of 18:2n-6 and 20:4n-6 increased. Groups whose dams received dietary 20:4n-6 had higher levels of tissue 20:4n-6 than the groups receiving 18:2n-6. Liver phospholipid levels of 22:6n-3 were

TABLE 2
Mean Number of Viable Pups and Weight Gain During Suckling[a]

	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
Total pups (number)	13.50 ± 1.41[a]	13.83 ± 1.14[a]	13.83 ± 0.87[a]	13.33 ± 1.45[a]	13.00 ± 1.69[a]	14.50 ± 1.06[a]	12.50 ± 0.56[a]
Live Pups (number)	13.50 ± 1.41[a]	13.33 ± 1.52[a]	13.67 ± 0.95[a]	12.67 ± 1.41[a]	13.00 ± 1.69[a]	13.83 ± 1.30[a]	12.50 ± 0.56[a]
Day 1 weight	7.05 ± 0.26[a]	6.99 ± 0.20[a]	6.96 ± 0.12[a]	6.81 ± 0.22[a]	7.02 ± 0.28[a]	6.90 ± 0.12[a]	7.09 ± 0.16[a]
Day 7 weight	14.29 ± 1.22[a]	14.78 ± 1.07[a]	13.86 ± 0.54[a]	15.54 ± 0.92[a]	14.76 ± 0.93[a]	14.48 ± 0.71[a]	14.48 ± 0.71[a]
Day 15 weight	31.51 ± 1.04[a]	31.12 ± 1.54[a]	32.08 ± 0.65[a]	37.20 ± 1.26[b]	34.14 ± 1.51[a,b]	33.65 ± 0.08[a,b]	34.06 ± 0.50[a,b]

[a]Values reported are means ± SEM ($n = 6$ litters). Values not bearing the same superscript are different at $P < 0.05$. For abbreviations see Table 1.

TABLE 3
Pup Stomach Contents[a]

Fatty acid	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
10:0	4.39 ± 0.41[a]	5.34 ± 0.67[a,b]	6.69 ± 0.62[a,b,c]	7.18 ± 0.28[a,b,c]	9.98 ± 0.54[c]	7.95 ± 1.30[b,c]	5.84 ± 0.62[a,b]
12:0	26.0 ± 0.07[a,b]	27.6 ± 0.04[b]	27.9 ± 0.03[b]	23.9 ± 0.09[a]	28.0 ± 0.04[b]	28.2 ± 0.07[b]	25.6 ± 0.04[a,b]
14:0	21.9 ± 0.03[c]	20.5 ± 0.04[b,c]	19.7 ± 0.06[b,c]	16.1 ± 0.07[a]	19.0 ± 0.02[b]	19.0 ± 0.10[b]	19.0 ± 0.02[b]
16:0	27.2 ± 0.70[c]	24.0 ± 0.11[b,c]	22.8 ± 0.07[a,b]	19.6 ± 0.05[a]	21.8 ± 0.04[a,b]	21.3 ± 0.14[a,b]	21.7 ± 0.08[a,b]
16:1n-7	1.10 ± 0.08[a,b]	1.09 ± 0.07[a,b]	1.44 ± 0.19[b]	1.47 ± 0.12[b]	0.96 ± 0.08[a,b]	1.09 ± 0.12[a,b]	0.84 ± 0.07[a]
18:0	8.65 ± 0.23	8.12 ± 0.37	7.29 ± 0.31	7.27 ± 0.31	7.11 ± 0.26	6.84 ± 0.30	8.14 ± 0.65
18:1n-9	5.60 ± 0.42[a]	7.10 ± 0.49[a]	7.15 ± 0.74[a]	12.7 ± 0.79[b]	5.47 ± 0.31[a]	6.17 ± 0.69[a]	6.05 ± 0.33[a]
18:2n-6	0.68 ± 0.08[a]	1.34 ± 0.16[b,c]	1.98 ± 0.18[c,d]	5.29 ± 0.33[e]	1.19 ± 0.16[b]	1.67 ± 0.20[b,c]	2.71 ± 0.11[d]
20:4n-6	0.21 ± 0.02[a]	0.28 ± 0.03[a]	0.29 ± 0.02[a]	0.54 ± 0.02[b]	0.75 ± 0.03[c]	1.40 ± 0.07[d]	3.31 ± 0.10[e]
20:5n-3	0.17 ± 0.00[a]	0.20 ± 0.01[a]	0.21 ± 0.01[a]	0.28 ± 0.01[b]	0.22 ± 0.01[a]	0.27 ± 0.01[b]	0.31 ± 0.01[b]
22:4n-6	0.03 ± 0.00[a]	0.04 ± 0.01[a]	0.05 ± 0.01[a]	0.10 ± 0.01[b]	0.12 ± 0.01[b]	0.23 ± 0.03[c]	0.48 ± 0.01[d]
22:5n-3	0.21 ± 0.01[a,b]	0.21 ± 0.01[a]	0.22 ± 0.02[a,b]	0.30 ± 0.02[a,b]	0.26 ± 0.02[a,b]	0.32 ± 0.04[b]	0.29 ± 0.01[a,b]
22:6n-3	1.07 ± 0.04	1.12 ± 0.05	1.07 ± 0.03	1.16 ± 0.03	1.12 ± 0.04	1.25 ± 0.05	1.17 ± 0.02

[a]Values reported are weight percentage and are means ± SEM ($n = 6$, that is, one representative animal from each of six litters). Values not bearing the same superscript letters are different at $P < 0.01$. For abbreviations see Table 1.

TABLE 4
Pup Liver Phospholipids[a]

Fatty acid	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
Phosphatidylethanolamine							
16:0	26.6 ± 0.70	26.5 ± 0.62	25.6 ± 1.25	22.9 ± 0.77	24.6 ± 0.47	24.8 ± 0.46	24.8 ± 1.28
18:0	25.1 ± 0.53	26.2 ± 1.18	25.0 ± 0.97	26.8 ± 0.61	26.4 ± 0.44	26.7 ± 0.78	29.4 ± 1.21
18:1n-9	1.11 ± 0.06	1.31 ± 0.07	1.20 ± 0.15	1.17 ± 0.08	1.11 ± 0.21	1.00 ± 0.10	1.27 ± 0.12
18:2n-6	1.22 ± 0.10^{a,b,c,d}	1.27 ± 0.07^{b,c,d}	1.36 ± 0.06^{c,d}	1.64 ± 0.11^{d}	0.95 ± 0.08^{a,b,c}	0.92 ± 0.05^{a,b}	0.87 ± 0.10^{a}
20:3n-6	0.42 ± 0.04	0.41 ± 0.02	0.49 ± 0.07	0.49 ± 0.03	0.42 ± 0.02	0.56 ± 0.06	0.60 ± 0.06
20:4n-6	9.05 ± 0.61^{a}	9.72 ± 0.44^{a}	9.73 ± 0.49^{a}	12.6 ± 0.28^{b}	12.8 ± 0.40^{b}	13.0 ± 0.20^{b}	15.3 ± 0.38^{c}
20:5n-3	0.99 ± 0.10^{e}	0.78 ± 0.11^{d,e}	0.84 ± 0.13^{d,e}	0.58 ± 0.04^{c,d}	0.44 ± 0.06^{c}	0.20 ± 0.01^{b}	—[b]
22:4n-6	0.28 ± 0.04^{a}	0.44 ± 0.05^{a,b}	0.49 ± 0.06^{a,b,c}	0.64 ± 0.19^{b,c,d}	0.61 ± 0.01^{b,c,d}	0.86 ± 0.07^{c,d}	1.00 ± 0.10^{d}
22:5n-6	0.18 ± 0.02	0.22 ± 0.04	0.11 ± 0.05	0.05 ± 0.04	0.17 ± 0.04	0.27 ± 0.02	0.14 ± 0.09
22:5n-3	1.96 ± 0.05^{a,b}	1.93 ± 0.13^{a,b}	2.12 ± 0.12^{b}	1.75 ± 0.11^{a,b}	2.02 ± 0.05^{a,b}	1.81 ± 01.0^{a,b}	1.55 ± 0.09^{a}
22:6n-3	30.1 ± 0.74^{b}	26.3 ± 1.12^{a,b}	27.7 ± 0.67^{a,b}	26.2 ± 1.20^{a,b}	27.1 ± 0.53^{a,b}	25.4 ± 0.46^{a,b}	23.0 ± 1.23^{a}
Phosphatidylcholine							
14:0	0.94 ± 0.09	1.34 ± 0.17	1.26 ± 0.21	1.42 ± 0.10	1.16 ± 0.16	1.04 ± 0.10	0.97 ± 0.11
16:0	34.0 ± 0.52	33.9 ± 0.97	34.0 ± 0.71	31.8 ± 0.86	33.3 ± 0.78	32.5 ± 0.43	30.3 ± 0.54
18:0	16.9 ± 0.53^{a}	16.9 ± 0.48^{a}	17.0 ± 1.06^{a}	17.3 ± 0.31^{a}	17.8 ± 0.83^{a}	18.5 ± 0.59^{a,b}	21.1 ± 0.72^{b}
18:1n-9	4.38 ± 0.29^{c}	4.66 ± 0.33^{c}	4.53 ± 0.26^{c}	4.27 ± 0.17^{c}	3.27 ± 0.20^{b}	2.63 ± 0.07^{b}	1.94 ± 0.12^{a}
18:2n-6	2.48 ± 0.21^{a}	3.84 ± 0.35^{b}	4.24 ± 0.17^{b}	5.44 ± 0.17^{c}	2.18 ± 0.17^{a}	1.87 ± 0.06^{a}	1.78 ± 0.22^{a}
20:3n-6	1.10 ± 0.05^{a}	1.20 ± 0.04^{a,b}	1.43 ± 0.06^{c}	1.30 ± 0.05^{b,c}	1.11 ± 0.03^{a,b}	1.17 ± 0.03^{a,b}	1.03 ± 0.02^{a}
20:4n-6	8.51 ± 0.59^{a}	10.0 ± 1.00^{a,b}	10.2 ± 0.28^{a,b}	11.9 ± 0.59^{b,c}	13.7 ± 0.37^{c,d}	15.7 ± 0.29^{d}	19.9 ± 0.60^{e}
20:5n-3	0.81 ± 0.06^{c}	0.66 ± 0.07^{c}	0.73 ± 0.14^{c}	0.44 ± 0.03^{c}	0.13 ± 0.08^{b}	—	—
22:4n-6	0.17 ± 0.01^{a}	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}	0.23 ± 0.02^{a,b}	0.29 ± 0.02^{b}	0.45 ± 0.05^{c}	0.67 ± 0.03^{d}
22:5n-6	0.16 ± 0.01^{b}	0.10 ± 0.04^{a,b}	0.04 ± 0.03^{a,b}	—	0.07 ± 0.05^{a,b}	0.06 ± 0.05^{a,b}	0.16 ± 0.08^{b}
22:5n-3	2.72 ± 0.07^{b}	2.22 ± 0.18^{a,b}	2.31 ± 0.15^{a,b}	1.79 ± 0.13^{a}	2.23 ± 0.14^{a,b}	2.34 ± 0.18^{a,b}	2.00 ± 0.07^{a,b}
22:6n-3	25.6 ± 0.47^{b}	22.2 ± 0.99^{a,b}	21.4 ± 0.74^{a,b}	19.2 ± 1.20^{a}	21.3 ± 1.04^{a,b}	21.4 ± 0.89^{a,b}	19.4 ± 0.46^{a}

[a]Values reported are weight percentage and are means ± SEM (n = 6, that is, one representative animal from each of six litters). Values not bearing the same superscript letters are different at $P < 0.01$. For abbreviations see Table 1.
[b]—, Below detection limit.

also influenced by diet. High levels of dietary 18:2n-6 resulted in modest reductions in liver PE and PC 22:6n-3 levels. Groups receiving dietary 20:4n-6 demonstrated more pronounced effects on hepatic PE tissue 22:6n-3 levels.

Plasma phospholipids. Plasma PC levels of 18:2n-6 increased in a dose-related manner as milk 18:2n-6 increased in LA1–LA5, whereas PE 18:2n-6 did not respond significantly to varying dietary levels of 18:2n-6 (Table 5). As in liver, the inclusion of dietary 20:4n-6 resulted in a reduction of plasma PE and PC 18:2n-6. Plasma phospholipid 20:4n-6 levels increased dramatically with increasing dietary 20:4n-6 levels. Plasma PC 20:4n-6 responded with a more pronounced increase to dietary 20:4n-6 than did plasma PE. Plasma 20:4n-6 may be derived from dietary 20:4n-6 or 18:2n-6 (following desaturation and chain elongation). The relationship of dietary 18:2n-6 and 20:4n-6 to plasma 20:4n-6 was assessed to determine the relative potency of the dams' dietary 18:2n-6 and 20:4n-6 in supplying pup plasma 20:4n-6. The relative potency of dietary 20:4n-6 to 18:2n-6 for supplying plasma 20:4n-6 was 5.1:1.0 (95% confidence limits = 3.7–9.2) for plasma PE and 56.2:1.0 (95% confidence limits = 14.5–356.3) for plasma PC. Decreases in the plasma PC 22:6n-3 levels occurred as 18:2n-6 and 20:4n-6 were added to the maternal diet and were related to the n-6 pup stomach contents. Decreases in 22:6n-3 also occurred in the plasma PE fraction, but were not related to the dietary level of n-6 fatty acids.

Brain phospholipids. Dietary manipulation did not alter brain levels of 20:4n-6 and 22:6n-3 at a significance level of $P < 0.01$, although marginal statistical significance was observed in a number of groups (for example the PE 20:4n-6 levels of AA1, AA2, and AA5 were all greater than n-3 LCP at $P < 0.05$, whereas the PE 22:6n-3 level of group AA5 was lower than the n-3 LCP group also at $P < 0.05$). Elevated dietary 20:4n-6 consumption resulted in significant alterations at $P < 0.01$ in several LCP fractions: PE and PC 22:4n-6 increased while PE 22:5n-3 decreased (Table 6). The ratio of major brain PE n-6 to n-3 LCP [(20:4n-6 + 22:4n-6)/22:6n-3] provided additional evidence of dietary control on the relative proportions of these fatty acids (Fig. 1). Increased levels of n-6 LCP (both in the dams' diets and in pup stomachs) resulted in a highly significant increase in this ratio. A similar effect was noted in brain PC (data not shown). Diet-related increases in this ratio for liver and plasma (Fig. 1) were proportionally greater than the increase in brain ratio. For example, the ratio increased 173% in plasma PE when the n-3 LCP group was compared to the AA5 group, while only a 57% increase was observed in the same groups in brain.

Correlation analysis. The relationship between plasma n-6 fatty acids and both liver and brain 20:4n-6 was evaluated. Plasma PE n-6 fatty acids (18:2n-6, 20:3n-6, and 20:4n-6) were not significantly predictive of 20:4n-6 levels in either liver or brain PE or PC. In contrast, plasma PC n-6 fatty acids (18:2n-6 and 20:4n-6) were highly predictive of liver PE and PC 20:4n-6, significantly so even after adjusting for plasma PE n-6 fatty acids (Table 7). Plasma 20:3n-6 was not required for an optimal correlation. Plasma PC 20:4n-6 was also pre-

TABLE 5
Pup Plasma Phospholipids[a]

Fatty acid	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
Phosphatidylethanolamine							
14:0	1.23 ± 0.25[a,b]	1.72 ± 0.22[a,b]	1.75 ± 0.21[a,b]	1.92 ± 0.25[a,b]	2.47 ± 0.39[b]	2.03 ± 0.31[a,b]	1.14 ± 0.05[a]
16:0	11.2 ± 0.56[a,b]	15.3 ± 0.96[b]	13.7 ± 1.03[a,b]	10.8 ± 0.85[a]	13.1 ± 0.74[a,b]	12.0 ± 0.59[a,b]	9.84 ± 0.73[a]
18:0	15.8 ± 0.53[a,b]	21.0 ± 1.35[c]	18.5 ± 1.15[b,c]	15.9 ± 0.71[a,b]	14.6 ± 0.76[a,b]	15.1 ± 0.90[a,b]	13.5 ± 0.67[a]
18:1n-9	2.66 ± 0.26[a]	3.86 ± 0.69	3.24 ± 0.30	2.80 ± 0.35	3.98 ± 0.72	2.65 ± 0.41	2.08 ± 0.27
18:2n-6	1.81 ± 0.17[a,b]	3.14 ± 0.52[b]	2.13 ± 0.25[a,b]	2.60 ± 0.27[b]	2.09 ± 0.50[a,b]	1.46 ± 0.22[a,b]	1.09 ± 0.05[a]
20:2n-6	0.94 ± 0.16	0.90 ± 0.12	0.83 ± 0.13	0.76 ± 0.10	1.02 ± 0.21	0.84 ± 0.13	0.70 ± 0.08
20:3n-6	3.34 ± 0.55	1.97 ± 0.19	3.04 ± 0.71	2.77 ± 0.39	2.79 ± 0.69	3.00 ± 0.78	1.99 ± 0.56
20:4n-6	7.77 ± 0.42[a,b]	7.51 ± 0.83[a,b]	6.94 ± 0.34[a]	9.55 ± 0.40[b]	9.69 ± 0.46[b]	12.7 ± 0.35[c]	17.5 ± 0.27[d]
22:3n-6	0.34 ± 0.05[a,b]	0.67 ± 0.27[a,b]	1.28 ± 0.32[b]	1.40 ± 0.26[b]	1.49 ± 0.27[b]	1.54 ± 0.42[b]	0.21 ± 0.07[a]
22:5n-3	1.19 ± 0.02[a]	2.93 ± 0.17[b,c]	3.18 ± 0.28[b,c]	2.85 ± 0.22[b]	3.80 ± 0.20[c]	3.44 ± 0.11[b,c]	0.88 ± 0.04[a]
22:6n-3	41.9 ± 0.82[c]	25.3 ± 1.11[a]	27.3 ± 1.96[a,b]	27.1 ± 1.60[a,b]	30.4 ± 1.56[a,b]	30.2 ± 0.97[a,b]	33.9 ± 1.20[b]
Phosphatidylcholine							
14:0	1.77 ± 0.11	1.43 ± 0.16	1.42 ± 0.12	1.25 ± 0.08	1.48 ± 0.07	1.39 ± 0.15	1.24 ± 0.07
16:0	27.8 ± 0.36[b]	26.1 ± 0.59[a,b]	26.8 ± 0.36[a,b]	25.2 ± 0.42[a]	26.2 ± 0.39[a,b]	26.7 ± 0.44[a,b]	25.6 ± 0.36[a,b]
18:0	19.2 ± 0.27	20.9 ± 0.35	21.1 ± 0.62	21.1 ± 0.45	20.6 ± 0.26	20.8 ± 0.37	20.8 ± 0.24
18:1n-9	10.3 ± 0.84[e]	7.57 ± 1.10[d]	6.69 ± 0.45[c,d]	4.57 ± 0.07[b,c]	5.37 ± 0.44[c,d]	3.00 ± 0.28[b]	1.32 ± 0.12[a]
18:2n-6	10.3 ± 0.92[b]	14.7 ± 1.06[c]	17.8 ± 0.31[d]	21.3 ± 0.22[e]	10.2 ± 0.53[b]	9.23 ± 0.18[b]	5.68 ± 0.19[a]
20:2n-6	0.48 ± 0.05[b]	0.48 ± 0.04[b]	0.44 ± 0.02[b]	0.37 ± 0.01[b]	0.43 ± 0.01[b]	0.13 ± 0.05[a]	0.16 ± 0.04[a]
20:3n-6	1.67 ± 0.11[a]	1.97 ± 0.17[a]	1.95 ± 0.08[a]	2.02 ± 0.05[a]	2.86 ± 0.07[b]	3.72 ± 0.07[c]	3.31 ± 0.11[b,c]
20:4n-6	7.89 ± 0.53[a]	9.15 ± 0.89[a,b]	8.38 ± 0.26[a,b]	10.2 ± 0.37[b]	16.2 ± 0.18[c]	20.0 ± 0.27[d]	29.7 ± 0.40[e]
20:5n-3	0.96 ± 0.02[c]	0.95 ± 0.13[c]	0.80 ± 0.04[c]	0.51 ± 0.02[b]	0.75 ± 0.02[c]	0.57 ± 0.01[b]	0.22 ± 0.01[a]
22:5n-3	0.92 ± 0.02[b]	0.95 ± 0.11[b]	0.73 ± 0.03[a,b]	0.62 ± 0.02[a]	0.87 ± 0.10[a,b]	0.72 ± 0.03[a,b]	0.60 ± 0.02[a]
22:6n-3	13.9 ± 0.23[e]	12.6 ± 0.56[d,e]	10.8 ± 0.40[b,c]	9.94 ± 0.20[a,b]	11.7 ± 0.57[c,d]	10.1 ± 0.14[a,b]	8.70 ± 0.14[a]

[a]Values reported are weight percentages and are means ± SEM (n = 6, that is, n equals pooled plasma from each of six litters). Values not bearing the same superscript letters are different at $P < 0.01$. For abbreviations see Table 1.

TABLE 6
Pup Brain Phospholipids[a]

Fatty acid	n-3 LCP	LA1	LA2	LA5	AA1	AA2	AA5
Phosphatidylethanolamine							
16:0	7.55 ± 0.23	7.72 ± 0.26	7.58 ± 0.32	7.23 ± 0.25	7.26 ± 0.19	8.46 ± 0.35	7.90 ± 0.14
18:0	22.4 ± 0.62	23.4 ± 0.67	23.3 ± 0.77	23.2 ± 0.49	22.4 ± 0.24	24.6 ± 0.48	23.5 ± 0.62
18:1n-9	7.92 ± 0.15	7.89 ± 0.20	7.63 ± 0.23	7.57 ± 0.32	7.15 ± 0.23	7.72 ± 0.16	7.25 ± 0.18
20:0	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
20:1n-9	0.31 ± 0.01	0.33 ± 0.04	0.29 ± 0.03	0.31 ± 0.02	0.27 ± 0.02	0.28 ± 0.01	0.24 ± 0.02
20:2n-6	0.22 ± 0.05	0.18 ± 0.04	0.13 ± 0.02	0.14 ± 0.02	0.30 ± 0.11	0.19 ± 0.04	0.13 ± 0.03
20:3n-6	0.75 ± 0.11	0.78 ± 0.08	0.74 ± 0.06	0.76 ± 0.06	0.52 ± 0.01	0.62 ± 0.07	0.54 ± 0.12
20:4n-6	12.9 ± 0.26	13.6 ± 0.47	13.6 ± 0.26	14.4 ± 0.56	15.1 ± 0.46	14.9 ± 0.56	15.3 ± 0.65
22:4n-6	3.16 ± 0.12[a]	3.52 ± 0.22[a,b]	3.62 ± 0.08[a,b]	4.31 ± 0.28[b,c]	4.45 ± 0.16[b,c]	4.58 ± 0.21[b,c]	5.05 ± 0.3[c]
22:5n-6	0.96 ± 0.04	1.04 ± 0.04	0.96 ± 0.04	0.99 ± 0.06	1.16 ± 0.05	1.12 ± 0.06	1.24 ± 0.09
22:5n-3	0.65 ± 0.04[c]	0.57 ± 0.05[b,c]	0.54 ± 0.02[b,c]	0.40 ± 0.04[a,b]	0.45 ± 0.02[b]	0.38 ± 0.02[a,b]	0.26 ± 0.05[a]
22:6n-3	21.3 ± 0.53	20.3 ± 0.62	20.0 ± 0.50	18.3 ± 1.32	19.9 ± 0.80	17.6 ± 1.07	17.1 ± 0.65
Phosphatidylcholine							
10:0	0.17 ± 0.06	0.15 ± 0.08	0.08 ± 0.01	0.17 ± 0.04	0.19 ± 0.11	0.12 ± 0.01	0.16 ± 0.03
14:0	1.60 ± 0.12	1.72 ± 0.06	1.62 ± 0.11	1.32 ± 0.16	1.59 ± 0.17	1.55 ± 0.20	1.59 ± 0.15
16:0	51.9 ± 0.37	53.4 ± 0.26	52.8 ± 0.57	50.8 ± 0.63	52.6 ± 0.88	52.1 ± 1.10	52.9 ± 0.37
16:1n-7	1.02 ± 0.03	1.03 ± 0.04	1.05 ± 0.03	0.90 ± 0.02	1.01 ± 0.06	0.99 ± 0.03	1.01 ± 0.01
18:0	9.51 ± 0.12	9.56 ± 0.13	9.45 ± 0.22	10.0 ± 0.28	9.87 ± 0.42	9.86 ± 0.20	9.55 ± 0.16
18:1n-9	18.3 ± 0.18[b]	17.5 ± 0.20[a,b]	17.8 ± 0.24[a,b]	17.4 ± 0.28[a,b]	17.3 ± 0.12[a,b]	17.4 ± 0.29[a,b]	16.9 ± 0.35[a]
18:2n-6	0.78 ± 0.03[b,c]	0.91 ± 0.03[b,c]	1.02 ± 0.03[c]	1.00 ± 0.15[c]	0.62 ± 0.05[a,b,c]	0.56 ± 0.03[a,b]	0.43 ± 0.11[a]
20:1n-9	0.44 ± 0.02	0.41 ± 0.02	0.44 ± 0.01	0.46 ± 0.01	0.42 ± 0.03	0.41 ± 0.02	0.41 ± 0.01
20:2n-6	0.22 ± 0.01	0.21 ± 0.01	0.23 ± 0.01	0.22 ± 0.03	0.16 ± 0.01	0.17 ± 0.03	0.16 ± 0.04
20:3n6	0.55 ± 0.03[b]	0.53 ± 0.01[b]	0.57 ± 0.03[b]	0.53 ± 0.03[b]	0.44 ± 0.03[a,b]	0.42 ± 0.03[a,b]	0.37 ± 0.04[a]
20:4n-6	5.23 ± 0.29	5.08 ± 0.10	5.10 ± 0.15	5.85 ± 0.53	5.89 ± 0.38	6.58 ± 0.39	6.44 ± 0.32
22:4n-6	0.39 ± 0.02[a]	0.39 ± 0.01[a]	0.40 ± 0.02[a]	0.55 ± 0.06[b]	0.52 ± 0.03[a,b]	0.62 ± 0.03[b]	0.64 ± 0.04[b]
22:6n-3	3.19 ± 0.17	2.54 ± 0.10	2.65 ± 0.17	2.63 ± 0.22	2.71 ± 0.26	2.78 ± 0.24	2.48 ± 0.19

[a]Values reported are weight percentage and are means ± SEM (n = 6, that is, one representative animal from each of six litters). Values not bearing the same superscript letters are different at $P < 0.01$. For abbreviations see Table 1

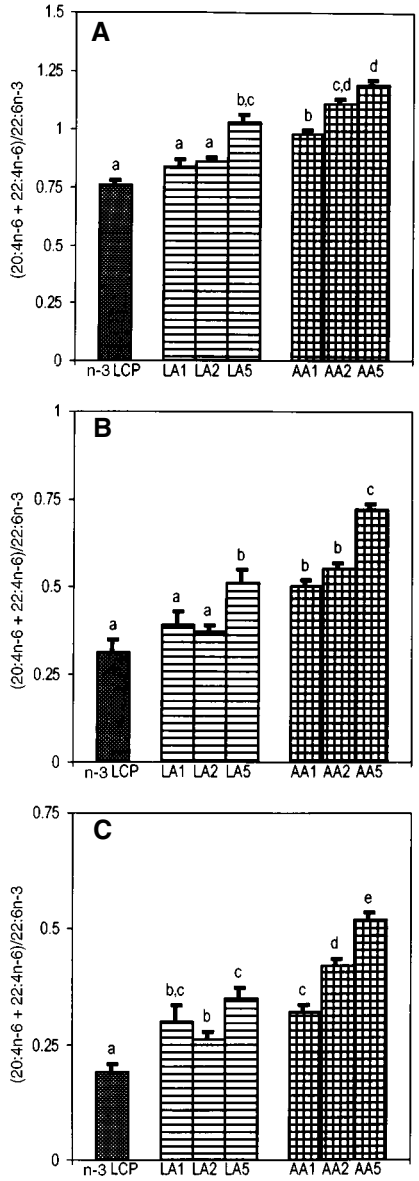

FIG. 1. Mean and SEM of the ratio of (20:4n-6 + 22:4n-6)/22:6n-3 in (A) brain, (B) liver, and (C) plasma phosphatidylethanolamine (PE); for brain and liver, *n* = one representative animal from each of six litters; for plasma, *n* = pooled samples from each of six litters. Bars not bearing the same letters are different at $P < 0.01$. Abbreviations: LCP, long-chain polyunsaturated fatty acids; n-3 LCP plus 1, 2, or 5% linoleic acid (referred to as LA1, LA2, and LA5); n-3 LCP plus 1, 2, or 5% arachidonic acid (referred to as AA1, AA2, and AA5).

dictive of brain PE and PC 20:4n-6. Because plasma PE n-6 fatty acids were highly correlated with plasma PC n-6 fatty acids, their effects were confounded and sometimes difficult to distinguish. Nevertheless, in all four cases, plasma PC n-6 fatty acids were more predictive than plasma PE n-6 fatty acids of liver and brain PC and PE n-6 fatty acids.

DISCUSSION

This study evaluated the complex interactions of n-6 and n-3 fatty acids in rat pups whose dams received diets containing a constant level of n-3 LCP and increasing levels of n-6 fatty acids. We chose rats pups as a model of the human preterm infant owing to the relative immaturity of the rat pup brain. The rat pup is born prior to its period of rapid brain growth (19); the relatively immature brain of the neonatal rat and subsequent rapid postpartum development may be analogous to the preterm human infant. The brain maturity of the 14-day-old pup is, in fact, similar to the term infant (20). The results of this experiment clearly demonstrate that the source of 20:4n-6 evaluated was bioavailable, being efficiently absorbed and incorporated into biological membranes. As reported previously (21), changes in maternal dietary fatty acid composition resulted in more significant fatty acid alterations of maternal milk, pup plasma, and pup liver than that of pup brain. The ability of dietary 18:2n-6 (as reflected in pup stomach fatty acid profiles) to influence pup n-6 LCP profiles was much less pronounced than the ability of 20:4n-6.

TABLE 7
Predictability of Liver and Brain 20:4n-6 from Plasma PC n-6 Fatty Acids[a]

Plasma n-6 species[b] (significant independent variables)	Tissue fraction (dependent variable)	R^{2c}	*P*
18:2n-6, 20:4n-6	Liver PE	0.87	0.0001
18:2n-6, 20:4n-6	Liver PC	0.96	0.0001
20:4n-6	Brain PE	0.41	0.0002
20:4n-6	Brain PC	0.36	0.0007

[a]Plasma phosphatidylethanolamine (PE) n-6 fatty acids were not predictive of liver or brain PE or phosphatidylcholine (PC).
[b]Fatty acids required for optimal correlation.
[c]All correlations are positive.

n-6 Accretion. The source of dietary 20:4n-6 employed in the current study contained modest levels of 18:2n-6. The levels of 20:4n-6 added to the maternal diets were designed to match the added level of 18:2n-6 (1, 2, or 5% of the fat blend). Owing to the presence of 18:2n-6 and other n-6 fatty acids in the single-cell oil employed in this study, the total level of dietary n-6 was higher for the dams receiving the 20:4n-6 oils than the groups receiving 18:2n-6 alone. However, when the stomach contents (milk) of the pups were examined, the total n-6 fatty acid levels were more closely matched. The striking differences in fatty acid profiles in some of the tissues examined (see below) clearly demonstrate that the observed effects were due to the presence of dietary 20:4n-6 aggressively inserted into biological membranes (22), and not to the relatively modest differences in stomach contents of total n-6 fatty acids.

Liver and plasma phospholipid 20:4n-6 levels were substantially higher when pups received preformed n-6 LCP (AA1–AA5) than when pups received 18:2n-6 as the predominant source of n-6 fatty acid (LA1–LA5). Plasma PC n-6 fatty acids were highly correlated with both liver PE and liver PC 20:4n-6 levels, suggesting a rapid exchange of fatty acids between these pools (a modest correlation was also observed between plasma 20:4n-6 and brain 20:4n-6 levels). In groups

with dams receiving 18:2n-6 as the only source of n-6 fatty acids, both pup hepatic and plasma 18:2n-6 levels tended to increase in a dose-related manner, while 20:4n-6 levels were significantly elevated only in groups receiving high 18:2n-6 levels. As a result of the presence of milk n-3 and n-6 LCP in LA1–LA5, the chain elongation and desaturation of 18:2n-6 to n-6 LCP by the pups may have been inhibited, since both n-3 and n-6 LCP can down-regulate elongation and desaturation of 18:2n-6 (9). Even in experimental systems which are not exposed to feedback inhibition, the accretion of hepatic 20:4n-6 from dietary 18:2n-6 is substantially less potent than if preformed 20:4n-6 is provided, as demonstrated by Mohrhauer and Holman (23) and Whelan *et al.* (24). Although the n-3 LCP, LA1, and LA2 groups received relatively low 18:2n-6 levels, no signs of essential fatty acid deficiency (elevated 20:3n-9) were observed. Milk in these groups contained sufficient 20:4n-6 to ensure adequate essential fatty acid status (approximately 0.25 energy percentage, see Ref. 23).

The present study also documents modest alterations in the n-6 fatty acid contents of brain phospholipids, with changes being observed in both PE and PC. Brain 20:4n-6 levels tended to be elevated in AA1–AA5 compared with the other groups, although statistical significance was observed only at a marginal level. Employing a similar model, Jumpsen *et al.* (25) evaluated the effects of dietary n-3 and n-6 LCP alone or in combination; milk LCP levels were within the range of the present study (25). Neuronal cell PE levels of LCP were not altered by dietary LCP at a pup age of 2 wk (the same as the current study), although differences were noted at 1, 3, and 6 wk of age. In addition, no significant differences were found at any time in neuronal PC 20:4n-6 levels. Mohrhauer and Holman (26) also studied the effect of increasing doses of 20:4n-6 in rats, but under different experimental conditions (weanling rat pups receiving various doses of 20:4n-6 ethyl ester for 100 d by oral gavage). They found no dose-related plateau of total brain 20:4n-6 levels over a wide range of 20:4n-6 levels (0.007–3.75 energy percentage) that bracketed the 20:4n-6 levels employed in the current study.

n-3 LCP accretion and interaction with n-6 fatty acids. Dramatic suppression of hepatic and brain 20:4n-6 levels occurs in weaned rats fed diets high in n-3 LCP and with 18:2n-6 as the only source of n-6 fatty acids, demonstrating the plasticity of tissue LCP content of these animals (27,28). The suppression of n-6 LCP tissue levels may be due to: (i) reduced chain elongation/desaturation rates resulting from n-3 LCP inhibition of the Δ6 desaturase, or (ii) competition of n-3 LCP for membrane insertion. Similarly, substantial reductions in hepatic, circulating, and brain n-6 LCP levels occur in rodent pups whose dams received diets containing n-3 LCP (fish oil) during pregnancy and nursing, although the levels of n-3 LCP administered in these studies were also relatively high (29–31). As expected, the n-3 LCP accretion in all tissues studied was significantly higher in LCP-treated groups than in control groups.

The present experiment provided dietary intervention with relatively low LCP levels only during the time of suckling, thus avoiding the prenatal complications of placental transfer of LCP to the fetus. The results of this experiment clearly demonstrate the sensitive reciprocal relationship of n-6 and n-3 fatty acids in liver, plasma, and brain. Although C18 precursors (in this case, 18:2n-6) may reduce n-3 LCP concentration, the present data clearly indicate that the provision of dietary n-6 LCP (to the pups) results in more pronounced alterations of n-3 LCP than does 18:2n-6. The ratio of major brain n-6 to n-3 LCP reflected this interaction, although relative responses were more pronounced in plasma and liver (Fig. 1).

Requirements for LCP. Human neonates may not possess adequate chain elongation/desaturation capacity to support maximal LCP levels, since both preterm and term formula-fed infants have lower plasma and red blood cell LCP levels than breast-fed infants (1–4). Carlson *et al.* (10) compared LCP status in preterm infants receiving either a marine oil-containing formula (20:5n-3 and 22:6n-3) or control formula for the first year of life. In this study, plasma and red blood cell 20:4n-6 levels were lower in the marine oil group compared to control, suggesting that n-3 LCP may inhibit 18:2n-6 chain elongation and desaturation to 20:4n-6 or may compete with n-6 LCP for insertion into phospholipids in human neonates. Growth parameters were correlated with 20:4n-6 status (11). Several other studies have also demonstrated an n-3 LCP mediated reduction in 20:4n-6 fatty acid levels in preterm and term infants, even when the n-3 LCP source high in 22:6n-3 and low in 20:5n-3 was employed (32,33). Thus, a more appropriate means of achieving optimal LCP levels may be to provide both n-3 and n-6 families of fatty acids as LCP.

In conclusion, we have explored the complex interactions of n-3 LCP with either 18:2n-6 or 20:4n-6 in rapidly growing neonatal rat pups employing single-cell oils as the primary source of LCP. Dietary 18:2n-6 was a less effective means of supplying n-6 LCP for tissue accretion than was preformed n-6 LCP. These observations were apparent not only in liver and plasma but, also to a modest extent, in brain.

ACKNOWLEDGMENT

The expert secretarial assistance of June Deitz is deeply appreciated.

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[Received April 23, 1997, and in final revised form January 14, 1998; revision accepted January 21, 1998]

Diabetes-Induced and Age-Related Changes in Fatty Acid Proportions of Plasma Lipids in Rats

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ABSTRACT: Diabetes-induced and age-related proportional changes in plasma fatty acids of triglycerides (TG), phospholipids (PL), and cholesteryl esters (CE) were investigated using streptozotocin-induced diabetic and control rats. Among n-6 fatty acids from diabetic rat plasma, increased proportions of 18:2n-6 and 20:3n-6 in all three lipid classes and of 18:3n-6 in PL at 1–3 months old and in TG at 3–5 months old were observed. The proportions of 20:4n-6 decreased in both PL and CE, but were unchanged in diabetic TG. Among the n-3 fatty acids, in the early stage, diabetes caused increases in the proportions of 18:3n-3 in PL and CE and of 20:5n-3 and 22:6n-3 in TG, while 22:5n-3 was decreased later in the disease course. These results suggest reduced Δ5-desaturase activities on 20:3n-6 but not on 20:4n-3, while Δ6-desaturase activity on 18:2n-6 was essentially unaffected. Furthermore, the reduction in Δ9-desaturase activity in diabetic rats may well explain the decreases in the proportions of 16:1n-7 and 18:1n-7. However, the proportion of 18:1n-9, another product of Δ9-desaturase, was significantly increased in CE and PL as compared to the controls. Thus, there was a discrepancy between our results and those of earlier studies with respect to the n-9, n-6, and n-3 fatty acid proportions of plasma lipids in diabetic rats. We also investigated age-related changes in the proportions of plasma fatty acids. Although rather small, age-related changes were evident in both diabetic and control rats.

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Several studies have shown that changes in the fatty acid compositions of phospholipid (PL) are observed in both humans (1–4) and animals (5–18) with diabetes. These alterations in PL have been reported in a variety of tissues including the liver, heart, kidney, aorta, and plasma. Decreases in palmitoleate (16:1n-7) and arachidonate (20:4n-6) and increases in linoleate (18:2n-6) and eicosatrienoate (20:3n-6) are common in these tissues, due mainly to suppressed Δ9, -6, and -5-desaturase activities in diabetes. Δ6-Desaturase (E.C. 1.14.99.5), the enzyme considered to be responsible for the first and rate-limiting step in arachidonic acid synthesis, catalyzes the conversion of 18:2n-6 to 18:3n-6. Its activity has been found to decrease rapidly in relation to aging in the rat testes (6) and more slowly in the liver (19–21). The activities of Δ9, -6, and -5-desaturases have been shown to be depressed in hepatic microsomes of rats with experimentally induced diabetes (10,11,16–18). These previous findings were obtained mainly from PL of humans and rats with diabetes mellitus, and the present results indicate that these enzyme activities are not suppressed in all tissues. Furthermore, some desaturase activities are undetectable in tissues such as platelets, endothelial cells, and skin (22–24). Dang *et al*. (25) showed an alternative mechanism by which the fatty acids in erythrocyte membrane PL were changed *via* decreased incorporation of long fatty acids. On the other hand, the proportions of fatty acids in PL were directly affected by the activities of PL synthetic and degradative enzymes (26,27). Iritani *et al*. (28) reported that acyl-CoA synthetase in rat liver exhibited high selectivity for both n-3 and n-6 polyunsaturated fatty acids and that acyl-CoA:1-acylglycerol 3-phosphocholine acyltransferase showed acyl-donor selectivity in the order of 20:4n-6-CoA > 20:5n-3-CoA ≥ 22:6n-3-CoA. Acyl-CoA:1-acylglycerol 3-phosphate acyltransferase and/or 1-acylglycerol 3-phosphocholine acyltransferase activities were reported to be increased in the livers of rats with experimentally induced diabetes, while mitochondrial phospholipase A_2 and lysophospholipase activities were decreased in diabetic rats (29). Changes in the activities of these enzymes on PL also may contribute to the altered fatty acid composition of PL from diabetic animals.

Furthermore, in rats with experimental diabetes, elongation of fatty acid chains may be affected along with changes in desaturase activities, possibly accounting, in part, for the altered fatty acid composition. Triglycerides (TG) and cholesteryl esters (CE), as well as PL, are the major components of plasma lipoproteins. TG in chylomicrons and very low density lipoproteins are hydrolyzed to fatty acids and glycerol by lipoprotein lipase, an insulin-sensitive enzyme activated by the apoC-I protein on the lipoprotein surface. The deacylation and acylation cycles of TG are therefore one factor potentially affecting the fatty acid composition of plasma lipids. To date, little attention has been paid to the effects of the stage of diabetes or aging on changes in the proportions of fatty acids associated with diabetes.

This report describes diabetes-induced and age-related

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Abbreviations: CE, cholesteryl ester; HbA_1, hemoglobin A_1; PL, phospholipid; STZ, streptozotocin; TG, triglyceride; TLC, thin-layer chromatography.

changes in the fatty acid compositions of three lipid fractions, TG, PL and CE, in plasma of diabetic and control rats.

MATERIALS AND METHODS

Animals. Diabetes was induced in male Wistar rats (5-wk old, initial weight ~124 g; Sankyo Laboservice, Tokyo, Japan) by administering streptozotocin (STZ; Sigma, St. Louis, MO) into the tail vein at a dose of 45 mg/kg body weight (30). Control rats were injected with 50 mM citrate buffer vehicle (pH 4.5). After STZ-administration, the diabetic rats were allowed access to 5% (wt/vol) sucrose solution for the next 20 h. The diabetic and age-matched rats were divided into three groups each, which were then maintained for 1, 3, and 5 mon and were given *ad libitum* access to normal laboratory chow and water until sacrifice.

Plasma glucose level. The plasma glucose levels of the nonfasted rats were measured by the electrode method using Antsense (Miles-Sankyo, Tokyo, Japan). Only rats with nonfasted blood glucose levels of at least 20 mM for the duration of the experimental period were considered diabetic.

Lipid extraction and separation. After 1, 3, or 5 mon, rats were anesthetized, and blood was collected by cardiac puncture. Plasma was obtained by centrifugation at 1,000 × *g* for 10 min at 4°C. Lipids were extracted from the plasma (100 μL) by the method of Folch *et al*. (31), and redissolved in 100 μL of chloroform/methanol (2:1) containing 0.02% (wt/vol) butylhydroxytoluene. An aliquot (40 μL) of the lipid solution was applied on a silica gel G thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany) using petroleum ether/diethylether/acetic acid (82:28:1, by vol) as the solvent. Three fractions, CE, TG and PL, were separated and identified on the TLC plates using authentic standards, and were employed for the fatty acid analyses.

Fatty acid analysis. The silica gel containing the lipid on the TLC plates was scraped off and inserted into a screw-capped test tube for methylation. Direct methylation of the fatty acids was carried out in 5% HCl–methanol (Wako, Osaka, Japan) or 14% boron trifluoride–methanol (Tokyo Kasei, Tokyo, Japan) at 90°C for 1 h with 29.3 nmol of 23:0 as the internal standard. Benzene (1 mL) was added to the CE samples owing to the poor solubility of CE in methanol during methylation. The methyl esters thus formed were extracted with hexane, dried under a N_2 stream, and identified and quantified using a gas–liquid chromatograph (Shimadzu GC-15A; Kyoto, Japan) with an Omegawax™ 320 capillary column (0.32 mm × 30 m, Supelco; Bellefonte, PA). The column was isothermally programmed at a temperature of 60°C for the initial 3 min, followed by a subsequent increase of 7°C/min from 60 to 180°C, then 3°C/min to 220°C, and finally 1°C/min to 255°C. Helium gas was used as the carrier gas at an approximate flow rate of 40 mL/min at a split ratio of 1:50 at 3 min after the injection. The injector and detector temperatures were 240 and 250°C, respectively. The sample volume applied to the column was 2 μL. The peaks were identified by comparison of the retention times with the standards of the Supelco 37 component FAME Mix™ or by gas chromatography–mass spectrometry analysis (JMS-DX 300 mass spectrometer; Jeol, Tokyo, Japan). At least 26 fatty acid methyl esters from 12:0 to 22:6n-3 could be identified and quantified by comparison with the internal standard (see Tables 2, 3, and 4). One batch of the experimental chow contained 5% (w/w) total lipids with the following molar percentages of fatty acids: 12:0, 0.01%; 14:0, 0.47%; 16:0, 15.7%; 16:1n-7, 0.86%; 18:0, 3.03%; 18:1n-9, 22.8%; 18:2n-6, 50.3%; 18:3n-3, 3.3%; 20:0, 0.30%; 20:1n-9, 1.33%; 20:2n-6, 0.06%; 20:4n-6, 0.05%; 20:5n-3, 0.75%; 22:0, 0.27%; 22:1n-9, 0.07%; 22:2n-6, 0.02%; 24:0, 0.06%; and 22:6n-3, 0.47%.

Blood analysis. Washed red blood cells (80 μL) from the rats were lysed by the addition of 0.1 mL of 0.1%(wt/vol) octylphenoxypolyethoxyethanol. The relative amounts of glycated hemoglobin (HbA_1) were determined in duplicate or triplicate by the affinity chromatography method with an *m*-aminophenylboronic acid–agarose column (Pierce, Rockford, IL). Total cholesterol, TG, and PL in the plasma were quantified enzymatically using commercial kits (Wako, Osaka, Japan). Each sample was tested in duplicate.

Statistical analysis. Data are presented as means ± SE. Data from the diabetic and control groups were compared by analysis of variance, and $P < 0.05$ was considered statistically significant.

RESULTS

The STZ-administered rats showed severe hyperglycemia and significant increases in HbA_1 levels at 1, 3, and 5 mon (Table 1). TG, PL, and total cholesterol levels were significantly elevated in diabetic plasma compared to controls.

To analyze the fatty acid alterations in lipid fractions, the proportions of fatty acids in diabetic plasma were compared with those obtained from nondiabetic plasma (Tables 2, 3, and 4). Diabetes caused significant increases in the proportions of TG fatty acids, 18:2n-6 (5.7–14.7%) and 20:3n-6 (0.3–0.5%) at 1–5 mon, 22:6n-3 (~1.2%) at 1–3 mon, 18:3n-6 (~0.2%) at 3–5 mon, and 20:5n-3 (0.34%) at 3 mon. As some of these changes were high in percentage but small in magnitude, the absolute changes are indicated in parentheses. Decreased proportions of fatty acids as compared to controls were found in diabetic rats as follows: 16:1n-7 [–(1.3–3.2%)], 18:1n-7 [–(0.5–1.8%)], 22:3n-6 [–(0.13–0.16%)], and 22:4n-6 [–(0.04–0.05%)] at 1–5 mon; 12:0 [–(0.4–0.5%)], 14:0 [–(0.8–1.2%)], 16:0 [–(2.6–3.4%)], 16:1n-9 [–(0.3–0.5%)], and 18:1n-9 [–(2.9–3.5%)] at 1–3 mon; 22:5n-3 [–(0.4–0.6%)] at 3–5 mon; 10:0 (–0.3%), 20:2n-6 (–1.9%), and 24:0 (–0.3%) at 1 mon (Table 2). In diabetic PL, the proportions of 18:2n-6 (7–8%) and 20:3n-6 (0.4–0.7%) at 1–5 mon; 18:3n-6 (~0.08%, 1–3 mon), 18:3n-3 (0.06%, 1 mon), 22:4n-6 (0.13%, 3 mon), and 18:1n-9 (0.6%, 5 mon) were significantly increased (Table 3). The following fatty acids were significantly decreased as compared to the controls: 16:1n-7 [–(0.4–0.6%)] and 20:4n-6 [–(3.2–5.5%)] at 1–5 mon; 14:0 (–0.4%) and 20:2n-6 [–(0.8–1.2%)] at 1–3

TABLE 1
Comparison of the Levels of Blood Glucose, HbA_1 and Plasma Lipid Concentrations in STZ-Induced Diabetic and Control Rats[a]

		1 Mon		3 Mon		5 Mon	
Blood glucose	Control	6.84 ± 0.54	(n = 5)	6.46 ± 0.37	(n = 6)	6.30 ± 0.56	(n = 7)
(mM)	Diabetic	33.5 ± 1.1 $	(n = 6)	35.5 ± 3.2 $	(n = 5)	30.4 ± 3.1 $	(n = 7)
HbA_1	Control	3.28 ± 0.30	(n = 5)	3.04 ± 0.09	(n = 6)	2.82 ± 0.13	(n = 7)
(%)	Diabetic	11.9 ± 0.57 #	(n = 6)	14.8 ± 0.47 #	(n = 5)	12.2 ± 0.56 #	(n = 7)
Triglyceride	Control	0.67 ± 0.14	(n = 5)	1.55 ± 0.27	(n = 6)	1.49 ± 0.25	(n = 7)
(mM)	Diabetic	3.20 ± 1.01	(n = 6)	7.29 ± 2.06 +	(n = 5)	7.85 ± 3.73 +	(n = 7)
Phospholipid	Control	1.29 ± 0.18	(n = 5)	1.62 ± 0.09	(n = 6)	1.77 ± 0.15	(n = 7)
(mM)	Diabetic	1.99 ± 0.48	(n = 6)	4.09 ± 0.69 +	(n = 5)	4.27 ± 1.36 *	(n = 7)
Total cholesterol	Control	1.74 ± 0.22	(n = 5)	1.54 ± 0.09	(n = 6)	2.07 ± 0.19	(n = 7)
(mM)	Diabetic	3.04 ± 0.40	(n = 6)	6.16 ± 1.88 #	(n = 5)	3.69 ± 0.65	(n = 7)

[a]Rats (124 ± 1 g initial weight) were injected (i.v.) with streptozotocin (STZ) at a dose of 45 mg/kg body weight. Values are expressed as means ± SE. Measurement of hemoglobin A_1 (HbA_1) and blood chemistry analyses were performed in duplicate on number of rats, as indicated in parentheses. #, $P < 0.001$; $, $P < 0.005$; *, $P < 0.01$; +, $P < 0.05$ vs. control.

mon; 18:1n-7 [–(1.5–1.8%), 3–5 mon]; 16:1n-9 (–0.2%) and 22:3n-6 (–0.13%) at 1 mon; 20:1n-9 (–0.14%) at 3 mon; 22:1n-9 (–0.18%), 22:4n-6 (–0.13%), 22:5n-3 (–0.1%), and 24:0 (–0.13%) at 5 mon. In diabetic CE, the most striking increases in the proportions of fatty acids were as follows: 18:2n-6 (12–16%), 20:1n-9 (0.04–0.11%), and 20:3n-6 (0.17–0.31%) at 1–5 mon; 18:1n-9 (4.3–4.6%) and 18:3n-3 (0.33–0.44%) at 1–3 mo; 18:0 (4.4%) and 20:0 (0.3%) at 1 mon, while fatty acids 16:1n-7 [–(1.2–2.2%)] and 20:4n-6 [–(12–17%)] at 1–5 mon; 12:0 [–(0.8–0.9%)] at 1–3 mon; 10:0 [–(0.3–0.5%)], 14:0 [–(0.4–0.6%)], 18:1n-7 [–(0.4–0.6%)], and 22:5n-3 [–(0.03–0.08%)] at 3–5 mon; 18:3n-6 (–0.2%), 22:0 (–0.1%), and 24:0 (–0.2%) at 1 mon; 20:2n-6 (–1.2%) at 3 mon were decreased (Table 4).

Figure 1 shows changes in the fatty acid profiles of the three lipid fractions from diabetic vs. control rats. Among the n-6 fatty acids (Fig. 1A–C), significant increases in the proportions of 18:2n-6 and 20:3n-6 were observed in all lipid fractions from the diabetic animals at all time points. The proportions of 18:3n-6, the product of Δ6-desaturase from 18:2n-6, showed complex changes in the diabetic rats: significant increases in PL at 1–3 mon and TG at 3–5 mon and a decrease in CE at 1 mon. In PL and CE, 20:4n-6 was significantly reduced over the entire study period, suggesting Δ5-desaturase activity to be reduced in diabetic rats. However, 20:4n-6 was unchanged in TG. The proportion of fatty acid 22:4n-6 in diabetic TG was clearly decreased at all time points compared to that in control rats, while that in PL was increased at 3 mon and then decreased at 5 mon. Thus, profiles of the n-6 fatty acids in PL and CE were similar except for 18:3n-6 at 1 mon.

Among the n-3 fatty acids (Fig. 1D–F), the proportion of initial precursor, 18:3n-3, was significantly higher in diabetic CE (1–3 mon) and PL (1 mon) than in controls, while there was no significant change in TG. The fatty acids 18:4n-3 and 20:4n-3 were not detected in any lipid fractions examined. The acid 20:5n-3, the product of Δ5-desaturase, was slightly but significantly increased in diabetic TG at 3 mon, but no significant changes were observed at other time points in TG or the other lipids as compared to the controls. The proportions of 22:5n-3 were decreased in the late stage of diabetes in all three lipids. The acid 22:6n-3 was significantly increased in TG (1–3 mon), while there were no significant changes in PL and CE as compared to controls.

Among the n-7 fatty acids, 16:1n-7 was significantly reduced in all fractions (Fig. 1G–I), suggesting reduced Δ9-desaturase activity in diabetic rats. The elongation product of 16:1n-7, 18:1n-7 was decreased in all three lipid classes at 3–5 mon.

Among the n-9 metabolites, a large increase in 18:0 was observed only in the diabetic CE at 1 mon. Owing to the high percentage of 18:1n-9 (23%) in the diet, the proportions of 18:1n-9 in TG from both groups were higher (15–20%) than those in other lipids. However, 18:1n-9 was decreased in TG from diabetic rats (1–3 mon), despite polyphagia, as compared to controls. On the contrary, significant increases in the proportions of 18:1n-9 in CE (1–3 mon) and PL (5 mon) were observed. The proportion of 20:1n-9, the elongation product of 18:1n-9, was significantly increased in CE but reduced in PL of diabetic rats at 3 mon (Table 4). The acid 16:1n-9 was significantly decreased in diabetic TG (1–3 mon) and PL (1 mon) as compared to controls, whereas CE showed no significant change. The longer-chain fatty acid 22:1n-9 did not differ markedly between the two groups (TG and CE).

Significant age-related decreases in the proportions of the following fatty acids were seen in control TG: 10:0, 12:0, 14:0, 16:0, 16:1n-9, 18:0, and 24:0, while there were significant increases in 18:2n-6, 18:3n-3, 22:1n-9, 22:5n-3, and 22:6n-3 (Table 2). In diabetic TG, significant age-related increases were observed in n-3 and particularly n-9 fatty acids, specifically, 18:1n-9, 18:3n-3, and 22:1n-9. The proportions of n-3 fatty acids were not increased in control TG, while those of 18:0, 20:4n-6, 22:5n-3, and 22:6n-3 were significantly decreased. The control PL showed significant age-related reductions in 16:0, 16:1n-9, 18:0, 20:2n-6, and 22:3n-6 and significant increases in 18:1n-7, 18:2n-6, 18:3n-6, 20:1n-9, 20:4n-6, 20:5n-3, and 22:1n-9 (Table 3). In diabetic PL, significant age-related reductions were seen in 18:0, 20:0, and 22:5n-3, significant increases in 18:1n-9, 18:2n-6, 20:1n-9, 20:5n-3, and 22:4n-6. The diabetic CE showed age-

TABLE 2
Fatty Acid Profile of Plasma Triglycerides in Diabetes Mellitus (DM) and Control Rats[a]

	1 Mon			3 Mon			5 Mon		
	Control (n = 5)	DM (n = 6)	Change (%)	Control (n = 6)	DM (n = 5)	Change (%)	Control (n = 7)	DM (n = 7)	Change (%)
10:0	0.40 ± 0.14	0.09 ± 0.02	−77 #	0.18 ± 0.00 b	0.06 ± 0.02	−65	0.12 ± 0.06 a	0.05 ± 0.02	−54
12:0	0.92 ± 0.21	0.37 ± 0.05	−60 $	0.67 ± 0.03	0.27 ± 0.06	−60 *	0.36 ± 0.14 b	0.26 ± 0.08	−28
14:0	1.82 ± 0.29	0.63 ± 0.03	−65 #	1.38 ± 0.06 b	0.57 ± 0.06	−59 #	0.83 ± 0.09 a,e	0.65 ± 0.03	−22
16:0	27.58 ± 1.22	25.01 ± 0.65	−9 +	26.67 ± 0.69	23.31 ± 0.96	−13 $	22.80 ± 0.88 a,f	23.55 ± 0.42	
16:1n-9	0.92 ± 0.05	0.42 ± 0.02	−54 #	0.85 ± 0.10	0.55 ± 0.07	−36 #	0.73 ± 0.05 d	0.64 ± 0.05	−13
16:1n-7	2.75 ± 0.27	0.61 ± 0.12	−78 #	3.87 ± 0.50 c	0.66 ± 0.06	−83 #	2.21 ± 0.29 e	0.89 ± 0.09	−60 #
18:0	9.41 ± 3.06	7.00 ± 1.00	−26	3.63 ± 0.33 a	4.75 ± 0.15	31	4.20 ± 0.36 a	4.42 ± 0.41 d	
18:1n-9	18.86 ± 1.52	15.38 ± 1.04	−18 *	20.79 ± 0.31	17.89 ± 0.60 d	−14 *	20.94 ± 0.63	19.48 ± 0.89 e	
18:1n-7	2.22 ± 0.31	1.69 ± 0.10	−24 +	3.34 ± 0.18 a	1.57 ± 0.04	−53 #	2.66 ± 0.19 f	1.60 ± 0.07	−40 #
18:2n-6	22.86 ± 2.19	37.60 ± 1.10	64 #	28.28 ± 0.40 a	39.11 ± 0.74	38 #	32.31 ± 1.04 a,g	38.02 ± 0.42	18 #
18:3n-6	0.38 ± 0.06	0.31 ± 0.03	−18	0.19 ± 0.03 d	0.44 ± 0.06	126 $	0.27 ± 0.02	0.47 ± 0.09	74 +
18:3n-3	1.09 ± 0.15	1.15 ± 0.25		1.71 ± 0.04 c	1.72 ± 0.13 c		1.80 ± 0.13 b	1.91 ± 0.16 a	
20:0	0.30 ± 0.06	0.24 ± 0.02	−18	0.17 ± 0.02 a	0.18 ± 0.02		0.24 ± 0.02 h	0.22 ± 0.01	
20:1n-9	0.36 ± 0.08	0.28 ± 0.03	−22	0.23 ± 0.02 b	0.30 ± 0.04	27	0.30 ± 0.03	0.28 ± 0.02	
20:2n-6	2.50 ± 0.79	0.62 ± 0.16	−75 #	0.89 ± 0.28 b	0.49 ± 0.20	−45	1.58 ± 0.35	0.99 ± 0.32	−38
20:3n-6	0.19 ± 0.05	0.64 ± 0.12	237 #	0.16 ± 0.02	0.58 ± 0.05	262 #	0.22 ± 0.02	0.51 ± 0.08	132 #
20:4n-6	2.13 ± 0.42	1.96 ± 0.45		1.16 ± 0.14 d	1.02 ± 0.14 d	−12	1.66 ± 0.21	1.24 ± 0.17 d	−26
20:5n-3	1.48 ± 0.20	1.54 ± 0.15		1.42 ± 0.09	1.76 ± 0.08	24 +	1.68 ± 0.14 h	1.57 ± 0.06	
22:0	0.10 ± 0.02	0.11 ± 0.02		0.08 ± 0.01	0.07 ± 0.01 d		0.12 ± 0.01 f	0.10 ± 0.00	−24
22:1n-9	0.05 ± 0.01	0.05 ± 0.01		0.04 ± 0.00	0.05 ± 0.01	32	0.12 ± 0.02 a,e	0.11 ± 0.02 a,f	−11
22:2	0.65 ± 0.31	0.29 ± 0.15	−55	0.18 ± 0.11	0.15 ± 0.09	−19	0.25 ± 0.13	0.08 ± 0.01	−70
22:3n-6	0.21 ± 0.00	0.09 ± 0.02	−59 *	0.22 ± 0.03	0.06 ± 0.01	−73 #	0.23 ± 0.03	0.07 ± 0.01	−69 #
22:4n-6	0.10 ± 0.05	0.05 ± 0.00	−49 +	0.08 ± 0.01	0.03 ± 0.00	−59 +	0.08 ± 0.02	0.03 ± 0.01	−57 *
22:5n-3	0.84 ± 0.19	0.70 ± 0.06	−16	1.21 ± 0.06 b	0.79 ± 0.09	−35 #	1.16 ± 0.09 c	0.56 ± 0.05 h	−52 #
24:0	0.38 ± 0.12	0.08 ± 0.01	−78 *	0.10 ± 0.02 b	0.06 ± 0.01	−32	0.14 ± 0.02 c	0.08 ± 0.01	−43
22:6n-3	1.90 ± 0.47	3.06 ± 0.34	62 *	2.50 ± 0.13	3.56 ± 0.37	42 *	2.97 ± 0.25 c	2.22 ± 0.21 f	−25
Total	100.0	100.0		100.0	100.0		100.0	100.0	
Saturated acids	40.91	33.54	−18	32.87	29.28	−11	28.82	29.33	
Unsaturated acids	59.50	66.46	12	67.13	70.72		71.18	70.67	
Monoenoic acids	25.17	18.43	−27	29.12	21.01	−28	26.95	23.00	−15
Polyenoic acids	34.33	48.03	40	38.00	49.71	31	44.23	47.68	
Concentration in plasma (mM)	1.48 ± 0.46	7.75 ± 2.81	424	3.96 ± 0.88	15.42 ± 3.85	289	3.62 ± 1.06	20.95 ± 12.2	479

[a]Data are expressed as molar percentages of each fatty acid over total fatty acids identified. Values are means ± SE. The numbers of rats are shown in parentheses. Fatty acids from plasma triglycerides obtained by thin-layer chromatography (TLC) were methylated with 1 mL of HCl–methanol as described in the Materials and Methods section. Individual fatty acids were identified and quantified by gas chromatography. Fatty acid concentrations were calculated by the addition of molar amounts of individual fatty acids. #, $P < 0.001$; $, $P < 0.005$; *, $P < 0.01$; +, $P < 0.05$, significantly different from corresponding control rats. a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.01$; d, $P < 0.05$, significantly different from corresponding 1-mon-diabetic rats. e, $P < 0.001$; f, $P < 0.005$; g, $P < 0.01$; h, $P < 0.05$, significantly different from corresponding 3-mon-diabetic rats.

TABLE 3
Fatty Acid Profile of Plasma Phospholipids in DM and Control Rats[a]

	1 Mon			3 Mon			5 Mon		
	Control (n = 5)	DM (n = 6)	Change (%)	Control (n = 6)	DM (n = 5)	Change (%)	Control (n = 7)	DM (n = 7)	Change (%)
10:0	0.10 ± 0.03	0.07 ± 0.02	–29	0.08 ± 0.02	0.04 ± 0.01	–48	0.10 ± 0.01	0.06 ± 0.02	–39
12:0	0.17 ± 0.01	0.22 ± 0.08	28	0.21 ± 0.04	0.10 ± 0.03	–50	0.29 ± 0.05	0.17 ± 0.05	–40
14:0	0.71 ± 0.05	0.35 ± 0.07	–51 +	0.67 ± 0.19	0.31 ± 0.09	–54 *	0.48 ± 0.03	0.33 ± 0.04	–32
16:0	32.66 ± 1.12	30.49 ± 1.08		29.87 ± 0.66 d	28.76 ± 0.76		29.76 ± 0.46 d	28.99 ± 0.88	
16:1n-9	0.46 ± 0.05	0.24 ± 0.03	–47 #	0.35 ± 0.04 d	0.32 ± 0.04		0.22 ± 0.03 a	0.31 ± 0.03	39
16:1n-7	0.63 ± 0.09	0.22 ± 0.04	–65 +	1.05 ± 0.07 d	0.46 ± 0.24	–56 $	0.61 ± 0.03 h	0.25 ± 0.02	–59 *
18:0	23.81 ± 0.57	23.63 ± 1.30		20.57 ± 0.70 d	21.04 ± 0.79 d		19.83 ± 1.12 b	19.60 ± 0.54 a	
18:1n-9	4.41 ± 0.14	4.58 ± 0.11		4.24 ± 0.11	4.65 ± 0.06	10	4.33 ± 0.24	4.95 ± 0.13 d	15 $
18:1n-7	2.17 ± 0.21	1.28 ± 0.03	–41	3.63 ± 0.35 d	1.79 ± 0.57	–51 $	3.13 ± 0.26 d	1.60 ± 0.15	–49 #
18:2n-6	18.74 ± 0.77	26.79 ± 1.42	43 +	22.18 ± 0.99 d	29.45 ± 2.08 d	33 *	22.16 ± 1.04	29.32 ± 1.49 d	32 #
18:3n-6	0.22 ± 0.02	0.30 ± 0.02	36 $	0.22 ± 0.01	0.29 ± 0.02	31 $	0.28 ± 0.01 b	0.29 ± 0.01	
18:3n-3	0.09 ± 0.02	0.15 ± 0.02	61 +	0.06 ± 0.01	0.19 ± 0.05	212	0.11 ± 0.01	0.15 ± 0.01	31
20:0	0.25 ± 0.01	0.26 ± 0.02		0.21 ± 0.01	0.20 ± 0.03 d		0.21 ± 0.01	0.21 ± 0.01 d	
20:1n-9	0.23 ± 0.05	0.18 ± 0.01	–23	0.34 ± 0.02 d	0.20 ± 0.03	–40 $	0.36 ± 0.04 b	0.31 ± 0.03 c,h	–15
20:2n-6	1.70 ± 0.47	0.51 ± 0.12	–70 #	1.32 ± 0.28	0.52 ± 0.10	–61 +	0.89 ± 0.15 d	0.77 ± 0.17	–13
20:3n-6	0.48 ± 0.07	1.18 ± 0.14	148 #	0.65 ± 0.08	1.08 ± 0.18	68 +	0.67 ± 0.04	1.05 ± 0.13	58 *
20:4n-6	8.40 ± 0.95	5.21 ± 0.63	–38 $	9.77 ± 0.36 b	5.41 ± 0.47	–45 #	11.39 ± 0.76 h	5.89 ± 0.66	–48 #
20:5n-3	0.36 ± 0.06	0.35 ± 0.04		0.52 ± 0.05 d	0.65 ± 0.09 a	26	0.55 ± 0.04 d	0.60 ± 0.04 b	
22:0	0.27 ± 0.05	0.24 ± 0.02	–11	0.25 ± 0.02	0.24 ± 0.02		0.29 ± 0.02	0.25 ± 0.02	–13
22:1n-9	0.07 ± 0.02	0.07 ± 0.03		0.06 ± 0.01	0.07 ± 0.02		0.26 ± 0.13 d,h	0.08 ± 0.01	–71 +
22:2	0.09 ± 0.02	0.05 ± 0.02	–38	0.09 ± 0.04	0.03 ± 0.03	–67	0.01 ± 0.01	0.00 ± 0.00	–100
22:3n-6	0.19 ± 0.10	0.06 ± 0.01	–70 *	0.07 ± 0.01 d	0.04 ± 0.01	–45	0.09 ± 0.01 d	0.05 ± 0.01	–41
22:4n-6	0.34 ± 0.05	0.30 ± 0.06	–10	0.22 ± 0.02 d	0.35 ± 0.03	58 +	0.60 ± 0.05 a,e	0.47 ± 0.04 d	–22 +
22:5n-3	0.39 ± 0.03	0.37 ± 0.03	–5	0.39 ± 0.04	0.36 ± 0.03		0.38 ± 0.02	0.27 ± 0.02 c,h	–27 $
24:0	0.54 ± 0.12	0.44 ± 0.04	–19	0.48 ± 0.04	0.45 ± 0.04		0.60 ± 0.03	0.47 ± 0.03	–22 +
22:6n-3	2.54 ± 0.13	2.52 ± 0.18		2.45 ± 0.21	3.03 ± 0.42	23	2.42 ± 0.26	2.26 ± 0.38	
Total	100.0	100.0		100.0	100.0		100.0	100.0	
Saturated acids	58.53	55.67		52.34	51.13		51.56	50.08	
Unsaturated acids	41.51	44.35		47.62	48.89		48.44	48.63	
Monoenoic acids	7.98	6.56	–18	9.68	7.49	–23	8.91	7.50	–16
Polyenoic acids	33.53	37.79	13	37.95	41.40		39.53	41.13	
Concentration in plasma (mM)	3.04 ± 1.90	3.78 ± 0.51	24	3.38 ± 0.51	7.55 ± 2.05	123	3.58 ± 0.53	7.74 ± 2.32	116

[a]Data are expressed as molar percentages of each fatty acid over total fatty acids identified. Values are means ± SE. The numbers of rats are shown in parentheses. Fatty acids from plasma phospholipids obtained by TLC were methylated with 1 mL of HCl–methanol as described in the Materials and Methods section. Individual fatty acids were identified and quantified by gas chromatography. Fatty acid concentrations were calculated by the addition of molar amounts of individual fatty acids. #, $P < 0.001$; $, $P < 0.005$; *, $P < 0.01$; +, $P < 0.05$, significantly different from corresponding control rats. a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.01$; d, $P < 0.05$, significantly different from corresponding 1-mon-diabetic rats. e, $P < 0.001$; f, $P < 0.005$; g, $P < 0.01$; h, $P < 0.05$, significantly different from corresponding 3-mon-diabetic rats. See Table 2 for abbreviations.

TABLE 4
Fatty Acid Profile of Plasma Cholesteryl Esters in DM and Control Rats[a]

	1 Mon			3 Mon			5 Mon		
	Control (*n* = 5)	DM (*n* = 6)	Change (%)	Control (*n* = 6)	DM (*n* = 5)	Change (%)	Control (*n* = 7)	DM (*n* = 7)	Change (%)
10:0	0.67 ± 0.08	0.51 ± 0.05	−24	0.89 ± 0.08	0.41 ± 0.13	−54 *	0.81 ± 0.17	0.49 ± 0.11	−40 +
12:0	1.78 ± 0.10	0.96 ± 0.13	−46 *	1.87 ± 0.21	1.01 ± 0.37	−46 $	1.15 ± 0.16 d,g	1.06 ± 0.20	
14:0	1.22 ± 0.09	0.83 ± 0.17	−32	1.31 ± 0.11	0.69 ± 0.17	−47 $	1.06 ± 0.19	0.68 ± 0.11	−36 +
16:0	12.22 ± 0.27	13.84 ± 1.21	13	14.31 ± 0.40	15.03 ± 0.70		13.24 ± 0.37	14.01 ± 1.14	
16:1n-9	1.12 ± 0.08	0.93 ± 0.25	−17	1.29 ± 0.15	0.95 ± 0.23	−26	0.97 ± 0.11	0.90 ± 0.10	
16:1n-7	2.67 ± 0.18	1.44 ± 0.39	−46 +	4.09 ± 0.63 d	1.89 ± 0.65	−54 #	2.47 ± 0.29 f	1.14 ± 0.12	−54 *
18:0	2.76 ± 0.28	7.14 ± 1.79	159 #	5.36 ± 0.49 d	4.48 ± 0.63 d	−17	4.49 ± 0.54	3.14 ± 0.56 a	−30
18:1n-9	8.49 ± 0.57	13.10 ± 1.12	54 +	8.48 ± 0.82	12.80 ± 3.70	51 +	10.19 ± 0.89	11.76 ± 1.14	15
18:1n-7	1.02 ± 0.09	1.01 ± 0.04		1.40 ± 0.10 b	0.83 ± 0.08	−41 #	1.26 ± 0.07 d	0.87 ± 0.05	−31 #
18:2n-6	25.76 ± 1.28	37.63 ± 2.34	46 #	25.82 ± 0.59	41.14 ± 2.23	63 #	25.75 ± 1.11	38.48 ± 2.62	49 #
18:3n-6	0.48 ± 0.12	0.31 ± 0.02	−35 +	0.37 ± 0.02	0.51 ± 0.04 d	37	0.46 ± 0.03	0.54 ± 0.05 b	19
18:3n-3	0.29 ± 0.05	0.62 ± 0.13	116 +	0.40 ± 0.03	0.82 ± 0.21	106 $	0.44 ± 0.04	0.55 ± 0.05 h	27
20:0	0.07 ± 0.00	0.36 ± 0.11	385 #	0.15 ± 0.02	0.12 ± 0.02 a	−18	0.12 ± 0.01	0.10 ± 0.01 a	−20
20:1n-9	0.05 ± 0.01	0.16 ± 0.04	203 #	0.05 ± 0.01	0.11 ± 0.02 d	98 +	0.05 ± 0.01	0.10 ± 0.02 b	77 +
20:2n-6	2.15 ± 0.43	1.06 ± 0.29	−51	1.67 ± 0.53	0.51 ± 0.13	−69 +	1.21 ± 0.41	1.59 ± 0.43	32
20:3n-6	0.24 ± 0.03	0.54 ± 0.11	129 #	0.24 ± 0.03	0.41 ± 0.06	73 +	0.27 ± 0.02	0.44 ± 0.06	67 *
20:4n-6	33.18 ± 2.13	15.84 ± 2.30	−52 #	28.79 ± 1.49	13.22 ± 3.02	−54 #	32.79 ± 2.43	20.51 ± 3.59 h	−37 #
20:5n-3	2.48 ± 0.45	2.22 ± 0.30	−10	2.47 ± 0.17	2.62 ± 0.30		2.20 ± 0.15	2.43 ± 0.26	10
22:0	0.20 ± 0.06	0.09 ± 0.01	−53 *	0.07 ± 0.01 a	0.04 ± 0.01	−44	0.07 ± 0.00 a	0.06 ± 0.01	−11
22:1n-9	0.06 ± 0.02	0.05 ± 0.01	−26	0.05 ± 0.01	0.02 ± 0.00	−46	0.04 ± 0.01	0.04 ± 0.01	
22:2	0.25 ± 0.15	0.19 ± 0.06	−24	0.07 ± 0.01	0.43 ± 0.39	495	0.15 ± 0.06	0.30 ± 0.18	109
22:3n-6	0.05 ± 0.01	0.03 ± 0.01	−38	0.04 ± 0.01	0.00 ± 0.00	−100	0.00 ± 0.00	0.00 ± 0.00	
22:4n-6	0.41 ± 0.37	0.05 ± 0.01	−88	0.02 ± 0.01	0.03 ± 0.02	22	0.00 ± 0.00	0.00 ± 0.00	
22:5n-3	0.03 ± 0.00	0.03 ± 0.01		0.07 ± 0.02 d	0.04 ± 0.01	−46 +	0.11 ± 0.02 b	0.03 ± 0.00	−74 $
24:0	0.34 ± 0.16	0.14 ± 0.05	−58 +	0.19 ± 0.05	0.13 ± 0.04	−31	0.24 ± 0.08	0.17 ± 0.03	−29
22:6n-3	1.11 ± 0.19	1.00 ± 0.07	−10	0.54 ± 0.03 a	0.79 ± 0.08	47	0.65 ± 0.09 b	0.67 ± 0.09 d	
Total	100.0	100.0		100.0	100.0		100.0	100.0	
Saturated acids	19.26	23.87	24	24.15	21.91		21.20	19.70	
Unsaturated acids	82.67	76.36		75.86	78.12		78.99	80.36	
Monoenoic acids	13.41	16.68	24	15.36	16.60		14.98	14.81	
Polyenoic acids	69.26	59.68	−14	60.51	61.52		64.00	65.55	
Concentration in plasma (mM)	1.13 ± 0.16	2.07 ± 0.35	83	1.59 ± 0.20	5.34 ± 1.71	236	1.84 ± 0.14	2.89 ± 0.81	57

[a]Data are expressed as molar percentages of each fatty acid over total fatty acids identified. Values are means ± SE. The numbers of rats are shown in parentheses. Fatty acids from plasma cholesteryl esters obtained by TLC were methylated with 1 mL of HCl–methanol as described in the Materials and Methods section. Individual fatty acids were identified and quantified by gas chromatography. Fatty acid concentrations were calculated by the addition of molar amounts of individual fatty acids. #, $P < 0.001$; $, $P < 0.005$; *, $P < 0.01$; +, $P < 0.05$, significantly different from corresponding control rats. a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.01$; d, $P < 0.05$, significantly different from corresponding 1-mon-diabetic rats. e, $P < 0.001$; f, $P < 0.005$; g, $P < 0.01$; h, $P < 0.05$, significantly different from corresponding 3-mon-diabetic rats. See Table 2 for abbreviations.

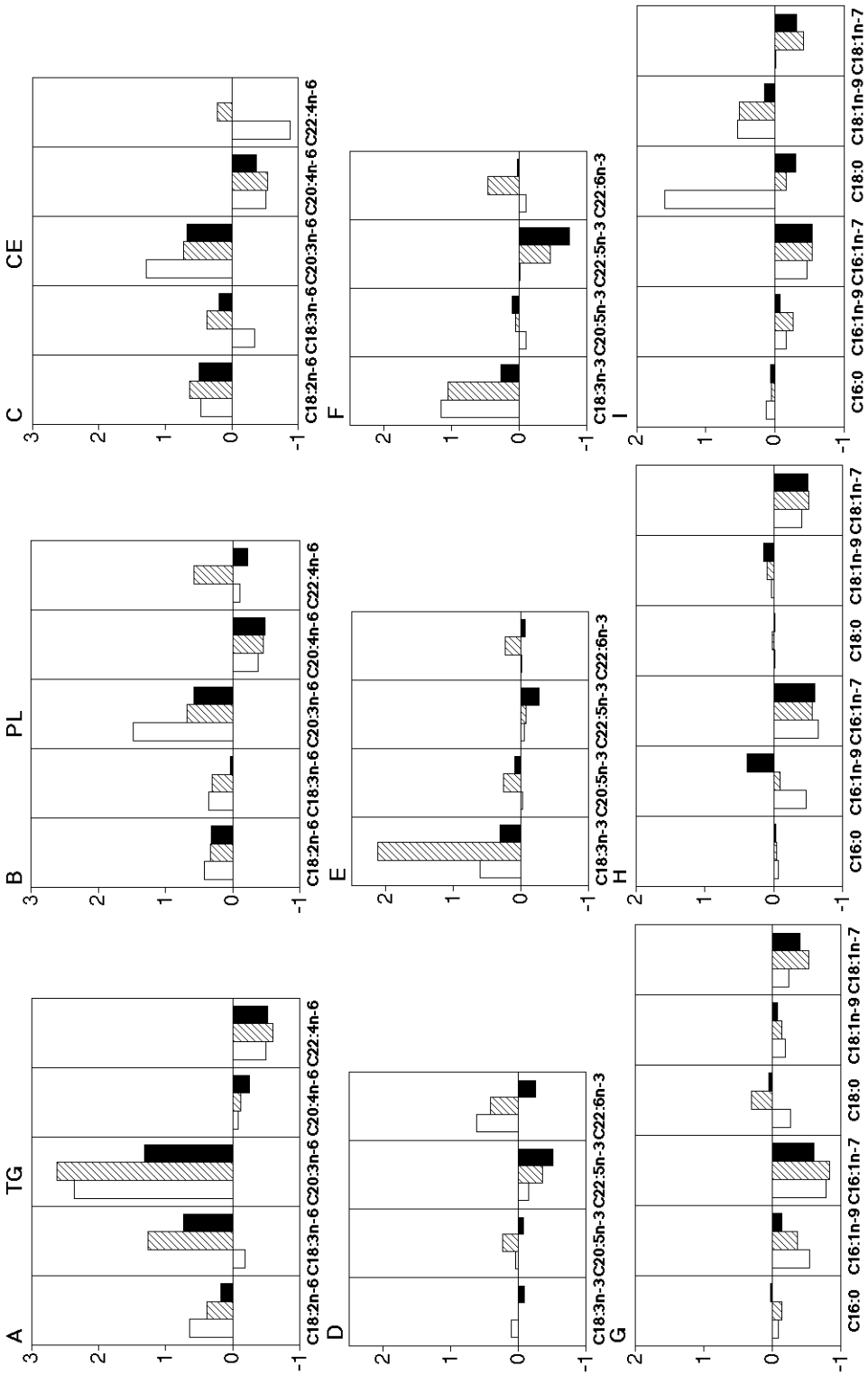

FIG. 1. Polyunsaturated fatty acid profiles of plasma triglycerides (TG) (A, D, and G), phospholipids (PL) (B, E, and H), and cholesteryl esters (CE) (C, F, and I) of diabetic rats compared to those of nondiabetic controls. Data are represented as ratios of the proportions of fatty acids in plasma lipid fractions from diabetic and control rats. Open, hatched, and solid bars refer to the ratios of fatty acid proportions at 1, 3, and 5 mon, respectively.

related reductions in 18:0, 20:0, 20:1n-9, and 22:6n-3, while 18:3n-6 and 20:4n-6 were increased. In control CE, 12:0, 22:0, and 22:6n-3 increased, while 18:1n-7 and 22:5n-3 decreased with aging (Table 4). In the late stage of diabetes, the fatty acid proportions in CE exhibited complex changes not seen in controls; saturated fatty acids were increased at 1 mon and then decreased, while unsaturated fatty acids showed the opposite changes. Monounsaturated acids were elevated at 1 and 3 mon in diabetic rats.

DISCUSSION

We found that rats with STZ-induced diabetes showed characteristic alterations in the proportions of plasma lipid fatty acids comparable to those in the diabetic rat liver. The proportions of 18:2n-6 and 20:3n-6 in diabetic plasma were increased over the entire experimental period in all three lipids, while 16:1n-7 and 20:4n-6 were reduced as compared to the control rats. The levels of 18:3n-6 formed by the action of Δ6-desaturase on 18:2n-6 decreased significantly in essentially the same manner as in diabetic PL and CE, showing Δ6-desaturase activity to be decreased in diabetic rats. Alterations in the fatty acid proportions of microsomal PL in rats with STZ-induced diabetes have been thoroughly investigated and well described. Direct evidence of fatty acid changes was obtained from *in vitro* experiments in which rat hepatic microsomal Δ9-, Δ6-, and probably Δ5-desaturase activities were decreased (10,11,16–18). These decreases in enzyme activities led to increases in 18:2n-6, 18:3n-6, and 20:3n-6 and decreases in 16:1n-9 and 20:4n-6 in diabetic human and rat hepatic PL (1–15). However, the results of the present study apparently contradict these earlier observations. We found that while one of the Δ9-desaturase products, 16:1n-7, was significantly decreased in all three lipids in diabetic plasma (1–5 mon), another Δ9-desaturase product, 18:1n-9, in CE in the early stage (1–3 mon) and in PL in the late stage of diabetes (5 mon), was unexpectedly increased. These findings indicate a discrepancy between diminished Δ9-desaturase activity and an increased proportion of one of its products. Because the chow given to the experimental rats contained a rather small amount of 16:1n-7 and an abundance of 18:1n-9, despite the higher chow intake of 18:1n-9 in the diabetic rats, the 18:1n-9 change was found to be smaller in diabetic TG. Furthermore, 18:3n-6, a product of Δ6-desaturase, was decreased as expected in CE only at 1 mon while being unexpectedly increased in PL in the early stage and TG in the late stage of diabetes, as compared to controls. Dang *et al*. (9) observed that the proportions of fatty acids in lipids isolated from plasma and red cells of rats (more than 2 mon old) with diabetes (for 2–3 wk) were significantly altered, while those in platelets and apparently the aorta were unchanged until a later stage (6 wk). These authors reported a significant 20:4n-6 increase in TG and significant 20:4n-6 decreases in PL and CE in 2–3-wk diabetic rats. Thus, the proportion of 20:4n-6 in diabetic plasma TG is not apparently reduced compared to that of controls. However, the mechanism underlying the stability of these fatty acid levels in plasma TG is unclear.

Voss *et al*. (32) have proposed a novel pathway in rat hepatocytes for the synthesis of 22:6n-3, a route independent of Δ4-desaturase, which involves elongation of 22:5n-3 and Δ6-desaturation of its product followed by chain shortening *via* β-oxidation. Δ6-Desaturase is the rate-limiting enzyme for the synthesis of 20:4n-6 from 18:2n-6. It is noteworthy that, despite the significant decrease in the proportion of 22:5n-3 in all three lipid fractions during the late stage of diabetes, the proportion of 22:6n-3 was significantly increased in TG at 1–3 mon while being unchanged in CE and PL. Furthermore, neither the proposed Δ6-desaturase product, 24:6n-3, nor 24:5n-3 was detected in our study. Δ5-Desaturase activities on the n-6 fatty acid 20:3n-6, but not the n-3 fatty acid 20:4n-3, were reduced. One possible explanation is that Δ5-desaturase exists as two distinct enzymes, as is the case with Δ9- and possibly Δ6-desaturase (33–35).

Elongation of 16:1n-7 to 18:1n-7 was significantly increased in CE and PL of diabetic animals. However, elongation of 18:1n-9 to 20:1n-9 was decreased in all three lipid fractions.

In diabetic rats, there was an overall decrease in the proportions of short-chain saturated fatty acids (10:0–16:0) as compared to controls. In TG, the proportions of 10:0, 12:0, 14:0, and 16:0 were reduced only in the early stage, whereas in CE, the proportions of 10:0 and 14:0 decreased in the late stage. Besides the saturated fatty acids discussed above, in control TG, the proportions of monounsaturated 16:1n-9 were age-dependently decreased, whereas the proportions of 18:2n-6 and 22:1n-9, and the n-3 fatty acids 18:3, 22:5 and 22:6, were increased. In diabetic TG, only the proportions of 18:0 and 20:4 showed age-related decreases, while 16:1n-9 and 18:1n-9 were increased, as were the fatty acids of which the proportions were increased in controls. In control PL, there were age-related decreases in the proportions of 16:0, 16:1n-9, 18:0, 20:2n-6, and 22:3n-6, the former three also being seen in control TG, and age-related increases in the proportions of 18:2n-6, 20:1n-9, and 20:5n-3 in both diabetic and control PL. In CE, only a few fatty acids showed age-related proportional changes.

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[Received July 8, 1997, and in final revised form and accepted January 26, 1998]

Effect of Continuous Enteral Medium-Chain Fatty Acid Infusion on Lipid Metabolism in Rats

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ABSTRACT: This study compared (i) the relative effects of long-chain triglycerides (LCT) and medium-chain triglycerides (MCT), (ii) the influence of amount of MCT, and (iii) the impact of medium-chain fatty acid position, on plasma and lymphatic triglycerides and portal vein free fatty acids. The animals were fed approximately at 250 kcal/kg · day for 20 h. The lymph from lymphatic duct and blood from portal vein and systemic circulation were collected. The results showed that feeding 100% MCT for 20 h was sufficiently long to reduce significantly the level of linoleic acid in portal vein fatty acids and plasma and lymph triglycerides. However, this alteration induced by MCT feeding was partially prevented by adding LCT to the diet. The level of arachidonic acid was significantly reduced in plasma triglycerides by any of the diets containing medium-chain fatty acids compared to 100% LCT. When feeding MCT only, palmitoleic acid, presumably reflecting *de novo* lipogenesis, was increased in lymphatic triglycerides and portal vein fatty acids. Total saturated fatty acids as a total percentage of total fatty acids were also significantly increased in plasma and lymphatic triglycerides and portal vein fatty acids. Thus, when linoleic acid is limiting, the conversion of MCT into long-chain fatty acids by *de novo* lipogenesis is likely to be an important metabolic route. Providing LCT with MCT or 2-monodecanoin appears to limit this pathway.

Lipids 33, 261–266 (1998).

It has long been known that the chain length and position of dietary fatty acids in the triglyceride structure are two important factors that influence lipid metabolism. For instance, medium-chain triglycerides (MCT) when consumed enterally are quickly hydrolyzed and rapidly absorbed into the intestinal cells, whereas long-chain triglycerides (LCT) are absorbed as free fatty acids and 2-monoglycerides after micellar formation (1–3). Moreover, after intestinal absorption, medium-chain fatty acids travel principally *via* the portal vein esterified to albumin, while LCT are largely reesterified in the intestinal cell into triglycerides by linking free fatty acids to the 2-monoglyceride and are incorporated into chylomicra to travel *via* the lymphatics into the circulation. MCT are also cleared more rapidly from the circulation compared to LCT. Given these metabolic differences, MCT can have a number of benefits in certain clinical situations.

For instance, a 50:50 physical mixture of LCT and MCT was found to be superior to 100% LCT emulsion owing to more rapid metabolic utilization and reduced deposition as fat in our previous animal study (4). Moreover, when subjects consumed a transesterified mixture of 50% MCT and 50% LCT as safflower oil, the mean serum cholesterol concentrations fell as compared to LCT alone (5). It appears that MCT or medium-chain fatty acids in the 2-position are favorable for displacing cholesterol-raising fatty acids, because it has been demonstrated that during reesterification the saturated long-chain fatty acids esterified to the 2-position raise cholesterol concentrations more than when esterified to the 1- or 3-position (5). Recent studies also demonstrate that MCT increase hepatic lipogenic enzyme activity in the liver (6) with the synthesis of saturated long-chain fatty acids when very large amounts of MCT are provided with very limited amounts of LCT or no LCT (7). Therefore, the various potential metabolic pathways of MCT are not completely known and could be influenced particularly by the amount of MCT feeding, the position of medium-chain fatty acids on the glycerol molecule, as well as the presence or absence of LCT.

The present study was designed to compare after enteral feeding: (i) the different effects of LCT and MCT when given alone; (ii) the influence of amount of isomolar mixture of MCT and LCT; and (iii) the impact of medium-chain fatty acid position on portal vein fatty acid and plasma and lymphatic triglyceride fatty acid composition in rats. L-Emental (Nutrition Medical, Buffalo, MN), a complete enteral nutrition formula, was used as the base feeding solution, because calories provided by fat are low (only 2.5%) in this diet. All the fat sources were added into the base feeding solution in isomolar amounts to reach total calories of approximately 250 kcal/kg · day with 25% of nonprotein from fat. Completely isocaloric comparison was not possible owing to the lower caloric content of medium-chain fatty acids and the presence of only one medium-chain fatty acid in 2-monodecanoin (2-mono). Animals were constantly fed for 20 h before blood samples were taken. The free fatty acids and triglyceride fatty acids were determined in the portal vein, lymphatic fluid, and plasma, respectively, to help define the effects of absorption pathway. The results suggest that by feeding MCT only, with

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Abbreviations: LCT, long-chain triglycerides; MCT, medium-chain triglycerides; 2-mono, 2-monodecanoin.

linoleic acid limiting, the conversion of MCT into long-chain fatty acids by *de novo* lipogenesis is likely to be an important metabolic route. Providing LCT with MCT or 2-mono appears to limit this pathway. This could be due either to greater amounts of linoleic acid or lesser amounts of medium-chain fatty acid or to the combination.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weight 280–300 g, were housed in stainless-steel wire-bottom cages and exposed to a 12-h light–dark cycle at ambient temperature for 5 d before experimentation. Animals were provided with standard laboratory chow and water *ad libitum* during this period. The experiment was approved by the Animal Care Committee of the Beth Israel Deaconess Medical Center, which follows guidelines prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the National Research Council.

Under ether anesthesia, a silicone catheter (0.025 mm i.d. × 0.047 mm o.d.; Silastic™, Dow-Corning Laboratories, Corning, NY) was inserted into the antrum of the stomach and advanced ~1 cm beyond the pylorus for enteral feeding. The catheters were tunneled subcutaneously and exteriorized at the midscapular region and connected to a flow-through swivel (Instech Laboratories, Philadelphia, PA) that allowed for uninterrupted infusion and free movement by the rats. Rats were housed individually in wire-bottomed cages during recovery and had free access to rat chow diet and tap water. Normal saline was infused at 2 mL/h through the catheter to maintain patency of the catheter.

After 24 h of recovery from surgery, the rats were randomly assigned to four different feeding groups. The L-Emental enteral nutrition (Nutrition Medical) was used as a base formula. The different fats were added to meet conditions of 25% nonprotein calories provided by fat source at approximate total of 250 kcal/kg · day. Four different lipids were included: a commercial sunflower oil (LCT group) (distributed by Sunfresh Inc., West Seneca, NY), a standard MCT mixture (Mead Johnson, Evansville, IN) (MCT group), an isomolar mixture of 50% LCT and 50% MCT (LCT/MCT group) and an isomolar mixture of 50% LCT and 50% 2-monodecanoic acid (Stepan Company, Maywood, NJ) (LCT/2-mono group). All the fat sources were in isomolar amounts based on the calculation from LCT. The fat was provided at approximately 0.29 g/h in LCT group, 0.30 g/h in MCT group, and 0.30 g/h in LCT/MCT and LCT/2-mono groups. Although the different fat sources were controlled at isomolar amounts, the caloric content per gram of fat in each diet was slightly different. All the animals were constantly fed with these diets for 20 h at 2.2 mL/h to reach a steady-fed state. At the end of feeding, under ether anesthesia all the animals had a lymph sample collected over one-half hour through silicone tubing inserted into the lymphatic duct while feeding was continued and a blood sample (1–1.5 mL) taken from the portal vein by direct venipuncture. During anesthesia, breathing and muscle tension were monitored. Finally, a systemic blood sample was collected at sacrifice. All the samples were stored under nitrogen at −20°C until assay to avoid oxidation of lipids.

Analysis. The components of plasma triglycerides or free fatty acids were extracted according to the method reported by Folch *et al.* (8) with modification. Systemic plasma (1 mL), portal plasma (0.5 mL), or lymph fluid (0.2 mL) was mixed with chloroform/methanol (2:1, vol/vol) solution and water (8 and 1 mL, respectively). Tritridecanoic acid or tridecanoic acid ($C_{13:0}$) (Sigma Chemical Co., St. Louis, MO) was added to each sample before lipid extraction. Triglyceride and free fatty acids were separated on thin-layer plates using a mobile phase of petroleum ether/diethyl ether/glacial acetic acid (80:20:1, by vol), and identified relative to the migration of standards using dichlorofluorescein spray. The triglyceride and free fatty acid bands were isolated, hydrolyzed, and methylated under nitrogen with 14% BF_3 in methanol for 45 min in a steam bath in a closed system. The fatty acid methyl esters were separated using a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA) after some evaporation of solvent to reduce the sample size. The column used was a 50-m fused-silica capillary column containing SP-2330 as the stationary phase with a 0.20-μm film thickness (Supelco, Bellefonte, PA). The relative mole percentage of individual fatty acids was identified and quantified using Chem Station software (Hewlett-Packard) based on the relative responses of an external standard of pure tridecanoic acid methyl esters (Nu-Chek-Prep, Elysian, MN). The recovery rate of known amounts (from approximately 0.005–0.04 μmol/mL) of 10:0 and 18:0 under our assay conditions was 86.5 ± 4.5% and 102.9 ± 4.8% (mean ± SEM), respectively.

Statistics. The data were presented as mean ± SEM. The significant comparison among four different groups was determined using one-way analysis of variance with LSD as the *post hoc* test. Significance was set at $P < 0.05$.

RESULTS

The free fatty acid profiles in portal vein plasma are listed in Table 1. No medium-chain free fatty acids were detected in the portal vein when feeding MCT-containing diets, including 100% MCT, 50% MCT, and 50% of 2-mono for 20 h, presumably owing to the continuous nature of the feeding. However, feeding with these three medium-chain fatty acid-containing diets significantly increased the amount of palmitoleic acid (16:1n-7) as compared to the LCT diet ($P < 0.01$). The greatest effect was observed in the MCT group, followed by LCT/2-mono and LCT/MCT groups. These changes in palmitoleic acid in the MCT group were associated with the greatest decreases in linoleic acid (18:2n-6) and arachidonic acid (20:4n-6). The total free fatty acids in the portal vein were higher but not significantly so in the MCT group compared to other groups.

Table 2 lists the changes in triglyceride fatty acids in the

TABLE 1
Free Fatty Acid Composition[a] in Portal Plasma

Fatty acids	LCT (5)	MCT (6)	LCT/MCT (6)	LCT/2-mono (7)
C12:0	5.37 ± 0.96	4.65 ± 1.38	4.87 ± 1.07	3.76 ± 0.62
C14:0	4.21 ± 0.35	4.88 ± 1.03	4.06 ± 0.59	5.09 ± 0.70
C16:0	33.56 ± 2.68	40.88 ± 3.25	34.48 ± 2.70	39.38 ± 2.58
C16:1n-7[b]	3.07 ± 0.73	13.82 ± 2.56[c]	5.75 ± 0.99[d]	10.89 ± 2.02[e]
C18:0	9.71 ± 0.47	10.35 ± 2.14	9.75 ± 1.33	9.34 ± 0.60
C18:1n-9	10.55 ± 2.68	13.44 ± 3.46	14.28 ± 2.93	12.54 ± 2.80
C18:2n-6[f]	26.31 ± 3.41	14.79 ± 1.49[g]	17.61 ± 2.56	23.85 ± 4.25
C20:4n-6[f]	4.23 ± 0.46	2.02 ± 0.50[g]	3.31 ± 0.60	4.57 ± 1.13
C22:6n-3	1.52 ± 0.01	1.01 ± 0.27	1.51 ± 0.06	Not detected
Total fat[h,i]	0.05 ± 0.01	0.11 ± 0.02	0.07 ± 0.01	0.07 ± 0.00

[a]Mean ± SEM: percentage calculated as (mol fatty acid/mol total fatty acid) × 100.
[b]$P < 0.01$, [f]$P = 0.05$, [h]$P = 0.07$ by one-way analysis of variance (ANOVA).
[c]$P < 0.05$ vs. LCT and LCT/MCT, [d]$P = 0.07$ vs. LCT/2-mono, [e]$P < 0.05$ vs. LCT.
[g]$P < 0.05$ vs. LCT and LCT/2-mono by least significant difference (LSD) test.
[i]Mean ± SEM: μmol/mL. LCT, long-chain triglycerides; MCT, medium-chain triglycerides; 2-mono, 2-monodecanoin.

lymph. Decanoic acid (10:0) was detected in all groups. Although substantially more 10:0 was found in medium-chain fatty acid-containing groups, there were statistically no significant differences among all groups owing to the great variability of the data. MCT feeding significantly increased the palmitoleic acid and oleic acid (18:1n-9) content compared to other groups ($P < 0.05$). In contrast, MCT feeding significantly decreased the content of linoleic acid. There were no differences in arachidonic acid, docosahexaenoic acid (22:6n-3) and total fat content among groups.

The changes in fatty acids in plasma triglycerides are listed in Table 3. No MCT was detected in the systemic circulation in any group. MCT feeding led to slight but not significant increases in palmitic acid (16:0), palmitoleic acid, and oleic acid (18:1n-9) as compared to other groups. The arachidonic acid content of triglycerides was significantly higher in the LCT group as compared to the three MCT groups, whereas linoleic acid was significantly different only from 100% MCT. The lowest linoleic acid content was in the MCT group, which was significantly lower than LCT/2-mono and marginally significant when compared to LCT/MCT. The total fatty acids were not significantly different among groups.

Figure 1 presents the percentage total saturated fatty acids of the total fatty acids in portal vein and lymph and plasma triglycerides. The saturated fatty acids (i.e., 12:0, 14:0, 16:0, and 18:0) in portal vein triglyceride were significantly higher in the MCT group compared to LCT and LCT/MCT groups. The total saturated fatty acids as a percentage of total fatty acids in the lymph triglyceride were significantly higher in the MCT group as compared to other groups. The total percentage of saturated fatty acid in plasma triglycerides was also significantly higher in the MCT group compared to other groups.

DISCUSSION

In the present study, feeding 100% MCT as the principal lipid source in the diet for 20 h significantly reduced the level of linoleic acid in plasma triglycerides in the systemic circulation

TABLE 2
Fatty Acid Composition in Lymph Triglyceride

Fatty acids[a]	LCT (5)	MCT (6)	LCT/MCT (6)	LCT/2-mono (7)
C10:0	0.03 ± 0.02	3.89 ± 1.98	1.08 ± 0.28	1.96 ± 0.96
C12:0	0.13 ± 0.04	0.34 ± 0.08	0.13 ± 0.01	0.29 ± 0.08
C14:0	0.33 ± 0.07	0.45 ± 0.08	0.39 ± 0.08	0.38 ± 0.10
C16:0	18.54 ± 2.27	18.22 ± 2.24	19.32 ± 2.55	18.28 ± 3.34
C16:1n-7[b]	0.87 ± 0.33	3.02 ± 0.84[c]	1.71 ± 0.32	1.00 ± 0.27
C18:0	5.80 ± 1.21	5.36 ± 0.95	5.45 ± 0.72	4.68 ± 0.76
C18:1n-9[d]	23.80 ± 2.25	36.27 ± 3.22[e]	23.86 ± 1.80	26.33 ± 1.75
C18:2n-6[b]	40.89 ± 3.65	23.05 ± 6.96[c]	38.78 ± 3.47	41.23 ± 2.99
C20:4n-6	4.41 ± 1.29	3.78 ± 0.84	4.09 ± 0.91	2.65 ± 0.95
C22:6n-3	1.39 ± 0.44	1.15 ± 0.32	1.34 ± 0.34	0.89 ± 0.33
Total fat[f]	2.15 ± 1.22	1.19 ± 0.35	2.03 ± 0.61	1.72 ± 0.84

[a]Mean ± SEM: percentage calculated as (mol fatty acid/mol total fatty acid) × 100.
[b]$P < 0.05$, [d]$P < 0.05$ by one-way ANOVA.
[c]$P < 0.005$ vs. LCT and LCT/2-mono, [e]$P < 0.005$ MCT vs. all other groups by LSD test.
[f]Mean ± SEM: μmol/mL. See Table 1 for abbreviations.

TABLE 3
Fatty Acid Composition in Plasma Triglyceride

Fatty acids[a]	LCT (5)	MCT (6)	LCT/MCT (6)	LCT/2-mono (7)
C12:0	Not detected	5.37 ± 4.27	0.29 ± 0.07	0.35 ± 0.06
C14:0	0.46 ± 0.09	1.93 ± 0.61	1.14 ± 0.15	1.45 ± 0.15
C16:0	28.57 ± 3.86	35.60 ± 3.89	24.67 ± 1.85	33.78 ± 3.27
C16:1n-7	6.05 ± 1.77	13.15 ± 3.08	9.02 ± 1.49	10.23 ± 2.83
C18:0	2.11 ± 0.15	3.02 ± 0.94	2.00 ± 0.26	1.73 ± 0.15
C18:1n-9	24.55 ± 3.54	26.93 ± 5.89	22.59 ± 1.87	20.96 ± 1.82
C18:2n-6[b]	30.14 ± 5.33	9.93 ± 1.57[c,d]	20.45 ± 2.18	23.11 ± 4.21
C20:4n-6[b]	5.79 ± 1.39[e]	3.05 ± 0.69	3.83 ± 0.84	2.76 ± 0.53
C22:6n-3	3.97 ± 0.72	1.82 ± 0.45	1.61 ± 0.19	2.28 ± 0.88
Total fat[f]	0.38 ± 0.18	0.33 ± 0.13	0.68 ± 0.40	0.17 ± 0.04

[a]Mean ± SEM: percentage calculated as (mol fatty acid/mol total fatty acid) × 100. [b]$P < 0.01$ by one-way ANOVA. [c]$P < 0.01$ vs. LCT and LCT/2-mono. [d]$P = 0.06$ vs. LCT/MCT. [e]$P < 0.05$ all by LSD test. [f]Mean ± SEM: μmol/mL. See Table 1 for abbreviations.

to a level one-third that observed in LCT. Moreover, the period of feeding (20 h) was also sufficient to significantly reduce the level of arachidonic acid, a principal end-product of linoleic acid. However, these alterations in linoleic acid induced by MCT feeding were partially prevented by adding LCT to the diet. In the current study, when half of MCT was replaced by LCT, the relative amount of linoleic acid was doubled in plasma in LCT/MCT as compared to MCT alone, although the content of linoleic acid was still lower in the LCT/MCT and LCT/2-mono group compared to the LCT group.

Our results also showed that the constant feeding with 100% MCT did not lead to the appearance of medium-chain fatty acids in portal vein fatty acids. The three medium-chain fatty acid-containing diets also did not lead to the appearance of medium-chain fatty acids in plasma triglycerides. These results are not consistent with previous findings. It was reported that decanoic acid (10:0) infused alone was essentially transported through the portal system (9). Bougneres *et al.* (10) found very high plasma concentrations of octanoic (8:0) plus decanoic acid (10:0) in full-term and preterm infants 1 h after a single intragastric dose of 2.8 mL MCT/kg body weight. In healthy adults, MCT also modestly elevated plasma concentrations of medium-chain fatty acids in triglycerides after oral administration of MCT oil (11,12). The following possibilities may contribute to the discrepancy. Most important, feeding parameters were different in each study. In the present study, the dietary MCT was given continuously as an emulsion, providing a relatively lower dose of MCT per unit time, and MCT was mixed with other nutrients. In the previous studies, MCT was given as either bolus injection or a higher dose of infusion alone. Therefore, the levels of MCT transported *via* the portal system would be expected to be lower in the present study, and even below the detectable range, when compared to those in earlier studies. It is also possible that metabolic transformation of medium-chain fatty acids by *de novo* lipogenesis and lymphatic transport as a long-chain triglyceride may become an important pathway under these conditions rather than portal transportation as medium-chain fatty acids. Certainly, further studies are needed to identify directly the different metabolic pathways between the steady-state feeding and the bolus feeding conditions.

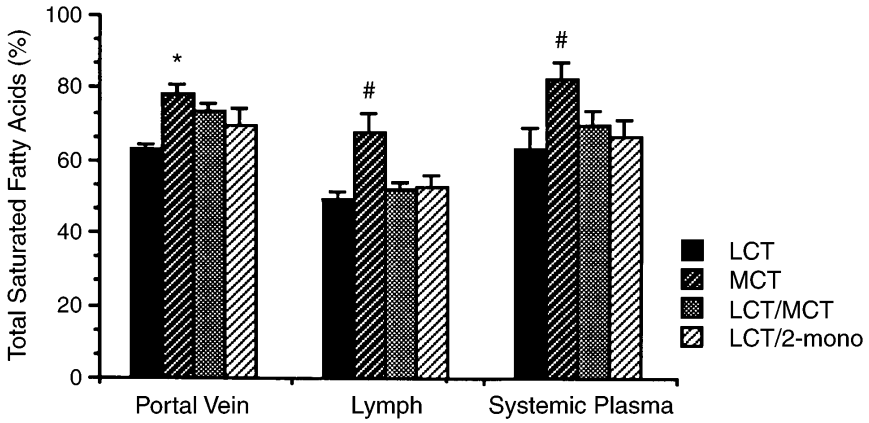

FIG. 1. Total saturated fatty acids as a percentage of total fatty acids in portal vein fatty acids, lymphatic, and plasma triglycerides. *$P < 0.05$ vs. long-chain triglycerides (LCT) and LCT/2-monodecanoic acid (2-mono); # $P < 0.05$ vs. all by one-way analysis of variance with least significant difference test. MCT, medium-chain triglycerides.

MCT feeding significantly increased the percentage of total saturated fatty acids in the portal vein fatty acids and palmitoleic and oleic acids in lymph triglyceride. It is well recognized that palmitoleic and oleic acids can derive from both dietary and endogenous origin. However, in the present study, MCT feeding did not provide these specific long-chain fatty acids. Although a possible source, it is unlikely that these two fatty acids were released from adipose tissues, given that the study was conducted in the fed-state at hypercaloric levels and feeding was continuous. Therefore, the higher levels of these fatty acids in the portal circulation and lymph are likely to be the results of *de novo* lipogenesis. Although *de novo* lipogenesis from MCT has been reported in premature infants with MCT feeding (13), the synthesis of fatty acids by this route is costly in terms of energy and is associated with a thermogenic cost of from 30 to as much as 100% of the MCT content of the diet (14,15). Thus, the present data provide further information that the conversion of MCT into long-chain fatty acids is likely to be an important route after its ingestion when linoleic acid intake is severely limited, especially in a well-fed state. However, that the contents of C_{16} and C_{18} saturated and monounsaturated fatty acids in portal vein fatty acids and lymph triglycerides in LCT/MCT and LCT/2-mono groups were not significantly different from the LCT group suggested the processes of *de novo* lipogenesis from medium-chain fatty acids are modulated by the long-chain fatty acid content of the diets (16–19).

It is also interesting to note that the changes in fatty acid appearance of palmitoleic acid induced by MCT feeding were most prominent in the portal vein, followed by the lymph triglyceride and least apparent in the systemic circulation. Presumably this endogenous fatty acid is largely synthesized in the intestine and then transported to some extent as a fatty acid by the portal vein, and in the lymph as a triglyceride, finally reaching the systemic circulation where fatty acid composition is influenced by the contributions from hepatic triglyceride production. Indeed, the incorporation of octanoic acid into fatty acids with chain length greater than 12 carbon atoms has been observed in intestinal slices (20,21). However, the relative contribution of liver and intestine to this *de novo* lipogenesis was not measured, and certainly the liver also contributes (6).

The percentage of total saturated fatty acids in lymphatic and systemic triglycerides was significantly higher in the MCT group when compared to each of the LCT-containing groups. However, there were no substantial differences found between LCT/MCT and LCT/2-mono except for the percentage of palmitoleic acid in the portal vein. LCT/2-mono increased the proportion of palmitoleic acid compared to LCT/MCT, although the difference was marginal ($P = 0.07$). The LCT/2-mono diet contains similar amounts of 10:0 but more 10:0 in the 2-position than the LCT/MCT diet. This is because one-third of the fatty acids in MCT are 10:0, but they are randomly distributed. The MCT diet would have six times as many medium-chain fatty acids as the LCT/2-mono diet but only twice as many 10:0, with two-thirds as many in the 2-position. One might anticipate that 10:0 fatty acids in the 2-position would be more likely to traverse the lymphatic route and might be metabolically more active in *de novo* lipogenesis. Although it is possible these differences contributed to the changes seen in palmitoleic acid in the portal vein, this is very speculative. Certainly, more study is needed to explore these possibilities.

In summary, the present study confirms previous findings that MCT feeding without linoleic acid appears to rapidly foster *de novo* lipogenesis, which might be considered early evidence of essential fatty acid deficiency. Providing LCT with either MCT or 2-mono effectively limits the former pathway. Our results thus suggest that continuous feeding is likely to produce markedly different pathways of medium-chain fatty acid utilization under conditions of energy surfeit than has been found with bolus or meal feeding.

ACKNOWLEDGMENTS

Authors thank Ross Products Division, Abbott Laboratories (Columbus, OH) for partial support of this research. The authors also thank Vickey Huang for technical asistance in gas chromatography analysis.

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[Received April 25, 1997, and in final revised form and accepted February 9, 1998]

Absorption, Excretion, and Distribution of Plant Sterols After Proximal Gut Resection and Autotransplantation of Porcine Ileum

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ABSTRACT: Contribution of different gut segments to plant sterol absorption, adaptation of plant sterol absorption after partial small bowel resection, and effects of gut transplantation (necessitates extrinsic autonomic denervation and lymphatic disruption) on plant sterol biodynamics are unclear. We studied the consequences of massive proximal small bowel resection and autotransplantation of the remaining ileum on the adaptive absorption and biodynamics of plant sterols. Dietary, fecal, biliary, hepatic and plasma plant sterols, fecal elimination and absorption of cholesterol, small bowel morphology, and intestinal transit were determined before (n = 5) and at 4, 8, and 14 wk after resection of the proximal 75% of the jejunoileum (n = 15) and autotransplantation of the remaining ileum (n = 15) or transection (n = 5). Proximal gut resection significantly reduced cholesterol absorption efficiency; percentage absorption and biliary secretion of plant sterols; plasma, biliary and hepatic campesterol-to-cholesterol proportions; and sitosterol proportions in plasma and bile. Autotransplantation of the remaining ileum further significantly decreased cholesterol absorption efficiency; percentage absorption and biliary secretion of campesterol; campesterol proportions in plasma, bile and liver; and plasma proportions of sitosterol while increasing fecal excretion of neutral and acidic steroids. Plasma proportions of the two plant sterols, but absorption of just campesterol, were gradually improved with increasing cholesterol absorption and villus height after proximal gut resection; the same result was observed to a lesser degree after ileal autotransplantation. In addition, significant positive correlations were found between percentage cholesterol and campesterol absorption and the plasma plant sterol proportions in both proximal resection groups, between campesterol absorption and ileal villus height in the resection group, and between campesterol absorption and intestinal transit time in the autotransplantation group. In conclusion, plasma campesterol and sitosterol closely reflect absorption of cholesterol and plant sterols from intact and autotransplanted ileum during adaptation to proximal gut resection. A loss of proximal gut absorptive surface impairs cholesterol and campesterol absorption more than sitosterol absorption, the latter being apparently less dependent on available jejunal villus surface area.

Lipids 33, 267–276 (1998).

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The major plant sterols normally found in small amounts in mammalian serum and tissues are campesterol and sitosterol. Except for differences in the side chain, these plant sterols are structurally similar to cholesterol with an identical sterol nucleus. Despite the structural similarities, plant sterols are absorbed to a much lesser extent than cholesterol, campesterol being absorbed more readily than sitosterol (1,2). The basis of the discrimination between cholesterol and plant sterol absorption is unclear, but the process may be mediated by differential uptake at the brush border membrane and a greater rate of cholesterol delivery from mixed micelle to the absorption site (3). Plant sterols are poorly absorbed from the intestine, are not synthesized endogenously, are poorly esterified in the liver, and are readily secreted into bile (4,5). They are detected in plasma only in low concentrations which are proportional to cholesterol absorption efficiency (6). As in healthy humans, serum plant sterol levels change in parallel with fractional cholesterol absorption in a variety of pathological conditions, including patients with untreated celiac disease or gut resections (6–9). Interestingly, at low cholesterol absorption rates in patients with celiac disease-induced villous atrophy of the proximal small bowel, the serum campesterol level is depressed relatively more than that of sitosterol (8), suggesting that the jejunum has a less central role in sitosterol absorption than in the absorption of cholesterol and campesterol.

Under physiological conditions absorption of cholesterol and plant sterols is believed to occur predominantly in the proximal small bowel through apical villus cells (10,11). Thus, removal of the proximal gut offers a useful model to examine the contribution of different small bowel segments to sterol absorption in general. On the other hand, after proximal small bowel resection, gut absorptive surface is reduced and sterol absorption occurs, abnormally, in the remaining ileum. The latter undergoes structural and functional adaptation to proximal small bowel resection, leading to increased absorption per unit gut length (12). As far as we know, adaptation of plant sterol absorption after partial small bowel resection has not been previously studied. Extrinsic denervation, lymphatic disruption, and ischemia, all necessitated by small bowel transplantation, may interfere with these compensatory changes

and further decrease overall intestinal absorption following resection (13,14). This is highly relevant, because intestinal transplantation has recently been applied to clinical use among patients with short bowel syndrome or end-stage intestinal failure. In our previous studies, 50% proximal resection of porcine small bowel, and especially autotransplantation of the remaining ileum, decreased plasma plant sterol-to-cholesterol proportions, a finding consistent with a loss of the major site for effective sterol absorption (15). The plasma plant sterol-to-cholesterol proportions improved gradually after the operations, campesterol more readily than sitosterol, indicating that ileal adaptation to plant sterol absorption occurred even after autotransplantation of the remaining ileum, and differently for campesterol and sitosterol.

We hypothesized that proximal small bowel resection and particularly autotransplantation of the remaining ileum would decrease absorption of campesterol more than of sitosterol. To this end and to further elucidate factors regulating absorption and plasma levels of plant sterols, to compare intestinal absorption of cholesterol, campesterol and sitosterol during postresectional adaptation of intact and autotransplanted ileum, and to ascertain whether plasma plant sterols reflect sterol absorption also under these conditions, plasma, biliary and hepatic contents, fecal excretion, absorption, and biliary secretion of plant sterols were determined before and after resection of the proximal 75% of the jejunoileum and autotransplantation of the remaining ileum or transection. Finally, the results were related to different variables of cholesterol biodynamics, small bowel morphology, and intestinal transit.

The results of the present study suggest first that a loss of proximal gut absorptive surface impairs cholesterol and campesterol absorption more than sitosterol absorption. Second, ileal autotransplantation in pigs with proximal gut resection further decreases cholesterol and campesterol absorption. Third, campesterol absorption in the ileum gradually increases, similarly to that of cholesterol, following massive proximal gut resection; and plasma campesterol and sitosterol closely reflect absorption of cholesterol and plant sterols from intact and autotransplanted ileum during adaptation to proximal gut resection.

MATERIALS AND METHODS

Experimental animals. Thirty-five growing female pigs purchased from a commercial supplier were housed individually in a light- and temperature-controlled environment, fed twice a day at standardized times, and offered water *ad libitum*. Standard pig chow (Suomen Rehu OY, Turku, Finland) containing 61.7% (w/w) carbohydrate, 17.0% protein, 3.5% fat, 4.7% crude fiber, and 12.0% aqueous substance was freely available for 1 h twice a day. Gas–liquid chromatographic (GLC) measurements of sterols in nonsaponifiable dietary material using a 50-m-long SE-30 capillary column (Hewlett-Packard, Palo Alto, CA) revealed that the diet contained 76.7 μg cholesterol, 133.7 μg campesterol, and 341.3 μg β-sitosterol per gram of chow. The animals used in this study received humane care in compliance with the principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985). An authorization (No. 154613) to perform these experiments was given by the Provincial Government of Uusimaa in accordance with Finnish legislation.

Surgical procedures. Animals randomly assigned into transection (n = 5), resection (n = 15), and autotransplantation (n = 15) groups underwent surgery after an overnight fast under general anesthesia with endotracheal intubation. A single dose of 500 mg ceftriaxone and 100 mg phenylbutazon were given intramuscularly for antibiotic prophylaxis and postoperative analgesia, respectively. Animals were kept on a liquid diet for the first postoperative day, after which the standard chow was introduced.

In the transection group, the small intestine (1286 ± 60 cm) was transected 10 cm distal to the ligament of Treitz, in the middle of the ileum and 5 cm proximal to the ileocecal junction (Fig. 1). In the resection group, the proximal 75% of the small intestine was resected 10 cm distal to the ligament of Treitz after transection of the ileum 5 cm proximal to the ileocecal junction, leaving the distal 25%, or 320 ± 10 cm, of small bowel functioning. Intestinal continuity was restored by end-to-end anastomoses. As described in detail previously (16), orthotopic autotransplantation of the ileum with proximal small bowel resection was carried out with some modifications. Briefly, the distal 25%, or 343 ± 7 cm, of the small bowel was isolated on a vascular pedicle composed of the branches of the superior mesenteric artery and vein. After the mesentery to the ileum was freed, the superior mesenteric vessels were divided, and the graft was perfused *via* the artery with cold (4°C) heparinized Ringer's solution (5000 IU/1000 mL) until the venous effluent was clear. The harvested small bowel was kept in cold isotonic saline solution, and a proximal 75% small bowel resection was performed as described above. Following systemic heparinization, the superior mesenteric vessels were anastomosed to the aorta and the inferior vena cava below the renal vessels. Total ischemic time averaged 60.7 ± 2.5 min with no significant differences among the three subgroups.

Study design. Preoperatively, and at 4, 8, and 14 wk after resection or transplantation and 14 wk after transection, intestinal transit time was measured with carmine red powder before a 3-d stool collection was performed at the end of an 8-d marker feeding period. Then, after an overnight fast, blood, gallbladder bile, liver, and small intestinal samples were obtained under aseptic conditions, and the animals, excluding those studied preoperatively, were killed with a lethal injection of thiopental. Five pigs subjected to small bowel resection were studied twice: preoperatively and 4 wk after resection. Thus, five animals were studied preoperatively; five resected and five transplanted animals were studied 4 and 8 wk after the operation; and five transected, five resected, and five autotransplanted animals 14 wk postoperatively. All ani-

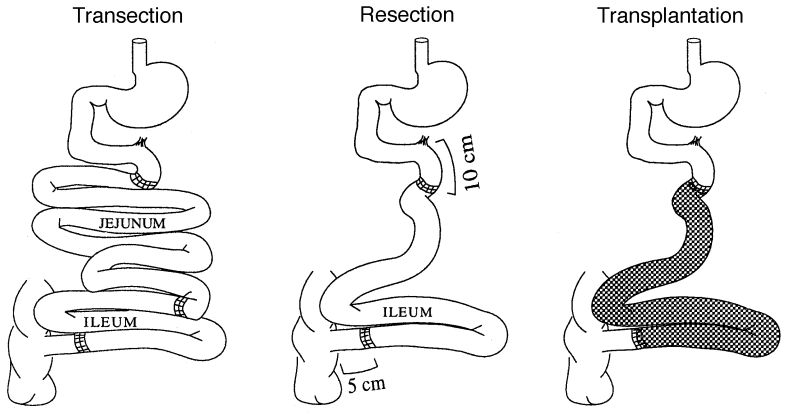

FIG. 1. Surgical procedures. In the transection group, the small bowel was transected and reanastomosed 10 cm distal to the ligament of Treitz, in the middle of the ileum and 5 cm proximal to the ileocecal valve. In the resection group, the proximal 75% of the small bowel, including the jejunum and proximal ileum, was resected after transection of the ileum 5 cm proximal to the ileocecal junction leaving the distal 25% of the small bowel functioning. In the autotransplantation group, the remaining distal ileum (shaded) underwent an additional autotransplantation. The graft mesenteric vessels were anastomosed to the aorta and the inferior vena cava below the renal vessels.

mals were weighed weekly and checked daily for chow intake.

Analytical methods. Fractional cholesterol absorption was measured using the double-isotope feeding method (17). For this and for determining fecal steroids of cholesterol origin, the pigs were fed capsules containing 0.09 μCi [^{14}C]cholesterol, 0.25 μCi [^{3}H]sitostanol (Radiochemical Centre, Amersham, United Kingdom), and 200 mg chromic oxide (Orion, Espoo, Finland) twice a day with each meal for 8 d under strict supervision. To measure intestinal transit time, the animals ingested 1 to 2 g of carmine red powder with the first capsules, and the appearance of the dye in feces was recorded.

Fecal neutral steroids of cholesterol origin, plant sterols, and bile acids were quantified with GLC on the 50-m-long SE-30 capillary column (Hewlett-Packard) (18–20). Fecal chromic oxide, used to correct the fecal flow, was determined from the 3-d fecal samples according to Bolin *et al.* (21).

Total (including free and esterified sterols) cholesterol, campesterol, and sitosterol concentrations in the diet, plasma, bile, and liver were determined by GLC on the 50-m cross-linked methyl silicone SE-30 capillary column (Hewlett-Packard) directly from nonsaponifiable material of respective lipid extracts (20,22–24). The bile and liver specimens were flash-frozen in liquid nitrogen and stored at −20°C along with plasma samples separated by centrifugation until analyzed. A weighed and homogenized sample of liver (200 mg) or chow (2000 mg), or an aliquot of plasma or bile (250 μL), was saponified by a mixture of 10 N KOH in 99.5% ethanol (1:9) after addition of internal standard 5α-cholestane (Sigma Chemical Co., St. Louis, MO). After a 2-h saponification, the mixtures were diluted to a 50% ethanolic solution and extracted at least twice with hexane, washed with 50% ethanol to remove adherents, dissolved with hexane/methanol, and evaporated to dryness. Finally, the sterol contents of the hexane extracts were quantified as trimethyl silyl ether derivatives. To eliminate the effects of varying cholesterol concentrations, plant sterols in plasma, bile, and liver are expressed in terms of μg/mg of cholesterol. The latter values are called proportions in the following text.

The small intestinal length was measured along the antimesenterial border, and small intestinal tissue samples were obtained from the proximal jejunum, mid-ileum, and distal ileum (Fig. 1). Samples were freed from mesentery, cut open along the mesenteric border, and measured for circumference. Separate full-thickness biopsies were fixed in formalin and embedded in paraffin. A pathologist determined villus height in a single-blind measurement from the villus–crypt junction to the tip of the villus using periodic acid–Schiff-stained slides. In every specimen an average of four well-oriented randomly chosen villi were measured and averaged to give single values representing mean villus height in the remaining small bowel.

Calculations and data analysis. Fractional cholesterol absorption was calculated from the difference between fecal and dietary ratios of [^{14}C]cholesterol and [^{3}H]sitostanol (17). Total intestinal cholesterol influx was calculated using an indirect method as follows (25): intestinal cholesterol influx = fecal neutral steroids/(1 − fractional cholesterol absorption). The difference between the intestinal cholesterol influx and dietary cholesterol equaled the biliary cholesterol secretion. The intestinal cholesterol influx multiplied by the fractional cholesterol absorption equaled the absorbed amount of total cholesterol. Biliary secretion of campesterol and sitosterol was calculated from their respective ratios to cholesterol, expressed as μg/mg of cholesterol in fasting pig gallbladder bile, by multiplying the ratios by the biliary cholesterol secretion (9,25). Fractional plant sterol absorption was calculated from the biliary plant sterol secretion divided by the

fecal excretion of campesterol and sitosterol, which represents the minimal absorption of plant sterols, because a small fraction of absorbed plant sterols may be eliminated as acidic steroids (26). The small bowel serosal surface area was obtained by multiplying the mean small intestinal circumference by its length.

Analysis of variance and the unpaired Student's *t*-test were used for comparisons between the groups. Fisher's protected least significant difference test was performed for multiple pairwise comparisons. Correlations were tested by linear regression analysis. The minimal level of statistical significance was defined as $P < 0.05$. All values are expressed as mean ± SEM.

RESULTS

Preoperative body weights (18.6 ± 0.6 kg) and small bowel lengths (1319 ± 23 cm) were similar in the three operated groups (data not shown). During the follow-up of 14 wk, weight gain was 65.0 ± 1.3 kg after transection, 38.6 ± 0.9 kg after resection, and only 4.8 ± 1.6 kg after transplantation. In Tables 1 and 2 data are given for all three experimental goups at 14 wk, and in Figures 2–5 preoperatively, 4, 8, and 14 wk, respectively, after resection and transplantation.

Small intestinal adaptation. Small intestinal serosal surface area, decreased by proximal small bowel resection, remained virtually unchanged during the follow-up in both proximal resection groups (Fig. 2). The values were over twofold higher in the resection than in the transplantation group, but remained about 50% lower than in the transected animals at 14 wk (Table 1). Villus height of the remaining small bowel increased markedly between 8 and 14 wk after resection and was significantly higher than in the transected or transplanted animals at the end of the study (Fig. 2 and Table 1). Intestinal transit time was similar in both proximal resection groups but considerably shorter when compared to the transected controls at the end of the follow-up (Table 1).

Cholesterol biodynamics. Table 1 displays the characteristics of cholesterol biodynamics 14 wk after the operations. Plasma and hepatic cholesterol contents were highest in the transected controls and lowest after ileal autotransplantation. Biliary cholesterol concentration was highest in the transplantation group and lowest in the transected controls. Fractional and total cholesterol absorption decreased markedly after resection and further after autotransplantation of the remaining ileum. Elimination of neutral and acidic steroids into feces was significantly increased after resection and even more so after autotransplantation of the remaining ileum.

Plant sterol biodynamics. Characteristics of plant sterol biodynamics 14 wk after the operations are shown in Table 2. In the transected controls, the plasma, biliary, and hepatic campesterol proportions were 2.2–3.3 times higher than the respective sitosterol proportions. Percentage absorption and biliary secretion of campesterol were, respectively, 5.6 and 2.2 times greater than those of sitosterol. When compared to the transection group at 14 wk, resection had greatly diminished campesterol proportions in plasma, bile and liver, sitosterol proportions in plasma and bile, and biliary secretion and percentage absorption of both plant sterols. In general, similar but even more pronounced changes were observed after transplantation. In both study groups, the decreases were markedly greater for campesterol than for sitosterol. Thus, the

TABLE 1
Effects of Massive (75%) Proximal Small Bowel Resection and Autotransplantation of the Remaining Ileum on Enteric Physiology and Cholesterol Biodynamics[a]

Variable (mean ± SEM)	Transection ($n = 5$)	Resection ($n = 5$)	Transplantation[b] ($n = 5$)
Enteric physiology			
Small intestinal serosal surface (dm^2)	66.1 ± 5.3	33.4 ± 1.9[a]	16.8 ± 1.2[a,b]
Small bowel villus height (μm)	569 ± 36	831 ± 15[a]	448 ± 11[a,b]
Intestinal transit time (h)	28.9 ± 0.2	22.6 ± 0.7[a]	21.4 ± 0.5[a]
Stool weight ($g \cdot kg^{-1} \cdot d^{-1}$)	11.2 ± 0.8	16.7 ± 1.4[a]	16.6 ± 1.9[a]
Cholesterol biodynamics			
Plasma cholesterol (mg/dL)	86.4 ± 5.3	62.8 ± 0.7[a]	47.0 ± 6.3[a,b]
Biliary cholesterol (mg/dL)	76.6 ± 11	82.3 ± 6.2	114 ± 12[a,b]
Hepatic cholesterol (mg/100 g)	171 ± 3.1	157 ± 4.9	154 ± 7.6
Cholesterol absorption			
Fractional	0.80 ± 0.03	0.27 ± 0.04[a]	0.10 ± 0.01[a,b]
Total ($mg \cdot kg^{-1} \cdot d^{-1}$)	27.5 ± 5.0	11.1 ± 2.2[a]	4.11 ± 0.5[a,b]
Intestinal cholesterol fluxes ($mg \cdot kg^{-1} \cdot d^{-1}$)			
Dietary	1.89 ± 0.1	2.45 ± 0.1	2.78 ± 0.3[a]
Endogenous	31.7 ± 5.3	37.6 ± 3.0	40.4 ± 1.4
Total	33.6 ± 5.4	40.1 ± 3.1	43.2 ± 1.5
Fecal steroid excretion ($mg \cdot kg^{-1} \cdot d^{-1}$)			
Neutral steroids	6.1 ± 0.4	29.0 ± 1.7[a]	39.1 ± 1.5[a,b]
Bile acids	21.5 ± 2.6	36.2 ± 4.7	96.6 ± 8.6[a,b]

[a]Determined 14 wk postoperatively.
[b]Superscript roman letters as follows: [a]$P < 0.05$ from transection in the same row; [b]$P < 0.05$ from resection in the same row.

TABLE 2
Effects of Massive (75%) Proximal Small Bowel Resection and Autotransplantation of the Remaining Ileum on Biodynamics of Campesterol and Sitosterol[a]

Variable (mean ± SEM)	Transection[b] (n = 5)	Resection[b] (n = 5)	Transplantation[b] (n = 5)
Plasma proportions (μg/mg cholesterol · 10^2)			
Campesterol	1425 ± 101	222 ± 27[a]	74.3 ± 14[a,b]
Sitosterol	432 ± 28	158 ± 14[a]	80.8 ± 9.9[a,b]
Biliary proportions (μg/mg cholesterol · 10^2)			
Campesterol	4947 ± 449	612 ± 89[a]	302 ± 87[a,b]
Sitosterol	2289 ± 267	741 ± 47[a]	775 ± 73[a]
Hepatic proportions (μg/mg cholesterol · 10^2)			
Campesterol	1283 ± 77	196 ± 32[a]	69.7 ± 13[a,b]
Sitosterol	394 ± 54	269 ± 49	196 ± 47[a]
Campe/sito proportion ratios			
Plasma	3.30 ± 0.10	1.39 ± 0.06[a]	0.89 ± 0.08[a,b]
Bile	2.20 ± 0.09	0.82 ± 0.08[a]	0.37 ± 0.08[a,b]
Liver	3.57 ± 0.57	0.80 ± 0.17[a]	0.38 ± 0.03[a,b]
Biliary secretion (μg · kg^{-1}· d^{-1})			
Campesterol	1573 ± 329	228 ± 31[a]	124 ± 38[a]
Sitosterol	724 ± 160	276 ± 17[a]	314 ± 34[a]
Fecal excretion (mg · kg^{-1}· d^{-1})			
Campesterol	3.46 ± 0.13	4.78 ± 0.45	5.48 ± 0.63[a]
Sitosterol	9.15 ± 0.35	11.9 ± 0.85	13.5 ± 1.41[a]
Percentage absorption			
Campesterol	44.6 ± 8.31	4.73 ± 0.37[a]	2.17 ± 0.44[a,b]
Sitosterol	7.96 ± 1.81	2.34 ± 0.12[a]	2.35 ± 0.18[a]

[a]Determined 14 wk postoperatively.
[b]Superscript roman letters as follows: [a]$P < 0.05$ from transection in the same row; [b]$P < 0.05$ from resection in the same row.

plasma proportions and percentage absorption of campesterol in the resected animals exceeded those of sitosterol at the end of the follow-up.

The plasma, biliary, and hepatic proportions and biliary secretion of campesterol, dramatically decreased by proximal small bowel resection, gradually increased between 4 and 14 postoperative weeks in the resection group and, to a lesser degree, in the transplantation group (Fig. 3). Similar changes occurred in plasma sitosterol proportions (Fig. 4). In contrast, the biliary proportions and secretion of sitosterol tended to be lower in the resection than in the transplantation group. Unexpectedly, hepatic sitosterol proportions were not significantly decreased in either of the study groups and showed a significant temporal increase after resection. The campesterol/sitosterol proportion ratios in plasma, bile, and liver underwent temporal increases in both proximal resection groups (Fig. 5). The values were significantly greater after resection than after transplantation.

Adaptation of sterol absorption. When compared to the preoperative values, the resection and transplantation groups showed substantial mean decreases in the percentage absorption of cholesterol and campesterol, about 10- and 14-fold, respectively (Fig. 2). The respective decrease was considerably smaller, about twofold, for sitosterol. Percentage absorption of cholesterol and campesterol, but not of sitosterol, increased gradually after resection and, albeit less so, after transplantation.

Correlations. Fractional absorption of campesterol was positively related to villus height and intestinal transit time in the unresected controls, to villus height in the resection group, and to intestinal transit time in the transplantation group (Table 3). Although insignificant and weaker, similar associations existed for sitosterol. In all the groups studied, plasma campesterol and sitosterol-to-cholesterol proportions displayed significant positive correlations with cholesterol absorption efficiency, and plasma campesterol proportions with campesterol absorption efficiency. However, the plasma sitosterol proportion was significantly related to fractional sitosterol absorption only in the unresected control animals. Plasma plant sterol proportions were interrelated with biliary and hepatic plant sterol proportions, with the exception of biliary sitosterol proportions in the transplantation group. In the transplanted animals with bile acid malabsorption, the higher the biliary sitosterol secretion, the higher was fecal excretion of bile acids (Fig. 6). No such association was found between fecal bile acids and biliary secretion of campesterol (r = 0.166, P = 0.55). However, biliary secretion of sitosterol showed only weak and insignificant correlation with biliary bile acid secretion (r = 0.301, P = 0.28).

DISCUSSION

In principle, the intact upper small intestine appears to be the major site for effective sterol absorption (10,11). The different absorption rates for the individual sterols were actually high in the control pigs of the present series, which absorbed

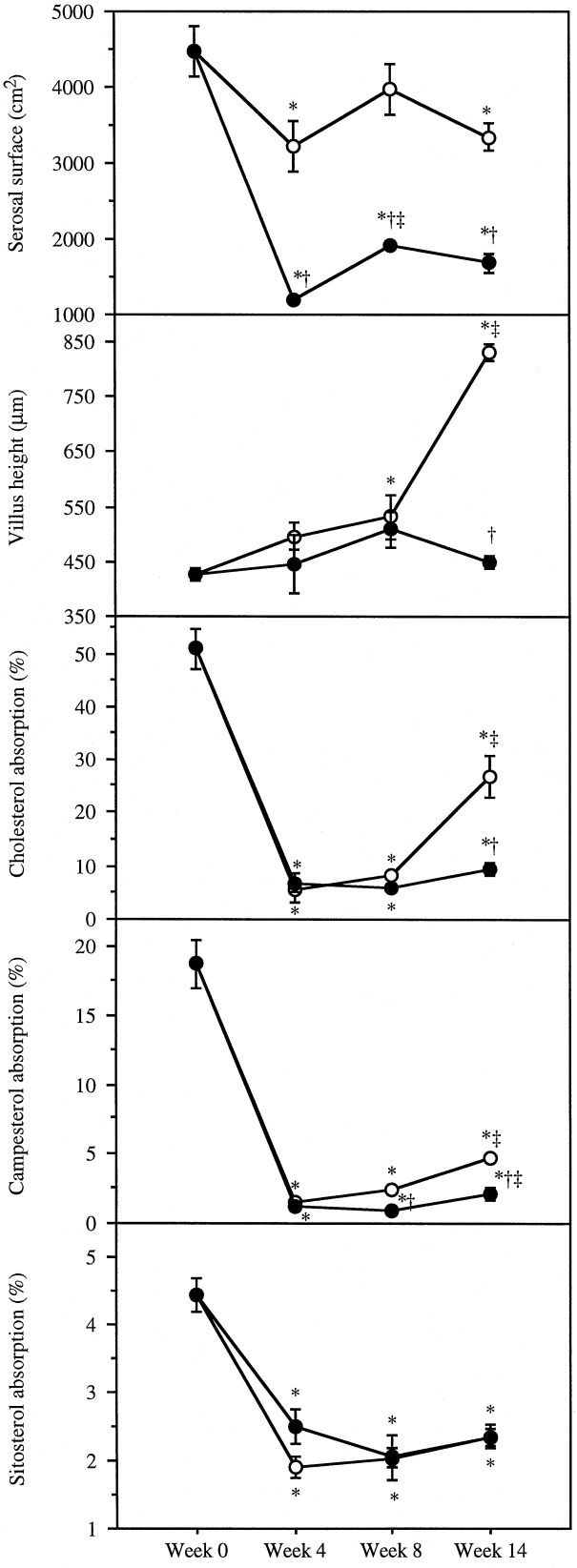

FIG. 2. Adaptation of small intestinal absorptive surface area and sterol absorption 4, 8, and 14 wk after proximal 75% gut resection (○) and autotransplantation (●) of the remaining ileum (mean ± SEM). *$P < 0.05$ from preoperative, †$P < 0.05$ from resection, ‡$P < 0.05$ from the respective 4- and/or 8-wk value.

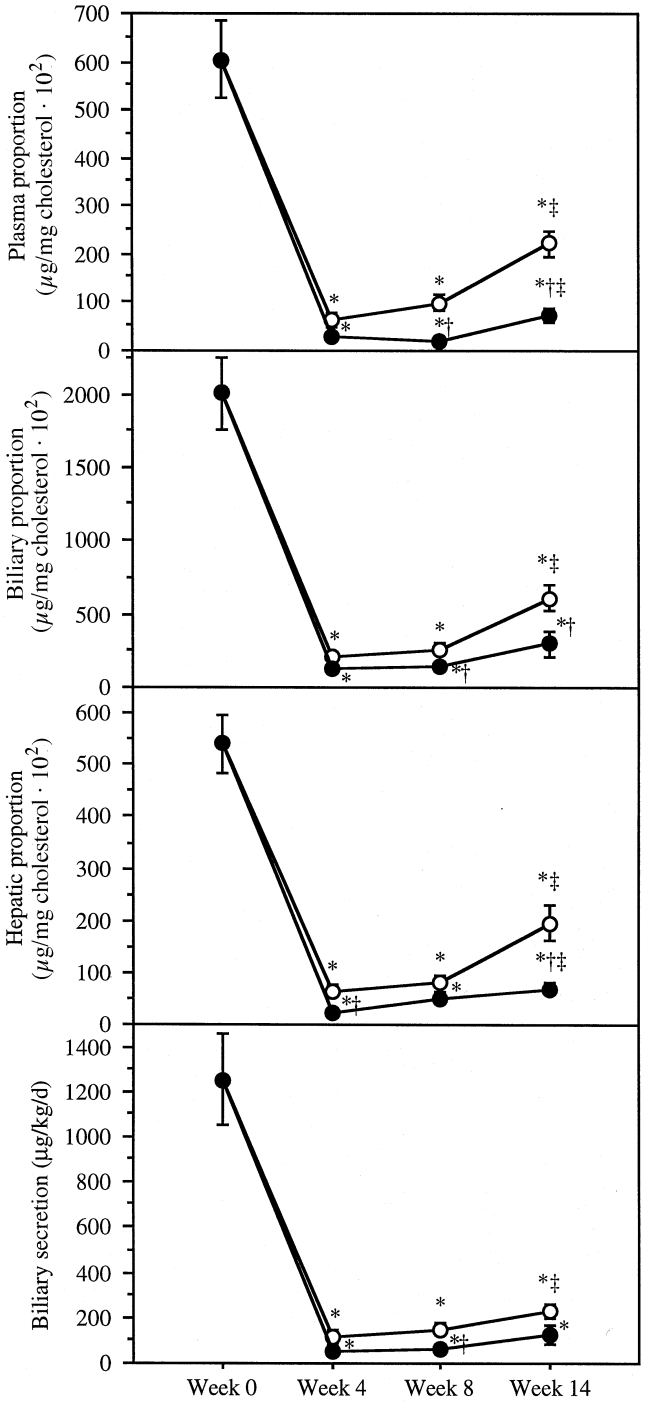

FIG. 3. The plasma, biliary, and hepatic proportions and biliary secretion of campesterol 4, 8, and 14 wk after proximal 75% gut resection (○) and autotransplantation (●) of the remaining ileum (mean ± SEM). *$P < 0.05$ from preoperative, †$P < 0.05$ from resection, ‡$P < 0.05$ from the respective 4- and/or 8-wk value.

cholesterol, campesterol, and sitosterol with respective percentages of 80–51, 45–19, and 8–4% at different ages. In general, these findings are in accord with previous studies in normal humans and rats, where higher intestinal absorption rates are reported for cholesterol (~40%) than for campesterol (~10%) or sitosterol (~5%) (1,2,4,27,28). As far as we know,

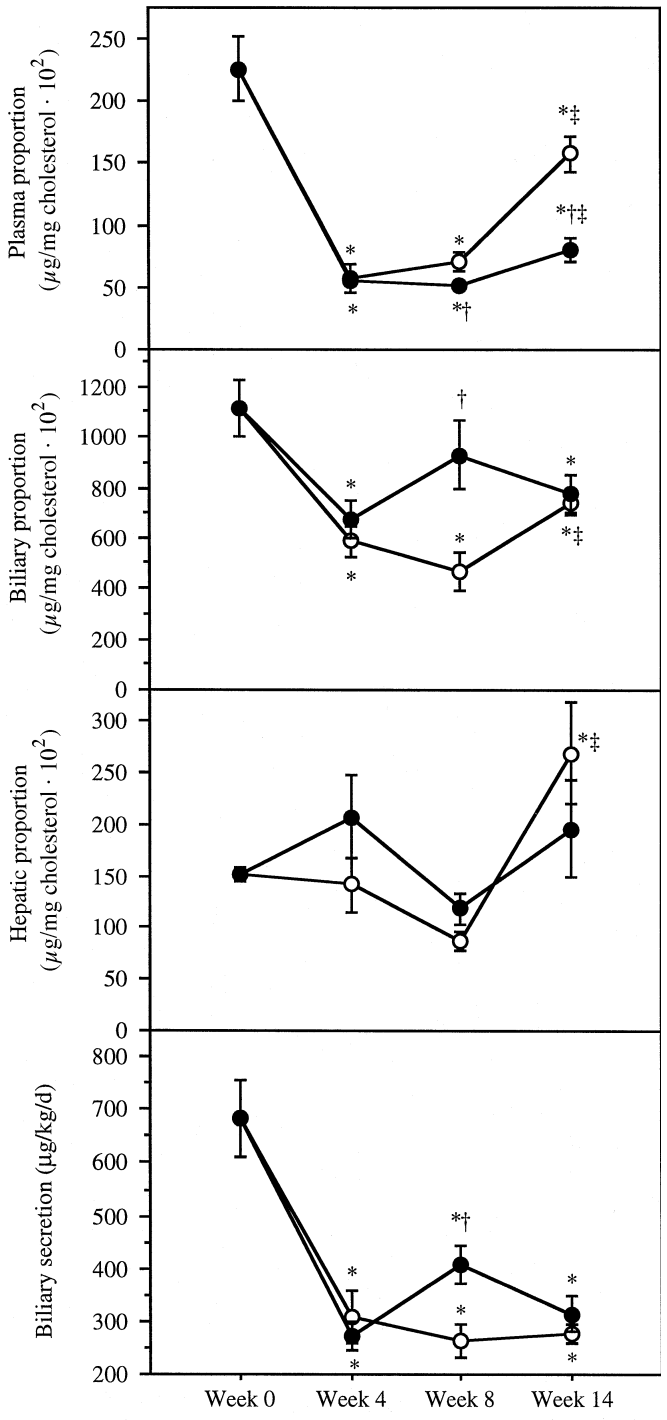

FIG. 4. The plasma, biliary, and hepatic proportions and biliary secretion of sitosterol 4, 8, and 14 wk after proximal 75% gut resection (○) and autotransplantation (●) of the remaining ileum (mean ± SEM). *$P < 0.05$ from preoperative, †$P < 0.05$ from resection, ‡$P < 0.05$ from the respective 4- and/or 8-wk value.

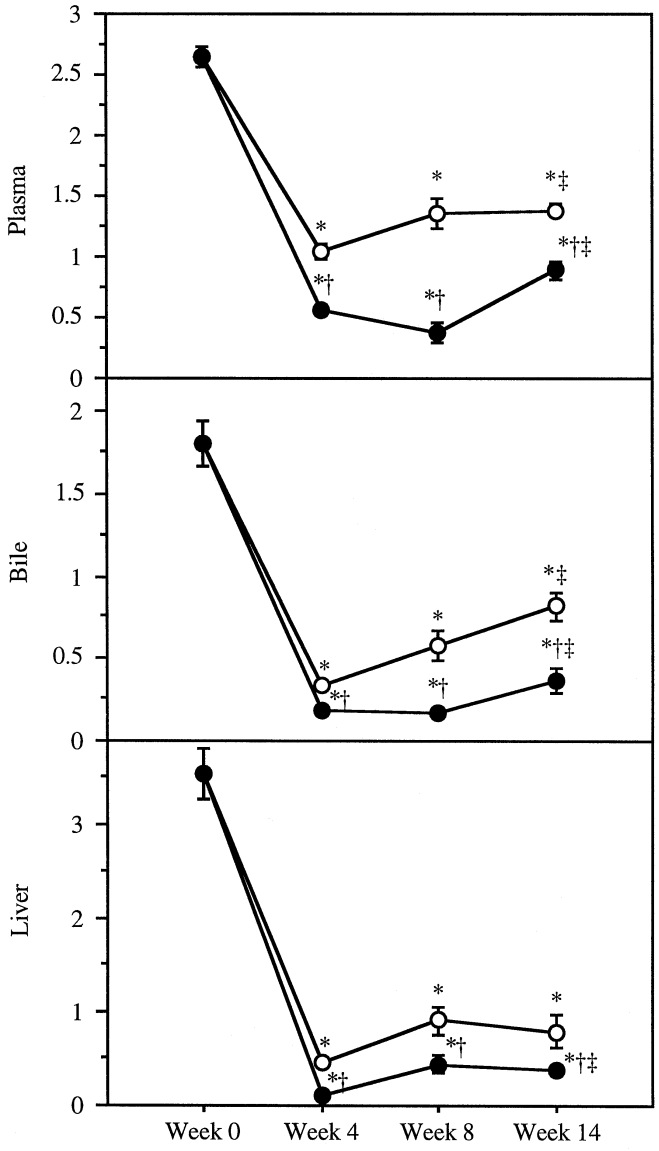

FIG. 5. Plasma, biliary, and hepatic campesterol/sitosterol proportion ratios 4, 8, and 14 wk after proximal 75% gut resection (○) and autotransplantation (●) of the remaining ileum (mean ± SEM). *$P < 0.05$ from preoperative, †$P < 0.05$ from resection, ‡$P < 0.05$ from the respective 4- and/or 8-wk value.

campesterol or sitosterol absorption rates have not previously been presented for pigs. However, the experiments performed here were primarily aimed at defining whether adaptation of plant sterol absorption occurs, analogously to that of cholesterol, after proximal gut resection and autotransplantation of the remaining ileum, whether this adaptation is different for the individual plant sterols, and whether plasma plant sterol proportions reflect their absorption efficiencies during ileal adaptation. In fact, close associations were demonstrated between cholesterol absorption efficiency and plasma proportions and absorption of plant sterols after massive proximal gut resection, with or without autotransplantation of the remaining distal small bowel.

The similarity in structure and physiology of the gastrointestinal tract of the pig to that of humans makes the pig an excellent animal model because of the important role of the gut in lipid metabolism (29). Lipoprotein distribution and composition are similar to that of humans, and low density lipoprotein is the major cholesterol-transporting lipoprotein in both species (30,31). The physical characteristics and

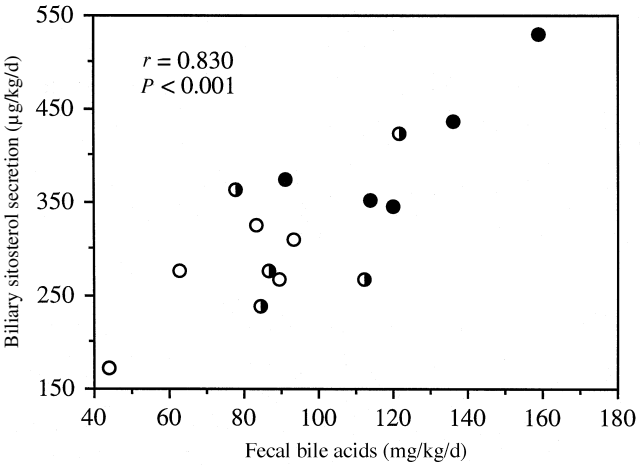

FIG. 6. Correlation of fecal bile acid excretion with biliary sitosterol secretion after proximal 75% small bowel resection with autotransplantation of the remaining ileum. ○, Week 4; ●, week 8; ◑, week 14.

turnover of pig low density lipoprotein resemble those of humans (30,32). Furthermore, interruption of the enterohepatic circulation of bile acids with cholestyramine and inhibition of hepatic cholesterol synthesis with mevinolin result in a decrease of plasma cholesterol, whereas enhanced intestinal absorption of cholesterol increases plasma cholesterol concentration as it does in humans (31,32).

Massive (75%) proximal resection of porcine small bowel dramatically decreased percentage absorption and biliary secretion of plant sterols, campesterol being affected much more severely than sitosterol. Because of the marked compensatory increase in cholesterol synthesis, the decrease in plasma cholesterol was only up to 46%, compared with 95% for campesterol and 81% for sitosterol concentrations by the respective absorption reductions of 88, 95, and 71% (Tables 1 and 2). At the end of the study, plasma proportions of campesterol and sitosterol in the two groups with proximal gut resection averaged about 13- and 4-fold lower than the corresponding values in the transection group. The approximately 15- and 3-fold higher average intestinal absorption of campesterol and sitosterol in the transection group could well explain these differences. Previous studies suggest that mucosal sitosterol uptake is well preserved throughout the length of the entire small intestine, whereas mucosal uptake of cholesterol and campesterol displays a precipitous proximodistal gradient in the intact small bowel, being most effective in the proximal jejunum (2,10,11). In agreement with this, the percentage absorption of both cholesterol and campesterol decreased about 10-fold or more after massive proximal gut resection, whereas the calculated fractional absorption of sitosterol was reduced relatively little, only about twofold (Fig. 2). In keeping with our findings, in untreated celiac disease with villous atrophy of the upper small intestine and low cholesterol absorption efficiency, serum concentration of campesterol is reduced more than that of sitosterol, and improved absorption during a gluten-free diet increases plasma campesterol more than sitosterol (8). Autotransplantation of the remaining ileum in pigs with proximal gut resection further decreased the various tissue proportions and absorption of campesterol (Fig. 3). In contrast, only the plasma proportions of sitosterol were decreased (Fig. 4). As shown in Table 3,

TABLE 3
Correlations of Plasma Proportions and Percentage Absorption of Campesterol and Sitosterol with Villus Height, Transit Time, and Different Variables of Cholesterol and Plant Sterol Biodynamics[a]

	Unresected controls (n = 10)				Resection (n = 15)				Transplantation (n = 15)			
	Plasma proportions (µg/mg chol)		Absorption (%)		Plasma proportions (µg/mg chol)		Absorption (%)		Plasma proportions (µg/mg chol)		Absorption (%)	
Variable	Campe	Sito	Campe	Sito	Campe	Sito	Campe	Sito	Campe	Sito	Campe	Sito
Villus height (µm)	0.85[b]	0.78[b]	0.71[a]	0.51	0.94[d]	0.95[d]	0.93[d]	0.34	–0.18	–0.23	–0.12	–0.38
Transit time (h)	0.92[c]	0.89[c]	0.75[a]	0.57	0.35	0.44	0.35	0.05	0.55[a]	0.53[a]	0.59[a]	0.51
Sterol absorption (%)												
Cholesterol	0.87[b]	0.89[c]	0.82[b]	0.62	0.83[c]	0.85[d]	0.89[d]	0.39	0.57[a]	0.59[a]	0.65[b]	0.67[b]
Campesterol	0.74[a]	0.78[b]	—	0.93[d]	0.96[d]	0.95d	—	0.41	0.91[d]	0.90[d]	—	0.43
Sitosterol	0.60	0.66[a]	0.93[d]	—	0.40	0.47	0.41	—	0.36	0.36	0.43	—
Fecal excretion (mg · kg^{-1}· d^{-1})												
Neutral steroids	–0.90[c]	–0.87[b]	–0.67[a]	–0.50	–0.58[a]	–0.61[a]	–0.63[a]	–0.27	–0.32	–0.31	–0.21	–0.53[a]
Bile acids	–0.68[a]	–0.67[a]	–0.50	–0.28	–0.44	–0.41	–0.46	–0.09	–0.20	–0.13	–0.20	–0.27
Plasma proportions (µg/mg cholesterol)												
Campesterol	—	0.99[d]	0.74[a]	0.60	—	0.98[d]	0.96[d]	0.40	—	0.97[d]	0.91[d]	0.36
Sitosterol	0.99[d]	—	0.78[b]	0.66[a]	0.98[d]	—	0.95[d]	0.47	0.97[d]	—	0.90[d]	0.36
Biliary proportions (µg/mg cholesterol)												
Campesterol	0.98[d]	0.98[d]	0.83[b]	0.73[a]	0.98[d]	0.96[d]	0.91[d]	0.44	0.80[c]	0.83[c]	0.90[d]	0.20
Sitosterol	0.93[d]	0.94[d]	0.78[b]	0.77[b]	0.56[a]	0.62[a]	0.49	0.59[a]	0.03	0.10	0.02	0.25
Hepatic proportions (µg/mg cholesterol)												
Campesterol	0.99[d]	0.98[d]	0.75[a]	0.63	0.96[d]	0.94[d]	0.89[d]	0.39	0.69[b]	0.71[b]	0.54[a]	–0.06
Sitosterol	0.64[a]	0.64[a]	0.45	0.24	0.63[a]	0.68[b]	0.65[c]	0.26	0.57[a]	0.54[a]	0.52[a]	0.58[a]

[a]Abbreviations: chol, cholesterol; Campe, campesterol; sito, sitosterol.
[b]Superscript roman letters as follows: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$.

one of the main factors determining plasma levels of plant sterols, especially that of campesterol, was their absorption efficiency. The percentage absorption and plasma proportions of campesterol were reduced more than those of sitosterol; consequently, the campesterol-to-sitosterol proportion ratios decreased (Fig. 5). Thus, a loss of proximal gut absorptive surface, due either to villous atrophy or to small bowel resection, apparently impairs campesterol absorption more than sitosterol absorption. Absorption of the latter appears less dependent on available villus surface area and occurs with comparable efficiencies in all parts of the small intestine (2). In fact, the percentage campesterol absorption showed close positive correlations with villus height in the control and resection groups, whereas no such association was found for sitosterol in any of the groups studied (Table 3).

In the current study, campesterol absorption gradually increased, similarly to cholesterol, following massive proximal small bowel resection with or without autotransplantation of the remaining ileum. However, ileal autotransplantation disturbed plant sterol absorption in pigs with proximal gut resection. The high positive correlation between intestinal villus height and percentage campesterol absorption in the control and resected animals indicates that campesterol absorption, analogously to that of cholesterol (33), changed in parallel to available absorptive (villus) surface area in these growing pigs. The relatively small but significant increase in campesterol absorption after ileal autotransplantation may reflect improved individual ileocyte absorptive capacity (34), because villus height was not increased in the autotransplanted ileum. Moreover, in the absence of villus, enlargement intestinal transit time appeared as a major factor determining plant sterol absorption efficiency from the autotransplanted ileum (Table 3). In the present study, the adaptive increase in ileal sterol absorption was blunted by addition of the ethyl group of sitosterol instead of the methyl group of campesterol at position 24 in the side chain. Normally, incorporation of the ethyl group into the side chain of sterols decreases jejunal sterol absorption more than incorporation of the methyl group (1). Thus, the mechanisms responsible for the different jejunal absorption rates of individual sterols appear to regulate sterol absorption also in the ileum during adaptation to proximal gut resection. These findings accord well with our previous studies, where 50% proximal resection of porcine small bowel, and especially autotransplantation of the remaining ileum, decreased plasma plant sterol-to-cholesterol proportions, and the plasma plant sterol-to-cholesterol proportions improved gradually after the operations, campesterol more readily than sitosterol (15).

Importantly, different solubilization properties in the gut lumen may greatly and unevenly affect intestinal absorption of cholesterol and plant sterols (3,35). In the present study, the intraluminal solubilities of different sterols were not tested, leaving a possibility that the observed changes in absorption of cholesterol, campesterol, and sitosterol were also affected by changes in solubilization rather than being solely due to physical alterations in the gut anatomy. The former hardly explains the several-fold increase in cholesterol and campesterol absorption in the resection group (Fig. 2), because biliary bile acid secretion and composition remain constant in these animals (36), whereas villus height showed nearly 70% increase (Fig. 2). However, increased deconjugation and altered composition of intestinal bile acids secondary to increased bacterial activity could certainly impair overall sterol absorption especially after transplantation (36).

In man, low cholesterol absorption is associated with increased synthesis and biliary secretion of cholesterol, which may, in turn, enhance cholesterol-dependent biliary secretion of plant sterols (37). In the present study in pigs with proximal gut resection with or without autotransplantation of the remaining ileum, these associations were not apparent, presumably owing to the extent of cholesterol malabsorption (Table 1). The high positive correlation between biliary sitosterol secretion and fecal excretion of bile acids in pigs with ileal autotransplantation-induced bile acid malabsorption is of particular interest (Fig. 6). A similar association has previously been reported in patients with gut resection-induced bile acid malabsorption (9), suggesting that increased hepatic bile acid synthesis may promote biliary secretion of sitosterol by reducing hepatic conversion of sitosterol into bile acids (4). However, in humans this pathway may be nonsignificant, whereas in some other mammalian species it may be active (26,38). Interestingly, the hepatic sitosterol proportion was negatively related to fecal excretion of bile acids ($r = -0.514$, $P = 0.05$) in the pigs with ileal autotransplantation, lending further support to the conclusion that increased bile acid synthesis may also contribute to increased biliary secretion of sitosterol. In fact, it is apparent from Figure 6 that the highest level of fecal bile acid excretion occurred 8 wk after ileal autotransplantation, which could certainly explain the relatively high biliary proportion and secretion of sitosterol measured at the same time (Fig. 4).

ACKNOWLEDGMENTS

This study was supported by the Clinical Research Institute of Helsinki University Central Hospital. The authors would like to thank Orvokki Ahlroos, Pia Hoffström, Leena Kaipiainen, Anja Salolainen, and Brian Fleming for their expert technical and editorial assistance.

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[Received October 2, 1997, and in revised form January 21, 1998; revision accepted January 28, 1998]

The Effect of Dietary Vitamin E Supply and a Moderately Oxidized Oil on Activities of Hepatic Lipogenic Enzymes in Rats

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ABSTRACT: Diets rich in polyunsaturated fatty acids (PUFA) are well known to suppress hepatic lipogenic enzymes compared to fat-free diets or diets rich in saturated fatty acids. However, the mechanism underlying suppression of lipogenic enzymes is not quite clear. The present study was undertaken to investigate whether lipid peroxidation products are involved in suppression of lipogenic enzymes. Therefore, an experiment with growing male rats assigned to six groups over a period of 40 d was carried out. Rats received semisynthetic diets containing 9.5% coconut oil and 0.5% fresh soybean oil (coconut oil diet, peroxide value 5.1 meq O_2/kg oil), 10% fresh soybean oil (fresh soybean oil diet, peroxide value 9.5 meq O_2/kg oil), or 10% thermally treated soybean oil (oxidized soybean oil diet, peroxide value 74 meq O_2/kg oil). To modify the antioxidant state of the rats, we varied the vitamin E supply (11 and 511 mg α-tocopherol equivalents per kg of diet) according to a bi-factorial design. Food intake and body weight gain were not influenced by dietary fat and vitamin E supply. Activities of hepatic lipogenic enzymes were markedly influenced by the dietary fat. Feeding either fresh or oxidized soybean oil diets markedly reduced activities of fatty acid synthase, (FAS), acetyl CoA-carboxylase, (AcCX), glucose-6-phosphate dehydrogenase, (G6PDH), 6-phosphogluconate dehydrogenase, and ATP citrate lyase (ACL) relative to feeding the coconut oil diet. Moreover, feeding oxidized soybean oil slightly, but significantly, lowered activities of FAS, AcCX, and ACL compared to feeding fresh soybean oil. Activities of hepatic lipogenic enzymes were reflected by concentrations of triglycerides in liver and plasma. Rats fed the coconut oil diet had markedly higher triglyceride concentrations in liver and plasma than rats consuming fresh or oxidized soybean oil diets, and rats fed oxidized soybean oil had lower concentrations than rats fed fresh soybean oil. The vitamin E supply of the rats markedly influenced concentrations of thiobarbituric acid-reactive substances in liver, but it did not influence activities of hepatic lipogenic enzymes. Because the vitamin E supply had no effect, and ingestion of an oxidized oil had only a minor effect, on activities of hepatic lipogenic enzymes, it is strongly suggested that neither exogenous nor endogenous lipid peroxidation products play a significant role in the suppression of hepatic lipogenic enzymes by diets rich in PUFA. Therefore, we assumed that dietary PUFA themselves are involved in regulation of hepatic lipogenic enzymes. Nevertheless, the study shows that ingestion of oxidized oils, regardless of the vitamin E supply, also affects hepatic lipogenesis, and hence influences triglyceride levels in liver and plasma.
Lipids 33, 277–283 (1998).

Several studies demonstrated that dietary polyunsaturated fatty acids (PUFA) suppress hepatic lipogenic enzymes by inhibiting enzyme protein synthesis (1,2). However, the mechanisms by which PUFA suppress hepatic lipogenic enzymes are not completely understood. Some experimental evidence suggests that the nutritionally important C18 PUFA, linoleic acid and α-linolenic acid, require Δ6 and Δ5 desaturation to C20 PUFA to exert their suppressive effect (3,4). According to a hypothesis of Clarke and Jump (5), those fatty acids interact directly at the genomic level to govern gene transcription. On the other hand, a recent study using primary hepatic monolayer culture indicated that PUFA require oxidative modification to exert their suppressive effect (6). If primary or secondary lipid peroxidation products are involved in lipogenic enzyme suppression, it might be expected that the effect of dietary PUFA on enzyme suppression would be modified by the antioxidant state of the animal. In this respect, feeding diets enriched with antioxidants could reduce, and feeding diets with low levels of antioxidants enhance the capability of dietary PUFA for lipogenic enzyme suppression. To test this hypothesis, we investigated the effect of PUFA from soybean oil accompanied by an insufficient and an excessive vitamin E supply in rats. Additionally, rats which were fed diets with thermally oxidized soybean oil were studied to ascertain the results from consumption of increased concentrations of lipid peroxidation products. According to the above hypothesis oxidized soybean oil should be a more potent inhibitor of lipogenic enzymes than fresh soybean oil.

MATERIALS AND METHODS

Animals and diets. Sixty male Sprague-Dawley rats weighing 51 (± 4, SD) g were assigned to six groups of 10 rats each.

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Abbreviations: PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid-reactive substances.

All the rats received a semisynthetic diet consisting of (in g/kg) casein (200), cornstarch (320), sugar (288), fat (100), fiber (30), minerals (40), vitamins (20), and DL-methionine (2). Minerals and vitamins with the exception of vitamin E were added in sufficient amounts as recommended by the American Institute of Nutrition (7). The dietary fat and the vitamin E supply were varied according to a bi-factorial experimental design. Dietary fats used were (I) a mixture of coconut oil and soybean oil (19:1, w/w; coconut oil diet), (II) fresh soybean oil (fresh soybean oil diet), (III) thermally treated soybean oil (oxidized soybean oil diet). Peroxide value (measured iodometrically) of the coconut oil/soybean oil mixture before inclusion into the diet was 0.8 meq O_2/kg, and that of fresh soybean oil was 3.7 meq O_2/kg. Oxidized soybean oil was prepared by bubbling air through fresh soybean oil at 130°C for 22 h. This treatment elevated the peroxide value to 23 meq O_2/kg and caused a loss of 83% of α-tocopherol, 93% of γ-tocopherol, and 38% of γ-tocopherol; percentages of PUFA were insignificantly reduced by heat treatment. Prior to inclusion in the diet, the fats were standardized to an identical vitamin E activity of 113 mg α-tocopherol equivalents per kg by supplementation with all-*rac*-α-tocopherol acetate. The fats were included into the diet either in this form or after supplementation with 7,450 mg all-*rac*-α-tocopherol acetate per kg. The calculated vitamin E concentrations of the diets were either 11 (low-vitamin E diet) or 511 mg (high vitamin E diet) α-tocopherol equivalents per kg. For preparing the diet, dietary components were mixed with water, homogenized, and dried at a temperature of 50°C for 4 d. During drying, the peroxide values of dietary oils increased. For assessing the peroxide value, aliquots of the diets were analyzed at different times within the experimental period. Average peroxide values of dietary fats over the whole experimental period are shown in Table 1. The fatty acid composition of dietary total lipids and analyzed concentrations of tocopherols are also given in Table 1. Both the fatty acid composition and tocopherol concentrations were not altered by storage at a temperature of 4°C during the experimental period. The experiment lasted 40 d. In order to stimulate activities of lipogenic enzymes, rats were given free access to food throughout until killing. Killing was carried out by decapitation after a slight anesthesia.

Analyses. After decapitation, the liver was excised and an aliquot was homogenized in a buffer containing 0.25 M sucrose and 0.1 M phosphate (pH 7.4) using a Potter-Elvehjem homogenizer. Homogenates were centrifuged (105,000 × *g* for 1 h at 4°C) and the supernatants were used for enzyme assays (8). All the enzymes were assayed by spectrophotometric methods. Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (9) and 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) (10) were determined by the rate of NADP reduction. In the assay of glucose-6-phosphate dehydrogenase, activity of 6-phosphogluconate dehydrogenase was inhibited by maleimide (9). Fatty acid synthase was determined from the rate of malonyl-CoA-dependent NADPH oxidation after the liver homogenate has been preincubated in a phosphate-buffered solution containing dithiothreitol to achieve maximal enzymatic activity (11). The activity of acetyl-CoA carboxylase (E.C. 6.4.1.2) was determined from NADH oxidation in an assay coupled with pyruvate kinase and lactate dehydrogenase (12). ATP citrate lyase (E.C. 4.1.3.8) was determined from NADH oxidation in an assay coupled with malate dehydrogenase (13).

For determination of triglycerides, cholesterol, and total

TABLE 1
Fatty Acid Composition, Tocopherol Concentrations, and Peroxide Values of the Experimental Diets[a]

	Parameter					
	CO		FSO		OSO	
	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E
Fatty acids (mol/100 moles)						
6:0	0.5	0.5	—	—	—	—
8:0	7.0	7.0	—	—	—	—
10:0	5.3	5.2	—	—	—	—
12:0	43.1	43.0	—	—	—	—
14:0	16.9	16.9	0.3	0.3	0.3	0.3
16:0	9.7	9.6	10.2	10.2	10.7	10.7
16:1	—	—	0.3	0.3	0.3	0.3
18:0	3.6	3.6	3.8	3.8	3.9	3.9
18:1n-9	8.7	8.6	23.9	24.0	24.0	24.0
18:2n-6	5.0	5.0	53.3	53.4	52.7	52.6
18:3n-3	0.6	0.6	8.1	8.1	7.7	7.7
Tocopherols (mg/kg)						
α-Tocopherol	9.1	646	7.1	623	13.2	665
γ-Tocopherol	4.0	4	58.0	58	3.8	4
γ-Tocopherol	3.3	3	27.0	27	13.6	14
Peroxide value (meq O_2/kg oil)	6.1	4.0	9.4	9.6	74	75

[a]Abbreviations: CO, coconut oil; FSO, fresh soybean oil; OSO, oxidized soybean oil.

fatty acids in liver tissue, lipids were extracted with a hexane/isopropanol mixture (3:2, vol/vol, containing butylated hydroxy toluene) (14). For determination of liver total cholesterol and triglycerides, an aliquot of the extract was dissolved in Triton X-100 (15). Total cholesterol and triglycerides then were determined using enzymatic reagent kits obtained from Boehringer Mannheim (Mannheim, Germany). Fatty acids of liver total lipids were converted into methyl esters by transesterification with boron fluoride/methanol reagent (16). Fatty acid methyl esters were separated by gas chromatography using a Hewlett-Packard HP 5890A gas chromatographic system (Taufkirchen, Germany) fitted with an automatic on-column injector, a flame-ionization detector, and a CP-Sil 88 capillary column (50 m × 0.25 mm internal diameter, film thickness 0.2 μm; Chrompack, Middleburg, The Netherlands). Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards and quantified with heptadecanoic acid methyl ester as internal standard (17). Concentrations of tocopherols in liver, oils, and diets were determined by high-performance liquid chromatography (18). Samples were mixed with 1 mL 1% pyrogallol solution (in ethanol, absolute) and 150 μL saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted three times with *n*-hexane. Individual tocopherols were separated using a mixture of *n*-hexane and 1,4-dioxane (94:6, vol/vol) as mobile phase and a LiChrosorb Si 60 column (5 μm particle size, 250 mm length, 4 mm internal diameter; Merck, Darmstadt, Germany) and detected by fluorescence (excitation wavelength: 295 nm, emission wavelength: 320 nm).

Thiobarbituric acid-reactive substances (TBARS) in liver were determined by high-performance liquid chromatography (19). Liver homogenates were mixed with 1 mL TBA reagent (10 mM TBA in 0.1 M phosphate buffer, pH 1) and 50 μL of a 0.5% solution of butylated hydroxytoluene (in ethanol, absolute). This mixture was heated for 45 min at 100°C. TBARS were extracted with *n*-butanol. The butanol phase was injected; the column used was a LiChrosorb RP-18 Cartridge (5 μm particle size, 250 mm length, 4 mm internal diameter; Merck). A water/acetonitrile mixture (80:20, vol/vol) was used for elution of TBARS; the fluorescence was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. 1,1,3,3-Tetramethoxypropane was used as standard. Plasma lipoproteins were separated by stepwise ultracentrifugation (230,000 × *g*, 20 h, 8°C). Plasma densities (δ) were adjusted by addition of potassium bromide. The lipoprotein classes (VLDL, $\delta < 1.019$ kg/L; LDL, $1.019 < \delta < 1.063$ kg/L; HDL, $1.063 < \delta < 1.21$ kg/L, where VLDL = very low density lipoprotein, LDL = low density lipoprotein; HDL = high density lipoprotein) were removed from each tube by suction with a pipette. The concentrations of triglycerides were measured enzymatically using an autoanalyzer (model 704; Hitachi, Tokyo, Japan) and a commercially available reagent kit (Boehringer Mannheim).

Statistics. Data were treated by analysis of variance. Classification factors were dietary fat, vitamin E supply and the interaction of those factors. For statistically significantly F-values ($P < 0.05$), means were compared by Fisher's multiple range test.

RESULTS

Daily food intake and body weight gains as well as concentrations of tocopherols and TBARS are shown in Table 2. Mean daily food intake and body weight gains were not influenced by either dietary fat or vitamin E supply and hence were similar within all the groups. Hepatic tocopherol concentrations were determined to assess the vitamin E status of the rats. As expected, hepatic tocopherol concentrations were markedly influenced by the dietary vitamin E supply. Rats fed the high-vitamin E diets on average had 13-fold higher tocopherol concentrations in liver than rats fed the low-vitamin E diets. The dietary fat also influenced the vitamin E status of the rats. Feeding the oxidized soybean oil reduced the tocopherol status relative to the other fat sources, regardless of the dietary vitamin E supply. Among groups receiving the high vitamin E supplement, rats fed the coconut oil diet had the

TABLE 2
Food Intake, Growth, and Concentrations of Tocopherols and TBARS in Liver[a,b]

	Parameter					
	CO		FSO		OSO	
	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E
Food intake (g/d)	17.9 ± 1.9	18.2 ± 2.0	17.7 ± 1.8	18.1 ± 1.9	18.1 ± 1.8	18.2 ± 1.5
Body weight gain (g/d)	7.94 ± 0.68	8.18 ± 0.97	7.97 ± 0.81	7.99 ± 0.54	8.23 ± 0.75	8.33 ± 0.62
Total tocopherols in liver[k]						
nmol/g dry matter[h,i,j]	69 ± 10[f]	1213 ± 309[c]	68 ± 10[f]	662 ± 152[d]	49 ± 5[g]	527 ± 98[e]
nmol/mg total lipid[h,i,j]	0.29 ± 0.05[e]	4.92 ± 0.92[c]	0.34 ± 0.09[e]	3.00 ± 0.61[d]	0.25 ± 0.04[e]	2.60 ± 0.50[d]
TBARS in liver						
pmol/mg dry matter[i]	282 ± 110[b]	145 ± 56[c]	411 ± 183[a]	167 ± 30[c]	338 ± 161[a,b]	144 ± 50[c]
nmol/mg total lipid[h,i]	1.28 ± 0.48[d]	0.56 ± 0.27[e]	2.01 ± 0.95[c]	0.76 ± 0.14[e]	1.76 ± 0.90[c,d]	0.72 ± 0.27[e]

[a]Values represent means ± SD with $n = 10$ for each group. Means with different superscript letters (c,d,e,f,g) within one row differ significantly by Fisher's multiple range test ($P < 0.05$).

[b]Significance of factors: [h]Fat: $P < 0.05$; [i]vitamin E: $P < 0.05$; [j]fat × vitamin E: $P < 0.05$. [k]Values were transferred into their logarithms prior to analyses of variance. TBARS, thiobarbituric acid-reactive substances. For other abbreviations see Table 1.

highest hepatic vitamin E content. In contrast, among groups receiving the low vitamin E diets, rats fed fresh soybean oil and those fed the coconut oil diet were similar on the basis of absolute tocopherol concentrations; on the basis of tocopherol concentrations expressed per mg of lipid, rats fed fresh soybean oil even exhibited a slightly higher vitamin E status than rats fed the coconut oil diet.

Concentrations of hepatic TBARS as an estimate of *in vivo* lipid peroxidation were also markedly influenced by vitamin E supply. Rats fed the low-vitamin E diets had on average 2.3-fold higher TBARS concentrations than rats fed the high-vitamin E diets. The effect of the dietary fat on TBARS concentrations was less pronounced. Rats fed the coconut oil diets had lower concentrations of TBARS than rats fed the soybean oil diets. The difference was more pronounced on the basis of concentrations related to hepatic lipids (30%) than in their absolute concentrations (20%). Between rats fed fresh soybean oil and rats fed oxidized soybean oil, hepatic TBARS concentrations did not differ.

Activities of hepatic lipogenic enzymes are shown in Figure 1. In general, activities of all the lipogenic enzymes were influenced by the type of fat, whereas the vitamin E supply did not affect those enzymes. A noticeable difference existed between rats fed the coconut oil diet and rats fed the soybean oil diets. Feeding fresh or oxidized soybean oil reduced activities of all the lipogenic enzymes by 30 to 50%. Besides this main effect, there existed significant differences in the activities of fatty acid synthase, acetyl-CoA carboxylase, and ATP citrate lyase between rats fed oxidized soybean oil and those fed fresh soybean oil. Oxidized soybean oil reduced activities of those enzymes by 20 to 30% relative to fresh soybean oil. In contrast, activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not different between rats fed fresh and those fed oxidized soybean oil.

There was a marked effect of the dietary fat on hepatic triglyceride concentrations, which reflected activities of lipogenic enzymes (Table 3). Rats fed the coconut oil diets had markedly higher triglyceride concentrations than rats fed the soybean oil diets. Moreover, the rats fed fresh soybean oil had slightly, but significantly, higher concentrations of triglycerides than rats fed oxidized soybean oil. In parallel with increased triglyceride concentrations, rats fed the coconut oil diets also had the highest concentrations of total lipids and fatty acids, and those fed oxidized soybean oil had the lowest concentrations. The dietary fat also influenced the fatty acid composition of hepatic total lipids. In rats fed coconut oil, saturated and monounsaturated fatty acids were predominant whereas rats fed fresh and oxidized soybean oil had high levels of n-6 PUFA. Moreover, rats fed oxidized soybean oil had lower concentrations of all types of fatty acids in liver than rats fed fresh soybean oil. The concentration of total cholesterol was not influenced by the dietary fat and by the vitamin E supply.

Triglyceride concentrations in plasma and lipoproteins also reflected activities of hepatic lipogenic enzymes (Table 4). Rats fed coconut oil had markedly higher concentrations of triglycerides in plasma, VLDL, and LDL than rats fed soybean oil, and rats fed fresh soybean oil had higher concentrations than rats fed oxidized soybean oil. According to the

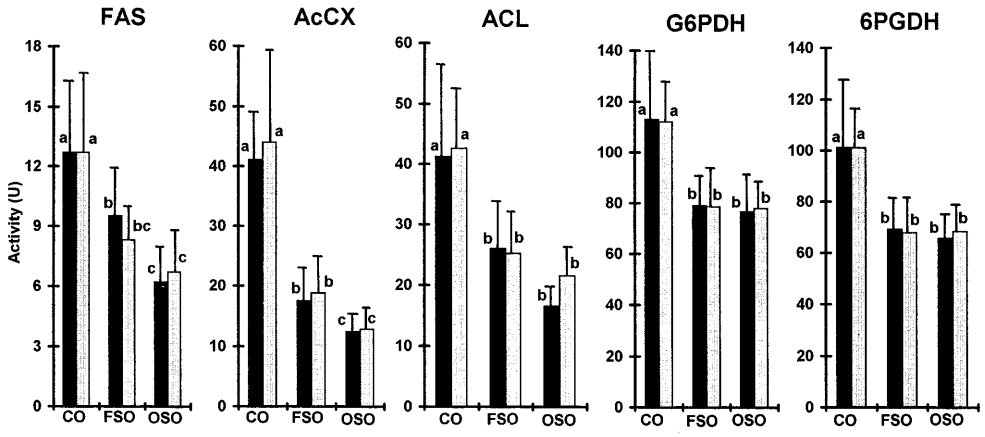

FIG. 1. The effect of dietary fat and vitamin E supply on activities of hepatic lipogenic enzymes (FAS = fatty acid synthase; AcCX = acetyl-CoA carboxylase; ACL = ATP citrate lyase; G6PDH = glucose-6-phosphate dehydrogenase; 6PGDH = 6-phosphogluconate dehydrogenase). Rats were fed diets containing predominantly coconut oil (CO), fresh soybean oil (FSO), or oxidized soybean oil (OSO). Vitamin E concentrations of the diets were: 11 mg α-tocopherol equivalents per kg (low-vitamin E diets, solid bars) and 511 mg α-tocopherol equivalents per kg (high-vitamin E diets, shaded bars). Results are means ± SD with $n = 10$ for each group. Units of activity are defined as: nmoles of NADH or NADPH oxidized per mg protein per minute at 37°C for AcCX and FAS, respectively; nmoles of NADP reduced per mg protein per minute at 37°C for G6PDH and 6PGDH; nmoles of NADH oxidized per mg protein per minute at 37°C for ACL. Significantly different means between the individual groups are marked by different letters (a,b,c). Statistical effects of factors: Dietary fat, $P < 0.05$ for all enzymes; vitamin E and vitamin E × dietary fat, nonsignificant for all enzymes.

TABLE 3
Concentrations of Hepatic Lipids[a,b]

	Parameter					
	CO		FSO		OSO	
	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E
Total lipids (mg/g)[f]	83.7 ± 22.4[c]	84.9 ± 28.9[c]	65.7 ± 9.5[d]	71.2 ± 7.9[c,d]	60.4 ± 5.3[d]	64.4 ± 7.4[d]
Triglycerides (μmol/g)[f]	53.9 ± 20.1[c]	52.2 ± 23.9[c]	17.5 ± 5.8[d]	18.1 ± 3.7[d]	13.6 ± 3.5[d]	14.0 ± 2.9[d]
Total cholesterol (μmol/g)	7.15 ± 0.94	6.81 ± 1.38	6.47 ± 0.52	7.12 ± 1.38	6.60 ± 0.68	6.03 ± 0.68
Total fatty acids (μmol/g)[f]	202 ± 58[c]	205 ± 70[c]	163 ± 32[c,d]	180 ± 28[c,d]	146 ± 23[d]	155 ± 22[d]
SFA (μmol/g)[f]	96.2 ± 31.9[c]	97.4 ± 34.3[c]	58.0 ± 10.5[d]	63.6 ± 8.2[d]	52.3 ± 5.5[d]	54.7 ± 6.0[d]
14:0[f]	7.23 ± 2.68[c]	7.44 ± 2.50[c]	1.20 ± 0.40[d,e]	1.47 ± 0.35[d]	0.87 ± 0.20[e]	1.05 ± 0.27[d,e]
16:0[f]	67.9 ± 27.4[c]	69.3 ± 32.1[c]	39.0 ± 9.0[d]	43.7 ± 7.3[d]	33.0 ± 5.2[d]	35.8 ± 5.3[d]
18:0[f]	18.6 ± 1.8	17.9 ± 1.6	17.4 ± 1.6	17.9 ± 1.4	18.0 ± 0.9	17.5 ± 1.1
MUFA (μmol/g)[f]	74.2 ± 26.7[c]	77.2 ± 36.1[c]	36.9 ± 12.1[d,e]	41.9 ± 9.0[d]	29.2 ± 4.9[e]	32.7 ± 7.4[e]
16:1[f]	15.4 ± 6.0[c]	16.9 ± 8.6[c]	5.29 ± 1.93[d,e]	6.07 ± 1.51[d]	3.65 ± 0.59[e]	4.32 ± 0.90[e]
18:1[f]	58.5 ± 20.8[c]	59.8 ± 27.6[c]	31.7 ± 10.3[d]	35.8 ± 7.7[d]	25.5 ± 4.6[d]	28.3 ± 6.5[d]
PUFA (μmol/g)[f]	31.2 ± 0.9[d]	30.5 ± 1.8[d]	68.2 ± 9.6[c]	74.8 ± 13.6[c]	63.8 ± 11.9[c]	67.1 ± 10.8[c]
18:2n-6[f]	7.89 ± 0.70[e]	8.42 ± 0.11[e]	36.4 ± 9.6[c,d]	40.9 ± 12.2[c]	30.3 ± 9.9[d]	33.6 ± 8.7[c,d]
18:3n-3[f]	0.24 ± 0.02[e]	0.27 ± 0.02[e]	3.03 ± 1.17[c]	3.32 ± 1.19[c]	2.09 ± 1.01[d]	2.62 ± 0.67[c,d]
20:4n-6[f]	14.1 ± 1.2[d]	13.4 ± 1.4[d]	19.1 ± 0.9[c]	20.0 ± 1.0[c]	21.0 ± 1.2[c]	20.1 ± 2.5[c]
22:6n-3[f]	4.10 ± 0.39[d]	3.89 ± 0.57[d]	5.98 ± 0.42[c]	6.40 ± 0.63[c]	6.42 ± 0.60[c]	6.04 ± 1.17[c]

[a]Values represent means ± SD with $n = 10$ for each group. Means with different superscript letters (c,d,e,) within one row differ significantly by Fisher's multiple range test ($P < 0.05$).

[b]Significance of factors: [f]Fat: $P < 0.05$. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. For other abbreviations see Table 1.

TABLE 4
Concentrations (mmol/L) of Triglycerides in Plasma and Lipoproteins[a,b]

	Parameter					
	CO		FSO		OSO	
	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E	Low vitamin E	High vitamin E
Plasma[f]	3.91 ± 1.03[c]	3.49 ± 0.85[c]	1.94 ± 0.93[e]	2.71 ± 0.98[d]	1.53 ± 0.47[e]	2.09 ± 0.69[d,e]
VLDL[f]	3.35 ± 0.90[c]	2.95 ± 0.68[c]	1.59 ± 0.85[e]	2.28 ± 0.85[d]	1.22 ± 0.46[e]	1.70 ± 0.65[d,e]
LDL[f]	0.38 ± 0.18[c]	0.38 ± 0.18[c]	0.20 ± 0.07[d]	0.25 ± 0.09[d]	0.16 ± 0.03[d]	0.23 ± 0.06[d]
HDL	0.18 ± 0.05	0.16 ± 0.02	0.15 ± 0.04	0.18 ± 0.08	0.14 ± 0.02	0.16 ± 0.02

[a]Values represent means ± SD with $n = 10$ for each group. Means with different superscript letters (c,d,e,) within one row differ significantly by Fisher's multiple range test ($P < 0.05$).

[b]Significance of factors: [f]Fat: $P < 0.05$. Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. For other abbreviations see Table 1.

analysis of variance, the vitamin E supply had no significant effect on concentrations of triglycerides in plasma and lipoproteins, although in the groups fed fresh and oxidized soybean oil diets, the group receiving a high vitamin E supplement had slightly higher concentrations in all the lipoproteins than those fed diets low in vitamin E.

DISCUSSION

The present study was carried out to investigate whether lipid peroxidation products are involved in the suppression of lipogenic enzyme activity by diets rich in PUFA. The effect of *in vivo* lipid peroxidation products was investigated by modifying the vitamin E status of the rats. The effect of exogenous lipid peroxidation products was investigated by feeding a thermally treated soybean oil. According to the peroxide values, the thermally treated soybean oil can be regarded as moderately oxidized. The relatively low level of oxidation of the oil used had two advantages: First, food intake was not suppressed by intake of lipid peroxidation products. This is an important demand in studying lipogenic enzymes because the food intake significantly influences the activities of lipogenic enzymes (20). In addition, the fatty acid composition of the oxidized oil was only negligibly different from that of the fresh soybean oil. Hence, the effect of oxidation products on lipogenic enzymes could be investigated without being confounded by a different fatty acid composition of dietary lipids. Most of the other studies dealing with dietary lipid peroxidation products used diets containing highly oxidized oils (21–24). Feeding such diets usually leads to lower consumption of diets containing highly oxidized oils by the animals than that of nonoxidized control diets, and rats fed highly oxidized oils also experience lower digestibility of fatty acids and lower dietary efficiency (22,23,25–27). Finally, highly oxidized oils contain less PUFA than the equivalent fresh oils (21–23,27,28). Therefore, dietary studies on the effects of

lipid peroxidation products are usually confounded by differences in nutritional status, growth rates, and fatty acid composition of dietary lipids, between the study groups.

Highly oxidized dietary oils have been reported to enhance *in vivo* lipid peroxidation as assessed by elevated levels of lipid peroxidation products in tissues (22–24). Ingestion of the moderately oxidized soybean oil in this study did not increase the generation of the malondialdehyde-TBAR adduct during the TBARS test from liver lipids, suggesting that there was not a marked increase of lipid peroxidation *in vivo*, regardless of the dietary vitamin E supply. It seems likely that an enhanced lipid peroxidation would be reduced by an elevated destruction of tocopherols. Destruction of tocopherols by peroxides could explain the lower vitamin E status of the rats fed oxidized soybean oil. Reduced tocopherol levels in animals fed oxidized oils, as observed in this study, have also been reported by other investigators (26,28–30). The effects of dietary fatty acids and vitamin E supply on lipid peroxidation products in liver were not unexpected. Other studies have also demonstrated reduced *in vivo* lipid peroxidation by feeding diets rich in saturated and monunsaturated fatty acids compared to diets rich in PUFA and by supplementing diets with high levels of vitamin E (31–33).

The present study strongly suggests that neither endogenous nor exogenous lipid peroxidation products play a significant role in the suppression of lipogenic enzymes by dietary oils rich in PUFA. This can be concluded from the observation that the suppression of lipogenic enzymes was completely independent of the vitamin E supply of the rats, and it was only slightly different between rats fed fresh and rats fed oxidized soybean oil. If lipid peroxidation products played an important role in suppression of lipogenic enzymes, it would be expected that both low vitamin E supply and ingestion of an oxidized oil would lead to suppression of lipogenic enzymes. Hence, it seems likely that PUFA *per se* exert a suppressive effect on hepatic lipogenic enzymes. This result agrees with a study by Iritani *et al.* (24) that also failed to demonstrate that autoxidation products of PUFA play a role in the suppression of lipogenic enzymes. An hypothesis on how PUFA *per se* could act on hepatic lipogenic enzymes recently has been given by Clarke and Jump (2,5). Triglyceride concentrations in liver and plasma reflected activities of hepatic lipogenic enzymes. Hence, the present study suggests that the effect of PUFA on triglycerides in liver and plasma is at least partially due to reduced hepatic lipogenesis. In the study of Iritani *et al.* (24), reducing activities of lipogenic enzymes by increasing dietary PUFA levels was also associated with lower concentrations of hepatic triglycerides.

Although the study suggests that lipid peroxidation products do not play a major role in regulation of lipogenic enzymes, such products potentially could affect hepatic lipogenesis. Rats fed oxidized soybean oil had lower activities of some key enzymes involved in lipogenesis than rats fed fresh soybean oil. As a consequence of reduced lipogenesis, rats fed the oxidized soybean oil exhibited reduced triglyceride concentrations in liver and plasma compared to rats fed the fresh soybean oil. This effect was independent of the vitamin E supply and TBARS generated from liver lipids, and hence probably is independent of *in vivo* lipid peroxidation processes, e.g., peroxidative damages to unsaturated fatty acids of membranes. From the results of the present study, the mechanism underlying reduced activities of lipogenic enzymes in rats fed oxidized oil cannot be determined. This phenomenon, however, may be due to general disturbances of energy production in rats fed oxidized oils. Ingestion of autoxidized oils has been shown to cause profound alterations in membrane composition and fluidity associated with alterations of some membrane-bound enzymes involved in energy metabolism (23,31). The study of Iritani *et al.* (24) did not observe differences in hepatic lipogenic enzymes and triglycerides between rats fed fresh and rats fed oxidized corn oil. This suggests that experimental factors other than different concentrations of primary and secondary lipid peroxidation products in the oxidized and control diets also may play a role in this regulatory process.

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[Received October 31, 1997, and in final revised form and accepted February 6, 1998]

Effects of Polyunsaturated Fatty Acids and Their n-6 Hydroperoxides on Growth of Five Malignant Cell Lines and the Significance of Culture Media

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ABSTRACT: We examined effects of polyunsaturated fatty acids (PUFA), their corresponding hydroperoxy fatty acids (hp-PUFA), as well as various pro- and antioxidants on the growth of tumor cells in culture. When cultured in RPMI 1640 medium, A-427 and WEHI clone 13 cells were both highly sensitive to hydroperoxy docosahexaenoic acid (hp-DHA), but they were far less sensitive in minimum essential medium (MEM). In contrast, A-427 cells were also sensitive to DHA in both culture media, while WEHI clone 13 cells, as well as other cell lines, tested in their respective media, were resistant. The lower sensitivity of the cell lines to hp-DHA in MEM-medium was apparently due to a more rapid reduction of hp-DHA to the corresponding hydroxy-DHA in MEM-medium. Addition of glutathione (GSH) to the culture medium abolished the effects of hp-DHA, but not the effects of DHA, while depletion of intracellular GSH levels by L-buthionine-*S,R*-sulfoximine strongly enhanced the cytotoxic effect of hp-DHA, but not the cytotoxic effect of DHA. α-Tocopherol protected A-427 cells against the toxic effect of DHA and abolished the induced lipid peroxidation, while it did not protect against the toxic effects of hp-DHA in A-427 or WEHI clone 13 cells. Ascorbic acid reduced the cytotoxic effect of DHA, but potentiated the toxic effect of hp-DHA while selenite essentially abolished the toxicity of both DHA and hp-DHA. These results indicate that sensitivity of tumor cell lines to PUFA and their oxidation products depends on their antioxidant defense mechanisms, as well as culture conditions, and establishes hp-DHA as a major, but probably not the sole, metabolite responsible for cytotoxicity of DHA.
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Diets enriched in n-3 fatty acids have been shown to delay the development and growth of experimentally induced tumors (1,2) and to reduce their invasiveness and metastatic potential (3). Growth inhibition of several tumor cell lines in culture has also been reported, indicating a direct cytotoxic effect of n-3 fatty acids (4–6). Several processes important for growth control, such as signal transduction (7), gene expression (8) and eicosanoid metabolism (9–12), may be affected by n-3 fatty acids, but the major mechanisms responsible for inhibition of tumor cell growth remain uncertain. However, it has become increasingly clear that oxidation products of polyunsaturated fatty acids (PUFA) contribute to their cytotoxicity (13–16), and recently deficiencies in cellular antioxidant defense mechanisms were shown to strongly enhance the sensitivity of tumor cells to n-3 fatty acids (17–19). Lipid peroxidation is a radical chain reaction, and the primary products of lipid peroxidation of PUFA are lipid hydroperoxides (20). Therefore, accumulation of hydroperoxides may amplify lipid peroxidation resulting in a range of products that may be deleterious to cells (20). Consistent with this, lipid hydroperoxides added to growth media inhibit cell growth and/or induce cell death in several different cell lines (21–27).

In the present study we have compared the effect of several PUFA and their hydroperoxy derivatives on the growth of different tumor cell lines. Among the cell lines tested, only one is significantly growth-inhibited by PUFA, docosahexaenoic acid (DHA) being one of the most potent. This sensitive cell line is also growth-inhibited by the primary lipid peroxidation product of DHA, hydroperoxy-DHA (hp-DHA), indicating that cytotoxicity of n-3 fatty acids may be mediated through the formation of this primary product. This is further supported by the fact that agents inhibiting lipid peroxidation or inactivating hydroperoxides also reduce cytotoxicity. We also find that cytotoxic effects of PUFA and their hydroperoxy derivatives may be modulated by factors in growth media and sera, underlining the importance of standardization of culture conditions when comparing effects of fatty acids on cultured cells.

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Abbreviations: AA, arachidonic acid (20:4n-6); ANOVA, analysis of variance; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSO, L-buthionine-*S,R*-sulfoximine; DEM, diethyl maleate; DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); FBS, fetal bovine serum; FCS, fetal calf serum; GSH, glutathione; hp-, n-6-hydroperoxy-; HPLC, high-performance liquid chromatography; LA, linoleic acid (18:2n-6); α-LNA, α-linolenic acid (18:3n-3); γ-LNA, γ-linolenic acid (18:3n-6); MDA, malondialdehyde; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances.

MATERIALS AND METHODS

Materials. Minimum essential medium with Earle's salts without L-glutamine (MEM), Dulbecco's modified Eagle's

medium (DMEM), sodium pyruvate, nonessential amino acids, Nutritient Mixture F-12 (HAM), L-glutamine, and trypsin were obtained from Gibco BRL, Life Technologies (Inchinnan, Scotland). RPMI-1640 medium was from Gibco BRL and from B.I. Bio Whittaker (Brussels, Belgium). Fetal bovine serum (FBS) was purchased from Hyclone (Cramlington, England), fetal calf serum (FCS) from Gibco BRL and Biological Industries (Kibbutz Beit Haemek, Israel). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-thiobarbituric acid (TBA), 1,1,3,3,-tetramethoxypropane, glutathione (GSH), α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), superoxide dismutase, sodium selenite, L-buthionine-*S,R*-sulfoximine (BSO), soybean lipoxidase type I-B, linoleic acid (LA), α-linolenic acid (α-LNA), γ-linolenic acid (γ-LNA), eicosapentaenoic acid (EPA), and DHA were obtained from Sigma (St. Louis, MO). Gentamicin sulfate was from Sigma and Schering Corp. (Kenilworth, NJ). Diethyl maleate (DEM) was purchased from Aldrich (Steinheim, Germany). Ascorbic acid was from E. Merck (Darmstadt, Germany). Hydroperoxy fatty acids were prepared by incubating the fatty acids with soybean lipoxygenase as previously described (27). They were purified by reversed-phase high-performance liquid chromatography (HPLC), and concentrations calculated as previously described (27).

Cell cultures. A-427, SK-LU-1, A-172, and A549 were purchased from American Type Culture Collection (Rockville, MD). WEHI clone 13 fibrosarcoma cell line (WEHI clone 13) was isolated as described previously (28). A-427 and SK-LU-1 were cultured in MEM supplemented with 10% (vol/vol) FCS, gentamicin (45 mg/L), L-glutamine (80 mg/L), sodium pyruvate (1 mM), and nonessential amino acids (100×, 10 mL/L). A549 was cultured in Nutritient Mixture F-12 (HAM) with L-glutamine (80 mg/L) supplemented with 10% (vol/vol) FCS and gentamicin (50 mg/L). A-172 was cultured in DMEM with 4.5 g/L glucose without L-glutamine, supplemented with 10% (vol/vol) FCS, gentamicin (50 mg/L), L-glutamine (80 mg/L), and sodium pyruvate (1 mM). WEHI clone 13 was cultured in RPMI-1640 medium containing 10% (vol/vol) FCS or FBS, 2 mM L-glutamine and 40 μg/mL gentamicin. All cell cultures were maintained in 5% CO_2 at 37°C.

Cell survival assay. Cell survival was measured in 96-well microplates (Costar 3598) seeding 3000 cells/well except for Figure 7 where 1500 cells/well were used. Cells were allowed to adhere for 4 h before additions were made. Stock solutions of parent fatty acids, hp-fatty acids, α-tocopherol, BHA, BHT, and DEM were prepared in ethanol, and the final ethanol concentration never exceeded 0.38% (vol/vol), which had no effect on cell survival alone. Stock solutions of GSH, ascorbic acid, sodium selenite, and BSO were prepared in purified, sterile water, and the final added water concentration never exceeded 2.0% (vol/vol), which had no effect on cell survival alone. Cell survival was measured at the times indicated, using MTT-assay, as previously described (29), measuring absorbance at 588 or 595 nm. Results are from one of at least two similar experiments with four parallels, and are given as mean with SD indicated as bars.

Reduction of hp-fatty acid in growth media alone. Hp-EPA was incubated with the growth media used for culturing A-427 cells and WEHI clone 13 cells (complete MEM and RPMI-medium, respectively) in 5% CO_2 at 37°C for the indicated time intervals. After incubation, lipids were extracted essentially according to the method described by Bligh and Dyer (30). Hp-EPA and hydroxy-EPA were then separated using reversed-phase HPLC as described in Reference 27. The peak area of hydroxy-EPA as percentage of the total peak area of hp-EPA and hydroxy-EPA combined was calculated. Results are given as mean of three experiments with SD indicated as bars.

Lipid peroxidation assay. The TBA test was used to measure the end products of lipid-peroxidation (31), mainly malondialdehyde (MDA). Cells were seeded at a concentration of 30,000 cells/mL (WEHI clone 13 cells) or 75,000 cells/mL (A-427 cells) in flasks (25 cm^2) with 4 mL medium. After 4 h, the medium was changed, and the cells were preincubated for 24 h with control medium or medium supplemented with antioxidant. The medium was then removed and control medium or medium supplemented with fatty acid added. After further 44 h, cells were killed by adding 2 mL 20% trichloroacetic acid to the flasks. Then 4 mL of 0.67% TBA was added, and the TBA test performed as previously described (32). Absorbance was converted to nmol MDA from a standard curve generated with 1,1,3,3,-tetramethoxypropane.

GSH assay. Cells were seeded at a concentration of 1.4×10^6 cells (A-427 cells) or 0.5×10^6 cells (WEHI clone 13 cells) in flasks (75 cm^2). After 4 h, medium was replaced with control medium or medium enriched with BSO (10 μM) or DEM (60 μM). After an additional 24 h of incubation, GSH analysis was performed as described below in one of the parallel flasks. In the remaining flasks, medium was changed to control medium or medium enriched with DHA (35 μM) or hp-DHA (35 μM). After a further 48 h of incubation, the cell layer was rinsed twice with cold phosphate buffered saline and harvested by scraping and then sonicated. The resulting homogenate was used as GSH source. Total GSH content was assayed using a GSH reductase assay (33). GSH content was quantitated by comparison with a standard curve generated with known amounts of GSH (reduced form), and expressed as nanomoles per milligram of protein.

Protein content was determined by the BioRad-assay (Bio-Rad Laboratories, Hercules, CA) using serum albumin as the standard.

Statistical analysis. If not otherwise stated, statistical analysis was carried out using one-way analysis of variance (ANOVA) with Student-Newman-Keuls method for multiple comparison or Kruskal-Wallis one-way ANOVA on ranks (when equal variance test failed), with a 0.05 level of significance. The analysis was performed using SigmaStat for Windows version 1.0 (Jandel Scientific Software, Jandel Corporation, Erkrath, Germany).

RESULTS

Effects of PUFA on growth of different malignant cell lines. The effects of the n-3 fatty acid DHA on growth of the murine fibrosarcoma cell line WEHI clone 13, the three human lung carcinoma cell lines A-427, A549 and SK-LU-1, and the human glioblastoma cell line A-172 are demonstrated in Figure 1. As previously shown (19), growth of the A-427 cell line was significantly inhibited in a time- and concentration-dependent manner by DHA, while DHA had little or no effect on growth of the other cell lines. Also γ-linolenic acid (γ-LNA) and arachidonic acid (AA) inhibited the growth of A-427 cells to approximately the same extent as DHA, while α-linolenic acid (α-LNA) and EPA had less effect (Fig. 2). The growth of the WEHI clone 13, SK-LU-1, A549, and A-172 cell lines were not affected by any of these fatty acids (results not shown).

Effects of the corresponding n-6-hydroperoxy fatty acids on growth of the malignant cell lines and the significance of growth media and sera. We have previously shown that lipid peroxidation is involved in the toxicity of DHA to A-427 cells (19). To investigate whether the toxicity of DHA could be explained by the primary lipid peroxidation product n-6 hp-DHA, we examined the effect of this product on different cell lines. Like the parent fatty acids, hp-DHA had no significant effect on growth of the SK-LU-1, A549 and A-172 cell lines (Fig. 3). The same was also the case for hp-γ-LNA, hp-AA, hp-α-LNA, and hp-EPA (results not shown). As previously shown (27), all the hp-fatty acids tested inhibited growth of WEHI clone 13 cells in a time- and concentration-dependent manner (Fig. 3, results shown for hp-DHA only).

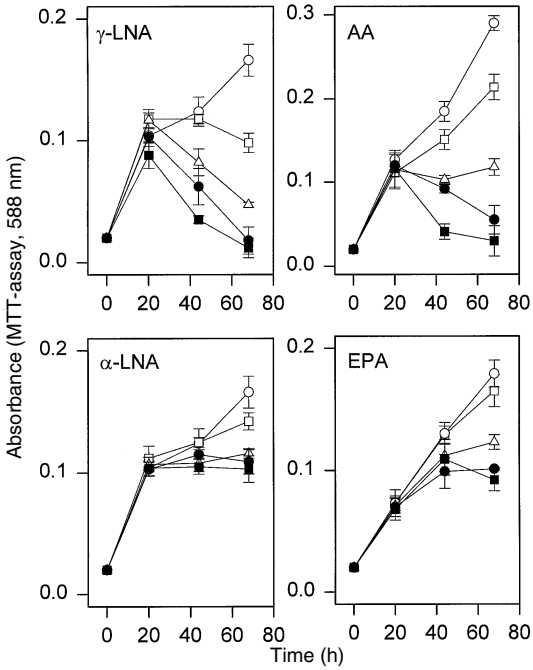

FIG. 2. Growth curves for the human lung carcinoma cell line A-427 cultured in the presence of different concentrations of γ-linolenic acid (γ–LNA), arachidonic acid (AA), α-LNA, or eicosapentaenoic acid (EPA). See Figure 1 for other abbreviations and symbol legend.

Somewhat surprisingly, A-427 cells seemed less sensitive to hp-DHA than to DHA (Figs. 1 and 3). Like hp-DHA, the other hydroperoxy fatty acids inhibited growth of A-427 cells only slightly, and significantly less than the corresponding parental fatty acid (results not shown). An exception was

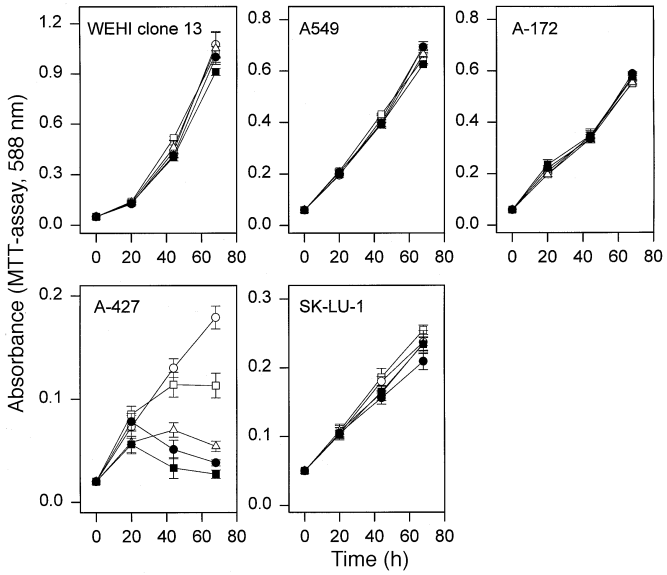

FIG. 1. Growth curves for the murine fibrosarcoma cell line WEHI clone 13, the three human lung carcinoma cell lines A-427, A549 and SK-LU-1, and the human glioblastoma cell line A-172 cultured in the presence of different concentrations of docosahexaenoic acid (DHA). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. ○, Control; □, 5 μM; △, 20 μM; ●, 35 μM; ■, 50 μM.

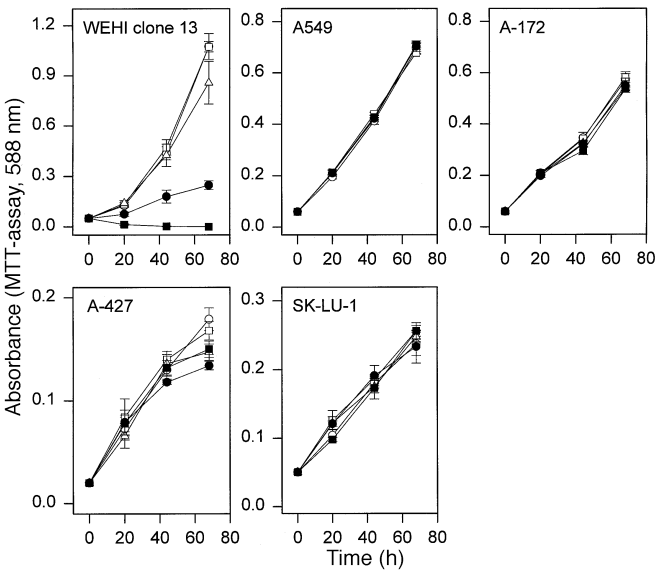

FIG. 3. Growth curves for the murine fibrosarcoma cell line WEHI clone 13, the three human lung carcinoma cell lines A-427, A549 and SK-LU-1, and the human glioblastoma cell line A-172 cultured in the presence of different concentrations of hydroperoxy (hp)-DHA. See Figure 1 for other abbreviations and symbol legend.

hp-α-LNA which inhibited growth of A-427 cells significantly more than the parental α-LNA in two out of four experiments (results not shown).

Also the degree of growth inhibition induced by parent fatty acid in A-427 cells showed some variation between experiments. We discovered that different batches of sera could explain much, if not all, of the variation in sensitivity between experiments due to a varying amount of selenium and/or α-tocopherol between batches (S. Schønberg, unpublished results). We also found that the synthetic part of the culture media was of importance for the growth inhibition observed. To investigate this in more detail, we compared the effect of DHA and hp-DHA on the growth of A-427 (DHA-sensitive) and WEHI clone 13 (hp-DHA sensitive) in complete MEM as well as complete RPMI medium using different batches of sera. The two batches of sera used in these experiments did not have any influence on the results (data not shown). Both the MEM and RPMI-medium used supported growth of the two cell lines to the same extent. We found that DHA and hp-DHA were considerably more toxic to A-427 cells when grown in RPMI medium than its recommended MEM, the effect of hp-DHA differing more than the effect of DHA (Fig. 4, results shown for hp-DHA only). Accordingly, hp-DHA was slightly more toxic to A-427 cells than DHA in RPMI medium (results not shown). Furthermore, hp-DHA was less toxic to WEHI clone 13 cells when grown in MEM instead of its standard RPMI medium (Fig. 4). Thus, when grown in the same medium, the effects of hp-DHA on the two cell lines were almost identical (Fig. 4). The hp-fatty acid was reduced to hydroxy fatty acid in a time-dependent way in both media (Fig. 5). However, the reduction was clearly faster in MEM compared to RPMI medium. Incubating hp-fatty acid in sterile water for 120 min only gave about 9% reduction (results not shown). In conclusion, A-427 cells and the WEHI-clone 13 cells are both highly sensitive to hp-DHA when cultured in RPMI- but not when cultured in MEM.

Effects of different antioxidants and GSH-modulating agents on DHA and hp-DHA-induced growth inhibition. To try to identify factors that may influence the toxicity of the parent fatty acids as well as the hp-fatty acids, we tested the effects of several antioxidants and GSH-modulating agents on the hp-DHA-induced growth inhibition in WEHI clone 13 cells and the DHA-induced growth inhibition in A-427 cells in their respective standard media. As shown in Figure 6, simultaneous addition of the GSH completely abolished the effect of hp-DHA, while GSH had little effect on DHA-induced growth inhibition. In contrast, the lipid-soluble, chain-breaking antioxidant α-tocopherol had no effect on hp-DHA-induced growth inhibition, but abolished the growth inhibitory effect of DHA almost completely (Fig. 6). The water-soluble antioxidant ascorbic acid also counteracted the DHA effect, while it potentiated the effect of hp-DHA (Fig. 6).

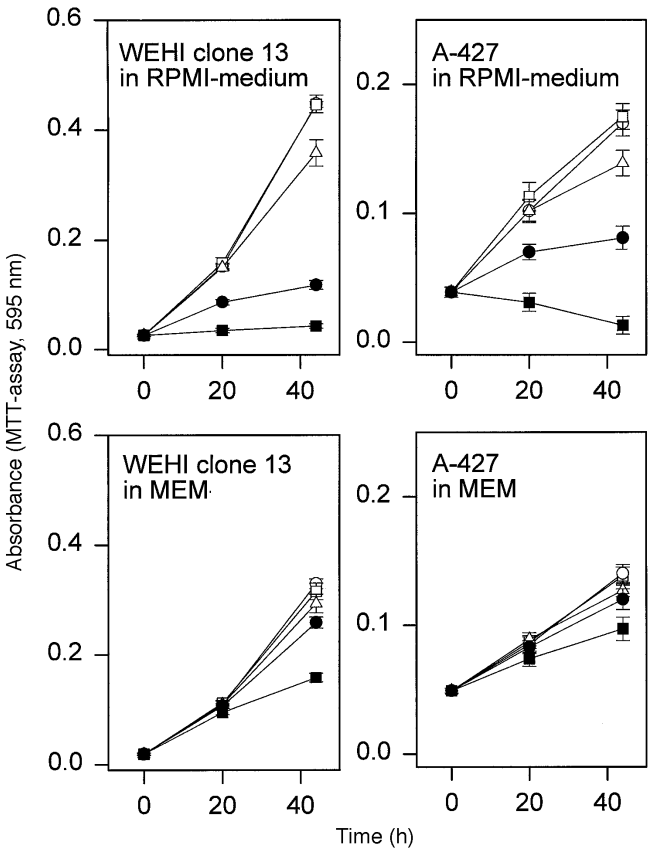

FIG. 4. Effect of different concentrations of hp-DHA on WEHI clone 13 cells and A-427 cells grown in both RPMI-based growth medium (used for the continuous culture of WEHI clone 13 cells) and minimum essential medium (MEM)-based growth medium (used for the continuous culture of A-427 and SK-LU-1 cells). See Figures 1 and 3 for other abbreviations. See Figure 1 for symbol legend.

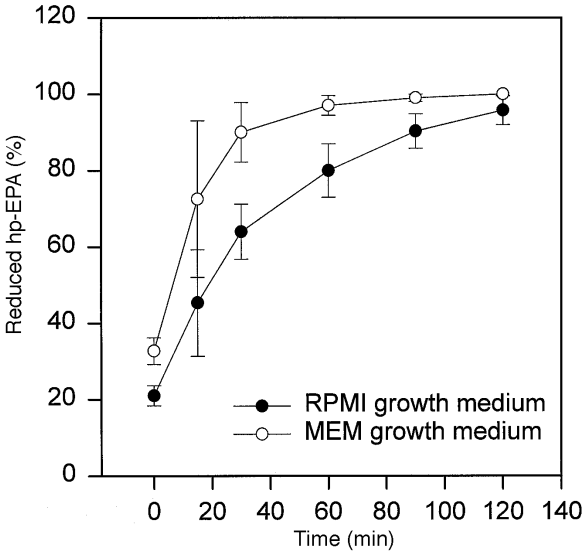

FIG. 5. Reduction of hp-EPA by growth media in the absence of cells. The hp-EPA was incubated with RPMI-based growth medium (used for the continuous culture of WEHI clone 13 cells) and MEM-based growth medium (used for the continuous culture of A-427 and SK-LU-1 cells) for the indicated time intervals. Lipids were then extracted, and amounts of hp-EPA and hydroxy-EPA were determined using reversed-phase high-performance liquid chromatography (HPLC). See Figures 2–4 for other abbreviations.

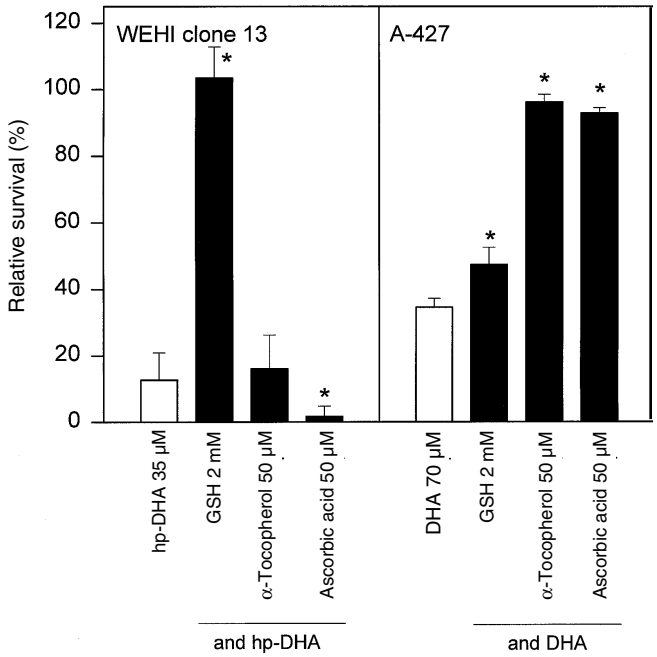

FIG. 6. Effect of various antioxidants on hp-DHA-induced (the WEHI clone 13 cell line) and DHA-induced (the A-427 cell line) growth inhibition. Fatty acid and antioxidant were added simultaneously, and cell survival was measured after 44 h using MTT assay. Relative survival was calculated as optical density (o.d.) (o.d. measured/o.d. control) × 100. *Significantly different from effects when cells are incubated with fatty acid alone. See Figures 1 and 3 for other abbreviations.

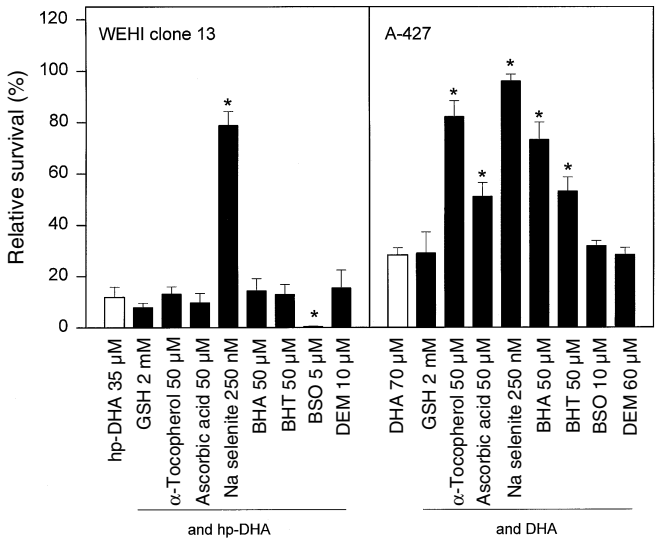

FIG. 7. Effect of preincubation with various antioxidants and GSH-modulating agents on hp-DHA-induced (the WEHI clone 13 cell line) and DHA-induced (the A-427 cell line) growth inhibition. Cells were preincubated for 24 h with the indicated additions before changing to medium containing hp-DHA (WEHI Clone 13) or DHA (A-427). Cell survival was measured after further 44 h using MTT assay. Relative survival was calculated as (o.d. measured/o.d. control) × 100. * Significantly different from effects when cells are incubated with fatty acid alone. See Figures 1, 3, and 6 for other abbreviations.

To further investigate the effects of antioxidant defense mechanisms, we preincubated the cells with antioxidants or GSH-modulating agents for 24 h before changing medium and challenging cells with DHA or hp-DHA for 44 h (Fig. 7). Preincubation with GSH had no effect on DHA or hp-DHA-induced toxicity, suggesting that the observed effect of GSH on hp-DHA-induced toxicity (Fig. 6) may be due to its ability to reduce the hp-fatty acid into the corresponding hydroxy fatty acid in the medium (27). We also investigated the importance of the intracellular GSH level and the GSH redox cycle on the growth inhibitory effect of DHA and hp-DHA by preincubating cells with BSO, a GSH synthesis inhibitor, and DEM, a GSH-depleting agent, as well as sodium selenite which has been shown to increase the activity of the selenium-dependent GSH peroxidase in A-427 cells (19). Figure 7 shows that preincubation with sodium selenite restored cell growth almost completely in A-427 cells, somewhat less in WEHI clone 13. BSO potentiated the effect of hp-DHA while the effect of DHA was unaffected.

Preincubation with α-tocopherol protected almost completely against DHA-induced toxicity in A-427 cells, while the cytotoxic effect of hp-DHA on WEHI clone 13 was unaffected. The same was true for the other lipid-soluble chain-breaking antioxidants that were included in this set of experiments, BHA and BHT, but to a lesser degree. In contrast, these lipid-soluble radical scavengers had no effect on hp-DHA-induced toxicity. Though to a lesser extent, preincubation with ascorbic acid still protected cells against DHA-induced toxicity, while the hp-DHA-induced toxicity was unaffected (Fig. 7).

Since we have shown that the effect of DHA, and in particular hp-DHA, depends on the synthetic part of the media, we also tested whether the media had any influence on the abolishing or potentiating effects of the antioxidants. This was done by incubating fatty acid in the presence of GSH, α-tocopherol, or ascorbic acid with A-427 cells for 44 h. To test the effect of antioxidants on hp-DHA-induced growth inhibition in MEM, we increased the hp-DHA concentration to 70 µM to obtain an effect. The effects of GSH and α-tocopherol on DHA-induced and hp-DHA-induced growth inhibition were the same in MEM and RPMI-medium. However, the effect of ascorbic acid varied depending on the growth medium, ascorbic acid being more protective in MEM than in RPMI medium (results not shown).

Effect of BSO and DEM on GSH level in A-427 and WEHI clone 13 cells. To further investigate the role of the intracellular GSH level for the cytotoxic effect of DHA and hp-DHA, we measured the GSH level after preincubation with BSO and DEM both before (Table 1) and after (Table 2) challenging cells with DHA or hp-DHA in their respective media. As shown in Table 1, after 24-h incubation, BSO reduced the GSH level in both A-427 and WEHI clone 13 cells. DEM also reduced the GSH level in WEHI clone 13 cells, but to a lesser extent than BSO. This difference may explain why preincubation with BSO, but not DEM, potentiated the hp-DHA effect in WEHI clone 13 cells. In contrast, DEM increased the GSH level in A-427 cells. An elevation in GSH level induced by DEM after an initial decrease has also been observed by

TABLE 1
Effect of 24-h Incubation with BSO and DEM on GSH Level in A-427 and WEHI Clone 13 Cells[a]

Cell line	Control	BSO (10 μM)	DEM (60 μM)
A-427	30.2 ± 2.2	9.2 ± 0.4*	40.4 ± 3.2*
WEHI clone 13	24.8 ± 3.7	9.2 ± 1.6*	15.5 ± 5.6*

[a]The values represent the mean ± SD of two separate experiments with each determination performed in at least triplicate, and are given as nmol GSH/mg protein. *Significantly different from control. BSO, L-buthionine-*S,R*-sulfoximine; DEM, diethyl maleate; GSH, glutathione.

others (34). Table 2 shows that when incubating with control medium for 24 h and then 48 h with the respective growth-inhibiting fatty acids, GSH levels in surviving cells of the two cell lines are not significantly different from the controls. When preincubating WEHI clone 13 cells with BSO, the GSH level in the surviving cells is still slightly reduced after 48-h incubation with hp-DHA (Table 2). When preincubating with DEM, the surviving WEHI clone 13 cells have regained normal GSH level after incubation with hp-DHA (Table 2). The GSH level in surviving A-427 cells when preincubating with DEM is not significantly different from the control, while GSH level when preincubating with BSO is still suppressed after 48-h incubation with DHA (Table 2). However, as shown in Figure 7, preincubating with BSO had no influence on DHA-induced toxicity, suggesting that the intracellular GSH level is of less importance for DHA-induced toxicity than hp-DHA-induced toxicity.

Effect of antioxidants on lipid peroxidation. To test whether the growth inhibitory effect of DHA and hp-DHA was correlated with an increase in lipid peroxidation products, we measured TBARS in A-427 and WEHI clone 13 cells incubated with DHA or hp-DHA in their respective media. When incubating cells with control medium only, TBARS were not detectable (Table 3). When incubating cells for 24 h with control medium and then 44 h with DHA or hp-DHA, the amount of TBARS increased, but was not significantly different either between cell type (using *T*-test) or between hp- and parent-fatty acid (using ANOVA, Table 3). This indicates that the amount of TBARS produced is not correlated to growth inhibition. We also tested whether the protective effects of some of the antioxidants were correlated to a reduction in lipid peroxidation products (Table 3). Preincubating with α-tocopherol protected A-427 cells against DHA-induced growth inhibition (Fig. 7), and also reduced the amount of TBARS after DHA exposure in these cells (Table 3). Preincubating WEHI clone 13 cells with α-tocopherol did not protect against hp-DHA-induced growth inhibition (Fig. 7) and had no effect on the TBARS level after hp-DHA exposure (Table 3). However, preincubating cells with sodium selenite, which protected both cell lines against growth inhibition (Fig. 7), had no effect on TBARS production in either of the cell lines after exposure to DHA or hp-DHA, again indicating that the amount of TBARS produced is not correlated to growth inhibition.

DISCUSSION

In an effort to elucidate mechanisms of toxicity of PUFA, we have studied the effects of n-3 and n-6 fatty acids and their hp derivatives on tumor cell lines. Our results indicate that only some tumor cell lines are appreciably sensitive to growth inhibition by PUFA. Further, the sensitivities vary not only depending on the properties of the cell line, but also depending upon medium composition. These results underline the importance of standardization of culture conditions when comparing sensitivities of different cell lines. However, the variation in sensitivities of cell lines in culture is not merely an *in vitro* culture phenomenon, since a correlation between *in vitro* and *in vivo* sensitivities has been documented (35).

Although it has become increasingly clear that lipid peroxidation induced by PUFA is involved in the cytotoxicity of such compounds to tumor cells (13–16), the molecular mechanisms have remained unclear. In agreement with reports from other laboratories (6,36), as well as recent work in our own laboratory (19), we have found that α-tocopherol essentially abolishes lipid peroxidation as well as the inhibitory effect of DHA on cell growth of A-427 cells. In addition, we found that ascorbic acid strongly reduces the toxicity of DHA to A-427 cells, possibly owing to recycling of α-tocopherol radicals to active α-tocopherol (37). The radical chain-breaking property of α-tocopherol prevents further formation of hp-PUFA (37). Consistent with this, ascorbic acid did not prevent the cytotoxic effect of hp-DHA, in fact, cytotoxicity was enhanced, the reason for which remains unclear. It is well known, however, that ascorbic acid may act both as a pro- and antioxidant depending on the given conditions. In the pres-

TABLE 2
Effect of 24-h Preincubation with BSO and DEM and Then 48-h Incubation with DHA or hp-DHA on GSH Level in A-427 and WEHI Clone 13 Cells, Respectively[a]

	Preincubation/incubation			
Cell line	Control/control	Control/fatty acid[b]	BSO 10 μM/fatty acid[b]	DEM 60 μM/fatty acid[b]
A-427	17.9 ± 1.7	16.4 ± 1.1	5.2 ± 0.5*	18.5 ± 2.9
WEHI clone 13	31.6 ± 4.6	38.2 ± 6.9	25.4 ± 5.2*	34.8 ± 2.0

[a]The values represent the mean ± SD of two separate experiments with each determination performed in at least triplicate, and are given as nmol GSH/mg protein.
[b]Docosahexaenoic acid (DHA) for A-427 and hydroperoxy (hp)-DHA for WEHI clone 13. *Significantly different from control/fatty acid. See Table 1 for other abbreviations.

TABLE 3
Lipid Peroxidation in Cells Supplemented with Antioxidant and/or Fatty Acid[a]

Agents added: preincubation/incubation	MDA (nmol/cell culture)	
	A-427	WEHI clone 13
Control/control	ND[b]	ND
Control/DHA	0.8 ± 0.2	1.0 ± 0.5
Control/hp-DHA	1.1 ± 0.3	1.2 ± 0.5
Sodium selenite/control	ND	ND
Sodium selenite/DHA	0.8 ± 0.2	—[c]
Sodium selenite/hp-DHA	—	1.0 ± 0.4
α-tocopherol/control	ND	ND
α-tocopherol/DHA	ND	—
α-tocopherol/hp-DHA	—	1.4 ± 0.1

[a]Cells were preincubated for 24 h with control medium or antioxidant before changing medium to medium with 35 μM DHA, 35 μM hp-DHA, or control medium as indicated. After a further 44 h, the TBA test was used to measure the end products of lipid peroxidation. The values represent the mean ± SD of two to four separate experiments each performed in duplicate or triplicate.
[b]ND, not detectable, which means <0.25 nmol.
[c]— Not performed. MDA, malondialdehyde. See Table 2 for other abbreviations.

ence of iron and hydroperoxide, it may function as a prooxidant by reducing ferric iron to ferrous iron which decompose hydroperoxide to alkoxyl radicals (37).

We have recently presented evidence that low levels of cellular Se-GSH peroxidase levels may explain the sensitivity to DHA of at least some tumor cell lines (19). Since hp-PUFA are known substrates for Se-GSH peroxidase (38), these results suggested that hp-DHA might be a major cytotoxic metabolite of DHA. Consistent with this, we have found that hp-DHA is highly toxic to both A-427 cells and WEHI clone 13 cells. Previously, an HIV-associated GSH peroxidase deficiency in transformed cultured cells was demonstrated to be associated with increased sensitivity and apoptotic cell death after exposure to 15-HPETE, probably as a result of reduced detoxification of 15-HPETE to 15-HETE (22). Together, these results indicate that Se-GSH peroxidase deficiency might be a common mechanism of cytotoxicity by PUFA. This is of considerable interest since tumor cells are frequently deficient in antioxidant defense systems (39,40). Although normal cells are generally insensitive to the cytotoxic effect of PUFA, the effects of hp-PUFA may not be restricted to transformed cells, since also normal endothelial cells in culture are injured by 15-HPETE (23). When cellular GSH levels are reduced by BSO, the susceptibility of these cells to 15-HPETE-induced injury is enhanced (41). These results are also consistent with our present observation that reduction of cellular GSH in tumor cells by BSO increases their sensitivity to hp-DHA. However, GSH depletion did not enhance the sensitivity of A-427 cells to DHA. This may indicate that the GSH level is less critical than the Se-GSH peroxidase activity, alternatively a part of the toxicity of DHA may be due to metabolites other than hp-DHA. Interestingly, WEHI clone 13 cells are essentially insensitive to DHA and other PUFA, but highly sensitive to hp-PUFA. This is somewhat intriguing since lipid peroxidation, measured as end point formation of TBARS, is essentially equal in WEHI clone 13 cells and the A-427 cells and since hp-PUFA are considered to be major intermediates in the lipid peroxidation process (20). We do not presently have a good explanation for this observation. It is possible, however, that this finding may be explained by kinetic differences in the lipid peroxidation process in the two cell lines leading to higher levels of certain transient intermediates, such as hp-DHA, in A-427 cells than in WEHI clone 13 cells.

Differences in the synthetic part of the media were apparently the reason for the lower sensitivity of cells to hp-fatty acids in MEM compared to RPMI medium, since in all experiments testing the growth inhibitory effect of fatty acids and their derivatives (Figs. 1–3), the different growth media were supplied with serum from the same batch. However, sera deficient in important antioxidants like vitamin E and selenium have been demonstrated to render cultured cells even more susceptible to oxidative damage (42). We also found that different batches of sera influenced the degree of cytotoxicity induced by different PUFA and hp-PUFA (R. Nøding, unpublished results). This emphasizes further the importance of a standardization of culture conditions when comparing effects of fatty acids on cultured cells. We found that preincubation with selenium almost totally restored growth in A-427 cells treated with DHA, and the toxicity of hp-DHA on WEHI clone 13 cells was also reduced, although to a lesser extent. The availability of selenium is known to regulate the activity of Se-GSH peroxidase (43). Consistent with this, we have previously reported that selenite added to the medium enhances Se-GSH peroxidase activity in A-427 cells (19) and this most likely explains the observed increase in resistance to both DHA and hp-DHA after addition of selenite to the culture medium.

The recovery of hp-fatty acid and hydroxy fatty acid was reduced with time up to about 60 min when incubated with the RPMI medium, while it remained at approximately the same level throughout the 120-min incubation period in the presence of MEM (results not shown). This may suggest that some of the increased effect of hp-DHA in RPMI medium is due to metabolites of hp-DHA that are too hydrophilic to be extracted with the lipids, or that have lost the conjugated double bond that gives the absorbance at 235 nm used to detect the hp- and hydroxy fatty acids.

In conclusion, our results demonstrate that tumor cell lines vary widely in their sensitivity to growth inhibition and cytotoxic effects of PUFA. This variation is apparently an intrinsic property of the cells and reflects their ability to withstand oxidative stress. Although lipid peroxidation products are clearly responsible for the cytotoxicity, variation in cytotoxicity of PUFA among cells in culture is not correlated to the degree of overall lipid peroxidation measured as the end point TBARS. Rather, the sensitivity is related to the ability of cells to detoxify specific peroxidation products, among which hp-PUFA are the most likely candidates. Furthermore, cytotoxicity of lipid peroxidation products depends upon culture conditions, underlining the importance of standardization of cul-

ture conditions when comparing the cytotoxicity of PUFA to different cell lines.

Gonzales *et al.* (13) showed that feeding fish oil to mice suppressed the growth of implanted breast carcinoma cells. They concluded that the growth suppression at least in part was a function of the accumulation of lipid peroxidation products. However, no clear association has been found between dietary intake of n-3 fatty acids and the incidence and staging of breast carcinoma in man (44–46). Differences between species could explain such discrepancies. However, the data presented here suggest that differences in the content of hydroperoxides and/or secondary peroxidation products could also explain some of the differences. Our data also suggest that when examining the effects of fats on carcinogenesis and cancer growth in experimental animal models, the content of hydroperoxides and secondary peroxidation products in the animal chow and oil supplements should be stated in addition to the fatty acid and total lipid content.

ACKNOWLEDGMENTS

The excellent technical assistance of Sylvia Nome Kvam and Merete Mack is gratefully acknowledged. We also thank Terje Espevik, Institute of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, Trondheim, for kindly providing the WEHI clone 13 cells. This work was supported by grants from The Norwegian Cancer Society, The Cancer Research Foundation at the University Hospital of Trondheim, The Novo Nordisk Foundation, and The Research Council of Norway.

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[Received July 9, 1997, and in final revised form and accepted January 22, 1998]

Cytotoxicity of Tocopherols and Their Quinones in Drug-Sensitive and Multidrug-Resistant Leukemia Cells

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ABSTRACT: Cytotoxicities of tocopherols (α-T, γ-T, δ-T), their para (α-TQ, γ-TQ, δ-TQ)- and ortho (Tocored)-quinone oxidation products, the synthetic quinone analog of γ-TQ containing a methyl group substituted for the phytyl side-chain (TMCQ) and the synthetic quinone analog of Tocored containing a methyl group substituted for the phytyl side-chain (PR) were measured in acute lymphoblastic leukemia cell lines that are drug-sensitive (CEM) and multidrug-resistant (CEM/VLB_{100}). Among tocopherols, only δ-T exhibited cytotoxicity. Among para quinones, α-TQ showed no cytotoxicity, while γ-TQ and δ-TQ were highly cytotoxic in both CEM and CEM/VLB_{100} cell lines (LD_{50} < 10 μM). δ-TQ and γ-TQ were more cytotoxic than the widely studied chemotherapeutic agent doxorubicin, which also showed selective cytotoxicity to CEM cells. The orthoquinone Tocored was less cytotoxic than doxorubicin in drug-sensitive cells but more cytotoxic than doxorubicin in multidrug-resistant cells. Cytotoxicity was not a function of the phytyl side-chain since both TMCQ and PR were cytotoxic in leukemia cells. Cytotoxic para and ortho quinones were electrophiles that formed adducts with nucleophilic thiol groups in glutathione and 2-mercaptoethanol. Cytotoxicity was enhanced when the glutathione pool was depleted by preincubation with buthionine-[*S,R*]-sulfoximine , but cytotoxicity was diminished by the addition of *N*-acetylcysteine to cultures. α-T also diminished the cytotoxicity of para- and orthoquinones. Buthionine-[*S,R*]-sulfoximine did not block the inhibitory effect of either *N*-acetylcysteine or α-T, showing that these agents did not act solely by maintaining the glutathione pool as an essential antioxidant system. In conclusion, tocopherylquinones represent a new class of alkylating electrophilic quinones that function as highly cytotoxic agents and escape multidrug resistance in acute lymphoblastic leukemia cell lines. *Lipids 33,* 295–301 (1998).

Tocopherols and tocotrienols have been studied extensively for their anticarcinogenic potential. Tocotrienols, unlike α-tocopherol (α-T), inhibit the proliferation of human breast cancer cells (1), and it was recently reported that only γ-tocotrienol and δ-tocotrienol exhibit this effect (2). We found in 1985 that γ-tocopheryl quinone (γ-TQ), a para quinone metabolite of γ-tocopherol (γ-T) (Fig. 1), was highly cytotoxic in smooth muscle cells, whereas α-T, γ-T and α-TQ did not exhibit this effect (3). We recently proposed that several quinone metabolites of the essential nutrient tocopherol family might have anticarcinogenic potential and, as naturally occurring compounds, these quinones might escape the immune surveillance imposed on xenobiotic quinones such as the chemotherapeutic agents doxorubicin and vinblastine by P-glycoprotein expression in multidrug-resistant tumors (4). This hypothesis was tested with γ-TQ, doxorubicin, and vinblastine in drug-sensitive acute lymphoblastic leukemia cell lines (CEM) and multidrug-resistant lymphoblastic leukemia cell lines (CEM/VLB_{100}), and we found that γ-TQ was highly cytotoxic in both CEM and CEM/VLB_{100} cells (4). We have now expanded these studies to compare parent tocopherols (α-T, γ-T, δ-T), para tocopheryl quinones (α-TQ, γ-TQ, δ-TQ), an ortho quinone 2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)-chroman-5,6-dione (Tocored), and the methyl side-chain analogs 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (TMCQ) and 2,2,7,8-tetramethylchroman-5,6-dione (PR) (Fig. 1).

Quinone cytotoxicity is explained either by the formation of reactive oxidant species through quinone redox cycling or by the formation of adducts between electrophilic quinones that act as alkylating agents (Fig. 1) and nucleophilic groups on proteins and DNA (4–8). In the present investigation, we examine the ability of para- and ortho tocopheryl quinones to form nucleophilic adducts with glutathione and 2-mercaptoethanol.

Quinone cytotoxicity is modified by intracellular glutathione, which functions both as a component of an essential antioxidant system and as a detoxifying nucleophile that blocks the formation of cytotoxic quinone adducts with nucleophilic groups on proteins and DNA (4–10). We have therefore examined the potential of a synergistic effect on quinone cytotoxicity with buthionine-[*S,R*]-sulfoximine (BSO), which depletes the intracellular glutathione pool by inhibiting γ-glutamyl-cysteine synthetase, the enzyme that catalyzes the rate-

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Abbreviations: ANOVA, analysis of variance; BSO, buthionine-[*S,R*]-sulfoximine; CEM, drug-sensitive lymphoblastic leukemia cells; CEM/VLB_{100}, multidrug-resistant lymphoblastic leukemia cells; FAB MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PR, 2,2,7,8-tetramethylchroman-5,6-dione; α-T, δ-T, and γ-T, α-, δ-, and γ-tocopherol; TLC, thin-layer chromatography; TMCQ, 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone; Tocored, 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-chroman-5,6-dione; TQ, tocopheryl quinone; α-TQ, γ-TQ, and δ-TQ, α-, γ-, and δ-tocopheryl quinone.

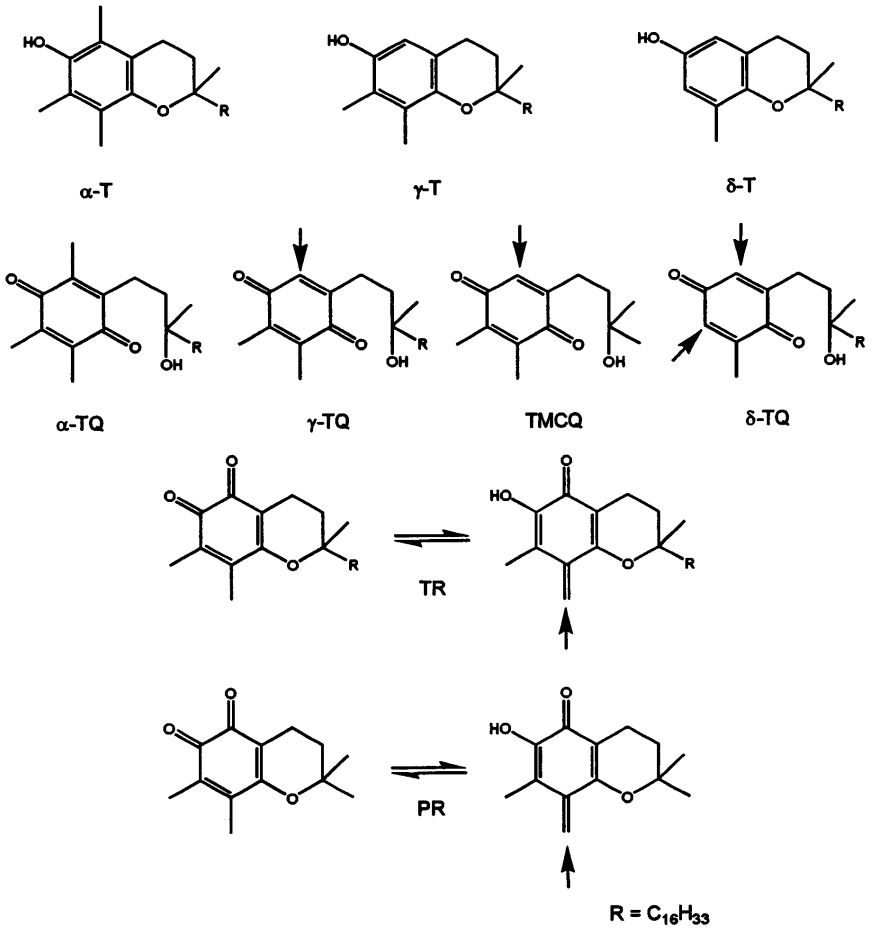

FIG. 1. Structural formulas for quinones and tautomeric methides showing (arrows) positions available for nucleophile addition.

limiting step in glutathione synthesis (11). We have also examined the potential of an antagonistic effect on quinone cytotoxicity with *N*-acetylcysteine (NAC), which may maintain the glutathione pool or function directly as a detoxifying nucleophile. Finally, we have examined the effect of the antioxidant α-T on the cytotoxicity of γ-TQ, δ-TQ, and PR.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: vinblastine from Lilly (Indianapolis, IN); doxorubicin from Cetus (Emeryville, CA); glutathione from Aldrich (Milwaukee, WI); (*d*)-δ-T (91.2%, isolated from soybean oil and containing a minor component, 5.6%, tentatively identified as γ-T), NAC, BSO, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (St. Louis, MO); (*d*)-α-T (99.6%) from Mann Research (New York, NY); (*d*)-γ-T (92.6%) from Tama (Tokyo, Japan).

Synthesis of tocopheryl quinones. α-TQ and γ-TQ were synthesized by oxidation with $FeCl_3$ or $AuCl_3$ from parent tocopherols as previously described (4). δ-TQ was synthesized by a modification of the general $AgNO_3$ oxidation procedure for tocopherols (12). δ-T, 130 mg, and 920 mg of $AgNO_3$ were dissolved in 6 mL of ethanol/water (85:15, vol/vol), heated at 60–70°C for 30 min, and the products extracted with diethyl ether. Yields were low in the absence of water, and overoxidation to red compounds, presumably ortho quinones, occurred when the water content was increased. δ-TQ was purified (yield 30%) on a silica gel 60 column using petroleum ether (bp 35–60°C)/diethyl ether (1:1, vol/vol). Fractions were monitored on a silica gel 60 thin-layer chromatography (TLC) plate (R_f α-TQ, 0.460; γ-TQ, 0.408; δ-TQ, 0.330). δ-TQ was identified by an ^1H nuclear magnetic resonance spectrum ($CDCl_3$, 250 MHz) characteristic of a paratocopheryl quinone (4) containing two aromatic protons at 6.35 ppm. Fast atom bombardment mass spectrometry (FAB MS) *m/z* [relative intensity] showed 418 $[M]^+$ [5], 402 [100], 401 [24] and no higher mass fragments. Tocored, PR and TMCQ were prepared as previously described (13,14).

Synthesis of thiol adducts. A modification of the procedure used previously with γ-TQ and glutathione (4) was adapted for sequential Michael addition and oxidation using δ-TQ and glutathione (15). δ-TQ, 0.4 mmol, was dissolved in methanol, cooled to 0°C, and 0.6 mmol glutathione in 1.2 mL water added dropwise with stirring. A yellow-brown precipitate formed initially and then changed to a white precipitate which dissolved in 1 mL water. Additional δ-TQ, 0.4 mmol dissolved in 2.5 mL methanol, was added dropwise with stirring.

Methanol was removed, the precipitate dissolved in water, reprecipitated overnight with acetone, then redissolved in water. The products were separated by preparative high-performance liquid chromatography (HPLC) on a Vydac C_4 column as described previously for the glutathione adduct of γ-TQ (4), and individual fractions identified by FAB MS.

A procedure described for the synthesis of a benzo[α]pyrene-7,8-quinone thiol adduct (16) was adapted for the synthesis of a PR/2-mercaptoethanol adduct. 2-Mercaptoethanol, 20 μL, was added to a 10-mL solution of 1.6 mg PR dissolved in 50 mM phosphate buffer (pH 7) containing 8% dimethyl sulfoxide. The reaction mixture was analyzed by TLC on silica gel 60 using diethyl ether/petroleum ether (3:1, vol/vol) and by FAB MS.

Cells. Cell lines were purchased from CENTOCOR (Malvern, PA). The multidrug-resistant cell line, CEM/VLB_{100}, is derived from the drug-sensitive CEM line and has the classic multidrug-resistant phenotype of overexpression of the *mdrl* gene and its product P-glycoprotein (4). Cells were grown from frozen stocks (−140°C) in suspension cultures or spinner flasks with medium containing RPMI 1640, 10% fetal bovine serum (heat-inactivated), 200 mM L-glutamine, and 0.5 μg/mL penicillin–streptomycin, at 37°C and 5% CO_2. The viability of cells at the start of the experiment was greater than 95% by Trypan blue exclusion.

Cytotoxicity studies were carried out in 96-well microtiter plates with 1×10^5 cells/culture well in 200 μL of complete medium. Tocopherols and quinones were dissolved in ethanol, diluted 1:6 with medium, and 12 μL added to each well. Other vehicles for addition such as incorporation into lipoproteins or liposomes were not explored. Control cultures received diluted ethanol alone with no effect on viability. Doxorubicin was dissolved in 0.9% saline, NAC was dissolved in medium (pH adjusted to 7.4), and BSO was dissolved in phosphate-buffered saline. Cultures were incubated for 24 h. Some cultures were preincubated with BSO for 18 h.

Viability assays. Cell viability was measured by the absorbance of blue Formazan produced in viable cells from the reduction of MTT by mitochondrial dehydrogenases (4,17,18), and cytotoxicity was estimated as diminished viability (decreased Formazan absorbance). MTT is used routinely in studies on the effects of BSO and NAC on quinone cytotoxicity (9). Cells grown in spinner flasks grew vigorously and generated almost twice as much Formazan in the MTT assay as cells grown in suspension cultures. However, the same patterns of relative toxicity were obtained with both cell preparations. Each data point represents the absorbance from a single culture well; 6–12 replicate cultures per dose were used in each experiment. MTT was dissolved in phosphate-buffered saline (5 mg/mL), added to cell suspensions (100 μg/well), and incubated for 4 h. The supernatant was removed, Formazan precipitate dissolved in dimethyl sulfoxide, and absorbance measured by dual filter at either 490 or 570 nm, reference filter 630 nm, using a microplate reader (Dynatech MR 7000, Chantilly, VA). Tocopherols, quinones, and other agents did not reduce MTT to Formazan in the absence of viable CEM and CEM/VLB_{100} cells.

The validity of the MTT assay for viability in cultures treated with TQ was established in a previous study by a comparison with the Neutral Red assay (4), and confirmed in the present study by cell counts (hemocytometer) of viable cells (Trypan blue exclusion). Data (mean ± SD as percentage of control) for cell count and Formazan absorbance, respectively, were: 10 μM α-T, 100 ± 12 and 100 ± 4; 50 μM α-TQ, 84 ± 15 and 83 ± 6; 1 μM γ-TQ, 93 ± 7 and 90 ± 14; 5 μM γ-TQ, 46 ± 12 and 27 ± 18; 10 μM γ-TQ, 8 ± 5 and 9 ± 5.

Statistics. Data from 3 to 10 experiments per treatment protocol were analyzed. Main effects for cell line, agent, and concentration and their interactions were calculated by two- and three-way analyses of variance (ANOVA). *Post-hoc* comparisons where appropriate used the highly conservative Scheffe test.

RESULTS

Cytotoxicity of tocopherols and of tocopheryl quinones compared to doxorubicin. The cytotoxicity of tocopherols was compared in drug-sensitive CEM and multidrug-resistant CEM/VLB_{100} leukemia cell lines using the MTT assay. Among parent tocopherols, only δ-T showed dose-dependent cytotoxicity and was cytotoxic in both cell lines (Table 1).

The cytotoxicity of TQ was then compared to that of doxorubicin in the drug-sensitive CEM and multidrug-resistant CEM/VLB_{100} leukemia cell lines. As expected, doxorubicin was more cytotoxic in CEM than CEM/VLB_{100} cells (Table 2). In contrast to doxorubicin, however, γ-TQ, δ-TQ, Tocored, and PR were equally cytotoxic in both the drug-sensitive and multidrug-resistant CEM cell lines in a dose-dependent manner. Unlike doxorubicin or the other TQ, α-TQ was not cytotoxic in either cell line. Analysis of the dose–response data showed that γ-TQ and δ-TQ were highly cytotoxic (LD_{50} between 1 and 10 μM) and that 1 μM δ-TQ was more cytotoxic than 1 μM γ-TQ.

The ortho quinone Tocored and its methyl side-chain analog PR were cytotoxic in both cell lines (Table 2). Tocored was less cytotoxic than either γ-TQ or δ-TQ. The difference in cytotoxicity between Tocored and PR compared to α-TQ was particularly striking in that neither ortho quinones nor α-TQ forms classic Michael adducts with nucleophiles. However, ortho quinones will form tautomeric quinone methides, and these reactive compounds may form adducts with nucleophiles; structural formulas for these quinones and tautomeric quinone methides showing (arrows) positions available for nucleophile addition are shown in Figure 1. PR (methyl side-chain) was more cytotoxic than Tocored (phytyl side-chain) (Table 2). The reason for this difference was not apparent since we found in another experiment (data not shown) that TMCQ, the methyl side-chain analog of γ-TQ (Fig. 1) was somewhat less cytotoxic than γ-TQ (F 15.01, P 0.0002) with a structure/concentration interaction (F 11.00, $P < 0.0001$) only at the 10 μM concentration.

Synthesis of nucleophile adducts of para- and ortho-quinones. We have synthesized monoglutathion-*S*-yl adducts of menadione (5) and γ-TQ (4) and shown that these adducts

TABLE 1
Effects of Tocopherols on Viability[a] in CEM and CEM/VLB$_{100}$ Leukemia Cells

	Tocopherol concentration (μM)							
	1	10	50	100	1	10	50	100
Agent	CEM				CEM/VLB$_{100}$			
α-T[b]	97 ± 4	100 ± 4	81 ± 8	110 ± 10	90 ± 12	92 ± 2	106 ± 19	119 ± 2
γ-T[b]	100 ± 7	97 ± 7	106 ± 6	110 ± 13	85 ± 9	88 ± 10	82 ± 13	88 ± 10
δ-T[b]	94 ± 9	100 ± 8	96 ± 7	84 ± 7	76 ± 18	89 ± 7	69 ± 24	56 ± 26

[a]Viability estimated as relative Formazan absorbance (mean ± S.D. in percentage). Cytotoxicity is reciprocal of viability.
[b]Three-way analysis of variance (ANOVA) (213 points) showed: cytotoxicity dose-dependent (F 6.00, P 0.0001); cytotoxicity structure-dependent (F 21.55, $P < 0.0001$) with δ-T cytotoxicity greater than either α-T or γ-T ($P < 0.001$), which did not differ from each other. Abbreviations: CEM, drug-sensitive lymphoblastic leukemia cells; CEM/VLB$_{100}$, multidrug-resistant lymphoblastic leukemia cells; α-T, γ-T, and δ-T, α-, γ-, and δ-tocopherol.

are, like other fully substituted glutathion-*S*-yl quinones (15), not cytotoxic agents. Studies with quinone adducts (6–8,15,19) predict that δ-TQ, with one less methyl group substitution than γ-TQ, will react even more rapidly than γ-TQ with thiols. The reactivity of δ-TQ was similar to 1,4-benzoquinone (15), with δ-TQ in excess glutathione depleting δ-TQ and rapidly forming a derivative with the appropriate molecular weight (FAB MS) for the monosodium salt of the monoglutathion-*S*-yl hydroquinone, *m/z* 748 $[M + H]^+$. The monoglutathion-*S*-yl hydroquinone was reoxidized by slowly adding excess δ-TQ, and a single product separated by HPLC. The product had the appropriate molecular weight (FAB MS) for the diglutathion-*S*-yl hydroquinone, *m/z* 1031 $[M + H]^+$. Purified hydroquinones did not reoxidize on standing.

Ortho quinones are in equilibrium with their tautomeric quinone methides (Fig. 1), which may both dimerize and form adducts with nucleophiles (7,8,16). Tocored is a reactive quinone that decomposed in alcohol solution to several compounds visualized on TLC (R_f 0.69, 0.53). PR dimers had been described previously (13). A new product (R_f 0.27) was formed when PR (R_f 0.52) reacted with 2-mercaptoethanol. FAB MS identified the hydroquinone adduct of PR and 2-mercaptoethanol, *m/z* 299 $[M + H]^+$. These studies showed that cytotoxic para- and ortho tocopheryl quinones shared a common property in their ability to react with thiol nucleophiles.

Thiol status and cytotoxicity. BSO alone had no effect on the viability of CEM cells. Data (mean ± SD as percentage of control) for Formazan absorbance were: media alone, 100 ± 5.7; 50 μM BSO, 102 ± 6.6. BSO increased the cytotoxicity of several TQ in CEM cell cultures (Table 3). Increasing concentrations of BSO enhanced the cytotoxicity of both γ-TQ and δ-TQ in the middle range of the quinone doses tested. δ-TQ was more cytotoxic than γ-TQ (Table 2), and the maximum BSO effect was found with 10 μM γ-TQ and 5 μm δ-TQ. Similarly, PR was less cytotoxic than para quinones (Table 2), and the maximal effect with BSO was found with 50 μM PR. As expected, BSO had no effect when the quinone concentrations were lower than their cytotoxic levels, nor did BSO add to the

TABLE 2
Effects of Doxorubicin (DOX) and Tocopheryl Quinones (TQ) on Viability[a] in CEM and CEM/VLB$_{100}$ Leukemia Cells

	Concentration (μM)							
	1	10	50	100	1	10	50	100
Quinone	CEM				CEM/VLB$_{100}$			
DOX[b]	60 ± 16	48 ± 19	28 ± 16	22 ± 15	100 ± 14	94 ± 20	78 ± 17	59 ± 19
α-TQ[c]	90 ± 8	85 ± 14	83 ± 6	76 ± 4	87 ± 2	93 ± 5	83 ± 3	86 ± 1
γ-TQ[c]	90 ± 14	15 ± 8	9 ± 3	8 ± 4	86 ± 18	17 ± 4	16 ± 4	13 ± 5
δ-TQ[c]	74 ± 17	14 ± 5	11 ± 2	14 ± 4	64 ± 9	21 ± 2	13 ± 2	15 ± 2
Tocored[d]	113 ± 23	86 ± 11	47 ± 6	22 ± 9	92 ± 5	98 ± 8	66 ± 8	25 ± 3
PR[d]	108 ± 28	23 ± 17	6 ± 1	7 ± 2	77 ± 12	13 ± 4	6 ± 2	8 ± 1

[a]See Table 1 for this footnote.
[b]Two-way ANOVA (187 points) showed: DOX more cytotoxic in CEM than CEM/VLB$_{100}$ cells (F 61.43, $P < 0.0001$); cytotoxicity dose-dependent (F 74.09, $P < 0.0001$); cell line/dose interaction (F 19.54, $P < 0.0001$) with DOX cytotoxicity greater in CEM at 10, 50, and 100 μM.
[c]Three-way ANOVA (284 points) showed: cytotoxicity dose-dependent (F 603.31, $P < 0.0001$); cytotoxicity structure-dependent (F 357.69, $P < 0.0001$); structure/dose interaction (F 76.96, $P < 0.0001$) with δ-TQ more cytotoxic than γ-TQ and α-TQ at 1 μM, δ-TQ and γ-TQ more cytotoxic than α-TQ at all other doses, and no difference in α-TQ cytotoxicity at all doses.
[d]Three-way ANOVA (96 points) showed: quinone cytotoxicity not affected by cell line (F 1.26, P 0.2656); cytotoxicity dose-dependent (F 167.63, $P < 0.0001$); cytotoxicity structure-dependent (F 126.80, $P < 0.0001$) with PR more cytotoxic than TR; structure/dose interaction (F 27.21, $P < 0.0001$) with 50 and 100 μM TR differing from medium and each other ($P < 0.01$), and 10, 50 and 100 μM PR differing from medium but not each other ($P < 0.01$). PR, 2,2,7,8-tetramethylchroman-5,6-dione; Tocored, 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-chroman-5,6-dione. For other abbreviations see Table 1.

TABLE 3
Buthionine-[*S,R*]-sulfoximine (BSO) Diminishes Viability[a] in CEM Cells Treated with TQ

	Quinone (μM)				
BSO (μM)	1	5	10	50	100
			α-TQ[b]		
0	100 ± 8	104 ± 8	102 ± 6	75 ± 13	71 ± 7
0.5	99 ± 11	99 ± 8	104 ± 5	77 ± 8	76 ± 6
5.0	99 ± 10	99 ± 8	103 ± 8	75 ± 9	76 ± 5
50.0	96 ± 7	104 ± 5	99 ± 3	71 ± 12	71 ± 7
			γ-TQ[c]		
0	103 ± 6	95 ± 9	64 ± 8	6 ± 1	
0.5	100 ± 8	92 ± 8	61 ± 6	3 ± 2	
5.0	99 ± 2	86 ± 12	50 ± 8	5 ± 1	
50.0	106 ± 4	87 ± 10	36 ± 13	5 ± 1	
			δ-TQ[d]		
0	101 ± 6	55 ± 18	11 ± 2	9 ± 1	
0.5	102 ± 6	42 ± 12	9 ± 3	10 ± 1	
5.0	100 ± 7	33 ± 11	10 ± 2	11 ± 1	
50.0	101 ± 7	29 ± 12	9 ± 3	10 ± 1	
			PR[e]		
0		102 ± 8	99 ± 6	82 ± 16	16 ± 9
0.5		98 ± 6	103 ± 7	53 ± 32	10 ± 6
5.0		95 ± 13	102 ± 4	25 ± 17	6 ± 1
50.0		96 ± 14	106 ± 4	8 ± 2	6 ± 1

[a]See Table 1 for this footnote.
[b]Two-way ANOVA (198 points) showed: cytotoxicity not affected by BSO (F 1.02, P 0.3862) and no α-TQ/BSO interaction (F 0.51, P 0.9066).
[c]Two-way ANOVA (370 points) showed: cytotoxicity enhanced by BSO (F 8.28, P <0.0001); a γ-TQ/BSO interaction (F 7.46, P < 0.0001) with 5 and 50 μM BSO affecting 10 μM γ-TQ.
[d]Two-way ANOVA (715 points) showed: cytotoxicity enhanced by BSO (F 6.07, P 0.0004); a δ-TQ/BSO interaction (F 16.66, P < 0.0001) with 0.5, 5, and 50 μM BSO affecting 5 μM δ-TQ.
[e]Two-way ANOVA (205 points) showed: cytotoxicity enhanced by BSO (F 26.60, P < 0.0001); a PR/BSO interaction (F 21.74, P < 0.0001) with 0.5, 5, and 50 μM BSO affecting 50 μM PR. For abbreviations see Tables 1 and 2.

cytotoxicity of the quinone when very high cytotoxic concentrations of quinone were tested. Unlike γ-TQ and δ-TQ, α-TQ was not cytotoxic (Table 2) and 50 μM BSO had no effect on cell viability with α-TQ even at a 100-μM α-TQ concentration.

NAC alone had no effect on the viability of CEM cells. Data (mean ± SD as percentage of control) for Formazan absorbance were: media alone, 100 ± 8.4; 0.5 mM NAC, 103 ± 6.2; 1 mM NAC, 103 ± 8.1. NAC had an effect on the cytotoxicity of γ-TQ and δ-TQ in CEM cell cultures (Table 4). Increasing concentrations of NAC diminished the cytotoxicity of 5 μM γ-TQ and 2.5 μM δ-TQ. NAC had no effect on higher concentrations of these quinones, which were very cytotoxic. Another study (data not shown) found that 1 mM NAC decreased the cytotoxicity of 2.5 and 5 μM δ-TQ in CEM cell cultures pretreated with 50 μM BSO (F 80.89, $P > 0.0001$). NAC/BSO data are consistent with a mechanism involving the detoxification of alkylating quinones through the formation of quinone NAC adducts rather than simply maintaining the glutathione pool as an essential antioxidant system.

α-T status and cytotoxicity. α-T alone had no effect on viability (Table 1), but α-T had a profound effect on the cytotoxicity of TQ in CEM cultures. Increasing concentrations of

TABLE 4
***N*-Acetylcysteine (NAC) Enhances Viability[a] in CEM Cells Treated with TQ**

	Quinone (μM)			
NAC (mM)	2.5	5.0	10	50
		γ-TQ[b]		
0		40 ± 14	6 ± 2	4 ± 1
0.5		52 ± 10	8 ± 2	5 ± 1
1.0		72 ± 17	19 ± 9	8 ± 2
		δ-TQ[c]		
0	49 ± 12	23 ± 12	8 ± 4	4 ± 1
0.5	54 ± 9	20 ± 7	8 ± 4	8 ± 2
1.0	61 ± 8	24 ± 6	8 ± 4	12 ± 1

[a]See Table 1 for this footnote.
[b]Two-way ANOVA (412 points) showed: cytotoxicity diminished by NAC (F 94.10, $P < 0.0001$); a γ-TQ/NAC interaction (F 25.73, $P < 0.0001$) with 0.5 and 1 mM NAC affecting 5μM γ-TQ and 1 mM NAC affecting 10 μM γ-TQ.
[c]Two-way ANOVA (1011 points) showed: cytotoxicity diminished by NAC (F 43.42, $P < 0.0001$); a δ-TQ/NAC interaction (F 15.09, $P < 0.0001$) with 1 mM NAC affecting 2.5 and 50 μM δ-TQ. For abbreviations see Tables 1 and 2.

α-T diminished the cytotoxicity of both γ-TQ and δ-TQ (Table 5). α-T also diminished the cytotoxicity of δ-TQ (Table 5) in cells pretreated with 50 or 100 μM BSO. A final experiment (data not shown) found that 100 μM α-T also diminished the cytotoxicity of PR in cells pretreated with 50 μM BSO (F 22.11, $P < 0.0001$). Another study (data not shown) found that α-TQ, the oxidation product of α-T, had

TABLE 5
α-T Enhances Viability[a] in CEM Cells Pretreated with/without BSO and then Treated with TQ

	Quinone (μM)		
α-T (μM)	2.5	5	10
		γ-TQ[b]	
0		15 ± 10	6 ± 4
50		46 ± 9	10 ± 5
100		75 ± 11	44 ± 12
		δ-TQ[c]	
0	50 ± 17	19 ± 8	9 ± 4
50	66 ± 16	31 ± 12	11 ± 2
100	74 ± 19	42 ± 11	16 ± 3
		δ-TQ (50 μM BSO)[d]	
0	44 ± 9	14 ± 3	8 ± 3
100	80 ± 19	59 ± 20	23 ± 6
		δ-TQ (100 μM BSO)[d]	
0	36 ± 6	14 ± 1	9 ± 2
100	76 ± 9	27 ± 3	15 ± 1

[a]See Table 1 for this footnote.
[b]Two-way ANOVA (634 points) showed: cytotoxicity diminished by α-T (F 271.97, $P < 0.0001$); a γ-TQ/α-T interaction (F 39.08, $P < 0.0001$) with 50 μM α-T affecting 5 μM γ-TQ and 100 μM α-T affecting 5 and 10 μM γ-TQ.
[c]Two-way ANOVA (1068 points) showed: cytotoxicity diminished by α-T (F 263.26, $P < 0.0001$); a δ-TQ/α-T interaction (F 23.54, $P < 0.0001$) with 50 μM α-T affecting 2.5 and 5 μM δ-TQ and 100 μM α-T affecting 2.5, 5, and 10 μM δ-TQ.
[d]Cells preincubated with 50 or 100 μM BSO. Three-way ANOVA (889 points) showed: cytotoxicity enhanced by increasing concentration of BSO (F 72.83, $P < 0.0001$); cytotoxicity diminished by α-T (F 193.44, $P < 0.0001$); a δ-TQ/α-T interaction (F 159.38, $P < 0.0001$) with 100 μM α-T diminishing the cytotoxicity of 2.5 and 5 μM δ-TQ. For abbreviations see Tables 1–3.

no effect on δ-TQ (F 0.70, P 0.4966). α-T/BSO data show that α-T does not exert its effect simply by maintaining the glutathione pool as an essential antioxidant system.

DISCUSSION

Physiologic concentrations (<1 μM) of α-TQ have been found in serum (20). High or pharmacologic concentrations (>1 μM) of several TQ metabolites are cytotoxic in acute lymphoblastic leukemia cell lines, with cytotoxicity decreasing in the order δ-TQ > γ-TQ > Tocored. Furthermore, δ-TQ and γ-TQ are more cytotoxic in acute lymphoblastic leukemia cells than the widely studied chemotherapeutic agent doxorubicin. The comparison between TQ and doxorubicin is striking, with doxorubicin being much less cytotoxic in multidrug-resistant CEM/VLB_{100} cells. Our results with TQ identify a new class of cytotoxic quinones that escape multidrug resistance without the necessity of other agents and strategies to overcome the multidrug-resistance effect.

Trace amounts of a number of TQ and quinone oxides have been found in cells, tissues, and tissue cultures (20–25). Several early studies showed that both α-T and γ-T were metabolized to their para quinones after ingestion or intraperitoneal injection in rats (21–24). After 27 h, liver contained 4% of labeled α-T as α-TQ and 1% of labeled γ-T as γ-TQ. α-T is also metabolized to Tocored in cell cultures that generate nitric oxides (26,27). Studies on the chemotherapeutic potential of pharmacologic concentrations of many metabolites of tocopherol oxidation are indeed warranted.

The cytotoxic TQ are electrophilic alkylating agents that form Michael adducts and quinone methide adducts with nucleophilic thiol groups. We propose that alkylation reactions with electrophilic TQ contribute to their cytotoxicity. α-TQ is not an alkylating agent, nor is it cytotoxic in our cell lines. Reactive oxidant species may contribute to TQ cytotoxicity, but the large step-function between α-TQ and γ-TQ cytotoxicity and the small difference between γ-TQ and δ-TQ cytotoxicity are not predicted from the effect of reduction potentials alone on the generation of reactive oxidant species. TQ are a new and interesting series of compounds to be included in the continuing discussion of mechanisms for the cytotoxicity of antitumor quinones (4,6–8).

Data that show BSO enhances and that NAC inhibits TQ cytotoxicity are consistent with many studies on the detoxification of quinones through the formation of fully substituted adducts with the nucleophiles glutathione and NAC (4–10,15). Detoxification by cellular thiols would help to explain why physiologic concentrations of γ-TQ, δ-TQ and Tocored are not *in vivo* cytotoxic agents.

Data showing that α-T inhibits γ-TQ and δ-TQ cytotoxicity could be interpreted as evidence for reactive oxidant species cytotoxicity. However, TQ are reduced by NADH + H^+ and $FADH_2$ (14) and by DT-diaphorase [NAD(P)H: quinone-acceptor oxidoreductase; E.C. 1.6.99.2] (28), an enzyme induced by phenolic antioxidants (29). The induction of DT-diaphorase by α-T should be explored. Hydroquinones are not alkylating electrophiles, and α-T may induce the reduction of alkylating TQ or prevent the autoxidation of hydroquinones to alkylating quinones. The body retains α-T selectively (30). For example, the α-T/γ-T ratio ranges from 6.5 in human serum (31) to 70 in mouse liver (32), and the protective effect of α-T in tissue cultures is found at α-T/γ-T ratios in the 10 to 20 range (Table 5). High α-T/γ-T ratios may help to explain why physiologic concentrations of γ-TQ are not cytotoxic *in vivo*.

Our data show that δ-T is the only parent tocopherol that is cytotoxic in leukemia cells. δ-T is oxidized to the most cytotoxic alkylating quinone, δ-TQ, suggesting that δ-T cytotoxicity is the result of oxidation. Recent studies show that γ- and δ-tocotrienols, unlike α-tocotrienol, are cytotoxic in several tumor cell lines (1,2). These data could be explained by the oxidation of γ- and δ-tocotrienols to alkylating quinones. The biological properties of these quinones should be explored.

ACKNOWLEDGMENTS

We thank Eman Sadoun for technical support, Dr. Pravin Kaumaya for the HPLC separation of glutathione adducts, Dr. Larry Sachs for statistical analyses, Dr. Pierre-Marie Robitaille for generous support, and Dr. Rex Munday for helpful discussions.

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[Received September 22, 1997, and in revised form and accepted February 9, 1998]

Conversion of Palmitate to Unsaturated Fatty Acids Differs in a *Neurospora crassa* Mutant with Impaired Fatty Acid Synthase Activity

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ABSTRACT: The *Neurospora crassa cel* (fatty acid *c*hain *el*ongation) mutant has impaired fatty acid synthase activity. The *cel* mutant requires exogenous 16:0 for growth and converts 16:0 to other fatty acids. In contrast to wild-type *N. crassa*, which converted only 42% of the exogenous [7,7,8,8-$^2H_4$]16:0 that was incorporated into cell lipids to unsaturated fatty acids, *cel* converted 72%. In addition, *cel* contains higher levels of 18:3$^{\Delta 9,12,15}$ than wild-type, and synthesizes two fatty acids, 20:2$^{\Delta 11,14}$ and 20:3$^{\Delta 11,14,17}$, found at only trace levels in wild-type. Thus, the Δ15-desaturase activity and elongation activity on 18-carbon polyunsaturated fatty acids are higher for *cel* than wild-type. This altered metabolism of exogenous 16:0 may be directly due to impaired flux through the endogenous fatty acid biosynthetic pathway, or may result from altered regulation of the synthesis of unsaturated fatty acids in the mutant.
Lipids 33, 303–306 (1998).

The filamentous fungus *Neurospora crassa* is a model system for the study of fatty acid metabolism. Several mutants in the fatty acid biosynthetic pathway exist; one such mutant is *cel* (fatty acid *c*hain *el*ongation). The *N. crassa cel* mutant has a defective cytosolic fatty acid synthase complex, with the complex containing only 2% of the wild-type content of 4′-phosphopantetheine prosthetic groups (1). The *cel* mutant synthesizes only minor amounts of fatty acid *de novo*, and requires exogenous fatty acids for normal growth. The optimal supplement is 16:0, an end-product of the fatty acid synthase, which allows near wild-type growth rates (2). In a key study using *cel*, fatty acid biosynthesis was first demonstrated to also occur in mitochondria by a prokaryotic-type fatty acid biosynthetic complex utilizing acyl carrier protein (3), a finding later confirmed (4).

In wild-type *N. crassa*, exogenous 16:0 is utilized only to a limited extent for the synthesis of unsaturated fatty acids (5). Previous studies of *cel* have given conflicting reports on the proportion of unsaturates in the mutant (2,6). If the fatty acid composition of *cel* is similar to wild-type *N. crassa*, it is likely to differ from wild-type in metabolism of exogenous 16:0 to unsaturated fatty acids. This study was undertaken to compare *cel* to wild-type in fatty acid composition and exogenous 16:0 metabolism.

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EXPERIMENTAL PROCEDURES

Neurospora crassa strains were obtained from the Fungal Genetics Stock Center (Kansas City, KS). Conidia (1×10^5) of wild-type strain 74-OR8-1 *a* or the mutant strain *cel a* (FGSC #819) were inoculated into liquid Vogel's medium N containing 30 μM [7,7,8,8-$^2H_4$]16:0 (98%; Cambridge Isotope Laboratories, Andover, MA). Cultures were incubated for 48 h at 34°C, with a single change of medium 24 h after inoculation, as described (5).

Cultures were harvested by filtration, lipids extracted, and fatty acids analyzed as previously described (7). Gas chromatography–mass spectrometry was used to identify fatty acid derivatives (20:2$^{\Delta 11,14}$ and 20:3$^{\Delta 11,14,17}$) and to determine the metabolic fate of the [7,7,8,8-$^2H_4$]16:0 (5). The percentage of each fatty acid which was deuterated was obtained from selective ion monitoring mass spectrometry data and, with the relative mass of each fatty acid, was used to calculate the percentage of total fatty acid which was derived *de novo* and from the supplement.

RESULTS AND DISCUSSION

The major fatty acids synthesized by vegetative cultures of wild-type *N. crassa* are 16:0, 18:0, 18:1$^{\Delta 9}$, 18:2$^{\Delta 9,12}$, and 18:3$^{\Delta 9,12,15}$. *Neurospora crassa* grown with supplemental [$^2H_4$]16:0 for 2 d had a typical fatty acid composition profile (composite of Fig. 1A and B, described below). Fatty acid profiles of *cel* grown with [$^2H_4$]16:0 differed from wild-type; *cel* had higher levels of 18:3 (26% of total fatty acids, compared to 6% in wild-type) and of total polyunsaturates (70%, compared to 57%). In addition, 20:2$^{\Delta 11,14}$ and 20:3$^{\Delta 11,14,17}$ made up 5% of the total fatty acid mass in *cel*, but these were detectable at only trace levels in wild-type.

By incubating the cultures with [$^2H_4$]16:0 throughout the culturing period, we were able to determine the fraction of fatty acids synthesized both *de novo* and from the deuterated

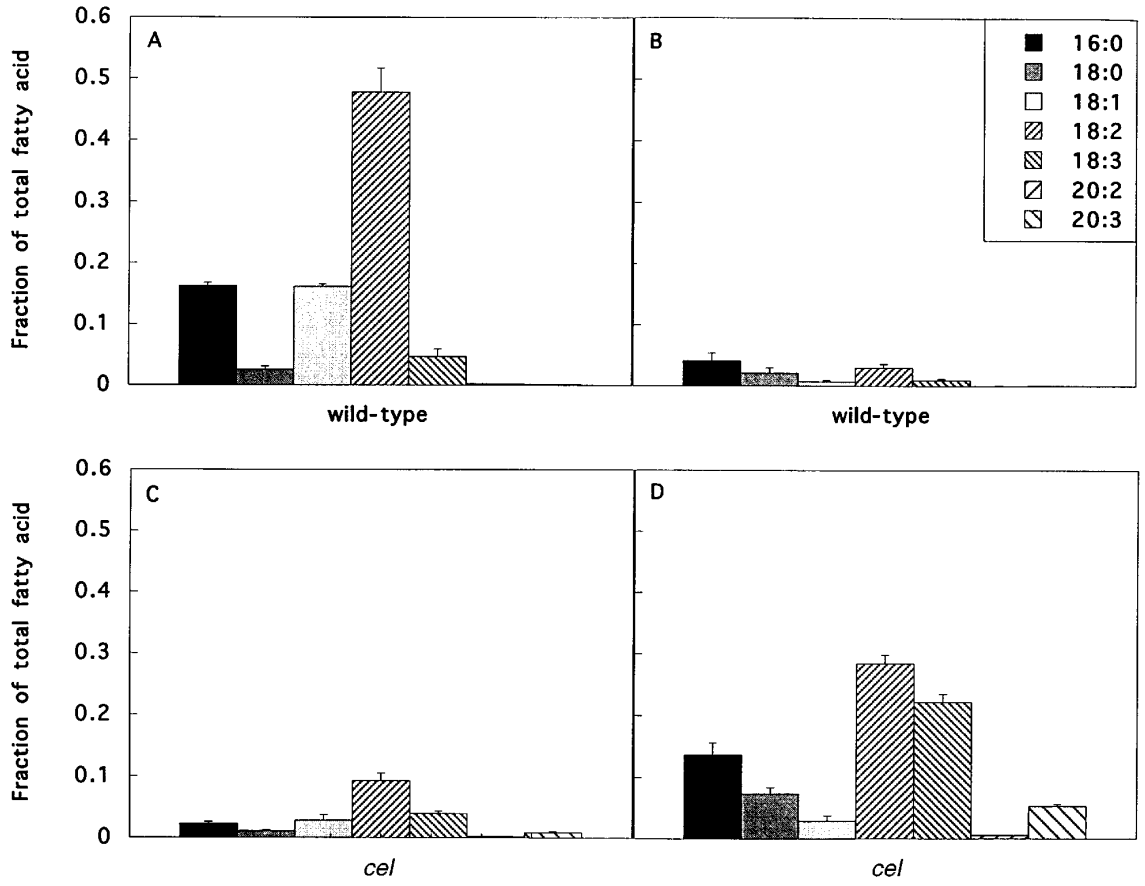

FIG. 1. Fatty acids synthesized by *Neurospora crassa de novo* and from supplemental [7,7,8,8-$^2H_4$]16:0. (A) Fatty acids synthesized *de novo* and (B) from exogenous [$^2H_4$]16:0 by wild-type; (C) fatty acids synthesized *de novo,* and (D) from [$^2H_4$]16:0 by *cel.* Cultures were grown for 2 d in presence of supplement, with the medium replenished after 1 d. ^2H incorporation into individual fatty acids was determined by gas chromatography–mass spectrometry (GC–MS) (selective ion monitoring), and used to calculate the fraction of total fatty acids derived from supplement. The fraction synthesized *de novo* was calculated by difference from the total. Data are presented as the fraction (out of 1.0) of the total fatty acid mass present in lipid extracts of the cultures. Values shown are averages ± SE for three wild-type cultures and six *cel* cultures. Average total fatty acid in wild-type cultures, 1440 ± 290 μg, and in *cel,* 264 ± 21 μg.

supplement. We compared fatty acids synthesized *de novo* and from exogenous [$^2H_4$]16:0 in wild-type and *cel* (Fig. 1). Wild-type synthesized the majority of its fatty acid *de novo* (Fig. 1A), with smaller amounts obtained from the supplement (Fig. 1B). Wild-type converted 42% of the exogenous [$^2H_4$]16:0 to unsaturates, comparable to the amount we observed earlier (37%) for a shorter uptake period (5). As expected, *cel*, because of its impairment in fatty acid biosynthesis, synthesized considerably less fatty acid *de novo* (Fig. 1C) than did wild-type (Fig. 1A). The majority of cel's fatty acids were obtained from the exogenous [$^2H_4$]16:0 (Fig. 1D). The *cel* mutant converted 72% of the [$^2H_4$]16:0 to unsaturates. It also utilized more of the [$^2H_4$]16:0 added to the medium in its lipids; *cel* incorporated 22% of the exogenous fatty acid into lipids, whereas wild-type incorporated only 14%, suggesting that less of the supplement enters the β-oxidation pathway in *cel*. Wild-type utilizes exogenous 16:0 preferentially for incorporation into triacylglycerols, and for elongation to longer-chain saturates (5). The *cel* mutant contained less triacylglycerol than wild-type (7% of total fatty acid *vs.* 18% for wild-type), but *cel* did not preferentially incorporate supplemental 16:0 into triacylglycerols (7% of fatty acid derived from the supplement, *vs.* 33% for wild-type). In addition, a greater proportion of the supplemental 16:0 incorporated into triacylglycerols had been converted to unsaturated fatty acids than in wild-type (Fig. 2).

The profiles of the fatty acids made by *cel* both *de novo* and from the supplement were similar (compare Figs. 1C, D), suggesting that, unlike wild-type (Figs. 1A, B), *cel* utilizes fatty acids obtained from the supplement similarly to fatty acids synthesized *de novo*. The single exception is the lower proportion of $18{:}1^{\Delta 9}$ relative to the other fatty acids derived from the supplement, compared to fatty acids synthesized by *cel de novo*. This effect is similar to that noted for wild-type (compare Fig. 1A, B, and Ref. 5). The *cel* mutant also produced a higher proportion of $18{:}3^{\Delta 9,12,15}$, $20{:}2^{\Delta 11,14}$ and $20{:}3^{\Delta 11,14,17}$, whether synthesized *de novo* or from the supplement, than did wild-type. The higher proportion of $18{:}3^{\Delta 9,12,15}$ indicates efficient desaturation of the $18{:}2^{\Delta 9,12}$ once formed.

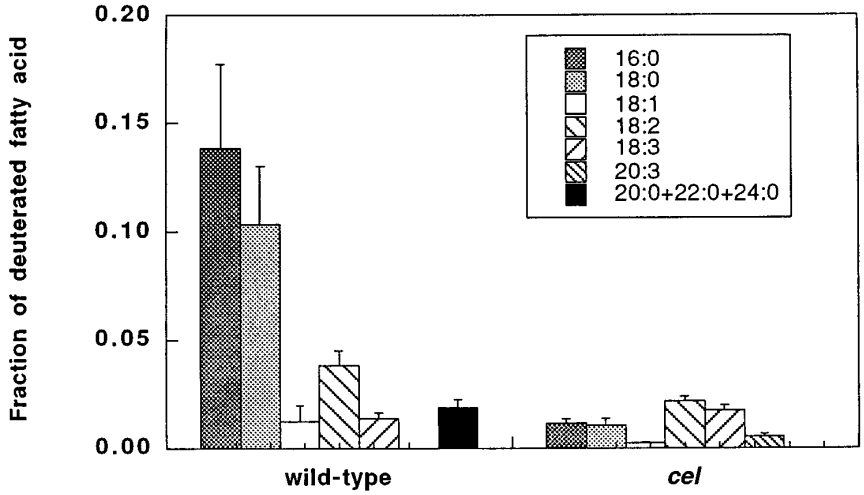

FIG. 2. Fraction of deuterated fatty acids incorporated into triacylglycerols by wild-type and *cel.* Triacylglycerols were separated from total lipids by thin-layer chromatography, and their fatty acid methyl esters analyzed by GC–MS as in Figure 1. Data are the fraction (out of 1.0) of the total deuterated fatty acids in each strain. Values are averages ± SE for three wild-type and two *cel* cultures. See Figure 1 for abbreviation.

Our earlier observation that exogenous 16:0 is poorly utilized for desaturation by wild-type, which has been confirmed here, suggested the presence of two pools of 16:0 in *N. crassa* (5): one that is used for synthesis of saturated fatty acids and one that leads into the unsaturated fatty acid pathway. Since the [$^2H_4$]16:0 is elongated to [9,9,10,10-$^2H_4$]18:0, an isotope effect on desaturation (18:0 to 18:1$^{\Delta 9}$) was considered as part of the reason for limited desaturation. However, lower levels of desaturation were also observed with 16:0 labeled in other positions. Although the isotope effect may have affected the fatty acid composition in wild-type cultures fed [$^2H_4$]16:0, exogenous 16:0, regardless of label position, was used preferentially for synthesis of saturated fatty acids while endogenously produced 16:0 was used for saturated and unsaturated fatty acids. On the other hand, because *cel* growth is dependent on supplemental fatty acid, *cel* must use exogenous 16:0 to produce saturated and unsaturated fatty acids in order to grow. We have found (Goodrich-Tanrikulu, M., Stafford, A.E., and McKeon, T.A., unpublished results), after feeding 16:0 labeled with deuterium in either the 2- or 16-position, that the isotope effect alters fatty acid composition in *cel*.

The production of the longer-chain polyunsaturated fatty acids 20:2$^{\Delta 11,14}$ and 20:3$^{\Delta 11,14,17}$ in *Neurospora* has not previously been reported (although they have been observed to be produced by *cel*; S. Brody, personal communication). The proportions of these fatty acids are also at least in part subject to culturing conditions, contributing as much as 20% of the total fatty acid in *cel* (not shown). The production of the longer-chain polyunsaturates presumably occurs by elongation of 18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$. In the yeast *Saccharomyces cerevisiae,* elongases are located in microsomes and mitochondria (8,9); various elongase systems optimally utilize fatty acid substrates ranging from 12 to 18 carbons (10). The formation of these fatty acids may be an indirect consequence of increased elongation activity of *cel*. We suspect that increased elongase activity is a mechanism for increasing the fatty acid biosynthetic capabilities of *cel*, assuming the elongases have some activity on short-chain fatty acids. Although *cel* derives the majority of its fatty acid from supplemental 16:0, the remaining fatty acid synthesized *de novo* is presumably derived from a combination of residual activity of the impaired cytosolic fatty acid synthase, mitochondrial fatty acid synthesis, and elongase activity. Elovson (1) assayed *cel* fatty acid synthase activity *in vitro* and found activity higher than expected based upon the content of 4′-phosphopantetheine in the impaired fatty acid synthase; however, the assay (based on oxidation of NADPH) would not have distinguished among these activities. Assays designed to distinguish among these activities will allow an estimate of the relative contributions of each to total fatty acid biosynthetic capability in *cel.*

ACKNOWLEDGMENTS

We thank Stuart Brody, for encouraging us to examine the metabolism of palmitate in the *cel* mutant and for helpful discussions and criticism throughout this study, and Charles Martin, for critical reading of the manuscript.

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[Received May 6, 1997; in final revised form and accepted February 10, 1998]

Identification of Ceramide-Phosphorylethanolamine in Oomycete Plant Pathogens: *Pythium ultimum, Phytophthora infestans,* and *Phytophthora capsici*

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ABSTRACT: Cellular lipids were extracted from three species of Oomycete plant pathogens (*Pythium ultimum, Phytophthora infestans,* and *Ph. capsici*) and analyzed *via* normal-phase high-performance liquid chromatography with flame-ionization detection. The most abundant polar lipids in each of the three species were the polar membrane lipids, phosphatidylethanolamine (PE), phosphatidylcholine, and a phosphosphingolipid that eluted soon after PE. Structural analysis *via* mass spectrometry and nuclear magnetic resonance spectrometry revealed that the phosphosphingolipid was ceramide phosphorylethanolamine (Cer-PE). The most abundant molecular species of Cer-PE in *P. ultimum* had a molecular weight of 670.5, contained an unusual 19-carbon branched triunsaturated sphingoid (C19-Δ4, 8, 10, 9-methyl long-chain base) and palmitic acid as the amide-linked fatty acid. The most abundant molecular species of Cer-PE in *Ph. infestans* had a molecular weight of 714.5, contained a common 16-carbon 1,3 di-OH sphingoid, and erucic (*cis* 13-docosenoic, C22-Δ13) acid as the amide-linked fatty acid. The Cer-PE in *Ph. capsici* comprised a mixture of each of the two molecular species found in *P. ultimum* and *Ph. infestans.*
Lipids 33, 307–317 (1998).

Formerly classified as fungi, the Oomycetes have so many physiological and biochemical peculiarities that they have recently been placed in a separate kindom, the Stramenopila (1). Unlike most fungi, the cell walls of Oomycetes contain no chitin, and instead contain structural carbohydrate polymers composed of glucans (2). Pythiaceous members of the Oomycetes (including the *Phytophthora* and *Pythium* species of this study) are unable to synthesize sterols and do not require them for growth, but must obtain sterols in their host or environment because sterols are necessary for their sexual reproduction (3,4). For this reason, fungicides that inhibit ergosterol biosynthesis are not effective against Oomycetes. There are several reports that certain Oomycetes contain high levels of an unusual sphingophospholipid, ceramide aminoethylphosphonate. This unusual sphingophospholipid was reported to occur in *P. prolatum* (5), *Ph. parasitica* (5), and *Ph. infestans* (6,7).

Our laboratory has recently developed sensitive high-performance liquid chromatography (HPLC) methodologies to quantitatively analyze phospholipids and other lipids in microorganisms (8,9)**.** This study was undertaken to employ the tools of modern HPLC methodology to rigorously reinvestigate the occurrence and structure of phosphosphingolipids in three plant pathogenic species of Oomycetes**,** *P. ultimum, Ph. infestans,* and *Ph. capsici.* These species infect many agricultural commodities and cause considerable economic losses.

EXPERIMENTAL PROCEDURES

Growth of Oomycetes. *Pythium ultimum* (ATCC 26083) from the American Type Culture Collection (Rockville, MD) was maintained on potato dextrose agar at 25°C. Mycelium for lipid isolation was prepared by inoculating 20-mL aliquots of a liquid asparagine–sucrose medium (10) in petri dishes (9 cm in diameter, 2 cm in depth) with a mycelial plug, 7 mm in diameter, taken from the growing edge of a culture. The dishes were incubated for 48 h at 25°C with shaking on a gyratory shaker at 60 rpm. Mycelium was harvested by filtration on glass fiber filters, washed with water, and freeze-dried.

Phytophthora infestans (A1 strain) was obtained from Dr. W. Fry (Cornell University, Ithaca, NY) and was maintained on pea juice agar, prepared by autoclaving 283 g of frozen peas in 1 L of distilled water and filtering through cheesecloth. Agar (20 g) was added to the filtrate, which was autoclaved again before pouring culture plates. Mycelium for lipid isolation was prepared as described above for *P. ultimum* except that cultures were grown for 20 d before harvesting the mycelium.

Phytophthora capsici (ATCC 15399) from the American Type Culture Collection was maintained on V-8 juice agar,

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Abbreviations: CAD, collisionally activated dissociation; CAEP, ceramide aminoethylphosphonate; Cer-PE, ceramide phosphorylethanolamine; Cer-PI, ceramide phosphorylinositol; 2D, two-dimensional; FAB, fast atom bombardment; FID, flame-ionization detector; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/MS; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

pH 7.0, containing 200 mL V-8 juice, 4 g $CaCO_3$, and 20 g agar per liter. Mycelium for lipid isolation was prepared as described above for *P. ultimum* except that cultures were grown for 96 h before harvesting the mycelium.

Lipid extraction and mild alkaline hydrolysis. Lyophilized hyphae (200 mg) were homogenized in chloroform/methanol/water (8 mL/16 mL/4.8 mL) with a Polytron Homogenizer (Brinkmann, Westbury, NY), and lipids were extracted with chloroform/methanol (11). Some lipid samples were subjected to mild alkaline hydrolysis by evaporating the solvent under N_2, adding about 5 mL of 1.5 M methanolic KOH/10 mg lipid, heating the mixture to 70°C for 30 min, acidification to pH 2, and reextraction of the hydrolysate with chloroform/methanol.

Analytical normal-phase HPLC. The lipids were separated and quantified using a method similar to one we have used for hopanoid analyses in other species (9). The column was a LiChrosorb 5 Si 60 (3 × 100 mm) from Chrompack, Inc. (Raritan, NJ), with a flow rate of 0.5 mL/min. The solvents were: A, hexane; B, isopropanol; and C, 0.04% triethylamine in water (C was prepared fresh daily). The linear gradient timetable was: at 0 min, 100/0/0; at 5 min, 95/5/0; at 10 min, 85/15/0; at 15 min, 40/60/0; at 53 min, 40/51/9; at 68 min, 40/51/9; at 73 min, 40/60/0; at 78 min, 100/0/0; at 100 min, 100/0/0; (%A/%B/%C, respectively). The HPLC system consisted of an Isco (Lincoln, NE) Model 2350 pump, an Isco Model 2360 gradient programmer, and a Tremetrics (Austin, TX) Model 945 flame-ionization detector (FID).

Semipreparative normal-phase HPLC. For purification of milligram quantities of ceramides, 10–20 mg of total lipid extract was injected in a volume of 1 mL on a column (10 × 250 mm, LiChrosorb 10 micron Silica 60A, packed by Phenomenex, Torrance, CA). The flow rate was 5.0 mL/min, and the ternary gradient was identical to that described above. The HPLC pumping system and detectors were also identical to those described above, except that the column effluent was split, using a Valco T (VICI Valco, Houston, TX), so that 10% of the flow entered the detector and 90% was collected.

Reverse-phase HPLC. Ceramide phosphorylethanolamine (Cer-PE) was purified by semipreparative normal-phase HPLC and analyzed by reverse-phase HPLC using a LiChrosorb 7 RP18 column (3 × 100 mm, Chrompack) and a mobile phase consisting of methanol/acetonitrile/water, 88:6:6, by vol, at a flow rate of 0.5 mL/min. The HPLC pumping system and detectors were as described above.

Mass spectrometry (MS). Liquid chromatography (LC)/MS measurements were performed on a Fisons Quattro SQ mass spectrometer (Beverly, MA) coupled to a Hewlett-Packard 1090 HPLC (Palo Alto, CA). The HPLC was operated with the same conditions as the reverse-phase HPLC method just described. The flow rate was 0.5 mL/min with approximately 12 μL/min of the eluant split post-column and directly introduced into the mass spectrometer. The analyte was ionized by electrospray ionization with detection of negative ions. The ion source was at 100°C, and the instrument scanned from 50–1000 *m/z* at 2.0 s/scan.

A Jeol HX-110 mass spectrometer (Peabody, MA) was employed for all other mass spectrometric measurements. The instrument was operated with 10 kV acceleration and detection of positive or negative ions. Mass resolution was 1000 (10% valley) except for high mass accuracy measurements where the resolution was 10,000 (10% valley). Analyte was ionized by fast atom bombardment (FAB) with a Xe beam and 3-nitrobenzyl alcohol matrix. For collisionally activated dissociation (CAD) measurements, helium was used as the target at a sufficient pressure to attenuate the primary beam to 30% of its original intensity. Linked scanning at constant B/E (magnetic field/electric field) provided spectra of the product ions. For high mass accuracy measurements, manual peak-matching was performed using a suitable calibrant with a mass similar to that of the unknown.

The sphingolipids were hydrolyzed by dissolution of a small amount in 3 N methanolic HCl and heating at 70°C for 4 h. FAB of the entire solution was performed for determination of the sphingoid and ceramide moieties. The fatty acid methyl esters were extracted with methylene chloride and analyzed by gas chromatography (GC)/MS to determine the fatty acid components, using a Hewlett-Packard 5970 for those of *P. ultimum* and a Hewlett-Packard 5890 GC connected to a Finnigan SSQ7000 (Sunnyvale, CA) for those of *Ph. infestans* and *Ph. capsici*. The analyses employed a J&W DB-5 column (30 m, 0.25 mm i.d., 0.025 mm film thickness; Folsom, CA), which was heated from 50 to 285°C at 8°C/min while the mass spectrometer scanned from 50 to 800 *m/z*.

Nuclear magnetic resonance (NMR) spectrometry. The NMR analyses were performed on a Bruker AMX500 spectrometer (Billerica, MA) at 25°C. The two-dimensional (2D) spectrum correlation spectroscopy experiment was acquired with 2K by 512 points which were zero-filled to make up a 2K × 2K set of data points. The projections along the two axes were from one-dimensional ^{1}H NMR data collected separately.

All experiments were repeated at least two times, and the data presented are the mean of duplicate samples from one experiment.

RESULTS

The yield of total lipid extract was 12–16 mg lipid per 200 mg dry weight of mycelia of *P. ultimum,* 17–20 mg lipid per 200 mg dry weight of mycelia of *Ph. infestans,* and 7–8 mg lipid per 200 mg dry weight of mycelia of *Ph. capsici*.

The total lipid extracts from each of the three organisms were analyzed with an analytical normal-phase HPLC–FID system (Fig. 1). The major phospholipids were phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The chromatogram of *Ph. infestans* lipids contained an unidentified peak at a retention time of 47.3 min; because this peak was resistant to mild alkaline hydrolysis, it was postulated to be a sphingolipid (probably a ceramide) and was labeled Cer 1. The chromatogram of *P. ultimum* lipids contained PE, PC, and an unidentified peak at a retention time of 48.3 min

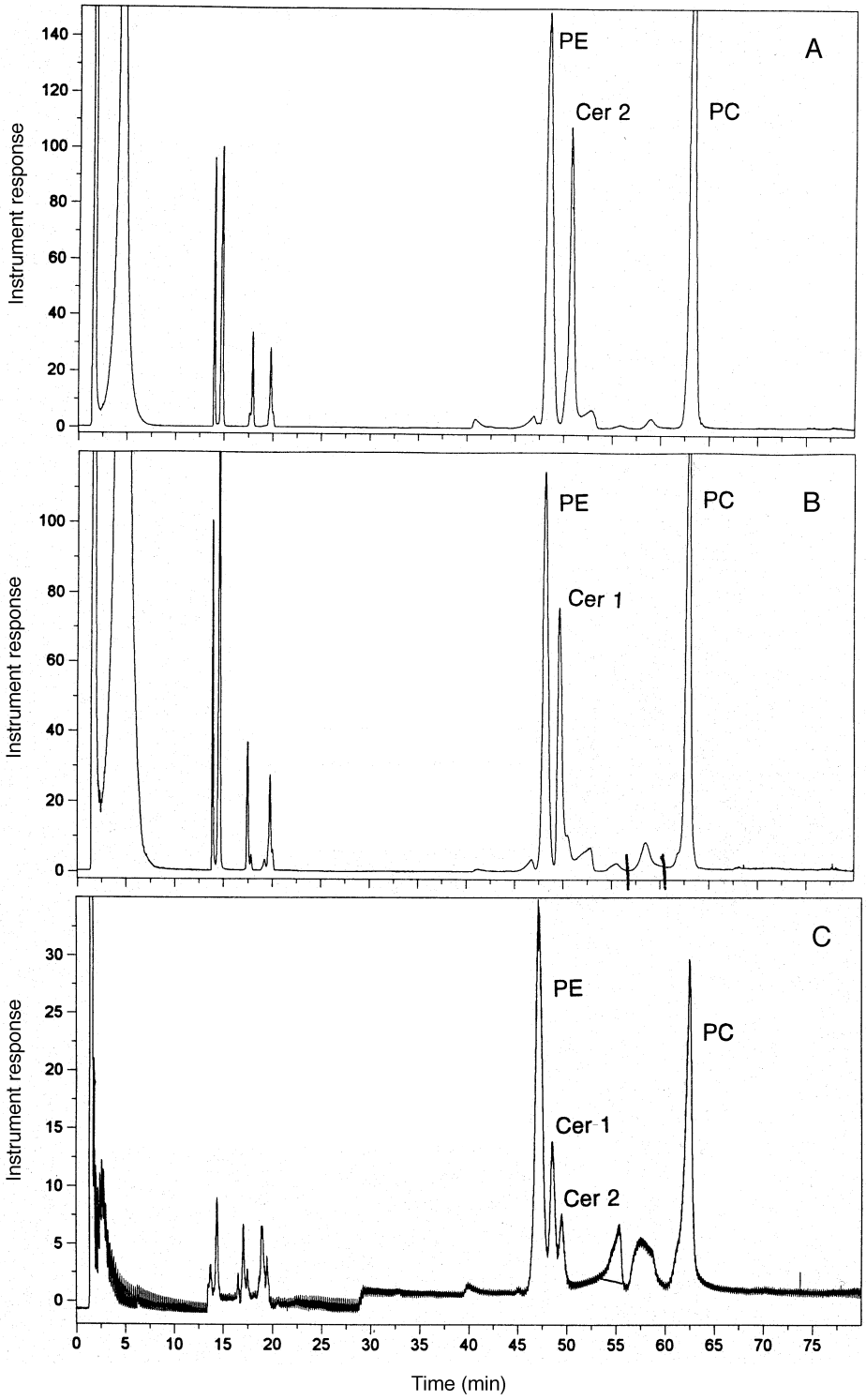


FIG. 1. Analytical normal-phase high-performance liquid chromatography–flame-ionization detector (HPLC–FID) chromatograms of the lipid classes in mycelial lipid extracts of (A) *Pythium ultimum,* (B) *Phytophthora infestans,* and (C) *Ph. capsici.* Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; Cer 1 and Cer 2, ceramide-1 and -2.

that was also resistant to mild alkaline hydrolysis. Thus it was also postulated to be a sphingolipid and was labeled Cer 2. The chromatogram of *Ph. capsici* lipids contained PE, PC, and unidentified peaks at the retention times of 47.3 and 48.3 min that were tentatively labeled Cer 1 and Cer 2.

To purify milligram quantities of Cer 1 and Cer 2 for structural analysis, we scaled up the above analytical normal-phase HPLC system to the semipreparative level, injecting larger samples (10–20 mg, in 1 mL) of total lipid extracts from each of the three species (Fig. 2). The semipreparative

chromatograms (Fig. 2) were very similar to those obtained with corresponding samples at the analytical level (Fig. 1). By using this system, several milligrams each of PE, PC, and Cer 1 from *Ph. infestans* and *Ph. capsici* and of Cer 2 from *P. ultimum* and *Ph. capsici* were purified.

Most phospholipid and sphingolipid classes that have been purified by normal-phase HPLC can be separated into their individual molecular species by reinjecting them in an appropriate reverse-phase HPLC system (12). When samples of purified PE and PC were injected in this reverse-phase system, multiple peaks of individual molecular species were observed with each (data not shown). To attempt to separate the molecular species of Cer 1 and Cer 2, we injected the four purified samples from the semipreparative normal-phase HPLC into an analytical reverse-phase HPLC system (Fig. 3). In this chromatographic system, injection of purified Cer 1 from both *Ph. infestans* and *Ph. capsici* yielded a major peak with a retention time of 27.8 min. Injection of purified Cer 2 from *P. ultimum* and *Ph. capsici* yielded a major peak with a retention time of 13.8 min. Because the chromatograms of the two

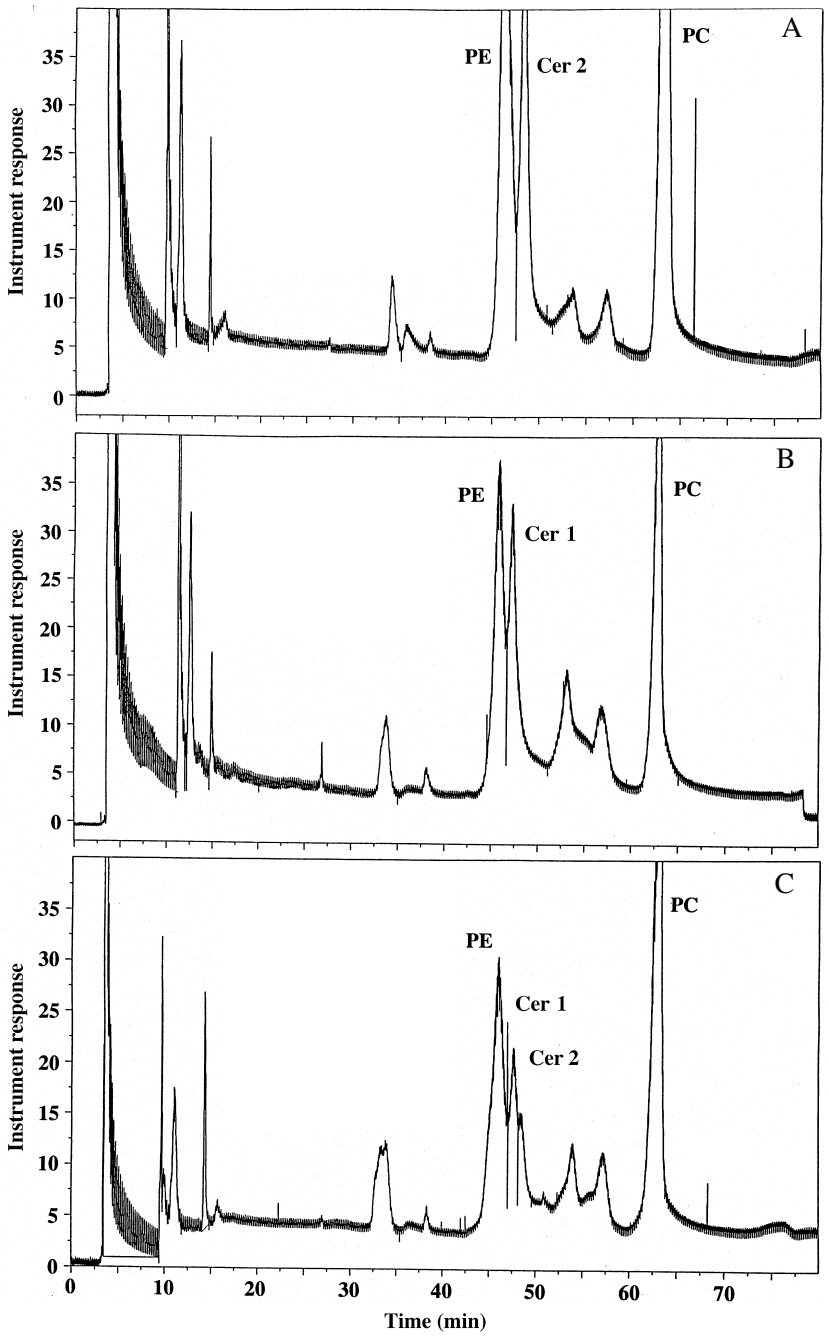


FIG. 2. Semipreparative normal-phase HPLC–FID chromatograms of the lipid classes in mycelial lipid extracts of (A) *Pythium ultimum,* (B) *Phytophthora infestans,* and (C) *Ph. capsici.* Abbreviations are defined in Figure 1.

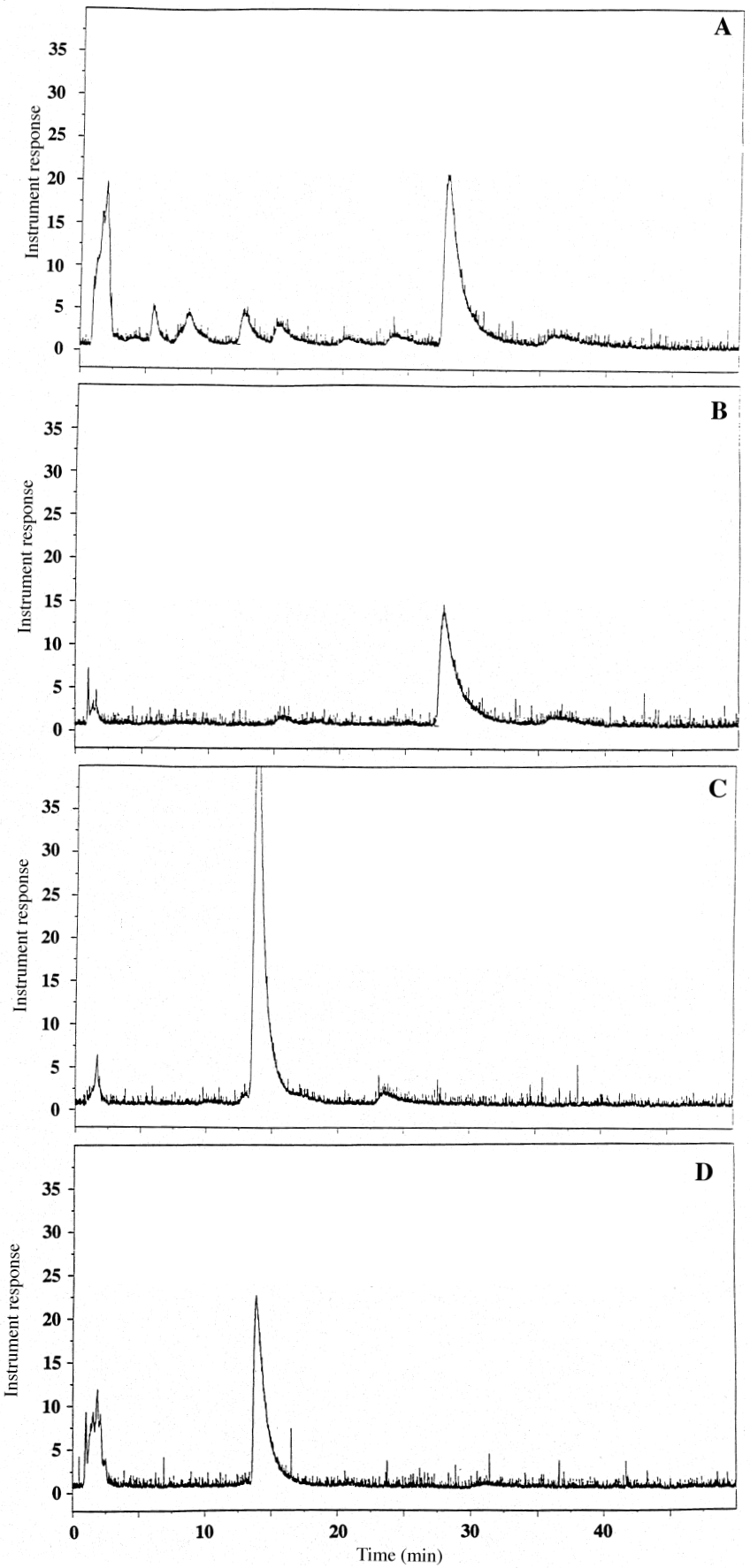


FIG. 3. Analytical reverse-phase HPLC–FID chromatograms of the molecular species of ceramides in the three species: (A) Cer 1 peak from *Phytophthora infestans*, (B) Cer 1 peak from *Ph. capsici*, (C) Cer 2 peak from *Pythium ultimum*, and (D) Cer 2 peak from *Ph. capsici*.

Cer 1 samples each show only one major peak and because the retention times are identical, it is likely that the samples contain the same major component. Similarly, because the two Cer 2 samples each only have one major peak, they apparently only contain one major molecular species; and because the retention times for these are identical, it is likely that their chemical structures are identical.

The Cer 1 and Cer 2 samples isolated by semipreparative normal-phase HPLC were then analyzed by electrospray LC/MS, using the above reverse-phase HPLC system, to determine the molecular weight of the major sphingolipid molecular species in the lipid fractions. The LC/MS chromatograms were similar to those from the reverse-phase HPLC–FID analyses shown in Figure 3. The mass spectra of the major components which corresponded to the intense peaks in Figure 3 are shown in Figure 4. The molecular weights of these components are readily determined as the negative ion electrospray produces the deprotonated molecular ions. In summary, the molecular weight of the major component in *P. ultimum* is 670.4 Da; in *Ph. infestans*, it is 714.5 Da; and in *Ph. capsici,* the major components are 670.4 and 714.5 Da. This supports the proposition that the sphingolipids in *Ph. capsici* are identical to those found in *P. ultimum* and *Ph. infestans*.

The FAB mass spectra, Figure 5, of the sphingolipid fractions, isolated by semipreparative normal-phase HPLC, confirmed the molecular weights of the major components. The mass spectrum for the *P. ultimum* sphingolipid shows the protonated molecular ion at *m/z* 671.4 while the *Ph. infestans* sphingolipid has *m/z* 715.5 as the protonated molecular ion. Additionally, the peaks at *m/z* 141 lower are seen in each of these spectra at *m/z* 530.4 and 574.5, respectively. This is indicative of loss of phosphorylethanolamine from the molecular ion (13). In the case of *Ph. capsici,* both the *m/z* 671.4 and 715.5 peaks and the *m/z* 530.4 and 574.5 peaks are observed.

Additional structural information was obtained from the FAB mass spectra of the total hydrolysate solution and is summarized in Schemes 1 and 2 (spectra not presented). The *P. ultimum* sphingolipid hydrolysate showed a major peak at *m/z* 433.3 which would correspond to the hydrolytic loss of a 16:0 fatty acid chain from the sphingolipid. An additional peak at *m/z* 310.3 shows further loss of phosphorylethanol-

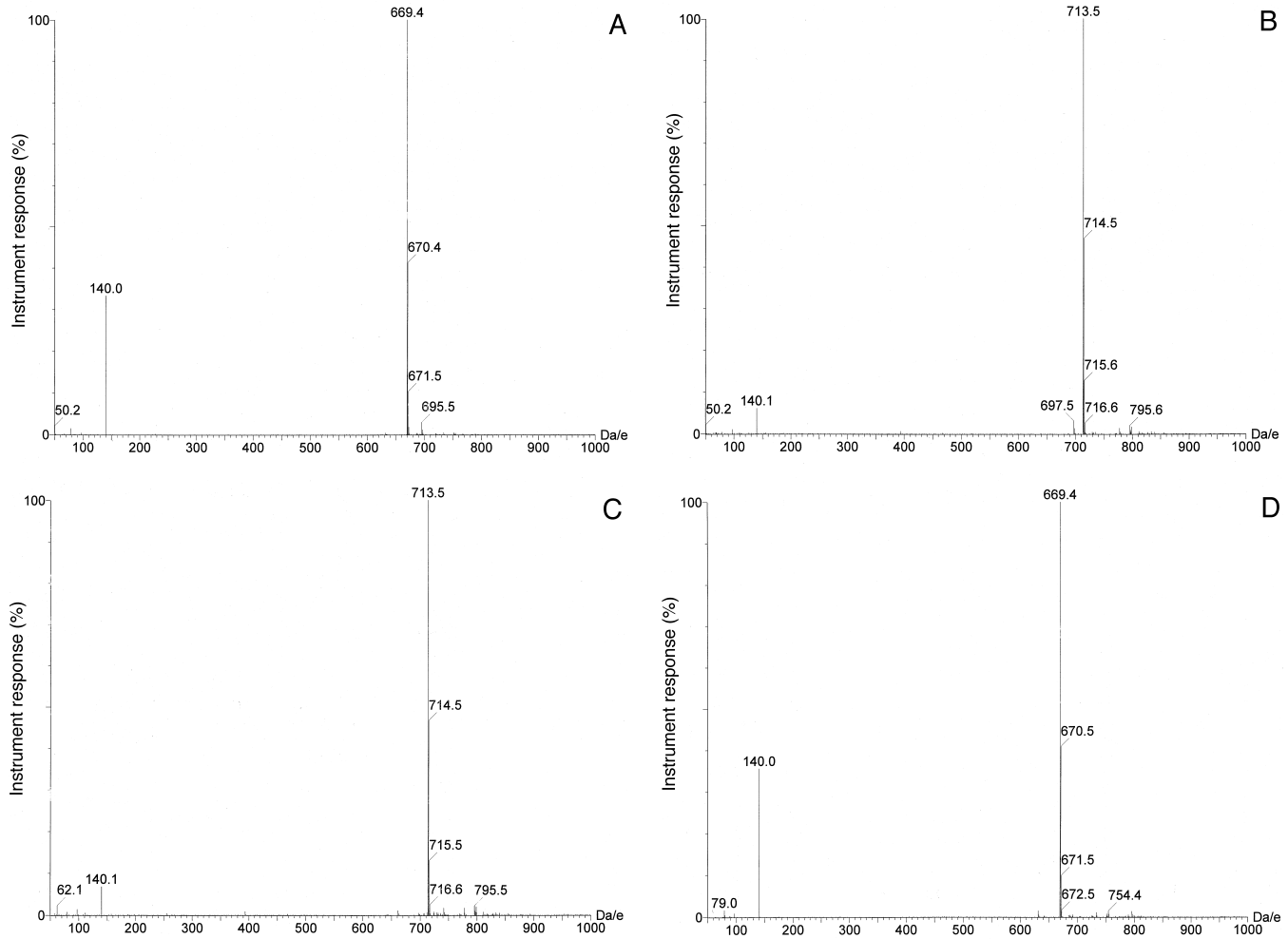


FIG. 4. Liquid chromatography–mass spectrometry electrospray analysis of (A) *Pythium ultimum,* (B) *Phytophthora infestans,* (C) *Ph. capsici* Cer 1, and (D) *Ph. capsici* Cer 2. The mass spectra of the major components were obtained using a micromass quadrupole mass spectrometer operating in the negative ion mode. For abbreviations see Figure 1.

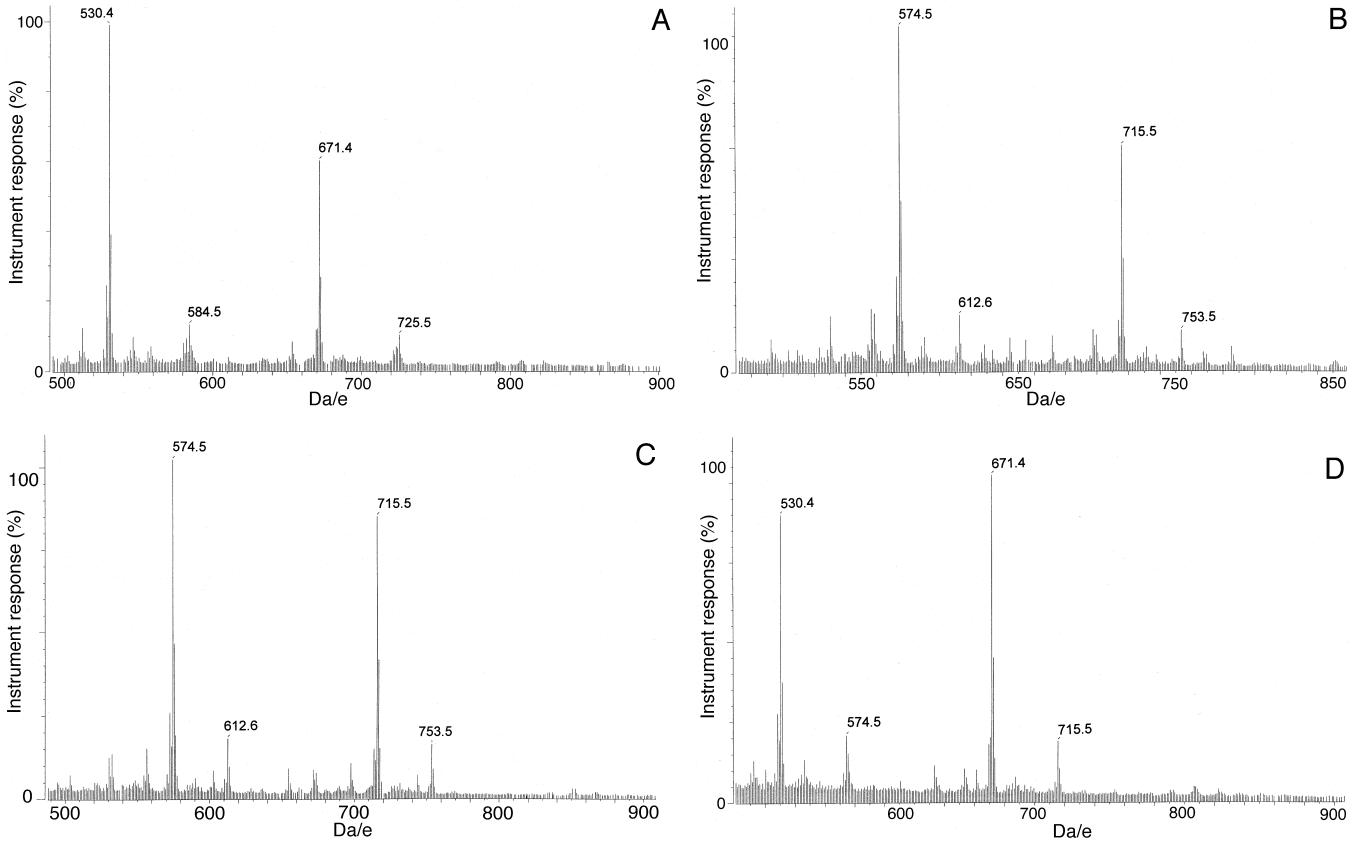
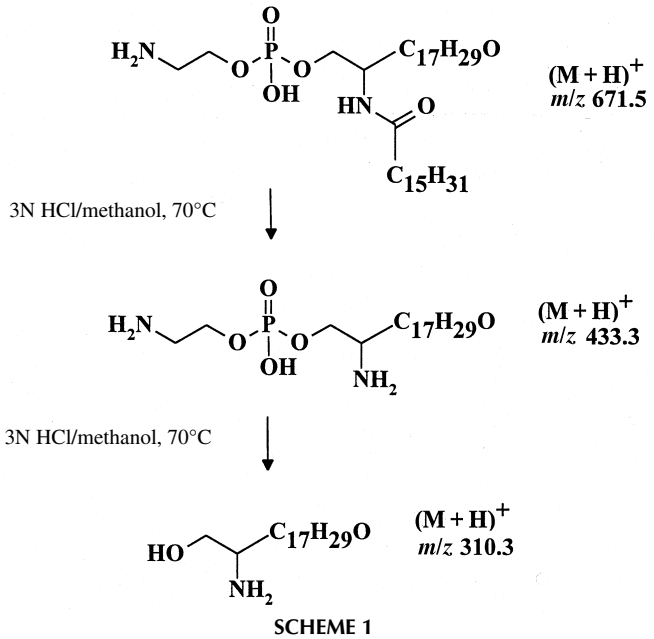
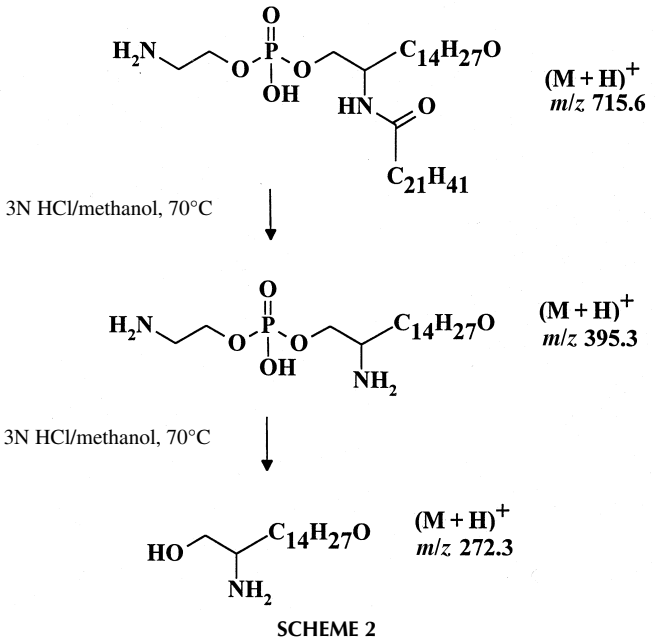


FIG. 5. The fast atom bombardment mass spectra of the sphingolipid fractions of (A) *Pythium ultimum,* (B) *Phytophthora infestans,* (C) *Ph. capsici* Cer 1, and (D) *Ph. capsici* Cer 2. The mass spectra were obtained using a Jeol HX-110 double focusing mass spectrometer (Peabody, MA) operating in the positive ion mode. Fractions were prepared in a 3-nitrobenzyl alcohol matrix. For abbreviations see Figure 1.

amine leaving *m/z* 310.3 as the protonated molecular ion of the sphingoid. The FAB mass spectrum of the *Ph. infestans* hydrolysate solution showed a major peak at *m/z* 395.3. This corresponds to the hydrolytic loss of a 22:1 fatty acid from the sphingolipid. The other significant hydrolysis product *m/z* 272 reflects further loss of phosphorylethanolamine. The *Ph. capsici* hydrolysate showed both pairs of hydrolysis products, further reinforcing the supposition



SCHEME 1



SCHEME 2

TABLE 1
Summary of High-Resolution Accurate Mass Measurements

Measured mass	Proposed formula	$\frac{\text{Measured mass} - \text{calculated mass}}{\text{Calculated mass}} \times 100$ (%)
Pythium ultimum[a]		
671.5136 $(M + H)^+$	$C_{37}H_{72}O_6N_2P$	0.00012
669.4983 $(M - H)^-$	$C_{37}H_{70}O_6N_2P$	0.00017
530.4919 $(M - PE)^+$	$C_{35}H_{64}O_2N$	–0.00034
140.0111 $(PE)^-$	$C_2H_7O_4NP$	–0.00012
P. ultimum hydrolysate		
433.2819 $(M + H)^+$	$C_{21}H_{42}O_5N_2P$	–0.00029
310.2729 $(M + H)^+$	$C_{19}H_{36}O_2N$	–0.00055
Phytophthora infestans		
715.5748 $(M + H)^+$	$C_{40}H_{80}O_6N_2P$	–0.00008
574.5532 $(M - PE)^+$	$C_{38}H_{72}O_2N$	–0.00054
Ph. infestans hydrolysate		
395.2658 $(M + H)^+$	$C_{18}H_{40}O_5N_2P$	–0.00043
272.2601 $(M + H)^+$	$C_{16}H_{34}O_2N$	0.00042

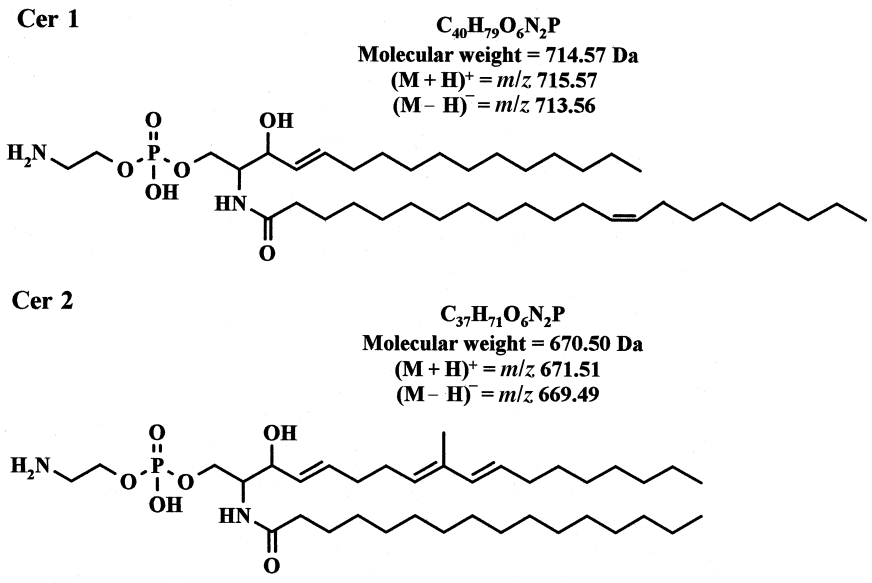
[a]PE, phosphatidylethanolamine.

that the sphingolipids in *P. ultimum* and *Ph. infestans* are both present in *Ph. capsici*.

The GC/MS analysis of the hydrolysate extract gave qualitative information on the fatty acid components of the sphingolipids. For *P. ultimum* the methyl esters of 14:0, 16:0, 18:0, 18:1, 20:0, and 20:1 were detected. For *Ph. infestans* the methyl esters of 16:0, 18:0, 22:0, and 22:1 fatty acids were found.

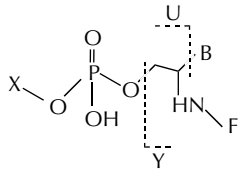
Strong structural evidence is provided by the accurate mass measurements of the sphingolipids and their hydrolysates. Table 1 summarizes the data showing the measured mass, the proposed formula, and the difference between the expected and measured mass. Mass errors of less than 0.001% are generally considered a good match. The difference in composition between the protonated molecular ion of the *P. ultimum* sphingolipid and the peak corresponding to the loss of *m/z* 141 almost exactly accounts for loss of phosphorylethanolamine. This strong indication of loss of phosphorylethanolamine is also shown for the *Ph. infestans* sphingolipid fragments. Similarly, the assignments of the hydrolysate products are very near the measured values. All of the assignments in Table 1 are within 0.00055% of the proposed masses, giving strong support for the proposed structures (Scheme 3).

The connectivity in these sphingolipids is further elucidated by the CAD spectra of the various molecular ions. These CAD spectra provide a fingerprint of the molecule and are a sensitive indicator of molecular structure. The major ions resulting from the fragmentation of the molecular ions are shown in Table 2. The notation is similar to that used in Pivot *et al.* (14). These data provide clear evidence of the connectivity of the various moieties mentioned in the above re-



SCHEME 3

TABLE 2
The Collisionally Activated Dissociation Spectra of Cer 1 and Cer 2



	Ion mode	$(M + H)^+$	$(M - H)^-$	Y	U	Y – B	Y – F – H_2O	XPO_4H_3	XPO_4
Cer 1	Positive	715.5		573		362	236	142	
	Negative		713.5		501				140
Cer 2	Positive	671.5		530		280	274	142	
	Negative		669.4		419				140

sults. Based on these CAD spectra and the above data, the structures of the Cer 1 and Cer 2 can be assigned with certainty and are shown in Scheme 3. Additionally the CAD spectra of Cer 1 and Cer 2 from *Ph. capsici* are essentially the same as these shown here, proving that the sphingolipids in *Ph. capsici* have the same structure as those in *Ph. infestans* and *P. ultimum* (Scheme 3).

The only ambiguities which are unresolved by the above MS analyses are in the position of the three double bonds and branching in the *P. ultimum* sphingoid moiety. Proton NMR was used to address this point and provide further evidence for the proposed structures.

The positions of the olefinic groups along the sphingoid (or sphingatriene) chain were determined in a 2D NMR spectrum correlation spectroscopy experiment (15) that was carried out on a 500 MHz NMR spectrometer. In this experiment, signals arising from protons three bonds apart are correlated in the 2D map as shown in Figure 6. There are two sets of signals in the 2D plot. The diagonal peaks running from the top right corner of the figure to the bottom left end are essentially the same as the normal one-dimensional proton NMR signals that are also shown on the top as well as left side of the 2D plot. The off-diagonal peaks indicate the connectivities among neighboring proton species. Horizontal and vertical dashed lines are drawn on the 2D spectrum to assist with these correlations. For example, the long vertical line at 5.34 ppm at the olefinic methine proton (**8** on the structure) shows two correlations to 1.69 and 2.16 ppm as highlighted by the two horizontal dashed lines. As indicated on the left side of the figure, these correspond to the methylene **7** and methyl group at **19**. The rest of the correlations are assigned in a similar fashion, as indicated with several dashed lines on the spectrum, and identify the *P. ultimum* sphingoid as a C19-Δ 4,8,10, nine-methyl-long chain base. A summary of the proton chemical shift assignment for the sphingoid (or sphingatriene) side chain is given in Table 3. The *J* coupling between the protons across the double bond for pairs 4–5 and 10–11 is 15.3 Hz—indicative of *trans* configuration of the two olefins. We could not accurately measure the *J* coupling between 8–9 (CH_3) owing to severe overlap and weak coupling. Thus, we could not determine its configuration.

Based on the above structural information, Cer 1 and Cer 2 appear to be molecular species of Cer-PE, with proposed structures shown in Scheme 3. The analytical HPLC data presented in Figure 1 were used for the quantitative analysis of each of the lipid classes in the lipid extracts of the three species (Table 4). To accomplish this quantification, several samples of each of the major peaks purified by semipreparative HPLC (Fig. 2) were injected, and the FID was calibrated to convert peak area to mass in the range of 1–50 µg for each of the major lipid classes. The levels of total lipids and nonpolar lipids varied significantly among the three species. Although no attempt was made to quantify the individual nonpolar lipid classes, they appeared to be mostly triacylglycerol, with some free fatty acids, and several other minor unidentified peaks. For *P. ultimum* and *Ph. infestans* the most abundant polar lipid class was PC, followed by PE, and then Cer-PE. For *Ph. capsici*, the most abundant polar lipid class was PE, followed by PC, and then Cer-PE.

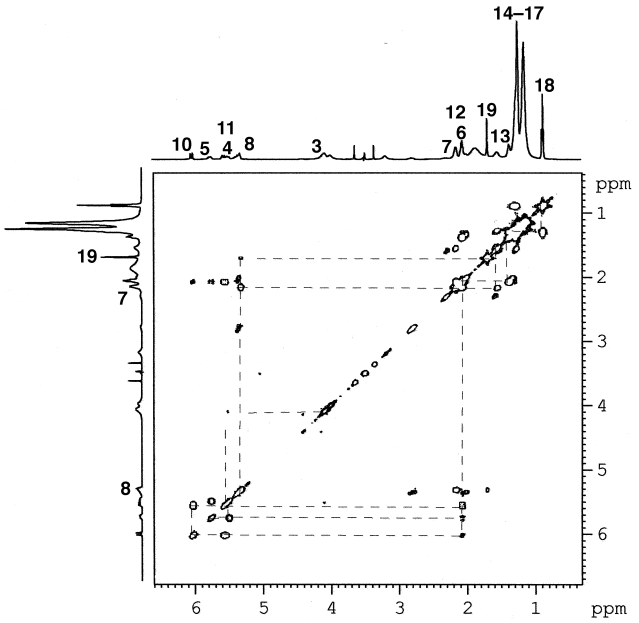
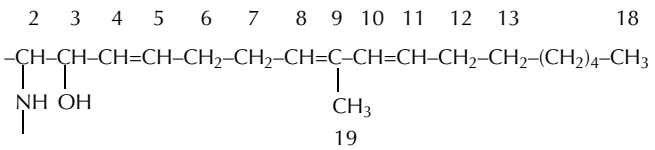


FIG. 6. The two-dimensional nuclear magnetic resonance spectrum correlation spectroscopy of *Pythium ultimum* ceramide phosphorylethanolamine.

TABLE 3
The Proton Chemical Nuclear Magnetic Resonance Shift Assignments for the *Pythium ultimum* Sphingatriene Sidechain



Position on structure	^{1}H chemical shift (ppm)	Position on structure	^{1}H chemical shift (ppm)
3	4.09	10	6.02
4	5.51	11	5.57
5	5.76	12	2.08
6	2.08	13	1.38
7	2.17	14–17	1.3
8	5.32	18	0.88
9			

DISCUSSION

This study is the first report of the occurrence of Cer-PE in a eukaryotic microorganism. It is also the first report of a 19-carbon-9-methyl-triunsaturated (Δ4, 8, 10) long chain base in a microbial sphingolipid. Several reports of the lipid composition of *P. ultimum* (16–18) failed to identify any sphingolipids. One of these reports noted the presence of a double peak of PE and several other unidentified phospholipid peaks, any of which could have been Cer-PE (16).

The current study now brings into question earlier reports of the occurrence of ceramide aminoethylphosphonate (CAEP) in *P. prolatum* (5), *Ph. palmitovora* (5), and *Ph. infestans* (6,7). In the cases of *P. prolatum* and *Ph. palmitovora*, it is not possible to comment on whether these species contain CAEP or Cer-PE, since these species were not examined in our current study. However, for *Ph. infestans*, all of the rigorous battery of mass spectral and NMR data that was employed in this study point to the occurrence of only Cer-PE (and no CAEP was detected). The two previous reports of CAEP in *Ph. infestans* (6,7) did not employ MS or NMR, and instead, the structural identification was based mainly on cochromatography of the hydrolyzed polar head group with commercial standards of aminoethylphosphonate. In one of the previous reports of CAEP in *Ph. infestans* (6) the major fatty acid moiety of CAEP was reported to be arachidonic acid. However, in the current study we found that erucic acid (*cis*-13-docosenoic, C22-Δ13) was the most abundant fatty acid in the Cer-PE of *Ph. infestans*. The identification of arachidonic acid in CAEP in the previous study (6) was performed by gas–liquid chromatography with packed columns. It is not known whether the arachidonic acid identified in the previous study could be adequately separated from erucic acid with the technique used. However, because the identification of erucic acid in Cer-PE in the current study is based on capillary gas–liquid chromatography with mass spectral analysis, we feel quite confident that this is the major fatty acid in the Cer-PE of *Ph. infestans*.

Ceramide phosphorylinositol (Cer-PI) was recently reported to be the major sphingolipid in *Ph. capsici* (14,19,20) and *Ph. parasitica* (21). Inositol sphingolipids such as these have been reported to occur in yeast and fungi (22,23). In our current study we did not see any evidence for the occurrence of Cer-PI or other inositol sphingolipids as major membrane lipids in *P. ultimum, Ph. infestans,* or *Ph. capsici*. While it is possible that Cer-PI is present in *Ph. parasitica* but absent from *Ph. infestans* and *P. ultimum*, we have no explanation for the failure to detect Cer-PI in *Ph. capsici* in our study except for the possibility of (i) differences in lipid composition between individual isolates, (ii) differences in the growth conditions, or (iii) differences in extraction procedures used in our study as compared to others (14,19,20).

Although this study is the first report of the occurrence of Cer-PE in a eukaryotic microorganism, Cer-PE has been reported to occur in *Sinotaia histrica*, a freshwater snail (24) and three genera of anaerobic Bacteroides (25).

The unusual 19-carbon-9-methyl-triunsaturated (Δ4, 8, 10) long chain base (sphingoid) that was found in the Cer-PE in *P. ultimum* (Scheme 3) has also been reported to occur in the glucosylceramides of *Asterias amurensis*, a starfish (26), and *Ophidiaster ophidiamus*, a sea star (27). Two fungal species, *Lentinus edodes* and *Schizophyllum commune*, have been reported to contain a somewhat similar 19-carbon-9-methyl, diunsaturated (Δ4, 8) long chain base in their glucosylceramides (28,29).

The subcellular localization and physiological significance of Cer-PE in Oomycetes are not known. However, because most of the sphingolipids in other species [including inositol sphingolipids in yeast (30), glucocerebrosides in plants (31), and sphingomyelin in animal cells (32)] are all localized in the plasma membrane, it is reasonable to expect that most of the Cer-PE in Oomycetes is localized in the plasma membrane. Cer-PE would be expected to be resistant to phospho-

TABLE 4
Quantitative Analysis (mg/g dry wt) of Ceramides and Other Lipid Classes in *Pythium ultimum, Phytophthora infestans,* and *Ph. capsici*[a]

Lipid class[b]	*Pythium ultimum*[b]	*Phytophthora infestans*	*Ph. capsici*
Nonpolar lipids	38.3	86.9	28.7
Polar lipids			
CL	trace	trace	trace
PE	9.44	5.29	3.66
Cer-PE 1	n.d.	3.03	1.39
Cer-PE 2	4.72	trace	0.84
PI	trace	trace	trace
PC	12.6	6.71	2.93
Total lipids	65.1	102.0	37.5

[a]Quantification is based on the normal-phase high-performance liquid chromatograms reported in Figure 1. Standard curves of each lipid class, injected as several concentrations, were used to calculate the mass of each lipid class.
[b]Abbreviations: n.d., not detected; CL, cardiolipin; Cer-PE 1 and 2, ceramide phosphorylethanolamine 1 and 2, with structures shown in Scheme 3; PI, phosphatidylinositol; PC, phosphatidylcholine. For other abbreviation see Table 1.

lipases A1, A2, and B, since these enzymes hydrolyze the fatty acid–oxygen ester bonds and there are no such bonds in Cer-PE. Since many plants (including potatoes and tomatoes, major hosts of *Ph. infestans*) contain high levels of phospholipase B [also called lipolytic acyl hydrolase (7)], the localization of Cer-PE in the plasma membrane could help protect the membrane from hydrolysis by plant phospholipase B. However, certain hydrolytic enzymes that are also common in certain plant tissues, phospholipase C and D, would presumably be capable of hydrolyzing Cer-PE.

The pathway leading to Cer-PE remains to be elucidated. Nevertheless, the discovery of Cer-PE as a novel class of sphingolipid found in *Phytophthora* and *Pythium* species but not in plants or mammals prompts the speculation that enzymes involved in biosynthesis of these lipids may represent potential biochemical targets for the discovery of new agricultural fungicides (Stramenopilocides).

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[Received September 19, 1997, and in final revised form and accepted January 28, 1998]

Lipids of *Thermococcus hydrothermalis*, An Archaea Isolated from a Deep-Sea Hydrothermal Vent

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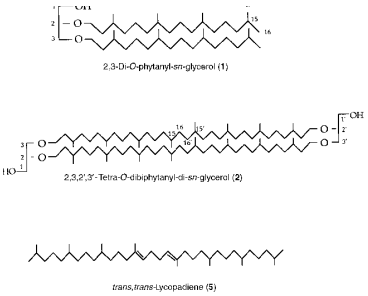
ABSTRACT: The membrane lipids of a deep-sea hydrothermal vent archaea, *Thermococcus hydrothermalis*, were isolated, purified, and structurally characterized. On the basis of acid methanolysis and spectroscopic studies, the polar lipids, amounting to 4.5% (w/w) of the dry cells, comprised diphytanyl glycerol diethers and dibiphytanyldiglycerol tetraethers, in a 45:55 ratio. No cyclopentane ring was present in the tetraethers. From the neutral lipids, accounting for 0.4% (w/w) of the dry cells, besides low amounts of di- and tetraethers occurring in a free form, four acyclic tetraterpenoid hydrocarbons, di- and triunsaturated were identified. All were structurally related to lycopane. The presence of these hydrocarbons provides some evidence that lycopane, widely distributed in oceans, could be derived, at least partially, from the hydrocarbons synthesized by some thermophilic Archaea. Finally, analysis of the uninoculated culture medium indicates that fatty acid derivatives and some steroid and triterpenoid compounds identified in the lipidic extract of the archaea originated from the culture medium. *Lipids 33*, 319–326 (1998).



SCHEME 1

All Archaea, including the halotolerant species, are characterized by the presence of isopranyl glycerol ether lipids in their membranes (1–6). Two types of complex ether lipids occur generally in Archaea: the diphytanyl glycerol diether, i.e., 2,3-di-*O*-phytanyl-*sn*-glycerol **1** and the dibiphytanyl diglycerol tetraether, i.e., 2,3,2′,3′-tetra-*O*-dibiphytanyl-di-*sn*-glycerol **2** (Scheme 1). In these latter, the C_{40} isoprenoid chains derive from ω,ω′ linkages of two *O*-phytanyl residues.

In the Archaea of the thermoacidophilic group, a typical feature is the presence of cyclopentane rings in the C_{40} chains (1). It has been suggested that the macrocycle and the cyclopentane rings occurring in tetraethers enhance the rigidity of the membranes of the microorganisms that are able to grow at elevated temperatures (7,8). However this assumption was reconsidered when new archaea, including some *Thermococcales* isolated from marine hydrothermal vent environments, were shown to contain very low amounts of dibiphytanyl diglycerol tetraethers with cyclopentane rings (2,9,10). In these cases, it has been proposed that the polar groups of the ether lipids, extensively enriched in carbohydrates, would ensure the rigidity of the membranes and reduce their permeability (11).

Within the *Thermococcales*, the genus *Thermococcus* is represented by eight species and some new isolates recognized from their morphological, physiological, and molecular features (12–20). The type species, *T. celer*, exhibits a rather simple lipid composition with phosphatidylmyoinositol ester of phytanyl diether being the most abundant lipid (2) but, in sharp contrast, *T. chitonophagus* is characterized by a more complex composition in core lipids with the presence of diphytanyl glycerol diether and acyclic and monocyclic dibiphytanyl diglycerol tetraether derivatives (16). Recently, we examined *T. hydrothermalis*, a new hyperthermophilic heterotrophic archaeal species isolated from a deep-sea hydrothermal vent on the East Pacific Rise, for its neutral lipid and ether lipid composition.

MATERIALS AND METHODS

Materials. Silica gels for column chromatography (CC) (silica gel 60, 70–230 mesh) and thin-layer chromatography (TLC) (silica gel PF) were from Merck (Darmstadt, Germany). In-

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Abbreviations: CC, column chromatography; DMDS, dimethyl disulfide; FAB–MS, fast atom bombardment–mass spectrometry; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography.

frared (IR) spectra were recorded from carbon tetrachloride solutions in NaCl cells (0.1 mm), using a Perkin-Elmer 1420 (Norwalk, CT) spectrometer. ^{13}C nuclear magnetic resonance (NMR) spectra were obtained from deuterochloroform solutions, with tetramethylsilane as internal reference, on a Bruker 250 spectrometer (Wissenbourg, France). Standard *trans,trans*-lycopadiene was isolated from the green microalga *Botryococcus braunii* as previously described (21), and lycopane standard was prepared by catalytic reduction of *trans,trans*-lycopadiene (21). 6,10,14-Trimethyl pentadecan-2-one was prepared *via* ozonolysis of phytol (21). Standards of isopropyl esters were synthesized by refluxing (1 h) myristic, palmitic, and oleic acids in propan-2-ol, added with a few drops of acetyl chloride to generate hydrochloric acid. Concentration of the reaction mixtures under reduced pressure afforded the pure isopropyl esters. Standards of fatty acid methyl esters were prepared by refluxing separately (1 h) corresponding fatty acids in methanol, added with a few drops of acetyl chloride to generate HCl. Concentration of the reaction mixtures under reduced pressure afforded the pure methyl esters. Standards of ether lipids were obtained from *Caldariella*.

*Microorganism origin and culture conditions***.** The strain AL662^T was isolated from an active chimney and was recovered from a hydrothermal site on the East Pacific Rise. On the basis of phenotypic and phylogenetic analyses and DNA/DNA relatedness, this strain was recognized as a new species of the genus *Thermococcus* and the name *T. hydrothermalis* was adopted (19). Growth medium contained the following nutrients per liter of sea water: tryptone (1 g) (from Biokar Diagnostics, Beauvais, France), yeast extract (4 g) (from Bio-Springer, Maisons-Alfort, France), cystine (5 g) (from Merck), and sea salt (30 g) (from Europrix, Lens, France). The archaea was grown at 79°C for 23 h at pH 6.8 under N_2. The cells were harvested by centrifugation. The yield was approximately 1.7 g lyophilized cells/I.

Lipid extraction and fractionation. A first extraction of the lipids was performed on the lyophilized archaeal biomass (10 g), using dichloromethane/methanol (2:1, vol/vol; 450 mL) with stirring at room temperature, 2 × 18 h. The combined extracts were fractionated, after concentration under reduced pressure, by CC on silica gel in the proportion of 15 g silica for 180 mg lipids. Neutral lipids were first eluted by 305 mL chloroform. Polar lipids were recovered by elution with 1220 mL of acetone and then with 305 mL methanol. Neutral lipids were separated into five fractions (frs. I–V) by CC on silica gel (12 g), using heptane (45 mL; fr. I), heptane/diethyl ether (19:1, vol/vol; 60 mL; fr. II), heptane/diethyl ether (23:2, vol/vol; 75 mL; fr. III), heptane/diethyl ether (17:3, vol/vol; 115 mL; fr. IV), and diethyl ether (45 mL; fr. V). The residual biomass from the first extraction was further extracted with 380 mL of chloroform/methanol/water (1:2:0.8, by vol), buffered with sodium acetate, according to the Bligh and Dyer procedure (22), 2 × 3 h. To the combined supernatants, 60 mL of chloroform and 48 mL of water were added to generate a biphasic system; the chloroform phase was concentrated under reduced pressure.

The presence of lipids in the starting culture medium was also examined by extraction of 40 g lyophilized fresh medium with 500 mL dichloromethane/methanol (2:1, vol/vol), 2 × 18 h. The combined crude extracts were separated on silica gel CC by elution with chloroform, acetone, and methanol, and the culture medium neutral lipids were further separated into five fractions by silica gel as above.

Methanolysis of polar lipid fractions. Acetone and methanol eluates and Bligh and Dyer extract were separately subjected to acid methanolysis (20 mL of dry methanol/HCl, 1 N), at 100°C for 18 h in closed tubes. The reaction mixture was extracted with dichloromethane and then diethyl ether, and the organic phases were washed with water until neutrality. Fractionation of the concentrated extracts by preparative silica gel TLC using heptane/diethyl ether/methanol (4:1:1, by vol) as eluent afforded fatty acid methyl esters (R_f = 0.66), glycerol diether (R_f = 0.34), and diglycerol tetraether (R_f = 0.19). Diphytanyl glycerol diether **1** and dibiphytanyl diglycerol tetraether **2** were analyzed by fast atom bombardment–mass spectrometry (FAB–MS) (Micromasse ZAB-SEQ; Manchester, England) in positive mode *via* inclusion of the products in a nitrobenzyl alcohol matrix and addition of lithium chloride to provide ionized $[M + Li]^+$ species. Diphytanyl glycerol diether, **1**: FAB–MS (low resolution): $[C_{43}H_{88}O_3 + Li]^+$, observed *m/z* 659.7, calculated *m/z* 660.1. Dibiphytanyl diglycerol tetraether, **2**: FAB–MS (high resolution): $[C_{86}H_{172}O_6 + Li]^+$, observed *m/z* 1308.3344, calculated *m/z* 1308.3313.

Fatty acid methyl esters. These were identified by gas chromatography–mass spectrometry (GC–MS) analysis with an HP 5890 chromatograph coupled with an HP 5989 mass spectrometer (Hewlett-Packard, Evry, France) and coinjection with standards. The chromatograph was equipped with a CPSil-5CB fused-silica capillary column (25 m × 0.25 mm) coated with polydimethylsiloxane (Chrompack, Middleburg, The Netherlands). The temperature program was from 150 to 300°C (4°C/min).

Neutral lipid analyses. Neutral lipid fractions, fractions I–V, were analyzed by GC–MS (same spectrometer and CPSil-5CB column). The temperature programs were as follows: fraction I from 140 to 300°C (4°C/min); fractions II–V, from 100 to 300°C (4°C/min).

Diphytanyl glycerol diether **1** and dibiphytanyl diglycerol tetraether **2** were identified as free compounds in fraction IV by silica gel TLC, using heptane/diethyl ether/methanol (4:1:1, by vol) as eluent, and by comparison of their solvent front solvent ratio values (R_f = 0.34 and R_f = 0.19, respectively) with those of standards.

Ratios and amounts of the compounds were evaluated by weighing (ether lipids) and/or GC analyses with an internal standard (lycopane and lycopadiene, fatty acid methyl esters).

Catalytic reduction of C_{40} hydrocarbons. Hydrocarbons (2 mg) dissolved in 5 mL heptane were reduced with H_2 (25 atm), in the presence of catalytic amounts of rhodium (5%) on charcoal, at 40°C for 24 h. Then the catalyst was removed by filtration and the products analyzed by GC–MS (same conditions as for fr. I). Lycopane was identified by coinjection

and comparison of its mass spectrum with that of an authentic standard.

Ozonolysis. Fraction I (2 mg), dissolved in 2 mL dichloromethane was treated by ozone at −78°C until the characteristic blue color of ozone persisted. The excess ozone was then removed by bubbling nitrogen into the cold solution and thereafter the ozonides formed were reduced with triphenylphosphine (3 mg). The resulting solution was allowed to warm to room temperature and, after concentration under reduced pressure, directly analyzed by GC–MS (CPSil-5CB column; temperature programmed from 80 to 300°C, 4°C/min).

Dimethyl disulfide (DMDS) adduction. DMDS adducts were prepared as previously described (23). A portion of fraction I (2 mg) was treated in 100 mL of *n*-heptane by addition of 100 mL of DMDS and 1.2 mg of iodine in 20 mL of diethyl ether. The reaction was carried out in a 10-mL closed tube and kept for over 48 h at 50°C. Thereafter, 200 mL *n*-heptane was added to the reaction mixture, and the excess of iodine was reduced by treatment with 200 mL of an aqueous solution of 5% Na_2SO_3. After recovery of the organic phase, the aqueous phase was extracted for a second time with 200 mL *n*-heptane. The combined extracts were concentrated under a stream of nitrogen and immediately analyzed by GC–MS; the temperature was programmed from 240 to 300°C at 4°C/min.

RESULTS

Lipids extracted from the dry archaeal biomass with dichloromethane/methanol accounted for 4% of the dry weight. Silica gel CC allowed us to fractionate this extract into neutral lipids by elution with chloroform (accounting for 10% of the total extract) and polar lipids by elution with acetone (7.5%) and methanol (70%). Additional lipids were obtained from the extracted biomass by the procedure of Bligh and Dyer (22). They accounted for 0.9% of the dry weight. Acid methanolysis of the residual biomass did not furnish any other lipidic material.

Polar lipids. The polar lipid eluates and the Bligh and Dyer extract were submitted to acid methanolysis in order to release ether lipids in a free form. From the methanol-eluted fraction and the Bligh and Dyer extract, three bands were detected by silica gel TLC (R_f values: 0.66, 0.34, and 0.19) and separated by preparative TLC. FAB–MS analyses (at low and high resolutions), IR and ^{13}C NMR spectroscopies, and comparison with literature data (24) allowed us to establish that compounds exhibiting R_f values of 0.34 and 0.19 on TLC plates were diphytanyl glycerol diether **1** and dibiphytanyl diglycerol tetraether **2**, respectively. Moreover, a comparison of the ^{13}C NMR spectrum of the compound exhibiting the lowest R_f with literature data relative to ^{13}C NMR of cyclopentane-containing tetraethers (24,25) established clearly that no cyclopentane ring occurs in the tetraethers of *T. hydrothermalis.* Dibiphytanyl diglycerol tetraether **2** accounted for *ca.* 43 and 63 wt% of the ether lipids of the methanol-eluted fraction and the Bligh and Dyer extract, respectively.

The farther eluted compounds (R_f = 0.66) consisted in low amounts of fatty acid methyl esters. Through GC–MS analysis, palmitate (16:0, 42.5%), oleate (18:1 *cis* Δ9, 17.5%), stearate (18:0, 15%), laurate (12:0, 7.5%), palmitoleate (16:1 *cis* Δ9, 5%), and myristate (14:0, 5%) were the main fatty acid components identified.

No ether lipid could be detected from the hydrolysis of the acetone-eluted fraction. In this case, only fatty acid methyl esters were isolated. They exhibited a composition similar to that found for the methanol-eluted fraction and the Bligh and Dyer extract. All these methyl esters were also obtained from the acid methanolysis of the polar lipidic fractions of the culture medium extract. This indicates that they are not biosynthesized by the archaea.

On a whole, the ether lipids recovered from methanolyses and purified by TLC accounted for 0.32% of the dry biomass, dibiphytanyl diglycerol tetraether **2** showing a slight predominance (55%).

Neutral lipids. Table 1 shows results concerning the fractionation of the neutral lipids by silica gel CC and the composition of the different fractions.

Fraction I exclusively contained hydrocarbons. GC–MS analysis showed the presence of a range of normal chain hydrocarbons (unsaturated C_{16} and C_{18}, saturated C_{16} to C_{36}) and four C_{40} isoprenoid compounds. Formation of DMDS adducts of the C_{16} and C_{18} alkenes, followed by GC–MS analysis of these products, allowed us to identify four *n*-hexadecenes (Δ2, Δ3, Δ4, and Δ5) and five *n*-octadecenes (Δ2, Δ3, Δ4, Δ5, and Δ6). The analysis of the neutral lipids isolated from the fresh culture medium showed, however, the presence of all these normal hydrocarbons. Thus, only the C_{40} isoprenoid compounds can be considered as metabolites of the *Thermococcus* strain.

Two $C_{40}H_{78}$ and two $C_{40}H_{76}$ hydrocarbons, with two and three degrees of unsaturation, respectively, were identified (Fig. 1). Catalytic reduction of fraction I led to a single $C_{40}H_{82}$ compound other than the nonarchaeal normal alkanes; this hydrocarbon was found to coelute on GC analysis with lycopane (2,6,10,14,19,23,27,31-octamethyldotriacontane), an acyclic tetraterpane. The formation of lycopane through hydrogenation was confirmed by the similarity of its mass spectrum with the one of an authentic standard. The C_{40} hydrocarbons in fraction I thus correspond to two tetradehydrolycopanes (lycopadienes; 35 and 27% of the C_{40} mixture) and two hexadehydrolycopanes (lycopatrienes; 24 and 14% of the C_{40} mixture).

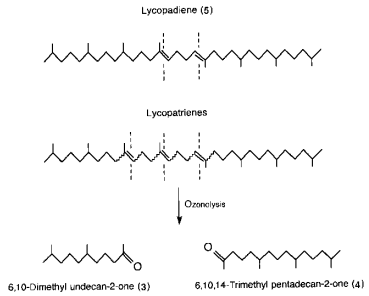
DMDS adducts of the unsaturated C_{40} afforded a very complex mixture of derivatives which did not allow us to determine the position of the unsaturations; instead, this was achieved by means of ozonolysis of the C_{40} dienoic and C_{40} trienoic mixture. GC–MS analysis of the compounds obtained from the reductive degradation of the ozonides indicated the formation of the 6,10-dimethyl undecan-2-one **3** and 6,10,14-trimethyl pentadecan-2-one **4** in a 4:1 ratio (Scheme 2). The nature of these ketones and the relative percentages of the dienic and trienic hydrocarbons strongly suggested that unsaturations occur at positions 14 and 18 in the lycopadienes and at positions 10, 14, and 18 in the lycopatrienes. (Nevertheless, ozonolysis does not allow for the identification of double-bond stereochemistry.)

TABLE 1
Composition of Neutral Lipids Extracted from the Archaeal Biomass[a]

Column chromatography fraction	Percentage of neutral lipids	Percentage of dry weight	Composition[b]
I	20.0	0.08	Normal hydrocarbons: (unsaturated C_{16}*–C_{18}*, saturated C_{16}*–C_{36}*), C_{40} isoprenoid hydrocarbons
II	22.5	0.09	Fatty acid isopropyl esters*, squalene*
III	10.0	0.04	Phytol*, DGD **1**
IV	8.7	0.03	DDT **2**, steroids*, triterpenoids*
V	11.3	0.05	Steroids*, triterpenoids*

[a]In addition to archaeal compounds (ether lipids, lycopadienes, and lycopatrienes), these fractions contain large amounts of nonarchaeal lipids (indicated by * in the Table) originating from the culture medium. Regarding nonarchaeal hydrocarbons, note that the growth medium contained a high amount of yeast extract; and normal hydrocarbons from C_{10} to C_{39} are known to occur commonly in yeasts (26). Moreover, the growth medium is composed of 40 g of nutrients per liter. Accordingly, even if the nutrients contain very low levels of lipidic contaminants, the latter should be present in substantial amounts when compared to the lipids of the archaea (*ca* 1.7 g of dry biomass containing less than 1 mg of free ether lipids and *ca*. 0.7 mg of lycopadienes and lycopatrienes from 1 L of culture). Moreover, to avoid cell lysis, the archaeal biomass was not washed after centrifugation, so nonarchaeal lipids may have been adsorbed on the cells. Additionally, lipids from the culture medium may have been incorporated by *Thermococcus hydrothermalis* as xenobiotic compounds.
[b]DGD **1** = diphytanyl glycerol diether, DDT **2** = dibiphytanyl diglycerol tetraether.

Furthermore, the major lycopadiene was found to coelute on GC analysis with the *trans*,*trans*-lycopadiene **5**, unsaturated at position C-14 and C-18 and previously isolated from a freshwater green microalga, *Botryococcus braunii* (21). Moreover, both compounds exhibited the same mass spectrum, thus demonstrating similar structures; the mass spectra of the derived lycopane, archaeal *trans*, *trans*-lycopadiene, and one lycopatriene are shown in Figure 2. Based on ozonolysis and on the identification of this C_{40} diene, the second diene should correspond to the *trans, cis*- or *cis, cis*-lycopadiene. The stereochemistry of the C_{40} trienes has not been determined. On the whole, the hydrocarbons structurally related to lycopane accounted for 54% of fraction I and *ca.* 0.04% of the dry archaeal biomass.



SCHEME 2

Based on IR and GC–MS analyses, fraction II was shown to comprise mainly a series of saturated and monounsaturated *n*-C_{14}, *n*-C_{16}, and *n*-C_{18} fatty acid isopropyl esters and low amounts of squalene. All these compounds were identified in the neutral lipids isolated from the culture medium extract, thus precluding an archaeal origin. Similarly, phytol, cholesterol, 4-cholesten-3-one, sitosterol and α-amyrin, all found in trace amounts in the more polar CC fractions, were shown to originate from the culture medium.

Finally, TLC of fractions III and IV revealed the presence of free diphytanyl glycerol diether **1** and dibiphytanyl diglycerol tetraether **2** in very low amounts.

DISCUSSION

The ether lipids of *T. hydrothermalis* are characterized by a slight dominance of dibiphytanyl diglycerol tetraethers over diphytanyl glycerol diethers. Similar ether lipid profiles have been reported to occur in several Archaea of the order of *Thermococcales*, including some *Thermococcus* species (6,27,28). It was considered that the tetraether/diether ratio tends to increase with the temperature of growth and could be

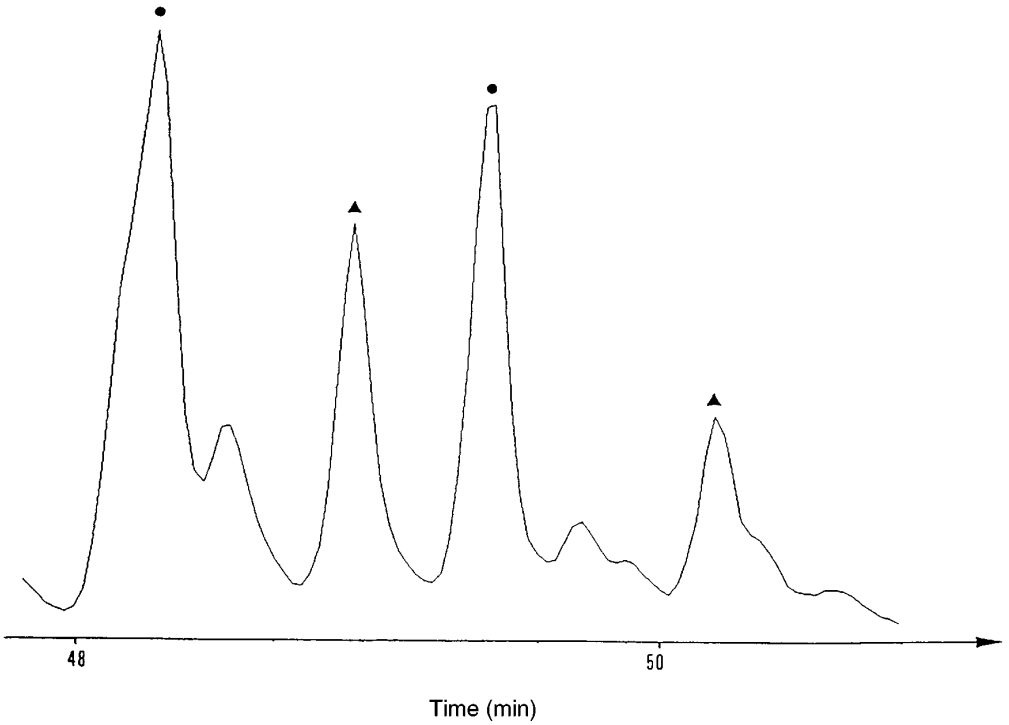


FIG. 1. Gas chromatographic trace of the C_{40} isoprenoid compounds. ●, $C_{40:2}$ isoprenoid; ▲, $C_{40:3}$ isoprenoid.

qualified as a temperature-related adaptative feature. However, as suggested by some authors (6), the exclusive presence of diethers in some other species of this genus precludes the use of the tetraether/diether ratio as a criterion for classification of this hyperthermophilic Archaea. Furthermore, no cyclopentane ring occurs in the tetraethers. Accordingly, as previously suggested for other Archaea isolated from deep-sea hydrothermal vent environments (11), it is very likely that the polar head groups of the ether lipids reinforce the membrane rigidity of these microorganisms growing at elevated temperatures in such environment.

Trace amounts of free diphytanyl glycerol diether and dibiphytanyl diglycerol tetraether were detected in the neutral lipids of *T. hydrothermalis*. This occurrence has previously been noticed for some other Archaea (29–32). However, the most important feature observed from neutral lipid analysis in this species is the identification of hydrocarbons structurally related to lycopane. To date, some hydocarbons have been clearly identified only in two groups of Archaea. Thus, in halophilic archaea, squalene and hydrosqualenes have been identified, and C_{15}, C_{20}, C_{25} and C_{30} isoprenoids in methanogens (8,31,33–35). The presence of compounds exhibiting a low polarity, which may thus correspond to hydrocarbons but for which the structure was not examined, has been previously reported in a very limited number of cases in the lipids of thermoacidophilic Archaea (29,36,37). The tetraterpenoid hydrocarbons, lycopadienes, and lycopatrienes identified in *T. hydrothermalis* arise, based on the structure of the central part of these molecules, from the tail-to-tail condensation of two diterpene derivatives. In sharp contrast, the C_{40} chains of the tetraethers are formed *via* a head-to-head linkage of two phytanyl residues. Two different biosynthetic pathways for the production of C_{40} isoprenoid chains seem therefore to coexist in *T. hydrothermalis*. The taxonomic value of these C_{40} hydrocarbons with a lycopane-type skeleton in Archaea, especially in the order of *Thermococcales*, remains to be established. To date, the dominant hydrocarbon of *T. hydrothermalis* had been identified only in a freshwater green microalga, *Botryococcus braunii* (21).

Unsaturated hydrocarbons show a well-known and general trend to be reduced into alkanes during the first step of fossilization in surface sediments. Accordingly, the occurrence of lycopane-related hydrocarbons in an Archaea is important from a geochemical point of view. Indeed, it is well documented that lycopane is widely distributed in oceans—concentrations up to 1.5 ng/L of seawater have been measured (38)—and its presence in some marine sediments has been noted (39–42); its origin, however, remains unknown.

The identification of potential geochemical precursors of lycopane in *T. hydrothermalis* gives some support to a previous assumption for an archaeal origin of this biomarker (39). In this respect, it is important to note that lycopane and archaeal ether lipids have been found together in a same sediment (43). The present finding does not rule out, however, a photoautotrophic source for lycopane (38), especially in oxic marine waters.

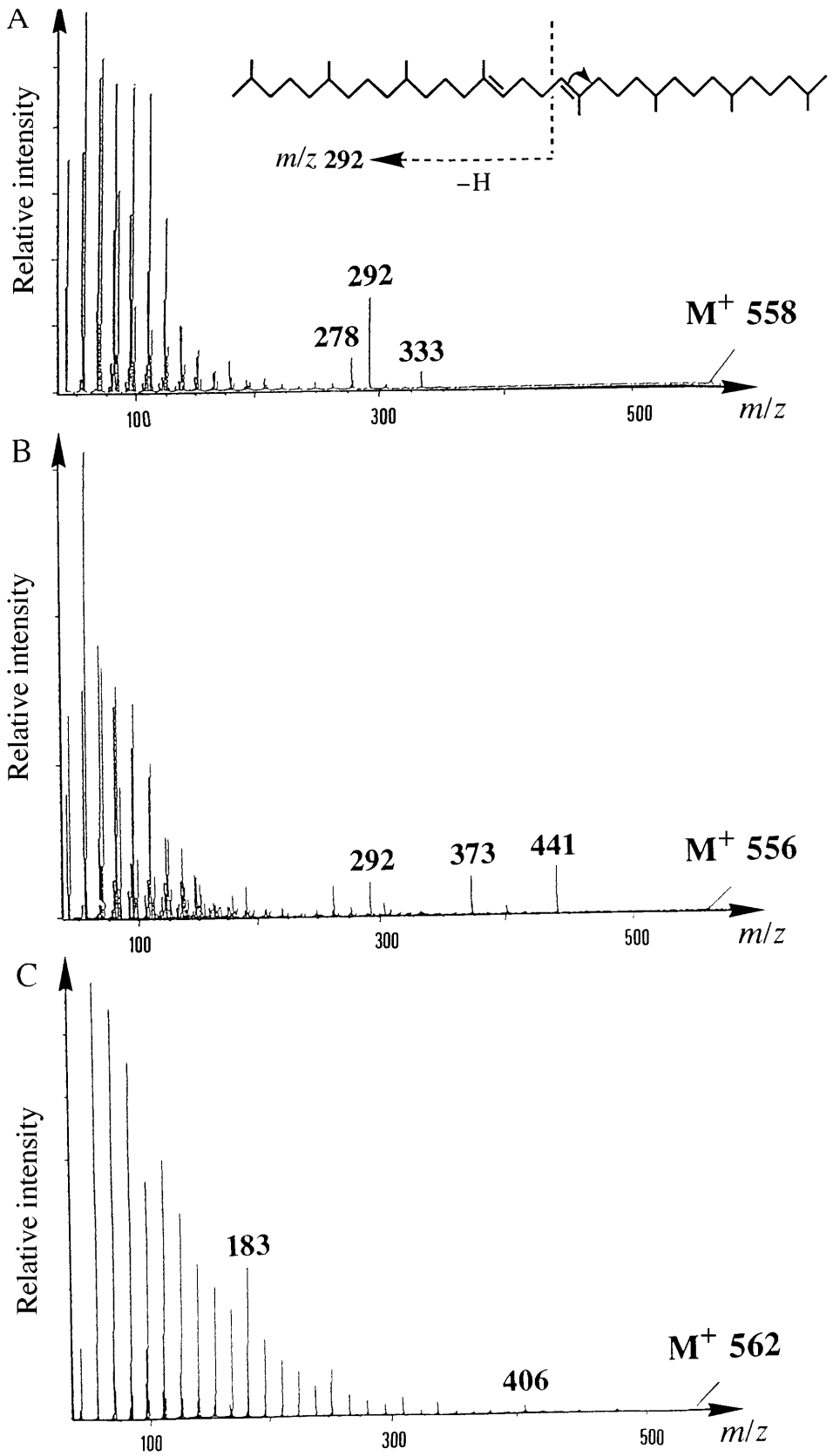


FIG. 2. Mass spectra of *trans,trans*-lycopadiene (A), first eluted lycopatriene (see Fig. 1) (B), and lycopane (C).

ACKNOWLEDGMENTS

The authors thank Yves Pouet for assistance in GC–MS experiments. We also thank Dr. Pierre Albrecht, University of Strasbourg, for core lipid standards.

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[Received October 14, 1997, and in final revised form January 19, 1998; revision accepted January 26, 1998]

Fatty Acid Composition of the Cellular Slime Mold *Polysphondylium pallidum*

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ABSTRACT: The cellular slime mold *Polysphondylium pallidum* was grown upon *Escherichia coli* B/r, and the fatty acid compositions of total lipids obtained from vegetative amebae and aggregation-competent cells were compared. Fatty acids isolated from vegetative cells included C-17 and C-19 cyclopropane fatty acids and also straight-chain, saturated fatty acids. The cyclopropane fatty acids were derived from the ingested bacteria. Development of amebae to aggregation-competent cells was accompanied by a substantial decrease in saturated cyclopropane fatty acids and a concomitant increase in unsaturated fatty acids and unsaturated cyclopropane fatty acids, mostly as 18:3 (5,9,12). We report here the fatty acid composition and identify the occurrence of Δ5 desaturation of cyclopropane fatty acids, namely, 9,10-methylene 5-hexadecenoic acid and 11,12-methylene 5-octadecenoic acid. These fatty acids have not been reported previously in the related species *Dictyostelium discoideum,* which also feeds on *E. coli* B/r and has Δ5-desaturation activity.

Lipids 33, 327–332 (1998).

A wild-type strain (WS320) of the cellular slime mold *Polysphondylium pallidum* grows upon *Escherichia coli* B/r as a food source; lack of food (starvation) triggers a well-characterized pattern of development which begins with the aggregation of individual amoebae and culminates in a multicellular fruiting body. *Polysphondylium pallidum* is an excellent model system for study of cell–cell interactions. Knowing the fatty acid composition is the first step in obtaining information with regard to membrane fluidity in relation to the cell–cell interaction. Many unusual fatty acids have been found in cellular slime molds, and the unsaturation patterns of fatty acids in *Dictyostelium discoideum* have been reported (1–3), but only a few chemical studies have been published on the fatty acids of a related species *P. pallidum* (4).

Cyclopropane fatty acids have been mainly noted in *E. coli* (5), and are formed by a postsynthetic modification of the unsaturated fatty acids of membrane phospholipids (6). The reaction involves the addition of a CH_2 group from *S*-adenosylmethionine to the double bond of a fatty acid to form the cyclopropane ring. To determine the membrane components of the cells involved in cell–cell interactions, we have now analyzed the fatty acids of *P. pallidum*. In this study, we determined the fatty acid composition and identified Δ5 desaturation in C-17 and C-19 cyclopropane fatty acids that also have a cyclopropane ring at Δ9 and Δ11 positions, respectively. These fatty acids have not been reported in *D. discoideum,* which also feeds on bacteria and has Δ5 desaturation.

EXPERIMENTAL PROCEDURES

Cell culture. Cells of *P. pallidum* strain WS320 were cultured with or without *E. coli* B/r as described previously (7,8) to the density of 2–5 × 10^6 cells/mL. At this stage, cells were harvested as vegetative cells (t_0). To obtain aggregation-competent cells ($t_{6.5}$), vegetative cells were washed with 17 mM phosphate buffer (pH 6.2) and resuspended at a density of 1 × 10^7 cells/mL and incubated for 6.5 h. The food source bacterium, *E. coli* B/r, was cultured in Luria-Bertani medium at 37°C, 150 rpm for at least 20 h to be a stationary phase.

Chemical materials. Silica gel plates for thin-layer chromatography (TLC) were purchased from E. Merck (Darmstadt, Germany). Hydrazine hydrate was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dimethyl disulfide and pyrrolidine were supplied from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade available.

Preparation of lipids and fatty acids. Total lipids were extracted by the method of Bligh and Dyer (9) from the vegetative-growth phase and 6.5-h starved wet cells as described above. Alkaline methanolysis of the total lipids released ester-linked fatty acids as methyl esters. The total fatty acid methyl esters (FAME) were separated according to the degree of unsaturation by argentation TLC (AgTLC) on a silica gel plate with a solvent system of chloroform/ethanol (99:1, vol/vol). After the plate had been developed, it was dried and sprayed with acetone/water (4:1, vol/vol) containing 0.01% primuline. The spots were detected under ultraviolet light. Each FAME was extracted with a mixture of hexane, methanol, and 500 mM NaCl in water (2:1:1, by vol) from the silica gel that had been scraped from the plate, dried, and then redissolved in hexane.

Derivatization and mass spectrometry. FAME were then

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Abbreviations: AgTLC, argentation TLC; DMDS, dimethyl disulfide; FAME, fatty acid methyl ester; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography.

analyzed by gas chromatography (GC) (model 263-30; Hitachi, Tokyo, Japan) using a flame-ionization detector and capillary column (CPS-1; 50 m × 0.25 mm, i.d.; Quadrex, New Haven, CT). The separated FAME were identified by comparing their retention times with those of authentic standards and by GC–MS, and were quantitated with a data analyzer (Chromato-Integrator D-2500; Hitachi) as previously described (10). Hydrogenation was carried out in glacial acetic acid with PtO_2 as a catalyst at room temperature for 6 h (11). The position of the double bond in the FAME was determined by the pyrrolidine method (12), and by an I_2-catalyzed reaction for the formation of the adducts with dimethyl disulfide (DMDS) according to a minor modification (13) of the procedure of Shibahara *et al.* (14). Pyrrolidine adducts and DMDS adducts were analyzed on a slightly polar HP-5 column (30 m × 0.32 mm, i.d.; Hewlett-Packard, Palo Alto, CA) in a Hewlett-Packard HP-5890 gas chromatograph coupled to a JMS-AX500 mass spectrometer (Jeol, Tokyo, Japan). The column temperature was increased from 260 to 300°C and from 230 to 330°C at a rate of 2°C/min for the pyrrolidine adducts and DMDS adducts, respectively. Hydrazine-reduced and hydrogenated fatty acids were analyzed by GC and GC–MS as described above.

RESULTS AND DISCUSSION

The lipid compositions from the cellular slime mold *P. pallidum* were examined at the different stages of development by two-dimensional TLC to see the changes in the composition of membrane lipids occurring during cell aggregation. We obtained 21–31 mg of total lipids from 1×10^9 cells by the method of Bligh and Dyer (9). The major phospholipid classes were shown to be phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and phosphatidic acid. These results are essentially the same with those of *D. discoideum* (15). For further analysis, total lipids were extracted from cells at both the growth and aggregation-competent phases, and subjected to alkaline methanolysis. In mixed cell suspensions, *D. discoideum* and *P. pallidum* form common aggregates, but the two cell types are sorted out within the aggregate during the aggregation-competent stage (16). This suggests that some membrane components may be involved in cell recognition during the aggregation-competent stage.

The resultant FAME were fractionated by AgTLC which separates the subcomponents according to the degree of unsaturation, and we were able to detect saturated, monoenoic, dienoic, and trienoic fractions in the cellular slime mold *P. pallidum*.

We analyzed the monoenoic fraction by a capillary GC (Fig. 1A) and by the pyrrolidine method (12) to determine the positions of double bonds. The mass spectra of the pyrrolidide derivatives of these acids disclosed the unexpected presence of two dienoic-like fatty acids in a monoenoic fraction (Fig. 1 A, peaks 1 and 2), which had co-migrated with *cis*-monoenoic fatty acids on an above-mentioned AgTLC plate. The pyrrolidine method enabled us to estimate peaks 1 and 2 as 17:2(5,9) and 19:2(5,11), respectively (data not shown). To confirm the position of the double bond, we subjected the monoenoic fraction to an I_2-catalyzed reaction for the formation of the adducts with DMDS (13,14). Figure 2 shows the mass spectra of the DMDS adducts of peak 1 and 2 methyl esters, respectively. The mass spectra indicated only Δ5 desaturation in peaks 1 and 2. If the reaction had been incomplete, other typical fragment ions that implicate the desaturation must have appeared at other positions (i.e., Δ9 or Δ11); actually, no other typical fragmentation was detected. To clar-

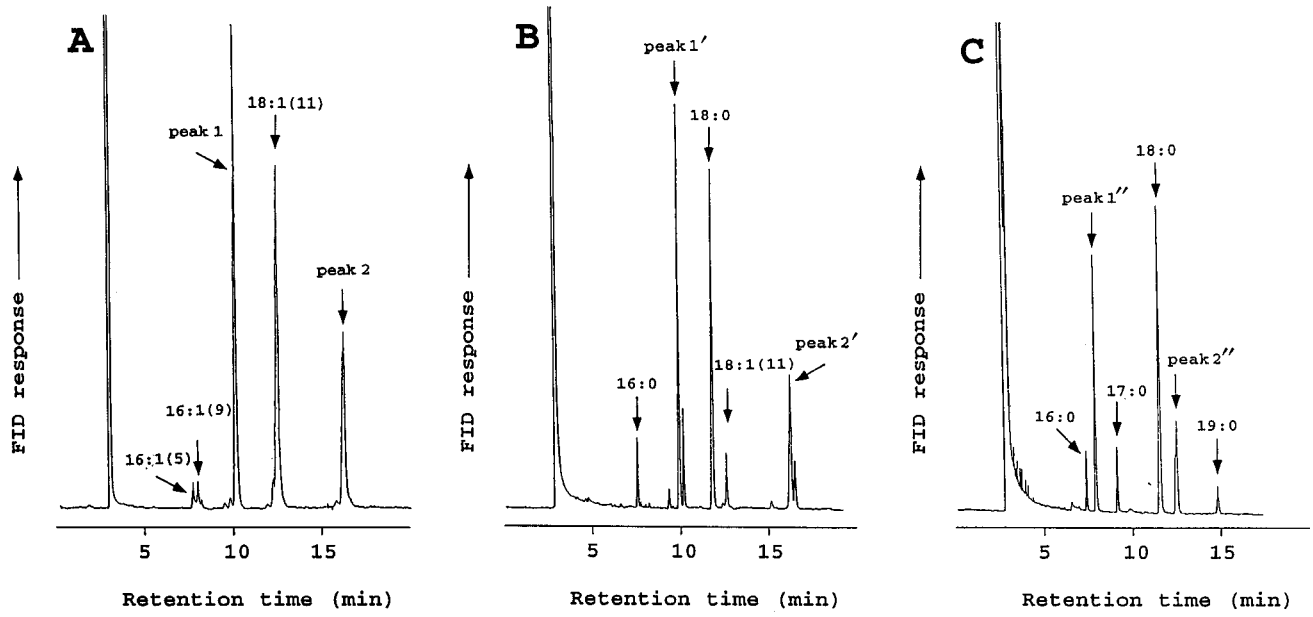

FIG. 1. Gas chromatograms of methyl esters of a monoenoic fraction. (A) Before hydrazine reduction, (B) after hydrazine reduction, and (C) after hydrogenation in glacial acetic acid of a hydrazine-reduced monoenoic fraction.

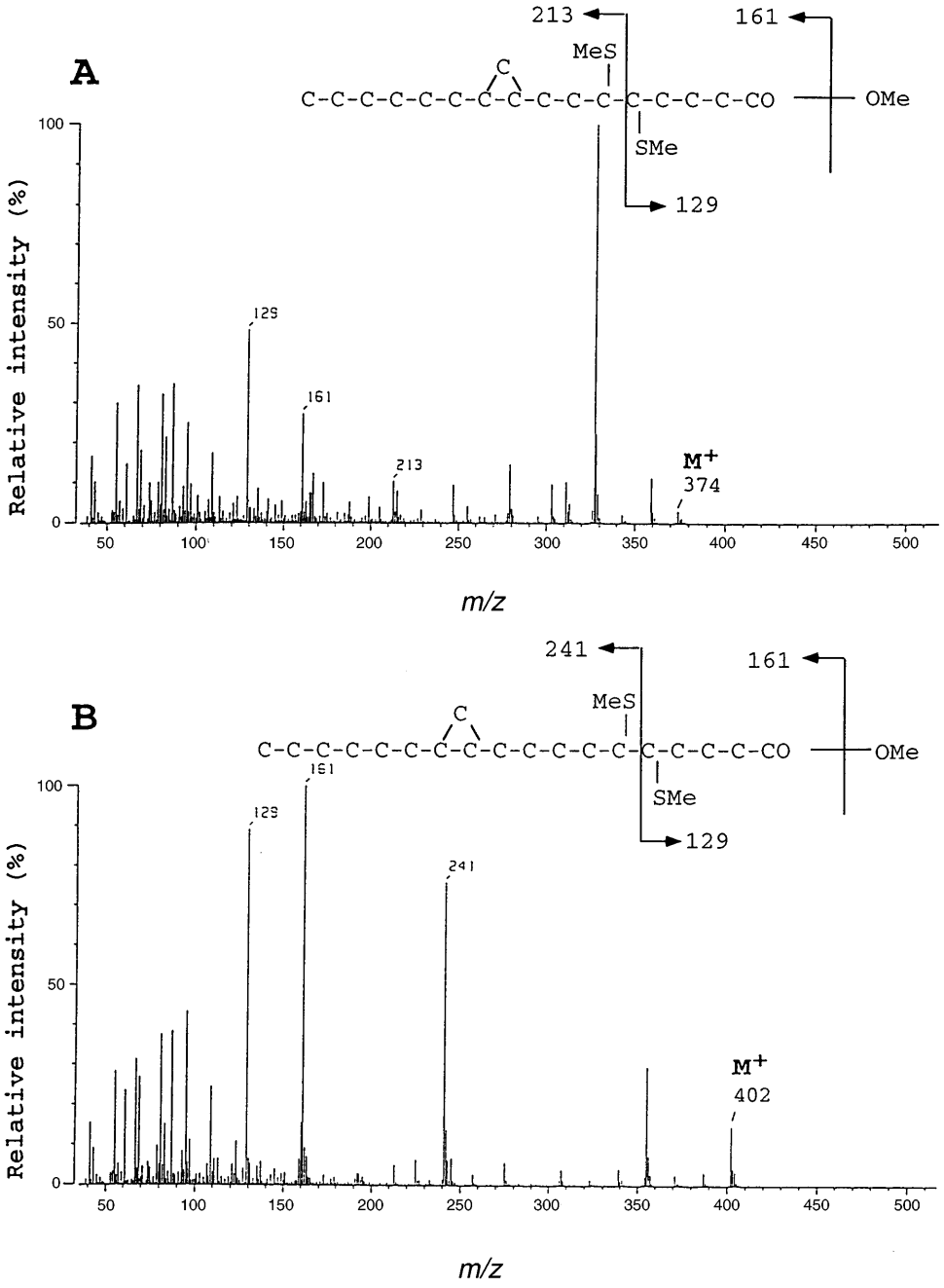

FIG. 2. Mass spectra of dimethyldisulfide (DMDS) adducts of peaks 1 (A) and 2 (B) acid methyl esters. Fragmentation scheme of DMDS adducts of these fatty acids is also indicated.

ify the inconsistency between these results, the monoenoic fraction was subjected to a hydrazine reduction, which reduces double bonds without geometrical or positional isomerization (14), to test whether a standard material (9,12-octadecadienoic fatty acid) can be reduced. It could. It was almost completely reduced to the saturated octadecanoic acid. The hydrazine-reduced monoenoic fraction was then subjected to capillary GC (Fig. 1). Peaks 1 and 2 shifted to the positions of cyclopropane or monoenoic acids, but not the saturated acids (Fig. 1B, peaks 1′ and 2′).

To clarify the issue further, we measured the mass spectra of peaks 1′ and 2′ reduced with hydrazine (data not shown). The results were similar to those recorded for *cis*-cyclopropane fatty acids described previously (17), and we therefore speculated that peaks 1 and 2 were cyclopropane monoenoic fatty acids. A method used to confirm the identity of this estimated structure was a combined procedure: a monoenoic fraction was reduced with hydrazine (14), and then subjected to hydrogenation in glacial acetic acid (11). Any cyclopropane fatty acid esters before the hydrogenation

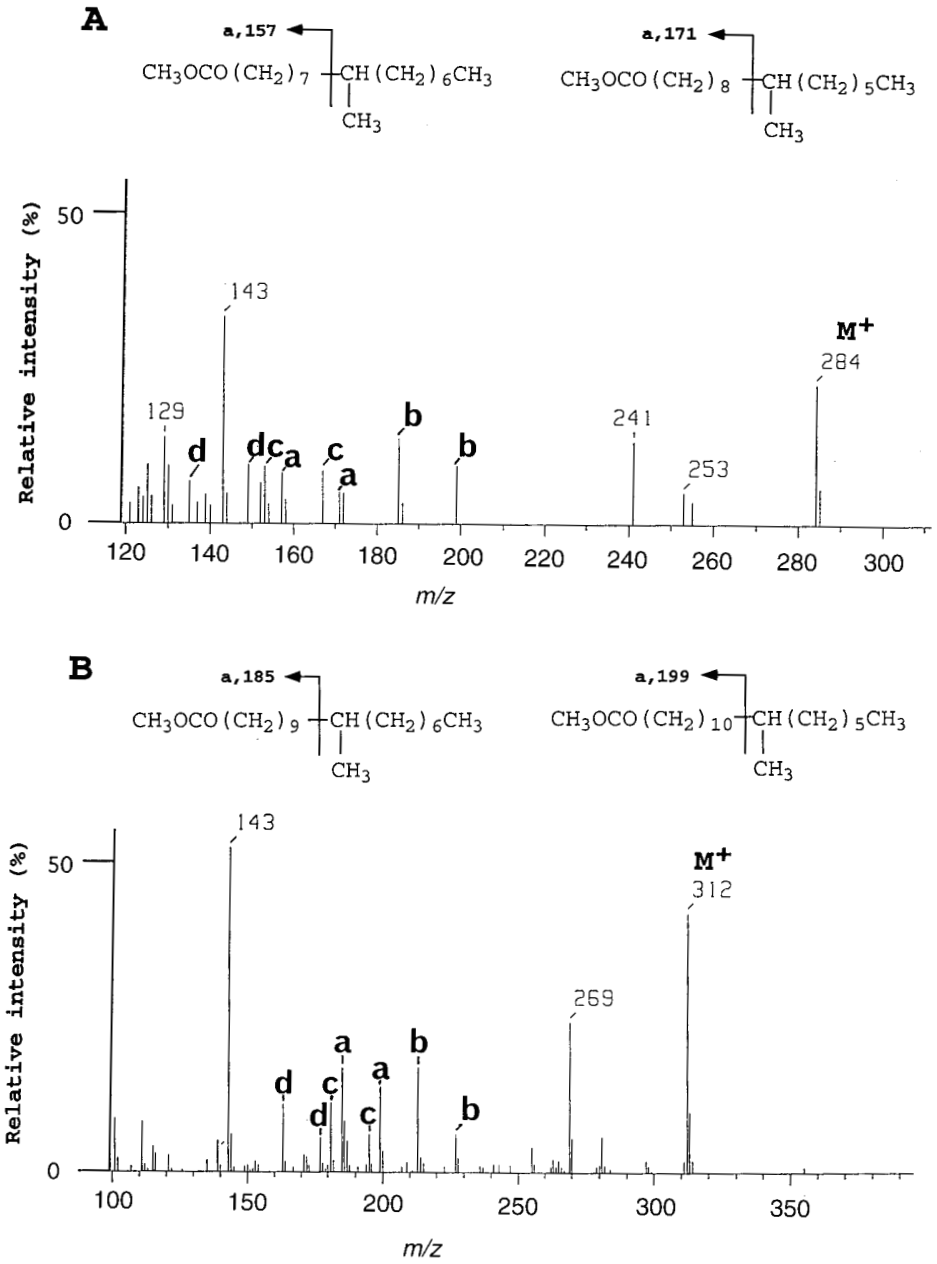

FIG. 3. Partial mass spectra of peaks 1″ and 2″ after hydrogenation in glacial acetic acid. Origins of the most common peaks in these spectra are summarized in Table 1. (A) The branched-acid esters derived from 9,10-methylene hexadecanoic acid. (B) The branched-acid esters derived from 11,12-methylene octadecanoic acid.

would now be a mixture of branched-chain and straight-chain fatty acid esters (11). Mass-spectral analysis of the mixture of branched-chain acid esters permits assignment of the position of the methyl groups: the position, that is, of the cyclopropane ring in the parent acids (11). Figure 1C shows a capillary GC trace of FAME from a hydrazine-reduced, hydrogenated monoenoic fraction. Peaks 1′ and 2′ shifted, respectively, to peaks 1″ and 2″, the positions of the branched-chain fatty acids, although about 15% of peaks 1′ and 2′ shifted to the saturated fatty acids. The partial mass spectra of peaks 1″ and 2″ are shown in Figure 3. The origins of the most common peaks in these spectra are summarized in Table 1. The location of the cyclopropane ring *via* the branch location is indicated by ions a, a+1 which were formed by α cleavage to the points of branching and abstraction of one hydrogen. The resulting characteristic sets of two adjacent peaks 14 mass units apart are easily recognized and used to establish the points of branching as shown by Figure 3 and Table 1. These results (Fig. 3 and Table 1) enabled us to identify peaks 1′ and 2′ *cis*-9,10-methylene hexadecanoic acid and *cis*-11,12-methylene octadecanoic acid. Finally, we conclude that peaks 1 and 2 in Figure 1 are all *cis*-9,10-methylene hexadeca-5-enoic acid

TABLE 1
The Origins of the Most Common Peaks

Designation in figures	Origin	*m/z*
a	$CH_3OCO(CH_2)n+$[a]	157, 171 185, 199
a + 1	Rearrangement of one and two hydrogens from the remainder of the molecule to a	158, 172 186, 200
b	$CH_3OCO(CH_2)nCH^+$ $\vert$ CH_3	185, 199 213, 227
c	Formed predominantly by elimination of CH_3OH from b	153, 167 181, 195
d	Due to elimination of H_2O from c	135, 149 163, 177

[a]*n,* Indicates the position of branching.

TABLE 2
Fatty Acid Compositions of *Polysphondylium pallidum* Total Lipid[a]

Fatty acids	Composition (%) t_0	$t_{6.5}$
12:0	0.2	trace
14:0	5.4	0.3
16:0	19.0	2.7
16:1(5)	0.1	0.6
16:1(9)	0.4	0.5
16:2(5.9)	1.1	2.4
17:0	0.8	0.7
cycl7:0	20.8	10.7
17:1(9)	0.1	0.2
cycl 7:1(5)	4.5	9.9
17:2(5,9)	0.2	1.2
17:3(5,9,12)	0.5	1.3
18:1(9)	1.1	0.9
18:1(11)	4.9	13.0
18:2(5,9)	1.7	0.6
18:2(5,11)	3.0	6.5
18:3(5,9,12)	12.5	25.8
cycl9:0	17.7	10.7
cycl9:1(5)	2.9	9.3
Othersb	3.1	2.7

[a]The above data were indicated by relative % (w/w) to the total lipid extracted from swollen cells. Vegetative stage cells (t_0) and aggregation competent cells ($t_{6.5}$) were analyzed according to the Experimental Procedures section.
[b]Indicates several unidentified minor components.

and all *cis*-11,12-methylene octadeca-5-enoic acid, and the scheme is shown in Figure 2. The percentage of these fatty acids in total fatty acids increased in the cells that had been starved for 6.5 h in shaken suspension without bacteria (Table 2), but not in just-harvested cells of bacterially grown, vegetative growth-phase cells, indicating that all *cis*-9,10-methylene hexadeca-5-enoic acid and all *cis*-11,12-methylene octadeca-5-enoic acid are actual metabolites of this organism.

To clarify the origin of these cyclopropane fatty acids, we also analyzed the fatty acids of axenically grown cells. Under these conditions, *Polysphondylium* cells grow normally and make aggregates. We could not, however, detect any cyclopropane fatty acids in the axenically grown cells (Fig. 4). The most reasonable explanation is that *P. pallidum* took saturated cyclopropane fatty acids from bacteria and converted them into unsaturated cyclopropane fatty acids using an endogenous Δ5 desaturase. This idea is supported by C-17 and C-19 cyclopropane fatty acids being the major lipid components of *E. coli*. We analyzed the fatty acid compositions of *E. coli* B/r and obtained results that were similar to previous reports (5).

On the other hand, it is known that *D. discoideum* wild-type strain, which contains Δ5-desaturated fatty acids, is also able to grow upon bacteria, and contains only saturated cyclopropane fatty acids but not desaturated ones (1), although we could not explain from where the difference between *D. discoideum* and *P. pallidum* was derived. In contrast, there are reports describing the inhibition of fatty acid Δ5- and Δ6-desaturation in liver microsomes of rats which were fed with baobab seed oil containing cyclopropane fatty acids but not heated oil practically devoid of cyclopropane fatty acids (18). Figure 1 shows that *Polysphondylium* contains an equal amount of 16:1 (5) and 16:1 (9). Taken together, it is speculated that *P. pallidum* cells have Δ5 desaturase whose specificity was not reported before. Cyclopropane fatty acids are one of the major types of fatty acids found in *E. coli* and other bacteria. These acids are formed by a postsynthetic modification of the unsaturated fatty acids of membrane phospholipids. Many functions have been proposed for cyclopropane fatty acids. However, *E. coli* Cfa-mutants completely defective with a cyclopropane synthetase grew and survived under various environmental stresses similar to strains which are able to synthesize cyclopropane fatty acids originally (19). It is not known, at present, whether the desaturation of cyclopropane fatty acids during aggregation is necessary or not.

Finally, it may be meaningful to address an evolutionary significance: when an organism feeds on some other organism as a new food source, the organism may be not able to employ a previously unencountered metabolite (in this case, cyclopropane), but during a long time the organism might acquire an ability (by addition of a new gene to the genome) to employ an ingested metabolite useful for the cells. The above result may be a mechanism promoting evolution. Although the obtained characteristics may have only a subtle effect on the survival of the cells, the mechanism may promote addition of a particular gene to a given species.

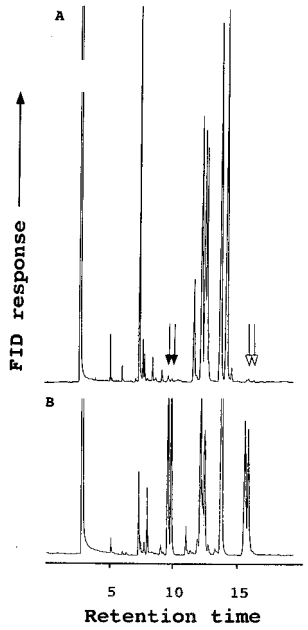

FIG. 4. Mass spectra of total fatty acid methyl esters of axenically grown cells (A) and bacterially grown cells (B). Arrows indicate the positions of the C17 cyclopropane fatty acid, 9,10-methylene hexadeca-5-enoic acid (closed) and C19 cyclopropane fatty acid, 11,12-methylene octadeca-5-enoic acid (open), respectively. In axenically grown cells, we could not detect any cyclopropane fatty acids, although we can see small peaks at the positions of closed arrow. The positions of C17 cyclopropane fatty acid and 9,10-methylene hexadeca-5-enoic acid are identical with those of 17:1 and 17:2.

ACKNOWLEDGMENTS

We thank Drs. A. Shibahara (Osaka Prefectural College of Health Science) and H. Okuyama (Hokkaido University) for helpful discussions.

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[Received October 27, 1997, and in final revised form February 2, 1998; revision accepted February 9, 1998]

Rapid Hydrolysis of Bile Acid Conjugates Using Microwaves: Retention of Absolute Stereochemistry in the Hydrolysis of (25*R*) 3α,7α,12α-Trihydroxy-5β-cholestan-26-oyltaurine

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ABSTRACT: In recent years, defects of bile acid synthesis caused by disorders of peroxisome biogenesis have led to increased interest in C_{27} bile acids. In humans, while the majority of bile acids are C_{24} carboxylic acids, the presence of increased concentrations of C_{27} bile acids and their metabolites in hereditary diseases associated with peroxisomal dysfunction can serve as a useful marker for the intensity of the metabolic disorder. Our present studies describe an efficient method for the rapid hydrolysis of C_{27} and C_{24} bile acid conjugates using a commercial microwave oven. The advantages of this method include freedom from racemization, minimal activation, mild reaction conditions, and the highly stereocontrolled nature of the reaction, thus allowing for free bile acid recovery in high yield. For example, when (25*R*) 3α,7α,12α-trihydroxy-5β-cholestan-26-oyl taurine, a major compound present in the bile of *Alligator mississippiensis*, was deconjugated with 4% NaOH/diethylene glycol or 1 M LiOH/propylene glycol in the microwave oven for 4–6 min, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) was obtained in 81% yield with retention of configuration at C-25. It is suggested that present studies will be helpful in delineating the absolute stereochemistry of 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase, the peroxisomal enzyme that catalyzes the first step in the oxidation of THCA.
Lipids 33, 333–338 (1998).

Several genetic diseases, including disorders of peroxisome biogenesis such as Zellweger syndrome, have been described (1–3) in which abnormal bile acid metabolism is characteristic of the primary defect (4,5). The presence of abnormally high plasma levels of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and of 3α,7α-dihydroxy-5β-cholestan-26-oic acid in Zellweger syndrome has resulted in increased interest in these C_{27} bile acid intermediates and their intact conjugates which are usually absent or present in very low concentrations in normal humans (4,5).

In mammals, the naturally occurring bile acids are C_{24} carboxylic acids which are formed from cholesterol in the liver. After their biosynthesis, their sidechain is amidated with glycine or taurine to form *N*-acyl conjugates which are secreted into the bile and stored in the gallbladder (6–8). However, in order to analyze them by gas–liquid chromatography (GLC), it is necessary to carry out the hydrolysis of the C_{24} and C_{27} amide bonds to give C_{24} and C_{27} free carboxylic acids. Hydrolysis of bile acid amides has traditionally been carried out by vigorous saponification in [15% NaOH/50% aqueous ethanol at 115–120°C,10 h in a Parr bomb, Compound **I**, Scheme 1, and Compound **I**, Scheme 2 (9)]. These drastic reaction conditions are unsuitable because many broken-down products are produced leading to decreased yield. Also, such reaction conditions frequently lead to isomerization of the stereogenic center at C_{25} in the taurine or glycine conjugates of THCA (Compound **III**, Scheme 2) (9,10). The commercially available bacterial enzyme, bile salt hydrolase or cholylglycine hydrolase (CGH), has been utilized in hydrolyzing an unactivated and sterically unhindered amide bond of tauro- and glyco-conjugates of the normally occurring C_{24} bile acids (Compound **I**, Scheme 1) (11). A careful and systematic study on the relationship of chemical structure (nuclear or sterically hindered side chain) to the enzymatic hydrolysis of conjugated bile salts recently has been reported by Huijghebaert and Hofmann (12). These results as well as the report of Batta *et al.* (13) suggest that a sterically hindered amide bond or alteration of the length of the side chain in bile acid conjugates causes significant resistance to hydrolysis by CGH (12–15). For example, this enzyme has been found to be ineffective in cleaving the taurine and glycine conjugates of THCA (Compound **III**, Scheme 2) (16), a major C_{27} bile acid found in patients with Zellweger syndrome (4,5) as well as a normal component in the bile of *Alligator mississippiensis* (17,18). Further studies by Whit-

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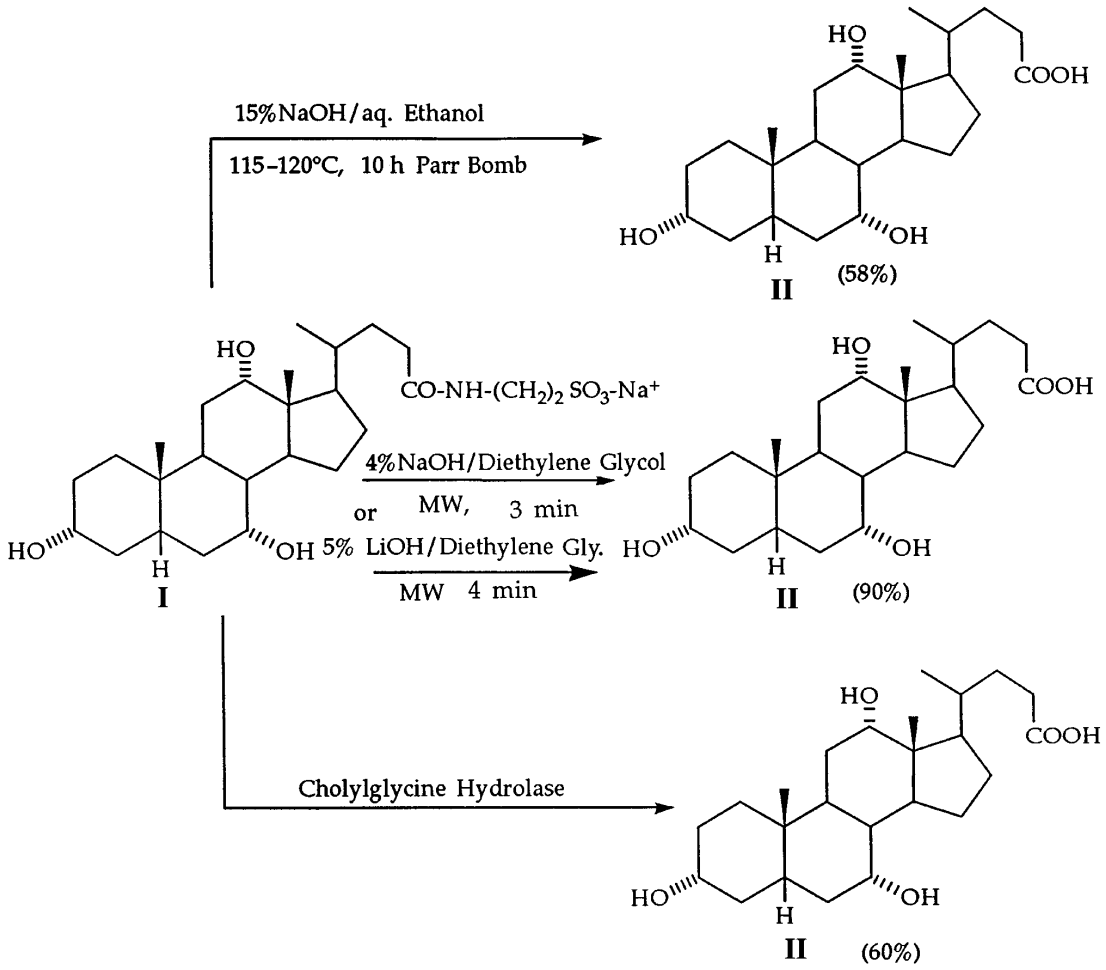
Abbreviations: CGH, cholylglycine hydrolase (E.C. 3.5.1.24); FAB-MS, fast atom bombardment-mass spectrometry; GLC, gas–liquid chromatography; MW, microwave oven; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine acid; TLC, thin-layer chromatography.



SCHEME 1

ney and Vessey (19) and Kimura *et al.* (20) have demonstrated that C_{23} nor-cholyltaurine and ursodeoxycholylsarcosine are resistant to hydrolysis by CGH.

Our interest in the hydrolysis of bile acid amides stems from ongoing research on the microwave-induced organic reactions of bile acids and bile alcohols (18,21–24). This paper describes an efficient method for the rapid hydrolysis of C_{24} and C_{27} amidated bile acids using a commercial microwave oven (MW). This methodology, which recently has been used for saponification, acetylation, amidation of free bile acids, and rapid hydrogenation of unsaturated sterols and bile alcohols (18,21–25), will be shown to hydrolyze the amidated C_{24} and C_{27} bile acids with high yield and with a high degree of purity. For example, the taurine conjugate of 25*R* diastereomer of THCA (C_{27} amidated bile acid) when subjected to microwave heating conditions yielded a single diastereoisomer of the C_{27} bile acid with retention of original configuration (Compound **II**, Scheme 2).

EXPERIMENTAL PROCEDURES

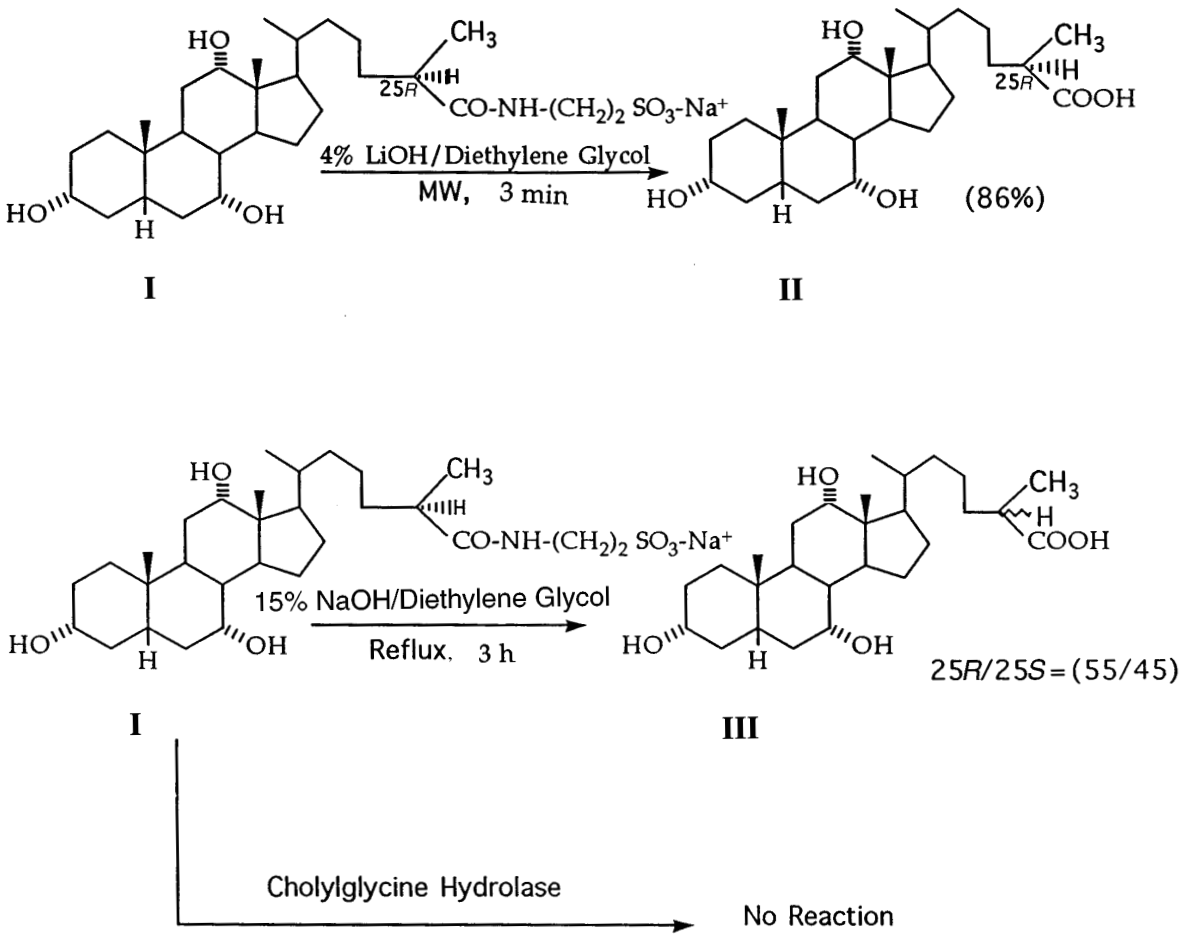
Melting points. These were determined on a Thermolyne apparatus (model MP-12600; Dubuque, IA) and are uncorrected.

Thin-layer chromatography (TLC) was performed in silica gel G plates (0.25 mm thickness, Brinkmann). The spots were detected with phosphomolybdic acid (3.5% in isopropanol) and sulfuric acid (10%).

Fourier transform infrared spectra were determined in $CHCl_3$ on a Perkin-Elmer model 421.

Optical measurements. The circular dichroism measurements were carried out on a Jasco-500A (Manchester, England) spectropolarimeter at 24°C, under a stream of high-purity, dry N_2. The coefficient of dichroic absorption, $\Delta\varepsilon$, was calculated from the molar ellipticity $[\Theta]$ by the following equation: $\Theta = 3300\ \Delta\varepsilon$. Both the Θ and $\Delta\varepsilon$ are expressed in degree $\times$ cm^2 $\times$ dmol^{-1} (26).

Positive ion fast atom bombardment mass–spectrometry (FAB–MS). Direct probe mass spectrum analysis of the con-



SCHEME 2

jugated bile acids was performed by FAB mass spectrometer using a ZAB-1F instrument (VG, Manchester, England) equipped with a standard FAB-source as described previously (26,27). In the FAB mode (positive ions), the sample was dissolved in thioglycerol, and a small aliquot (1 to 2 µL) was placed on the mass spectrometer direct probe. After insertion into the mass spectrometer, the sample was bombarded with a neutral atom beam of xenon with approximately 6 kV of translational energy.

GLC. Capillary GLC analysis of bile acid methyl esters (as the trimethyl silyl derivatives) was performed on Hewlett-Packard model No. 4890 (Palo Alto, CA) (equipped with a flame-ionization detector) and a split column injector using a CPsil 5 (CB) WCOT capillary column (25 m × 0.22 mm with 0.13-mm film thickness) as described previously (18–22,26,27). Helium was used as a carrier gas at a flow rate of 20.2 mL/min (135 kPa).

The MW used in these experiments was a domestic Whirlpool Commercial Microwave (model number 3600XS; Whirlpool Corporation, Benton Harbor, MI) operating at 2450 MHz (maximum power: 650 watts). The reactions described in this study were carried out in Erlenmeyer flasks or scintillation vials covered with a funnel or watch glass at ambient pressure in the MW as described previously (18–25). Taurocholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oyl taurine), taurochenodeoxycholic acid, glycoursodeoxycholic acid, glycocholic acid, and CGH (E.C. 3.5.1.24) were purchased from Sigma Chemical Company (St. Louis, MO) (Product No. C-4018). Taurine conjugates of THCA (Compound **I**, Scheme 2) were isolated from the bile of *A. mississippiensis* (17,18).

Isolation and characterization of 3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine. Gallbladder bile of *A. mississippiensis* (5 mL) was deproteinized (17,18) with 12 mL of methanol, the supernatant decanted and evaporated, and the greenish-yellow residue thus obtained was dissolved in 0.5 mL of methanol and applied on a 20 × 20 cm preparative TLC silica gel G plate and developed twice with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (26:8:4:2, by vol) showing two major components with R_f values of 0.45 and 0.39. The less polar component had mobility as the taurine conjugate of the 25*R* diastereoisomer of 3α,7α-di-

hydroxy-5β-cholestan-26-oic acid and the more polar component had mobility as the taurine conjugate of the 25*R* diastereoisomer of THCA (Compound **I**, Scheme 2). The infrared spectrum in chloroform (Scheme 2, Compound **I**, R_f 0.39) gave a broad band at 3420 cm^{-1}, indicative of OH and NH groups and a strong peak of the amide bond at 1650 cm^{-1}. The FAB mass spectrum of the nonderivatized native molecule of the taurine conjugate of THCA gave only one significant peak at *m/z* (602, 100%) which is $[M + Na]^+$ for the structure shown in Scheme 2, Compound **I**. The protonated and deprotonated molecules of this amide were also observed at *m/z* (580, 10%) and (578, 8%), respectively. The chirality at C-25 was assigned as *R* on the basis of circular dichroism spectra ($\Delta\varepsilon_{195} = -0.58$ degree $\times$ $cm^2 \times dmol^{-1}$) as described by Dayal *et al.* (26).

Representative procedure for the hydrolysis of bile acid conjugates by MW. The procedure described below was applied to all of the following conjugated bile acids: taurocholic acid, taurochenodeoxycholic acid, glycocholic acid, glycochenodeoxycholic acid, glyco- and tauroursodeoxycholic acids and taurine conjugate of THCA (Compound **I**, Scheme 2). Approximately the same substrate–reagent and substrate–solvent ratios were used in each case. The deconjugations of taurocholic acid and taurine conjugate of THCA (Schemes 1,2) are described below.

(i) 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (from Compound **I**, Scheme 1): Cleavage of the amide bond in 3α,7α,12α-trihydroxy-5β-cholan-24-oyltaurine (Compound **I**, Scheme 1) involved heating a solution of the bile salt (50 mg dissolved in 2 mL of 4% NaOH/diethylene glycol (or 1 M LiOH solution (eight equivalents) in propylene glycol in an Erlenmeyer flask covered with an inverted funnel (or sealed Teflon vessel) in a commercial MW for 3–4 min. Completion of the reaction was monitored by TLC and GLC. Workup of the reaction mixture involved acidifying to pH 2 with 10% HCl and extracting with 20 mL of ether twice. The ether layer was then dried over anhydrous $MgSO_4$ and filtered and, following solvent removal, yielded 43 mg of solid residue (86%). The authenticity of cholic acid was proven by comparison of its physical characteristics, TLC, infrared, and GLC–MS with those of an authentic reference standard (18,21–23).

In certain cases a saturated solution of $Ba(OH)_2$ also was used for 4–7 min for hydrolysis, but the reactions were not cleaner as compared to using a saturated solution of LiOH for the same reaction.

(ii) (25*R*) THCA (from Compound **I**, Scheme 2): 3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine (15 mg) was deconjugated with 5% NaOH/diethylene glycol (0.5 mL) in the MW for 4–5 min according to the general protocol detailed above to afford THCA (Compound **III**, Scheme 2), in 86% yield. After crystallization with ethyl acetate/acetone (95:5, vol/vol) at 0°C this material provided colorless prisms (mp 181–183°C, lit. mp 180–182°C) (17,18,28). This crystalline material was found by TLC analysis using silica gel G plates and a solvent system of chloroform/acetone/methanol 70:50:10, by vol; developed twice), to have one component only with R_f= 0.41. This compound had identical TLC, GLC, infrared spectra, and showed identical mass spectral fragmentation patterns which agreed with that obtained from *A. mississippiensis* (17). ^{1}H Nuclear magnetic resonance ($CDCl_3$ + CD_3OD) 400 MHz *d*: 0.68 (*s*, 3H, Me − 18), 0.89 (*s*, 3H, Me − 19), 0.97 (*d,J* = 6.5 Hz, 3H, Me − 21), 1.15 (*d,J* = 6.5 Hz, 3H, Me − 27), 3.40 (*m*, 1H, H − 3), 3.85 (*br s*, 1H, H − 7), 3.95 (*br s*, 1H, H − 12), 3.45 (*m*, 1H, 25 − H). The chirality at C-25 was assigned as *R* on the basis of the circular dichroism spectra ($\Delta\varepsilon_{212} = -0.174$ degree $\times$ $cm^2 \times dmol^{-1}$) and was found to be in agreement with the x-ray diffraction studies described previously (18,28).

Some of the reactions also were carried out in sealed 5-mL capacity Teflon vessels. In such experiments, the reagents were thoroughly mixed in the Teflon vessel, the lid screwed on tightly, and the mixture heated in the oven for 90–120 s. After the heating was complete, the vessel was left to stand in the oven for 5 min to reduce the pressure in the vessel. Then the vessel was removed from the oven and cooled to room temperature, unscrewed, and worked up as described in the Experimental Procedures section.

We must emphasize that great care is to be exercised when adopting the microwave technique to new reactions in sealed or open vessels. Longer reaction times and chemical reactions using larger volumes of reactants must be avoided because these conditions generate very high pressures in the Teflon vessels and may result in an explosive reaction.

DISCUSSION

Recently, several laboratories, including our own, have reported the use of MW for rapid organic synthesis (18,21–25,29–36). We have reported rapid and efficient saponification, deacetylation, hydrogenation, and esterification reactions of bile acids and sterols (21–25). More recently Bose and his coworkers (29,30) have utilized microwave technology for the hydrolysis of peptides using dihydrogen phosphate (pH < 2.8). Microwave-induced vapor phase hydrolysis of proteins and a rapid hydrolysis of phosphoanhydride bonds in nucleotide triphosphates also have been reported recently (37,38). In this report we have further expanded the usefulness of the MW technique for the rapid hydrolysis of C_{24} and C_{27} bile acid conjugates (Schemes 1,2). This method provides rapid, convenient, and inexpensive access to very high temperatures and pressures, leading to a dramatic decrease in reaction times and increased yields. As shown in Schemes 1 and 2, the bile acid conjugates were hydrolyzed in 4–6 min, and the free bile acids were isolated in almost quantitative yields (see Experimental Procedures section).

CGH isolated from *Clostridium perfringens* is routinely used by many laboratories to deconjugate bile salts prior to GLC (11–14,39). This enzyme has been found to be ineffective in the hydrolysis of the taurine conjugate of the 25*R* and 25*S* diastereomer of THCA (12,13). Furthermore, drastic alkaline hydrolytic conditions yield a mixture of diastereomers (Compound **III**, 25*R*/25*S* = 55/45, Scheme 2) that are diffi-

cult to separate on a preparative scale (9,10,16,28). In contrast, complete hydrolysis of the taurine conjugate of THCA, (25*R*) 3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine, was achieved in 3–4 min in the MW (Compound **I**, Scheme 2) with the retention of configuration. Of particular importance was the fact that only the original diastereomer of THCA was isolated from the reaction mixture, indicating that heating the substrate in the MW effectively split the amide linkage of the taurine conjugate of THCA without isomerizing the stereogenic center at C-25 (Compound **II**, Scheme 2).

THCA (Compound **I**, Scheme 2), the major component of the bile of *A. mississippiensis* (17), is a key intermediate in the biosynthesis of cholic acid from cholesterol (8–10,40). In human bile, the natural isomer is (25*R*)-THCA, while both conjugates of (25*R*)- and (25*S*)-THCA have been found to be excreted in urine in patients with Zellweger syndrome (5,40). Furthermore, the enzyme 5β-cholestane-3α,7α,12α-triol-27-hydroxylase, responsible for the formation of THCA, has been found to have *R* absolute stereochemistry (40). Therefore, the retention of absolute configuration at C-25 *via* the rapid hydrolysis of the 27-amide bond may be helpful in delineating the absolute stereochemistry of the peroxisomal enzyme, 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase (THCA-CoA oxidase) that catalyzes the first step in the oxidation of THCA.

In summary, our present studies illustrate the great usefulness of microwave technology in providing rapid and high-yielding hydrolysis of bile acid amides. Recent studies by Setchell *et al.* (41) have elucidated a new and unique inborn error in bile acid conjugation involving a deficiency in amidation. The present methodology may further help in the rapid identification and diagnosis of this and other inborn errors of bile acid metabolism. Our laboratory is currently investigating such hydrolytic reactions utilizing microwaves.

ACKNOWLEDGMENTS

We are indebted to Santy Mathew, undergraduate student from Stevens Institute of Technology, for her skillful technical assistance. This work was supported in part by a grant from the New Foundation for Diabetes and a grant US-PHS, HL-17818. We also wish to thank Kenneth Mizrach, Director, V.A. New Jersey Health Care System, for his encouragement and continuing support. This paper is dedicated to Prof. Ajay K. Bose (Stevens Institute of Technology) on the occasion of his 70th birthday.

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[Received December 4, 1997, and in final revised form and accepted January 21, 1998]

REVIEW

Gas Chromatography–Mass Spectrometry Methods for Structural Analysis of Fatty Acids

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ABSTRACT: Procedures for structural analysis of fatty acids are reviewed. The emphasis is on methods that involve gas chromatography–mass spectrometry and, in particular, the use of picolinyl ester and dimethyloxazoline derivatives. These should be considered as complementing each other, not simply as alternatives. However, additional derivatization procedures can be of value, including hydrogenation and deuteration, and preparation of dimethyl disulfide and 4-methyl-1,2,4-triazoline-3,5-dione adducts. Sometimes complex mixtures must be separated into simpler fractions prior to analysis by gas chromatography–mass spectrometry. Silver ion and reversed-phase high-performance liquid chromatography are then of special value. In particular, a novel application of the latter technique, involving a base-deactivated stationary phase and acetonitrile as mobile phase, is described that is suited to the separation of fatty acids in the form of picolinyl ester and dimethyloxazoline derivatives, as well as methyl esters.
Lipids 33, 343–353 (1998).

The common fatty acids of animal and plant origin have even-numbered chains of 16 to 22 carbon atoms, with zero to six double bonds of the *cis* configuration; methylene-interrupted double-bond systems predominate. Nature provides countless exceptions, however, and odd- and even-numbered fatty acids with up to nearly 100 carbon atoms exist. In addition, double bonds can be of the *trans* configuration, acetylenic and allenic bonds occur, and there can be innumerable other structural features, including branch points, rings, oxygenated functions, and many more (1,2). More than a thousand different fatty acids of natural origin must exist, as well as others produced as artifacts when fats are used in commerce and in cooking, for example.

It is essential to have simple, rapid methods for determination of fatty acid structures. In particular, new methods involving gas chromatography–mass spectrometry (GC–MS), GC linked to Fourier-transform infrared spectroscopy (FTIR), and silver ion and reversed-phase high-performance liquid chromatography (HPLC) are available, among others. I described the current state of the methodology in a recent review (3). This paper is much less comprehensive in its coverage of the literature, and it lays particular emphasis on those GC–MS methods that the author has found especially useful. All of the derivatization procedures described utilize readily available reagents and have simple glassware requirements, so they should be accessible to most laboratories.

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Abbreviations: DMOX, dimethyloxazoline; FTIR, Fourier-transform infrared spectroscopy; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography.

MASS SPECTROMETRY OF PICOLINYL ESTER AND DIMETHYLOXAZOLINE DERIVATIVES

General comments. Fatty acids are usually analyzed by GC as methyl ester derivatives, but their mass spectra rarely contain ions indicative of structural features; the positions of double bonds in the aliphatic chain, for example, cannot be determined. Instead, the carboxyl group must be derivatized with a reagent containing a nitrogen atom. When the molecule is ionized in the mass spectrometer, the nitrogen atom, not the alkyl chain, carries the charge, and double-bond ionization and migration are minimized. Radical-induced cleavage occurs evenly along the chain and gives a series of relatively abundant ions of high mass from the cleavage of each C-C bond. When a double bond or other functional group is reached, diagnostic ions tend to occur. The first useful nitrogen-containing derivatives, i.e., pyrrolidides, were described more than 20 yr ago, and their mass spectral properties were reviewed by Andersson (4). Although they give useful spectra and should not be discounted, most analysts now prefer either picolinyl (3-hydroxymethylpyridinyl) ester or 4,4-dimethyloxazoline (DMOX) derivatives. A definitive review of mass spectral fragmentation properties of such compounds has been published by Harvey (5), and other reviews have appeared (6,7). Here, I will concentrate on practical applications of the derivatives to structure determination of natural fatty acid mixtures as opposed to mechanistic concepts.

Both picolinyl ester and DMOX derivatives have their merits in MS terms, and neither should be neglected. Each has advantages for particular types of fatty acid, and they are best considered as complementary.

In choosing a derivative for MS, good chromatographic properties are also important. One advantage of DMOX derivatives is that they are only slightly less volatile than methyl esters; they can be subjected to GC analysis on polar stationary phases under comparable conditions and give equivalent

resolution (8). Picolinyl esters, on the other hand, require column temperatures about 50°C higher than methyl esters (9), meaning initially that they had to be separated on nonpolar phases, which gave relatively poor resolution. With the introduction of new polar phases that are stable to high column temperatures and have low-bleed characteristics for MS analysis, such as BPX-70™ (SGE Europe Ltd. Milton Keynes, United Kingdom) (10,11) or even some of those of the Carbowax type, such as Supelcowax 10™ (Supelco UK, Poole, United Kingdom) (12), the problem of GC resolution of picolinyl esters is greatly lessened and only very long chain fatty acids (>C_{20}) tend to cause problems.

Picolinyl esters must be prepared from free fatty acids, and intact lipid or methyl ester samples must first be hydrolyzed. The author (unpublished) then prepares the acid chloride by reaction with oxalyl chloride overnight and reacts this with 3-hydroxymethylpyridine in dichloromethane to form the picolinyl ester. Alternatively, a mild quantitative method developed for derivatizing sensitive polyunsaturated fatty acids containing epoxy groups may be preferred, involving an imidazolide intermediate (13). Dry solvents and fresh reagents are required because the reactions are sensitive to moisture.

DMOX derivatives are prepared in a simple one-pot reaction. Lipids are reacted with 2-amino-2-methyl-1-propanol in a nitrogen atmosphere at 180°C [2 h for free acids (14), or 18 h for methyl esters (15,16) and intact lipids (17)]. Unfortunately, we have often observed incomplete reaction and appearance of an intermediate that elutes later from GC columns (and gives a mass spectrum almost identical to that of the required derivative). The prolonged high temperature required for the preparation of DMOX derivatives gives cause for concern, and there must be some risk to polyunsaturated fatty acids or any other compound with a labile functional group. However, the only important problem reported to date was with *trans*-3-hexadecenoic acid, common in plant photosynthetic tissue, which was found to have isomerized largely to *cis*-2-hexadecenoic acid during the reaction (18). Although an alternative two-step reaction has been described, it uses a strong acid as catalyst and may not be safer (14).

Saturated and branched-chain fatty acids. The mass spectrum of the picolinyl ester derivative of 14-methylhexadecanoic acid is illustrated in Figure 1. It is typical in that it has prominent ions at *m/z* = 92, 108, 151 (the McLafferty ion), and 164, which are all fragments about the pyridine ring. The molecular ion (*m/z* = 361) is easily distinguished and is always odd-numbered, because of the presence of the nitrogen atom, but most other ions are even-numbered. In interpreting such spectra, the simplest approach is to start with the molecular ion and progress downward, as if one were unzipping the molecule one methylene group at a time. Thus, there is loss

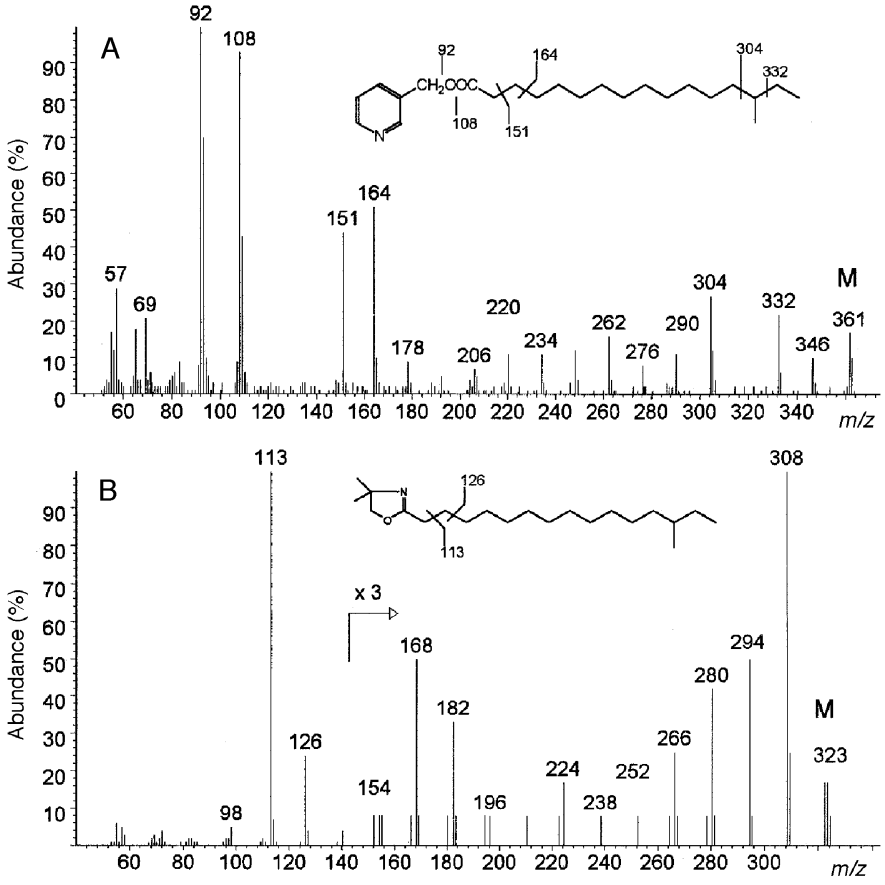


FIG. 1. Mass spectrum of 14-methylhexadecanoic acid as the picolinyl ester (A) and dimethyloxazoline derivative (B).

of a methyl group to *m/z* = 346, and a further methylene to *m/z* = 332. Then there is a gap of 28 amu to *m/z* = 304, representing loss of the carbon 14 with the associated methyl group, affording definitive identification. Finally a series of ions is observed 14 amu apart for loss of successive methylene groups. There is little sign of the complex rearrangement ions that can be found with such fatty acid derivatives as methyl esters, in which minor changes in ion intensities must be used in an attempt to identify branch points, so interpretation of spectra can be very difficult.

The mass spectrum of the DMOX derivative of this acid is also illustrated in Figure 1. Prominent ions at *m/z* = 113 and 126 are invariably seen, the former representing cleavage between carbons 2 and 3 (the McLafferty ion). With DMOX derivatives of saturated fatty acids, the fragments of higher molecular weight are often in relatively low abundance and have been magnified here. The molecular ion (*m/z* = 323) is easily recognized, as is the loss of the terminal methyl group (*m/z* = 308). Then there is a series of ions 14 amu apart for loss of successive methylene groups, but nothing that defines the loss of the methyl group on carbon 14. This is one of a few instances where picolinyl esters are demonstrably greatly superior to DMOX derivatives. However, when methyl groups are further from the terminal end of the molecule, some small differences in intensity are seen that it is claimed may be used for diagnostic purposes (14). The author is not convinced that such small differences will have practical value with unknown compounds.

Unsaturated fatty acids. As an example, the mass spectrum of the picolinyl ester of 11-octadecenoic acid is illustrated in Figure 2. There is a large molecular ion (*m/z* = 373), and then an ion at *m/z* = 358 representing loss of the terminal methyl group, followed by a series of ions 14 amu apart for cleavage at successive methylene groups. When a double bond is reached, there is a gap of 26 amu (between *m/z* = 262 and 288). This gap can sometimes be difficult to locate precisely, but a further distinctive feature of clear diagnostic value is two abundant ions 14 amu apart, representing cleavage on the distal side of the double bond at *m/z* = 302 and 316 in this instance (5,19,20). In practice, these two ions can be picked out for identification purposes even when isomeric fatty acid derivatives are imperfectly resolved by GC. Formation of the ions has been rationalized in mechanistic terms as an initial abstraction of allylic hydrogens on each side of the double bond with the production of conjugated diene systems, which form relatively stable ions (5,19). Similar series of ions are seen in most monoenes, but it is advantageous to have access to spectra of authentic standards when the double bond

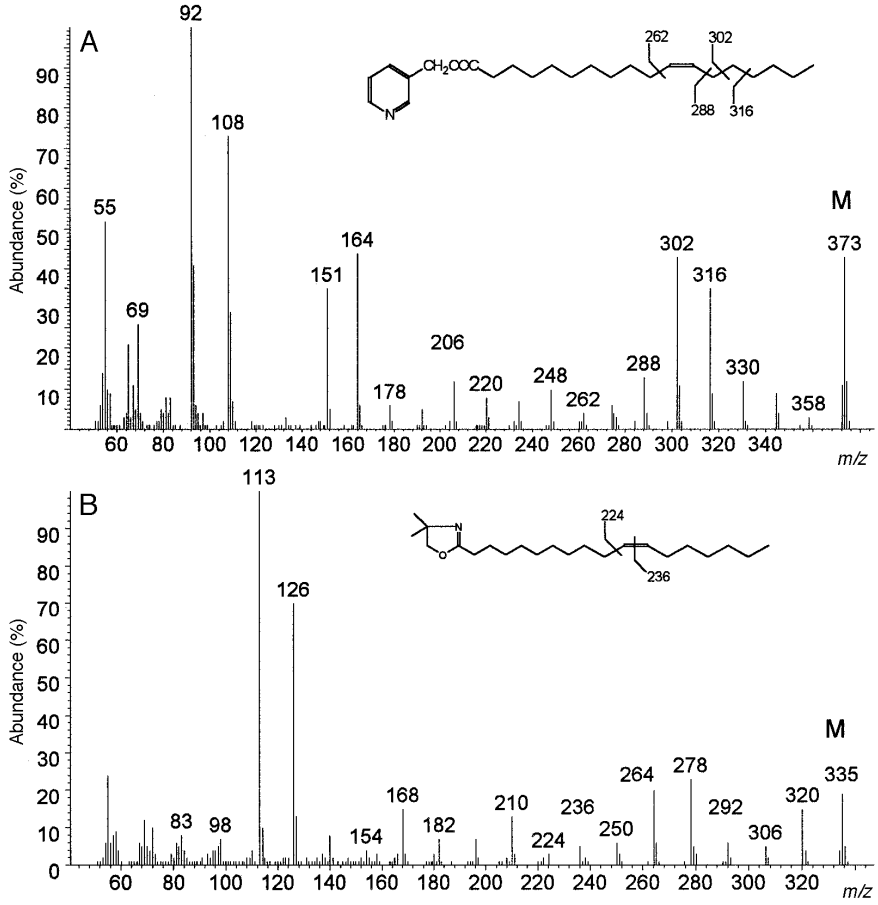


FIG. 2. Mass spectrum of 11-octadecenoic acid as the picolinyl ester (A) and dimethyloxazoline derivative (B).

is close to either end of the molecule to avoid any confusion (20). For example, when the double bond is closer to the carboxyl group, the second ion of the two is relatively more abundant.

In the case of the DMOX derivative of vaccenic acid, illustrated also in Figure 2B, the molecular ion is at *m/z* = 335, and there are characteristic ions at *m/z* = 320, 306, 292, 278, and so forth for the loss of each successive methylene group. A gap of 12 amu, instead of the usual 14, between *m/z* = 224 (C-10) and 236 (C-11) is indicative of a double bond between carbons 11 and 12. Although interpretation of the spectrum differs in principle from that with picolinyl esters, the result is equally clear. The author (unpublished) has mass spectra for DMOX derivatives of the complete series of octadecenoic acid isomers, and again it is apparent that anomalies can arise when the double bond is close to either end of the molecule. Indeed, it has been demonstrated that odd-numbered ions may be found in spectra of polyunsaturated fatty acids with double bonds in positions 4 to 6 (21).

Similar principles apply to interpretation of spectra from polyunsaturated fatty acids. As an example, the mass spectrum of the picolinyl ester of 5,9,12-octadecatrienoic acid, a common constituent of the seed oils of conifers, is illustrated in Figure 3. The molecular ion is at *m/z* = 369, and again there is a regular series of ions 14 amu apart until the double bond in position 12 is reached, recognized by the gap of 26 amu between *m/z* = 272 and 298. As a practical point with polyenes, a double bond on carbon atom *n* is sometimes more easily distinguished from a gap of 40 amu between carbons *n* − 1 and *n* + 1, in this instance from *m/z* = 258 to 298. The double bonds in positions 9 and 5 are recognized by gaps of 26 amu between *m/z* = 232 and 258 and between *m/z* = 178 and 204, respectively. However, an even more characteristic feature for a bis-methylene-interrupted diene is an ion at *m/z* = 219 (actually 218 + 1, which unusually is odd-numbered). This has been found in a number of fatty acids with 5,9-double bonds (22–26), including those in sponges, and analogous ions are found with other *bis*-methylene interrupted dienes (27).

The mass spectrum of the DMOX derivative of 5,9,12-octadecatrienoic acid is also illustrated in Figure 3B. In this instance, the double bonds in positions 9 and 12 are recognized by gaps of 12 amu between *m/z* = 194 and 206 and *m/z* = 234 and 246, respectively. The base peak is at *m/z* = 180 and represents a cleavage between carbons 7 and 8, i.e., at the center of the bis-methylene-interrupted system, and is a key diagnostic ion for this feature (15,17,28). Analogous ions are present for DMOX derivatives of other fatty acids containing bis-methylene-interrupted systems (author,

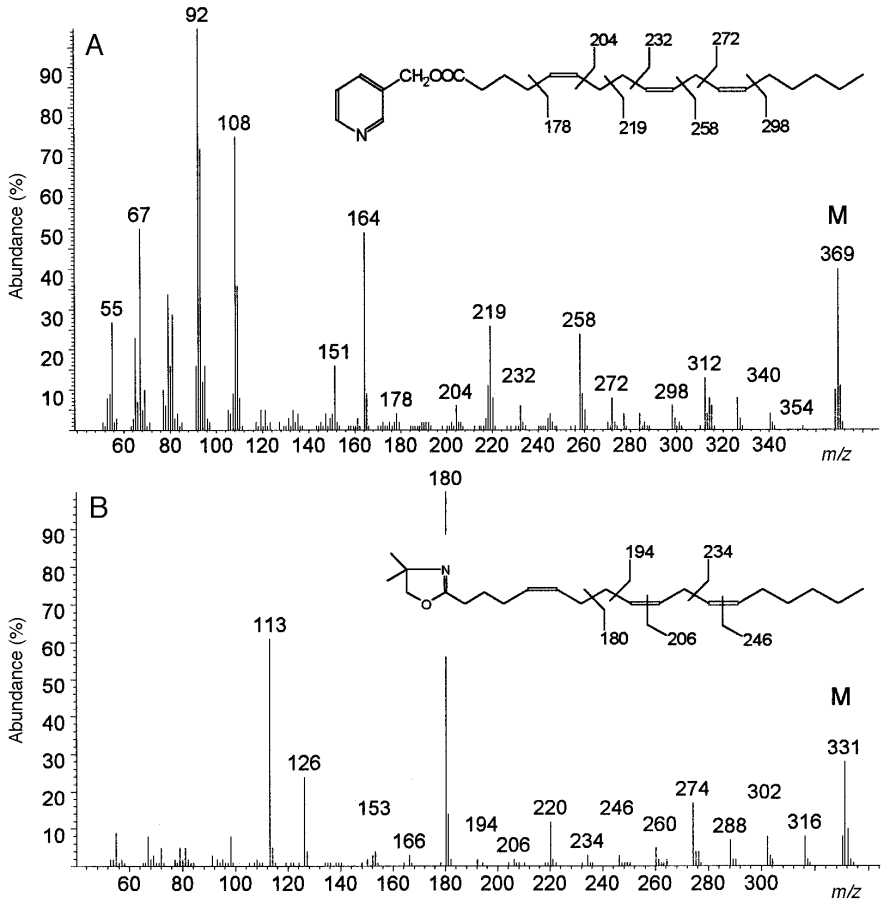


FIG. 3. Mass spectrum of 5,9,12-octadecatrienoic acid as the picolinyl ester (A) and dimethyloxazoline derivative (B).

unpublished). DMOX derivatives also give excellent spectra when conjugated double-bond systems are present (15,29).

From the practical standpoint of identification of unknown unsaturated fatty acids, the author has observed that DMOX derivatives tend to give spectra which are easier to interpret, provided the GC peak contains a single fatty acid. With poorly resolved peaks, picolinyl esters are often best, provided there is access to standard spectra. Again, this illustrates that the two types of derivatives complement each other.

Cyclic fatty acids. The picolinyl esters of cyclopropanoid fatty acids gave distinctive spectra with a diagnostic ion that included the first carbon of the ring, and unusually were odd-numbered (30). In contrast, the mass spectra of the DMOX derivatives of cyclopropyl fatty acids appear to be much less distinctive. They lack a diagnostic odd mass ion, corresponding to that of the picolinyl esters, and essentially resemble the spectra of monoenes (as is the case for methyl esters), the only differences being the relative intensities of certain ions (7,31). Although it has been argued that these minor differences can be used diagnostically, there is evidence to contradict this (6). In contrast, both types of derivative gave definitive spectra for cyclopropenoid fatty acids. For example, the picolinyl ester derivatives gave mass spectra with diagnostic ions representing cleavage beta to the ring on each side (32). Similarly, characteristic ions were obtained with DMOX derivatives (33). With the DMOX derivative of sterculic acid, a gap of 10 amu between ions at *m/z* = 196 and 206 (carbons 8 and 9) located the cyclopropene ring between carbons 9 and 10 (33).

Both picolinyl ester (34) and DMOX derivatives (35) gave excellent spectra with natural cyclopentenoid fatty acids, enabling identification of a number of novel components in certain seed oils. When a double bond occurred also in the aliphatic chain, the expected mass spectral features were present and located its position, but there were no ions to indicate the position of a double bond in the ring and this remains an unsolved problem in MS. Similarly, the complex range of cyclic fatty acids, with internal 5- and 6-membered rings and double bonds in many different positions, which are formed by free-radical mechanisms when vegetable oils are heated to high temperatures as during frying of food or the commercial physical refining process, has been characterized both as DMOX and picolinyl ester derivatives (36–42). Here DMOX derivatives may be marginally better.

SOME ANCILLARY MASS SPECTROMETRIC TECHNIQUES

Hydrogenation. Catalytic hydrogenation is a simple procedure that provides invaluable structural information regarding fatty acid identity, when combined with GC or GC–MS analysis. Some needlessly complex procedures are sometimes described, but details of a convenient practical procedure are available (43). At its simplest, hydrogenation is used merely to determine the chain length of components. By eliminating all unsaturated centers in fatty acid methyl esters from most samples of natural origin, a simple set of peaks is obtained for the saturated even-numbered homologous series and can be compared with authentic standards. However, the presence of anomalous peaks may be an indication of novel structures. In samples of animal origin, small amounts of odd-chain fatty acids may be detected in the GC trace, together with methyl-branched fatty acids, usually *iso*- closely followed by *anteiso*-isomers, which can be identified as described above. Similarly, this technique was of great value in characterizing the cyclic fatty acids in the papers cited above. It is best carried out with methyl esters prior to conversion to picolinyl ester or DMOX derivatives.

Deuteration. Deuteration has been used since the early days of MS of lipids as a means of locating double bonds, but the value of the procedure for structure determinations was limited with methyl ester derivatives, as the wide range of rearrangement ions formed led to some scrambling of the deuterium atoms in the alkyl chain. However, by using nitrogen-containing derivatives, which give clean radical-induced fragmentations with minimal rearrangement, most problems have been eliminated.

Deuteration with deuterium gas and Wilkinson's catalyst [tris(triphenylphosphine)rhodium(I) chloride] is usually employed for the purpose, including in the author's laboratory, and can be recommended. Gaseous deuterium is available commercially in small cylinders, or it can be generated *in situ* by reaction of deuterium chloride with sodium borodeuteride (44). It is essential to have a good excess of deuterium so that the reaction goes rapidly to completion, otherwise some isomerization of the double bonds and scrambling of the hydrogen atoms is possible (author, unpublished). Again, the reaction is best carried out with methyl esters prior to conversion to picolinyl ester or DMOX derivatives.

The technique was first used in conjunction with pyrrolidide derivatives and has also been used with DMOX derivatives, but picolinyl esters appear best for the purpose, as they give cleaner radical-induced fragmentations with fewer rearrangement ions. Also, they give more abundant ions of high molecular weight with saturated fatty acids. Lie Ken Jie and Choi (45) were first to demonstrate this with mono- to hexaenoic fatty acids and for some acetylenic fatty acids (46). On mass spectral analysis, clear diagnostic ion fragments were obtained that permitted the determination of the positions of the original double bonds in the alkyl chain.

As an example, the mass spectrum of the picolinyl ester of 12-oxo-octadec-9-enoic acid is shown in Figure 4 (11). In addition to the ions characteristic of picolinyl esters, there was a small molecular ion (*m/z* = 387). The gap of 26 amu between *m/z* = 234 and 260 was diagnostic for the double bond, the gap of 28 amu between the base ion at *m/z* = 274 and the prominent ion at *m/z* = 302 represented fragmentation involving loss of the 12-oxo group. However, it was noted that in this instance cleavage occurred alpha (as opposed to beta as is more normal) to the oxo group, presumably because of the directing

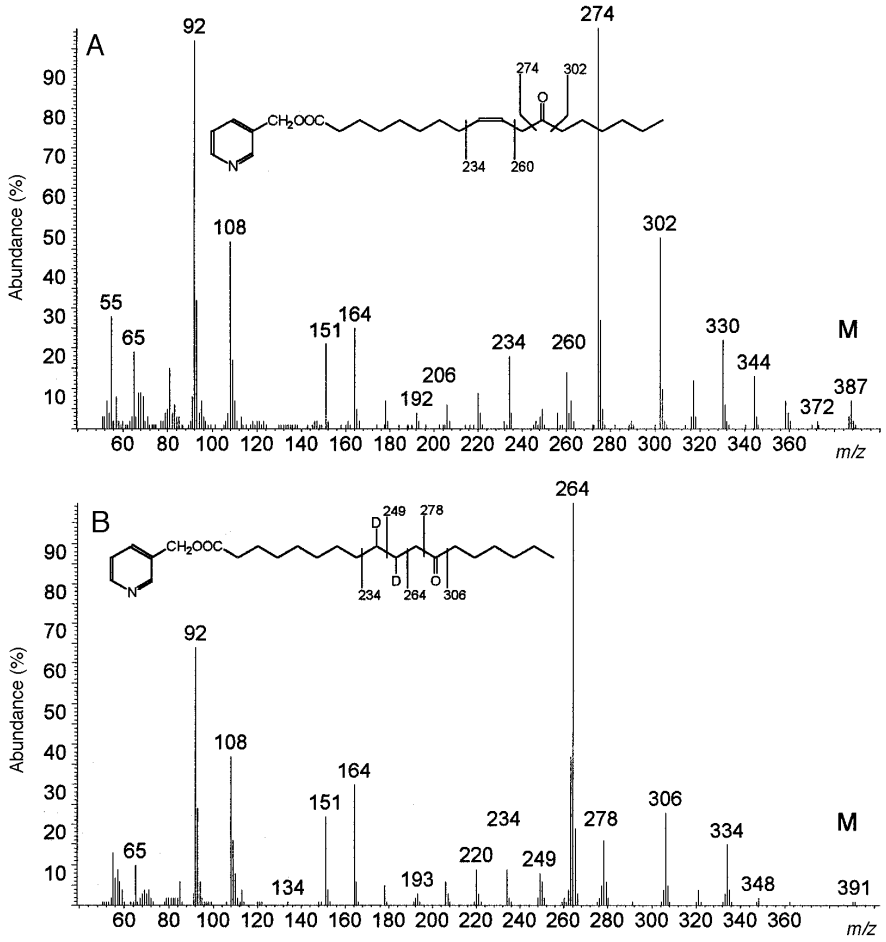


FIG. 4. Mass spectra of picolinyl 12-oxo-octadec-9-enoate (A) and 9,10-d_2,12-oxo-octadecanoate (B) (redrawn from Ref. 11).

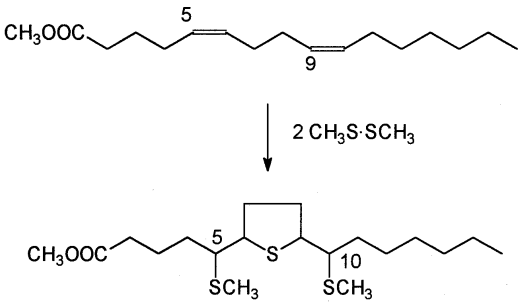
influence of the double bond in position 9. This could greatly hinder interpretation of the spectra of unknowns, so deuteration was performed. The mass spectrum of picolinyl 9,10-d_2,12-oxo-octadecanoate is also illustrated in Figure 4. Now, the base peak at *m/z* = 264 represents cleavage beta to the oxo group, with a gap of 28 amu between ions at *m/z* = 278 and 306 for the loss of the oxo group. Gaps of 15 (rather than 14) from *m/z* = 234 to 249 and from 249 to 264 confirm the presence of deuterium atoms on carbons 9 and 10.

Such methodology enabled identification of 15 novel fatty acids of this type in milk fat and cheese (11). It was also invaluable for characterizing the cyclic fatty acids produced in heated vegetable oils, enabling location of double bonds both in the aliphatic chain and in the ring structures, both with picolinyl ester and DMOX derivatives (36,37), and for fatty acids with two bis-methylene-interrupted double-bond systems from a sponge (26).

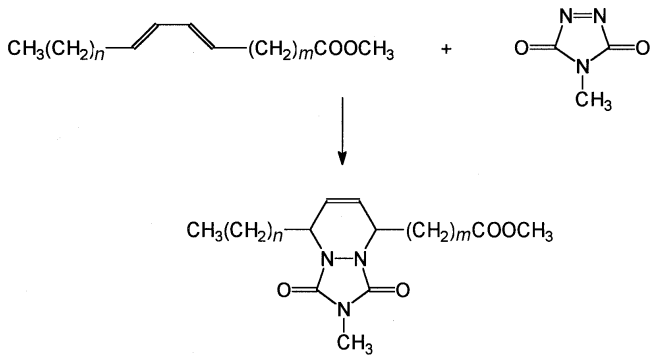
Dimethyl disulfide adducts. A large number of further reagents that involve addition to a double bond have been described, but the only one to have stood the test of time involves preparation of dimethyl disulfide adducts. A single reagent and a one-step reaction are required for the preparation, which is carried out simply by dissolving the methyl ester of the fatty acid in dimethyl disulfide with a trace of iodine (47). Adduct formation is entirely stereospecific, presumably by *trans* addition, so that *threo*- and *erythro*-derivatives are formed from *cis*- and *trans*-isomers, respectively. Although the different geometrical isomers have indistinguishable spectra, they are eluted separately from GC columns containing either polar or nonpolar phases, that derived from the *cis*-isomer eluting first (48).

The reaction has proved especially useful for monoenes (see Ref. 3 for a full list of references). Dienoic fatty acids present more of a problem for the technique, partly because comparatively high temperatures are required for GC analysis, but mainly from complications in the reaction with dimethyl disulfide. There is no problem when double bonds are separated by more than four carbon atoms, but methylene-interrupted double bonds give products with thietane, tetrahydrothiophene, and tetrahydrothiopyran structures (4-, 5-, and 6-membered rings) (49,50). However, such compounds can give characteristic and diagnostic spectra, so that the technique continues to have some practical value. Only conjugated fatty acids cannot be analyzed usefully by this means (50).

Fatty acids with a bis-methylene-interrupted double-bond system, as in a 5,9-diene systems, are common constituents



SCHEME 1



SCHEME 2

of marine sponges and of seed oils in many species of Gymnosperms. Reaction of these with dimethyl disulfide under appropriate conditions gives a 5-membered cyclic thioether with methylthio groups on the carbons immediately adjacent to the ring as illustrated in Scheme 1 (50). These compounds give characteristic spectra permitting location of the double bonds, and many different demospongic acids of this type have been identified in this way, including some containing bromine atoms and methyl branches in addition to the 5,9-double-bond system (51,52).

Diels-Alder adducts for conjugated double bonds. As shown in Scheme 2, a useful derivative specific for determination of double-bond positions in conjugated dienes is to form the Diels-Alder adduct of the fatty acid methyl ester by reaction with the reagent 4-methyl-1,2,4-triazoline-3,5-dione (53). Such derivatives have excellent mass spectrometric properties, enabling determination of structures in such samples as commercial conjugated linoleic acid (CLA) (53,54) and metabolites formed from this in animal tissues (55). It was also used to establish the absence of conjugated double-bond systems in cyclic dienes produced in heated vegetable oils (37).

SIMPLIFICATION OF COMPLEX MIXTURES PRIOR TO ANALYSIS BY GC–MS

Reversed-phase HPLC. When samples are subjected to GC–MS in the form of picolinyl esters or DMOX derivatives, there may be loss of resolution, and minor components may not give definitive spectra. Sometimes, natural mixtures are so complex that it is unreasonable to expect adequate resolution. In such circumstances, some subdivision into simpler fractions by chromatographic means is desirable. Also, if a technique such as deuteration is to be used, it must be applied to relatively pure compounds.

Reversed-phase HPLC [reviewed by Nikolova-Damyanova (56)] is a mild method in that it is carried out at ambient temperature and involves only liquid–liquid interactions. The principle is well known in that separation is based both on the chain length and degree of unsaturation of components, each double bond reducing the retention time by the equivalent of about two methylene groups. It was one of the first techniques to be used for micropreparative separation of picolinyl esters *per se* in 1987 (57). In this instance, a stationary phase of the octadecylsilyl type was employed with a gradient of water/pyridine/acetic acid into methanol and evaporative light-scattering detection. The polar mobile phase was necessary to overcome the attraction between residual silanol groups on the stationary phase and the nitrogen moiety of the derivative.

New deactivated stationary phases of the octadecylsilyl type are now available that permit elution of basic compounds as sharp peaks without addition of ionic species to the mobile phase. Figure 5 illustrates a separation of picolinyl ester derivatives, prepared from borage oil, on a column of Hichrom RPB™ (Hichrom Ltd., Reading, United Kingdom) using acetonitrile only as the mobile phase with a flow gradient of 0.5 to 1.5 mL/min (author, unpublished). If the column temperature is kept constant, the elution times are highly reproducible, facilitating automated fraction collection. In this instance, evaporative light-scattering detection was used for convenience, but ultraviolet detection should also be possible by using the specific absorbance of the pyridine ring. By avoiding water as a component of the mobile phase, it is easy to recover fatty acid derivatives from fractions that are collected. It is noteworthy that the 18:1 derivative eluted before rather than after 16:0, as is more usual in reversed-phase HPLC.

Methyl esters and DMOX derivatives can be chromatographed under comparable conditions, except that corresponding components elute a little earlier than for picolinyl esters. Again, ultraviolet detection should be possible, but at 206 nm.

Silver ion HPLC. Silver ion chromatography has been widely used for separation of fatty acid derivatives, and the technique has been reviewed comprehensively (58,59). Only a few papers relating to the main theme of this review will be cited here. The principle is well known, and involves transient formation of polar charge-transfer complexes so that components are separated by degree of unsaturation. In addition, it can be used to separate fatty acids with *cis*- from *trans*-double bond, at the same time providing confirmatory evidence as to geometry, and to separate positional isomers.

Until recently the most useful manifestation was silver ion thin-layer chromatography, but silver ion HPLC affords more efficient separations and cleaner fractions in micropreparative applications. The author's approach was to convert a

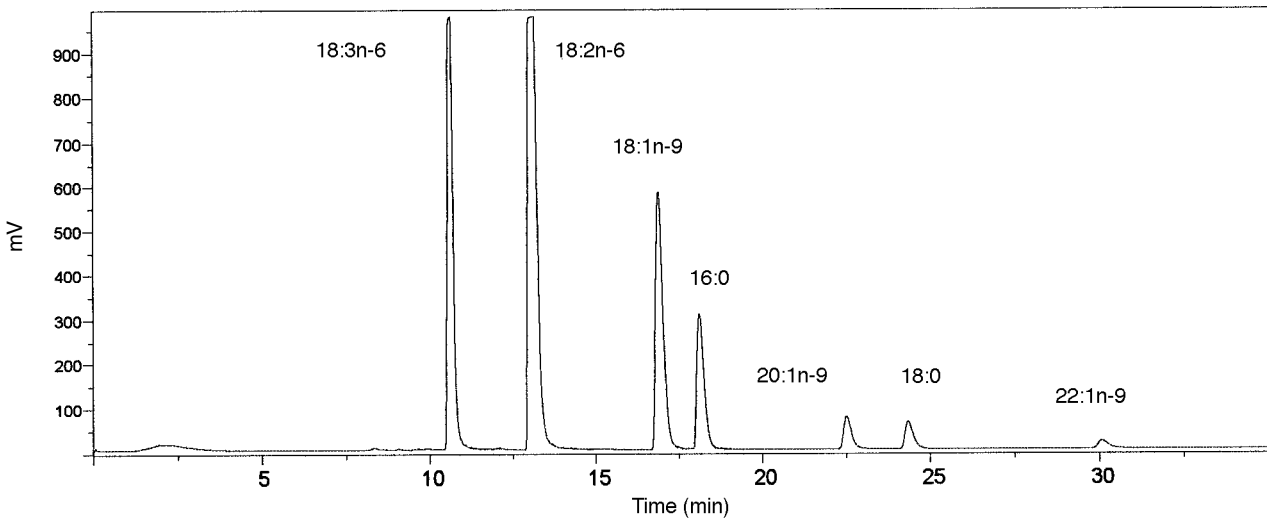


FIG. 5. Separation of picolinyl esters prepared from borage oil by reversed-phase high-performance liquid chromatography (HPLC). A column of Hichrom RPB™ (250 × 4.6 mm; Hichrom Ltd., Reading, United Kingdom) was employed with acetonitrile as mobile phase, with the flow rate programmed from 0.5 to 1.5 mL/min over 30 min, and held at this for a further 5 min. The temperature of the column was maintained at 20°C. The sample (5 μL) was injected in a solution of acetone/acetonitrile (1:9, vol/vol). An evaporative light-scattering detector was used.

commercial ion-exchange column (Nucleosil 5SA™; Hichrom Ltd., Reading, United Kingdom) to the silver ion form by the simple process of pumping water through the column into which silver nitrate was introduced *via* the rheodyne valve, before the water was replaced by organic solvents of decreasing polarity (60). Pre-silvered columns are now available commercially (Chromspher Lipids™ from Chrompack BV, Netherlands).

It should be noted that methyl and other simple esters of fatty acids must be used for this technique, and not DMOX derivatives or picolinyl esters. Apparently, the nitrogen-containing moieties are held so strongly that they cannot be recovered from the column (3).

For group separations of components differing in degree of unsaturation, satisfactory resolution of fatty acid derivatives into fractions with zero to six *cis* double bonds was achieved on silver-loaded Nucleosil™ 5SA columns by using binary gradient elution schemes. For example, good resolution was obtained with a gradient of dichloromethane/1,2-dichloroethane (1:1, vol/vol) to dichloromethane/1,2-dichloroethane/methanol/acetonitrile (47:47:3:3, by vol), as illustrated in Figure 6 (23,24,26). An evaporative light-scattering detector was employed with a stream-splitter inserted to enable collection of samples. In this example, seven fractions were collected, some differing according to geometry and others according to position, as well as to the number of double bonds. This procedure has been applied to many complex natural mixtures as fatty acid methyl esters, as a means of simplification before proceeding to structural analysis by GC–MS. For example, in studies of sponges (23,24, 26), up to 10 fractions were collected, and subsequent analysis by GC–MS revealed as many as a hundred fatty acids including cyclic and mono- and multimethyl-branched isomers, as well as methylene- and multimethylene-interrupted unsaturated components of several different biosynthetic families. Many of these were minor components that would have been hidden by more abundant fatty acids if this simplification step were not undertaken. My recent review (3) should be consulted for a full list of applications.

If the HPLC separation is carefully optimized and the type of fatty acid derivative is chosen correctly, much better resolution is possible, and individual positional, as well as configurational, isomers can be separated. Phenacyl derivatives of fatty acids were used in early work on separation of positional isomers, the choice being determined by a requirement for ultraviolet detection. However, it has become evident that these are especially useful in that formation of a chelate complex between a silver ion, a double bond, and the additional carbonyl moiety of the phenacyl moiety facilitated separation of positional isomers; indeed, *p*-methoxyphenacyl esters may prove to be even better (61). Among many applications that could be cited is the work on structure determination of cyclic fatty acids formed in heated vegetable oils (36,37).

In conclusion, this has been a selective review of the present methodology for structural analysis of fatty acids, but it is my experience that those procedures discussed are adequate for most purposes. Some important techniques have been neglected here. For example, GC coupled to FTIR is an enormously powerful technique that has been well utilized in some laboratories. Similarly, nuclear magnetic resonance spectroscopy can give valuable structural information, but at present it requires relatively large amounts of fatty acids in a pure state. The GC–MS methods described above can give satisfactory results with submilligram amounts of lipid.

ACKNOWLEDGMENT

This paper is published as part of a program funded by the Scottish Office Agriculture, Environment and Fisheries Dept.

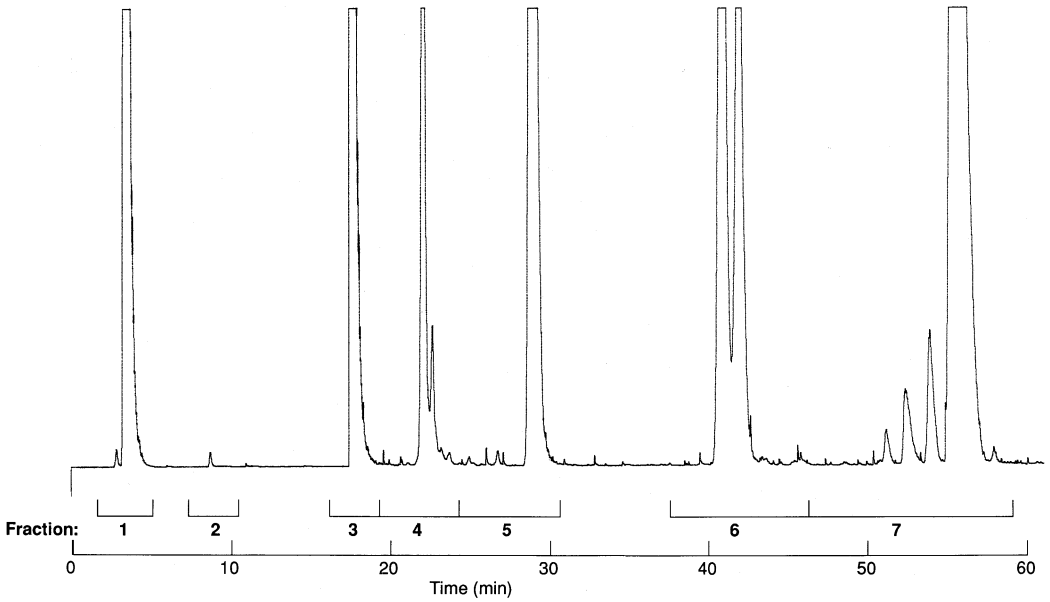


FIG. 6. Silver ion HPLC of methyl esters from the sponge *Hymeniacidon sanguinea*. A column (4.6 × 250 mm) of Nucleosil™ 5SA was utilized in the silver ion form, and a gradient of dichloromethane/dichloroethane (1:1, vol/vol) to dichloromethane/dichloroethane/acetonitrile/methanol (47:47:3:3, by vol) was utilized with evaporative light-scattering detection. Fraction 1 = saturated; 2 = *trans*-monoenes; 3 = *cis*-monoenes; 4 = dienes; 5 = 5,9-dienes; 6 = trienes and tetraenes; 7 = pentaenes and hexaene. (Reproduced from Ref. 23 with the permission of *Comparative Biochemistry and Physiology*.)

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[Received October 17, 1997, and in final revised form February 18, 1998; revision accepted February 20, 1998]

ARTICLE

Isolation and Structure of a New Galactolipid from Oat Seeds

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ABSTRACT: Seeds of oat (*Avena sativa* L.) were recently shown to contain significant quantities of a new hydroxy acid, (15*R*)-hydroxy-(9*Z*),(12*Z*)-octadecadienoic acid (trivial name, avenoleic acid). In the present work, avenoleate was found to be mainly (63%) localized in the glycolipid fraction of oat seed lipids. Fractionation of the glycolipids by thin-layer chromatography and reversed-phase high-performance liquid chromatography revealed the presence of a main molecular species which accounted for 20% of the total avenoleate content of oat seeds. Structural studies by chemical methods and mass spectrometry demonstrated that the avenoleate-containing glycolipid was a galactolipid assembled of one molecule of avenoleic acid, two molecules of linoleic acid, two molecules of D-galactose, and one molecule of glycerol. Degradation of the new galactolipid by chemical and enzymatic methods demonstrated the localization of acyl chains, i.e., linoleate at *sn*-1 and linoleoyl-avenoleate at *sn*-2. Nuclear magnetic resonance spectroscopy gave independent support for this structure and also demonstrated that the two galactoses formed an α-D-galactopyranosyl-1-6-β-D-galactopyranosyl moiety which was bound to the *sn*-3 position. Based on these experiments, the new galactolipid could be formulated as 1-[(9′*Z*),(12′*Z*)-octadecadienoyl]-2-[(15″*R*)-{(9‴*Z*),(12‴*Z*)-octadecadienoyloxy}-(9″*Z*),(12″*Z*)-octadecadienoyl]-3-(α-D-galactopyranosyl-1-6-β-D-galactopyranosyl)-glycerol. Quantitatively, the amount of the avenoleate-containing galactolipid was of the same order of magnitude as those of individual molecular species of digalactosyldiacylglycerol containing nonoxygenated acyl chains. The content of the new galactolipid in oat seeds was 0.5–0.6 mg per g of seed.

Lipids 33, 355–363 (1998).

A new oxylipin, i.e., 15(*R*)-hydroxy-(9*Z*),(12*Z*)-octadecadienoic acid, was recently isolated from seeds of oat (*Avena sativa* L.) (1). The trivial name "avenoleic acid" was given to the new hydroxy acid. Quantitative determination of avenoleic acid in oats showed a content of 0.6–0.7 mg per g of dry seed (1). Avenoleic acid was also present in seeds of wild oats (*A. fatua*) (Hamberg, M., and Hamberg, G., unpublished observations); however, it was not detectable in seeds of barley, rye, or wheat (1). Avenoleic acid is chemically related to ricinoleic acid. Both compounds possess a homoallylic alcohol group, and in both compounds the alcohol group is located at a position that in the corresponding nonhydroxylated compound can undergo desaturation. Thus, oleate, precursor of ricinoleate [(12*R*)-hydroxyoleate], can be transformed into linoleate by desaturation at C-12/C-13 whereas linoleate, the putative precursor of avenoleate [(15*R*)-hydroxylinoleate], can be transformed into α-linolenate by desaturation at C-15/C-16. Interestingly, two enzymes catalyzing hydroxylation and desaturation of oleate, i.e., oleoyl 12-hydroxylase from *Ricinus communis* and n-6-oleoyl desaturase from *Arabidopsis*, have been shown to be homologous (2). It seems possible that the putative linoleoyl 15-hydroxylase responsible for biosynthesis of avenoleate from linoleate is related to n-3-linoleoyl desaturase involved in the formation of α-linolenic acid from linoleic acid.

The present paper is concerned with the localization of avenoleic acid in lipids of oat seeds and with the isolation of an unusual galactolipid which serves as a quantitatively important storage form of avenoleic acid in oat seeds. The structure of the new galactolipid was determined by chemical, physical, and enzymatic methods.

EXPERIMENTAL PROCEDURES

Plant material. Seeds of oat (*A. sativa* L., cv. Vital) were obtained from Svalöf Weibull, Svalöv, Sweden.

Chemicals. Lipase from *Rhizopus arrhizus* was purchased from Boehringer Mannheim (Mannheim, Germany). D-Glucose, D-galactose, L-galactose, methyl α-D-galactopyranoside, and methyl β-D-galactopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO). *S*-(+)-2-Butanol was purchased from Aldrich Chemical Co. (Gillingham, United Kingdom). A mixture of methyl α- and β-D-glucopyranosides was prepared by refluxing D-glucose in methanol/concentrated hydrochloric acid (2.6:0.4, vol/vol) for 1 h. Digalactosyldiacylglycerol from wheat flour was purchased from Sigma Chemical Co. This material was subjected to preparative reversed-phase high-performance liquid chromatography

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Abbreviations: Avenoleic acid, (15*R*)-hydroxy-(9*Z*),(12*Z*)-octadecadienoic acid; ES, electrospray; FT-IR, Fourier-transform infrared; GC–MS, gas–liquid chromatography–mass spectrometry; GLC, gas–liquid chromatography; HMBC, heteronuclear multiple bond correlation spectroscopy; HPLC, high-performance liquid chromatography; Me_3Si, trimethylsilyl; NMR, nuclear magnetic resonance; RP, reversed-phase; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy.

(RP-HPLC) [solvent: methanol/water (95:5, vol/vol)] to provide a major molecular species having an effluent volume of 89.4–98.4 mL. Alkaline hydrolysis of this material afforded linoleic acid as the sole fatty acid, demonstrating that the parent galactolipid was identical to 1,2-dilinoleoyl-3-(α-galactopyranosyl-1-6-β-galactopyranosyl)-glycerol.

Methyl 3-(8′-carbomethoxyoctanoyloxy)-hexanoate. To a solution of methyl 3-hydroxyhexanoate (2 mmol) and pyridine (0.5 mL) in toluene (5 mL) was added 8-carbomethoxyoctanoyl chloride (2.2 mmol). The mixture was stirred at 23°C for 18 h and subsequently extracted with hexane. The hexane layer was washed with dilute NaOH and taken to dryness. Purification by SiO_2 chromatography and preparative thin-layer chromatography (TLC) (solvent system A) afforded methyl 3-(8′-carbomethoxyoctanoyloxy)-hexanoate which was pure as judged by gas–liquid chromatography (GLC) analysis. The Fourier-transform infrared (FT-IR) spectrum (film) showed bands at *inter alia* 2936, 2860, and 1739 cm^{-1} (ester carbonyls). Mass spectrometric analysis showed prominent ions at *m/z* 299 (2%; M − 31; loss of $\cdot OCH_3$), 257 (5; M − 73; loss of $\cdot CH_2–COOCH_3$), 185 [100; $CH_3OOC–(CH_2)_7–C{\equiv}O^+$], 145 [37; M − 185; loss of $CH_3OOC–(CH_2)_7–(CO)\cdot$], and 128 [48; M − 202; elimination of $CH_3OOC(CH_2)_7–COOH$]. As expected, alkaline hydrolysis of an aliquot of the diester resulted in the formation of 3-hydroxyhexanoic acid and azelaic acid as determined by gas chromatography–mass spectrometry (GC–MS).

Chemical methods. Acetylation was carried out by treating samples with acetic anhydride/pyridine (1:1, vol/vol) at 23°C for 15 h. Alkaline hydrolysis was performed by treatment with 0.2 M NaOH in 80% aqueous ethanol under an atmosphere of argon at 23°C for 18 h. Oxidative ozonolysis (3) was performed as described.

Method for identification of carbohydrate. For identification of carbohydrate, the galactolipid sample (1.8 mg) was dissolved in methanol (2.6 mL), and concentrated hydrochloric acid (0.4 mL; final concentration of HCl, *ca.* 5%) was added. The mixture was refluxed for 2 h. One-tenth of the residue obtained after evaporation was trimethylsilylated and analyzed by GLC and GC–MS. The remaining material was dissolved in water (2.6 mL), and concentrated hydrochloric acid (0.4 mL; final concentration of HCl, *ca.* 5%) was added. The mixture was refluxed for 2 h, taken to dryness, trimethylsilylated, and analyzed by GLC and GC–MS.

Steric analysis of galactose. Configurational assignment of galactose was performed by modification of the method described by Gerwig *et al.* (4). Thus, samples of galactolipid or D- or L-galactose (10 μmol) were heated at 85°C under argon for 1 h with *S*-(+)-2-butanol (450 μL) and concentrated hydrochloric acid (50 μL). The mixtures were taken to dryness under a stream of argon and trimethylsilylated. Separation of the trimethylsilyl (Me_3Si) derivatives of the diastereomeric *S*-(+)-2-butyl-galactopyranosides was carried out by GLC using a methyl silicone capillary column operated at 190°C. The retention times of the derivatives of the two anomers of D-galactose were 8.57 and 10.50 min whereas the retention times recorded for the derivatives of the anomers of L-galactose were 8.98 and 9.90 min. The α- and β-configurations of the anomers were not determined (4).

Enzymatic hydrolysis. Galactolipid (1.3 mg) was dissolved in 0.2 mL of chloroform/methanol (2:1, vol/vol) containing 3 mg of Triton X-100. The solvent was removed under a stream of argon, and 1 mL of 0.04 M Tris buffer pH 7.7 was added to the residue. After careful vortex mixing, a clear solution was obtained. Lipase from *R. arrhizus* (2500 units) was added and the mixture was stirred at 23°C for 1 h. The mixture was acidified to pH 3 and extracted with 6 mL of chloroform/methanol (2:1, vol/vol). An aliquot of the extracted material was esterified, trimethylsilylated, and analyzed by GLC and GC–MS. The lysolipid was isolated by TLC using solvent system D.

Extraction of lipids from oat seeds. Batches of oat seeds were ground in an electric rotating knife coffee mill and the powder (10 g) was homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in chloroform/methanol (2:1, vol/vol; 100 mL) containing 2,6-di-*tert*-butyl-4-methylphenol (1 mg). The mixture was filtered through cheesecloth and centrifuged at $1200 \times g$ for 10 min. The supernatant was taken to dryness *in vacuo* and the residue subjected to open column SiO_2 chromatography.

Preparation of galactolipid B_1. For large-scale preparations of galactolipid B_1, the major avenoleate-containing galactolipid in oat seeds, batches of oat seed powder (100 g) were homogenized in acetone (1 L) containing 2,6-di-*tert*-butyl-4-methylphenol (10 mg). The mixture was centrifuged and the supernatant taken to dryness. The residue (weight, 3.0–3.2 g) was subjected to SiO_2 chromatography (column, 25 g of SiO_2; elution with 300 mL of chloroform followed by 400 mL of acetone). The residue obtained after evaporation of the fraction eluted with acetone (280–330 mg) was dissolved in methanol (5 mL) and subjected to preparative RP-HPLC. Effluent containing galactolipid B_1 (75–83 mL) was collected and taken to dryness. The specimen of galactolipid B_1 thus obtained (25–39 mg) contained contaminants and was further purified by preparative TLC (solvent system B) followed by a second step of RP-HPLC. In this way galactolipid B_1 was obtained as a semitransparent solid (10–14 mg). The preparation was homogenous upon TLC analysis (solvent system B; $R_f = 0.34$; reference, 1,2-dilinoleoyl-3-(α-galactopyranosyl-1-6-β-galactopyranosyl)-glycerol, $R_f = 0.28$).

Preparation of the acetate of galactolipid B_1. Galactolipid B_1 (20 mg) was treated with acetic anhydride (1 mL) and pyridine (1 mL) at 23°C for 18 h. The residue obtained following evaporation (24 mg) was subjected to preparative straight-phase HPLC to provide the pure acetate of galactolipid B_1 as a colorless oil (19.5 mg; 10.8–12.5 mL effluent).

Quantitative determination of avenoleic acid in lipid fractions of oat seeds. Methyl 17-hydroxystearate (124.7 μg) was added to the lipid fraction to be analyzed and the mixture was subjected to alkaline hydrolysis. Material obtained after extraction was methyl-esterified and trimethylsilylated and analyzed by GLC (temperature, 230°C). The amount of avenoleic acid was calculated from the areas of the peaks correspond-

ing to the Me_3Si derivatives of methyl avenoleate (retention time, 8.2 min) and 17-hydroxystearate (retention time, 10.5 min) and from the amount of the 17-hydroxystearate internal standard added.

Chromatographic and instrumental methods. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany). The following solvent systems were used: A, ethyl acetate/hexane (7:93, vol/vol); B, chloroform/methanol/water (50:10:1, by vol); C, chloroform/methanol/water/acetic acid (50:10:1:1, by vol); D, chloroform/methanol/water (50:20:2, by vol). Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under ultraviolet light. RP-HPLC was performed with a column of Nucleosil C_{18} 100-7 (250 × 10 mm) purchased from Macherey-Nagel (Düren, Germany). If not otherwise indicated, methanol at a flow rate of 4 mL/min was used as the mobile phase. The detector was set at 210 nm. Straight-phase HPLC was carried out with a column of Nucleosil 50-5 (200 × 4.6 mm; Macherey-Nagel). The solvent system used was 2-propanol/hexane (5:95, vol/vol) and the flow rate was 1.5 mL/min. GLC was performed with a Hewlett-Packard model 5890 gas chromatograph (Avondale, PA) equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. GC–MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. Infrared spectroscopy was carried out with a Perkin-Elmer model 1650 FT-IR spectrophotometer (Norwalk, CT).

Electrospray (ES) MS. Positive- and negative-ion ES mass spectra were recorded on an AutoSpec OATOFFPD mass spectrometer (5) (Micromass, Manchester, United Kingdom) equipped with a conventional ES interface. Samples were introduced in a methanol solution at a flow rate of 2 μL/min, and the interface was tuned on the $(M + Na)^+$ or $(M - H)^-$ ion. Low resolution (2000 Resolution, 10% valley definiton) mass spectra were recorded over a *m/z* range of 2000–100 at a rate of 15 s/decade. Accurate mass measurements were made at moderately high resolution (5000 Resolution, 10% valley definition) by voltage scanning at a rate of 15 s/scan; the mass range scanned was just sufficient to include two calibrant ion peaks on either side of the ion of interest.

Nuclear magnetic resonance (NMR) spectroscopy. A Bruker DMX-600 instrument (Karlsruhe, Germany) was used for the NMR analyses. Deuteriobenzene was used as the solvent. Proton NMR spectra were recorded at 600 MHz and ^{13}C spectra at 150 MHz. Analyses included double-quantum filtered two-dimensional correlaton spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), ^{13}C-heteronuclear single-quantum correlation spectroscopy, and ^{13}C-heteronuclear multiple bond correlation spectroscopy (HMBC), all recorded with 512 × 8192 data points, t_{1max} = 70–85 ms, t_{2max} = 560 ms. The TOCSY spectrum was recorded with an unusually long mixing time, i.e., 2 s. The ^{13}C-HMBC spectrum was recorded with a ^1H-^{13}C *J*-evolution delay of 80 ms. Scalar coupling constants were measured from the 1D ^1H NMR spectrum and the DQF-COSY spectrum. Chemical shifts are referred to tetramethylsilane.

RESULTS

Detection and isolation of galactolipid B_1. Total lipids obtained from 10 g of oat seeds were subjected to open column chromatography using SiO_2 (20 g) as the adsorbent. Elution was accomplished with chloroform (300 mL), acetone (400 mL), and methanol (400 mL). An aliquot (10 mL) of each fraction was removed for quantitative determination of avenoleate. As seen in Table 1, the main part (62–63%) of the total amount of avenoleic acid was present in the glycolipid fraction eluted with acetone. Of total acyl chains in this fraction, 16.6–18.5% were identical to avenoleate.

An aliquot of the material present in the glycolipid fraction (Table 1) was subjected to TLC using solvent system C. As reference, 1,2-dilinoleoyl-3-(α-galactopyranosyl-1-6-β-galactopyranosyl)-glycerol was used. Several bands, labeled A–H, were visualized by spraying with dichlorofluorescein (Table 2). The materials forming these bands were eluted with chloroform, subjected to alkaline hydrolysis, and analyzed by GLC following methyl-esterification and trimethylsilylation. Material in band A, which cochromatographed with the reference and was essentially devoid of avenoleate, was provisionally identified as digalactosyldiacylglycerol containing only nonhydroxylated acyl chains. Hydrolysis of material in band B liberated a fatty acid mixture containing 33.1% avenoleate, suggesting the identity of this material with glycolipid containing one avenoleate and two nonhydroxylated fatty acid residues. Hydrolysis of material forming the less intense bands C and D afforded a fatty acid mixture comprising a higher proportion (41.2–47.8%) of avenoleate.

In another experiment, an aliquot of the glycolipid fraction was subjected to TLC, and the amounts of avenoleate present in band B and in the remaining zones of the plate were separately determined. It was found that the amount of avenoleate associated with band B accounted for 46% of the total amount of avenoleate present in the glycolipid fraction.

Analysis of material forming band B by RP-HPLC showed two components, i.e., galactolipid B_1 (68%; effluent volume, 72.0–77.0 mL) and galactolipid B_2 (32%; effluent volume,

TABLE 1
Separation of Lipids from 10 g of Oat Seeds by SiO_2 Chromatography

Experiment	Eluent	Weight (mg)	Avenoleic acid[a] (mg)	Avenoleic acid[b] / total fatty acids
1	Chloroform	231	1.48 (29%)	0.009
	Acetone	74	3.24 (63%)	0.185
	Methanol	142	0.41 (8%)	0.031
2	Chloroform	250	1.52 (29%)	0.008
	Acetone	64	3.19 (62%)	0.166
	Methanol	148	0.46 (9%)	0.030

[a]Determined by gas–liquid chromatography (GLC) using methyl 17-hydroxystearate as internal standard.
[b]Ratio between avenoleic acid and total fatty acids as determined by GLC.

TABLE 2
Separation of Glycolipid Fraction from SiO_2 Chromatography by Thin-Layer Chromatography[a]

	Band							
	A	B	C	D	E	F	G	H
Intensity	Strong	Strong	Medium	Medium	Medium	Weak	Weak	Weak
R_f value	0.30	0.40	0.46	0.57	0.66	0.80	0.87	0.96
Avenoleic acid[b]	<0.8	33.1	47.8	41.2	7.2	9.2	3.9	3.8

[a]Solvent system C (described in the Experimental Procedures section); reference, 1,2-dilinoleoyl-3-(α-galactopyranosyl-1-6-β-galactopyranosyl)-glycerol, R_f = 0.30.
[b](Avenoleic acid/total fatty acids) × 100 as determined by GLC. For abbreviation see Table 1.

86.5–94.6 mL). Alkaline hydrolysis performed on galactolipid B_1 produced avenoleic acid (33.7%) and linoleic acid (66.3%), suggesting that galactolipid B_1 contained one ester-bound avenoleate and two ester-bound linoleates. Hydrolysis of galactolipid B_2 produced avenoleic acid (34.2%), palmitic acid (13.5%), linoleic acid (31.8%), and oleic acid (20.5%). Obviously, galactolipid B_2 was due to more than one molecular species, probably a mixture of two compounds containing: 1 avenoleate + 1 linoleate + 1 oleate, and 1 avenoleate + 1 linoleate + 1 palmitate. This material was not further characterized.

IR and mass spectrometric analysis of galactolipid B_1. The FT-IR spectrum of galactolipid B_1 showed absorption bands at *inter alia* 3408 (OH), 2927 (CH_2), 1733 (ester carbonyl), 1653 (C=C), 1071 (C–O), and 916 cm^{-1} (type 1 band of sugars: ring vibration). Acetylation of galactolipid B_1 by treatment with acetic anhydride/pyridine abolished the hydroxyl absorption band and produced a strong absorption band at 1752 cm^{-1} (ester carbonyl). In addition, the FT-IR spectrum of the acetate of galactolipid B_1 showed prominent bands at 2929 (CH_2), 1653 (C=C), 1371 (CH_3), 1223 (asymmetric stretching of C–O–C of acetate), 1069 (symmetric stretching of C–O–C of acetate), and 911 cm^{-1} (type 1 band of sugars: ring vibration).

The negative-ion ES mass spectrum of galactolipid B_1 revealed an intense ion at *m/z* 1217.8 corresponding to the $(M - H)^-$ ion. Accurate mass measurements gave an *m/z* value of 1217.835 compatible with the chemical formula $C_{69}H_{117}O_{17}$. The positive-ion ES mass spectrum of galactolipid B_1 showed an intense ion at *m/z* 1241.8 corresponding to the $(M + Na)^+$ ion. Accurate mass measurement gave an *m/z* value of 1241.828 compatible to within 1 ppm of the chemical formula $C_{69}H_{118}O_{17}Na$. The data mentioned thus strongly indicated that the empirical formula of galactolipid B_1 was $C_{69}H_{118}O_{17}$, i.e., identical to the formula calculated for a molecule assembled of one avenoleate, two linoleates, one glycerol, and two galactose residues.

Identification of the carbohydrate moiety of galactolipid B_1. Galactolipid B_1 was subjected to acid methanolysis as well as acid hydrolysis, and the trimethylsilylated products were analyzed by GLC and GC–MS. GLC analysis (column temperature, 200°C) of the acid methanolysis product of galactolipid B_1 showed peaks due to methyl linoleate (retention time, 10.08 min) and to the Me_3Si derivative of methyl avenoleate (22.99 min). In addition, four peaks due to the carbohydrate moiety of galactolipid B_1 were observed with retention times of 4.46 (63%), 5.05 (27%), 5.36 (4%), and 6.35 min (6%). As references, the Me_3Si derivatives of the following carbohydrates were used: anomers of D-glucopyranose (retention times, 6.06 and 8.33 min), anomers of D-galactopyranose (5.36 and 6.35 min), anomers of methyl D-glucopyranoside (5.43 and 5.85 min), methyl α-D-galactopyranoside (4.46 min), and methyl β-D-galactopyranoside (5.05 min). The mass spectrum recorded on the peak eluting at 4.46 min in the analysis of the acid methanolysis product of galactolipid B_1 was identical to that of the Me_3Si derivative of methyl α-D-galactopyranoside and showed a prominent ion at *m/z* 361 [3%; M − (31 + 90); loss of $\cdot OCH_3 + Me_3SiOH$], 217 (26; $Me_3SiO^+{=}CH{-}CH{=}CH{-}OSiMe_3$), 204 [100; $(Me_3SiO{-}CH{=}CH{-}OSiMe_3)^+$ or its equivalent], 147 (18; $Me_3SiO^+{=}SiMe_2$), and 133 ($CH_3O{-}CH{=}O^+SiMe_3$). Weak ions indicating the molecular weight were observed at *m/z* 482 (0.04; M^+) and 435 (0.1; $M^+ - (15 + 32)$; loss of $\cdot CH_3 + CH_3OH$). In the same way, the mass spectrum recorded on the material eluting at 5.05 min in the analysis of the acid methanolysis product of galactolipid B_1 was identical to that of the Me_3Si derivative of authentic methyl β-D-galactopyranoside.

GLC analysis of the material obtained following acid hydrolysis of galactolipid B_1 showed peaks at 5.36 (37%) and 6.35 min (63%), cochromatographing with the Me_3Si derivatives of the anomers of D-galactopyranose. The mass spectra recorded on the materials produced from galactolipid B_1 were identical to those recorded on the corresponding peaks of the Me_3Si derivatives of the two anomers of D-galactopyranose.

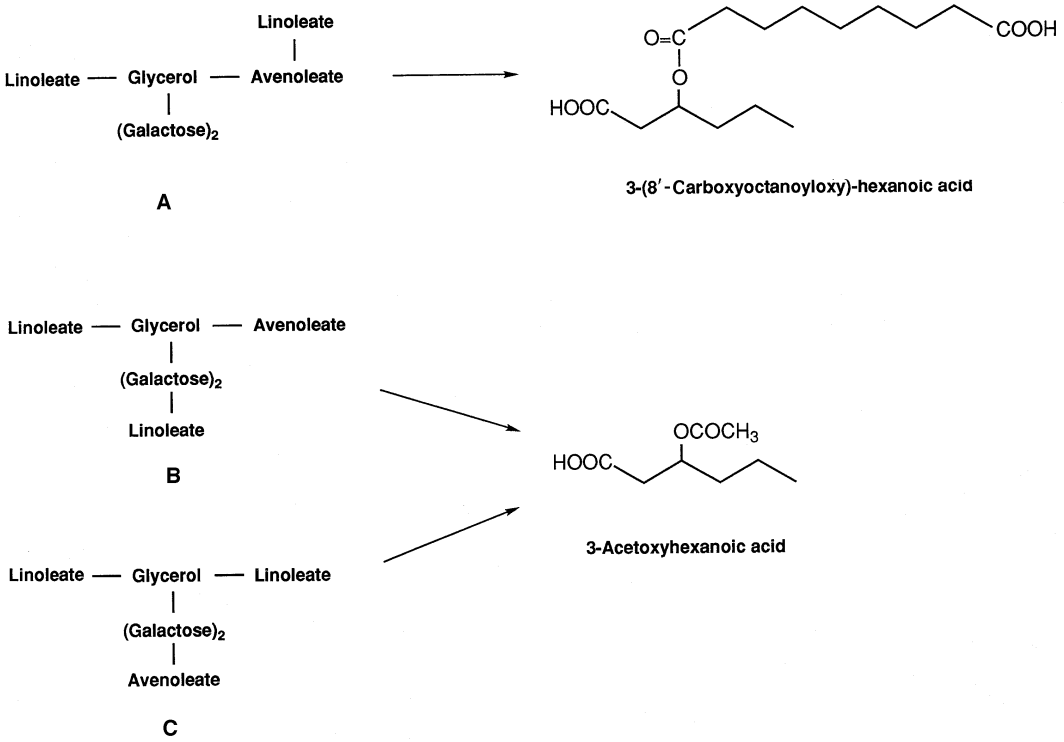
Configurational assignment of the galactopyranose moieties of galactolipid B_1 was accomplished by GLC analysis of the trimethylsilylated *S*-(+)-2-butyl galactopyranoside obtained by treatment of galactolipid B_1 (10 μmol) with *S*-(+)-2-butanol/concentrated hydrochloric acid (9:1, vol/vol). GLC performed using a column temperature of 190°C demonstrated two major peaks coinciding with the peaks of trimethylsilylated *S*-(+)-2-butyl D-galactopyranoside (8.57 and 10.50 min) but separated from the peaks of the corresponding derivative of L-galactopyranoside (8.98 min and 9.90 min). The identity of the derivatives was confirmed by MS. Thus, the mass spectra of the earlier-eluting derivative obtained from galactolipid B_1 and D-galactose were identical

and showed prominent ions at *m/z* 451 [0.2%; $M^+ - 73$; loss of $CH_3–CH_2–CH(O\cdot)–CH_3$], 419 (1; $M^+ - (15 + 90)$; loss of $\cdot CH_3 + Me_3SiOH$), 361 [6; $M^+ - (73 + 90)$], 305 (4), 217 (19; $Me_3SiO^+=CH–CH=CH–OSiMe_3$), 204 [100; $(Me_3SiO–CH=CH–OSiMe_3)^+$ or its equivalent], 175 [$CH_3–CH_2–CH(CH_3)–O–CH=O^+SiMe_3$], 147 (18; $Me_3SiO^+=SiMe_2$), 129 (6; $CH_2=CH–CH=O^+SiMe_3$), and 73 (41; Me_3Si^+).

From these data, combined with the results of the mass-spectrometric analysis of galactolipid B_1, it was concluded that galactolipid B_1 contained two D-galactopyranose residues.

Identification of the acyl chains of galactolipid B_1. As already mentioned, alkaline hydrolysis performed on galactolipid B_1 liberated avenoleic acid (33.7%) and linoleic acid (66.3%), suggesting the presence in galactolipid B_1 of one avenoleate and two linoleates. Assuming that two of the fatty acid residues were esterified to the *sn*-1 and *sn*-2 positions of the glycerol backbone, it was conceivable that the third acyl chain was esterified either to the alcohol group of the avenoleate residue (structure **A** in Scheme 1) or to one of the alcohol groups of the galactose residues (structures **B** and **C**). To distinguish between these possibilities, a sample (1 mg) of galactolipid B_1 was treated with acetic anhydride/pyridine and subjected to oxidative ozonolysis. Analysis of the esterified product by GLC showed the presence of one major oxidation product which was identified as methyl 3-(8′-carbomethoxyoctanoyloxy)-hexanoate by its retention time and its mass spectrum using the authentic compound as reference. Methyl 3-acetoxyhexanoate was undetectable in spite of scrupulous search using temperature programming from 100°C with the authentic compound as reference. On the basis of these results, it was concluded that galactolipid B_1 contained one linoleate residue and one linoleoyl-avenoleate residue esterified to glycerol (structure **A**, Scheme 1).

Localization of acyl chains in galactolipid B_1. Lipase from *R. arrhizus* catalyzes selective hydrolysis of the ester bond at *sn*-1 of galactolipids (6). To identify the acyl chain which was esterified at *sn*-1 of galactolipid B_1, a sample of the galactolipid was treated with lipase from *R. arrhizus*. An aliquot of the hydrolysis product was esterified, trimethylsilylated, and subjected to GLC (230°C). A major peak (96%; 4.1 min) coeluting with methyl linoleate, and a minor peak (4%; 8.2 min) coeluting with the Me_3Si derivative of methyl avenoleate were observed. Analysis by GC–MS confirmed the identities of these compounds. The remaining part of the product obtained following the lipase treatment was subjected to TLC using solvent system D. This analysis showed that galactolipid B_1 ($R_f = 0.68$) had been completely hydrolyzed and converted to a major ($R_f = 0.44$) and a minor ($R_f = 0.29$) lysolipid. The proportion of the less abundant material was reduced when smaller amounts of enzyme were used or when Triton X-100 was excluded from the reaction mixture. The nature of this material was not further investigated. Part of the major lysolipid was subjected to alkaline hydrolysis to afford a 1:1 mixture of avenoleic acid and linoleic acid. In another experiment, the major lysolipid was acetylated and subjected to oxidative ozonolysis. Analysis of the esterified product by GLC demonstrated the presence of methyl 3-(8′-carbomethoxyoctanoyloxy)-hexanoate and the absence of



SCHEME 1

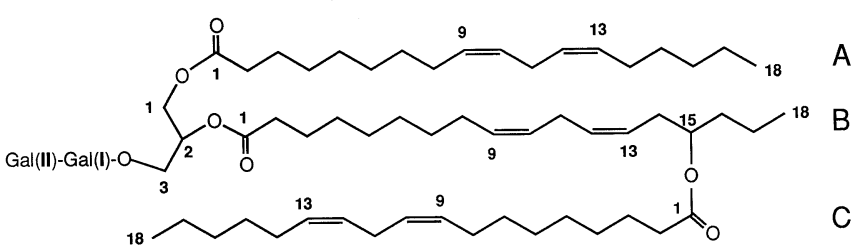
methyl 3-acetoxyhexanoate. This result demonstrated that the lysolipid retained the linoleoyl-avenoleate residue. On the basis of these findings, it was concluded that galactolipid B_1 contained a linoleate residue esterified at the *sn*-1 position and a linoleoyl-avenoleate residue esterified at the *sn*-2 position (Scheme 2). In Scheme 2, letters A, B, and C indicate acyl chains, i.e., linoleate esterified to *sn*-1 of glycerol, avenoleate esterified to *sn*-2 of glycerol, and linoleate esterified to the C-15 alcohol group of avenoleate, respectively; Gal = galactose.

Analysis of galactolipid B_1 by NMR. NMR spectroscopy was used in order to verify the structure of galactolipid B_1 established by chemical and enzymatic methods and MS (Scheme 2). A challenging problem in the NMR analysis was posed by the question, whether the linoleate (A) and linoleoyl-avenoleate (C-B) residues of Scheme 2 were connected to *sn*-1 and *sn*-2, respectively, or *vice versa*, since the acyl chains A and B differ chemically only at a position remote from the glycerol moiety. The ^{1}H NMR resonances of the methylene protons at position 2 of acyl chains A, B, and C were assigned by a ^{13}C-HMBC spectrum, correlating the ^{13}C NMR signals of the C-1 carbons with the protons on both sides of the ester bonds. The spectral region with the cross peaks C-1 to H_2-2 for all three acyl chains is shown in the lower panel of Figure 1. The figure shows that the ^{13}C chemical shifts of the carboxy groups are well resolved, but for the three C-2 methylene groups only the ^{1}H chemical shift of acyl chain A was clearly different from those of the acyl chains B and C. Furthermore, the ^{1}H chemical shifts of the protons within each methylene group are indistinguishable. The multiplet fine structure of the H_2-2 (C) resonance is much better resolved than for the H_2-2 (A) and H_2-2 (B) resonances. Quite generally, narrower lineshapes were observed for the linoleoyl residue C than for the other acyl chains, and broader signals were observed for the carbohydrate and glycerol moieties. This effect may be explained by reduced mobility caused by intermolecular hydrogen bonds transiently formed between the hydroxyl groups of different molecules in the apolar solvent used. The ^{1}H and ^{13}C NMR signals from the 4, 5, 6, and 7 positions of acyl chains A, B, and C were indistinguishable (Table 3). For the protons at positions 13 to 17, the ^{1}H NMR signals of the avenoleoyl chain (B) could be resolved from the corresponding signals from the linoleoyl chains (A and C). To determine whether the H_2-2 (A) or the H_2-2 (B) resonance belongs to the avenoleoyl chain requires the correlation of the H_2-2 signals with the H-13 signals. This correlation was obtained by a TOCSY experiment recorded with a very long mixing time (2 s), using the scalar proton–proton couplings for magnetization transfer within each acyl chain. The cross peak between the resolved H-13 resonance of the avenoleoyl chain and the H_2-2 resonance of the same acyl chain is identified by a horizontal rectangle in the upper panel of Figure 1. The locations of the corresponding cross peaks of the acyl chains A and C are identified by an oval and a vertical rectangle, respectively. The ^{1}H chemical shift of the H_2-2 signal of the avenoleate residue revealed in this experiment matched the ^{1}H chemical shift observed with the ^{13}C-HMBC cross peak at 173.8 ppm, but not the ^{1}H chemical shift of the cross peak at 173.6 ppm (Fig. 1). The match is complicated by the different cross peak multiplicities and lineshapes of the TOCSY and ^{13}C-heteronuclear single-quantum correlation spectroscopy cross peaks. Thus, the H_2-2 resonance of

TABLE 3
^{1}H and ^{13}C Nuclear Magnetic Resonance Data for Galactolipid B_1[a]

Carbon number	^{1}H	^{13}C	Carbon number	^{1}H	^{13}C
Glycerol			14(A)	2.16	28.3
1	4.54, 4.46	63.6	14 (B)	2.49, 2.38	33.2
2	5.37	71.3	14 (C)	2.15	28.3
3	4.02, 3.80	68.7	15 (A, C)	1.40	30.4
			15 (B)	5.19	73.8
Acyl chains[b]			16 (A, C)	1.34	32.5
1(A)	—	173.6	16 (B)	1.63, 1.51	36.7
1 (B)	—	173.8	17 (A, C)	1.34	23.6
1 (C)	—	173.2	17 (B)	1.43, 1.37	30.7
2 (A)	2.27	34.9	18 (A, C)	0.96	14.9
2 (B)	2.29	35.1	18 (B)	0.94	14.7
2 (C)	2.29	35.4			
3 (A, B)	1.71	26.1	Galactoses[c]		
3 (C)	1.68	25.8	1 (**I**)	4.13	104.3
4–7 (A–C)	1.25–1.45	30.2–30.4	1 (**II**)	5.12	100.3
8 (A, B)	2.16	28.2	2 (**I**)	3.67	74.5
8 (C)	2.15	28.2	2 (**II**)	4.13	70.9
9 (A, C)	5.56	76.4	3 (**I**)	4.08	80.2
9 (B)	5.56	76.0	3 (**II**)	4.05	70.6
10 (A, C)	5.56	78.4	4 (**I**)	3.75	74.6
10 (B)	5.56	78.7	4 (**II**)	4.15	69.8
11 (A–C)	2.96	26.8	5 (**I**)	3.68	72.8
12 (A, C)	5.56	78.4	5 (**II**)	3.52	64.0
12 (B)	5.56	79.0	6 (**I**)	4.21, 3.62	68.0
13 (A, C)	5.56	76.4	6 (**II**)	3.79, 3.73	63.2
13 (B)	5.64	73.3			

[a]Spectra were recorded in deuteriobenzene at 36°C.
[b]Letters A, B, and C refer to acyl chains as indicated in Scheme 2.
[c]Roman numerals **I** and **II** refer to galactose residues as indicated in Scheme 2.



SCHEME 2

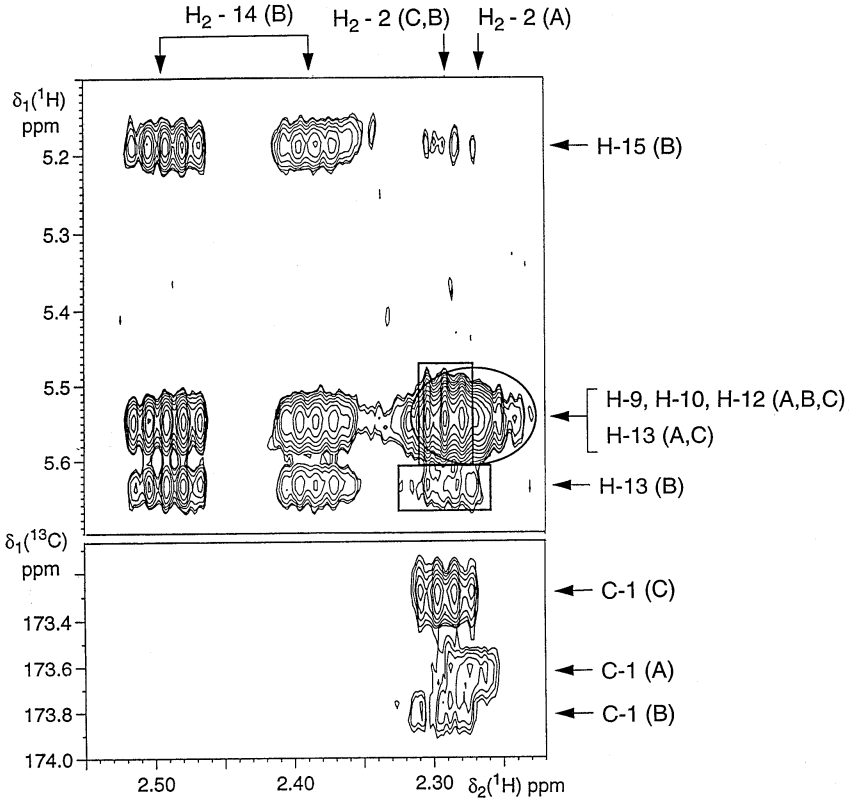


FIG. 1. Assignment of the acyl chains by nuclear magnetic resonance (NMR). Top panel: spectral region from the total correlation spectroscopy (TOCSY) experiment showing the ^{1}H-^{1}H cross peaks between H-13 in the δ_1 dimension and H_2-2 in the δ_2 dimension for the acyl chains A–C of Scheme 2. The chemical shifts of all protons involved in the cross peaks are indicated in the margins. The oval, the horizontal box, and the vertical box identify the location of the H-13–H_2-2 cross peak for the acyl chain A, B, and C, respectively. Bottom panel: spectral region from the ^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) spectrum showing the ^{13}C-^{1}H cross peaks between C-1 in the δ_1 dimension and H_2-2 in the δ_2 dimension for the acyl chains A–C. Note that the multiplet fine structures of the H_2-2 resonances in the δ_2 dimension are different in the TOCSY and ^{13}C-HMBC spectrum because of additional ^{1}H-^{13}C couplings in the ^{1}H NMR multiplets of the ^{13}C-HMBC spectrum and different, mixed lineshapes in both experiments.

acyl chain C appears as a quartet in the ^{13}C-HMBC spectrum but as a triplet in the TOCSY spectrum. Furthermore, the H-13 (B)–H_2-2 (B) cross peak in the TOCSY spectrum appears asymmetric with higher intensity toward the low ppm end of the cross peak and a larger total width than the C-1 (B)–H_2-2 (B) cross peak in the ^{13}C-HMBC spectrum. However, there is clearly no cross peak intensity at 2.26 ppm, which would be expected if the match were with the H_2-2 (A) resonance. The H_2-2 resonance of acyl chain A appears at a lower ppm value in the TOCSY spectrum, as illustrated by the cross peak between the degenerate protons 9, 10, 12, 13 and the H_2-2 resonance (identified by an oval in Fig. 1). The successful correlation of the H-13 (B) with the H_2-2 (B) resonance, which was nontrivial because of the long distance between the protons, completes the assignment of the avenoleoyl acyl chain to the position 2 of the glycerol moiety.

The ^{13}C-HMBC experiment further confirmed that the disaccharide residue was bound at the terminal position of the glycerol moiety. Analysis of vicinal ^{1}H-^{1}H coupling constants recorded on acetylated galactolipid B_1 showed that the galactose ring proximal to the glycerol moiety is a β-anomer and that the terminal galactose ring is an α-anomer (Table 4).

Quantity of galactolipid B_1 in oat seeds. On the basis of the facts that 63% of the total amount of avenoleic acid of oat seeds was present in the glycolipid fraction obtained by SiO_2

TABLE 4
^{1}H-^{1}H Coupling Constants in the Galactose Rings of Acetylated Galactolipid B_1[a]

Protons (carbon number)	*J*, galactose **I** (Hz)	*J*, galactose **II** (Hz)
1-2	7.9	3.7
2-3	10.6	10.9
3-4	3.5	3.2
4-5	1.0	1.0
5-6	5.4, 7.5	6.2, 6.9
6-6	10.2	11.2

[a]Roman numerals **I** and **II** indicate galactose residues which are proximal and distal, respectively, to the glycerol moiety.

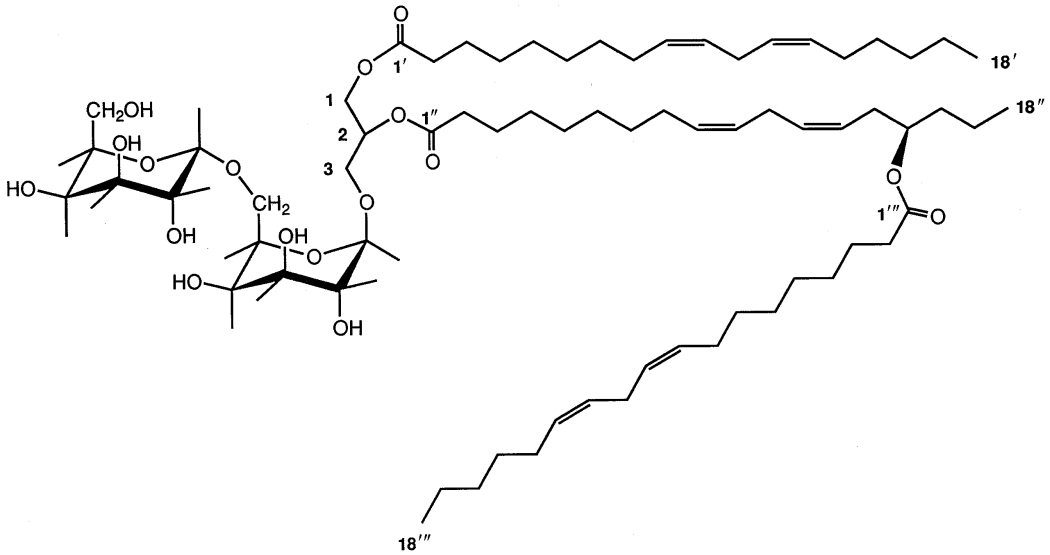
chromatography, that 46% of avenoleic acid of the glycolipid fraction was associated with band B found upon TLC, and that 68% of avenoleic acid of band B was associated with galactolipid B_1 isolated by RP-HPLC, it was concluded that 20% of the total amount of avenoleate of oat seeds was associated with galactolipid B_1. On the basis of the previously determined content of avenoleic acid in oat seeds, i.e., 593–725 μg/g (1), and the molecular weights of avenoleic acid (296) and galactolipid B_1 (1218), it was further calculated that the amount of galactolipid B_1 in oat seeds was 0.5–0.6 mg/g.

The quantitative relationship between galactolipid B_1 and digalactosyldiacylglycerol, the major galactolipid of oat seeds (7,8), was determined in the following way. An aliquot of the material present in the glycolipid fraction isolated by SiO_2 chromatography (10 mg; see Table 1) was subjected to TLC using solvent system C. The zones corresponding to digalactosyldiacylglycerol (R_f = 0.30) and galactolipids $B_1 + B_2$ (R_f = 0.40) were recovered, and methyl 17-hydroxystearate (124.7 μg) was added as an internal standard. The two samples were subjected to alkaline hydrolysis and the methyl-esterified and trimethylsilylated products analyzed by GLC. From the relative amounts of fatty acids in the two fractions, making correction for the presence of three acyl chains per molecule of galactolipid in the galactolipid B sample, it was found that the amount of galactolipids $B_1 + B_2$ was 26% of the amount of digalactosyldiacylglycerol. Because galactolipid B_1 constituted 68% of galactolipids $B_1 + B_2$, it was calculated that the percentage of galactolipid B_1 relative to digalactosyldiacylglycerol was 18%. Digalactosyldiacylglycerol in oat seeds exists as a mixture of molecular species, the major ones being molecules containing two linoleates (37%) and one linoleate plus one palmitate (31%) (9). From these percentages it was apparent that the amount of galactolipid B_1 in oat seeds was of the same order of magnitude as those of previously recognized molecular species of digalactosyldiacylglycerol. Oat seed lipids have been thoroughly studied (7–10), and it was surprising that galactolipid B_1 and related avenoleate-containing galactolipids had not been previously observed.

DISCUSSION

Avenoleic acid, a new oxylipin present in oat seeds (1), was found to be largely localized in the galactolipid fraction of oat seed total lipids. Fractionation of the galactolipids by TLC and HPLC afforded a major molecular species (galactolipid B_1) which accounted for 20% of the avenoleate content of oat seeds. Chemical methods and MS revealed that galactolipid B_1 was assembled of linoleoyl-avenoleate, linoleate, galactose, and glycerol. An enzymatic method demonstrated that the linoleoyl-avenoleate residue was positioned at *sn*-2 and the linoleate residue at *sn*-1. NMR spectroscopy confirmed these results and also showed that the proximal and distal galactopyranosyl residues had the β and α configurations, respectively. Steric analysis of diastereomeric S-(+)-2-butyl galactopyranoside derivatives by GLC demonstrated that the galactopyranosyl residues of galactolipid B_1 had the D absolute configurations. From these data, the new galactolipid was assigned the structure 1-[(9′*Z*),(12′*Z*)-octadecadienoyl]-2-[(15″*R*)-{(9‴*Z*),(12‴*Z*)-octadecadienoyloxy}-(9″*Z*),(12″*Z*)-octadecadienoyl]-3-(α-D-galactopyranosyl-1-6-β-D-galactopyranosyl)-glycerol (Scheme 3).

Galactolipids containing oxygenated fatty acids are rare. To the best of our knowledge, the only previous examples are galactolipids containing 12(*R*),13(*S*)-dihydroxy-5(*Z*),8(*Z*),-10(*E*),14(*Z*),17(*Z*)-eicosapentaenoic acid (11) and 5-hydroxy-12-oxo-6(*E*),8(*E*),10(*E*)-dodecatrienoic acid (12) isolated from the red alga *Gracilariopsis lemaneiformis*. Another unusual feature of the oat seed galactolipid was its content of an estolide, i.e., an acylated, not free, hydroxy acid residue. Ricinoleic acid [12(*R*)-hydroxy-9(*Z*)-octadecenoic acid], to which avenoleic acid is structurally related, is present in its



SCHEME 3

nonacylated form in the triacylglycerol fraction of castor oil; however, the occurrence of acylated ricinoleate residues (mono-, di-, and triestolides) in lipids from ergot oil has been described (13).

Galactolipid-bound linoleate undergoes desaturation into α-linolenate in the presence of linoleate n-3 desaturase (14). In our ongoing studies of the biosynthesis of avenoleate in preparations of oat seeds, attention is being paid to the possibility that galactolipid-bound linoleate may serve as the substrate for the putative linoleate 15-hydroxylase.

ACKNOWLEDGMENTS

The expert technical assistance of Mrs. Gunvor Hamberg is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (proj. no 03X-05170) and by the Swedish Natural Science Research Council (proj. no. 10161).

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[Received December 22, 1997, and in final revised form March 2, 1998; revision accepted March 9, 1998]

Stoichiometric and Kinetic Studies on *Ginkgo biloba* Extract and Related Antioxidants

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ABSTRACT: Owing to increasing evidence showing the importance of lipid peroxidation in oxidative stress *in vivo*, the role and evaluation of antioxidants have received much attention. *Ginkgo biloba* extract (GBE), well-known as an efficient drug against diseases induced by free radicals, has been suggested to exert its effect by antioxidant action. A method was established to determine the activity of GBE as a hydrogen donor by stoichiometric and kinetic studies, and GBE was compared with several other antioxidants such as α-tocopherol, propyl gallate, and two kinds of flavonoids which are found in GBE, quercetin, and kaempferol. It was found that there were 6.62×10^{19} active hydrogens in 1 g of GBE. Stoichiometric studies showed that one molecule of α-tocopherol reacted with one molecule of galvinoxyl radical. For quercetin, kaempferol and propyl gallate, the experimental stoichiometric numbers were 4.0, 1.9, and 3.1, respectively. The rates of reaction of antioxidants with galvinoxyl in ethanol were determined spectrophotometrically, using a stopped-flow technique. The second-order rate constant, k_2, obtained at 25°C was 0.13 $(g/L)^{-1}s^{-1}$ for GBE and 5.9×10^3, 2.1×10^3, 1.2×10^4, and 2.4×10^3 $M^{-1}s^{-1}$ for quercetin, kaempferol, propyl gallate, and α-tocopherol, respectively. The second-order rate constant, k_2', on the molar basis of active hydroxyl groups in the tested substances obtained at 25°C decreased in the order of propyl gallate > α-tocopherol > quercetin > GBE ≈ kaempferol. This is the first study on GBE as an antioxidant which reports both stoichiometric and kinetic results.

Lipids 33, 365–370 (1998).

Extracts from the leaves of *Ginkgo biloba* trees have been used therapeutically in China from ancient times and in Western countries such as France and Germany from the 1960s for the treatment of atherosclerotic diseases of peripheral vascular and cerebrovascular insufficiency (1). The well-defined but complex product consists of two major groups of substances, flavonoid glycosides and terpenoids (2,3). The flavonoid fraction is composed of three flavonols, quercetin, kaempferol and isorhamnetin, which are linked to a sugar (2,4,5). The terpenoid fraction is composed of ginkgolides and bilobalides (6). The antioxidant action has been claimed to be one of the molecular mechanisms underlying the beneficial effects of *Ginkgo biloba* extract (GBE). GBE has been reported to scavenge superoxide (7) and hydroxyl radicals (8,9), to quench peroxyl radical (10,11), and to interact with nitric oxide (12) produced from cell or biochemical reaction systems. GBE attenuates oxidative stress in macrophages, endothelial cells, and cardiomyocytes (13–15). It was also reported that hydroxyl radical-induced apoptosis and death in rat cerebellar neuronal cells could be prevented by GBE (16,17). The antioxidant activity of GBE has been attributed to the free radical-scavenging activity of the flavonoid fraction in GBE, and confirmed experimentally (18).

However, because GBE is a mixture, it is difficult to evaluate the antioxidant effectiveness of GBE and to compare the scavenging and inhibiting effects of GBE on free radicals and lipid peroxidation with those of other antioxidants. It is important to understand the behavior of GBE as an antioxidant and to find a way to measure the rates at which it reacts with radicals. The lack of published studies on the efficiency and kinetics of GBE prompted us to carry out the present work.

For comparison, we studied several other well-known antioxidants that are pure chemicals and whose antioxidant activity is ascribed to the phenolic hydroxyl groups. Two of them are quercetin and kaempferol, the two main flavonoids in GBE and the most important flavonoids studied as antioxidants. A third is propyl gallate, a polyphenol used as a food additive. Another is α-tocopherol, an important chain-breaking antioxidant that has been extensively studied *in vitro* and *in vivo.* In the present work, we have established a method to determine the stoichiometric number of active phenolic hydroxyl groups. Furthermore, we used a stopped-flow technique to measure the reaction rate constants for related antioxidants with galvinoxyl.

MATERIALS AND METHODS

Reagents. Galvinoxyl, quercetin, and *n*-propyl gallate were obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Kaempferol was purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI) GBE was provided by Takehaya Co., Ltd. (Tokyo, Japan). α-Tocopherol was kindly supplied by Eisai Company Ltd. (Tokyo, Japan). All of the substances were dissolved in ethanol and used as received. Ethanol was of analytical reagent grade. Experiments were always performed on freshly made up solutions. The structures of tested substances

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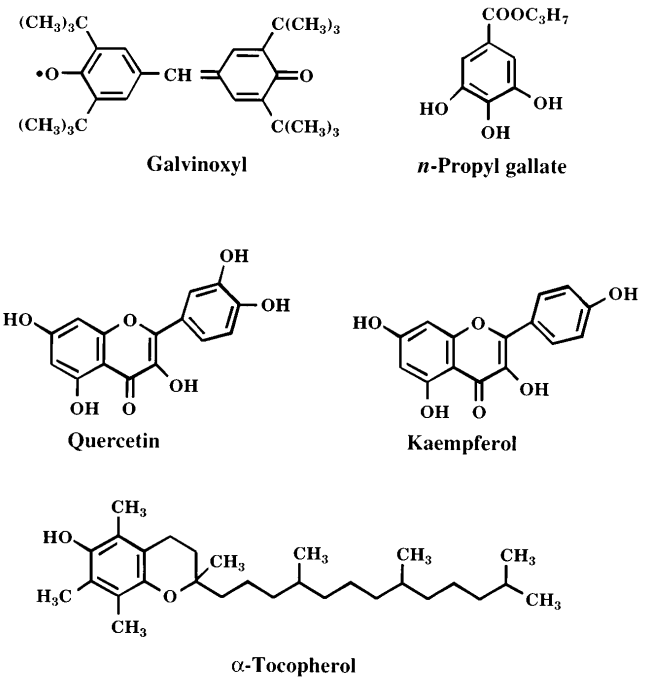
Abbreviations: GBE, *Ginkgo biloba* extract; k_1, first-order rate constant; k_2, second-order rate constant; TAA, total antioxidant activity; TEAC, Trolox-equivalent antioxidant activity; XOH, hydrogen-donating antioxidant.



SCHEME 1

are shown in Scheme 1.

Stoichiometric study. Galvinoxyl, a stable phenoxyl radical, has a strong absorption peak at 428 nm in ethanol. It can be reduced by hydrogen-donating free-radical scavengers and has been used to assess the antioxidative activity of various compounds (19,20). The reaction of galvinoxyl with a hydrogen-donating antioxidant is represented by Equation 1:

$$G\cdot + XOH \rightarrow GH + XO\cdot \quad [1]$$

where G· is the galvinoxyl radical, XOH, the hydrogen-donating free-radical scavenger, GH, the reduced galvinoxyl, and XO·, the corresponding radical derived from XOH.

On the basis that one galvinoxyl molecule reacts with one active hydroxyl group, we can determine the quantity of active phenolic hydrogens in the reaction with galvinoxyl by the absorbance decrease of galvinoxyl in the reaction solution under the condition of [G·] > [XOH], which allows all of the XOH to take part in the reaction with galvinoxyl. The stoichiometric factor may be calculated as follows from the decrease in absorbance of galvinoxyl and the concentration of XOH.

From the definition of molar absorption of a substance (Equation 2), Equation 3 can be obtained for the reaction in Equation 1:

$$A = \varepsilon C l \quad [2]$$

(A: absorbance; ε: molar extinction coefficient; C: concentration, l: cell length);

$$\Delta N = V \Delta A/(\varepsilon l) \quad [3]$$

where, ΔN is the molar quantity of galvinoxyl reduced in the reaction solution, ΔA is the absorbance difference of galvinoxyl at 428 nm between the initial and the end reaction, and V is the reaction volume.

Here we assume that one mole of XOH reacts with n moles of galvinoxyl. In Equation 1, where XOH is consumed completely Equation 4 can be obtained:

$$\Delta N = n V C_x \quad [4]$$

where C_x is the concentration of XOH (for pure substances, c is expressed as mol/L; for GBE, c is in g/L).

From Equations 3 and 4, the stoichiometric number of radicals trapped by each antioxidant, n, is given by Equation 5.

$$n = \Delta A/(\varepsilon l C_x) \quad [5]$$

In our experiments, the concentration of galvinoxyl was set at 10 μmol/L (final concentration). For pure compounds, the concentrations were set between 0.1–2 μmol/L (final concentration). The concentration of GBE was selected as 0–0.02 g/L so that considerable galvinoxyl would remain at the end of reaction. The reaction was started by mixing the galvinoxyl ethanol solution with the hydrogen-donating compound ethanol solution. The decrease in absorbance of galvinoxyl at 428 nm was traced for 20 min after mixing by using a spectrophotometer (DU 640; Beckman, Fullerton, CA) at 25°C.

Kinetic study. A stopped-flow spectrophotometer (RA-2000; Photal Otsuka Electronics, Osaka, Japan) equipped with an advanced data acquisition and processing system was used for the fast reaction kinetics. Kinetic runs were carried out under pseudo-first-order conditions, [XOH] » [G·]. The reaction was monitored at 428 nm, the absorption maximum for galvinoxyl in ethanol. Appropriate volumes of the reactants were taken in two cells: cell A containing galvinoxyl in ethanol, and cell B containing test substance in ethanol. All kinetic runs were performed with a sweep-time longer than 10 half-lives of the corresponding reaction. The data acquisition system acquired absorbance vs. time data and computed the rate constants from log $(A_t - A_\infty)$ vs. time plots, where A_t and A_∞ correspond to the absorbances at time t and infinite time (after completion of the reaction), respectively. Each kinetic run was performed five times, and the average first-order rate constants, k_1, were evaluated. The measurements were performed at 25°C.

RESULTS

Molar extinction coefficient of galvinoxyl. Galvinoxyl was stable in ethanol. Its molar extinction coefficient was determined as 1.50×10^5 M^{-1} cm^{-1} at 428 nm in ethanol.

Stoichiometric studies of tested substances in the reaction with galvinoxyl. In Figure 1 we see that the fall in absorbance attributable to galvinoxyl (10 μmol/L) at 428 nm was proportional to the concentrations of the antioxidant (inset is an example of time course in the reaction between quercetin and galvinoxyl). Excellent linear correlation was obtained in plots for all the antioxidants studied. By using Equation 5, n values could be determined. The value used for ε for galvinoxyl at

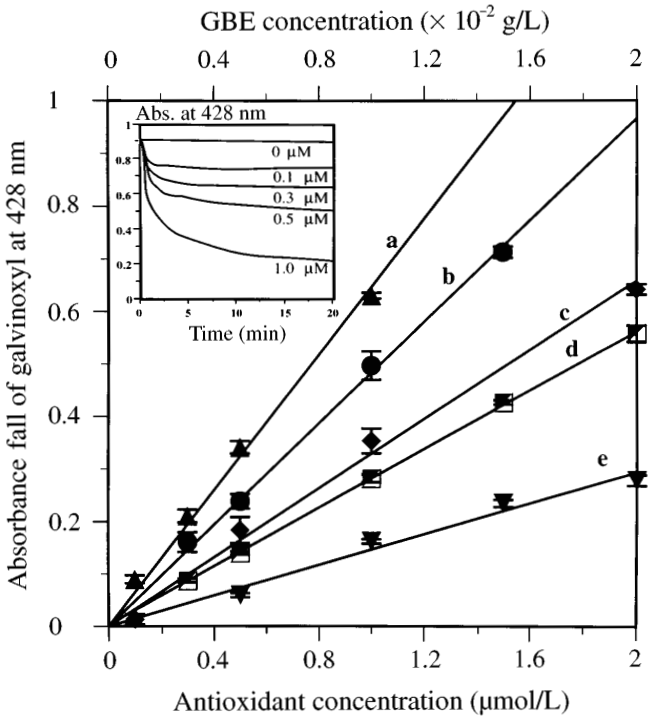


FIG. 1. Plots of absorbance falls of galvinoxyl at 428 nm against concentrations of hydrogen-donating antioxidants. Upper abscissa represents the concentration of *Gingko biloba* extract (GBE) whereas lower abscissa represents concentrations of pure compounds tested. Line a: quercetin; b: propyl gallate; c: GBE; d: kaempferol; and e: α-tocopherol. Data points represent means ± SD of five individual experiments.

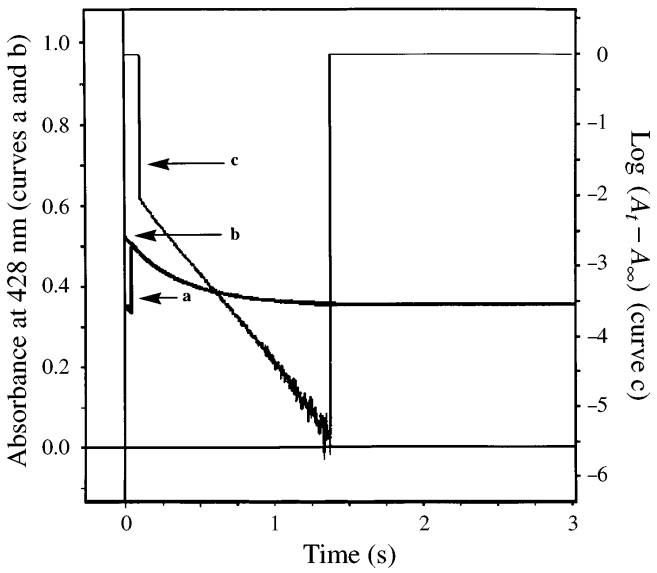


FIG. 2. Representative experiment of the pseudo-first-order study. Galvinoxyl (20 μmol/L ethanol) was mixed with propyl gallate (0.4 mmol/L ethanol) (curve a). Curve b is a theoretical fit to curve a. A typical log plot vs. time is also shown (curve c) which gives a pseudo-first-order rate constant of 2.4 s^{-1}. See the Methods section for the definition of log ($A_t - A_\infty$).

428 nm in Equation 5 was 1.50×10^5 M^{-1} cm^{-1}, and the cell length, *l*, was 1 cm. The slopes of the lines in Figure 1 correspond to $n/\varepsilon l$, from which *n* was calculated (Table 1). The *n* value for GBE was determined as 1.1×10^{-4} mol/g, meaning 1 g GBE could react with 1.1×10^{-4} mol galvinoxyl. In other words, there are 1.1×10^{-4} mol (6.62×10^{19} molecules) of active phenolic hydroxyl groups (OH) in 1 g GBE on the basis that one molecule of galvinoxyl reacts with one active OH. Similarly, the results show that quercetin, kaempferol, propyl gallate, and α-tocopherol contain 4.0, 1.9, 3.1, and 1.0 mole active hydrogens per mole, respectively, to reduce galvinoxyl.

Kinetic study of tested substances in the reaction with galvinoxyl by stopped-flow spectrophotometer. The kinetics of the oxidation of tested substances by galvinoxyl was carried out by following the decrease in absorbance of galvinoxyl at 428 nm. The concentration of galvinoxyl radical was always more than five times lower than those of tested substances (in the case of GBE, the number of active OH groups was taken into account) to obtain the pseudo-first-order rate constant, k_1. The second-order rate constant, k_2, may be calculated from the k_1 values by using Equation 6:

$$-d\,[G\cdot]/dt = k_1\,[G\cdot] = k_2\,[XOH]\,[G\cdot] \qquad [6]$$

Figure 2 is a typical time course of the decrease in absorbance at 428 nm when an ethanol solution of galvinoxyl is mixed with that of an antioxidant. The theoretical curve b fits very well with an experimentally obtained curve a which increased rapidly and then decreased. A typical plot of log ($A_t - A_\infty$) vs. time (curve c) gives a pseudo-first-order rate constant. The first-order rate constants observed at 428 nm were linearly dependent on the concentrations of the substances tested (Fig. 3). The plots of k_1 against the concentrations of tested substances yielded the second-order rate constants k_2 (Eq. 6) for quercetin, kaempferol, propyl gallate, and α-tocopherol in the reaction with galvinoxyl. These data are also summarized in

TABLE 1
Stoichiometric Numbers and the Second-Order Rate Constants for the Reactions Between Galvinoxyl and Antioxidants

	GBE	Quercetin	Kaempferol	Propyl gallate	α-tocopherol
n	1.1×10^{-4} [a]	4.0	1.9	3.1	1.0
k_2 [b]	0.13 [c]	5.9×10^3	2.1×10^3	1.2×10^4	2.4×10^3
k_2' [b]	1.2×10^3	1.5×10^3	1.1×10^3	3.9×10^3	2.4×10^3

[a]Mol active hydroxyl groups/g.
[b]Second-order rate constant for molecule (k_2) and per active hydrogen (k_2') in M^{-1} s^{-1}.
[c]$(g/L)^{-1}$ s^{-1}. GBE, *Gingko biloba* extract.

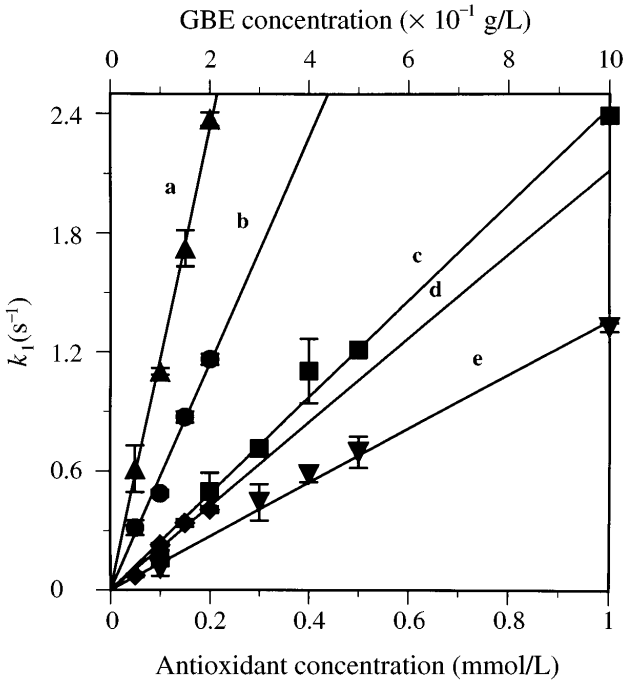


FIG. 3. Linear plots of first-order rate constants (k_1) as a function of concentrations of the antioxidant tested. Upper abscissa represents the concentration of GBE whereas lower abscissa represents concentrations of pure compounds tested. Line a: propyl gallate; b: quercetin; c: α-tocopherol; d: kaempferol; and e: GBE. The data points represent means ± SD of five individual experiments. The measurements were carried out at 25°C. See Figure 1 for abbreviations.

Table 1. The k_2 value obtained for GBE was 0.13 (g/L)$^{-1}$ s^{-1}. For the pure substances, the values decreased in the order of propyl gallate > quercetin > α-tocopherol > kaempferol.

We may rewrite Equation 1 in the following form by taking the stoichiometric factor into consideration:

$$G\cdot + (1/n)\ XOH \rightarrow GH + (1/n)\ XO\cdot \quad [7]$$

and rewrite Equation 6 as Equation 8:

$$-d\ [G\cdot]/dt = k_1\ [G\cdot] = (1/n)\ k_2\ [XOH]\ [G\cdot] = k_2'[XOH]\ [G\cdot] \quad [8]$$

where $k_2' = k_2/n$. The term k_2' may be defined as the rate constant of a single active hydroxyl group in the hydrogen-donating compound.

The rate constants k_2 and k_2' may be considered as the total reactive potential and the average reactive potential of a single active hydroxyl group, respectively, for these reactions. From k_2', we may compare the relative reactivities of active hydroxyl groups in different substances or mixtures of several substances like GBE. The results in Table 1 reflect that the active phenolic hydrogen of GBE has a similar reactivity to that of quercetin and kaempferol and a slightly smaller reactivity than that of α-tocopherol and propyl gallate.

DISCUSSION

The previous suggestion (20) that galvinoxyl reacts with phenolic antioxidant by a hydrogen atom abstraction was confirmed by our result that one molecule of galvinoxyl reacted with one molecule of α-tocopherol. The potency of antioxidant will be discussed below in terms of both stoichiometric and kinetic factors.

As expected from Equation 5, the decline in absorbance attributable to galvinoxyl by every antioxidant was directly proportional to the concentration of the antioxidant over the concentration range selected. If a higher concentration of an antioxidant than that of galvinoxyl was selected, the reduction of galvinoxyl was saturated (data not shown). A preliminary experiment was carried out to determine the range of GBE concentration in which the plot was linear. The stoichiometric study gave the number of active hydroxyl groups in 1 g of GBE as 1.1×10^{-4} mol. The stoichiometric numbers (n) of the tested pure substances in the reaction with galvinoxyl were determined experimentally as 4.0, 1.9, 3.1, and 1.0 for quercetin, kaempferol, propyl gallate, and α-tocopherol, respectively. We may consider the numbers strictly or theoretically as 4, 2, 3, and 1 for these compounds reacting with galvinoxyl. These values indicate that in the reaction with galvinoxyl in ethanol, quercetin provides four of its five hydroxyl groups, kaempferol two of its four hydroxyl groups, and that all of the three hydroxyl groups of propyl gallate take part in the reaction. The stoichiometric number of α-tocopherol for the reaction with peroxyl radicals is two (21,22), rather than one observed for galvinoxyl. This difference may be ascribed to the fact that α-tocopherol reacts with the first peroxyl radical to donate its hydrogen, and the resulting α-tocopheroxyl radical reacts with the second peroxyl radical to give an adduct (23), whereas α-tocopheroxyl radical does not react with galvinoxyl.

For measuring antioxidant potentials, the total antioxidant activity (TAA) or the Trolox-equivalent antioxidant activity (TEAC) has been reported by Rice-Evans *et al.* (24–28) as measuring the concentration of Trolox solution with an equivalent antioxidant potential to a standard concentration of the compound or the mixture under investigation. The TEAC or TAA reported for quercetin, kaempferol, gallic acid, and α-tocopherol were 4.7, 1.3, 3.0, and 1.0 (24–28), respectively, which gave the order of antioxidant potential as quercetin > gallic acid > kaempferol > α-tocopherol. Our stoichiometric studies gave the order quercetin (4) > propyl gallate (3.1) > kaempferol (1.9) > α-tocopherol (1.0). The orders obtained by using different methods are in accord. However, the TAA method was designed to compare the ability of a hydrogen-donating antioxidant to scavenge $ABTS^{\cdot+}$ radical, which is generated from the reaction of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with ferrylmyoglobin radical species (29), with that of Trolox. The value of TAA or TEAC may be considered as the same as the stoichiometric factor defined in the text because TAA or TEAC for Trolox is set at 1.0. In fact, our result showed that the TAA or TEAC values of gallic acid and α-tocopherol are equal to their stoichiometric numbers. The TAA or TEAC values of quercetin and kaempferol are not strictly the same as the stoichiometric

numbers we determined, but they are similar. Here we may say that the antioxidant activity defined by Rice-Evans is actually the number of active OH groups of substances.

Whether a certain substance acts as a radical scavenger may be revealed by determining the rate constant for reaction with a specific radical. Although stoichiometric number determines the duration of the inhibition period or lag time, the rate constant can indicate the extent of oxidation inhibition, that is, how much the antioxidant reduces the rate of oxidation. The results obtained from our kinetic studies and summarized in Table 1 show that the total reactive potentials of pure substances decrease in the order propyl gallate > quercetin > α-tocopherol > kaempferol, but we could not compare GBE with the pure chemicals. Based on the average reactive potential, however, the reactivity of GBE could be compared with those of the pure compounds, and the results in Table 1 show that the reactivity of phenolic hydroxy groups decreases in the order of propyl gallate > α-tocopherol > quercetin > GBE ≈ kaempferol.

Structure–activity relationships have been used to explain the role and potency of flavonoids as antioxidants (25,30,31). On the basis of spectral and kinetic evidence for the formation and decay of flavonoid aroxyl radicals, there are three mainly chemical criteria for flavonoids as effective antioxidants: (i) the *o*-dihydroxy structure in the B ring confers higher stability to the radical formed and participates in electron delocalization; (ii) a 2,3-double bond in conjugation with the 4-oxo function in the C ring contributes through participation in electron delocalization from the B ring; and (iii) the 3- and 5-OH groups with 4-oxo function in A and C rings provide maximal radical scavenging potential. Quercetin, satisfying all three criteria, shows higher reactivity than kaempferol with a lone B-ring hydroxyl group in the 4′ position. A catechol moiety in ring B, required for good scavenging activity, has also been confirmed by van Acker *et al.* (32) from quantum chemical explanation and electron spin resonance data on spin densities. They also showed the importance of intramolecular hydrogen bonding (32). Propyl gallate was found to possess the highest reaction potential with galvinoxyl among the substances studied. This activity could also be ascribed to the catechol moiety. The phenoxyl radical derived from polyphenol in the reaction with galvinoxyl could be stabilized by hydrogen bonding with an adjacent phenolic hydroxyl as well as by the aromatic ring (32,33); this stabilization promotes polyphenols to react with more galvinoxyl radicals, giving a stoichiometry greater than 1.

The order in relative activities of quercetin and α-tocopherol is reversed in the total reactive potential and the average reactive potential. This reversal indicates that the total activity of scavenging galvinoxyl of α-tocopherol is lower than that of quercetin, whereas the activity of the phenolic hydroxyl group of α-tocopherol is higher than that of the active hydroxyl groups of quercetin. The hydroxyl group activity of GBE is equal to that of kaempferol and lower than those of propyl gallate, α-tocopherol, and quercetin. [The two reactive potential orders of pure compounds (k_2 and k_2' in Table 1) are different from their TAA order (25) or our stoichiometric order.]

The antioxidative potentials compared here are the reactivities of substances toward galvinoxyl radical. To understand the whole activity of GBE as an antioxidant will be very complex because GBE may act not only by scavenging radicals but also (i) by chelating metal ions *via* the phenolic *o*-dihydroxy structure of the flavonoids, although it has been recognized that the antioxidative and lipid peroxidation-inhibiting potential of flavonoid predominantly resides in the radical-scavenging capacity rather than the chelation of metals (34,35) or (ii) by regenerating α-tocopherol through reduction of the α-tocopheroxyl radical. To act as a hydrogen-donating antioxidant is one of the mechanisms not only for GBE but also for flavonoids such as quercetin and kaempferol. Furthermore, the total antioxidant efficiency of radical-scavenging antioxidants is determined not only by their reactivities toward radicals but also by other factors such as fate of antioxidant-derived radicals, interactions with other antioxidants, and physical factors such as location and mobility at the microenvironment (36).

In conclusion, we have reported the quantity of active hydroxyl groups in GBE and in other pure substances which are contained in GBE or well-known antioxidants in the reaction with galvinoxyl. Taking the stoichiometric number into consideration, we have compared the second-order rate constant of GBE with other pure substances on the molar basis of active hydroxyl groups. We propose that the method devised in this study will be a useful one to evaluate and understand stoichiometrically and kinetically the antioxidative activity of hydrogen-donating compounds, pure substances, or mixtures.

ACKNOWLEDGMENTS

We are very grateful for the many valuable comments from the reviewers. We thank Dr. Mareyuki Takahashi and Dr. Noriko Noguchi for providing kind help. This study was supported in part by Grant-in Aid for Scientific Research and COE Research from the Ministry of Education, Science, Sports and Culture, Japan.

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Lipid Molecular Order in Liver Mitochondrial Outer Membranes, and Sensitivity of Carnitine Palmitoyltransferase I to Malonyl-CoA

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A**BSTRACT:** Mitochondrial outer membranes were prepared from livers of rats that were in the normal fed state, starved for 48 h, or made diabetic by injection of streptozotocin. Membranes were also prepared from starved late-pregnant rats. The latter three conditions have previously been shown to induce varying degrees of desensitization of mitochondrial overt carnitine palmitoyltransferase (CPT I) to malonyl-CoA inhibition. We measured the fluorescence polarization anisotropy of two probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate (TMA-DPH) which, when incorporated into membranes, report on the hydrophobic core and on the peripheral regions of the bilayer, respectively. The corresponding polarization indices ($\mathbf{r}_{DPH}$ and $\mathbf{r}_{TMA\text{-}DPH}$) were calculated. In membranes of all three conditions characterized by CPT I desensitization to malonyl-CoA, $\mathbf{r}_{DPH}$ was decreased, whereas there was no change in $\mathbf{r}_{TMA\text{-}DPH}$, indicating that CPT I is sensitive to changes in membrane core, rather than peripheral, lipid order. The major lipid components of the membranes were analyzed. Although significant changes with physiological state were observed, there was no consistent pattern of changes in gross lipid composition accompanying the changes to membrane fluidity and CPT I sensitivity to malonyl-CoA. We conclude that CPT I kinetic characteristics are sensitive to changes in lipid composition that are localized to specific membrane microdomains.
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Under most physiological conditions, carnitine palmitoyltransferase of the mitochondrial outer membrane (CPT I) catalyzes the reaction at which most of the control over β-oxidation of fatty acids is exerted (1–3). In the liver, this is particularly pertinent under basal conditions and during the onset of ketogenic states, whereas control over the pathway shifts to step(s) proximal to CPT I during the early phase of the reversal of these conditions. The property of CPT I that makes it possible for the reaction it catalyzes to exert such a level of control is its inhibition by malonyl-CoA (4). This inhibition diminishes its activity sufficiently *in vivo* so that, coupled with the efficient removal of the product (acylcarnitine) by its transport into the mitochondria (or the lumina of other membrane systems), it makes the reaction virtually unidirectional. Although this role of malonyl-CoA is central to the regulation of the rate of fatty acid oxidation in the liver (4–6), the decrease in the hepatic concentration of malonyl-CoA observed in ketogenic states, e.g., starvation, diabetes (7–11), does not appear to be sufficient by itself to ensure that the rate of acylcarnitine formation is increased to the extent required during the establishment of maximal rates of ketogenesis, even when the availability of fatty acid substrate has been maximally achieved (11). The additional feature of malonyl-CoA inhibition that enables full activation of ketogenesis to occur is the severalfold desensitization of CPT I to malonyl-CoA inhibition that occurs during the development of ketogenic conditions (12–15). This has been demonstrated through the monitoring of changes of different parameters *in vivo* during the onset of the pharmacological effects of streptozotocin-induced diabetes (16) and starvation (11). Conversely, studies in which the fate of fatty acids specifically directed to the liver *in vivo* (17) were monitored during the refeeding of starved rats (18) or insulin treatment of diabetic rats (19) have shown that only when the sensitivity of CPT I to malonyl-CoA is reestablished is the control of CPT I over the rate of acylcarnitine formation recovered.

In all instances, the changes in CPT I sensitivity to malonyl-CoA occur over a relatively slow time scale, requiring several hours for completion. This has been confirmed most recently through studies *in vivo* (11,18,20) and on cultured rat liver cells treated with insulin (21). This time scale has prompted us previously to suggest (5,6,22) that it is changes in the properties of the membrane (e.g., in the order of the membrane lipids) and the consequent alterations in the interactions between protein and membrane that mediate the changes in CPT I kinetic characteristics. This suggestion was made in light of previous data which showed that the changes in CPT I sensitivity observed in mitochondria isolated from rats in different physiological states can be mimicked *in vitro* by temperature- or chemically induced modulation of outer membrane lipid bilayer fluidity (23–25). In general, conditions that increase membrane fluidity *in vitro* result in decreased malonyl-CoA sensitivity of CPT I. A plausible basis for this strong effect of outer membrane fluidity on CPT I has recently been obtained through the elucidation of the topol-

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Abbreviations: CPT I, the outer-membrane carnitine palmitoyltransferase of mitochondria; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfonate.

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ogy of the protein within the membrane (26,27). However, direct demonstration that changes in membrane fluidity actually occur *in vivo* in parallel with changes in the sensitivity of CPT I to malonyl-CoA has not hitherto been available.

MATERIALS AND METHODS

Animals. Wistar rats (A. Tuck & Sons, Battlesbridge, Kent, United Kingdom) were either fed *ad libitum* on a standard laboratory chow diet (see Ref. 11 for details) or starved for 48 h. Diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg body weight); the animals were used 4 d later when their blood glucose was >20 mM. Rats used in the pregnant state were mated when they weighed about 180–200 g, and their pregnancy was allowed to proceed to day 19, when they were starved for 24 h before being used.

Preparation of mitochondrial outer membranes. These were prepared as described previously (28). Briefly, a crude preparation of mitochondria was obtained by differential centrifugation of liver homogenates prepared in a medium containing 300 mM sucrose, 5 mM Tris, 1 mM EGTA (pH 7.4 at 0°C). The mitochondria were further purified on self-forming Percoll gradients as described (28). After washing, the mitochondria were swollen in hypotonic medium and homogenized lightly to release outer membrane vesicles which were further purified by incubation with cytochrome C-Sepharose to minimize inner membrane contamination. Although, as expected, this resulted in the loss of some outer membrane material, routinely it increased the enrichment of monoamine oxidase (outer membrane marker) over that of cytochrome oxidase (inner membrane) by >50-fold compared to that in intact mitochondria. After high-speed sedimentation at 100,000 × *g* for 45 min, membranes were finally resuspended in a buffer containing 150 mM KCl, 5 mM Tris, and 1 mM EGTA (pH 7.4 at 0°C) and frozen at –80°C until used.

Measurement of fluorescence anisotropy. The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfonate (TMA-DPH) were used. They were dissolved in tetrahydrofuran to a concentration of 2 mM and then dispersed in degassed phosphate-buffered saline (pH 7.1) to give a final concentration of 2 μM. The solutions were purged for 20 min with nitrogen gas to remove the tetrahydrofuran and mixed with equal volumes of membrane suspensions prepared in the same medium. The final protein concentration was 0.7 mg protein/mL. The mixture was kept at 37°C, and fluorescence (excitation at 360 nm, emission at 430 nm) was measured at 30-min intervals over the following 2 h, to ensure attainment of stable readings. A Perkin-Elmer fluorimeter (EL-5), equipped with a 400-mm filter, temperature-controlled cell holder and polarizer, was used. Fluorescence was measured with the emission polarizer in the vertical ($I_{//}$) and horizontal ($I_{\perp}$) positions. The intensities of the fluorescence by membranes incubated without either of the probes were subtracted from the corresponding experimental values before calculation of the fluorescence polarization index, **r**, as defined by Equation 1 (29):

$$\mathbf{r} = (I_{//} - GI_{\perp}) / (I_{//} + 2GI_{\perp}) \quad [1]$$

where *G* (the correction factor for instrument polarization) is the ratio of the vertical and horizontal polarized emission components with the excitation polarizer in the horizontal position. Routinely, the value of *G* was always very close to unity.

Lipid analyses. These were performed as described by Christie (30). Aliquots of membrane suspensions containing 0.4–1.0 mg protein were homogenized by hand, using a 10-mL glass–glass homogenizer, in 5 mL chloroform/methanol (3:2, vol/vol) to which had been added 100 μL of D,L-α-phosphatidyl-*N*,*N*-dimethylethanolamine, dipalmitoyl as standard. After the addition of a further 4 mL of chloroform, the samples were rehomogenized to ensure full extraction of total lipids. The extract was washed with 2.25 mL of 0.88% KCl and left on ice for 30 min. The organic phase was collected, dried under nitrogen at 40°C, solubilized in 0.5 mL methanol, and taken to dryness under nitrogen. The lipids were finally stored in 1 mL of chloroform under nitrogen at –20°C. Samples were filtered (polytetrafluoroethylene, 0.45 μm) and dried to <100 μL for injection onto a high-performance liquid chromatography column (10 cm × 5 mm) packed with Spherisorb™ 3-μ silica (Hichrom Ltd., Reading, Berkshire, United Kingdom). The solvent systems used and the mass detection of the eluted lipids were as described previously (30). Calibration curves for the major lipid classes were constructed using purified lipids and were used to quantitate individual lipids in the samples after correction for losses calculated from the recovery of phosphatidyl-*N*,*N*-diethanolamine, dipalmitoyl. The fractions containing the main lipid classes were collected and their constituent fatty acids quantified by gas chromatography after formation of their methyl esters using standard methods (30).

Enzyme assays. The activity of CPT I in purified mitochondrial outer membrane preparations was measured exactly as described (28). The concentration of malonyl-CoA required to achieve 50% inhibition of CPT I activity was calculated from a series of activity measurements conducted in the presence of increasing concentrations (1–200 μM) of malonyl-CoA. Marker enzyme activities were also measured as described (28).

Materials. DPH and TMA-DPH were obtained from Molecular Probes Inc. (Cambridge Bioscience, Cambridge, United Kingdom). Solvents (high-performance liquid chromatography grade) were obtained from Fisher Scientific UK (Loughborough, Leicestershire, United Kingdom). Percoll, cytochrome C, cholesterol and phospholipid standards, and phosphatidyl-*N*,*N*-dimethylethanol-amine, dipalmitoyl were obtained from Sigma (Poole, Dorset, United Kingdom).

RESULTS AND DISCUSSION

Many instances are documented in the literature of the desensitization of hepatic CPT I to malonyl-CoA in starvation and diabetes. To test the hypothesis that a link exists *in vivo* between mitochondrial outer membrane lipid order and CPT I sensitivity to malonyl-CoA, we prepared purified outer membranes of mitochondria isolated from normal-fed, 48 h-starved, and 4-d

streptozotocin diabetic rats. In addition, we studied membranes isolated from 24-h starved 20-d pregnant rats, as in (31) and found that CPT I in mitochondria isolated from the livers of these animals (in which the metabolic demands placed by the fetuses result in pronounced starvation effects) is extremely insensitive to malonyl-CoA. The relative sensitivities of CPT I to malonyl-CoA inhibition in membranes prepared from rats in these four conditions were expressed as IC_{50} values (concentration of inhibitor required to achieve 50% inhibition of CPT I activity; see the Materials and Methods section) in Table 1.

The fluidity of the acyl chain core of the membranes and of the outer regions of the leaflets of the bilayer was assessed, respectively, by the measurement of the fluorescence polarization of two probes: (i) DPH, a rodshaped probe which partitions between the two acyl chain layers of the membrane core, and (ii) a charged derivative of DPH, namely, TMA-DPH which, by virtue of its positively charged tertiary ammonium group, partitions with this group alongside the phospholipid headgroups in the membrane, with its aliphatic chain alongside the outer regions of the acyl chains of the bilayer (32). Consequently, TMA-DPH monitors the fluidity of the outer regions of the membrane leaflets (33).

The polarization indices (**r** values) for the two probes (calculated as described in the Materials and Methods section) in the four types of membrane studied are also given in Table 1. The value of $\mathbf{r}_{DPH}$ is decreased in membranes from rats in all the ketogenic states studied, compared to that for membranes from normal-fed rats. Preliminary experiments established that the value of $\mathbf{r}_{DPH}$ for mitochondrial outer membranes was decreased by approximately 0.01 of a unit when membranes were fluidized by incubation with 20 mM-benzyl alcohol (not shown)—a treatment which mimics the magnitude of the effects of starvation with respect to the desensitization of CPT I to malonyl-CoA (23). Conversely, when the order of the mitochondrial outer membrane lipids was increased by lowering the incubation temperature from 37 to 30°C, $\mathbf{r}_{DPH}$ increased by about the same amount [c.f. (34,35) for plasma membrane]. The data in Table 1 indicate that decreases in the sensitivity of CPT I to malonyl-CoA are accompanied by increases in fluidity of the membrane core lipids (decreased $\mathbf{r}_{DPH}$ value) induced by the same physiological condition. By contrast, $\mathbf{r}_{TMA\text{-}DPH}$ was invariant for membranes from different physiological states, suggesting that differences in the restriction of the movement of this probe in the more peripheral regions of the bilayer are not significant in membranes prepared from the range of physiological conditions studied. Consequently, CPT I kinetic characteristics appear to respond primarily to changes in membrane core lipid order, as would be expected from the existence of its two highly hydrophobic transmembrane segments (26).

TABLE 1
Values of Fluorescence Polarization Indices *r* for DPH and TMA-DPH Incorporated into Mitochondrial Outer Membranes Prepared from Livers of Rats in Different Physiological Conditions[a]

Animals	$\mathbf{r}_{DPH}$	$\mathbf{r}_{TMA\text{-}DPH}$	IC_{50} for malonyl-CoA (μM)
Fed (n = 4)	0.079 ± 0.005	0.121 ± 0.007	14 ± 2
48-h-starved (n = 4)	0.071 ± 0.008	0.131 ± 0.006	39 ± 3*
Streptozotocin-diabetic (n = 4)	0.069 ± 0.002*	0.121 ± 0.001	42 ± 5*
20-d pregnant , 24-h starved (n = 5)	0.057 ± 0.006*	0.122 ± 0.007	71 ± 12*

[a]The IC_{50} values for CPT I inhibition by malonyl-CoA are also given. Values are means (± SEM) for the number of determinations on separate membrane preparations indicated in parentheses. Values significantly different from those for fed, normal animals ($P < 0.05$) are indicated by asterisks. Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-sulfonate.

Membrane lipid composition changes associated with changes in fluidity. The motion of the DPH molecule within membranes can be described as wobbling within a double cone about axes which lie in the plane of the membrane. This motion is influenced mainly by the hindrance offered to free molecular wobbling and, to a lesser extent, by dynamic considerations, with lipid order being the most important influence (36). Since membrane proteins have to undergo conformational changes in order to achieve optimal catalytic activity and regulatory function, the rate of shift between conformations and the probability of the attainment of these conformations would be expected to be affected by the hindrance offered by membrane lipids (i.e., their fluidity). This is particularly applicable to CPT I which appears to undergo large conformational changes upon interaction with malonyl-CoA, as evidenced by its hysteretic behavior (23,24,37,38) and the ability of malonyl-CoA and other enzyme ligands to render it very resistant to proteolysis (39,40). We have recently shown that CPT I is likely to adopt a highly folded and compact conformation, as evidenced by its remarkable resistance to proteolysis in intact mitochondria (26). Moreover, loss of the extreme N-terminus of the protein is sufficient to disrupt both catalytic activity and malonyl-CoA sensitivity of the enzyme (26). Therefore, it would be anticipated that restriction of the motion of the enzyme molecule would favor malonyl-CoA sensitivity (27).

Three aspects of membrane lipid composition are particularly important in determining the molecular order of the acyl chains that make up the hydrophobic core of membranes: (i) the cholesterol/phospholipid ratio; (ii) the phospholipid head group composition, and (iii) the degree of saturation of the acyl chains (41). All these parameters potentially affect the ordered packing of the hydrocarbon (acyl) chains. Thus, cholesterol rigidifies membrane lipids above their transition temperature as it intercalates perpendicularly to the plane of the membrane, whereas phospholipids differ in their ability to achieve tight packing by virtue of the respective properties of their different headgroups. Of the three main phospholipids, phosphatidylethanolamine (PE) is capable of the closest packing of its acyl chains because of its small neutral headgroup. By contrast, the negatively charged headgroup of phosphatidylinositol (PI) packs much less readily, whereas the packing ability of phosphatidylcholine (PC) is intermediate. The more tightly

the headgroups of the constituent phospholipids (and hence the attached acyl chains) are packed, the higher the molecular order (rigidity) of the membrane lipids. The relative packing ability of the three phospholipid classes is illustrated by the large differences in capacity of artificial membranes made from the respective lipids to swell to accommodate water molecules (PI>PC>PE, 4:2:1) (41). Consequently, low PE/PC and high PI/PC ratios both result in looser packing of the acyl chains (i.e., fluidization of the core of the membrane). Finally, increased unsaturation of the acyl chains (decreased saturation index, see legend to Table 3) would also be expected to fluidize membranes, as *cis*-double bonds antagonize ordered packing, although previous observations have suggested that there is little correlation between the saturation index of the component fatty acyl chains of phospholipids and the fluidity of naturally occurring membranes (see below).

The lipid composition of the purified outer membrane vesicles prepared from rats in the four conditions studied is given in Tables 2 and 3. The data indicate that the decreases in $\mathbf{r}_{DPH}$ (increased fluidity) observed for membranes in which CPT I is less sensitive to malonyl-CoA are not accompanied by marked or consistent changes in the gross lipid composition of the membranes.

Diabetic rats and rats starved for 48 h. The only significant change observed for membranes prepared from these two groups of rats was a 50% decrease in their cholesterol content compared to membranes from normal, fed rats. Neither the relative contents of the different classes of phospholipids nor the overall saturation indices of the component acyl chains were altered by starvation or streptozotocin-induced diabetes. However, as expected from the well-established effects of insulin deficiency to decrease Δ6 desaturase activity (42–45), the 20:4/18:2 ratio was markedly decreased (Table 3). Moreover, even if the decrease in the already low cholesterol content of these membranes had any fluidizing effect in membranes from diabetic rats, it would be expected to be antagonized by the decrease in PI content of these membranes. The latter was expected from the fact that short-term streptozotocin treatment is known to result in decreased myoinositol content in the liver and other tissues (46,47).

Starved pregnant rats. Membranes prepared from these animals showed the largest decrease in $\mathbf{r}_{DPH}$ and, correspondingly, the largest increase in the IC_{50} value for malonyl-CoA (Table 1). However, this increased fluidity appeared not to be related to changes in either overall cholesterol content of the membrane or the saturation index of the component acyl chains of the phosphoplipids. However, there was an increase in the PI/PE ratio from a value of 0.34 in membranes of control animals to 0.74 in membranes obtained from starved pregnant rats. As already mentioned, such a change in the head group composition of phospholipids would be expected to result in increased fluidity of the membrane. However, only starved pregnant rats showed this change. Indeed, in diabetic rats the PI content decreased (see preceding paragraph). Late

TABLE 2
Lipid Composition of Mitochondrial Outer Membranes Prepared from Livers of Rats in Different Physiological States[a]

	Animals			
Lipid	Fed (n = 3)	Starved (n = 5)	Diabetic (n = 3)	Pregnant starved (n = 3)
Cholesterol	4.3 ± 1.0	2.2 ± 0.5*	2.3 ± 0.7*	4.0 ± 0.1
PC	60.5 ± 4.5	58.3 ± 2.5	64.9 ± 1.5	61.7 ± 2.8
PE	25.7 ± 1.7	29.0 ± 0.3	29.2 ± 0.9	19.7 ± 1.4*
PI	9.5 ± 2.5	8.8 ± 1.8	3.5 ± 0.3*	14.5 ± 3.3*
PS	1.0 ± 0.4	0.5 ± 0.4	0.5 ± 0.5	0.5 ± 0.3
CL	0.5 ± 0.1	0.9 ± 0.4	0.5 ± 0.4	0.5 ± 0.4
SM	1.8 ± 0.5	1.2 ± 0.6	0.5 ± 0.4	0.7 ± 0.4

[a]Values are expressed as means of the percentage composition (± SEM) for the number of separate outer membrane preparations analyzed. Those that are significantly different from "fed" values ($P < 0.05$) are indicated by asterisks. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin.

TABLE 3
Fatty Acid Composition (%) of the Acyl Chains of the Major Phospholipids of Mitochondrial Outer Membranes Prepared from Livers of Rats in Different Physiological Conditions

	Animals			
	Fed (n = 3)	48-h starved (n = 5)	Diabetic (n = 3)	Pregnant starved (n = 3)
PC				
16:0	26.4 ± 2.1	26.3 ± 1.7	32.5 ± 3.9	31.8 ± 0.9
18:0	16.2 ± 2.5	14.5 ± 5.7	17.1 ± 2.1	28.8 ± 0.4*
18:1	8.1 ± 1.5	5.3 ± 0.6	7.0 ± 0.8	6.8 ± 0.3
18:2	16.8 ± 1.5	14.9 ± 1.2	23.7 ± 0.7*	12.7 ± 0.2*
20:4	19.8 ± 2.1	26.3 ± 1.5	12.4 ± 2.1*	11.6 ± 1.6*
22:6	7.8 ± 0.8	6.2 ± 0.6	6.0 ± 1.5	3.2 ± 1.0*
Sat. index	0.27	0.23	0.35	0.61
18:1/18:0	0.50	0.37	0.40	0.24
20:4/18:2	1.18	1.77	0.52	0.91
PE				
16:0	27.9 ± 1.5	24.7 ± 2.0	32.9 ± 0.6	26.5 ± 2.6
18:0	18.3 ± 1.1	22.4 ± 0.6	19.6 ± 0.8	32.2 ± 2.5*
18:1	5.3 ± 1.4	3.7 ± 0.4	4.0 ± 0.4	5.2 ± 0.6
18:2	9.3 ± 2.1	9.8 ± 1.3	15.5 ± 1.3*	9.8 ± 1.7
20:4	13.3 ± 4.4	18.2 ± 3.0	11.8 ± 1.5	12.0 ± 2.7
22:6	15.4 ± 2.9	11.4 ± 2.2	14.1 ± 1.7	5.6 ± 0.6*
Sat. index	0.27	0.29	0.31	0.55
18:1/18:0	0.29	0.17	0.20	0.16
20:4/18:2	1.43	1.85	0.76	1.22
PI				
16:0	19.1 ± 2.5	9.9 ± 2.0*	26.9 ± 7.0	7.2 ± 1.7*
18:0	36.5 ± 1.5	42.6 ± 1.3	36.5 ± 4.7	47.9 ± 2.0*
18:1	1.5 ± 0.3	1.1 ± 0.3	3.7 ± 1.5	4.1 ± 0.4*
18:2	2.5 ± 2.0	37.3 ± 2.8	21.9 ± 1.7*	23.2 ± 1.5*
20:4	34.6 ± 2.0	37.3 ± 2.8	2.1 ± 0.3	2.3 ± 0.2
22:6	3.2 ± 1.0	2.6 ± 0.4	2.1 ± 0.3	2.3 ± 0.2
Sat. index	0.34	0.31	0.54	0.47
18:1/18:0	0.04	0.03	0.10	0.09
20:4/18:2	13.8	13.8	3.7	7.0

[a]Only the major fatty acyl species are listed. Values are means (± SEM). Statistically significantly different values (compared to controls, $P < 0.05$) are indicated by asterisks. The saturation (Sat.) index is defined as the number of saturated acyl chains divided by the sum of each unsaturated chain multiplied by its number of double bonds. For abbreviations see Table 2.

pregnancy is characterized by hyperinsulinemia and insulin-resistance (48,49), as well as by other specific hormonal changes (e.g., high levels of progesterone and placental lactogen) which, in addition to inducing changes in insulin signaling (50,51) may exert their own effects on fatty acid and phospholipid metabolism. This may explain the very marked changes in mitochondrial outer membrane lipid composition observed in the liver of pregnant animals.

In all the membrane preparations studied, sphingomyelin (which occurs in association with cholesterol) and cardiolipin (a mainly inner membrane lipid) were present only in trace concentrations (0.5–1.0%). In particular, we could not confirm the changes in sphingomyelin content of membranes described (52) for 48-h-starved and streptozotocin-diabetic rat liver mitochondrial outer membranes. However, it is to be noted that the preparations used in Reference 52 appear to have contained inordinately high amounts of cholesterol and sphingomyelin for the mitochondrial outer membrane (see, e.g., Ref. 53), suggesting that the apparent increase in sphingomyelin content may have resulted from the use of less pure preparations of mitochondrial outer membranes.

The results of this study have shown that the relationship between mitochondrial outer membrane fluidity and desensitization of CPT I to malonyl-CoA, previously deduced from *in vitro* studies (23), also occurs *in vivo*. Membranes isolated from liver mitochondria of animals displaying a wide range of CPT I sensitivity to malonyl-CoA display an equally wide (inverse) range of fluidity of the core of the membrane (accessed by DPH). However, these parallel changes in the two parameters are not accompanied by a consistent pattern of changes in gross lipid composition of the membranes. It is noteworthy that a lack of correlation of saturation index of the acyl chain components of phospholipids and of the lipid molecular order has been observed previously for several other types of cell membrane (see, e.g., Ref. 54). Changes in the fluidity of the core of the lipid bilayer sensed by the transmembrane segments of CPT I may be associated with changes in composition that are more subtle than can be discerned by gross analysis of membrane lipids, possibly because of the specialized distribution of the enzyme within the mitochondrial contact sites (55). Transmembrane segments of integral membrane proteins are known to favour helix–helix packing in preference to interaction with the hydrophobic core of the membrane (56). CPT I has two closely located transmembrane segments (26), the packing of which may be affected specifically by the microdomain in which the enzyme resides (55). It would appear from our data that changes in the composition of these lipids may occur in parallel to those that occur in the bulk of the membrane lipids and which are reflected in changes in DPH fluorescence. However, any specific changes that occur in the vicinity of CPT I do not appear to be reflected in the gross lipid composition of the isolated mitochondrial outer membranes.

ACKNOWLEDGMENTS

We thank the British Diabetic Association and the Scottish Office Agriculture, Fisheries and Environment Department for support.

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Biochemical and Structural Characterization of Triacylglycerol Lipase from *Penicillium cyclopium*

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ABSTRACT: An extracellular lipase, active on water-insoluble triacylglycerols, has been isolated from *Penicillium cyclopium*. The purified enzyme has a molecular mass of 29 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis. It hydrolyzes emulsions of tributyrin, trioctanoin, and olive oil at the same rate as pancreatic lipase and shows very low activity against partial acylglycerols (monooctanoin and dioctanoin) and methyl esters. It is stable at 35°C for 60 min and has maximal activity in a pH range of 8–10. Hydrolysis of triacylglycerols by *P. cyclopium* lipase is inhibited by detergents such as Triton X-100. Comparison of the sequence of the 20 first amino acid residues of *P. cyclopium* triacylglycerol lipase with other *Penicillium* lipases indicates a high homology with previously characterized lipases produced by *P. expansum* and *P. solitum* which are enzymes of comparable size and substrate specificity. Conversely, homology between *P. cyclopium* lipase and *P. simplicissimum* lipase, a nonspecific lipolytic enzyme, is low. *Penicillium cyclopium* triacylglycerol lipase shows no homology with *P. camembertii* lipase which is specific to monoacylglycerol and diacylglycerol.
Lipids 33, 377–384 (1998).

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are found in all organisms (1). Early studies with pancreatic lipase have shown that enzyme activity is related to the physical state of the substrate (2,3). Pancreatic lipase binds to the lipid–water interface and hydrolyzes water-insoluble tributyrin and triolein or water-soluble triacetin and tripropionin at concentration beyond the solubility limit. This particular mode of action was originally proposed to distinguish between lipases and nonspecific carboxylesterases which show maximal activity on aqueous dispersion of water-soluble short-chain triacylglycerols. These observations have led to the speculative proposal that lipase activity could result from conformational changes of the enzyme protein upon binding at interface, a phenomenon known as interfacial activation (4–6). In recent years, the elucidation of the three-dimensional structure of mammalian pancreatic lipase as well as lipases from microorganisms such as *Mucor miehei*, *Geotrichum candidum* and, more recently, from *Pseudomonas cepacia* have confirmed that these lipases are serine enzymes in which access of triacylglycerol to the catalytic site is impaired by an amphiphilic α-helical peptide segment (lid) that lies over the catalytic site (7–12). Further studies have provided evidence that lipases crystallized in the presence of substrate analog or covalently bound to a serine-specific inhibitor show conformational rearrangements which include movement of the lid giving free access to the catalytic site (13–17). However, structural and catalytic studies with cutinase from *Fusarium solani* (18) and with lipase from guinea pig pancreas which contain a mini-lid structure have challenged the classical distinction between lipases and nonspecific esterases and called for a reassessment of the structure–function relationships of triacylglycerol-hydrolyzing enzymes with respect to interfacial activation, substrate specificity, and conformational changes (19–22).

Lipases also have received a great deal of interest because of the potential applications of these enzymes in many industrial domains. The molecular and biochemical properties of lipases isolated from a large variety of microorganisms, including fungi of the *Penicillium* species such as *P. expansum* (23), *P. solitum* (P. sp. UZLMA-4) (24,25), *P. simplicissimum* (26), *P. camembertii* (27), *P. citrinum* (28,29), and *P. crustosum* (30) have been reported. The primary structure of *P. camembertii* lipase has been established (31). In the case of *P. cyclopium*, several reports indicate that this fungus produces various types of lipolytic enzymes with preference for triacylglycerol or partial acylglycerol, respectively (32–35).

In this report, we describe the biochemical characterization of an extracellular lipase produced by *P. cyclopium* which specifically hydrolyzes triacylglycerol in the alkaline pH range. A comparison between *P. cyclopium* triacylglycerol lipase and other *Penicillium* lipases based on molecular mass, substrate specificity, and sequence of the first 20 amino acid residues is presented in this communication.

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Abbreviations: IEF, isoelectric focusing; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Microorganism and cultivation. The *Penicillium* strain was isolated from walnut in our laboratory and identified as *P. cy-*

clopium. The strain was maintained in Sabouraud medium (Merck, Nogent-sur-Marne, France) and produced extracellular lipolytic activity. Lipase production was estimated with the tributyrin assay each day during cultivation. The medium used for highest lipase production consisted of 2% corn steep (Roquette, Lille, France) with 1% commercial olive oil. This medium was adjusted to pH 6 with NaOH and sterilized at 120°C for 20 min. Cultivation was carried out at the constant temperature of 25°C, without shaking, in 1-L Erlenmeyer flasks containing 100 mL of medium to which was added 1 mL of spore suspension containing around 10^2 spores. After 5 d, lipase activity sharply increased fourfold and, 2 d later, the mycelia were separated by filtration. The culture filtrate was used as enzyme source for lipase purification.

Assay of lipase activity. Lipase activity was determined titrimetrically with the pH-stat method by using an emulsion of tributyrin (tributanoylglycerol), trioctanoin (trioctanoylglycerol) purchased from Fluka (Saint-Quentin-Fallavier, France), or commercial olive oil. All triacylglycerols were previously chromatographed on a silica gel column (2 × 30 cm) developed in hexane/diethyl ether (95:5, vol/vol). Monooctanoin and dioctanoin were separated chromatographically from a mixture of acylglycerols obtained by incomplete hydrolysis of trioctanoin by *Candida cylindracea* lipase (Meite Sangyo, Nagoya, Japan) as follows: 10 g of trioctanoin was mixed with 400 mg of crude enzyme suspended in 60 mL of 2.5 mM phosphate buffer pH 7 and incubated at 30°C. After 2 h, the pH was raised to 10 with NaOH, and the reaction mixture was extracted twice with 60 mL of diethyl ether. The extract was evaporated and analyzed by thin-layer chromatography (TLC) and reversed-phase liquid chromatography (RP-HPLC). TLC was performed on silica gel plates (20 × 10 cm). The hexane/diethyl ether/acetic acid mixture (80:20:1, by vol) was used for plate development. Acylglycerols were visualized by spraying with 9 M H_2SO_4 followed by heating at 120°C. Analytical RP-HPLC of the acylglycerol mixture was performed on a Shimadzu Liquid Chromatography System (Shimadzu, Kyoto, Japan) equipped with a C18 Beckman column (0.4 cm internal diameter × 25 cm). The solvent used for elution of the column was the acetone/acetonitrile mixture (60:40, vol/vol). Under the conditions used for triacylglycerol hydrolysis, the relative proportions of monooctanoin, dioctanoin, and trioctanoin in the acylglycerol mixture were 12:38:50, respectively. Acylglycerols were separated by chromatography on a silica gel column (2 × 30 cm). Trioctanoin and dioctanoin were recovered from the column with 500 mL of the hexane/diethyl ether mixture (95:5 and 50:50, vol/vol, respectively). Monooctanoin was eluted by washing the column with 500 mL of diethyl ether. Purity of each acylglycerol fraction was checked by TLC and HPLC. Other substrates, namely, methyl butyrate and methyl propionate, were purchased from Fluka and used without further purification.

Lipase activity against tributyrin was determined at 30°C and pH 8 as follows: 0.2 mL of tributyrin was suspended under stirring in 20 mL of 2.5 mM Tris buffer pH 8 with 100 mM NaCl. Kinetics of hydrolysis were zero-order within the first 5 min of the reaction. The rate of the reaction was measured at constant pH by adding 20 mM NaOH. Activity against long-chain triacylglycerol (olive oil) was determined at pH 9 with 20 mL of substrate emulsion prepared in a blender (Sofraca Turmix, Vitrolles, France) by mixing 40 mL of olive oil with 400 mL of a 10% solution of gum acacia in distilled water. No bile salt was added. Finally, activity against monooctanoin, dioctanoin, or trioctanoin was determined under the same conditions as above but with 25, 50, and 200 µL of substrate, respectively. Assays were performed in duplicate and averaged. In all cases, results of duplicate assays differed by less than 5%. Activity was expressed as lipase units. One lipase unit corresponds to the release of one microequivalent of fatty acid per minute under standard assay conditions.

Determination of protein concentration. Protein concentration in culture filtrate and lipase samples was estimated with the colorimetric method of Lowry *et al.* (36) using serum albumin as standard protein. In chromatography experiments, the protein content of fractions was routinely estimated by measuring absorbance at 280 nm.

Purification. After 1 wk cultivation of *P. cyclopium*, the culture broth from eight flasks was separated from the mycelia by filtration. The average lipolytic activity of the filtrate (650 mL), determined with the standard tributyrin assay, was 210 units per mL. No lipase was released by washing mycelia with distilled water after filtration. Detergent that might interfere in the following purification steps was not used during extraction. Proteins of the ice-cold filtrate were precipitated with solid ammonium sulfate at 80% saturation. Insoluble proteins were collected by centrifugation and resuspended in a minimal volume of 25 mM Tris buffer pH 7.5. The protein solution (30 mL) was placed on a G 75 Sephadex column (Pharmacia Biotech, Saint-Quentin-Fallavier, France) (2.5 × 100 cm) in the same Tris

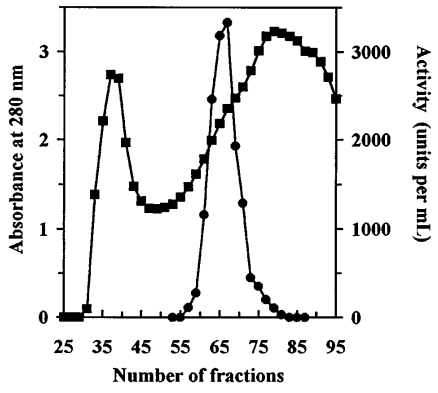

FIG. 1. Gel filtration of a crude sample of *Penicillium cyclopium* lipase. Proteins of the culture broth were separated by precipitation with ammonium sulfate and filtered through a Sephadex G 75 column (2.5 × 100 cm) in 25 mM Tris buffer pH 7.5 at 4°C . Flow rate: 15 mL per hour. Fraction volume: 3 mL. Void volume (V_o) of the column: 93 mL. Lipase-containing fractions (59 to 74) were pooled for further separation by ion-exchange chromatography. ■: Absorbance at 280 nm; ●: lipase activity against tributyrin (units per mL).

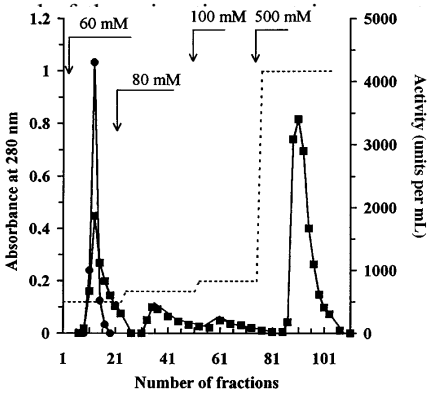

FIG. 2. Ion-exchange chromatography of *P. cyclopium* lipase. Lipase fractions from the Sephadex G 75 column were pooled and placed on a DEAE-Sephadex column (2 × 10 cm) equilibrated at 4°C in 25 mM Tris buffer at pH 7.5. The column was washed with 100 mL of buffer to separate nonadsorbed proteins. Stepwise elution was carried out by successive passage of 100 mL of NaCl solution in buffer at the concentration of 60, 80, 100 and 500 mM, respectively. Flow rate: 60 mL per hour. Fraction volume: 5 mL. Dotted line: stepwise NaCl gradient. See Figure 1 for abbreviation and symbols.

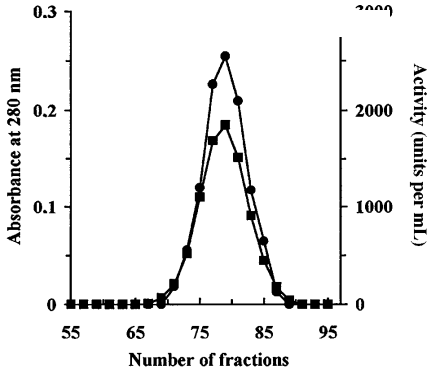

FIG. 3. Gel filtration of partially purified *P. cyclopium* lipase. The enzyme sample was a pool of lipase fractions collected from the DEAE-Sephadex column and concentrated by ultrafiltration. Chromatography was performed on a column (2.5 × 100 cm) of Sephadex G 75 in 25 mM Tris buffer at pH 7.5 with 100 mM NaCl. Flow rate: 15 mL per hour. Fraction volume: 2.3 mL. Fractions 75 to 85 were pooled, concentrated to a final volume of 5 mL by ultrafiltration, and stored at –20°C. See Figure 1 for abbreviation and symbols.

mined on 10 µL aliquots after 15 and 60 min incubation. The of *P. cyclopium* lipase by RP-HPLC (Fig. 5) shows one major buffer at 4°C. From the elution diagram (Fig. 1), it appears that all lipase activity against tributyrin emerged in one peak eluted with 1.9 void volume. Lipase-containing fractions were pooled and submitted to ion-exchange chromatography on a DEAE-Sephadex column (Pharmacia Biotech) (2 × 10 cm) at 4°C in the same Tris buffer. The column was washed with 100 mL of buffer, and adsorbed proteins were eluted by stepwise increase of NaCl concentration in Tris buffer. All lipase recovered from the column was eluted with 60 mM NaCl (Fig. 2). Lipase fractions were pooled, concentrated to 5 mL by ultrafiltration (Amicon membrane PM_{10}; Millipore S.A., Saint-Quentin-en Yvelines, France), and filtered through a Sephadex G 75 column (2.5 × 100 cm) at 4°C in 25 mM Tris buffer with 100 mM NaCl (Fig. 3). Specific activity of lipase and enzyme recovery at each step of purification are shown in Table 1. Purified lipase was concentrated to 3 mL by ultrafiltration and kept at –20°C for months without loss of activity.

protein eluted with acetonitrile/water (55:45, vol/vol) to-

Gel electrophoresis and isoelectric focusing (IEF). Homogeneity of the purified enzyme was checked using 12% polyacrylamide gel electrophoresis with 1% SDS (SDS-PAGE). IEF was performed in the pH range 3–10. In both cases, proteins were detected with the silver staining method. The SDS-PAGE gel was also stained with Coomassie Blue. The molecular mass of *P. cylopium* lipase was estimated by SDS-PAGE and by gel filtration on a G 75 Sephadex column (2.5 × 100 cm) carried out in 25 mM Tris buffer pH 7.5 with 100 mL NaCl.

Analytical RP-HPLC. The purified lipase preparation was analyzed under conditions previously described (37). Elution was achieved with a linear gradient (total volume: 70 mL) from 2 to 72% acetonitrile in 0.1% trifluoroacetic acid in water at a flow rate of 1 mL per min. Proteins were detected at 230 nm.

Carbohydrate detection, amino acid composition, and amino terminal sequence determination. The method of Zacharius *et al.* (38) was used to detect carbohydrate in the purified enzyme. A sample of lipase for amino acid analysis was prepared as follows: *P. cyclopium* lipase (15 µg) was submitted to SDS-PAGE electrophoresis as described above. At

TABLE 1
Purification of *Penicillium cyclopium* Triacylglycerol Lipase

Step	Total protein (mg)	Total activity[a] (units)	Specific activity (units mg^{-1})	Yield (%)	Purification (fold)
Culture filtrate	1592	136,500	86	100	1
Ammonium sulfate	536	96,900	180	71	2
Sephadex G 75	59	87,200	1,478	63	17
DEAE-Sephadex	15	54,000	3,600	39	41
Sephadex G 75	6	48,000	8,000	35	93

[a]Activity determined against tributyrin.

gether with minor contaminants absorbing at 230 nm. The acetonitrile concentration required for lipase elution is indicative of the strong affinity of *P. cyclopium* lipase for the hydrophobic phase. Actually, under the same chromatographic conditions, *M. miehei* lipase is eluted with 40% acetonitrile.

The molecular mass of *P. cyclopium* lipase was estimated to be around 28 kDa by SDS-PAGE (Fig. 4, diagrams 1 and 2) and 29 kDa by gel filtration (not shown). No carbohydrate was detected in the protein. The amino acid composition of *P. cyclopium* lipase is reported in Table 2 together with other *Penicillium* lipases for the sake of comparison. Values in Table 2 show large similarities in amino acid composition of *P. cyclopium* and *P. expansum* lipases, in particular with respect to the content of hydrophobic residues. As shown by the curves of Figure 6, purified *P. cyclopium* lipase is stable at 30°C and pH 8 and loses about 10% activity after 1 h incubation at 35°C at the same pH. The stability of the enzyme decreases markedly at 40°C. Activity is fully lost after 10-min incubation at 45°C.

The pH activity dependence curve (Fig. 7) indicates that the activity of the enzyme sharply increases from pH 5 to pH 8 and remains constant from pH 8 to pH 10.

The activity of *P. cyclopium* lipase against tributyrin (Fig. 8A) and olive oil (Fig. 8B) was determined in the presence of increasing concentrations of sodium taurodeoxycholate, sodium taurocholate, or SDS. It appears from the curves that lipase activity is not affected by sodium taurodeoxycholate at concentrations below 8 mM and that the inhibitory effect of sodium taurocholate or SDS is dependent on the triacylglycerol used as substrate, as observed earlier in the case of pancreatic lipase (39,40). Moreover, hydrolysis of emulsified tributyrin or olive oil was fully inhibited by Triton X-100 (Fig. 9) and by other detergents such as Brij 35, Tween 20, or Tween 80 (not shown).

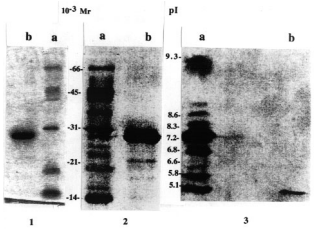

FIG. 4. Analytical gel electrophoresis and isoelectric focusing of *P. cyclopium* lipase. (1) and (2): SDS-polyacrylamide gel electrophoresis. Purified lipase (a) and reference proteins (b) were detected with Coomassie Blue (1) or with the silver staining method (2). Both analyses were performed with 5 µg of protein. (3): Isoelectric focusing. Proteins were detected as in (2). See Figure 1 for abbreviation.

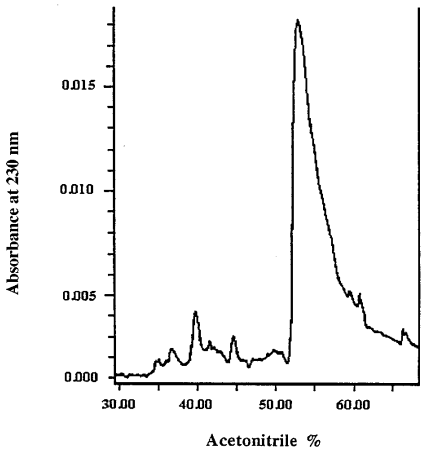

FIG. 5. Analytical reversed-phase liquid chromatography of purified *P. cyclopium* lipase. A sample of purified lipase (30 µg in 50 µL) was chromatographed at room temperature. Elution was achieved with a linear gradient from 2 to 72% acetonitrile in 0.1% trifluoroacetic acid in water (total volume: 70 mL) at a flow rate of 1 mL per min. No protein was eluted at concentration of acetonitrile below 30%. Ordinates: absorbance at 230 nm. Abscissae: percentage of acetonitrile.

Substrate specificity. Rates of hydrolysis of tributyrin, trioctanoin, olive oil, monooctanoin, dioctanoin, methyl propionate, and methyl butyrate by purified *P. cyclopium* lipase are listed in Table 3. It can be observed that the enzyme is highly active against all triacylglycerols and shows very low activity against partial acylglycerols or monoesters. Further, the specific activity of *P. cyclopium* lipase, measured against tributyrin (8000 units per mg) and medium- or long-chain triacylglycerols, is in the same range as that of mammalian pancreatic lipases.

Several groups (32–35) reported that three extracellular lipolytic enzymes are produced by *P. cyclopium*. Amounts of each of the enzymes depend upon cultivation conditions of the mold. So far, these lipases have been identified and characterized only by their specific activity against various ester substrates. Lipase I and Lipase II are proteins with molecular mass around 30 kDa and act specifically on triacylglycerols and partial acylglycerols, respectively. Lipase III hydrolyzes *p*-nitrophenyl laurate and rapidly releases glycerol from triacylglycerols in serum in the presence of Triton X-100. Lipase III is a dimer of molecular mass of 108 kDa. Our results show that lipase isolated from the cuture medium is identical with Lipase I.

For the first time, we have determined the amino terminal sequence of *P. cyclopium* lipase over 20 amino acid residues. This sequence is presented in Table 4 together with the corresponding sequence of other *Penicillium* lipases and lipases from *M. miehei* (41) and *Rhizopus delemar* (42). This offers

TABLE 2
Amino Acid Composition of *P. cyclopium* Triacylglycerol Lipase. Comparison with Other *Penicillium* Lipases

	Amino acid composition (mol%)			
Amino acid	*P. cyclopium*	*P. expansum*	*P. simplicissimum*	*P. camembertii*
Gly	13.2	10.3	15.3	8.9
Ser	9.2	7.1	7.5	7.6
Asp + Asn	10.1	11.0	9.3	11.4
Glu + Gln	7.5	8.3	9.5	7.1
Lys	4.5	4.9	6.6	4.4
Arg	3.1	4.1	6.0	2.7
Tyr	4.3	3.3	2.7	5.6
His	2.3	2.8	5.4	2.4
Trp	n.d.[a]	n.d.[a]	1.4	2.7
Ala	9.9	11.2	9.8	11.4
Thr	7.6	9.0	5.8	8.0
Pro	3.6	4.1	6.7	5.7
Cys	n.d.[a]	n.d.[a]	2.2	3.1
Val	6.7	6.8	6.1	9.4
Ile	5.5	5.3	1.5	3.0
Leu	6.9	7.4	2.5	7.9
Met	1.5	0.5	1.7	0.2
Phe	4.1	3.9	3.6	4.4

[a]n.d., Not determined.

the opportunity to compare the triacylglycerol-hydrolyzing lipase produced by *P. cyclopium* with lipases previously isolated from other *Penicillium* species, on a structural basis. From the alignment of the sequences, *P. cyclopium* lipase is homologous to lipases from *P. expansum* and *P. solitum* (Table 4), with 15 out of the 20 residues at the *N*-terminus conserved in the three proteins. Conversely, it may be noticed that only three residues of alanine of the amino terminal region of these three enzymes are conserved in *P. simplicissimum* lipase, at positions 1, 3, and 14. On the other hand, only two residues of the amino terminal sequence of *P. camembertii* lipase, namely, valine at position 2 and serine at position 5, are common with *P. expansum* lipase. *Penicillium camembertii* lipase shows no homology with lipases from *P. cyclopium* or *P. solitum*. Moreover, lipases from *P. cyclopium*, *P. expansum,* and *P. solitum* have a molecular mass in the 25–35 kDa range and comparable substrate specificity, with preference for tributyrin and olive oil, with a tributyrin/olive oil rate ratio of 2. Instead, lipase from *P. simplicissimum* has been characterized as nonspecific with the capacity to hydrolyze each of the three ester bonds of triolein (26), and lipase from *P. camembertii* is specific for monoacylglycerols and diacylglycerols and has no activity on triacylglycerols (27). Then, from the comparison of the molecular and biochemical properties and amino-terminal amino

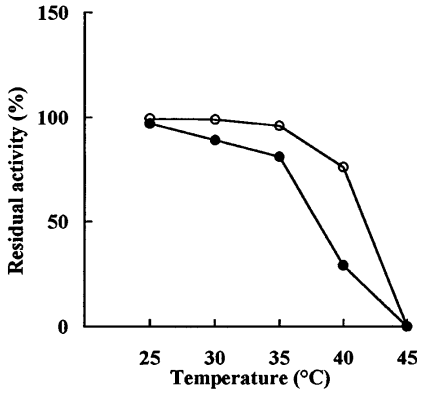

FIG. 6. Thermal stability of *P. cyclopium* lipase. The purified enzyme (0.1 mg per mL in 2.5 mM Tris buffer pH 8 with 100 mM NaCl) was incubated at 25, 30, 35, 40 and 45°C, and residual activity was measured on 10 µL after 15 and 60 min with the standard tributyrin assay. ○: Residual activity after 15 min incubation; ●: residual activity after 60-min incubation. Residual activity is expressed as percentage of initial activity. See Figure 1 for abbreviation.

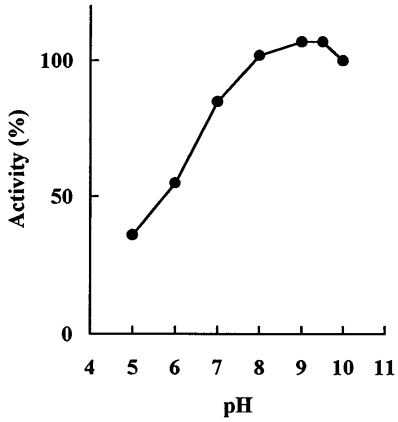

FIG. 7. Effect of pH on *P. cyclopium* lipase activity. Hydrolysis of triacylglycerol substrate (emulsified tributyrin) was measured potentiometrically under standard conditions, at constant pH ranging from pH 5 to pH 10. Correction was made for butanoic acid ionization at pH below 6 and for nonenzymatic hydrolysis of tributyrin at pH above 8. Activity is expressed as percentage of hydrolysis at pH 8 (100%). See Figure 1 for abbreviation.

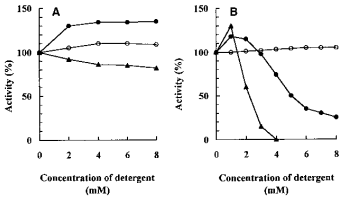

FIG. 8. Effect of increasing concentration of bile salt and SDS on *P. cyclopium* lipase activity against triacylglycerol. Residual activity is expressed as percentage of the initial rate of hydrolysis (100%) of tributyrin (curves A) and olive oil (curves B) determined at pH 8 and 9, respectively, in absence of detergent. ●: Sodium taurocholate; ○: sodium taurodeoxycholate; ▲: SDS. See Figure 1 for abbreviation.

acid sequence, it appears that triacylglycerol lipases (Lipase I) from *P. cyclopium*, *P. solitum*, and *P. expansum* belong to the same lipase gene family. Since no structural data are presently available for *P. cyclopium* lipase II, a comparison between Lipase I and Lipase II is not yet possible. From previous reports (32–35) and from our experience (data not shown), it appears that the relative amounts of lipases of types I and II produced by *P. cyclopium* are dependent upon the cultivation conditions. In this respect, notice can be made that the production by *P. camembertii* of an extracellular triacylglycerol-hydrolyzing enzyme has never been reported. More work then remains to be done to know if all fungi of the *Penicillium* species can produce both types of lipases and how production of these enzymes is regulated at the gene level.

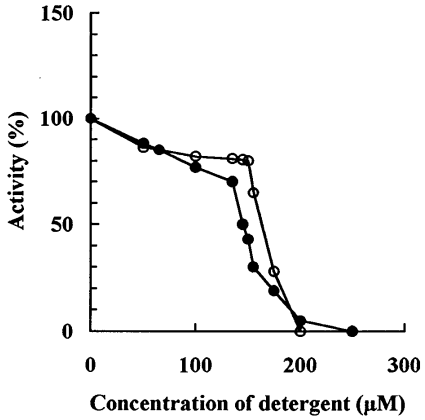

FIG. 9. Effect of increasing concentration of Triton × 100 on the activity of *P. cyclopium* lipase against emulsified triacylglycerol. Residual activity against tributyrin (●) and olive oil (○) is expressed as in Figure 8. See Figure 1 for abbreviation.

TABLE 3
Substrate Specificity of *Penicillium cyclopium* Triacylglycerol Lipase[a]

Tributyrin	100	Dioctanoin	6.5	Methyl propionate	0
Trioctanoin	53	Monooctanoin	1	Methyl butyrate	1
Olive oil	46				

[a]Activities are expressed as per cent of activity determined against tributyrin (100%) under standard assay conditions. All assays are performed at 30°C and pH 8 except for hydrolysis of olive oil performed at pH 9.

TABLE 4
Comparison of the *N*-Terminal Sequence of *Penicillium cyclopium* Triacylglycerol Lipase with other *Penicillium* Lipases and Lipases from *Rhizopus delemar* and *Mucor miehei*

		Reference
P. cyclopium	A T A D A A A F P D L H R A A K L S S A	(this work)
P. expansum	A V A A S A A F P D L X R A A K L S S A	16
P. solitum (sp. UZLM-4)	A T A A T A A F P D L N X A A K L S S A	18
P. simplicissimum	A I A P C V V L S K P Q A A V I G	19
P. camembertii	D V S T S E L D Q F E F W V Q Y A A A S	34
M. miehei	S I D G G I R A A T S Q E I N E L T Y Y	35
R. delemar	S D G G K V V A A T T A Q I Q E F T K Y	36

ACKNOWLEDGMENTS

The authors thank Dr. G. Vanot, Dr. J. Bonicel, and Dr. V. Desseaux for useful assistance and advice in part of the experiments. This work was carried out, in part, with the financial support of the BRIDGE-T-Lipase program of the European Community. One of us (A.I.) is grateful to the Hariri Foundation for a grant.

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[Received May 5, 1997, and in final revised form and accepted March 12, 1998]

Enhanced Macrophage Uptake of Lipoprotein(a) After Ca^{2+}-Induced Aggregate-Formation

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ABSTRACT: We tested the hypothesis that aggregated lipoprotein(a) [Lp(a)] is avidly taken up by macrophages. Lp(a) was isolated by sequential centrifugations and gel chromatography from a patient with high plasma levels of Lp(a) who was being treated with low density lipoprotein (LDL)-apheresis. Aggregated Lp(a) was prepared by mixing native Lp(a) with 2.5 mmol/L $CaCl_2$, and 54% of the ^{125}I-Lp(a) aggregated after interacting with $CaCl_2$. The binding and degradation of aggregated Lp(a) in macrophages were 4.6- and 4.7-fold higher than those of native Lp(a), respectively. An excess amount of LDL did not inhibit either increase. Cholesterol esterification in macrophages was markedly stimulated by aggregated Lp(a), and macrophages were transformed into foam cells. Cytochalasin B, a phagocytosis inhibitor, strongly inhibited the degradation and cholesterol esterification (78 and 83%, respectively). These findings suggested that aggregation may be partially involved in Lp(a) accumulation, thereby contributing to the acceleration of atherosclerosis.

Lipids 33, 385–392 (1998).

Lipoprotein(a) [Lp(a)] is now recognized as a strong independent risk factor for coronary artery disease (CAD). This unique lipoprotein consists of low density lipoprotein (LDL)-like particles containing apolipoprotein B-100 and highly negatively charged apolipoprotein(a) [apo(a)] (1). The apo(a) moiety is formed by three different structural domains having high homology with kringle 4, 5, and the protease domain of plasminogen (2). The kringle 4 domain in apo(a) is present in a sequence consisting of various numbers of multiple copies, thereby giving rise to different sizes of apo(a) isoform and consequently of Lp(a) (3,4). In subjects with high plasma levels of Lp(a), apo(a) isoforms of smaller size or smaller number of kringle 4 domains is commonly observed (5–7).

Histochemical studies suggest that Lp(a) contributes to the occurrence of CAD by accelerating atherosclerotic lesions through accumulation in the arterial wall (8–10). The observation of colocalization of Lp(a) with cholesteryl ester-rich cells (foam cells) in atherosclerotic lesions strongly supports this notion. Despite these data, previous *in vitro* studies were unable to demonstrate significant internalization and degradation of native Lp(a) by cultured macrophages (11–13). Unlike other lipoproteins, Lp(a) possesses a unique property of self-aggregation under *in vitro* condition. In addition, Hoff *et al*. (14) showed that in human atherosclerotic lesions apo(a) is present in some possibly aggregated forms. These findings suggest a possibility that aggregation of Lp(a) is related to the mechanism of foam cell formation.

In the present study, to address this issue, we tested macrophage uptake of Ca^{2+}-induced Lp(a) aggregates, a model of self-aggregation of Lp(a) (15).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) without $CaCl_2$ was obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (BSA) was purchased from Intergen Company (Purchase, NY). Fetal bovine serum (FBS) was obtained from Cansera International Inc. (Rexdale, Ontario, Canada). Sephacryl 400 HR, premade 4–15% gradient polyacrylamide Phast Gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a silver stain kit for PAGE were obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Na^{125}I (100 mCi/mL of iodide) and [9,10(n)-^{3}H]oleic acid (370 GBq/mmol) were purchased from Amersham International plc. (Bucks, United Kingdom). Silica gel on aluminum sheets for thin-layer chromatography was obtained from Merck (Darmstadt, Germany). An apo(a) phenotype analyzing kit was purchased from Sanwa Chem. Co. (Tokyo, Japan).

Isolation of mouse peritoneal macrophages. Macrophages were elicited by intraperitoneal injection of 2 mL of thioglycolate medium 4 d before harvesting. The macrophages were plated in 12-well tissue culture plates (area/well = 4.5 cm^2) at a density of 1.0×10^6 cells in 1 mL of DMEM containing FBS (20%), streptomycin (0.1 mg/mL), and penicillin (100 U/mL). Plated macrophages were then incubated for 2–3 h at 37°C in a humidified-air incubator (95% air, 5% CO_2). After incuba-

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Abbreviations: apo(a), apolipoprotein(a); BSA, bovine serum albumin; CAD, coronary artery disease; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LDL, low density lipoprotein; Lp(a), lipoprotein(a); PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy.

tion, they were washed twice with serum-free DMEM to remove any contaminating lymphocytes and platelets. Before the cellular assay, the macrophages were incubated for 24 h at 37°C in 1 mL of DMEM containing lipoprotein-deficient serum (5 mg/mL), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (medium A).

Preparation and iodination of lipoproteins. LDL (d = 1.020 to 1.050) was isolated by preparative ultracentrifugations from fresh plasma of a healthy young male (16). These were dialyzed against 0.15 mol/L NaCl (pH 7.4) containing Na_2EDTA (0.3 mmol/L) followed by sterilization using a 0.45 μm-filter and stored at 4°C. Lp(a) was isolated from a patient who was being treated with LDL-apheresis. The Lp(a) which adsorbed to the apheresis column was eluted with NaCl solution (0.5 mol/L) and collected in the presence of Na_2EDTA (1 mg/mL) and aprotinin (100 kallikrein-inactivating units/mL) immediately after LDL-apheresis. The solution containing Lp(a) was subjected to sequential ultracentrifugations to isolate the density fraction of $1.050 < d < 1.120$ g/mL. To separate Lp(a) from high density lipoprotein and LDL, this density fraction was filtered on Sephacryl 400 HR (2.5 × 90 cm) at a flow rate of 120 mL/h with 1 mol/L NaCl and 5 mmol/L Tris-HCl (pH 7.4) containing Na_2EDTA (1 mg/mL) and NaN_3 (0.01%) (buffer A). For the present study, only Lp(a) from a 63-year-old female patient with high levels of plasma Lp(a) (127 mg/dL) was used unless mentioned otherwise. The purity of Lp(a) was monitored by SDS-PAGE under a nonreduced condition. Isolated Lp(a) was stored at 4°C in buffer A and was extensively dialyzed against 0.15 mol/L NaCl and 5 mmol/L Tris-HCl (pH 7.4) containing 0.3 mmol/L Na_2EDTA before iodination. One day before the experiments were performed, isolated Lp(a) was labeled with $Na^{125}I$ using the iodine monochloride procedure of MacFarlane (17) as modified by Bilheimer *et al*. (18). Free iodine was removed by subsequent dialysis against 200-fold volumes of calcium-free DMEM three times. The specific activities of labeled Lp(a) were 400 to 700 cpm/ng of protein.

Preparation of aggregated Lp(a). Aggregated Lp(a) was made by mixing 1 mL of native Lp(a) with 2.5 mmol/L $CaCl_2$ in calcium-free DMEM according to the method of Yashiro *et al*. (15). To determine the extent of Lp(a) aggregation, ^{125}I-Lp(a) mixed with 2.5 mmol/L $CaCl_2$ in calcium-free DMEM was pipetted into 1.5 mL microtubes and centrifuged immediately at 10,000 rpm for 10 min. The percentage of ^{125}I-Lp(a) which precipitated was determined by counting the radioactivity in the supernatant before and after mixing with $CaCl_2$. Fifty-four percent of the ^{125}I-Lp(a) aggregated under this condition. In our experiments a mixture of aggregates and remaining soluble material was provided for cells, because the precipitated Lp(a) aggregates were difficult to resuspend in DMEM.

Evaluation of aggregated Lp(a) morphology. The size and degree of aggregated Lp(a) after mixing native Lp(a) with 2.5 mmol/L $CaCl_2$ in calcium-free DMEM were examined using transmission electron microscopy (TEM) after negative staining with 2% Na-phosphotungstate (19). The area and diameter profile of aggregates were quantified using NIH Image Version 1.52 (Wayne Rasband, National Institutes of Health, Research Service Branch, NIMH, Washington, D.C.). These results were expressed as the mean ± SD.

Binding of ^{125}I-Lp(a). The binding assays of lipoproteins with macrophages were carried out at 4°C essentially as described by Innerarity *et al.* (20). Macrophage monolayers were washed once with serum-free DMEM, then twice with calcium-free DMEM containing BSA (0.2%), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (medium B), and cooled to 4°C with medium B. The medium was then removed, and 1 mL of medium B containing indicated concentrations of native or aggregated ^{125}I-Lp(a) with or without a 20-fold excess of corresponding unlabeled Lp(a) was added. After incubation at 4°C for 4 h, the cells were washed four times with 1 mL/well of ice-cold phosphate-buffered saline (PBS) containing 0.2% BSA and once with 1 mL/well of ice-cold PBS; the first wash was allowed to sit 10 min before proceeding with the remaining three washes in rapid succession as described by Haberland *et al*. (21). Then, after washing them, each cell sheet was lysed with 0.5 mL of 0.1 N NaOH, and the radioactivity was measured. An aliquot of the lysate was removed to determine cellular protein by the method of Lowry *et al*. (22).

Degradation of ^{125}I-Lp(a). The degradation experiments were performed essentially as described by Goldstein *et al.* (23). Macrophage monolayers were washed once with serum-free DMEM then twice with medium B. Medium B containing indicated concentrations of native or aggregated ^{125}I-Lp(a) with or without a 20-fold excess of the corresponding unlabeled Lp(a) was added to the cells. Cells were then returned to the 37°C incubator for 5 h. Proteolytic degradation of ^{125}I-Lp(a) was measured by assaying the amount of ^{125}I-labeled trichloroacetic acid-soluble (noniodide) material formed by the cells and excreted into the medium. Each cell sheet was lysed after washing, and protein content was measured as described for the binding assays.

Incorporation of [^{3}H]oleate into cholesteryl esters. To assay cholesterol esterification in macrophages, the incorporation of [^{3}H]oleate into cellular cholesteryl [^{3}H]oleate was determined as described by Miyazaki *et al*. (24). The [^{3}H]oleate was conjugated with BSA as described by Brown *et al*. (25). Macrophage monolayers were washed once with serum-free DMEM then twice with medium B. Each well was incubated with 0.1 mmol/L of [^{3}H]oleate (10,000 dpm/nmol) in the presence of indicated concentrations of aggregated Lp(a) in 1.0 mL of medium B. After a 5-h incubation, cells were washed five times with 1.0 mL of ice-cold PBS and extracted twice with 0.5 mL of hexane/isopropanol (3:2, vol/vol). The extract was evaporated to dryness, resuspended with 200 μL of isopropanol, spotted on a silica gel thin-layer plate, and developed in hexane/diethyl ether/methanol/acetic acid (85:20:1:1, by vol). A band corresponding to cholesteryl oleate was cut out, and the radioactivities were measured. Each cell sheet was lysed after washing, and protein was mea-

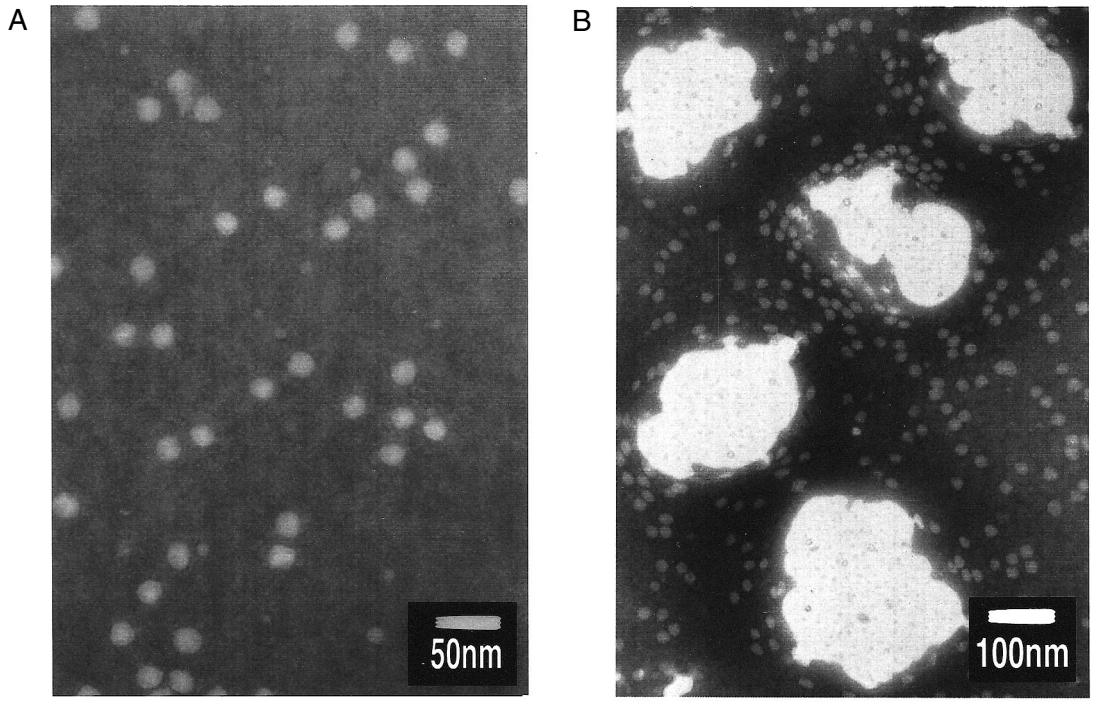

FIG. 1. Morphology of native and aggregated lipoprotein(a) [Lp(a)]. Aggregated Lp(a) was made by mixing native Lp(a) with 2.5 mmol/L $CaCl_2$ in calcium-free Dulbecco's modified Eagle's medium. Samples of native Lp(a) (A) and aggregated Lp(a) (B) were examined by transmission electron microscopy after negative staining with 2% Na-phosphotungstate.

sured as described for the binding assays. All assays were performed in triplicate.

Oil Red O staining. Macrophages (5×10^5 cells) were plated on 11-mm circular coverslips within a 12-well tissue culture plate. Each coverslip received 1.0 mL of medium A and was incubated at 37°C for 24 h. Then 1.0 mL of medium B containing 100 µg protein/mL of native or aggregated Lp(a) was added to the cells. After incubation at 37°C for 15 h, the cells were stained with Oil Red O, followed by counter-staining with Mayer's hematoxylin, as described by Brown *et al.* (25). The coverslips were mounted face-down on glass microscope slides using pre-warmed, liquefied 20% glycerol. The stained cells were photographed with a Nikon photomicroscope (Nippon Kogaku K.K., Tokyo, Japan) at 250× magnification.

Apo(a) isoform phenotype determinations. Apo(a) isoform phenotyping was performed with SDS-PAGE of plasma from peripheral venous blood under reducing conditions followed by immunoblotting using a commercially available apo(a) isoform analyzing kit (Sanwa Chem. Co.). This analysis basically follows the method of Abe and Noma (26). Using the terminology proposed by Utermann *et al.* (5), the various isoforms of apo(a) have been designated according to the electrophoretic mobility relative to that of apolipoprotein B-100.

Lipid analysis. Plasma Lp(a) was quantified using the latex-enhanced turbidimetric immunoassay by the method described by Abe *et al.* (27). Total cholesterol, free cholesterol, phospholipids, and triglycerides were determined using standard enzymatic kits (28). Protein was measured by the method of Lowry *et al.* (22).

RESULTS

Morphology of aggregated Lp(a). To assess the morphology of aggregated Lp(a), we subjected native Lp(a) and these aggregates to TEM after negative staining. As shown in Figure 1, native Lp(a) particles after isolation are monomeric and did not coalesce. In contrast, aggregated Lp(a) consisted of numerous monomeric particles. The mean profile area and diameter of Lp(a) aggregates were $3.7 \pm 1.2 \times 10^4$ nm^2 and 255.0 ± 9.4 nm, respectively.

Concentration dependency of binding and degradation of Lp(a). Figures 2A and 2B illustrate the binding of native and aggregated ^{125}I-Lp(a) to macrophages held at 4°C. Specific binding for native ^{125}I-Lp(a) reached saturation, and 0.49 µg protein of ^{125}I-Lp(a) was bound per mg cell protein when 200 µg/mL lipoprotein was added. In contrast, for aggregated ^{125}I-Lp(a), specific binding was markedly enhanced compared with that for native ^{125}I-Lp(a), and there was a greater than 4.6-fold increase in binding when 200 µg/mL lipoprotein was added [2.27 µg protein/mg cell protein for aggregated Lp(a)].

Figures 2C and 2D illustrate the degradation of native and aggregated ^{125}I-Lp(a) in macrophages at 37°C. Reflecting enhanced binding, specific degradation for aggregated

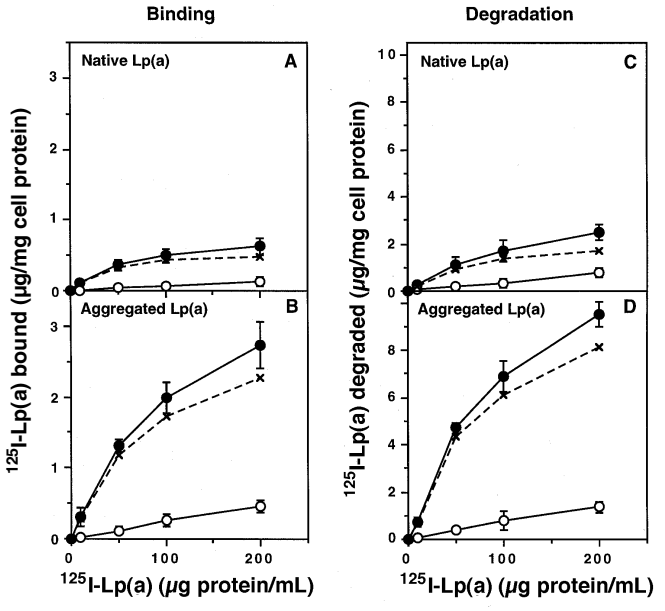

FIG. 2. Concentration dependency of the binding and degradation of native or aggregated Lp(a). Macrophage monolayers were incubated with the indicated concentrations of native or aggregated ^{125}I-Lp(a) with or without a 20-fold molar excess of the corresponding unlabeled Lp(a), respectively. Cellular binding or degradation was analyzed after incubation at 4°C for 4 h or at 37°C for 5 h, respectively. Specific binding or degradation (x) of native or aggregated ^{125}I-Lp(a) was defined as total binding or degradation (●) minus nonspecific binding or degradation (○) seen in the presence of the corresponding unlabeled Lp(a). Each data point represents the mean value of three experiments with the indicated SD. See Figure 1 for abbreviation.

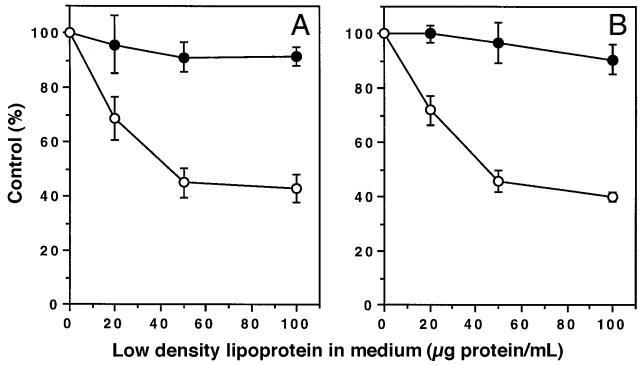

FIG. 3. Ability of unlabeled native low density lipoprotein (LDL) to compete for the binding or the degradation of native or aggregated ^{125}I-Lp(a). Macrophage monolayers were incubated with 5 μg protein/mL of native or aggregated ^{125}I-Lp(a) and the indicated concentrations of native LDL. Cellular binding or degradation was analyzed after incubation at 4°C for 4 h or at 37°C for 5 h, respectively. The results were expressed as percentages of the (A) binding or (B) degradation of native or aggregated ^{125}I-Lp(a) in the absence of native LDL. In the binding study, the 100% values for native and aggregated ^{125}I-Lp(a) were 0.10 and 0.32 μg protein/mg cell protein, respectively. In the degradation study, the 100% values for native and aggregated ^{125}I-Lp(a) were 0.18 and 0.47 μg protein/mg cell protein, respectively; ○, represent the total binding and degradation of native ^{125}I-Lp(a); ●, represent the total binding and degradation of aggregated ^{125}I-Lp(a). Each data point represents the mean value of three experiments with the indicated SD. See Figure 1 for other abbreviation.

^{125}I-Lp(a) was markedly elevated, and the extent of degradation was 4.7-fold higher than that of native ^{125}I-Lp(a) when 200 μg/mL lipoprotein was added [8.13 μg protein/mg cell protein for aggregated Lp(a) and 1.70 μg protein/mg cell protein for native Lp(a)].

To test whether the LDL receptor is involved in the uptake of native or aggregated Lp(a), we examined the extent to which unlabeled native LDL competed for the binding and degradation of native or aggregated ^{125}I-Lp(a). As shown in Figure 3, native LDL inhibited, although incompletely, the binding and degradation of native ^{125}I-Lp(a) (57 and 60%, respectively) at a 20-fold excess concentration. On the other hand, very little competition was observed with native LDL at a concentration 20-fold in excess of that of aggregated ^{125}I-Lp(a) both in the binding and degradation (9 and 10%, respectively).

Stimulation of cholesteryl ester synthesis by aggregated Lp(a). Consistent with the results of degradation, cholesteryl ester synthesis in macrophages was markedly enhanced by aggregated Lp(a), and 16.4 ± 1.2 nmol of [^{3}H]oleate was incorporated into cholesteryl ester per milligram of cell protein (Fig. 4). On the other hand, native Lp(a) showed almost no stimulation of cholesteryl ester synthesis. This considerable difference in intracellular cholesterol accumulation also was confirmed by Oil Red O staining. As shown in Figure 5, in the presence of aggregated Lp(a), massive lipid droplets were observed in the cytoplasm indicating the transformation of macrophages into foam cells. In contrast, there was very little lipid deposition in the presence of native Lp(a). These findings strongly indicated that aggregated Lp(a) could lead macrophages to change into foam cells and that the process of aggregation may play an important role in the mechanism of Lp(a) deposition in macrophages.

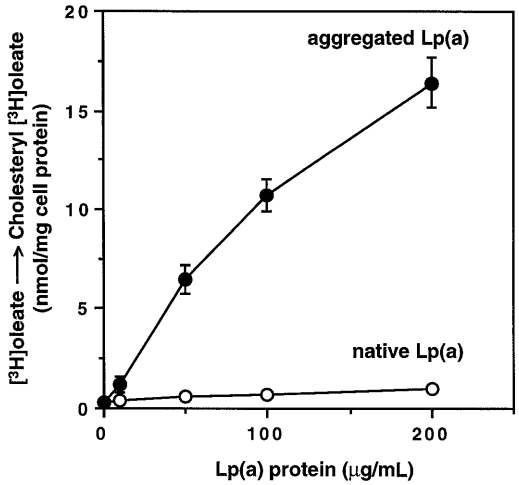

FIG. 4. Concentration dependency of cholesterol esterification induced by aggregated Lp(a). Macrophage monolayers were incubated with 0.1 mmol/L [^{3}H]oleate and increasing concentrations of native (○) or aggregated ^{125}I-Lp(a) (●). After incubation at 37°C for 5 h, the cellular content of cholesteryl [^{3}H]oleate was determined. Each data point represents the mean value of three experiments with the indicated SD. See Figure 1 for abbreviation.

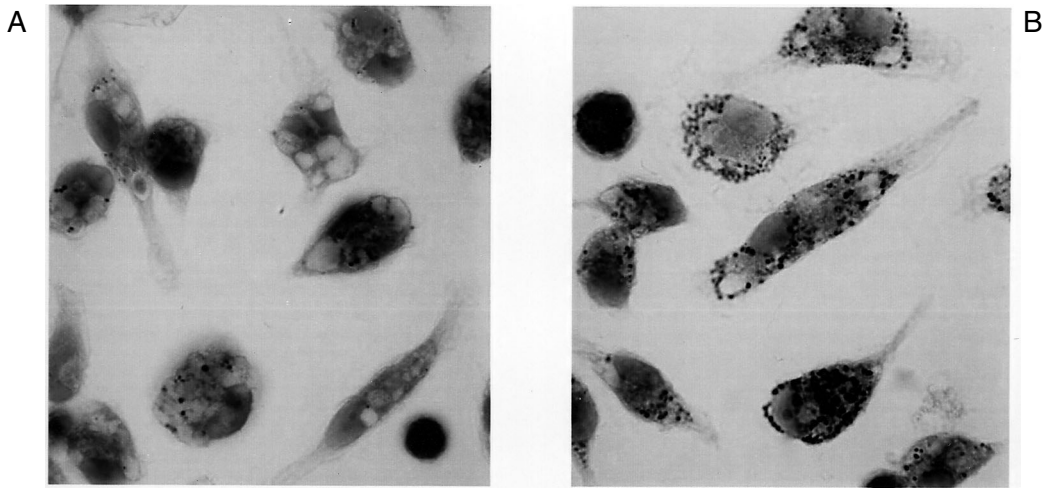

FIG. 5. Appearance of Oil Red O-stained macrophages observed under light microscope; 5×10^5 cells plated on 11-mm circular coverslips incubated with 100 µg protein/mL of native (A) or aggregated Lp(a) (B) at 37°C for 15 h. The cells on the coverslips were then washed with phosphate buffered saline and stained with Oil Red O as described in the Materials and Methods section.

Effects of cytochalasin B on degradation and cholesterol esterification induced by aggregated Lp(a) in macrophages. There have been several reports showing that aggregated lipoproteins can be avidly uptaken by macrophages by phagocytosis. To determine the involvement of this process in the enhanced uptake of aggregated Lp(a) observed, phagocytic inhibitor cytochalasin B was employed. As shown in Figures 6A and 6B, in case of aggregated ^{125}I-Lp(a), cytochalasin B dramatically inhibited degradation and cholesterol esterification by 78 and 83%, respectively, but no significant inhibition was observed in that of native ^{125}I-Lp(a), suggesting that the phagocytosis is involved in the internalization of aggregated Lp(a) by macrophages.

Cholesterol esterification induced by aggregation of Lp(a) of two phenotypes. We compared the rate of cholesterol esterification obtained with two Lp(a) of different phenotypes to observe if there is a significant difference between them. Lp(a) were isolated from two patients treated with LDL-apheresis. The patient with the S_3/B isoform is the same from whom all data presented above were obtained. The patient with the S_3/S_2 isoform was a 75-year-old female with high levels of plasma Lp(a) (47 mg/dL). The extent of Lp(a) aggregation of the two phenotypes was almost equivalent (54 vs. 49%). As shown in Table 1, aggregates of S_3/B-Lp(a) were 2.6-fold larger in profile area than those of S_3/S_2-Lp(a) ($n = 20$, $P < 0.012$, S_3/B vs. S_3/S_2, analysis of variance). In addition the former induced 2.4 times more cholesterol esterification than the latter.

DISCUSSION

This is the first study showing that Ca^{2+}-induced Lp(a) aggregates, a model of self-aggregation of Lp(a), were avidly uptaken by macrophages, possibly by phagocytosis, leading to the transformation of macrophages into cholesteryl ester-rich foam cells.

Documented *in vitro* modifications of Lp(a) that may lead to the formation of macrophage-derived foam cells are malondialdehydration (29), oxidation (30), and association with

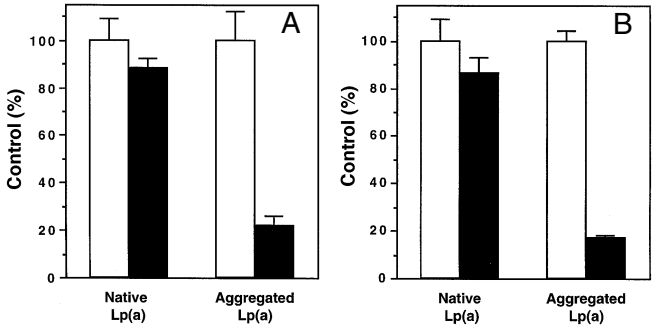

FIG. 6. Effects of cytochalasin B on (A) degradation and (B) cholesterol esterification induced by aggregated Lp(a) in macrophages. (A) Macrophage monolayers were incubated with 50 µg protein/mL of native or aggregated ^{125}I-Lp(a) with or without 10 µg/mL of cytochalasin B at 37°C for 5 h. After incubation, the amount of ^{125}I-labeled trichloroacetic acid-soluble material in the medium was determined. The 100% values for native and aggregated ^{125}I-Lp(a) were 1.12 and 3.80 µg protein/mg cell protein/5 h, respectively. (B) Macrophage monolayers were incubated with 0.1 mmol/L [^{3}H]oleate and 50 µg protein/mL of native or aggregated Lp(a) in the presence or absence of 10 µg/mL of cytochalasin B at 37°C for 5 h. After incubation, the cellular content of cholesteryl [^{3}H]oleate was determined. The 100% values for native and aggregated Lp(a) were 0.63 and 6.35 nmol of cholesteryl oleate/mg cell protein/5 h, respectively. The results were expressed relative to the amount of ^{125}I-Lp(a) degraded or cholesteryl oleate formed in the presence of cytochalasin B. □, 10 µL DMSO/mL medium. ■, 10 µg of cytochalasin B in 10 µL DMSO/mL medium. Each bar represents the mean value ± SD of three experiments. See Figure 1 for abbreviation.

TABLE 1
Comparison Between Two Lp(a)[a] and Their Aggregates

Lp(a) phenotype	Composition (wt%)					Profile area of aggregates[b] ($\times 10^4$ nm^2)	Incorporation of [^{3}H]oleate into CE induced by aggregates[c] (nmol/mg cell protein)
	Pro	PhL	Cho	TG	CE		
S_3/B	26.3	20.2	11.4	5.2	36.9	3.7 ± 1.2	1.68 ± 0.09
S_3/S_2	30.7	19.8	9.8	4.1	35.6	1.4 ± 0.4	0.69 ± 0.02

[a]Lp(a), lipoprotein(a); Pro, protein; PhL, phospholipids; Cho, free cholesterol; TG, triglycerides; CE, cholesteryl esters.
[b]The profile areas of aggregates were quantified after mixing native Lp(a) with 2.5 mmol/L $CaCl_2$ in calcium-free Dulbecco's modified Eagle's medium under transmission electron microscopy.
[c]Macrophage monolayers were incubated with 20 μg cholesterol/mL of aggregated Lp(a) in the presence of 0.1 mmol/L [^{3}H]oleate at 37°C for 5 h. After incubation, the [^{3}H]oleate incorporation into cholesteryl esters was determined as described in the Materials and Methods section. Each value represents the mean value ± SD of three sets of experiment from the same donor, except composition of Lp(a).

extracellular matrix (31). Lp(a) modified in such ways has been shown to be internalized through a scavenger pathway. However, all these studies were conducted with the focus on proposed mechanisms of LDL-induced lipid deposition, and did not focus on the unique property of Lp(a), i.e., self-aggregation. Although aggregated Lp(a) might not precipitate in circulation *in vivo*, one cannot rule out the possibility that it might undergo aggregation in an atherosclerotic milieu. In fact, Hoff *et al.* (14) characterized tissue Lp(a) extracted from human atherosclerotic lesions and found that some of the tissue Lp(a) formed aggregates. Similar to our results, Tertov *et al.* (32) demonstrated, using Lp(a) isolated from a patient with CAD, that aggregates formed after 6-h incubation under cell culture conditions induced massive lipid deposition in macrophages, indicating a significant role of self-aggregation in cellular uptake of Lp(a). Taking these findings and our data into consideration, it can be said that aggregation may be related to the mechanisms of Lp(a) accumulation in the artery wall, or may even be a crucial step for accelerating the development of atherosclerotic lesions.

At the present time, the metabolism of native Lp(a) is still unclear. Studies with cultured cells (33), partially purified receptors (34), and transgenic mice overexpressing the LDL receptor (35) provided evidence of a specific binding of Lp(a) to the LDL receptor. Our findings that LDL in excess concentration inhibited the binding and degradation of native Lp(a) in macrophages are consistent with the findings of previous studies and support the significant role of the LDL receptor in native Lp(a) metabolism. On the other hand, the failure of LDL to inhibit the binding and degradation of aggregated Lp(a) indicated that the LDL receptor was not involved in the metabolism of aggregated Lp(a). This is possibly because conformational changes of apo(a) and/or Lp(a) induced by aggregation sterically hinder the receptor binding domain of apolipoprotein B-100 in Lp(a) from interacting with the LDL receptor of macrophages. Earlier, Yashiro *et al.* (15) reported that monomeric LDL can bind to Ca^{2+}-induced aggregated Lp(a) under *in vitro* conditions. This evidence raises the possibility that aggregated Lp(a) internalization was not inhibited because of LDL sequestration by aggregated Lp(a). However, this is unlikely because monomeric LDL showed little binding to aggregated Lp(a) in the culture medium used in these experiments (data not shown). Although the precise reason for this is not known, a higher ionic strength of the medium compared with that of previously reported interacting solutions (15) may inhibit the ionic binding between LDL and aggregated Lp(a).

As shown in the results, the inhibition of aggregated Lp(a) uptake by cytochalasin B suggested that a phagocytic pathway was possibly involved in its metabolism. Several reports have suggested that Fc receptor, one of the phagocytosis-promoting receptors, is related to enhanced uptake of modified LDL such as immune complexes of aggregated LDL or oxidized LDL (36,37). Therefore, we tested the contribution of the Fc receptor to the uptake of aggregated Lp(a) using heat-aggregated gamma globulin as a competitor. Although the results were not shown in the results section, the Fc receptor was only slightly involved in the uptake of aggregated Lp(a). Cholesterol esterification in macrophages was reduced by 15% in the presence of heat-aggregated gamma globulin. This is consistent with the previous report by Khoo *et al.* (36) using vortex-induced aggregated LDL, and suggested that aggregated Lp(a) may be taken up *via* non-Fc receptor-mediated phagocytosis.

Recently, a novel endocytic pathway such as sequestration or macropinocytosis has been proposed for the mechanism of macrophage uptake (38,39). These pathways are actin-dependent and inhibited by cytochalasin as phagocytosis is. Therefore, we cannot rule out the possibility that such pathways are associated with the aggregated Lp(a) metabolism observed in this study. To better understand the precise mechanism by which aggregated Lp(a) is taken up by macrophages, further studies will be needed.

It has been already reported for LDL that self-aggregation is one of the important steps leading to foam cell formation of macrophages (40). Therefore, a comparison of cholesterol esterification between aggregated LDL and Lp(a) may be valuable. However, a direct comparison cannot be made because, unlike Lp(a), LDL does not undergo self-aggregation by calcium as reported by Yashiro *et al.* (15). Although many

different aggregation models have been proposed for LDL (32,40–44), it is meaningless to compare these models with ours because results obtained using different aggregation models may be biased by different properties of aggregates derived from the method used.

In this study, the ability of Lp(a) of two different phenotypes to stimulate cholesterol esterification in macrophages was also examined, and we found a significant difference between them. This difference might be a clue for explaining differences in atherogenicity between Lp(a) phenotypes as previously suggested (45–47); however, our data are too preliminary to justify a conclusion. To clarify this issue, further studies using a greater number of donors and Lp(a) phenotypes must be performed.

The present study proposed one potential mechanism by which Lp(a) accumulates in the artery wall, and thereby supports the significant role of Lp(a) in atherosclerotic diseases.

ACKNOWLEDGMENTS

The authors thank Dr. Hirofumi Matsuda, Dr. Hideki Hakamada, and Dr. Seikoh Horiuchi, Kumamoto University Medical School, for valuable advice. The authors also thank Chizuko Ohba, Mikako Yotsuhashi, and Tomoko Fujita for technical assistance.

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[Received November 3, 1997, and in final revised form February 17, 1998; revision accepted March 10, 1998]

Effect of Selenium and Vitamin E Supplements on Tissue Lipids, Peroxides, and Fatty Acid Distribution in Experimental Diabetes

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ABSTRACT: The protective role of selenium (Se), given as a Se-rich yeast, selenomethionine or selenomethionine + vitamin E supplement, toward changes in lipid, peroxide, and fatty acid distribution in tissues of streptozotocin-induced diabetic rats, was investigated, after 24 wk of disease. Diabetes increased liver thiobarbituric acid-reactive substances and conjugated dienes; Se supplement completely corrected these changes. In kidney, as in heart, the peroxide levels were not significantly changed by diabetes. In diabetic rat liver, a significant drop in triglycerides and phospholipids ($P < 0.05$) was observed; this was modulated by Se + vitamin E supplementation. Se + vitamin E supplementation also inhibited the decrease in 18:2n-6 and the increase in 22:6n-3 observed in liver of diabetic rats, changes which reflect altered glycemic control. In kidney, heart, and aorta, diabetes produced some changes in lipid content and fatty acid distribution, especially an increase in heart triglycerides which was also corrected by the Se supplement. Se supplementation to diabetic rats also increased 18:0 ether-linked alcohol, 20:4 n-6, and 22:5 n-3 in cardiac lipids. In aorta, Se + vitamin E significantly increased 20:5 n-3. These polyunsaturated fatty acids are precursors, *in situ*, of prostaglandin I_2 (PGI_2) and PGI_3 which may protect against cardiovascular dysfunction. In kidney, conversely, Se decreased 20:4 n-6, the precursor of thromboxane A_2 implicated in diabetic glomerular injury. Thus Se, and more efficiently Se + Vitamin E supplementation, in experimental diabetes could play a role in controlling oxidative status and altered lipid metabolism in liver, thereby maintaining favorable fatty acid distribution in the major tissues affected by diabetic complications.
Lipids 33, 393–399 (1998).

Diabetes mellitus is associated with characteristic vascular complications. The capillaries, arterioles and large arteries can be affected, leading to clinical manifestations of micro- or macroangiopathy (1). Lipid abnormalities such as hypertriglyceridemia (2) and fatty acid distribution changes (3) could participate in the development of vascular lesions in diabetes. However, hyperglycemia alone has been reported to have a causal link with diabetic microangiopathy complications (4). The toxicity of glucose has been confirmed by studies on nonenzymatic glycosylation of proteins, on the polyol pathway, and on autooxidation of sugars and sugar-adduct proteins. These products and oxidized unsaturated lipids generate free radicals and may induce an oxidative stress *in vivo* possibly indicating a common pathway linking diverse mechanisms for the pathogenesis of vascular diabetic complications (5). However, to confirm the importance of oxidative stress in this pathology, it is important to show that its inhibition prevents or delays abnormalities in diabetic tissues.

In previous studies, we have shown that antioxidant supplementation could modulate platelet hyperactivity and fatty acid changes and also delay cataract development in diabetic rats (6). More recently, we have shown that a pharmacologic dose of vitamin E could decrease cholesterol in the aorta of diabetic rats (7) and that a selenium (and more efficiently a selenium + vitamin E) supplement delayed renal lesions in streptozotocin-induced diabetic rats (8). This study was undertaken to show whether selenium, a constituent of glutathione peroxidase and selenoproteins with antioxidant properties (9), along with vitamin E could modulate peroxide and lipid levels in different diabetic rat tissues.

MATERIALS AND METHODS

Animals and treatments. Seventy-six male Sprague-Dawley rats (Charles River, Saint Aubin les Elbeuf, France) with initial body weight of 525 ± 80 g were used for the study. Sixty-four rats were injected iv with 30 mg/kg streptozotocin dissolved in a buffer (7). All rats with glycemia >2.5 g/L were considered diabetic. Six rats died 48 h after streptozotocin injection. Twelve rats were used as a control group and injected with buffer alone (Group C). Before experimentation, all rats were fed with a standard laboratory chow (A04 from UAR, Villemoison sur Orge, France) containing selenium (Se) at 200 µg/kg of diet, and had a normal blood selenium of 524 ± 14.5 µg/L. A week after streptozotocin injection, all rats received a diet according to the current recommendations for

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Abbreviations: DMA, dimethyl acetal; GSH, glutathione; MDA, malondialdehyde; TBARS, thiobarbituric-acid reactive substances.

diabetes (6). Briefly, the diet contained 15% protein, 52% carbohydrate, and 33% lipid in percentages of total calories. The lipids were a mixture of butter and hydrogenated coconut, canola, and sunflower oils (4, 1.7, 7, and 4.5 g/100 g of diet, respectively). The ratio of polyunsaturated/saturated fatty acids was 1. Purified diet without supplementation contained 20 µg of Se and 9.6 mg of vitamin E per 100 g of diet [vitamin E consisted of 5 mg of α-tocopherol acetate from a vitamin mixture added to basal diet (ICN, Aurora, OH), and 4.6 mg of natural tocopherols (α, β, γ) from the vegetable oils]. Diabetic rats were separated into four groups. Group D (14 rats) received the purified diet without supplementation as given to the control group. The other groups received the same diet supplemented either with Se-rich yeast (i.e., Selenion; Cellife Intern No. 4537; Montrouge, France) (63.2 mg/100 g of diet for 14 rats, group: DSel), with selenomethionine (194.5 µg/100 g, for 14 rats, group: DSm), or with a double supplementation, i.e. selenomethionine + α-tocopherol acetate (Sigma, Isle d'Abeau, France) (194.5 µg/100 g and 62.5 mg/100 g, respectively, for 16 rats, group: DSmE). The elemental Se in the three groups was 78.2 µg/100 g of diet. Diabetic rats were injected subcutaneously four times per week with 3.2 IU ultra-slow insulin (Novo, Boulogne, France). After 24 wk of diet, following a fast of 7 h and 24 h of insulin deprivation, blood was removed from the jugular vein of each rat and the animals were sacrificied by iv pentobarbital injection. The different organs (liver, heart, aorta, and kidney) were quickly collected, surrounding adipose tisssue was carefully removed, and the tissues were lyophilized.

Biochemical determinations. Tissue lipids were extracted by the technique of Folch *et al.* (10), and total cholesterol levels were evaluated in accurately measured aliquots of lyophilized lipid tissue extract according to the technique of Omodeo Sale *et al.* (11). Phospholipids and triglycerides were determined by enzymatic kits: Biomérieux (Marcy l'Etoile, France), and Merck (Darmstadt, Germany). Aorta vitamin E was measured in lipid extracts by high-performance liquid chromatography (7). Kidney, heart, and liver vitamin E levels were determined using the same technique preceded by a saponification step according to the technique reported by Rittenmaier and Schuep (12) with some modifications (7). Tissue Se was determined by atomic electrothermal absorption spectroscopy with a Varian model Spectra AA 300 (Tokyo, Japan) (8). Thiobarbituric acid-reactive substances (TBARS) and conjugated dienes were measured in aliquots of lipid extract (8).

Fatty acid distribution in lipid extracts was studied. Fatty acids were transesterified using BF_3-methanol by the technique of Morrison and Smith (13) and purified by thin-layer chromatography on silica gel G with a solvent composed of petroleum ether and diethyl ether (90:30, vol/vol.). The spots of methylated fatty acids were revealed by a spray of dichlorofluorescein dissolved in ethanol/acetone (1:1, vol/vol). Fatty acid methyl ester distribution was studied by gas–liquid chromatography. Liver and heart fatty acid methyl esters were separated on an Intersmat instrument IGC 120 DFL (Courtry, France) using a flame-ionization detector on a semi-capillary column Restek CRTX 2330 (30 m × 0.52 mm) (Restek Corporation, Bellefonte PA). The oven temperature was 85°C at injection and was maintained for 10 min, then raised by 4°C/min to 235°C. Injector and detector temperatures were 260 and 230°C, respectively. Helium was used as carrier gas under a pressure of 0.6 bars. Kidney and aorta methylated fatty acids were obtained as described before and gas–liquid chromatographic analysis was performed on a Carlo Erba HRCG 5160 instrument (Milan, Italy) and a capillary column SP 2340 (30 m × 0.32 mm) (Supelco Company, Bellefonte, PA). The oven temperature was 90°C for 1 min at injection and raised by 6°C/min to 145°C, 2°C/min to 200°C , then 3°C/min to 220°C and isothermal maintained for 17 min.

Statistical analysis. Unpaired Student's *t*-test preceded by analysis of variance was used to evaluate the significance of differences.

RESULTS

Tissue lipids levels are reported in Table 1. Liver phospholipids and triglycerides significantly decreased in group D, diabetic rats without supplements compared to group C, controls ($P < 0.05$). This decrease of phospholipids was prevented only in group DSmE, diabetic rats supplemented with selenomethionine and α-tocopherol acetate. Triglyceride levels were more than three times less in group D than in group C. A double supplementation with Se + vitamin E restored the liver triglyceride level in group DSmE nearly to that in group C, the control. The different lipids in heart and kidney were not significantly changed in diabetic animals supplemented or not with Se; only a significant increase in heart triglycerides for diabetic rats without supplements (group D) compared to controls was noted ($P < 0.05$). Results for tissue peroxides, evaluated by measurement of TBARS and conjugated dienes, are given in Figure 1. A significant increase in TBARS was noted in the hepatic tissue of group D, diabetic rats without supplements compared to controls ($P < 0.05$); antioxidant supplementation decreased this effect. Heart and kidney tissues presented higher TBARS levels than liver, but these were not significantly changed in diabetic groups compared to group C (controls). For conjugated dienes, we observed the same effects in liver tissue as for TBARS: a significant increase in diabetic rats without supplements compared to controls ($P < 0.05$), corrected by Se supplementation. These compounds were also higher in heart and kidney than in liver but were not significantly changed by the disease.

Tissue antioxidant concentrations are given in Figure 2. Liver vitamin E level was very significantly decreased in (group D) compared to controls (group C) ($P < 0.0005$); this was completely corrected in the group DSmE. Vitamin E concentrations in group DSmE were very significantly increased compared to group D ($P < 0.0005$). Liver Se level in group D was only slightly decreased compared to group C. In Se-supplemented diabetic groups, liver Se increased significantly compared to groups C (control) and D (diabetic rats without supplements). In heart, vitamin E and Se levels were lower

TABLE 1
Tissue Lipid Levels (nmol/mg tissue) in Control and Diabetic Rats Fed with a Purified Diet or the Same Diet Supplemented with Selenium[a]

Groups	C	D	DSel	DSm	DSmE
Phospholipids					
Liver	80.4 ± 2[b]	67.0 ± 3[c]	65.9 ± 2[c]	66.6 ± 2[c]	70.3 ± 2[c]
Heart	51.0 ± 1[b]	49.8 ± 1.6[b]	47.8 ± 2[b]	48.9 ± 2[b]	49.3 ± 1[b]
Kidney	63.8 ± 2[b]	70.9 ± 5[b]	61.1 ± 3[b]	60.7 ± 2[b]	62.0 ± 2[b]
Cholesterol					
Liver	23.9 ± 1[b]	20.6 ± 4[b]	17.6 ± 1[b]	18 ± 1[b]	20.3 ± 2[b]
Heart	10.9 ± 0.2[b]	10.4 ± 0.3[b]	10.6 ± 0.3[b]	10.6 ± 0.4[b]	10.3 ± 0.2[b]
Kidney	57.9 ± 1[b]	57.8 ± 3[b]	56.7 ± 3[b]	57.2 ± 2[b]	58.6 ± 2[b]
Aorta					
Triglycerides					
Liver	280 ± 37[b]	83 ± 20[c]	125 ± 23[c]	141 ± 18[b]	207 ± 64[b]
Heart	4 ± 0.5[b]	4.8 ± 0.8[c]	3.4 ± 0.6[b]	2.7 ± 0.4[b]	3.5 ± 0.6[b]
Kidney	3.6 ± 0.4[b]	3 ± 0.4[b]	3.2 ± 0.7[b]	2.6 ± 0.3[b]	2.3 ± 0.2[b]

[a]Values are means ± SD of 7–11 rats in each group. [b,c]Values not bearing the same superscript letters in the same row are significantly different from each other at $P < 0.05$. Abbreviations: C, control; D, diabetic rat with no supplements; DSel, diabetic rat supplemented with Se-rich yeast; DSm, diabetic rats supplemented with selenomethionine; DSmE, diabetic rat supplemented with selenomethionine + vitamin E.

than those observed in liver in all groups. Heart vitamin E level was significantly increased in group DSmE (diabetic rats supplemented with selenomethionine + α-tocopherol acetate) compared to groups C (control), D (diabetic rats without supplements), DSel (diabetic rats with Se-rich yeast) ($P < 0.005$, $P < 0.0005$, $P < 0.0005$, respectively). Concerning heart Se concentration, a significant increase was observed, as for liver, in all diabetic Se-supplemented groups. Kidney tissue had a lower vitamin E level compared with the other tissues studied; this was not changed by disease, nor supplementation. On the contrary, kidney had a higher Se content than did other tissues. Diabetes did not modify kidney Se level, but the Se supplementation very significantly increased kidney Se content in diabetic groups ($P < 0.0005$).

Changes in liver fatty acid distribution are given in Table 2. A significant decrease in linoleic acid (18:2 n-6) was observed

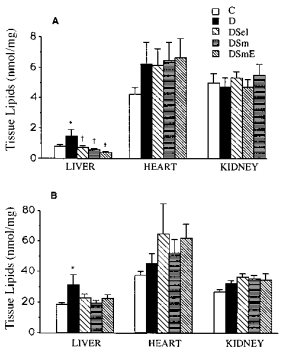

FIG. 1. (A) Tissue thiobarbituric acid-reactive substances (TBARS) and (B) conjugated diene levels in control rats (C) and diabetic rats without supplements (D); and diabetic rats supplemented with a Se-rich yeast (DSel), selenomethionine (DSm), or selenomethionine + vitamin E (DSmE). Values are means ± SD for 7 to 11 animals in each group. Symbols denote statistically significant differences vs. C, control group * = $P < 0.05$; vs. unsupplemented diabetic rats, group D † = $P < 0.05$.

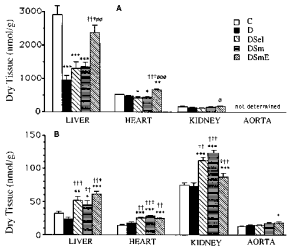

FIG. 2. Tissue vitamin E (A) and Se (B) levels in control and diabetic rats (C, D) and diabetic rats supplemented with a Se-rich yeast (DSel), selenomethionine (DSm), or selenomethionine + vitamin E (DSmE). Values are means ± SD for 7 to 11 animals in each group. The symbols denote statistically significant differences vs. control group C, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.0005$; vs. unsupplemented diabetic rats, group D †, ††, †††; and vs. DSel, ø, øø, øøø idem. Vitamin E and Se levels in kidney are reprinted by permission of Blackwell Science Inc. from Douillet, C. *et al.* A selenium supplement associated or not with vitamin E delays early renal lesions in experimental diabetes, *Proc. Soc. Exp. Biol. Med. 21,* 323–331.

TABLE 2
Main Liver Fatty Acids (%) and Main Fatty Acid Changes in Control and Diabetic Rats Fed with a Purified Diet or the Same Diet Supplemented with Selenium[a]

Fatty acids	C	D	DSel	DSm	DSmE
16:0	22.3 ± 0.56[b]	20.9 ± 0.71[b]	21.4 ± 0.70[b]	22.2 ± 1.33[b]	22.2 ± 0.54[b]
18:0	8.3 ± 0.62[b]	9.5 ± 0.60[b]	8.3 ± 0.25[b]	8.3 ± 0.29[b]	8.6 ± 0.72[b]
18:0 DMA	2.6 ± 0.5[b]	3.6 ± 0.4[b]	3.3 ± 0.5[b]	3 ± 0.5[b]	3 ± 0.6[b]
18:1(n-9 + n-7)	19.9 ± 1.05[b]	18.8 ± 1.04[b]	21.3 ± 0.54[b]	20.3 ± 0.81[b]	19.3 ± 1.4[b]
18:2n-6	22.9 ± 0.72[b]	20.2 ± 0.75[c]	19.3 ± 0.86[c]	19.3 ± 1.0[c]	21.9 ± 0.75[b]
20:3n-6	0.6 ± 0.04[b]	1.1 ± 0.0[c]	0.9 ± 0.05[c]	1.0 ± 0.08[c]	0.9 ± 0.08[c]
20:4n-6	11.2 ± 0.99[b]	13.8 ± 0.7[b]	12.9 ± 0.73[b]	12.9 ± 0.82[b]	12.4 ± 0.95[b]
22:4n-6	0.40 ± 0.02[b]	0.4 ± 0.04[b]	0.3 ± 0.02[b]	0.4 ± 0.07[b]	0.3 ± 0.03[b]
18:3n-3	1.1 ± 0.07[b]	0.6 ± 0.05[c]	0.6 ± 0.06[c]	0.7 ± 0.09[c]	0.8 ± 0.08[c]
20:5n-3	0.2 ± 0.02[b]	0.2 ± 0.06[b]	0.2 ± 0.01[b]	0.2 ± 0.02[b]	0.2 ± 0.02[b]
22:5n-3	0.6 ± 0.07[b]	0.4 ± 0.04[c]	0.4 ± 0.03[c]	0.5 ± 0.08[b]	0.4 ± 0.03[c]
22:6n-3	2.5 ± 0.22[b]	3.4 ± 0.26[c]	3.3 ± 0.12[c]	3.2 ± 0.25[c]	2.8 ± 0.24[b]

[a]Values reported are weight percentage and are means ± SD (n = 7–13 rats in each group). [b,c]Values not bearing the same superscript letters in the same row are significantly different from each other at $P < 0.05$. DMA: dimethyl acetal. For other abbreviations see Table 1.

in groups D, DSel, and DSm (diabetic rats supplemented with selenomethionine) compared to group C ($P < 0.05$); this decrease was corrected in group DSmE. A significant increase in dihomo-γ-linoleic acid (20:3n-6) was noted in all diabetic groups compared to control group C. A decrease in linolenic acid (18:3n-3) ($P < 0.05$) and docosapentaenoic acid (22:5n-3) associated with an increase in docosahexaenoic acid (22:6n-3) ($P < 0.05$) were observed in diabetic groups compared to group C except for group DSmE, which was supplemented with both selenomethionine and α-tocopherol acetate.

Changes in cardiac fatty acids are shown in Table 3. In rat heart tissue, diabetes induced a significant decrease in arachidonic acid (20:4n-6) and docosapentaenoic acid (22:5n-3) ($P < 0.05$) compared to control. These changes in diabetic rats were prevented by Se supplementation. Moreover, an increase in ether-linked 18:0 alcohol was observed in all diabetic Se-supplemented rat groups compared to control group C, but this was only significant in groups DSm and DSmE ($P < 0.05$).

Changes in kidney fatty acids are indicated in Table 4. Conversely, in kidney, a significant increase in 20:4n-6 was noted in Group D (diabetic rats with no supplementation) compared to C, which was corrected in groups DSm and DSmE. We also observed in this tissue a significant decrease in monounsaturated fatty acids (18:1n-9 + 18:1n-7) and a significant increase in 22:6n-3 in all diabetic groups compared to control group C.

Fatty acid changes in aorta are noted in Table 5. An increase in ether-linked 18:0 alcohol was observed in diabetic Se-supplemented groups compared to control group C but, as in heart, this was only significant in groups DSm (supplemented with selenomethionine) and DSmE (supplemented with selenomethionine and α-tocopherol acetate). A significant increase in 20:3n-6 was noted in all diabetic groups compared to control group C. In group DSmE, 20:5n-3 also was significantly increased ($P < 0.05$) compared to groups D (unsupplemented) and C (control). Moreover, 22:5n-3 and 22:6n-3 were increased in the diabetic groups compared to C, but this was significant only for groups DSel and DSmE ($P < 0.05$).

TABLE 3
Main Heart Fatty Acids and Main Fatty Acid Changes in Control and Diabetic Rats Fed with a Purified Diet or the Same Diet Supplemented with Selenium[a]

Fatty acids	C	D	DSel	DSm	DSmE
16:0	13 ± 0.43[b]	13.4 ± 0.55[b]	14.8 ± 1.10[b]	12.3 ± 0.44[b]	12.6 ± 0.41[b]
18:0	18.3 ± 0.94[b]	19.2 ± 1.36[b]	19.6 ± 0.64[b]	19.4 ± 0.37[b]	18.8 ± 0.74[b]
18:0 DMA	0.4 ± 0.03[b]	0.4 ± 0.05[b]	0.5 ± 0.10[b,d]	0.7 ± 0.16[c,d]	0.6 ± 0.08[c,d]
18:1(n-9 + n-7)	15.7 ± 1.07[b]	17.4 ± 1.41[b]	14.8 ± 1.60[b]	13.7 ± 0.98[b]	15.1 ± 1.16[b]
18:2n-6	21.7 ± 0.47[b]	22.8 ± 0.66[b]	20.6 ± 0.57[b]	21.7 ± 0.8[b]	21.2 ± 0.8[b]
20:3n-6	0.3 ± 0.04[b]	0.4 ± 0.03[b]	0.4 ± 0.05[b]	0.5 ± 0.04[b]	0.5 ± 0.08[b]
20:4n-6	15.8 ± 0.74[b]	12.9 ± 1.13[c]	14.5 ± 0.86[b]	15.8 ± 0.56[b]	14.7 ± 0.85[b]
22:4n-6	0.6 ± 0.06[b]	0.6 ± 0.04[b]	0.6 ± 0.04[b]	0.7 ± 0.04[b]	0.7 ± 0.06[b]
18:3n-3	0.5 ± 0.03[b]	0.5 ± 0.09[b]	0.4 ± 0.04[b]	0.4 ± 0.03[b]	0.5 ± 0.04[b]
20:5n-3	0.06 ± 0.00[b]	0.07 ± 0.00[b]	0.08 ± 0.01[b]	0.08 ± 0.02[b]	0.08 ± 0.01[b]
22:5n-3	1.3 ± 0.1[b]	0.9 ± 0.1[c]	1.2 ± 0.1[b]	1.5 ± 0.1[b]	1.4 ± 0.1[b]
22:6n-3	5.3 ± 0.5[b]	5.1 ± 0.7[b]	6 ± 0.5[b]	5.9 ± 0.4[b]	6.1 ± 0.4[b]

[a]Values reported are weight percentage and are means ± SD (n = 7–13 rats in each group). [b–d]Values not bearing the same superscript letters in the same row are significantly different from each other at $P < 0.05$. For abbreviations see Tables 1 and 2.

TABLE 4
Main Kidney Fatty Acids and Main Fatty Acid Changes in Control and Diabetic Rats Fed with a Purified Diet or the Same Diet Supplemented with Selenium[a]

Fatty acids	C	D	DSel	DSm	DSmE
16:0	30.4 ± 1.25[b]	27.8 ± 2.16[b]	28.2 ± 0.83[b]	27 ± 1.39[b]	28.6 ± 0.87[b]
18:0	28.4 ± 1.51[b]	26.7 ± 0.91[b]	28.7 ± 1.26[b]	30 ± 0.93[b]	30.7 ± 1.34[b]
18:0 DMA	0.3 ± 0.03[b]	0.3 ± 0.05[b]	0.3 ± 0.04[b]	0.3 ± 0.03[b]	0.3 ± 0.04[b]
18:1(n-9 + n-7)	18 ± 0.84[b]	15.7 ± 0.69[c]	15.5 ± 0.60[c]	15.6 ± 0.81[c]	14.2 ± 0.82[c]
18:2n-6	10.6 ± 0.6[b]	12.4 ± 0.93[b]	11.5 ± 0.69[b]	11.8 ± 0.76[b]	11.1 ± 0.77[b]
20:3n-6	0.16 ± 0.01[b]	0.18 ± 0.02[b]	0.18 ± 0.01[b]	0.18 ± 0.02[b]	0.21 ± 0.02[b]
20:4n-6	8 ± 1.04[b]	13.2 ± 1.58[c,d]	11.9 ± 1.26[c,d]	11.5 ± 1.44[b,d]	10.6 ± 0.71[b,d]
22:4n-6	1.2 ± 0.21[b]	1.1 ± 0.16[b]	1 ± 0.16[b]	1 ± 0.16[b]	1.2 ± 0.14[b]
18:3n-3	0.09 ± 0.01[b]	0.07 ± 0.00[b]	0.09 ± 0.01[b]	0.08 ± 0.01[b]	0.09 ± 0.01[b]
20:5n-3	0.04 ± 0.01[b]	0.03 ± 0.00[b]	0.04 ± 0.00[b]	0.05 ± 0.01[b]	0.05 ± 0.00[b]
22:5n-3	0.05 ± 0.01[b]	0.04 ± 0.01[b]	0.04 ± 0.00[b]	0.04 ± 0.01[b]	0.04 ± 0.01[b]
22:6n-3	0.10 ± 0.01[b]	0.2 ± 0.03[c]	0.2 ± 0.02[c]	0.15 ± 0.02[c]	0.2 ± 0.02[c]

[a]Values reported are weight percentage and are means ± SD (n = 7–13 rats in each group). [b–d]Values not bearing the same superscript letters in the same row are significantly different from each other at $P < 0.05$. For abbreviations see Tables 1 and 2.

TABLE 5
Main Aorta Fatty Acids and Main Fatty Acid Changes in Control and Diabetic Rats Fed with a Purified Diet or the Same Diet Supplemented with Selenium[a]

Fatty acids	C	D	DSel	DSm	DSmE
16:0	21 ± 0.72[b]	19.2 ± 0.4[b]	20.2 ± 1.1[b]	18.4 ± 1.1[b]	19.5 ± 0.5[b]
18:0	9.4 ± 0.7[b]	11.8 ± 1[b,d]	12.2 ± 0.6[c,d]	11.3 ± 0.5[b,d]	13.2 ± 0.8[c,d]
18:0 DMA	0.9 ± 0.1[b]	1.2 ± 0.2[b,d]	1.3 ± 0.2[b,d]	1.3 ± 0.1[c,d]	1.6 ± 0.04[c,d]
18:1(n-9 + n-7)	33.6 ± 1.6[b]	32.9 ± 1.2[b]	31.6 ± 1.3[b]	31.8 ± 1.4[b]	30.9 ± 1.4[b]
18:2n-6	19.1 ± 1[b]	17.4 ± 1.1[b]	15 ± 0.7[c]	16.2 ± 1.5[b]	14.3 ± 0.9[c]
20:3n-6	0.16 ± 0.02[b]	0.20 ± 0.03[b]	0.24 ± 0.03[c]	0.22 ± 0.01[c]	0.26 ± 0.03[c]
20:4n-6	6.4 ± 0.08[b]	9.1 ± 1.1[b]	10 ± 0.9[b]	9.1 ± 0.4[b]	10.4 ± 1[b]
22:4n-6	0.95 ± 0.1[b]	1.34 ± 0.2[b,d]	1.35 ± 0.2[b,d]	1.29 ± 0.1[b,d]	1.4 ± 0.2[c,d]
18:3n-3	0.2 ± 0.02[b]	0.22 ± 0.02[b]	0.2 ± 0.02[b]	0.18 ± 0.03[b]	0.2 ± 0.02[b]
20:5n-3	0.04 ± 0.01[b]	0.03 ± 0.00[b]	0.02 ± 0.00[b]	0.05 ± 0.01[b,d]	0.09 ± 0.04[c,d]
22:5n-3	0.18 ± 0.02[b]	0.23 ± 0.001[b,d]	0.26 ± 0.00[c,d]	0.27 ± 0.02[c,d]	0.28 ± 0.04[c,d]
22:6n-3	0.42 ± 0.06[b]	0.65 ± 0.03[c,d]	0.64 ± 0.02[c,d]	0.52 ± 0.02[b,d]	0.65 ± 0.06[c,d]

[a]Values reported are weight percentage and are means ± SD (n = 7–13 rats in each group). [b–d]Values not bearing the same superscript letters in the same row are significantly different from each other at $P < 0.05$. For other abbreviations see Tables 1 and 2.

DISCUSSION

Two forms of Se supplementation were utilized in the present work: a selenium-rich yeast representing a natural organic-bound form, and selenomethionine. Previous studies showed that the Se-rich yeast steadily increased plasma selenium (14) and prevented secondary myocardial infarction (15). Supplementation with selenomethionine was studied, as the major Se constituent of yeast, to determine whether it had biological activity.

Se supplementation in streptozotocin-diabetic rats provoked a significant increase of this element in liver, heart, and kidney, but this increase only occurred in aorta when Se was given in association with vitamin E. Se-cysteine is an active site of glutathione (GSH) peroxidase. GSH peroxidase has antioxidant activity since it catalyzes the conversion of hydroperoxides to stable nonradical products (9). One could hypothesize that a Se increase in tissue could prevent oxidative damage. In diabetic rats, serum oxidative markers are raised (16). Hence, it seemed interesting to observe changes in lipids, polyunsaturated fatty acids (targets for peroxidation), and peroxide levels in different tissues, after Se supplementation.

In the present study, a significant decrease in liver lipid levels, especially triglycerides, was observed in diabetic rats without supplements compared to controls. In contrast, hepatic triglycerides have been reported to accumulate after only a few hours in experimental diabetes; this correlated with ketosis and was partially reversed by insulin treatment (17). In our study, diabetic rats were treated with insulin for a long period (24 wk), to avoid ketoacidosis or hyperosmolarity, and fed with a lipid-rich diet. Decrease in liver triglycerides, observed in this study, could be a result of these hormonal and dietary changes. It has been reported that chronic deficiency of insulin provokes a lowering of triglyceride synthesis (18). Both selenium and vitamin E have been associated with insulin-like properties (19,20). In the present study, Se supplementation tended to normalize liver triglyceride levels. Moreover, in group DSmE, triglyceride levels were close

to control. The large increase in liver TBARS observed in diabetic rats could promote DNA and protein alterations (21), inducing changes in the enzyme activities implicated in lipid metabolism. In Se- supplemented diabetic rats, these alterations could be partly diminished. This point would be worthy of further study.

Liver phospholipid levels also decreased in all diabetic rats, supplemented whether with selenium or not. As seen with triglycerides, this decrease could be a result of altered liver phospholipid synthesis, or perhaps due to liver peroxide levels. It has been reported that lipid peroxidation stimulates expression of phospholipase A_2 in liver which preferentially hydrolyzes peroxidized phospholipids (22). In group DSmE (supplemented with selenomethionine and α-tocopherol acetate) only, liver phospholipid loss was weakly prevented, perhaps because membrane phospholipid unsaturated fatty acids are highly susceptible to oxygen free-radical attack (23).

An increase in triglycerides and cholesterol in rat myocardium was reported after streptozotocin injection but was completely corrected by insulin after 8 wk of diabetes (24). Under our experimental conditions, after 24 wk of diabetes, an increase in heart triglycerides persisted in group D. Accumulation of cholesterol and triglycerides in the left ventricle and septum in diabetic patients has been associated with cardiomyopathy (25). Thus, decrease in heart triglyceride observed in diabetic Se-supplemented rats after 24 wk could represent a protective effect of selenium on cardiac function.

Concerning liver antioxidant and peroxide levels, diabetes induced a large decrease in vitamin E which tended to be prevented with Se supplementation and was completely corrected in group DSmE. In contrast, liver Se decreased slightly in group D (diabetic rats with no supplementation) compared to control group C but was significantly increased in all Se supplemented groups; this increase was amplified by concomitant vitamin E administration as observed previously in plasma and platelets (26). In liver, TBARS and conjugated dienes were increased in group D compared to group C. Se or Se + vitamin E supplementation in diabetic rats significantly decreased liver TBARS. The same effect of selenium and vitamin E was also observed in liver of rats submitted to a diet inducing oxidative stress (27). Liver conjugated dienes were also reduced by antioxidant supplements. TBARS partly reflect generation of malondialdehyde (MDA), a cytotoxic aldehyde, formed in the peroxidative degradation of membrane lipids. The excretion of urinary MDA-conjugated metabolites (28) was reported as being increased in diabetic rats (29). Accelerated detoxification of MDA by liver in Se- and vitamin E-supplemented rats may play a role in the preservation of oxidative status and polyunsaturated content of other tissues.

Diabetes under our conditions of diet and insulin treatment did not change Se or vitamin E levels in heart and kidney, nor consequently peroxide levels measured as TBARS and conjugated dienes. However, basal peroxide levels are more elevated in these tissues than in liver. This could reflect a decreased capacity to destroy these compounds, perhaps in relation to a relatively weak vitamin E content and lower activity of antioxidant enzymes than in liver.

For tissue fatty acid distributions in diabetic rats (groups D, DSel, DSm), the main changes were observed in liver: a decrease in 18:2n-6 associated with an increase in 20:3n-6 which could suggest an upregulation of Δ-6 desaturase by insulin as described (3). The decrease in 18:2n-6 was prevented by Se + vitamin E supplementation. In the liver of diabetic rats, a decrease in fatty acids of the n-3 series was observed: 18:3n-3, 22:5n-3, whereas, paradoxically, the 22:6n-3 was increased compared to control, except for group DSmE. Increase in 22:6n-3 has been associated with insufficient metabolic control (30).

In heart, an increase in ether-linked 18:0 alcohol in groups DSm and DSmE compared to C and D could indicate an improved possibility for long-term preservation of cardiac function. There has been a proposed link between lethal ischemic damage to myocardial tissue and plasmalogen degradation (31). Plasmalogen ethanolamine has also been described as the storage form of myocardial arachidonic acid (32). Increased plasmalogen through Se supplementation could help to maintain the myocardium. In the same way, a significant decrease in 20:4n-6 in group D (unsupplemented), compared to C, was also noted which disappeared in groups DSel, DSm, and DSmE. Prostacyclin is the major prostaglandin formed from arachidonic acid in the heart of most animals (33). Prostacyclin increases coronary flow, and protects myocardium *in vivo* from ischemic damage (34). Se treatment tended also to increase polyunsaturated fatty acids of the n-3 series in heart and aorta, 22:5n-3 and 20:5n-3, respectively. The latter of these may serve as the precursor of PGI_3, a vasodilator and a platelet antiaggregant (35).

In the kidney, diabetes mainly induced a significant increase in 20:4n-6. This increase was partially reduced by Se supplementation. This observation could partly explain the delay in development of renal lesions in diabetic streptozotocin-induced rats treated with vitamine E (8). Elevation of 20:4n-6 could play a role in the formation of thromboxane A_2, implicated in diabetic glomerulopathy (36).

In conclusion, in streptozotocin-induced diabetic rats, Se or Se+ vitamin E supplementation for 24 wk normalized lipid metabolism in liver and enhanced its capacity to destroy peroxidant compounds as observed before *in vitro* in liver slices (37). The effects of this supplementation were to modulate fatty acid composition in heart, kidney, and aorta and thus to help maintain normal tissue functions *in situ*. The treatment of diabetic patients with free radical scavengers such as Se and vitamin E with the aim of reducing oxidative stress is a question for further study.

ACKNOWLEDGMENTS

This work was supported by INSERM and a grant from Labcatal Company, Montrouge, France. We thank Susan Gamon for editorial assistance and Christiane Mouterde for technical assistance.

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[Received May 13, 1997, and in final revised form January 19, 1998; revision accepted March 10, 1998]

Chronic Dietary n-3 Polyunsaturated Fatty Acids Deficiency Affects the Fatty Acid Composition of Plasmenylethanolamine and Phosphatidylethanolamine Differently in Rat Frontal Cortex, Striatum, and Cerebellum

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ABSTRACT: As chronic consumption of a diet devoid of n-3 fatty acid induced modification of neurotransmission pathways in the frontal cortex of rats, plasmalogen alteration could occur in this area. Because of the propensity to facilitate membrane fusion, plasmenylethanolamine (PmE), a major plasmalogen of brain, may be involved in synaptic transmission. Female rats were fed diet containing peanut oil [(n-3)-deficient diet] through two generations. Two weeks before mating, half of the female rats of the second generation received a diet containing peanut oil and rapeseed oil (control group). The distribution and acyl composition of major phospholipids, phosphatidylethanolamine and PmE, were measured in the frontal cortex, striatum, and cerebellum of the male progeny of the two groups at 60 d of age. The n-3 polyunsaturated fatty acid (PUFA) deficiency had no effect on the distribution of phospholipids in all brain regions but affected their acyl composition differently. The level of 22:6n-3 was significantly lower and compensated for by higher levels of n-6 fatty acids in all regions and phospholipids studied. However, docosahexaenoic acid, being more concentrated in the PmE of frontal cortex, is also more decreased in the n-3-deficient rats compared to the striatum. By contrast, striatum PmE has retained more 22:6n-3 than PmE of the other regions. In addition, the increase of n-6 PUFA was significantly lower in frontal cortex PmE compared to the striatum and cerebellum PmE. In association with altered neurotransmission observed in frontal cortex of n-3-deficient rats, our results suggest that frontal cortex PmE might be more affected in chronically α-linolenic-deficient rats. However, by retaining 22:6n-3, striatum PmE could be most resilient.

Lipids 33, 401–407 (1998).

Polyunsaturated fatty acids (PUFA) derived from the n-6 or n-3 series of fatty acids (FA) are fundamental components of the structural membrane lipids of the brain. They are derived from the essential FA precursors, linoleic (18:2n-6) and α-linolenic acids (18:3n-3), which must be obtained from an exogenous source, i.e., the diet. It has been previously shown that a chronic dietary n-3 PUFA deficiency dramatically alters the FA composition of brain membranes, decreasing brain levels of n-3 PUFA which are compensated for by increased amounts of n-6 PUFA. This diet also altered learning ability in rats (1–7). Moreover, it has been shown that a lesion of dopaminergic neurons disturbed brain integrative functions, i.e., learning and memory, cognitive functions, and reinforcement processes (8–10). On the assumption that impairment of this neuronal system can occur as a result of this specific diet deficiency, Delion *et al.* (11) evaluated the influence of a diet deficient in α-linolenic acid on neurotransmission in the different regions under serotoninergic and dopaminergic regulation: frontal cortex and striatum. Chronic dietary α-linolenic acid deficiency led to a reduced docosahexaenoic acid (DHA), or 22:6n-3, level in these two cerebral regions (11), as already described by Bourre *et al.* (3) in the whole brain. However, a modification of several specific factors of the dopaminergic and the serotoninergic neurotransmission was observed only in the frontal cortex, a region which may be more specifically affected by the FA composition of the diet. The level of DHA in control rats was found to be slightly higher in the frontal cortex than in the striatum (11), which could make the frontal cortex more sensitive to a n-3 PUFA deficiency. PUFA, specifically DHA, are fundamental components of ethanolamine glycerophospholipids (EGP). Plasmalogens, as a subclass of EGP, are often not distinguished from diacyl components, even though they constitute 50% of EGP total levels and about 17% of total phospholipids in brain. These ether-lipids exhibit a different molecular structure as compared to diacylphospholipid. They are characterized by the presence of 1-*O*-alk-1′-enyl ether (vinyl ether)

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Abbreviations: ANOVA, analysis of variance; 20:4n-6, arachidonic acid; DHA, docosahexaenoic acid; 22:6n-3, docosahexaenoic acid; EGP, ethanolamine glycerophospholipids; FA, fatty acid; 18:3n-3, α-linolenic acid; 18:1n-9, oleic acid; PE, phosphatidylethanolamine; PmE, plasmenylethanolamine; PUFA, polyunsaturated fatty acid; Pi, total phosphorus; TLC, thin-layer chromatography.

linkage at the *sn*-1 position as compared to the 1-*O*-acyl ester linkage present in diacylphospholipids. Furthermore, the *sn*-2 hydroxyl group is usually esterified to highly unsaturated FA such as 20:4n-6 and 22:6n-3. Cellular functions of plasmalogens are poorly understood. However, several hypotheses have been proposed. Because of reactivity of the alkenyl ether bond of plasmalogens with singlet oxygen and other reactive oxygen species, plasmalogens may function as effective antioxidants (12,13). They may be a reservoir of PUFA that serve as precursors or modulators in eicosanoid production (14–16). In addition, the high vinyl ether content of membrane phospholipids seems to be a ubiquitous characteristic of electrically active tissues such as heart and brain (17). Their presence in significant amounts in these tissues suggests that they may be involved in the regulation of transmembrane ion movements. Indeed, specific interactions of plasmalogens with membrane proteins have been observed, i.e., rabbit skeletal muscle calcium ATPase (18) and Na^+/K^+ ATPase (19). Moreover, plasmenylethanolamine (PmE), the major brain plasmalogen, is abundant in several subcellular membranes which undergo rapid membrane fusion, e.g., synaptic vesicles and plasma membranes (20). Because of the greater propensity of this phospholipid to adopt a hexagonal phase, vesicles containing PmE undergo fusion six times more rapidly than those containing phosphatidylethanolamine (PE). This suggests that PmE may be involved in membrane fusion, a process occurring during synaptic transmission (21). On the basis of these previous experiments, we hypothesized that plasmalogens play a role in neurotransmission processes. Thus, the aim of this study was to evaluate the influence of chronic dietary n-3 PUFA deficiency on plasmalogens in different brain regions under monoaminergic regulation by determining whether modifications could be obtained in their composition in parallel with changes in neurotransmission observed in the frontal cortex of rats deficient in n-3 PUFA. Accordingly, we measured the distribution and FA composition of major phospholipids of the brain, i.e., PE and PmE in rats chronically fed a diet deficient in n-3 PUFA. These components were studied in frontal cortex, striatum, and cerebellum. This last region was chosen as a reference area since it is poor in monoaminergic innervation (22).

MATERIALS AND METHODS

Animals and diets. The experimental protocol was in accordance with the procedure previously described by Delion *et al.* (11) and was detailed as follows: two generations of female Wistar rats (Laboratoire de Nutrition et Sécurité Alimentaire, Jouy-en-josas, France) were fed all their life a diet containing 6% fat as African peanut oil specifically deficient in α-linolenic acid. This deficient diet provided around 1200 mg of linoleic acid but less than 6 mg of linolenic acid per 100 g of diet. Two weeks before mating, at 8 wk of age, female rats originating from the second generation of the α-linolenic acid-deficient rats were divided into two equal groups. The first group continued to receive the deficient diet, and the second a diet in which peanut oil was replaced by a 60/40% mixture of peanut oil and rapeseed oil. The latter (control) diet provided the same amount of linoleic acid as the deficient diet and also around 200 mg of α-linolenic acid per 100 g of diet [(n-6)/(n-3) = 6]. Diets were consumed *ad libitum* by both groups. At weaning, the male progeny (third generation) of these groups of female rats received the same diet as their respective dams until sacrifice. Each measurement was realized on 6 male rats per group. Animals were killed at 60 d of age. The composition of diets and the composition in FA are reported in Tables 1 and 2. This protocol was in compliance with applicable guidelines from "Ministère de l'Agriculture, France."

Isolation of different brain regions. The animals were anesthetized with diethyl ether and infused with 0.9% saline sodium. The brain regions from each rat were rapidly removed and dissected, and the frontal cortex, striatum, and cerebellum were weighed and frozen at −80°C until use. Each tissue from each rat was homogenized in 10 vol of Tris-HCl buffer, pH 7.4.

Lipid analyses. Total lipids were extracted according to the method of Bligh and Dyer (23). A part of total lipid extract was used for total phosphorus (Pi) determination and another part for separation of the phospholipid classes by thin-layer chromatography (TLC) on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany), as described by Owens (24). The method is based on the lability of plasmalogens toward mercuric chloride. Lipid samples containing around 250 nmoles of lipid phosphorus were fractionated by TLC. The plates were then developed in freshly prepared chloroform/methanol/water/acetic acid (65:43:3:1, by vol). In order to separate PmE and PE, the left lipid track of the chromatogram was sprayed with 5 mM mercuric chloride in 10 mM

TABLE 1
Diet Composition

	Composition (g/kg diet)	
	Control	(n-3) Deficient
Casein	220	220
DL-Methionine	1.6	1.6
Cornstarch	432.4	432.4
Saccharose	216	216
Cellulose	20	20
Mineral mixture[a]	40	40
Vitamin mixture[b]	10	10
Peanut oil[c]	23.6	60
Rapeseed oil[c]	36.4	—

[a]Composition of mineral mixture (mg/kg diet): $CaHPO_4 \cdot 2H_2O$, 15200; K_2HPO_4, 9600; $CaCO_3$, 7200; NaCl, 2760; MgO, 800; $MgSO_4 \cdot 7H_2O$, 3600; $FeSO_4 \cdot 7H_2O$, 340; $ZnSO_4 \cdot H_2O$, 200; $CuSO_4 \cdot H_2O$, 40; NaF, 32; $CrK(SO_4)_2 \cdot H_2O$, 20; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 8; KI, 1.6; $CoCO_3$, 8; $Na_2SeO_3 \cdot 5H_2O$, 20.

[b]Composition of vitamin supplement, triturated in dextrose (mg/kg diet): retinyl acetate, 10; cholecalciferol, 0.0625; acetate all-*rac*-α-tocopherol, 50; menadione, 1; thiamine HCl, 10; riboflavin, 10; nicotinic acid, 45; D-calcium pantothenate, 30; pyridoxine HCl, 10; inositol, 50; D-biotin, 0.2; folic acid, 2; cyanocobalamin, 0.0135; L-ascorbic acid, 100; *p*-aminobenzoic acid, 50; choline chlorhydrate, 750.

[c]Total dietary lipids: 6 g/100 g diet.

TABLE 2
Fatty Acid Composition of Dietary Lipids

Fatty acid[a]	Composition (mg/100 mg fatty acids)	
	Control[b]	(n-3) Deficient[c]
16:0	8.1	9.9
18:0	2.4	3.1
20:0	0.9	1.2
22:0	1.2	1.8
24:0	0.6	0.8
∑ SFA	13.1	16.8
16:1n-7	1.1	0.0
18:1n-9	60.9	60.8
18:1n-7	0.0	0.0
20:1n-9	1.1	1.1
∑ MUFA	60.2	61.9
18:2n-6	21.2	21.3
∑ (n-6) PUFA	21.2	21.3
18:3n-3	3.6	<0.1
∑ (n-3) PUFA	3.6	<0.1
∑ (n-6) + (n-3)	24.8	21.3
(n-6)/(n-3)	5.9	>213
PUFA, mg/100 g diet		
18:2n-6	1196	1201
18:3n-3	203	<6

[a]Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]African peanut–rapeseed oil mixture (60.5%–39.5%).
[c]African peanut oil.

acetic acid, while the remainder of the chromatogram was screened. After being evaporated to dryness under a stream of nitrogen for 1 h, the plates were developed in the second dimension with chloroform/methanol/water (60:35:8, by vol). The chromatogram was allowed to dry and then sprayed with diphenylhexatriene (Sigma Chemical, St. Louis, MO) to locate phospholipid spots under ultraviolet light. Appropriate blank areas also were marked. Each spot was scraped off into tubes containing appropriate chloroform/methanol (2:1, vol/vol) solvents. Phospholipid fractions were extracted with chloroform/methanol (2:1) and then quantified for phosphorus content using the procedure of Bartlett (25) modified by Böttcher (26). Results are expressed in nmoles/100 nmoles of Pi. To determine the FA composition of the phospholipids studied, around 600 nmoles of Pi were used to separate the different phospholipids by the procedure described above. FA were saponified and then methylated with 6% methanolic sulfuric acid. Separation of fatty acid methyl esters was done by capillary gas chromatography on a Hewlett-Packard model N 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a capillary column (length, 50 m; diameter, 0.25 mm; CP-Sil 88; Chrompack, Les Ulis, France). The oven temperature was programmed to rise from 170 to 230°C at a rate of 2°C/min. The injector temperature was 230°C and the detector temperature 270°C. Peaks were integrated by a programmable integrator-calculator (D 2500A chromato-integrator; Merck, Nogent sur Marne, France). The relative concentration of each FA was expressed as percentage of identified FA equal or greater than 16 carbon atoms for tissue samples. For statistical analyses, a one-way analysis of variance (ANOVA) was performed, and comparisons of means were determined using the Student's *t*-test. Values between control and deficient rats were considered significantly different when $P < 0.05$.

RESULTS

Weights of body and brain regions. The difference in mean body weight of the deficient (248 ± 8 g) and control (246 ± 8 g) groups was not significant ($P > 0.05$, ANOVA) at the time of the study. The dietary α-linolenic acid deficiency had no effect on the brain region weight of the animals. The weights in control vs. deficient rats were 272 ± 45 vs. 239 ± 26 mg, 50 ± 9 vs. 53 ± 8 mg, and 275 ± 25 vs. 288 ± 7 mg in frontal cortex, striatum, and cerebellum, respectively. The difference was not significant ($P > 0.05$, ANOVA).

Proportions of phospholipids classes (Table 3). Analysis of phospholipid composition in all three areas studied concerned only PE and PmE. The EGP levels, i.e., sum of PE and PmE levels, were similar for the three brain regions in the control group but the proportions of PE and PmE in this phospholipid class varied according to the region. In frontal cortex and striatum, PE levels were slightly higher than PmE levels and represented 55 and 52% of EGP levels, respectively. In contrast, cerebellum contained higher amounts of PmE than PE, since data showed PE levels about 47% of EGP total levels. In all brain regions studied, the proportions of PE and PmE were not modified by the n-3-deficient diet.

Fatty acid composition of PE (Table 4). In control rats, total PUFA levels were rather similar between the three re-

TABLE 3
Phosphatidylethanolamine (PE) and Plasmenylethanolamine (PmE) Composition (nmoles/100 nmoles Pi) in the Frontal Cortex, Striatum, and Cerebellum of Rats Fed Either a Control or a n-3 PUFA-Deficient Diet[a]

Phospholipid classes	Frontal cortex		Striatum		Cerebellum	
	Control	Deficient	Control	Deficient	Control	Deficient
PE	16.5 ± 1.9	16.1 ± 1.3	16.3 ± 2.9	16.6 ± 1.6	13.2 ± 2.5	14.9 ± 2.6
PmE	13.5 ± 1.8	13.8 ± 1.7	14.8 ± 1.3	13.7 ± 1.5	15.2 ± 2.0	16.5 ± 1.7
EGP	29.9 ± 1.6	29.8 ± 2.4	31.1 ± 3.5	30.5 ± 2.8	28.4 ± 3.1	31.4 ± 3.2

[a]Values represent means ± SD, for six animals/group. Pi, total phosphorus; PE, phosphatidylethanolamine; PmE, plasmenylethanolamine; EGP, ethanolamine glycerophospholipids. For other abbreviation see Table 2.

TABLE 4
Acyl Chain Composition (% of identified fatty acids) of PE in the Frontal Cortex, Striatum, and Cerebellum of Rats Fed Either Control or n-3 PUFA-Deficient Diet[a]

	Frontal cortex		Striatum		Cerebellum	
Acyl chain	Control	Deficient	Control	Deficient	Control	Deficient
16:0	10.5 ± 0.9	7.9 ± 2.2	10.7 ± 2.0	9.7 ± 3.2	9.6 ± 1.5	10.1 ± 1.4
18:0	41.6 ± 1.7	34.1 ± 1.4[c]	36.0 ± 6.5	35.7 ± 2.4	32.8 ± 0.8	31.6 ± 1.5
∑ SFA	**52.7 ± 2.3**	**42.7 ± 2.7[b]**	**48.7 ± 4.5**	**46.5 ± 5.3**	**43.0 ± 2.7**	**42.6 ± 2.3**
18:1n-9	12.1 ± 1.3	10.7 ± 1.9	15.9 ± 3.3	12.8 ± 1.9	15.4 ± 1.4	14.9 ± 2.3
18:1n-7	2.2 ± 0.3	2.2 ± 0.1	2.1 ± 1.4	1.8 ± 1.1	3.8 ± 1.0	2.7 ± 1.0
∑ MUFA	**16.3 ± 1.7**	**14.8 ± 0.9**	**20.5 ± 2.5**	**16.3 ± 1.6[a]**	**20.9 ± 2.3**	**18.2 ± 1.5**
18:2n-6	0.5 ± 0.1	0.4 ± 0.1	1.4 ± 0.4	0.5 ± 0.2[a]	0.8 ± 0.3	0.7 ± 0.3
20:4n-6	11.4 ± 1.1	15.6 ± 0.5[c]	11.8 ± 0.4	14.4 ± 2.5	9.6 ± 1.0	11.0 ± 0.5[a]
22:4n-6	2.8 ± 0.3	4.8 ± 0.4[c]	3.8 ± 2.2	4.2 ± 0.7	2.7 ± 0.4	5.0 ± 0.6[c]
22:5n-6	0.9 ± 0.2	16.6 ± 1.6[c]	1.1 ± 0.9	13.3 ± 3.1[c]	0.7 ± 0.3	15.3 ± 1.5[c]
∑ (n-6) PUFA	**15.7 ± 1.7**	**37.6 ± 1.8[c]**	**18.4 ± 3.5**	**32.6 ± 5.9[b]**	**14.3 ± 0.9**	**33.2 ± 1.8[c]**
22:6n-3	14.4 ± 2.6	3.8 ± 0.5[c]	11.9 ± 1.9	3.4 ± 0.5[c]	21.0 ± 2.6	4.6 ± 0.7[c]
∑ (n-3) PUFA	**14.8 ± 2.2**	**4.1 ± 0.7[c]**	**12.2 ± 2.0**	**3.9 ± 0.5[c]**	**21.4 ± 2.4**	**5.2 ± 0.7[c]**
(n-6) + (n-3)	**30.6 ± 3.4**	**41.8 ± 2.5[b]**	**30.6 ± 5.2**	**36.6 ± 5.9**	**35.7 ± 3.2**	**38.4 ± 2.4**
(n-6)/(n-3)	1.0 ± 0.2	9.2 ± 1.2[c]	1.5 ± 0.2	8.4 ± 1.9[c]	0.7 ± 0.1	6.6 ± 0.2[c]
20:4n-6/22:6n-3	0.8 ± 0.1	4.1 ± 0.4[c]	1.0 ± 0.2	4.2 ± 0.6[c]	0.5 ± 0.1	2.4 ± 0.3[c]
22:5n-6/22:6n-3	0.06 ± 0.01	4.37 ± 0.47[c]	0.20 ± 0.10	3.79 ± 0.56[c]	0.03 ± 0.01	3.30 ± 0.16[c]

[a]Values represent means ± SD for six animals/group. See Tables 2 and 3 for abbreviations. ∑ (n-6) PUFA: sum of linoleic series. ∑ (n-3) PUFA: sum of linolenic series. (n-6) + (n-3): sum of linoleic and linolenic series. Minor fatty acids of the sum as 16:1n-9, 16:1n-7, 17:0, 18:3n-3, 20:0, 20:1n-9, 20:1n-7, 20:3n-9, 20:3n-6, 20:5n-3, 22:0, 22:1n-9, 24:0, and 22:5n-3 have been excluded from the table. [a]Significant difference from the control group at the level of $P < 0.05$. [b]Significant difference from the control group at the level of $P < 0.01$. [c]Significant difference from the control group at the level of $P < 0.001$.

gions, i.e., 31% in the frontal cortex and striatum and 36% in the cerebellum. However, the relative distribution of n-3 and n-6 PUFA varied from one brain area to another. The PUFA composition of frontal cortex and striatum PE did not differ significantly, but great differences were observed between cerebellum and the other two regions. The cerebellum was significantly enriched in 22:6n-3 compared to the other regions, by 46% ($P < 0.05$) and by 76% ($P < 0.01$) compared to frontal cortex and striatum, respectively, and the proportions of n-6 PUFA were less than the striatum, by 22% ($P < 0.05$).

Differences in FA composition of PE between the two dietary groups involved principally the PUFA. DHA levels were significantly decreased in all three regions compared to controls ($P < 0.001$). The decrease was significantly higher in cerebellum than in the striatum and in the frontal cortex, i.e., 78% in the cerebellum, 71% ($P < 0.01$) in the striatum, and 73% in the frontal cortex ($P < 0.05$). In the deficient group, the reduction in DHA was compensated for by the increase in level of n-6 PUFA including 22:5n-6, 22:4n-6, and 20:4n-6. The largest change occurred for 22:5n-6 for all brain regions studied. The overall results were that the total (n-6 + n-3) PUFA level in the deficient group was equivalent to that of the control group only in the striatum and the cerebellum. Total PUFA levels in the frontal cortex were significantly increased in the deficient group compared to the control group ($P < 0.01$), by 37%. Highest 22:5n-6/22:6n-3 ratio was observed in the frontal cortex compared to striatum and cerebellum in the deficient group. Although increased, 20:4n-6/22:6n-3 and 22:5n-6/22:6n-3 ratios were lower in the cerebellum of deficient rats than in the other two regions.

Fatty acid composition of PmE (Table 5). In the control group, PUFA proportions were dramatically different for each region. The level was significantly higher in the frontal cortex than in the striatum and the cerebellum ($P < 0.01$). DHA was the major fatty acid in frontal cortex PmE, since levels were significantly higher than in the striatum and the cerebellum by 31% ($P < 0.05$) and 33% ($P < 0.01$), respectively. Oleic acid (18:1n-9) was the predominant FA in striatum and cerebellum, and the level was 44% ($P < 0.001$) higher in the cerebellum than in the frontal cortex. So, in contrast with PE, significant differences were observed between frontal cortex and striatum in FA composition of PmE in control group. They involved 22:6n-3 and 18:1n-9.

n-3 PUFA deficiency induced profound modifications in PUFA proportions. As expected, DHA levels were significantly lower in the PmE of all regions for the deficient group, but the decrease was significantly greater in the frontal cortex and cerebellum PmE, i.e., 81 and 79%, respectively, than in the striatum (74%) ($P < 0.01$). Moreover, the increase of n-6 PUFA, which compensated for the low levels of n-3 PUFA, was significantly lower in the frontal cortex ($P < 0.05$) compared to the other two regions, i.e., 66, 82, and 85% increases were measured in the frontal cortex, striatum, and cerebellum, respectively. Total (n-6 + n-3) PUFA levels did not vary significantly in frontal cortex and cerebellum PmE, whereas this value increased significantly in striatum ($P < 0.01$).

In frontal cortex, as in cerebellum, the increase of n-6 PUFA was significantly higher in PE than in PmE in the deficient group ($P < 0.001$), whereas in the striatum, n-6 PUFA levels were increased in the same proportions in PE and PmE.

TABLE 5
Acyl Chain Composition (% of identified fatty acids) of PmE in the Frontal Cortex, Striatum, and Cerebellum of Rats Fed Either Control or n-3 PUFA-Deficient Diet[a]

	Frontal cortex		Striatum		Cerebellum	
Acyl chain	Control	Deficient	Control	Deficient	Control	Deficient
16:0	7.5 ± 1.8	8.5 ± 2.6	5.1 ± 2.2	2.6 ± 1.5	3.6 ± 1.7	5.9 ± 3.5
18:0	3.1 ± 1.9	4.9 ± 2.2	2.3 ± 1.0	2.7 ± 0.6	2.7 ± 1.1	2.8 ± 0.2
∑ SFA	**10.7 ± 3.7**	**16.3 ± 4.1**	**8.8 ± 2.7**	**7.7 ± 1.9**	**7.2 ± 2.6**	**9.3 ± 3.2**
18:1n-9	21.4 ± 1.8	17.8 ± 2.9	33.4 ± 6.1	24.3 ± 2.6[a]	38.0 ± 2.8	38.2 ± 5.9
18:1n-7	1.8 ± 0.6	1.7 ± 0.6	3.5 ± 2.1	2.6 ± 0.7	4.9 ± 2.7	2.6 ± 1.1
∑ MUFA	**25.6 ± 2.4**	**21.6 ± 3.5**	**39.2 ± 6.9**	**27.8 ± 2.9[a]**	**45.8 ± 1.9**	**43.2 ± 4.7**
18:2n-6	0.7 ± 0.3	1.0 ± 0.5	0.7 ± 0.3	0.3 ± 0.2	0.5 ± 0.1	1.5 ± 0.7[a]
20:4n-6	20.6 ± 0.3	21.8 ± 2.1	19.7 ± 2.4	24.5 ± 1.0[b]	13.4 ± 0.9	15.7 ± 0.9[b]
22:4n-6	9.6 ± 0.8	12.8 ± 1.0[b]	9.3 ± 2.5	13.4 ± 1.5[a]	7.8 ± 0.4	11.8 ± 2.2[b]
22:5n-6	1.2 ± 0.4	18.2 ± 3.3[c]	1.0 ± 0.4	18.4 ± 2.0[c]	0.6 ± 0.1	12.5 ± 1.6[c]
∑ (n-6) PUFA	**32.8 ± 1.5**	**54.4 ± 5.5[c]**	**31.3 ± 4.9**	**57.0 ± 3.7[c]**	**23.2 ± 0.8**	**42.8 ± 4.4[c]**
22:6n-3	28.5 ± 2.1	5.4 ± 0.8c	21.8 ± 4.0	5.3 ± 0.8[c]	21.4 ± 0.5	4.5 ± 1.0[c]
∑ (n-3) PUFA	**29.1 ± 1.9**	**6.7 ± 1.3[c]**	**22.4 ± 3.5**	**5.8 ± 0.4[c]**	**22.6 ± 1.3**	**5.6 ± 1.1[c]**
(n-6) + (n-3)	**62.0 ± 0.8**	**61.0 ± 5.8**	**51.7 ± 5.5**	**62.9 ± 4.4[a]**	**45.8 ± 1.0**	**48.4 ± 5.4**
(n-6)/(n-3)	1.1 ± 0.1	8.3 ± 1.4[c]	1.4 ± 0.2	9.9 ± 1.1[c]	1.0 ± 0.1	7.8 ± 1.1[c]
20:4n-6/22:6n-3	0.7 ± 0.1	4.0 ± 0.6[c]	0.9 ± 0.1	4.6 ± 0.6[c]	0.6 ± 0.1	2.8 ± 0.3[c]
22:5n-6/22:6n-3	0.04 ± 0.01	3.30 ± 1.41[c]	0.12 ± 0.10	3.45 ± 0.37[c]	0.02 ± 0.01	2.80 ± 0.33[c]

[a]Values represent means ± SD for six animals/group. Abbreviations as in Tables 2 and 3. Minor fatty acids of the sum as 16:1n-9, 16:1n-7, 17:0, 18:3n-3, 20:0, 20:1n-9, 20:1n-7, 20:3n-9, 20:3n-6, 20:5n-3, 22:0, 22:1n-9, 24:0, and 22:5n-3 have been excluded from the table. [a]Significant difference from the control group at the level of $P < 0.05$. [b]Significant difference from the control group at the level of $P < 0.01$. [c]Significant difference from the control group at the level of $P < 0.001$.

DISCUSSION

It appeared from the study that a chronic n-3 PUFA-deficient diet altered the PUFA composition of PmE and PE differently in the three brain regions.

Delion *et al.* (11) have reported that the proportions of different phospholipid classes in frontal cortex, striatum, and cerebellum were not modified by α-linolenic acid deficiency. However, these authors did not analyze the composition of plasmalogen phospholipids, important constituents in cerebral membranes. The role of these ether-lipids has not been well established, but their high concentrations in the brain tissue suggest an involvement in cerebral function. We analyzed plasmalogen content in the same regions and with the same animal model as Delion *et al.* (11), i.e., frontal cortex, striatum, and cerebellum in rats fed control diet and rats fed n-3 PUFA-deficient diet. Plasmalogen composition already has been analyzed in the whole rat brain (17), in the grey matter, white matter, and myelin in human brain (27), but no information is available on the distribution and acyl composition of plasmalogen of different brain regions. It appeared from this study that the proportions of EGP were similar in the three brain regions and were in accordance with proportions already observed in the whole rat brain (17). However, we showed that PmE and PE levels varied from one region to another, essentially from cerebellum to the other two regions. It has been previously shown that PmE are particularly enriched in the white matter and myelin (28,29). It can be speculated that PmE levels obtained in various regions of the central nervous system depend on the degree of myelination and proportion of white matter of the region. This explains the relatively high values of PmE for cerebellum, which contained larger amounts of white matter compared to striatum and frontal cortex. It is already interesting to note that the FA composition of cerebellum EGP differs from the FA composition of EGP observed in striatum and frontal cortex in control group. The great differences concerned PmE which contained larger amounts of monounsaturated fatty acids and low levels of n-6 PUFA compared to the other two regions. Almost 40% of cerebellum PmE contained oleic acid (18:1n-9) esterified in position *sn*-2. It is believed that plasmalogen species with 18:1 acyl chains participate exclusively in the activity of oligodendrial cells and myelin sheets (30).

PUFA composition of PE did not differ between the two regions under monoaminergic regulation, i.e., frontal cortex and striatum in the control groups, but marked differences were observed in PUFA composition of PmE: a greater proportion of DHA was found in the frontal cortex than in the striatum. Delion *et al.* (11) have demonstrated the greater richness of DHA in this region, and our study indicates the more specific implication of plasmalogen content. The present study also showed that the different cerebral regions studied seemed to have a specific need for n-6 and n-3 PUFA. It is interesting to note that striatum EGP are rich in n-6 PUFA and poor in n-3 PUFA compared to those present in the frontal cortex and cerebellum. By contrast, cerebellum EGP contained higher proportions of n-3 PUFA than n-6 PUFA. The proportions of n-6 PUFA were lowest in the cerebellum EGP. In the frontal cortex, n-6 PUFA and n-3 PUFA were equally distributed in the PE and in the PmE, but PUFA were present in larger amounts than in the other two regions. This area contained a higher concentration of 22:6n-3 compared to the

striatum, and highest levels of DHA were observed particularly in PmE.

Dietary n-3 PUFA deficiency did not modify plasmalogen proportions in the three brain regions. This diet seemed to have no influence on the synthesis of these phospholipids. However, this deficiency induced marked changes in FA content of PmE and PE. In all areas and phospholipids studied, our results showed a reduced 22:6n-3 level and compensatory higher levels of n-6 FA, essentially 22:5n-6 and 22:4n-6, in the deficient group. These results have been previously observed in the whole brain (12) and in total phospholipids of the same cerebral regions (11). In a recent study, this has been confirmed in the brain phosphatidylcholine, -ethanolamine and -serine of n-3-deficient rats. The proportion of 22:6n-3 was partially compensated for by an increase in the 22:5n-6 level (31). However, no studies have been performed on the effect of this diet in the FA composition of PmE and PE separately in brain. Our study showed that the total (n-6 + n-3) PUFA level could be increased or maintained according to the phospholipid and to the cerebral region. This specific phenomenon has not been observed in total phospholipids (11). Moreover, frontal cortex and striatum underwent different changes not only in PmE but also in PE. The decrease in DHA was significantly greater ($P < 0.05$) in frontal cortex PmE (81%) than in the striatum PmE (74%). This decrease level was similar in PE of these two regions. The increase of n-6 PUFA which compensated for the loss of n-3 PUFA was significantly lower ($P < 0.01$) in frontal cortex PmE (66%) than in PE of this region (140%), whereas in the striatum, n-6 levels were increased in the same proportions in PE and PmE, i.e., 94% in PE and 92% in PmE. In addition, this increase was significantly lower in the frontal cortex PmE than in the striatum PmE ($P < 0.05$) and in the cerebellum PmE ($P < 0.01$). On the basis of these observations, we could hypothesize that frontal cortex might be more specifically affected by n-3 PUFA deficiency than striatum. The frontal cortex contained a higher concentration of 22:6n-3 compared to the striatum, particularly in PmE. In deficient group, n-6 PUFA were incorporated preferentially in PE of the frontal cortex in comparison with PmE of this same region and with PmE of the other regions. This could correspond to the greater need for n-3 PUFA in frontal cortex PmE. The n-3 diet-induced deficiency in DHA may have modified the physicochemical properties of plasmalogen in the frontal cortex. By contrast, striatum could be most resilient to a n-3 deficiency by retaining more 22:6n-3 in PmE than frontal cortex. The decrease in DHA was significantly lower than that in frontal cortex ($P < 0.01$) and cerebellum ($P < 0.05$). So, striatum could be least affected by the n-3 PUFA deficiency.

A link has already been established between DHA and plasmalogen in human brain. DHA deficiency has been a constant finding in early-onset peroxisomal disorders in brain (32). These diseases are characterized by severe deterioration of the central nervous system. In addition, patients with impaired peroxisomal biogenesis have a deficient plasmalogen synthesis. Treatment with pure DHA ethyl ester of two patients with peroxisomal disease has increased the erythrocyte levels of 18:0 plasmalogen in both patients (33). This observation suggests a cause–effect relationship between the DHA deficiency and the defective plasmalogen synthesis in peroxisomal disorder. DHA is, in fact, an important factor in cerebral development, and it appears that this specific role could be linked to its presence in plasmalogen. Moreover, the ethanolamine plasmalogens are decreased in plasma membrane phospholipid in affected regions in Alzheimer's disease (34). The characteristic symptoms of this disease are progressive loss of memory and cognitive function. Behavior studies have shown that cognitive processes are altered in a dietary α-linolenic acid deficiency rat model. Enrichment of frontal cortex with plasmalogen species with 22:6n-3 acyl chains could be in accordance with a specific implication for this phospholipid in learning and cognitive functions.

In conclusion, in association with neurotransmission changes occurring only in the frontal cortex and not in the striatum, we show different modifications of the acyl composition of PmE and PE of the frontal cortex and the striatum. Different changes in plasmalogen composition of cerebral membranes in frontal cortex compared to striatum could contribute to alterations in synaptic transmission. In frontal cortex, PmE might be more affected by the n-3 deficient-diet. The striatum seems to be most resilient to the deficiency by retaining more 22:6n-3 than the two other areas in PmE.

We did not observe any modification of the proportions of PmE in the frontal cortex of deficient rats. However, PmE is concentrated in synaptic membranes. Studies focused on these membranes should provide more precise analysis, and we expect that changes in phospholipid composition will be observed specifically in these membranes.

DHA is preponderant in synaptic membranes (35) which are particularly rich in plasmalogens. DHA could have an influence on the mechanisms of neurotransmission through plasmalogen mediation. Studies focused on these synaptosomal membranes should determine whether the changes observed in frontal cortex plasmalogen relate specifically to these synaptic membranes.

ACKNOWLEDGMENTS

We thank Annie Guettier and Raymond Pontcharraud for excellent technical assistance.

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[Received September 17, 1997, and in final revised form January 14, 1998; revision accepted March 4, 1998]

Inhibitory Effect of Linoleic Acid on Chain Elongation and Desaturation of 18:2 *c,t* Isomers in Lactating and Neonatal Rats

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ABSTRACT: The previous studies showed that dietary 18:2 *c,t* isomers could be chain-elongated and desaturated to produce unusual 20:4 isomers. The present study was undertaken to determine the minimal amount of 18:2n-6 required to suppress the chain elongation and desaturation of 18:2 *c,t* isomers in the lactating and neonatal rats when animals were fed 15% partially hydrogenated canola oil diet containing 1.72% energy as 18:2 *c,t* isomers and varying amounts of free 18:2n-6. These diets induced marginal essential fatty acid (EFA) deficiency states (0.56% energy 18:2n-6) to EFA adequacy (2.56% energy 18:2n-6). After feeding for 50 d, the female animals were mated with males by overnight pairing. After conception, the lactating rats were killed, together with one pup from each dam, at term and day 26 of lactation. Two unusual 20:4 isomers in both maternal and neonatal liver phospholipids were identified as 20:4Δ5*c*,8*c*,11*c*,14*t* and 20:4Δ5*c*,8*c*,11*c*,15*t*, which were derived from 18:2Δ9*c*,12*t* and 18:2Δ9*c*,13*t*, respectively. The results showed that 18:2n-6 at about 2.0% of total energy in maternal diet was required to block the production of 20:4Δ5*c*,8*c*,11*c*,14*t* and 20:4Δ5*c*,8*c*,11*c*,15*t* in the maternal liver, whereas 18:2n-6 at about 2.5% of total energy in maternal diet was required to suppress production of these unusual 20:4 isomers in the neonatal liver.

Lipids 33, 409–416 (1998).

The health effects of dietary *trans* fatty acids in humans are controversial. Dietary *trans* isomers of oleic acid (18:1n-9) have been shown to raise serum low density lipoprotein cholesterol and lower high density lipoprotein cholesterol (1–3). Some epidemiological studies have suggested that high intake of *trans* fatty acids may be a significant risk factor in development of cardiovascular disease (4–7). However, other studies have not proved a health risk associated with their intake (8–11).

Several isomers of linoleic acid (18:2n-6) present in the Western diet are formed during refining or partial hydrogenation of vegetable oils. These isomers in the partially hydrogenated vegetable oil mainly include 18:2Δ9*c*,13*t*, 18:2Δ9*c*,12*t*, and 18:2Δ9*t*,12*c*, with the former being most abundant (12,13). We have previously found that human adipose tissue contains these 18:2 isomers and has a similar isomeric distribution as that in partially hydrogenated vegetable oils (14). We also have demonstrated that 10% of the breast milk samples of 198 Canadian lactating women have as much as 1.7% of total milk fat as these 18:2 isomers (15). Interest in these 18:2 *c,t* isomers is related to their possible inhibition of synthesis of arachidonic acid (20:4n-6) and formation of unusual 20:4 isomers *via* chain elongation and desaturation pathway (16,17). Subsequently, the unusual isomers of 20:4n-6 may, *via* lipoxygenase and cycloxygenase pathways, serve as precursors of uncommon eicosanoids with unknown structures and functions (18). In fact, dietary 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t*, *via* processes similar to the conversion of 18:2n-6 to 20:4n-6, have been shown to be desaturated and elongated to form 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* in the rat liver, respectively (19).

Trans fatty acids may interfere with biosynthesis of long-chain polyunsaturated fatty acids (20). One study showed that human infant birth weight was inversely correlated to *trans* fatty acids in cord blood (21). Both 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t* seem to be ideal substrates for Δ6 desaturase and compete for this desaturation and elongation pathway with 18:2n-6 since their first double bond is at Δ9 position and is in *cis* configuration. Thus, high dietary intake of these isomers may increase the requirement of 18:2n-6, especially during early life when adequate synthesis of very long chain polyunsaturated fatty acids such as 20:4n-6 and docosahexaenoic acid (22:6n-3) is essential for normal brain development. On the other hand, dietary 18:2n-6 should exhibit an inhibitory effect on the production of unusual 20:4 isomers. The present study was therefore undertaken to determine the minimal amount of dietary 18:2n-6 required to suppress the chain elongation and desaturation of these unusual 20:4 isomers in the neonatal and lactating rats fed partially hydrogenated canola oil (PHCO).

MATERIALS AND METHODS

Diet. The animals were fed one of six diets containing either 15% canola oil (Diet F) or PHCO (Diet A) with supplementa-

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Abbreviations: DMOX, 2-alkenyl-4,4-dimethyloxazoline; EFA, essential fatty acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; PHCO, partially hydrogenated canola oil; PL, phospholipid; TLC, thin-layer chromatography.

tion of free 18:2n-6 at 0.25% energy (Diet B), 0.50% energy (Diet C), 1.25% energy (Diet D), and 2.00% energy (Diet E). The other ingredients in the diets were, in g/kg, cornstarch, 288; sucrose, 243; casein, 235; cellulose, 32; AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10; choline bitartrate, 4; and DL-methionine, 3. The diets were prepared biweekly and stored at –4°C. The fatty acid composition of each diet was determined using gas–liquid chromatography (GLC, Table 1)

Animals. Female Wistar rats (200 g) were randomly divided into six groups and housed individually in an animal house maintained at 23°C with 12-h light and dark cycles. Diets and water were provided *ad libitum*. The females were mated with male rats by overnight pairing. The animals were fed one of six diets prior to pregnancy (50 d) and through gestation (21 d) and lactation (26 d). The litter size varied from 8–13 pups. The lactating rats were killed at day 26 of lactation under carbon dioxide anesthesia. One neonate from each dam was killed at term and day 26 of lactation. The maternal and neonatal livers were removed, washed with saline, and immediately frozen in liquid nitrogen and stored at –80°C.

Lipid separation. Total lipids of maternal and neonatal liver were extracted using chloroform/methanol (2:1, vol/vol) containing 0.02% butylated hydroxytoluene (Sigma Chemical, St. Louis, MO) as an antioxidant. L-Phosphatidylcholine (diheptadecanoyl) was added as an internal standard to an aliquot of the total lipid extract to quantify total phospholipids (PL). Lipid classes were separated by neutral lipid thin-layer chromatography (TLC; 20 × 20 cm plate precoated with 250 µm silica gel 60A; Macherey-Nagel, Duren, Germany) using a developing solvent system of hexane/diethyl ether/acetic acid (80:20:1, by vol). PL band was recovered from the TLC plate, and its fatty acids were converted to the corresponding fatty acid methyl esters (FAME) using 14% boron trifluoride in methanol (Sigma Chemical) under nitrogen gas.

Fatty acid analysis. FAME were analyzed by GLC using a SP-2560 flexible fused silica capillary column (100 m × 0.25 mm i.d., 20 µm film thickness; Supelco, Inc., Bellefonte, PA) in a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA). The column temperature was programmed from 180 to 220°C at a rate of 1°C/min and then held for 20 min. Injector and detector temperatures were set at 250°C. Hydrogen gas was used as the carrier gas at a column head pressure of 15 psi.

Determination of 18:1 trans, *18:2* trans*, and 20:4* trans *isomers.* The total 18:1 *trans* fatty acids were determined using $AgNO_3$-TLC in conjunction with capillary GLC as described previously (15,22). The geometric and positional isomers of 18:2 *trans* were identified and determined as described by Ratnayake and Pelletier (12). Determination and identification of 20:4 isomers were made as we previously described (19). In brief, the total FAME from the liver PL were saponified and acidified to the free fatty acids and then converted to 2-alkenyl-4,4-dimethyloxazoline (DMOX) derivatives (23). The double-bond positions were confirmed by GLC/mass spectrometry analysis of DMOX derivative. GLC separation of the DMOX derivatives was performed in the same SP-2560 capillary column, and helium was the carrier gas. Mass spectra of the DMOX derivatives were obtained on a VG Analytical MS system (Model 7070 EQ; VG Analytical, Manchester, England) equipped with an 11/250 data system interfaced to a Varian GC (Model Vista 6000; Varian Associates, Palo Alto, CA) operated at an ionization energy of 70 eV. Mass DMOX spectra of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* have been reported in detail elsewhere (19).

TABLE 1
Fatty Acid Composition (g/kg diet) of Dietary Fats

	Diet[a]					
Fatty acid	A	B	C	D	E	F
16:0	8.4	8.4	8.4	8.4	8.4	8.6
18:0	10.1	10.1	10.1	10.1	10.1	3.8
20:0	0.4	0.4	0.4	0.4	0.4	1.1
22:0	0.4	0.4	0.4	0.4	0.4	<0.1
Total saturates	19.2	19.2	19.2	19.2	19.2	13.5
16:1n-7	0.3	0.3	0.3	0.3	0.3	0.4
18:1n-9	73.1	73.1	73.1	73.1	73.1	83.5
Other 18:1	10.4	10.4	10.4	10.4	10.4	4.9
20:1n-9	1.4	1.4	1.4	1.4	1.4	2.9
22:1n-9	0.5	0.5	0.5	0.5	0.5	0.5
Total monounsaturates	85.7	85.7	85.7	85.7	85.7	92.2
18:2n-6	2.6	4.2	5.5	9.3	14.1	30.9
18:3n-3	<0.1	<0.1	<0.1	<0.1	<0.1	10.9
18:1*t*	33.3	33.3	33.3	33.3	33.3	<0.1
18:2Δ9*t*,12*t*	0.4	0.4	0.4	0.4	0.4	<0.1
18:2Δ9*c*,12*t*	1.6	1.6	1.6	1.6	1.6	<0.1
18:2Δ9*t*,12*c*	1.9	1.9	1.9	1.9	1.9	0.3
18:2Δ9*c*,13*t*	2.1	2.1	2.1	2.1	2.1	0.3
Total 18:2*c*,*t*	8.0	8.0	8.0	8.0	8.0	0.6

[a]Diet A, 15% partially hydrogenated canola oil (PHCO; 0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; and Diet F, 15% canola oil (reference diet).

RESULTS

Fatty acid composition of dietary fats. The fatty acid composition (g/kg diet) of the six diets is summarized in Table 1. Diets A–E had similar fatty acid composition except for 18:2n-6, which was increased from 2.6 to 14.1 g/kg diet. Dietary 18:2n-6 as percentage of total energy was 0.56% in Diet A, 0.81% in Diet B, 1.06% in Diet C, 1.81% in Diet D, and 2.56% in Diet E. The 18:1 *t* isomer in Diets A–E was 33.3 g/kg diet (7.17% energy), whereas 18:1n-9 was 73.1 g/kg diet (15.74% energy). The total 18:2 *c,t* isomers accounted for 8.0 g/kg diet (1.72% energy), with 18:2Δ9*c*,13*t* being a major isomer followed by 18:2Δ9*t*,12*c* and 18:2Δ9*c*,12*t*.

Food consumption, body and organ weights. Maternal rats fed different diets with varying levels of 18:2n-6 supplementation consumed similar amounts of food (18.1–20.8 g dry weight/rat/d). However, maternal rats in Group A fed PHCO diet appeared to gain less body weight than the rest of the

TABLE 2
Food Intake and Body Weight Gain[a]

	Group[b]					
	A	B	C	D	E	F
Maternal						
Food intake (g/d)	20.0 ± 1.5	19.0 ± 1.0	18.8 ± 0.7	18.1 ± 0.7	19.6 ± 1.7	20.8 ± 1.2
Body weight (g)						
Initial	183 ± 7	185 ± 12	183 ± 7	186 ± 7	191 ± 14	182 ± 7
Final	302 ± 27	324 ± 23	314 ± 31	310 ± 18	318 ± 13	322 ± 25
Neonatal body weight (g)						
At term	5.4 ± 0.5	5.9 ± 0.6	5.6 ± 0.7	6.8 ± 0.6	5.3 ± 0.5	6.7 ± 0.4
At day 26	64.9 ± 10.3	60.3 ± 6.6	69.7 ± 4.9	65.8 ± 4.9	68.9 ± 3.9	61.7 ± 8.9

[a]Data are mean ± SD/n = 6.
[b]Diet A, 15% PHCO (0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; Diet F, 15% canola oil. See Table 1 for abbreviations.

groups although a significant difference was not found statistically (Table 2).

Fatty acid composition of maternal liver and brain PL. The fatty acid composition of maternal liver at day 26 of lactation is shown in Table 3. In contrast to that observed with feeding the canola oil diet (Diet F), the feeding PHCO diet (Diet A) led to the incorporation of 18:1 *trans* and 18:2 *trans* fatty acids in maternal liver PL (Table 3).

TABLE 3
Fatty Acid Composition (% total) of Liver Phospholipids of Maternal Rats Fed PHCO with Varying Level of 18:2n-6 Supplement[a]

	Group[b]					
Fatty acids	A	B	C	D	E	F
16:0	9.68 ± 0.22[a]	8.14 ± 0.80[b]	8.23 ± 1.01[b]	7.63 ± 0.92[b]	9.31 ± 2.61[a]	10.75 ± 1.06[a]
18:0	21.83 ± 0.99	24.18 ± 1.54	23.43 ± 1.31	23.74 ± 1.03	23.25 ± 2.21	30.48 ± 1.31
16:1n-9	0.18 ± 0.04[a]	0.12 ± 0.03[b]	0.12 ± 0.04[b]	0.08 ± 0.04[b]	0.08 ± 0.04[b]	0.13 ± 0.03[b]
16:1n-7	0.53 ± 0.13[a]	0.41 ± 0.08[a]	0.42 ± 0.18[a]	0.27 ± 0.07[b]	0.27 ± 0.18[b]	0.21 ± 0.03[b]
18:1n-9	12.48 ± 2.00[a]	8.45 ± 1.79[b]	9.37 ± 1.22[a,b]	7.28 ± 1.27[b]	6.93 ± 2.45[b]	5.16 ± 0.83[c]
18:1n-7	1.57 ± 0.12	1.61 ± 0.30	1.57 ± 0.21	1.54 ± 0.16	1.41 ± 0.16	1.69 ± 0.15
Other 18:1*c*	1.88 ± 0.14[a]	1.54 ± 0.18[b]	1.46 ± 0.17[b]	1.35 ± 0.12[b]	1.20 ± 0.28[b]	0.22 ± 0.05[c]
20:1n-9	0.17 ± 0.02[b]	0.19 ± 0.03[a,b]	0.16 ± 0.04[b]	0.14 ± 0.03[b]	0.14 ± 0.04[b]	0.22 ± 0.02[a]
20:3n-9	4.85 ± 0.55[a]	2.81 ± 1.18[b]	1.99 ± 0.79[b]	0.53 ± 0.31[c]	0.59 ± 0.29[c]	0.02 ± 0.04[d]
18:2n-6	6.95 ± 1.32[a]	5.41 ± 1.53[a,b]	6.47 ± 1.26[a,b]	4.83 ± 1.40[b]	6.30 ± 2.11[a,b]	7.75 ± 0.95[a]
20:3n-6	1.35 ± 0.18[a]	1.29 ± 0.33[a]	1.34 ± 0.41[a]	0.78 ± 0.36[b]	0.87 ± 0.43[b]	0.66 ± 0.15[b]
20:4n-6	17.32 ± 2.66[c]	23.48 ± 2.98[b]	24.04 ± 2.77[a,b]	29.65 ± 1.53[a]	28.17 ± 2.91[a]	25.28 ± 0.63[a,b]
22:4n-6	0.14 ± 0.02[c]	0.21 ± 0.04[b]	0.24 ± 0.05[b]	0.38 ± 0.07[a,b]	0.63 ± 0.34[a]	0.10 ± 0.05[c]
22:5n-6	1.83 ± 0.75[c]	3.28 ± 1.15[b,c]	2.62 ± 0.69[c]	3.90 ± 0.46[b]	7.18 ± 2.90[a]	<0.01
18:3n-3	0.06 ± 0.04[b]	0.03 ± 0.04[b]	0.03 ± 0.04[b]	0.04 ± 0.03[b]	0.07 ± 0.04[b]	0.13 ± 0.02[a]
20:5n-3	0.23 ± 0.03[b]	0.01 ± 0.01[d]	0.09 ± 0.01[c]	<0.01	<0.01	0.94 ± 0.14[a]
22:5n-3	0.25 ± 0.06[b]	0.13 ± 0.10[c]	0.15 ± 0.11[c]	0.15 ± 0.08[c]	0.14 ± 0.09[c]	0.98 ± 0.21[a]
22:6n-3	4.68 ± 0.73[b]	5.11 ± 0.72[b]	4.36 ± 0.79[b]	4.25 ± 0.78[b]	4.11 ± 1.32[b]	11.06 ± 0.62[a]
18:1*t*	7.86 ± 0.36	8.18 ± 1.11	8.62 ± 1.03	9.18 ± 0.87	7.34 ± 1.55	<0.01
18:2Δ9*t*,12*t*	0.06 ± 0.00	0.02 ± 0.03	0.04 ± 0.03	0.07 ± 0.05	<0.01	<0.01
18:2Δ*c*,12*t*	0.18 ± 0.02[a]	0.16 ± 0.02[a]	0.17 ± 0.02[a]	0.15 ± 0.02[a]	0.11 ± 0.05[b]	<0.01
18:2Δ9*t*,12*c*	0.68 ± 0.11[a]	0.47 ± 0.11[b]	0.42 ± 0.11[b]	0.25 ± 0.05[c]	0.20 ± 0.07[c]	<0.01
18:2Δ(9*c*,13*t*/8*t*,12*c*)	0.28 ± 0.06[a]	0.19 ± 0.05[a]	0.19 ± 0.04[a]	0.13 ± 0.02[b]	0.11 ± 0.05[b]	<0.01
20:2Δ8*c*,14*c*	0.06 ± 0.04[a]	<0.01	<0.01	<0.01	0.01 ± 0.02[b]	<0.01
20:3Δ5*c*,8*c*,14*c*	0.13 ± 0.02[a]	0.05 ± 0.05[b]	<0.01	<0.01	<0.01	<0.01
20:3Δ5*c*,8*c*,11*c*	0.32 ± 0.03[a]	0.32 ± 0.06[a]	0.29 ± 0.08[a]	0.10 ± 0.03[b]	0.17 ± 0.09[b]	0.01 ± 0.04[c]
20:4Δ5*c*,8*c*,11*c*,15*t*	0.35 ± 0.08[a]	0.20 ± 0.09[a,b]	0.18 ± 0.07[b]	0.02 ± 0.04[c]	0.02 ± 0.03[c]	<0.01
20:4Δ5*c*,8*c*,11*c*,14*t*	0.50 ± 0.07[a]	0.40 ± 0.12[a,b]	0.36 ± 0.14[b]	0.10 ± 0.10[c]	0.05 ± 0.06[c]	<0.01

[a]Data are mean ± SD/n = 6.
[b]Diet A, 15% PHCO (0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; Diet F, 15% canola oil. Means in the same row with different superscript letters (a–d) differ significantly at $P < 0.05$. See Table 1 for abbreviations.

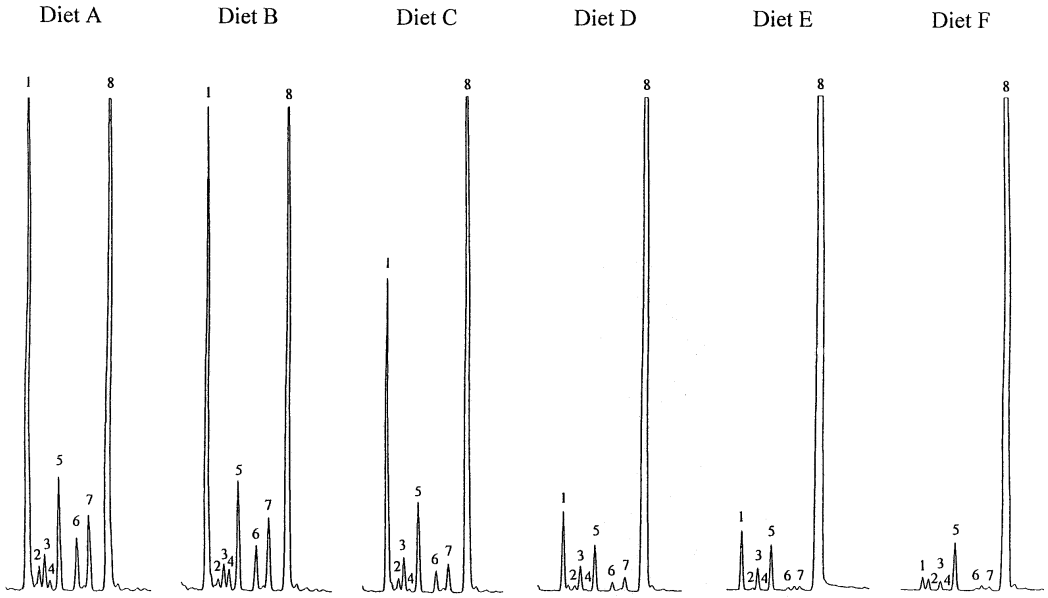

FIG. 1. The C20 region of gas chromatograms of the fatty acid methyl esters from the liver phospholipids of rats fed partially hydrogenated canola oil (PHCO). Peak identification: 1, 20:3n-9; 2, 20:3Δ5*c*,8*c*,14*c*; 3, 20:3Δ5*c*,11*c*,14*c*; 4, unknown; 5, 20:3n-6; 6, 20:4Δ5*c*,8*c*,11*c*,15*t*; 7, 20:4Δ5*c*,8*c*,11*c*,14*t*; 8, 20:4n-6. Diet A, 15% (PHCO; 0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; and Diet F, 15% canola oil.

Among 18:2 unusual isomers, the relative proportions of 18:2Δ(9*c*,13t/8*t*,12*c*), 18:2Δ9*c*,12*t*, 18:2Δ9*t*,12*c*, and 18:2Δ9*t*,12*t* were lower in the maternal liver PL than in the dietary PHCO. On the other hand, the proportion of these 18:2 *trans* isomers incorporated into the liver PL was different from that in the diet, with 18:2Δ9*t*,12*c* being highest followed by 18:2Δ(9*c*,13*t*/8*t*,12*c*). The feeding of PHCO diet (Diet A) also led to the formation of two unusual 20:4 isomers, namely, 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*, which collectively accounted for 0.85% of total liver PL. These isomers were not observed in Diet F, the reference diet. Supplementation with unesterified 18:2n-6 suppressed the formation of these two unusual 20:4 isomers in the maternal liver PL (Figs. 1 and 2; Table 3). The ratio of 20:3n-9 to 20:4n-6 in the liver PL of Group A maternal rats was 0.28, indicating borderline essential fatty acid (EFA) deficiency (24).

In addition, supplementation of dietary free 18:2n-6 (Diets B–E) did not result in an increase in the content of 18:2n-6 and 20:3n-6 in the maternal liver PL. However, it increased the metabolites of 18:2n-6 with four or more double bonds, including 20:4n-6, 22:4n-6, and 22:5n-6 (Table 3). In contrast, supplementation with dietary 18:2n-6 generally decreased the content of n-3 metabolites, mainly 20:5n-3, 22:5n-3, and 22:6n-3 in the maternal liver PL. Similarly, the content of monounsaturated fatty acids including 18:1n-9, 16:1n-9, and 16:1n-7 was decreased with supplementation of dietary 18:2n-6.

Feeding Diet A (PHCO) did not lead to the formation of the two unusual 20:4 isomers, 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*, in the brain PL of maternal rats. However, feeding PHCO (Diet A) increased the amount of 20:3n-9 in the brain PL. Supplementation with increasing amounts of dietary 18:2n-6 gradually decreased 20:3n-9 formation while increasing 20:4n-6, 22:4n-6, and 22:5n-6 in the maternal brain PL, but the levels of docosahexaenoic acid (22:6n-3) were unaffected (data not shown).

Fatty acid composition of neonatal liver and brain PL at day 26 of lactation. After feeding Diet A (PHCO) to the maternal rats, a higher incorporation of 18:1 *trans* and 18:2 *trans* fatty acids was observed in the neonatal liver PL at day 26 of lactation than that found in the maternal PL (Tables 3 and 4).

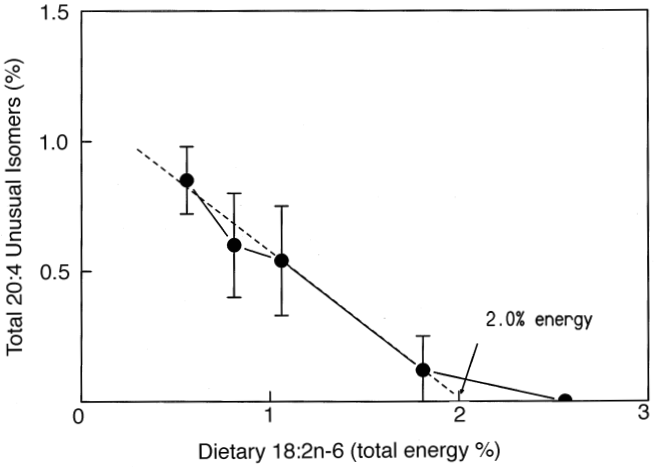

FIG. 2. Effect of dietary 18:2n-6 on biosynthesis of unusual 20:4 isomers (sum of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*) in the maternal liver. Data are expressed as mean ± SD/*n* = 6. Diet A, 0.56% energy 18:n-6; Diet B, 0.81% energy 18:2n-6; Diet C, 1.06% energy 18:2n-6; Diet D, 1.81% energy 18:2n-6; Diet E, 2.56% energy 18:2n-6.

TABLE 4
Fatty Acid Composition (% total) of Liver Phospholipids of Neonatal Rats from the Dam Fed PHCO with Varying Levels of 18:2n-6 Supplement[a]

	Group[b]					
Fatty acids	A	B	C	D	E	F
16:0	10.34 ± 0.80[b]	11.13 ± 0.95[b]	8.67 ± 0.57[b]	9.66 ± 0.62[b]	11.20 ± 0.68[b]	14.29 ± 0.55[a]
18:0	13.94 ± 1.48[c]	13.06 ± 1.19[c]	16.58 ± 1.48[b]	18.20 ± 1.97[a,b]	20.46 ± 0.33[a]	20.22 ± 1.81[a]
16:1n-9	0.60 ± 0.05[a]	0.57 ± 0.10[a]	0.59 ± 0.05[a]	0.30 ± 0.15[b]	0.18 ± 0.05[c]	<0.01
16:1n-7	0.12 ± 0.02[a]	0.08 ± 0.05[a]	0.12 ± 0.01[a]	0.02 ± 0.05[b]	0.08 ± 0.02[a]	<0.01
18:1n-9	15.67 ± 1.54[a]	16.96 ± 1.99[a]	12.24 ± 1.32[b]	8.78 ± 1.36[c]	7.88 ± 0.48[c]	8.79 ± 0.72[c]
18:1n-7	2.79 ± 0.20[b]	3.88 ± 0.35[a]	2.32 ± 0.25[b,c]	2.01 ± 0.23[c]	1.40 ± 0.07[d]	3.23 ± 0.32[a]
Other 18:1*c*	4.79 ± 0.47[a]	4.05 ± 0.17[a]	3.42 ± 0.48[b]	2.37 ± 0.33[c]	1.40 ± 0.19[d]	0.29 ± 0.05[e]
20:1n-9	0.37 ± 0.03[b]	0.30 ± 0.03[b]	0.32 ± 0.02[b]	0.19 ± 0.04[c]	0.19 ± 0.01[c]	0.79 ± 0.19[a]
20:3n-9	7.85 ± 0.40[a]	6.38 ± 0.31[b]	5.42 ± 0.18[c]	1.76 ± 0.45[d]	0.63 ± 0.09[e]	0.14 ± 0.03[f]
18:2n-6	6.72 ± 0.22[c]	8.49 ± 0.31[b]	8.25 ± 0.43[b]	5.53 ± 0.96[c]	6.69 ± 0.56[c]	10.15 ± 0.68[a]
20:3n-6	1.28 ± 0.09[b]	1.36 ± 0.12[b]	1.60 ± 0.08[a]	0.87 ± 0.16[c]	0.46 ± 0.08[d]	0.73 ± 0.09[c]
20:4n-6	9.55 ± 0.97[e]	8.75 ± 0.69[e]	15.42 ± 1.25[d]	24.52 ± 1.56[b]	28.19 ± 0.64[a]	19.90 ± 0.93[c]
22:4n-6	0.12 ± 0.01[d]	<0.01	0.16 ± 0.00[c]	0.38 ± 0.05[b]	0.85 ± 0.08[a]	0.17 ± 0.01[c]
22:5n-6	1.71 ± 0.31[c]	1.39 ± 0.27[c]	2.75 ± 0.30[b]	6.03 ± 1.00[a]	7.32 ± 0.88[a]	0.03 ± 0.07[d]
18:3n-3	0.06 ± 0.04[c]	0.03 ± 0.06[c]	0.02 ± 0.03[c]	0.02 ± 0.03[c]	0.10 ± 0.02[b]	0.34 ± 0.06[a]
20:5n-3	0.09 ± 0.01[b]	0.08 ± 0.01[b]	<0.01	<0.01	<0.01	1.37 ± 0.28[a]
22:5n-3	<0.01	<0.01	<0.01	0.14 ± 0.09[c]	0.25 ± 0.03[b]	1.29 ± 0.15[a]
22:6n-3	2.18 ± 0.37[c]	1.60 ± 0.16[d]	2.11 ± 0.13[c]	2.28 ± 0.24[c]	3.35 ± 0.21[b]	12.90 ± 0.55[a]
18:1*t*	11.20 ± 0.86[a]	9.81 ± 0.61[a,b]	11.79 ± 0.26[a]	11.73 ± 0.43[a]	6.76 ± 0.66[b]	<0.01
18:2Δ9*t*,12*t*	0.17 ± 0.23[a,b]	0.26 ± 0.24[a]	<0.01	<0.01	0.07 ± 0.11[b]	<0.01
18:2Δ9*c*,12*t*	0.38 ± 0.03[a]	0.40 ± 0.03[a]	0.29 ± 0.06[b]	0.25 ± 0.05[b]	0.11 ± 0.01[c]	<0.01
18:2Δ9*t*,12*c*	1.53 ± 0.09[a]	1.68 ± 0.09[a]	1.08 ± 0.10[b]	0.54 ± 0.16[c]	0.20 ± 0.02[d]	0.10 ± 0.06[e]
18:2Δ(9*c*,13*t*/8*t*,12*c*)	0.77 ± 0.07[a]	0.79 ± 0.07[a]	0.45 ± 0.10[b]	0.25 ± 0.07[c]	0.13 ± 0.01[d]	<0.01
20:2Δ8*c*,14*c*	0.22 ± 0.06[a]	0.18 ± 0.02[a]	0.13 ± 0.01[b]	<0.01	0.01 ± 0.02[c]	<0.01
20:3Δ5*c*,8c,14*c*	0.33 ± 0.04[a]	0.16 ± 0.10[c]	0.26 ± 0.01[b]	0.09 ± 0.05[d]	<0.01	<0.01
20:3Δ5*c*,8*c*,11*c*	0.38 ± 0.05[c]	0.31 ± 0.04[c]	0.63 ± 0.06[a]	0.45 ± 0.05[b]	0.26 ± 0.07[d]	0.12 ± 0.02[e]
20:4Δ5*c*,8*c*,11*c*,15*t*	0.66 ± 0.06[a]	0.56 ± 0.05[b]	0.43 ± 0.05[c]	0.19 ± 0.03[d]	<0.01	0.01 ± 0.03[e]
20:4Δ5*c*,8*c*,11*c*,14*t*	0.74 ± 0.13[a]	0.84 ± 0.20[a]	0.62 ± 0.07[b]	0.37 ± 0.07[a]	0.02 ± 0.03[d]	0.02 ± 0.05[d]

[a]Data are mean ± SD/*n* = 6.
[b]Diet A, 15% PHCO (0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; Diet F, 15% canola oil. Means in the same row with different superscript letters (a–f) differ significantly at $P < 0.05$. See Table 1 for abbreviation.

Dietary supplementation with unesterified 18:2n-6 in the maternal diet increased most of the longer-chain n-6 metabolites in the neonatal liver PL (20:4n-6, 22:4n-6, and 22:5n-6) except for 20:3n-6, which decreased. In contrast to the maternal liver, supplementation with dietary 18:2n-6 did not decrease the longer-chain n-3 metabolites. Instead, 22:6n-3 was increased in the liver PL of pups from the dams fed Diet E. Similar to that found in the maternal liver PL, supplementation of the maternal diet with 18:2n-6 decreased the incorporation of 18:2Δ(9*c*,13*t*/8*t*,12*c*), 18:2Δ9*c*,12*t*, 18:2Δ9*t*,12*c*, and 18:2Δ9*t*,12*t* in the neonatal liver PL. Among these unusual 18:2 isomers, 18:2Δ9*t*,12*c* exhibited the highest incorporation followed by 18:2Δ(9*c*,13*t*/8*t*,12*c*) and 18:2Δ9*c*,12*t* (Table 4). Compared with the maternal PL, feeding Diet A (PHCO) to the lactating rats produced a greater amount of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* in the neonatal liver PL (Table 4). In addition, supplementation of the maternal diet with 18:2n-6 gradually decreased the biosynthesis of these two 20:4 isomers in the neonatal rats (Fig. 3). The ratio of 20:3n-9 to 20:4n-6 in the liver PL of neonatal rats whose

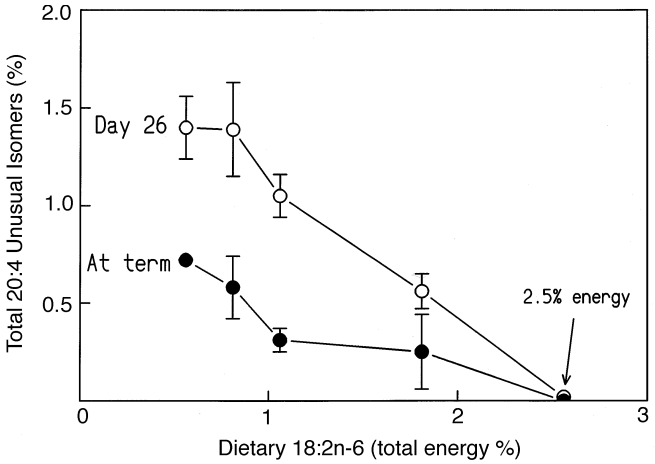

FIG. 3. Effect of dietary 18:2n-6 on biosynthesis of unusual 20:4 isomers (sum of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*) in the neonatal liver at term and day 26 of lactation. Data are expressed as mean ± SD/*n* = 6. Diet A, 0.56% energy 18:n-6; Diet B, 0.81% energy 18:2n-6; Diet C, 1.06% energy 18:2n-6; Diet D, 1.81% energy 18:2n-6; Diet E, 2.56% energy 18:2n-6.

TABLE 5
Fatty Acid Composition (% total) of Liver Phospholipids of Neonatal Rats (at term) from the Dam Fed PHCO with Varying Levels of 18:2n-6 Supplement[a]

	Group*					
Fatty acids	A	B	C	D	E	F
16:0	17.71 ± 0.42^{b}	16.72 ± 0.82^{b,c}	15.99 ± 2.46^{b,c}	15.83 ± 1.14^{c}	16.35 ± 0.66^{b,c}	19.80 ± 0.65^{a}
18:0	16.22 ± 0.63^{a}	14.82 ± 1.22^{b}	17.81 ± 3.22^{a}	15.59 ± 1.43^{a,b}	15.61 ± 1.08^{a,b}	17.67 ± 2.51^{a}
16:1n-9	3.39 ± 0.28^{a}	3.42 ± 0.30^{a}	2.50 ± 0.56^{b}	3.26 ± 0.45^{a}	1.62 ± 0.73^{c}	2.65 ± 0.43^{b}
16:1n-7	1.63 ± 0.18^{a}	1.45 ± 0.10^{a}	0.99 ± 0.22^{b}	1.51 ± 0.20^{a}	0.81 ± 0.33^{b}	1.25 ± 0.16^{a}
18:1n-9	23.02 ± 1.08^{a}	23.54 ± 2.21^{a}	18.16 ± 1.59^{b}	19.61 ± 1.25^{b}	16.35 ± 3.54^{b,c}	14.45 ± 0.75^{c}
18:1n-7	3.84 ± 0.23	3.49 ± 0.28	3.30 ± 0.38	3.51 ± 0.25	3.05 ± 0.48	3.37 ± 0.25
Other 18:1*c*	1.77 ± 0.11^{a}	1.90 ± 0.29^{a}	1.32 ± 0.17^{b}	1.48 ± 0.59^{a,b}	1.12 ± 0.17^{b}	0.22 ± 0.03^{c}
20:1n-9	0.18 ± 0.12	0.34 ± 0.09	0.17 ± 0.27	0.16 ± 0.09	0.25 ± 0.17	0.25 ± 0.14
20:3n-9	6.57 ± 0.37^{a}	5.64 ± 0.56^{b}	5.41 ± 0.43^{c}	2.64 ± 1.27^{d}	2.13 ± 0.45^{e}	1.20 ± 0.36^{f}
18:2n-6	3.67 ± 0.27^{b}	3.29 ± 0.35^{b}	4.98 ± 0.43^{a,b}	4.08 ± 0.60^{b}	6.02 ± 0.61^{a}	5.68 ± 0.50^{a}
20:3n-6	0.61 ± 0.04^{b}	0.54 ± 0.05^{b}	0.79 ± 0.07^{a}	0.07 ± 0.61^{c}	0.06 ± 0.79^{c}	0.71 ± 0.07^{a}
20:4n-6	7.92 ± 0.34^{c}	8.56 ± 0.82^{c}	12.72 ± 0.58^{b}	13.98 ± 1.51^{b}	19.13 ± 3.60^{a}	15.58 ± 0.89^{a}
22:4n-6	<0.01	0.47 ± 0.12^{c}	0.72 ± 0.31^{b}	0.65 ± 0.07^{b}	1.06 ± 0.42^{a}	0.67 ± 0.05^{b}
22:5n-6	1.34 ± 0.04^{e}	2.19 ± 0.22^{d}	2.97 ± 0.45^{c}	4.22 ± 0.63^{b}	6.58 ± 1.74^{a}	0.72 ± 0.13^{f}
18:3n-3	<0.01	0.04 ± 0.06	<0.01	0.03 ± 0.04	<0.01	<0.01
20:5n-3	<0.01	0.04 ± 0.02^{b}	0.06 ± 0.03^{b}	0.05 ± 0.04^{b}	0.04 ± 0.02^{b}	0.74 ± 0.07^{a}
22:5n-3	<0.01	<0.01	<0.01	<0.01	<0.01	0.54 ± 0.05
22:6n-3	2.32 ± 0.14^{c}	1.86 ± 0.17^{c}	2.76 ± 0.13^{b,c}	2.09 ± 0.31^{c}	3.44 ± 0.89^{b}	11.29 ± 0.94^{a}
18:1*t*	2.08 ± 0.12^{b}	2.87 ± 0.98^{a}	2.43 ± 1.30^{a,b}	1.90 ± 0.24^{b}	2.73 ± 0.53^{a}	<0.01
18:2Δ9*t*,12*t*	<0.01	<0.01	<0.01	0.19 ± 0.18	<0.01	<0.01
18:2Δ9*c*,12*t*	0.27 ± 0.19^{a,b}	0.39 ± 0.09^{a}	0.05 ± 0.11^{c}	0.36 ± 0.04^{a}	0.19 ± 0.21^{b}	<0.01
18:2Δ9*t*,12*c*	1.25 ± 0.07^{a}	1.12 ± 0.11^{a}	0.76 ± 0.10^{b}	0.80 ± 0.07^{b}	0.24 ± 0.25^{c}	0.05 ± 0.12^{d}
18:2Δ(9*c*,13*t*/8*t*,12*c*)	0.73 ± 0.04^{a}	0.78 ± 0.12^{a}	0.48 ± 0.05^{b}	0.49 ± 0.11^{b}	0.07 ± 0.17^{c}	<0.01
20:2Δ8*c*,14*c*	0.20 ± 0.13^{a}	0.23 ± 0.05^{a}	0.21 ± 0.21^{a}	0.06 ± 0.06^{b}	<0.01	<0.01
20:3Δ5*c*,8*c*,14*c*	<0.01	0.17 ± 0.05	<0.01	<0.01	<0.01	<0.01
20:3Δ5*c*,8*c*,11*c*	0.05 ± 0.09^{b}	0.25 ± 0.04^{a}	0.33 ± 0.08^{a}	0.15 ± 0.09^{a,b}	0.20 ± 0.11^{a,b}	<0.01
20:4Δ5*c*,8*c*,11*c*,15*t*	0.28 ± 0.02^{a}	0.18 ± 0.10^{b}	0.04 ± 0.08^{c}	0.11 ± 0.10^{b}	<0.01	<0.01
20:4Δ5*c*,8*c*,11*c*,14*t*	0.45 ± 0.02^{a}	0.40 ± 0.11^{a}	0.27 ± 0.06^{b}	0.14 ± 0.10^{c}	<0.01	<0.01

[a]Data are mean ± SD/n = 6.
[b]Diet A, 15% PHCO (0.56% energy 18:2n-6); Diet B, 15% PHCO + 0.25% energy 18:2n-6; Diet C, 15% PHCO + 0.50% energy 18:2n-6; Diet D, 15% PHCO + 1.25% energy 18:2n-6; Diet E, 15% PHCO + 2.00% energy 18:2n-6; Diet F, 15% canola oil. Means in the same row with different superscript letters (a–f) differ significantly at $P < 0.05$. See Table 1 for abbreviation.

mothers had been fed Diets A and B was 0.82 and 0.73, respectively, suggesting induction of EFA deficiency. Further increases in the proportion of dietary 18:2n-6 in the maternal diet (C–E) gradually decreased the ratio to less than 0.4 (24).

As observed in the maternal rats, 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* were not detected in the brain PL of neonatal rats at day 26 of lactation. Supplementation of dietary 18:2n-6 increased 22:4n-6 and 22:5n-6 without affecting the level of 20:4n-6. In contrast, the inclusion of unesterified 18:2n-6 in the diets decreased both 20:3n-9 and 22:6n-3 in the brain PL of neonatal rats (data not shown).

Fatty acid composition of neonatal liver and brain PL at term. Feeding Diet A (PHCO) to the maternal rats also resulted in an incorporation of 18:1 *trans* and 18:2 *trans* fatty acids in the neonatal liver at term. However, the degree of incorporation of these isomers in the neonatal liver PL at term was less than that at day 26 of lactation but more than that in the maternal liver PL (Table 5). Similarly, the longer-chain n-6 metabolites in the neonatal liver PL at term increased with supplementation of dietary 18:2n-6 in the maternal diet except for 20:3n-6 (Table 5). Compared with that in the maternal liver PL, a similar amount of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* also was found in the neonatal liver PL at term. Supplementation with 18:2n-6 at the level of 2.0% energy in the maternal diet completely blocked the production of these two unusual isomers (Fig. 3; Table 5).

The change caused by supplementation of varying levels of 18:2n-6 in the brain PL of neonatal rat at term was similar to that observed at day 26 of lactation. The ratio of 20:3n-9 to 20:4n-6 in the liver PL of Group A, B, and C neonatal rats at term was more than 0.4, and supplementation of dietary 18:2n-6 in the maternal diet gradually decreased the ratio to the level less than 0.1 (24).

DISCUSSION

Many geometric and positional isomers of 18:2n-6 are formed during partial hydrogenation of vegetable oils (12,13) or during the deodorization of oils (25,26); 18:2Δ9*c*,13*t*, 18:2Δ9*c*,12*t*, and 18:2Δ9*t*,12*c* are the major *trans* isomers of 18:2n-6. A significant amount of these isomers is present in margarine and other dietary fats (12). The present results support the previous observation that some of these 18:2 isomers can be chain-elongated and desaturated to form unusual 20:4 isomers in the rats fed PHCO. This occurred not only in male rats (19) but also in neonatal and pregnant/lactating rats fed PHCO diet. Two unusual 20:4 isomers, 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t,* have been previously identified and are most likely derived from two corresponding precursors, 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t, via* alternating sequence of desaturation, chain elongation, and desaturation (19).

It is known that dietary 18:2n-6 exhibits an inhibitory effect on production of unusual 20:4 isomers in the liver because 18:2 *c,t* isomers and 18:2n-6 compete for the same desaturase and elongase pathways (27). In a study by Holman *et al.* (17), the formation of these unusual 20:4 isomers was extensive and was even more than 20:4n-6 in the liver PL of rats fed partially hydrogenated soybean oil. However, the partially hydrogenated soybean oil used in their study contained very low 18:2n-6 (<0.08% of total fatty acids) and should be regarded as an EFA-deficient diet (28). In another study (19), it was found that 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t* were metabolized to 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t*, respectively, even under sufficient intake of 18:2n-6 (1.46% energy). This led us to determine the minimal amount of dietary 18:2n-6 required to completely suppress chain-elongation and desaturation of 18:2 *c,t* isomers. As shown in Figure 1, it is clear that formation of unusual 20:4 isomers in the rats fed PHCO is inversely associated with dietary supplementation of 18:2n-6. The present results suggest that 18:2n-6 at about 2.0% of total energy is required to block completely the production of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* in the maternal liver PL (Fig. 2), whereas 18:2n-6 intake at about 2.5% of total energy in the maternal diet is minimal to suppress synthesis of these unusual 20:4 isomers in the neonatal rats (Fig. 3).

In parallel with formation of 20:4Δ5*c*,8*c*,11*c*,15*t* and 20:4Δ5*c*,8*c*,11*c*,14*t* in both neonatal and maternal rats of Group A (0.56% energy 18:2n-6) fed PHCO diet, the ratio of 20:3n-9 to 20:4n-9 was relatively higher (maternal rats: 0.28; neonatal rats: 0.82), indicating a marginal EFA deficiency in maternal rats and EFA deficiency in neonatal rats (24). When 18:2n-6 in maternal diet was increased to 1.06% total energy (Diet C), the ratio was correspondingly reduced to 0.10 in maternal rats while in the neonatal rats it was only reduced to 0.35, indicating the level of 18:2n-6 in diet C was not adequate for neonatal rats but it was adequate for maternal rats fed PHCO diet with supplementation of 0.50% energy 18:2n-6. The present data demonstrated clearly not only that 20:4 *trans* isomers were formed but also that the requirement for dietary 18:2n-6 was increased in rats fed PHCO diet, particularly the neonatal rats.

The present study, together with previous work (19), has demonstrated clearly that formation of unusual 20:4 isomers is possible in the rats fed PHCO when dietary 18:2 is well above 0.6% energy recommended by the National Research Council for EFA adequacy (29). However, it still remains unclear whether these unusual 20:4 isomers can be precursors of uncommon eicosanoids and exert physiological effect despite being quantitatively minor (18). However, a *trans* isomer of 22:6n-3 derived from a mono *trans* isomer of linolenic acid (18:3n-3) *via* desaturation and elongation system has been found to incorporate into the retina of rats (30) and consequently alter the electroretinography (31).

The examination of incorporation pattern of all 18:2 *c,t* isomers into the liver PL indicates a preferential accumulation of 18:2Δ9*t*,12*c* to 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t*. This observation was in agreement with many previous studies (27,32,33). In a study using deuterium labeling, 18:2Δ9*t*,12*c* was found to incorporate into the mouse liver several times faster than 18:2Δ9*c*,12*t* (27). However, the mechanism involved remains poorly understood. Perhaps a lower accumulation of 18:2Δ9*c*,13*t* and 18:2Δ9*c*,12*t* than 18:2Δ9*t*,12*c* is due to their faster catabolic rate. In fact, only the former two isomers but not the latter can be desaturated and elongated to form corresponding longer-chain metabolites. This is most likely because the Δ6 desaturase recognizes an 18:2 isomer with the first double bond at Δ9 position with a *cis* configuration better than the other isomers (34).

In conclusion, the present study was the first to determine the minimal amount of dietary 18:2n-6 required for complete suppression of chain-elongation and desaturation of the geometric and positional isomers of 18:2n-6 in the neonatal and lactating rats fed a PHCO diet. The present results indicate that dietary 18:2n-6 intake at the recommended 0.6% energy for EFA adequacy is insufficient to inhibit biosynthesis of unusual 20:4 isomers derived from 18:2 *c,t* isomers. In view of the higher requirement for longer-chain polyunsaturated fatty acids during fetal/neonatal development, the overconsumption of partially hydrogenated vegetable oils containing a significant amount of 18:2 *c,t* isomers should be questioned although the physiological effect of these unusual 20:4 isomers remains undetermined at the present time.

ACKNOWLEDGMENTS

We thank the Hong Kong Research Grant Council for support of this project (CUHK 352/95M). David Forster and Greg Hatfield (CanAmera Foods, Toronto, Canada) are thanked for preparation of PHCO. Li-Ya Wang is from Luoyang Normal College, Luoyang, Henan, China.

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[Received October 30, 1997, and in final revised form January 22, 1998; revision accepted February 10, 1998]

Conjugated Linoleic Acids Alter Bone Fatty Acid Composition and Reduce *ex vivo* Prostaglandin E_2 Biosynthesis in Rats Fed n-6 or n-3 Fatty Acids

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ABSTRACT: This study evaluated the effects of conjugated linoleic acids (CLA) on tissue fatty acid composition and *ex vivo* prostaglandin E_2 (PGE_2) production in rats given diets varying in n-6 and n-3 fatty acids. Four groups of rats were given a basal semipurified diet (AIN-93G) containing 70 g/kg of added fat for 42 d. The fat treatments were formulated to contain CLA (0 vs. 10 g/kg of diet) and n-6 (soybean oil having an n-6/n-3 ratio of 7.3) and n-3 fatty acids (menhaden oil + safflower oil having an n-6/n-3 ratio of 1.8) in different ratios in a 2×2 factorial design. Fatty acids in liver, serum, muscle, heart, brain, spleen, and bone (cortical, marrow, and periosteum) were analyzed by capillary gas–liquid chromatography. The various dietary lipid treatments did not affect growth; however, CLA improved feed efficiency. The CLA isomers were found in all rat tissues analyzed although their concentrations varied. Dietary CLA decreased the concentrations of 16:1n-7, 18:1, total monounsaturates and n-6 fatty acids, but increased the concentrations of n-3 fatty acids (22:5n-3 and 22:6n-3), and saturates in the tissues analyzed. *Ex vivo* PGE_2 production in bone organ culture was decreased by n-3 fatty acids and CLA. We speculate that CLA reduced the concentration of 18:1 fatty acids by inhibiting liver Δ9-desaturase activity. The fact that CLA lowered *ex vivo* PGE_2 production in bone organ culture suggests that these conjugated fatty acids have the potential to influence bone formation and resorption.

Lipids 33, 417–425 (1998).

Conjugated linoleic acids (CLA) are positional and geometric isomers of linoleic acid that occur naturally in several foods but are highest in dairy products and beef (1). The CLA isomers have been suggested to possess potent beneficial health and biological effects (2–6). CLA isolated from extracts of grilled ground beef were found to exhibit anticarcinogenic activity against chemically induced skin cancer in mice (7). Recent experiments on prostate cancer cell lines demonstrated that CLA isomers are incorporated into cell lipids and that CLA compared to linoleic acid decreased cell proliferation (8). Further, CLA were reported to reduce prostaglandin E_2 (PGE_2) concentration in rat serum and spleen (9).

CLA isomers become enriched in tissue and cell lipids when administered in a diet or supplemented in a cell culture medium (8–10). As CLA incorporates into membrane phospholipids, it may compete in elongation and desaturation steps with other unsaturated fatty acids that are precursors of arachidonic acid (the precursor of PGE_2), and such competition in arachidonate synthesis may alter eicosanoid biosynthesis (11,12). The *cis*-9, *trans*-11 isomer of CLA was preferentially incorporated into the membrane phospholipids of mice (13) and rats (14), suggesting that *cis*-9, *trans*-11 is the biologically active isomer. Therefore, CLA may exert physiological effects by altering the formation of phospholipid-derived hormones, e.g., eicosanoids, or by modifying the signal transduction system (15).

This laboratory recently reported that milk fat stimulated bone formation rate in growing chicks possibly by modulating *ex vivo* PGE_2 production in bone (16). Having a concentration up to 30 mg CLA/g fat (17), milk fat is a primary dietary source of CLA. With regard to bone metabolism, PGE_2 is recognized as a mediator of bone formation and bone resorption *in vivo* (18). Although CLA recently has been shown to affect PGE_2 production (9) and to modulate cytokine production (19), the purpose of the present investigation was twofold: first, to quantify the effects of CLA on the fatty acid composition of tissue lipids in the presence of relatively high dietary n-6 or n-3 fatty acids; second, to document that CLA could alter PGE_2 production in mammalian bone in the presence of dietary lipids varying in an n-6/n-3 fatty acid ratio.

MATERIALS AND METHODS

Animals and diets. Forty male weanling rats (21-d-old, mean body weight 46 ± 2.0 g; Harlan Sprague-Dawley, Indianapolis, IN) were housed in individual cages under a 12-h light-dark cycle. Animal care was in compliance with applicable guidelines from the Purdue University (West Lafayette, IN) policy on animal care and use. The rats were randomly assigned to four groups and fed *ad libitum* the basal diet (AIN-93G without fat; Dyets, Inc., Bethlehem, PA) with one of the following fat treatments at 70 g/kg (Table 1): soybean oil (SBO, diet rich in n-6 fatty acids); soybean oil + CLA

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Abbreviations: BF_3, boron trifluoride; CLA, conjugated linoleic acids; FAME, fatty acid methyl esters; MSO, menhaden oil + safflower oil diet; MSOC, menhaden oil and safflower oil + CLA diet; PGE_2, prostaglandin E_2; PUFA, polyunsaturated fatty acid; SBO, soybean oil diet; SBOC, soybean oil + CLA diet.

TABLE 1
Fatty Acid (wt%) and Ingredient Composition of the Diets Given to Rats[a,b,c]

	Dietary lipid treatments[d]			
Fatty acid	SBO	SBOC	MSO	MSOC
12:0 (lauric)	Trace	Trace	0.08	0.03
14:0 (myristic)	0.20	0.19	3.64	3.07
15:0 (pentadecylic)	n.d.[e]	n.d.	0.34	0.29
16:0 (palmitic)	10.32	8.80	12.19	10.23
16:1n-7 (palmitoleic)	0.09	Trace	4.83	4.05
17:0 (margaric)	0.07	Trace	0.41	0.35
18:0 (stearic)	4.63	3.96	3.11	2.60
18:1 (octadecenoic)	23.18	19.75	12.25	10.47
18:2n-6 (linoleic)	52.84	45.33	36.67	32.38
18:3n-3 (α-linolenic)	7.26	6.24	1.03	0.85
18:4n-3 (stearidonic)	n.d.	n.d.	1.89	1.64
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	n.d.	6.52	n.d.	7.11
18:2(*t*-10,*c*-12)	n.d.	6.84	n.d.	7.34
18:2(*c*-9,*c*-11/*c*-10,*c*-12)	n.d.	0.55	n.d.	Trace
18:2(*t*-9,*t*-11/*t*-10,*t*-12)	n.d.	0.42	n.d.	0.43
20:0 (arachidic)	0.35	0.34	0.27	Trace
20:1n-9 (gondoic)	0.16	n.d.	0.73	0.65
20:4n-6 (arachidonic)	n.d.	n.d.	0.42	0.35
20:5n-3 (eicosapentaenoic)	n.d.	n.d.	8.02	6.72
22:0 (behenic)	0.47	0.42	Trace	Trace
22:5n-3 (docosapentaenoic)	n.d.	n.d.	1.51	1.27
22:6n-3 (docosahexaenoic)	n.d.	n.d.	8.57	7.20
SAT[f]	16.04	13.71	20.04	16.57
MONO[g]	23.44	19.75	17.81	15.17
PUFA[h]	60.09	51.57	58.10	50.41
n-6 PUFA	52.84	45.33	37.09	32.73
n-3 PUFA	7.26	6.24	21.01	17.67
n-6/n-3	7.28	7.27	1.77	1.85

[a]The semipurified basal diet contained the following (g/kg): casein, 200; corn starch, 397.486; DYETROSE, 132; sucrose, 100; cellulose, 50; L-lysine, 3; choline bitartrate, 2.5; salt mix, 35; vitamin mix, 10.
[b]Salt mix provided (mg/kg diet): $CaCO_3$, 12495; K_2HPO_4, 6860; $C_6H_5O_7K_3 \cdot H_2O$, 2477; NaCl, 2590; K_2SO_4, 1631; MgO, 840; $C_6H_5O_7Fe$, U.S.P., 212.1; $ZnCO_3$, 57.75; $MnCO_3$, 22.05; $CuCO_3$, 10.5; KIO_3, 0.35; Na_2SeO_4, 0.359; $(NH_4)_2MoO_4 \cdot H_2O$, 0.278; $Na_2O_3Si \cdot 9H_2O$, 50.75; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; LiCl, 0.609; H_3BO_3, 2.853; NaF, 2.223; $NiCO_3$, 1.113; NH_4VO_3, 0.231.
[c]Vitamin mix provided (mg/kg diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 7; niacin, 30; calcium pantothenate, 16; folic acid, 2; biotin, 0.2; cyanocobalamin (B-12) (0.1%), 25; vitamin A palmitate (500,000 IU/g), 4000; vitamin E acetate (500 IU/g), 75; vitamin D_3, 1000; vitamin K_1, 0.75.
[d]Dietary treatments included soybean oil (SBO), or menhaden oil + safflower oil (MSO) and with (+) or without (–) conjugated linoleic acids [CLA; 41.9% 18:2(*c*-9,*t*-11/*t*-9,*c*-11), 44.3% 18:2(*t*-10,*c*-12), 13.6% 18:2(*t*-9,*t*-11/*t*-10,*t*-12), and <1% 18:2(*c*-9,*c*-11/*c*-10,*c*-12)]. Total fat content in each diet was 70 g/kg of diet and CLA was added (10 g/kg of diet) at the expense of SBO or MSO.
[e]n.d., not detected (peak detection at 10 ng).
[f]SAT, total saturated fatty acids.
[g]MONO, total monounsaturated fatty acids.

(SBOC); menhaden oil + safflower oil mixture (MSO, menhaden/safflower = 56:44 weight basis, diet rich in n-3 fatty acids); MSO + CLA (MSOC). The CLA was added at 10 g/kg at the expense of the SBO or MSO. All dietary lipids were stored at –80°C before use. Four 49-d-old rats were used in a preliminary study to evaluate the acute enrichment of tissues with CLA. Two rats were fed SBO and the other two SBOC, and the rats on the SBOC diet also were administered CLA intragastrically twice (0.5 g CLA mixed with 0.5 g SBO, at day six and eight) before being killed after 9 d of feeding. Fresh diets were mixed every 14 d, and all the diets were kept at –20°C until fed. Food cups were refilled three times weekly and feed consumption measured at the same time. Body weights were recorded weekly and feed efficiency (total gram weight gain/total gram feed consumed) calculated for each treatment group.

Sample collections. After 42 d of feeding (9 d of feeding for the four rats in the preliminary study), the rats were killed and tissue samples (liver, heart, thigh muscle, spleen, femur, blood, and brain) collected for lipid analysis. Blood was collected by bleeding from axillary vessels. All samples were kept on ice at the time of collection and then frozen at –20°C. Serum was separated by centrifuging the clotted (room temperature) blood at $1200 \times g$ for 20 min (Beckman GS-6; Palo Alto, CA) and stored at –20°C until analyzed.

Analysis of fatty acids. Lipids in the diet and tissue samples were extracted with chloroform/methanol (2:1, vol/vol). Fatty acid methyl esters (FAME) were prepared from dietary lipids by transesterification using boron trifluoride (BF_3) in methanol (14%, w/w; Supelco Inc., Bellefonte, PA). Cortical bone, freed of periosteum and marrow, was cooled with liquid nitrogen and pulverized to a fine powder with a mortar and pestle, placed in 7 mL methanol, and sonicated for 10 min to extract lipids (16). Lipids extracted from rat tissue samples were methylated (sodium methoxide) as follows: sample was dissolved in dry toluene (1 mL) in a test tube with a Teflon-lined screw cap, 0.5 M sodium methoxide in anhydrous methanol (2 mL) added, the solution maintained at 50°C for 10 min, and glacial acetic acid (0.1 mL) added followed by deionized water (5 mL). The FAME were extracted into hexane (2×3 mL), dried over anhydrous sodium sulfate, and filtered. For comparison, lipids from several replicate samples were methylated using BF_3-methanol. All FAME were analyzed using a gas chromatograph (HP 5890 series II, autosampler 7673, HP 3365 ChemStation; Hewlett-Packard Co., Avondale, PA) equipped with a DB 225 or DB 23 column (30 m, 0.53 mm i.d., 0.5 μm film thickness; J&W Scientific Co., Folsom, CA) and operated at 140°C for 2 min, temperature programmed 1.5°C/min to 198°C and held for 7 min. The injector and flame-ionization detector temperatures were 225 and 250°C, respectively. FAME were identified by comparison of their retention times with authentic standards [GLC-422, CLA (UC-59-A and UC-59-M); Nu-Chek-Prep, Elysian, MN; CLA (Cat# 1245, *c*-9,*t*-11 and Cat# 1181, *t*-9,*t*-11); Matreya Inc., Pleasant Gap, PA] and FAME prepared from menhaden oil (Matreya Inc.).

Ex vivo *PGE_2 production.* PGE_2 production by bone organ cultures was performed as previously described (16,20). Shafts from the right tibia and femur bones were removed and carefully flushed with 0.9% NaCl to remove marrow cells. A section of bone shaft was immersed in 20 mL of Hank's balanced salt solution (Sigma Chemical Co., St. Louis, MO) and incubated with shaking for 2 h at 37°C. After incubation, the bone culture medium was collected and stored at –80°C until analyzed for PGE_2 by radioimmunoassay. Values for PGE_2 were expressed per unit of bone wet or dry weight.

Statistical analysis. Data were statistically analyzed by a one-way or two-way analysis of variance, and where significant differences were found, a Tukey's range test was performed at a probability of $P < 0.05$ (SAS software package for UNIX; SAS Institute Inc., Cary, NC). Variation between treatment groups was expressed as the pooled standard error of the mean (SEM).

RESULTS

Body weights between the four treatment groups were not significantly different throughout the feeding period. Final mean body weights at 42 d ranged from 305–313 g (average weight gain in grams: 260 ± 8 SBO, 264 ± 4 SBOC, 267 ± 9 MSO, 263 ± 5 MSOC). Feed efficiency (total grams weight gain/total grams feed consumed), however, was significantly improved ($P < 0.01$) in rats fed CLA compared with those not given CLA (0.42 vs. 0.39). Dietary levels of n-6 and n-3 fatty acids did not influence mean body weights nor feed efficiencies.

Upon analysis of FAME by gas chromatography, the sodium methoxide method gave superior results for CLA isomer distribution compared with those obtained by the BF_3 method. The latter tended to overestimate the concentrations of 18:2(*c,c*) and 18:2(*t,t*) but underestimate those for 18:2(*c*-9,*t*-11/*t*-9,*c*-11) and 18:2(*t*-10,*c*-12). Therefore, all rat tissue fatty acid values reported herein were obtained by the sodium methoxide method.

The short-term feeding and gavage of rats with CLA enriched cortical bone (femur and tibia) with 18:2(*c*-9,*t*-11/*t*-9,*c*-11) and 18:2(*t*-10,*c*-12). The CLA content reached 2.57% of the total fatty acids in 9 d of treatment (data not shown). The enrichment of CLA in bone was accompanied by an increase in n-3 fatty acids, primarily 18:3n-3, and a decrease in 16:0.

After 42 d of feeding, CLA isomers were detected in femur (cortical bone, marrow, and periosteum), brain, heart, liver, muscle, serum, and spleen in rats given CLA (Table 2). The concentration of CLA isomers varied across the rat tissues analyzed with bone containing the greatest amounts. For bone, CLA were highest in bone periosteum (5.46% of total fatty acids), while only trace amounts were detected in brain. The 18:2(*c*-9,*t*-11/*t*-9,*c*-11) and 18:2(*t*-10,*c*-12) isomers were incorporated into all tissues of rats given CLA independent of the n-6 and n-3 fatty acid content of the diet. In contrast, the 18:2(*t*-9,*t*-11) isomer was only observed in bone marrow and periosteum. Liver, serum, bone, marrow, and periosteum had more 18:2(*c*-9,*t*-11/*t*-9,*c*-11) than 18:2(*t*-10,*c*-12), while spleen, muscle, and heart had more 18:2(*t*-10,*c*-12).

Significant differences in tissue fatty acid composition were observed between the dietary treatment groups. For example, rats given CLA had lower concentrations of 16:1n-7, 18:1, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, and total monounsaturates in liver regardless of the level of n-6 and n-3 fatty acids in the diet (Table 3). Total liver n-6 fatty acid concentration was lower in the MSOC group compared to that in the MSO group, but it was not different between the SBO and SBOC groups. The concentrations of 22:5n-3, 22:6n-3, and total saturates were increased by CLA in liver of rats independent of the n-6 and n-3 fatty acid treatments. However, CLA increased the concentration of 18:0 in liver of rats given the n-6 but not n-3 fatty acid diet. CLA had a similar effect on serum and spleen fatty acid composition (data not shown) compared with liver except that the concentrations of 18:2n-6, 18:3n-6, 20:2n-6, and 18:0 were not affected.

When given CLA, the fatty acid composition of rat skeletal muscle (Table 4) and heart (data not shown) showed similar changes, that is, decreased 16:1n-7, 18:1, and total monounsaturates. Both tissues had increased 22:5n-3, but heart contained reduced concentrations of 18:0 and 22:6n-3. The biceps femoris of rats given diets rich in n-3 fatty acids (MSO and MSOC) had increased 20:5n-3 and 22:6n-3, but lower 20:4n-6 and total n-6 fatty acid concentrations.

Rat brain fatty acid composition was least affected by 42 d of CLA feeding. CLA decreased 16:1n-7 and 20:2n-6, but increased 22:5n-3, 22:5n-6 in rats given the n-6 fatty acid treatment (data not shown). The total n-3 fatty acid concentration was greatest in brain of rats on the MSO and MSOC treatments. Total monounsaturates, saturates, and polyunsaturated fatty acids (PUFA) were not affected by either CLA or n-6

TABLE 2
Tissue Level of CLA Isomers in Rats[a]

	n-6 PUFA + CLA			n-3 PUFA + CLA		
Tissue	CLA (*c*-9,*t*-11/*t*-9,*c*-11)	CLA (*t*-10,*c*-12)	CLA (*t*-9,*t*-11/*t*-10,*t*-12)	CLA (*c*-9,*t*-11/*t*-9,*c*-11)	CLA (*t*-10,*c*-12)	CLA (*t*-9,*t*-11/*t*-10,*t*-12)
Femur cortical bone	1.03 ± 0.20	0.81 ± 0.13	n.d.[b]	0.95 ± 0.13	0.86 ± 0.12	n.d.
Femur marrow	2.02 ± 0.03	1.34 ± 0.05	0.16 ± 0.01	2.13 ± 0.20	1.46 ± 0.20	0.19 ± 0.01
Femur periosteum	2.31 ± 0.06	1.98 ± 0.08	0.12 ± 0.04	2.86 ± 0.13	2.42 ± 0.13	0.18 ± 0.04
Brain	Trace[c]	Trace	n.d.	Trace	Trace	n.d.
Heart	0.31 ± 0.02	1.42 ± 0.05	n.d.	0.32 ± 0.02	1.34 ± 0.03	n.d.
Liver	0.95 ± 0.05	0.69 ± 0.03	n.d.	1.45 ± 0.15	0.70 ± 0.09	n.d.
Muscle	0.68 ± 0.15	1.32 ± 0.12	n.d.	0.56 ± 0.10	1.19 ± 0.06	n.d.
Serum	1.06 ± 0.13	0.78 ± 0.09	n.d.	1.08 ± 0.10	0.53 ± 0.10	n.d.
Spleen	0.67 ± 0.04	1.30 ± 0.03	n.d.	0.76 ± 0.07	1.24 ± 0.04	n.d.

[a]The enrichment of CLA in tissues is expressed as wt% ± SEM.
[b]n.d., not detected.
[c]Trace, less than 0.01% of total fatty acids. See Table 1 for abbreviations.

TABLE 3
Fatty Acid Composition (wt%) of Liver from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary group[b]							
	SBO		MSO		Pooled	ANOVA *P* value		
Fatty acid	CLA +	CLA –	CLA +	CLA –	SEM	CLA	PUFA	CLA × PUFA
14:0	0.45^{n}	0.49^{n}	0.72^{m}	0.56^{m}	0.06	NS[c]	0.007	NS
16:0	21.37^{n}	21.45^{n}	23.77^{m}	22.41^{m}	0.34	NS	0.0001	NS
16:1n-7	$1.43^{n,y}$	$2.65^{n,x}$	$2.51^{m,y}$	$2.95^{m,x}$	0.27	0.007	0.02	NS
17:0	0.30^{n}	0.26^{n}	0.38^{m}	0.39^{m}	0.03	NS	0.002	NS
18:0	14.42^{A}	11.72^{B}	11.63^{B}	11.77^{B}	0.53	0.03	0.02	0.01
18:1	$10.81^{m,y}$	$14.69^{m,x}$	$9.27^{n,y}$	$10.93^{n,x}$	0.65	0.0004	0.0006	NS
18:2n-6	18.14^{y}	19.78^{x}	17.24^{y}	18.69^{x}	0.61	0.02	NS	NS
18:3n-6	$0.24^{m,y}$	$0.28^{m,x}$	$0.17^{n,y}$	$0.21^{n,x}$	0.01	0.001	0.0001	NS
18:3n-3	0.66^{B}	0.89^{A}	0.22^{C}	0.22^{C}	0.05	0.04	0.0001	0.046
20:2n-6	$0.48^{m,y}$	$0.54^{m,x}$	$0.23^{n,y}$	$0.30^{n,x}$	0.03	0.03	0.0001	NS
20:3n-6	$0.88^{n,y}$	$0.93^{n,x}$	$1.08^{m,y}$	$1.21^{m,x}$	0.04	0.04	0.0001	NS
20:4n-6	19.21^{A}	18.30^{A}	6.74^{B}	8.45^{B}	0.59	NS	0.0001	0.04
20:5n-3	0.37^{n}	0.41^{n}	5.68^{m}	5.70^{m}	0.10	NS	0.0001	NS
22:4n-6	0.61^{m}	0.52^{m}	$n.d.^{n}$	$n.d.^{n}$	0.03	NS	0.0001	NS
22:5n-6	0.13^{m}	0.04^{m}	$n.d.^{n}$	$n.d.^{n}$	0.04	NS	0.03	NS
22:5n-3	$2.29^{n,x}$	$1.60^{n,y}$	$5.06^{m,x}$	$4.01^{m,y}$	0.11	0.0001	0.0001	NS
22:6n-3	$5.94^{n,x}$	$4.77^{n,y}$	$11.92^{m,x}$	$10.86^{m,y}$	0.47	0.03	0.0001	NS
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	0.95^{B}	$n.d.^{C}$	1.45^{A}	$n.d.^{C}$	0.08	0.0001	0.006	0.006
18:2(*t*-10,*c*-12)	0.69^{x}	$n.d.^{y}$	0.70^{x}	$n.d.^{y}$	0.04	0.0001	NS	NS
SAT	36.53^{x}	33.91^{y}	36.50^{x}	35.14^{y}	0.55	0.002	NS	NS
MONO	$12.24^{m,y}$	$17.34^{m,x}$	$11.78^{n,y}$	$13.88^{n,x}$	0.89	0.0006	0.04	NS
PUFA	48.95	48.06	48.33	49.64	0.74	NS	NS	NS
n-6 PUFA	39.70^{A}	40.39^{A}	25.45^{C}	28.85^{B}	0.57	0.002	0.0001	0.02
n-3 PUFA	$9.26^{m,x}$	$7.67^{m,y}$	$22.88^{n,x}$	$20.80^{n,y}$	0.51	0.002	0.0001	NS
n-6/n-3	4.31^{A}	5.31^{A}	1.12^{C}	1.39^{B}	0.12	0.0001	0.0001	0.01

[a]Mean values for liver fatty acid composition ($n = 6$) within a row having different superscripts (m,n for PUFA effect; x, y for CLA effect; A, B, C for interaction effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 12$ in the statistical analysis of main effects.
[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of SBO or MSO.
[c]NS, not significant. See Table 1 for other abbreviations.

and n-3 fatty acids. Generally, rats fed high n-3 fatty acids (MSO and MSOC) had more 16:1n-7, 20:3n-6, 20:5n-3, 22:5n-3, and 22:6n-3. In contrast, rats fed high n-6 fatty acids (SBO and SBOC) had higher 20:2n-6, 20:4n-6, and 22:4n-6. The 18:2n-6 concentration in brain was not affected by the fatty acid treatments.

Consumption of CLA lowered the concentrations of 20:3n-6, 22:6n-3, and total n-6 in cortical bone (Table 5). The rats given n-3 fatty acids (MSO and MSOC) had higher concentrations of 16:0, 20:5n-3, 22:5n-3, 22:6n-3, total n-3, and saturates but lower 18:1, 18:3n-3, 22:4n-6, total n-6, and monounsaturates compared to those given n-6 fatty acids (SBO and SBOC). In bone marrow of rats supplemented with CLA, the concentrations of 12:0, 14:0, 16:0, and total saturates were higher while those for 16:1n-7, 18:1, 18:2n-6, 20:2n-6, 20:3n-6, 22:5n-6, total monounsaturates, PUFA, and n-6, were lower (Table 6). The n-3 fatty acid diets (MSO and MSOC) resulted in higher 12:0, 14:0, 15:0, 16:0, 16:1n-7, 17:0, 20:3n-6, 20:5n-3, 22:5n-3, 22:6n-3, total saturates, and n-3, but lower 18:1, 18:2n-6, 20:2n-6, 20:4n-6, 22:4n-6, total monounsaturates, PUFA, and n-6 concentrations in marrow. A CLA–PUFA interaction influenced the 18:3n-6, 18:3n-3, 20:1n-9, and 22:6n-3 concentrations in bone marrow. The periosteum of rats given CLA had higher 12:0, 14:0, 16:0, and total saturates but lower 18:1, 18:2n-6, total monounsaturates, PUFA, and n-6 (Table 7). Rats given n-3 fatty acids compared with those on the n-6 fatty acid diets (SBO and SBOC) had higher 14:0, 15:0, 16:0, 16:1n-7, 20:1n-9, 20:3n-6, 20:5n-3, 22:5n-3, 22:6n-3, total saturates, and n-3 although lower 12:0, 18:1, 18:2n-6, 18:3n-3, 20:2n-6, 20:4n-6, 22:4n-6, 22:5n-6, total monounsaturates, PUFA, and n-6 concentrations in periosteum.

In rats, CLA and n-3 fatty acids both reduced *ex vivo* PGE_2 production (Table 8). The *ex vivo* PGE_2 production by bone organ cultures from rats given n-3 fatty acids (MSO and MSOC) was lower than the values from those given n-6 fatty acids (SBO and SBOC). Interestingly, CLA consumption by rats further reduced *ex vivo* PGE_2 production in bone (femur and tibia) independent of n-6 and n-3 fatty acid intake.

DISCUSSION

The results from the feeding experiment showed that neither CLA nor n-6 and n-3 fatty acids (SBO high in n-6 and MSO high in n-3 fatty acids) influenced final body weight or weight gain of the rats. However, feed efficiency was significantly

TABLE 4
Fatty Acid Composition (wt%) of Muscle (biceps femoris) from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary group[b]							
	SBO		MSO		Pooled	ANOVA *P* value		
Fatty acid	CLA +	CLA –	CLA +	CLA –	SEM	CLA	PUFA	CLA × PUFA
14:0	0.66	0.78	0.84	0.85	0.08	NS	NS	NS
16:0	21.96	21.62	23.36	22.41	0.30	NS	NS	NS
16:1n-7	1.17^{y}	2.74^{x}	1.71^{y}	2.28^{x}	0.20	0.02	NS	NS
17:0	0.13^{n}	0.15^{n}	0.28^{m}	0.31^{m}	0.01	NS	0.0001	NS
18:0	12.73	10.38	11.47	11.56	0.32	NS	NS	NS
18:1	9.91^{B}	16.63^{A}	7.44^{B}	9.76^{B}	0.52	0.0003	0.0002	0.046
18:2n-6	25.30^{m}	25.03^{m}	19.67^{n}	19.86^{n}	0.23	NS	0.0001	NS
18:3n-3	0.68^{B}	1.31^{A}	n.d.[C]	0.06^{C}	0.05	0.004	0.0001	0.02
20:3n-6	0.37	0.41	0.44	0.46	0.02	NS	NS	NS
20:4n-6	12.50^{A}	10.67^{A}	4.41^{B}	5.09^{B}	0.27	NS	0.0001	0.03
20:5n-3	n.d.[n]	n.d.[n]	1.74^{m}	1.80^{m}	0.03	NS	0.0001	NS
22:4n-6	0.83^{m}	0.07^{m}	0.10^{n}	0.05^{n}	0.03	NS	0.0001	NS
22:5n-6	0.46^{m}	0.43^{m}	n.d.[n]	n.d.[n]	0.02	NS	0.0001	NS
22:5n-3	3.11^{x}	2.32^{y}	2.90^{x}	2.51^{y}	0.09	0.004	NS	NS
22:6n-3	8.07^{n}	6.32^{n}	23.66^{m}	22.24^{m}	0.63	NS	0.0001	NS
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	0.68^{x}	n.d.[y]	0.56^{x}	n.d.[y]	0.05	0.0001	NS	NS
18:2(*t*-10,*c*-12)	1.32^{x}	n.d.[y]	1.19^{x}	n.d.[y]	0.03	0.0001	NS	NS
SAT	$35.48^{n,x}$	$32.94^{n,y}$	$35.96^{m,x}$	$35.14^{m,y}$	0.21	0.001	0.005	NS
MONO	$11.08^{m,y}$	$19.37^{m,x}$	$9.15^{n,y}$	$12.04^{n,x}$	0.70	0.0007	0.003	NS
PUFA	51.33	47.34	52.92	52.06	0.80	NS	NS	NS
n-6 PUFA	39.46^{A}	37.39^{B}	24.62^{C}	25.45^{C}	0.25	NS	0.0001	0.009
n-3 PUFA	11.86^{n}	9.95^{n}	28.30^{m}	26.61^{m}	0.68	NS	0.0001	NS
n-6/n-3	3.37^{m}	3.83^{m}	0.87^{n}	1.02^{n}	0.16	NS	0.0001	NS

[a]Mean values for muscle fatty acid composition ($n = 6$) within a row having different superscripts (m,n for PUFA effect; x, y for CLA effect; A, B, C for interaction effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 12$ in the statistical analysis of main effects.
[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of SBO or MSO. See Tables 1 and 3 for other abbreviations.

improved for rats given CLA independent of the dietary n-6 and n-3 fatty acid treatments. Our results corroborate those of Chin *et al.* (21) who observed that CLA given to rats improved feed efficiency. Sugano *et al.* (9) also found that CLA feeding to rats did not influence growth, but an experiment in mice revealed that CLA reduced body weights without influencing feed disappearance (22). Unfortunately, the authors of study in mice did not measure feed wastage (22) which could have explained the discrepancy in food disappearance and the 50% depression in growth.

CLA was reported to reduce the catabolic response from immune stimulation in animals without adversely affecting immune function (23,24). Cytokines and PGE_2 (21) are believed to mediate this catabolic response, and recently, CLA was found to lower *in vivo* and *ex vivo* production of cytokines in rats (9,19). The present investigation with rats is consistent with other findings that dietary CLA did not affect body weight but decreased feed consumption to improve feed efficiency. Further research is needed to characterize the biochemical and physiological actions of CLA on energy metabolism.

The pattern of CLA enrichment in rats showed certain tissue selectivity for the various isomers. For example, liver, serum, bone, marrow, and periosteum contained more 18:2(*c*-9, *t*-11/*t*-9,*c*-11) than 18:2(*t*-10,*c*-12), the two predominant isomers. Spleen, muscle, and heart, however, contained more 18:2(*t*-10,*c*-12) than the 9,11 isomer. Furthermore, only trace amounts of 18:2(*c*-9,*t*-11/*t*-9,*c*-11) and 18:2(*t*-10,*c*-12) were detected in brain. To our knowledge this is the first report demonstrating that CLA are found in bone tissue. Surprisingly, concentrations of CLA in bone tissues (cortical, marrow, and periosteum) were the highest of all tissues analyzed. Moreover, the various isomers of CLA incorporated into bone tissue lipids equally well for diets high in n-6 or n-3 fatty acids.

The fatty acid composition of rat tissues was significantly modified by the dietary lipids. The effects of dietary n-6 and n-3 enrichment (SBO and MSO, respectively) on fatty acid composition of bone in rats were similar to the results in chicks we have reported previously (20). Linoleate (18:2n-6) and its chain elongation and desaturation products (20:4n-6, 22:4n-6, and 22:5n-6) were higher in liver, spleen, serum, muscle, heart, bone (marrow and periosteum), and brain in rats given SBO. CLA consumption decreased the concentrations of 16:1n-7, 18:1, total monounsaturates, and n-6, but increased those for 22:5n-3, 22:6n-3, total n-3, and saturates in most tissues analyzed. CLA decreased the concentration of 18:2n-6 in bone marrow and periosteum, 18:3n-6, 20:2n-6, 20:3n-6, 22:5n-6 in marrow, and 20:3n-6 in cortical bone. Further, CLA decreased the ratio of n-6/n-3 in liver, brain,

TABLE 5
Fatty Acid Composition (wt%) of Femur Cortical Bone from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary group[b]							
	SBO		MSO		Pooled	ANOVA *P* value		
Fatty acid	CLA +	CLA –	CLA +	CLA –	SEM	CLA	PUFA	CLA × PUFA
14:0	1.43	1.08	1.30	1.43	0.16	NS	NS	NS
15:0	0.27^{n}	0.31^{n}	0.41^{m}	0.51^{m}	0.04	NS	0.001	NS
16:0	27.78^{n}	26.06^{n}	28.66^{m}	30.18^{m}	1.12	NS	0.04	NS
16:1n-7	1.64	2.52	2.08	2.66	0.40	NS	NS	NS
17:0	0.30	0.14	0.13	0.20	0.12	NS	NS	NS
18:0	9.76	7.96	8.47	9.42	0.50	NS	NS	NS
18:1	18.65^{m}	16.09^{m}	11.69^{n}	13.83^{n}	1.23	NS	0.002	NS
18:2n-6	12.36	15.49	11.72	12.64	1.28	NS	NS	NS
18:3n-4	2.65	3.28	3.54	1.27	0.89	NS	NS	NS
18:3n-3	0.61^{m}	1.15^{m}	n.d.[n]	n.d.[n]	0.16	NS	0.0001	NS
20:2n-6	0.40	0.68	0.42	0.34	0.11	NS	NS	NS
20:3n-6	0.23^{y}	0.89^{x}	0.72^{y}	0.88^{x}	0.16	0.02	NS	NS
20:4n-6	7.91	9.28	6.87	8.59	0.80	NS	NS	NS
20:3n-3	0.83	0.90	1.31	0.44	0.36	NS	NS	NS
20:5n-3	0.75^{n}	0.54^{n}	2.76^{m}	2.52^{m}	0.16	NS	0.0001	NS
22:0	0.15	0.25	0.36	0.14	0.12	NS	NS	NS
22:1n-9	0.34	0.16	0.37	0.08	0.21	NS	NS	NS
22:4n-6	2.45^{m}	2.62^{m}	1.33^{n}	1.48^{n}	0.30	NS	0.002	NS
22:5n-3	0.58^{n}	0.80^{n}	3.16^{m}	3.10^{m}	0.15	NS	0.0001	NS
22:6n-3	$0.64^{n,y}$	$11.12^{n,x}$	$3.35^{m,y}$	$4.14^{m,x}$	0.20	0.007	0.0001	NS
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	1.03^{x}	n.d.[y]	0.95^{x}	n.d.[y]	0.13	0.0001	NS	NS
18:2(*t*-10,*c*-12)	0.81^{x}	n.d.[y]	0.86^{x}	n.d.[y]	0.09	0.0001	NS	NS
SAT	39.70^{n}	35.80^{n}	39.34^{m}	41.88^{m}	1.33	NS	0.046	NS
MONO	20.63^{m}	18.77^{m}	14.14^{n}	16.56^{n}	1.49	NS	0.01	NS
PUFA	29.42^{y}	36.73^{x}	35.18^{y}	35.40^{x}	1.77	0.049	NS	NS
n-6 PUFA	$23.36^{m,y}$	$28.95^{m,x}$	$21.06^{n,y}$	$23.93^{n,x}$	1.71	0.02	0.048	NS
n-3 PUFA	3.41^{n}	4.50^{n}	10.58^{m}	10.20^{m}	0.35	NS	0.0001	NS
n-6/n-3	7.33^{m}	6.74^{m}	1.99^{n}	2.35^{n}	0.85	NS	0.0001	NS

[a]Mean values for femur cortical bone fatty acid composition ($n = 5$) within a row having different superscripts (m, n for PUFA effect; x, y for CLA effect; A, B, C for interaction effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 10$ in the statistical analysis of main effects.
[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of SBO or MSO. See Table 1 for other abbreviations.

spleen, and serum of rats given MSO. The relative sparing of n-3 fatty acids in rats given CLA might be explained as either an increase in the utilization of n-6 or a conservation of n-3 fatty acids. The increase of total saturates in liver, marrow, periosteum, spleen, muscle, and serum of rats consuming CLA could indicate a depression of desaturase activity. Since liver is a major organ for fatty acid synthesis in mammals, and we observed a decrease of 18:1n-9 in rat tissues (liver, marrow, and periosteum), one would speculate that CLA affects the saturates/monounsaturates ratio by inhibiting liver Δ9-desaturase activity as proposed by Lee *et al.* (10).

The n-3 fatty acid treatment (MSO) significantly reduced *ex vivo* PGE_2 production in bone organ culture (tibia and femur) compared to the n-6 dietary treatment (SBO). In bone, dietary CLA lowered *ex vivo* PGE_2 production beyond that of n-3 fatty acid feeding. Sugano *et al.* (9) also reported that the concentration of PGE_2 in spleen tended to be reduced by CLA. Dietary supplementation with CLA may impact bone modeling in the young since PGE_2 mediates bone formation and bone resorption (18). Recent work from our laboratory demonstrated that modulation of *ex vivo* PGE_2 production in bone with dietary lipids was associated with changes in bone formation rate (16,20). PGE_2 is a potent agent regulating both bone modeling and remodeling, but its effect in bone may be concentration-dependent. For example, Raisz and Koolemans-Beynen (25) showed that PGE_2 inhibits matrix formation at high concentrations in bone organ culture. However, at lower doses, PGE_2 can stimulate bone formation *in vitro* and *in vivo* (26–29). Therefore, excessive production of PGE_2 may adversely affect bone modeling, and a lower level of PGE_2 is believed to stimulate bone formation in animals fed diets containing moderate levels of n-6 fatty acids. Chicks fed a diet containing a lower n-6/n-3 fatty acid ratio demonstrated reduced PGE_2 production in bone that was accompanied by an increased bone formation rate when compared with those given a higher n-6/n-3 ratio (20). Although different mechanisms may be operative for CLA and n-3 fatty acids to reduce *ex vivo* PGE_2 production, the fact that both fatty acids lowered PGE_2 by cultured bone may be significant in understanding their potential effects on bone health.

In summary, supplementing the diets of rats with CLA led to differences in CLA enrichment in various tissues, with

TABLE 6
Fatty Acid Composition (wt%) of Femur Bone Marrow from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary group[b]							
	SBO		MSO		Pooled	ANOVA *P* value		
Fatty acid	CLA +	CLA –	CLA +	CLA –	SEM	CLA	PUFA	CLA × PUFA
12:0	$0.21^{n,x}$	$0.14^{n,y}$	$0.27^{m,x}$	$0.21^{m,y}$	0.01	0.0002	0.0002	NS
14:0	$2.37^{n,x}$	$1.76^{n,y}$	$3.24^{m,x}$	$2.90^{m,y}$	0.11	0.0004	0.0001	NS
15:0	0.25^{n}	0.23^{n}	0.35^{m}	0.37^{m}	0.01	NS	0.0001	NS
16:0	$29.15^{n,x}$	$25.17^{n,y}$	$31.48^{m,x}$	$28.32^{m,y}$	0.51	0.0001	0.0001	NS
16:1n-7	$4.12^{n,y}$	$4.94^{n,x}$	$5.02^{m,y}$	$6.10^{m,x}$	0.38	0.02	0.01	NS
17:0	0.21^{n}	0.19^{n}	0.30^{m}	0.32^{m}	0.01	NS	0.0001	NS
18:0	6.53	6.39	7.15	6.74	0.39	NS	NS	NS
18:1	$18.89^{m,y}$	$23.07^{m,x}$	$14.95^{n,y}$	$18.11^{n,x}$	0.42	0.0001	0.0001	NS
18:2n-6	$22.73^{m,y}$	$24.63^{m,x}$	$18.52^{n,y}$	$19.62^{n,x}$	0.48	0.006	0.0001	NS
18:3n-6	0.17^{C}	0.25^{A}	0.17^{C}	0.21^{B}	0.01	0.0001	0.003	0.003
18:3n-3	1.78^{A}	2.13^{A}	0.50^{B}	0.42^{B}	0.10	NS	0.0001	0.04
20:1n-9	0.19^{B}	0.25^{A}	0.20^{B}	0.24^{A}	0.01	0.0001	NS	0.007
20:2n-6	$0.62^{m,y}$	$0.82^{m,x}$	$0.54^{n,y}$	$0.60^{n,x}$	0.04	0.002	0.0004	NS
20:3n-6	$0.24^{n,y}$	$0.36^{n,x}$	$0.42^{m,y}$	$0.49^{m,x}$	0.03	0.01	0.0002	NS
20:4n-6	4.68^{m}	5.75^{m}	3.76^{n}	3.80^{n}	0.41	NS	0.002	NS
20:5n-3	0.13^{n}	0.06^{n}	1.44^{m}	1.82^{m}	0.13	NS	0.0001	NS
22:4n-6	0.77^{m}	0.96^{m}	0.27^{n}	0.26^{n}	0.06	NS	0.0001	NS
22:5n-6	0.07^{y}	0.14^{x}	0.07^{y}	0.13^{x}	0.02	0.01	NS	NS
22:5n-3	0.58^{n}	0.58^{n}	2.06^{m}	2.27^{m}	0.16	NS	0.0001	NS
22:6n-3	0.59^{C}	0.59^{C}	2.49^{B}	3.30^{A}	0.20	0.047	0.0001	0.048
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	2.02^{x}	n.d.^{y}	2.13^{x}	0.10^{y}	0.11	0.0001	NS	NS
18:2(*t*-10,*c*-12)	1.34^{x}	n.d.^{y}	1.46^{x}	0.08^{y}	0.11	0.0001	NS	NS
18:2(*t*-9,*t*-11/*t*-10,*t*-12)	0.16^{x}	n.d.^{y}	0.19^{x}	n.d.^{y}	0.01	0.0001	NS	NS
SAT	$38.73^{n,x}$	$33.87^{n,y}$	$42.80^{m,x}$	$38.86^{m,y}$	0.48	0.0001	0.0001	NS
MONO	$23.32^{m,y}$	$28.40^{m,x}$	$20.32^{n,y}$	$24.63^{n,x}$	0.70	0.0001	0.0001	NS
PUFA	$32.35^{m,y}$	$36.27^{m,x}$	$30.24^{n,y}$	$32.95^{n,x}$	0.93	0.002	0.008	NS
n-6 PUFA	$29.27^{m,y}$	$32.91^{m,x}$	$23.74^{n,y}$	$25.10^{n,x}$	0.77	0.004	0.0001	NS
n-3 PUFA	3.08^{n}	3.36^{n}	6.50^{m}	7.85^{m}	0.43	NS	0.0001	NS
n-6/n-3	9.87^{m}	9.83^{m}	3.84^{n}	3.26^{n}	0.49	NS	0.0001	0.01

[a]Mean values for femur bone marrow fatty acid composition ($n = 6$) within a row having different superscripts (m, n for PUFA effect; x, y for CLA effect; A, B, C for interaction effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 12$ in the statistical analysis of main effects.
[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of SBO or MSO. See Tables 1 and 3 for other abbreviations.

brain having the lowest concentrations of isomers but bone tissue containing the highest. Furthermore, CLA influenced the fatty acid composition of rat tissues leading to reduced 18:1 in liver, muscle, heart, and bone (marrow and periosteum). The data on tissue fatty acid composition presented here suggest that CLA may affect the formation of 18:1n-9 and metabolism of n-6 and n-3 fatty acids. Consistent with the hypothesis that CLA modifies arachidonate-derived eicosanoid production, we observed that CLA depressed *ex vivo* PGE_2 production in bone organ cultures. The reduction in PGE_2 by CLA might be explained as a competitive inhibition of n-6 fatty acid formation (30) that would lower substrate availability for cyclooxygenase. Since PGE_2 is a potent agent that stimulates both bone formation and resorption, research on CLA will be relevant to understanding the potential role of essential fatty acids in bone biology.

ACKNOWLEDGMENTS

Special thanks to J.A. Scimeca (Nutrition Department, Kraft General Foods, Glenview, IL) for providing the conjugated linoleic acid and K.D.G. Allen for technical assistance. Supported by USDA NRI grant no. 96-35200-3137. Approved as Journal Paper Number 15559 of the Purdue Agricultural Experiment Station.

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TABLE 7
Fatty Acid Composition (wt%) of Femur Bone Periosteum from Rats Fed Different Dietary Lipid Treatments[a]

	Dietary group[b]							
	SBO		MSO		Pooled	ANOVA *P* value		
Fatty acid	CLA +	CLA –	CLA +	CLA –	SEM	CLA	PUFA	CLA × PUFA
12:0	$0.15^{m,x}$	$0.09^{m,y}$	$0.17^{n,x}$	$0.14^{n,y}$	0.02	0.01	0.03	NS
14:0	$1.65^{n,x}$	$1.12^{n,y}$	$2.57^{m,x}$	$2.33^{m,y}$	0.12	0.004	0.0001	NS
15:0	0.18^{n}	0.14^{n}	0.28^{m}	0.31^{m}	0.02	NS	0.0001	NS
16:0	$25.68^{n,x}$	$22.18^{n,y}$	$28.42^{m,x}$	$24.97^{m,y}$	0.54	0.0001	0.0001	NS
16:1n-7	2.80^{n}	2.77^{n}	3.94^{m}	4.57^{m}	0.30	NS	0.0001	NS
17:0	$0.21^{B,C}$	0.16^{C}	$0.31^{A,B}$	0.38^{A}	0.03	NS	0.0001	NS
18:0	8.38	8.92	7.86	8.29	0.47	NS	NS	NS
18:1	$17.14^{m,y}$	$20.70^{m,x}$	$13.46^{n,y}$	$17.10^{n,x}$	0.63	0.0001	0.0001	NS
18:2n-6	$25.38^{m,y}$	$28.16^{m,x}$	$20.85^{n,y}$	$22.54^{n,x}$	0.60	0.001	0.0001	NS
18:3n-3	1.69^{A}	2.07^{A}	0.50^{B}	0.42^{B}	0.10	NS	0.0001	0.04
20:1n-9	$n.d.^{n}$	$n.d.^{n}$	0.10^{m}	0.18^{m}	0.03	NS	0.0001	NS
20:2n-6	0.25^{m}	0.26^{m}	0.14^{n}	0.15^{n}	0.04	NS	0.01	NS
20:3n-6	0.27^{n}	0.30^{n}	0.34^{m}	0.42^{m}	0.04	NS	0.02	NS
20:4n-6	5.43^{m}	6.88^{m}	2.93^{n}	2.94^{n}	0.47	NS	0.0001	NS
20:5n-3	$n.d.^{C}$	$n.d.^{C}$	1.30^{B}	1.72^{A}	0.08	0.01	0.0001	0.01
22:4n-6	0.81^{m}	0.73^{m}	0.36^{n}	0.33^{n}	0.09	NS	0.0002	NS
22:5n-6	0.28^{m}	0.20^{m}	$n.d.^{n}$	0.10^{n}	0.06	NS	0.002	NS
22:5n-3	1.41^{n}	1.44^{n}	2.01^{m}	2.00^{m}	0.13	NS	0.0003	NS
22:6n-3	3.25^{n}	3.53^{n}	7.46^{m}	8.98^{m}	0.62	NS	0.0001	NS
18:2(*c*-9,*t*-11/*t*-9,*c*-11)	2.31^{x}	$n.d.^{y}$	2.86^{x}	$n.d.^{y}$	0.13	0.0001	NS	NS
18:2(*t*-10,*c*-12)	1.98^{x}	$n.d.^{y}$	2.42^{x}	$n.d.^{y}$	0.13	0.0001	NS	NS
18:2(*t*-9,*t*-11/*t*-10,*t*-12)	0.12^{x}	$n.d.^{y}$	0.18^{x}	$n.d.^{y}$	0.04	0.0003	NS	NS
SAT	$36.25^{n,x}$	$32.61^{n,y}$	$39.61^{m,x}$	$36.43^{m,y}$	0.52	0.0001	0.0001	NS
MONO	$19.94^{m,y}$	$23.47^{m,x}$	$17.50^{n,y}$	$21.85^{n,x}$	0.81	0.0001	0.02	NS
PUFA	$38.76^{m,y}$	$43.57^{m,x}$	$35.88^{n,y}$	$39.61^{n,x}$	1.17	0.002	0.008	NS
n-6 PUFA	$32.42^{m,y}$	$36.52^{m,x}$	$24.62^{n,y}$	$26.48^{n,x}$	0.86	0.003	0.0001	NS
n-3 PUFA	6.34^{n}	7.05^{n}	11.26^{m}	13.12^{m}	0.72	NS	0.0001	NS
n-6/n-3	5.29^{m}	5.28^{m}	2.24^{n}	2.06^{n}	0.28	NS	0.0001	NS

[a]Mean values for femur bone periosteum fatty acid composition ($n = 6$) within a row having different superscripts (m, n for PUFA effect; x, y for CLA effect; A, B, C for interaction effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 12$ in the statistical analysis of main effects.
[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of SBO or MSO. See Tables 1 and 3 for other abbreviations.

TABLE 8
Effect of Dietary CLA and PUFA Treatments on PGE_2 Production (mean ± SE) in Bone Organ Culture[a]

	Dietary groups[b]						
	SBO		MSO		ANOVA *P* value		
Tissue	CLA +	CLA –	CLA +	CLA –	CLA	PUFA	CLA × PUFA
Tibia, ng/g (dry weight)	$19.1 \pm 2.1^{m,y}$	$42.9 \pm 11.6^{m,x}$	$6.6 \pm 2.6^{n,y}$	$15.8 \pm 2.0^{n,x}$	0.02	0.007	0.25
Tibia, ng/g (wet weight)	$17.6 \pm 1.5^{m,y}$	$34.5 \pm 8.8^{m,x}$	$5.0 \pm 1.7^{n,y}$	$12.8 \pm 1.3^{n,x}$	0.02	0.003	0.35
Femur, ng/g (dry weight)	$21.2 \pm 3.6^{m,y}$	$30.8 \pm 4.7^{m,x}$	$6.6 \pm 0.8^{n,y}$	$16.0 \pm 4.3^{n,x}$	0.02	0.002	0.98
Femur, ng/g (wet weight)	19.8 ± 3.6^{m}	25.7 ± 4.2^{m}	5.6 ± 1.1^{n}	14.5 ± 4.3^{n}	0.06	0.004	0.69

[a]Mean values for *ex vivo* prostaglandin E_2 (PGE_2) production ($n = 6$) having different superscripts (m, n for PUFA effect; x, y for CLA effect) are significantly different by two-way ANOVA and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an $n = 12$ in the statistical analysis of main effects.

[b]Dietary treatments included SBO, or MSO and with (+) or without (–) CLA. Total fat content in each diet was 70 g/kg of diet, and CLA was added (10 g/kg of diet) at the expense of kSBO or MSO. See Tables 1 and 3 for other abbreviations.

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[Received October 16, 1997, and in final revised form February 11, 1998; revision accepted February 17, 1998]

Effects of Dietary Marine Oils and Olive Oil on Fatty Acid Composition, Platelet Membrane Fluidity, Platelet Responses, and Serum Lipids in Healthy Humans

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ABSTRACT: The influence of various dietary marine oils and olive oil on fatty acid composition of serum and platelets and effects on platelets and serum lipids were investigated as part of an extensive study of the effects of these oils on parameters associated with cardiovascular/thrombotic diseases. Healthy volunteers (266) consumed 15 mL/d of cod liver oil (CLO); whale blubber oil (refined or unrefined); mixtures of seal blubber oil and CLO; or olive oil/CLO for 12 wk. In the CLO, seal oil/CLO, and whale oil groups, serum levels of eicosapentaenoic acid (EPA) were increased. In platelets, EPA was increased in the CLO, seal/CLO, and olive oil/CLO groups. The localization of n-3 polyunsaturated fatty acids in the triacylglycerols did not seem to influence their absorption. Intake of oleic acid is poorly reflected in serum and platelets. No significant differences in triacylglycerols (TG), total cholesterol, or high density lipoprotein cholesterol were observed, even though TG were reduced in the CLO, CLO/seal oil, and whale oil groups. Mean platelet volume increased significantly in both whale oil groups and the CLO/olive oil group. Platelet count was significantly reduced in the refined whale oil group only. Lipopolysaccharide-stimulated blood tended to generate less thromboxane B_2 in CLO, CLO/seal, and CLO/olive groups. The whale oils tended to reduce *in vivo* release of β-thromboglobulin. In conclusion, intake of various marine oils causes changes in platelet membranes that are favorably antithrombotic. The combination of CLO and olive oil may produce better effects than these oils given separately. The changes in platelet function are directly associated with alterations of fatty acid composition in platelet membranes.

Lipids 33, 427–436 (1998).

In the 1970s, Bang, Dyerberg, and coworkers (1–3) suggested that the low incidence of death attributable to coronary heart disease among Greenland Eskimos was due to their high intake of n-3 polyunsaturated fatty acids (PUFA) derived from fish and other seafoods. These studies created the basis for the great increase in the interest in cardiovascular benefits of fish oils, and the effects of the consumption of fish and fish oils have been examined in various important biological systems.

Dietary supplementation with fish and fish oils is reported to modify platelet and leukocyte function in several ways. Suggested modes of action are through their modulation of eicosanoid synthesis (4–6) and reduction in plasma triacylglycerol (TG) concentration (for review see Ref. 7). n-3 Fatty acids as a dietary supplement decrease platelet aggregation and release thromboxane A_2 (TxA_2). Another mechanism for the effect of n-3 PUFA has been suggested to be associated with the change in fluidity by incorporation of PUFA in the cell membranes and an influence on the activities of membrane-associated enzymes or receptors (8). Because of variable results, additional human clinical studies are still needed.

Attention has been directed on n-3 PUFA, although it was evident from Bang and Dyerberg's studies that the traditional Eskimo diet consists of substantial quantities of meat and blubber from seals and whales (1). The fat composition of seal, and of whale in particular, differs significantly from that of fish (9). In marine mammals eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are found mainly in the *sn*-1 and *sn*-3 positions of the TG, whereas in fish these fatty acids are positioned in *sn*-2 (10,11). However, the diet of Greenland Eskimos also differs from the Western diet in other respects, especially in the intake of saturated fatty acids (2). The favorable disease pattern in Eskimos may be caused by the combination of a diet low in saturated fat together with high n-3 PUFA intake, but the beneficial effects of this diet also may be due to components other than the fatty acids. A previous study in our laboratory indicated that oils from the blubber of seal and whale have beneficial effects on some selected parameters thought to play a role in cardiovascular disease, and it was hypothesized that the effect of whale oil is not mediated by n-3 fatty acids alone (12).

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Abbreviations: AA, arachidonic acid; CLO, cod liver oil; CV, coefficient of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; HDL, high density lipoprotein; LPS, lipopolysaccharide; MPV, mean platelet volume; PGA_1, $-A_2$, $-D_2$, $-E_2$, $-F_{1\alpha}$, $-F_{2\alpha}$, prostaglandin A_1, $-A_2$, $-D_2$, $-E_2$, $-F_{1\alpha}$, $-F_{2\alpha}$; PUFA, polyunsaturated fatty acid; r_s, steady-state fluorescence anisotropy; TG, triacylglycerol; β-TG, β-thromboglobulin; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate; TxA_2, $-B_2$, thromboxane A_2, B_2.

The so-called Mediterranean diet is characterized, among other things, by a moderately low intake of PUFA and by a high intake of monounsaturated fatty acids from olive oil. Since Mediterranean populations have shown a low mortality from cardiovascular diseases, which is negatively correlated to the percentage of dietary energy supplied by monounsaturated fatty acids (13,14) in addition to findings of favorable effects of n-3 fatty acid supplementation to a diet rich in olive oil (15), it was of major interest to include olive oil in this dietary study.

With the traditional diets of Mediterranean populations and Greenland Eskimos in mind, we have focused on the effects of marine oils and olive oil on important parameters concerning the physical and functional properties of blood platelets. Platelets play an essential role in arterial thrombosis and atherosclerosis, but the mechanisms for the effects of dietary oils on platelet function are not well understood. In this controlled study we investigated whether any of the dietary oils contributed to changes in structural order (membrane fluidity) of the platelets and related membrane fluidity to platelet function by measuring eicosanoid production thromboxane [(TXB_2 and prostaglandin E_2) (PGE_2)] and release of the α-granule protein β-thromboglobulin (β-TG) from platelets. In addition, we have related these results to plasma lipids and to the corresponding fatty acid composition in serum and platelets and compared the changes in fatty acid patterns of serum and platelet membranes to the composition of the dietary oils. The results are also discussed in relation to the intramolecular TG structures of the oils.

MATERIALS AND METHODS

Subjects and dietary protocol. Healthy volunteers (266) living in Tromsø, Norway (124 women and 149 men), aged 16–69 yr (median 36), were randomly selected to consume 15 mL/d of cod (*Gadus morhua*) liver oil (CLO), olive (*Olea europea*) oil (extra virgin), refined (steam refined by heating to 170°C with N_2) and unrefined oil from the blubber of minke whale (*Balaenoptera acutorostrata*), mixtures of CLO and harp seal (*Phoca groenlandica*) blubber oil (1:1, vol/vol) and CLO and olive oil (1:1, vol/vol), and no oil (control group). Table 1 shows the number of subjects in each group and some characteristics of the groups regarding distribution of gender, age, and smoking habits. The oils were a supplement to their normal dietary habits, and the subjects were counseled to continue eating their regular diet. The study was double-blinded for the groups given various dietary oils. No placebo oil was given in the control group, and the study of this group could thus not be blinded to volunteers or investigators. The seven groups were run in parallel for a period of 12 wk. The procedures followed were in accordance with the Helsinki declaration of 1964 as revised in 1989.

The fatty acid composition of the dietary oils, determined by gas–liquid chromatography as described shortly, is given in Table 2. There were no changes detected during storage over the period of 12 wk. The vitamin E concentrations of the oils were analyzed according to Lie *et al.* (16) and were as follows: CLO: 1.52 mg/g; olive oil: 0.23 mg/g; refined whale oil: 0.17 mg/g; whale oil: 0.25 mg/g; seal oil/CLO: 1.38 mg/g; and olive oil/CLO: 0.85 mg/g.

Blood sampling. Blood samples were taken before the start of the study, after 8 wk, and after 12 wk of supplementation. The data after 12 wk of supplementation are presented in this work. The participants were informed not to drink alcohol or perform any strenuous exercise for 48 h prior to each blood sampling. They fasted overnight, and all the blood samples were taken between 8:00 and 10.00 A.M. Venous blood (25 mL) was drawn into a plastic syringe with a 19-gauge needle and immediately distributed into serum tubes or tubes containing anticoagulants as specified for each procedure described.

Mean platelet volume (MPV) and platelet counts. MPV and platelet counts were measured in whole blood anticoagulated with Na_2-EDTA (final concentration 0.2%) with a Coulter Counter (STRK, Luton, England).

Platelet preparation. Platelet-rich plasma was obtained by centrifugation of citrated blood (5 mL, final concentration of Na_3-citrate: 0.38%) at $180 \times g$ for 15 min at room temperature with the brake off. For fatty acid analysis, the platelets were washed once in 0.15 mol/L NaCl, centrifuged at $1500 \times g$ for 10 min and resuspended in 0.15 mol/L NaCl. The platelet preparations were kept at −70°C until extraction of the fatty acids was performed for the gas chromatographic analysis. For membrane fluidity studies, the platelet pellets were washed twice with 0.15 mol/L NaCl, centrifuged at $1500 \times g$ for 10 min and resuspended in potassium phosphate buffer (0.01 mol/L, pH 7.4). The platelet suspensions were

TABLE 1
Characteristics of the Test Groups Regarding Smoking Habits, Age, and Sex

Oil consumed	Number of females/males	Age (yr)[a]	Smokers (%)
Control (n = 38)	20/18	29.0 (21–57)	18
CLO[b] (n = 37)	14/23	33.0 (20–59)	22
Olive oil (n = 34)	18/16	38.0 (16–66)	26
Whale oil, refined (n = 39)	17/22	36.0 (20–69)	21
Whale oil (n = 40)	17/23	36.5 (22–59)	40
Seal oil/CLO (n = 40)	21/19	35.0 (21–64)	28
Olive oil/CLO (n = 38)	17/21	34.5 (22–59)	39

[a]Age data are median and total range.
[b]CLO = cod liver oil.

TABLE 2
Fatty Acid Profile of the Dietary Oils (wt/wt% of total fatty acids)[a]

Fatty acid	CLO	Olive oil	Whale (refined)	Whale oil (crude)	Seal/CLO	Olive/CLO
14:0	3.9	0.0	5.9	6.2	4.9	2.4
15:0	0.3	0.0	0.4	0.4	0.6	0.2
16:0	9.9	11.1	9.6	9.8	9.4	10.5
18:0	1.9	2.0	1.8	1.9	1.4	2.0
20:0	0.2	0.3	0.0	0.2	0.1	0.1
22:0	0.1	0.1	0.0	0.0	0.0	0.1
Saturated	16.1	13.6	17.6	18.5	16.4	15.3
14:1	0.3	0.0	0.0	0.3	0.0	0.2
16:1n-7	8.1	0.6	9.6	9.6	10.6	5.4
18:1n-9	21.3	77.4	21.9	23.0	20.7	43.3
20:1n-9	11.7	0.6	17.0	16.9	10.7	7.2
22:1n-11	6.5	0.0	10.7	10.6	5.0	3.9
24:1	0.5	0.0	0.3	0.3	0.2	0.3
MUFA	48.6	78.6	59.5	60.7	47.2	60.3
16:2	0.4	0.0	0 4	0.3	0.5	0.3
18:2n-6	1.8	6.0	1.4	1.8	1.7	3.4
20:2n-6	0.3	0.1	0.4	0.4	0.4	0.2
18:3n-6	0.1	0.0	0.3	0.3	0.1	0.1
18:3n-3	1.3	0.7	1.2	1 2	1.3	1.1
16:4	0.1	0.0	0.0	0.1	0.2	0.1
18:4n-3	2.7	0.0	1.7	1 7	3.0	1.7
20:4n-6	0.4	0.0	0.3	0.2	0.3	0.2
20:4n-3	0.7	0.0	0.9	0.9	0.6	0.4
20:5n-3	9.5	0.0	3.9	3.3	7.7	5.8
21:5n-3	0.4	0 0	0 2	0.2	0.4	0.3
22:5n-3	1.0	0.0	1.7	1.7	2.2	0.6
22:6n-3	13.5	0.0	5.9	4.2	11.3	7.8
PUFA	32.2	6.8	18.3	16.3	29.7	22.0
n-3 PUFA	29.1	0.7	15.5	13.2	26.5	17.7
Total	96.8	98.7	95.9	95.7	97.6	97.4

[a]MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. For other abbreviation, see Table 1.

frozen at −70°C until anisotropy measurements were performed (17). Before labeling with the fluorescent probe, the suspensions were sonicated in an MSE sonicator (Sussex, United Kingdom) (high power, setting 4, 10 s × 2 with 10-s interval, on ice). The platelet preparations were adjusted to a final optical density of 0.03 at 600 nm to minimize the effect of light scattering on the anisotropy measurements (18). Platelet counts were performed both in whole blood and in platelet-rich plasma.

Fatty acid analysis. The fatty acids were extracted from serum and platelet membrane preparations using chloroform/methanol (1:1 vol/vol; Merck, Darmstadt, Germany) by a modified method of Folch and coworkers (19), esterified with 2 mL boron trifluoride in methanol (Supelco, Bellefonte, PA) at 100°C for 90 min (20). Fatty acid methyl esters (FAME) were analyzed by capillary gas–liquid chromatography using a Fisons Carlo Erba 8340 gas chromatograph (Carlo Erba, Rodano, Italy). FAME were separated on a 50-m × 0.25-mm CP-Sil 88 (FAME) capillary column with 0.2 μm film thickness (Chrompack, Middelburg, Holland) after splitless injection of a 1-μL sample at a column temperature of 80°C. The operating conditions were as follows: The initial temperature was maintained for 1 min and then raised by 20°C/min to 160°C, followed by a gradient of 2°C/min to 177°C and 20°C/min to 230°C, and it was kept there for 15 min. The injector and the flame-ionization detector temperatures were 250 and 270°C, respectively. The carrier gas was hydrogen, with an inlet pressure of 110 kPa. The fatty acid composition is expressed as percentage FAME with respect to the total peak surface (area/area).

Analysis of the stereochemistry of TG in the marine oils. The positional distribution of EPA (20:5n-3) and DHA (22:6 n-3) in CLO, seal blubber, and whale blubber oils was analyzed by high-resolution ^{13}C nuclear magnetic resonance spectroscopy as previously described (21).

Measurements of platelet membrane fluidity. The membrane fluidity was assessed by measurements of steady-state fluorescence anisotropy (r_S) of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH; Molecular Probes. Inc., Eugene, OR) (22–24). A stock solution of TMA-DPH in *N*,*N*-dimethylformamide (Uvasol®; spectroscopy grade, Merck) was prepared at a con-

centration of 10 mmol/L, stored at –20°C and diluted before use. The diluted platelet preparation (2 mL) was incubated for 10 min at 37°C in the dark with TMA-DPH at a final concentration of 1.0 μmol/L. The r_s measurements were carried out with a Perkin-Elmer LS 50 Luminescence Spectrometer (Buckinghamshire, United Kingdom) equipped with polarizers. The measurements were made at excitation and emission wavelengths of 360 and 430 nm, respectively, with slit widths of 5 nm in both excitation and emission. The r_S-value for each sample is an average of five readings (integration time, 10 s), measured at a constant temperature of 37.0 ± 0.1°C, controlled with a digital thermometer. The r_S is automatically calculated from the following equation:

$$r_S = \frac{IV_V - GIv_H}{IV_V + 2GIV_H} \text{ with a correcting factor } G = \frac{IH_V}{IH_H} \quad [1]$$

where r_S is the corrected anisotropy, and IV_V and IH_V are the emission intensities of vertically polarized light parallel and perpendicular to the plane of excitation, respectively. Likewise, IV_H and IH_H are the emission intensities of horizontally polarized light perpendicular and parallel to the plane of excitation. Fluorescence anisotropy values are inversely related to the rotational rate of the probe in the membrane, thus low anisotropy values indicate high fluidity of the membrane.

Lipids in serum. Serum samples were analyzed for TG, cholesterol, and high density lipoprotein (HDL) cholesterol using commercially available enzyme kits from Boehringer (Mannheim, Germany) in combination with an RA-1000 automatic analyzer (Technion RA-100; Bayer Norway, Hagan, Norway).

Quantitation of β-TG. β-TG was measured in heparinized plasma with a β-TG radioimmunoassay kit according to the manufacturer's instructions (Kodak Clinical Diagnostics Ltd., Amersham, United Kingdom). At 45 ng/mL the within-assay coefficient of variation (CV) was 7.5%, and the between-assay CV was 9.9–15.3% in the range 19.6–108 ng/mL.

Stimulation of whole blood in vitro. Lipopolysaccharide (LPS; *Escherichia coli* 026:B6; Difco, Detroit, MI) was added to heparinized blood (1.0 mL) to a final concentration of 5 ng/mL, followed by incubation at 37°C in an incubator shaker (180 rpm) for 2 h. The stimulation was stopped by adding 0.1 mL 2% Na_2-EDTA/mL blood, and the blood was immediately centrifuged at 1400 × *g* for 10 min. The plasma was pipetted off and frozen at −70°C until quantitation of TxB_2 and PGE_2 was performed.

Quantitation of TxB_2. TxB_2, the stable metabolite of TxA_2, was measured in plasma of LPS-stimulated blood with a TxB_2 enzyme immunoassay system (Biotrak, Amersham, United Kingdom), as described by the manufacturer. The cross reactivities to related compounds were: 2,3-dinor-TxB_2: 60.50%; 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$: <0.40%; 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$; <0.01%; 11-dehydro-TxB_2, 0.10%; 6-keto-PGE_2, <0.01%; PGD_2, 0.18%; PGE_2, <0.01%; $PGF_{1\alpha}$, 1.6%; $PGF_{2\alpha}$, 0.06%; and arachidonic acid (AA), <0.01%. The sensitivity of this assay was 3.6 pg/mL, and the within and between assay CV were 2.5–9.2% (range 21.4–254 pg/mL) and 9.9–13.9% (range 46.2–222 pg/mL), respectively.

Quantitation of PGE_2. PGE_2 was measured in plasma of LPS-stimulated blood in a PGE_2 [^{125}I] RIA (radioimmunoassay) Kit (Du Pont, NEN Research Products, Boston, MA). The following compounds have been tested for cross reactivity: PGE_1, 30%; 13,14-dihydro-15-keto-PGE_2, 0.02%; PGA_2, 0.8%; $PGF_{1\alpha}$, 0.7%; TxB_2, 0.01%; $PGF_{2\alpha}$, 0.9%; AA, 0.01%; 13,14-dihydro-15-keto-$PG_{2\alpha}$, 0.005%; PGA_1; 0.08%; PGB_2, 0.07%; 6-keto-$PGF_{1\alpha}$, 1%; linoleic acid, 0.002%; and PGD_2, 0.3%. The sensitivity of the system was 4.4 pg/mL.

Measurements of antioxidant capacity. Antioxidant capacity was measured in citrated plasma by enhanced chemiluminescent assay as described elsewhere (25). The enhanced chemiluminescent immunoassay signal reagent and horseradish peroxidase conjugate were obtained from Amersham International (Amersham, United Kingdom). The signal reagent consisted of assay buffer and tablets A and B containing luminol, enhancer, and oxidant. The tocopherol analog standard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was obtained from Aldrich (Gillingham, United Kingdom). Chemiluminescence was measured in a Biocounter (Schaesberg, The Netherlands) (Lumac/3M, M2010, Switzerland).

Statistical analysis. The data are presented as mean ± standard error of mean (SEM). Differences between gender groups in baseline values of various parameters were determined by Student's unpaired *t*- or Mann-Whitney *U*-test. For each parameter tested, the differences between the values before and after intake of dietary oils were calculated within the groups. One-way analysis of variance followed by Dunnett's *t*-test was used for simultaneous, multiple comparisons of the test groups with the control group. When data are presented as percentages, statistical analysis was performed on absolute values, unless otherwise stated. Correlations were calculated by means of the Spearman's rank correlation coefficient (*r*). *P*-values <0.05 were considered as statistically significant. All analyses were performed on an Apple Macintosh using StatView™ SE+ Graphics version 1.04 statistical software (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Baseline characteristics. Statistical analysis (one-way analysis of variance and Dunnett's *t*-test) of baseline characteristics in the different groups of oils showed that TG in the whale oil group were significantly higher than the values observed in the control group ($P < 0.05$). For the other clinical and biochemical variables, there were no significant differences between the groups of oils.

Comparisons of differences in clinical and biochemical characteristics between the gender groups showed that men had higher mean values for age (37.9 and 34.3 yr for men and women, respectively), TG (1.5 ± 0.1 and 1.1 ± 0.1 mmol/L), and cholesterol (6.3 ± 0.1 and 5.7 ± 0.1 mmol/L). Women had

significantly higher HDL cholesterol (1.6 ± 0.03 and 1.2 ± 0.03 mmol/L) and TxB_2 values (12.5 ± 0.6 and 10.5 ± 0.5 ng/mL) than men.

Fatty acid composition in serum. The composition of total fatty acids in serum before and after 12 wk of supplementation with different dietary oils is given in Table 3. After 12 wk of daily intake, CLO and the mixture of CLO and seal oil both produced highly significant rises ($P < 0.01$) in serum EPA (20:5n-3), by 188 and 115%, respectively, and DHA (22:6n-3), by 56 and 50%. The same groups experienced a significant reduction (50–60%) of dihomo-γ-linolenic acid (20:3n-6) compared with the control group. The changes in EPA (110% increase, $P < 0.01$) and dihomo-γ-linolenic acid (43% decrease, $P < 0.05$) levels were also seen in the whale oil group. In addition, we observed an increase in the serum concentration of oleic acid (18:1n-9) in the group given olive oil (from 18.8 to 20.8%), but this change was not statistically significant.

There was a dose–response relationship between the relative contents of n-3 fatty acids in the various dietary oils and the change in total n-3 fatty acids in serum.

Fatty acid composition in platelets. When comparing the baseline fatty acid composition of serum (Table 3) and platelets (Table 4), we found that the relative composition of fatty acids did not differ much for all except three fatty acids: There were striking differences between stearic acid (18:0), linoleic acid (18:2n-6), and AA (20:4n-6) in serum and platelets. The relative contents of these fatty acids were as follows: 7.5 and 20.4% (18:0), 31.6 and 6.1% (18:2n-6), and 4.9 and 18.1% (20:4n-6) for serum and platelets, respectively. The strongest positive correlation between fatty acids in serum and platelets was found for 20:5n-3 ($r = 0.84$, $P < 0.0001$).

Table 4 shows that in all three groups given CLO (CLO alone or in mixtures with seal oil or olive oil) the concentration of EPA in platelet membrane preparations was significantly raised (by 118, 92, and 64%, respectively). In addition, in the CLO group, there were increases in 22:5n-3 and 22:6n-3 and a reduction in the relative content of AA (20:4n-6) by 21%.

Intramolecular fatty acid distributions in the marine oils. Distribution of the main n-3 PUFA in the TG molecule in the oils of marine origin was dependent on their source. Nuclear magnetic resonance data showed that 81% of EPA and 93.7% of DHA were localized in *sn*-1/3 positions of the TG in minke whale oil. Corresponding values for harp seal oil were 97.7 and 98.1%, and for CLO 58.2 and 22.3% for EPA and DHA, respectively.

Serum lipids. Neither the TG, total cholesterol, nor the HDL-cholesterol concentrations in any of the groups were significantly altered after 12 wk of supplementation with dietary oils, even though the TG were reduced by 10–12% in the CLO, CLO/seal, and whale oil groups (data not shown). Although men and women differed in baseline values of the serum lipids, no difference was observed between the two groups concerning effects of the oils when tested separately. Still, there was a strong trend to reduction of TG in the CLO and seal/CLO groups in women.

Platelet membrane fluidity. The r_S of the platelet membrane preparations labeled with TMA-DPH was unchanged after intake of marine oils and olive oil (data not shown).

MPV and platelet counts. MPV increased in both whale oil groups as well as in the CLO/olive oil group after 12 wk of dietary supplementation (Fig. 1), with the most pronounced effect in the refined whale oil group with a rise from 8.7 to 9.4 μm^3 (8%, $P < 0.01$). Baseline values of MPV correlated negatively with platelet counts ($r = -0.41$, $P < 0.0001$). The platelet count was significantly reduced only in the refined whale oil group (7%, $P < 0.01$).

Platelet products. TxA_2 is generated mainly from platelets in LPS-stimulated blood, and the measurement of its stable metabolite TxB_2 showed no significant changes after intake of dietary oils, both when the results were analyzed for the two genders together and separately. However, there was a tendency to reduction in all groups given CLO: TxB_2 was reduced by 39, 34, and 28% in the CLO, seal/CLO, and olive/CLO groups, respectively.

There were significant changes in the concentration of PGE_2 ($P < 0.01$) in all the groups given CLO [CLO only (28%) or CLO in mixture with seal oil (22%) or olive oil (32%)] when compared with the control group. However, the rise in PGE_2 was most pronounced in the control group (81%), so that the significant changes in PGE_2 in those three groups mean less rise in the concentrations of PGE_2 when compared with the control group.

The release of β-TG from platelets was not affected by any of the dietary oils (data not shown), even though the whale oils tended to reduce the concentrations of β-TG from 7.59 ± 0.41 to 6.43 ± 0.43 ng/mL (15%) in the unrefined whale oil group and from 7.63 ± 0.49 to 5.84 ± 0.34 ng/mL (23%) in the group receiving refined whale oil.

Antioxidant capacity. Analyses of antioxidant capacity in citrated plasma were preliminary studies and only performed on a limited number of samples in some of the test groups. There were no significant changes in the antioxidant capacity following supplementation with CLO, whale oil, or olive oil. In the control group ($n = 15$), the antioxidant capacities were 564 ± 34 (before) and 550 ± 35 μmol/L (after 12 wk). The baseline values were significantly ($P < 0.0001$) higher in men (628 ± 24 μmol/L, $n = 30$) than in women (464 ± l5 μmol/L, $n = 30$), as revealed by Student's unpaired *t*-test.

DISCUSSION

The main interest in the present work was to investigate the effects on physical and functional properties of platelets and possibly find any associations between the parameters measured and the fatty acid composition in serum and platelets. Changes in the distribution of total serum fatty acids partly reflected the fatty acid composition of the ingested dietary oils as seen in Tables 3 and 4. Serum ordinarily contains low levels of EPA (20:5n-3) and DHA (22:6n-3), but after admin-

TABLE 3
Fatty Acid Composition in Serum Before and After 12 wk of Supplementation with Dietary Oils (wt/wt% of total fatty acids)[a]

	Control ($n = 35$)		Cod liver oil ($n = 25$)		Olive oil ($n = 29$)		Whale oil, refined ($n = 38$)		Whale oil ($n = 31$)		Seal oil/CLO ($n = 36$)		Olive oil/CLO ($n = 33$)	
Fatty acid	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
14:0	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
16:0	23.8 ± 0.3	24.1 ± 0.3	23.4 ± 0.3	23.2 ± 0.3	24.3 ± 0.4	23.4 ± 0.3	23.7 ± 0.4	23.0 ± 0.3	24.0 ± 0.2	24.2 ± 0.3	23.4 ± 0.2	23.3 ± 0.3	23.7 ± 0.3	24.0 ± 0.3
18:0	7.5 ± 0.1	7.4 ± 0.1	7.5 ± 0.2	7.8 ± 0.2	7.8 ± 0.1	7.3 ± 0.1	7.2 ± 0.1	7.7 ± 0.1	7.6 ± 0.1	7.3 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.5 ± 0.1	7.6 ± 0.1
16:1n-7	2.1 ± 0.1	1.8 ± 0.2	2.0 ± 0.1	1.6 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	1.7 ± 0.1
18:1n-9	17.9 ± 0.5	18.2 ± 0.4	18.2 ± 0.3	16.4 ± 0.5	18.8 ± 0.8	20.8 ± 0.5	17.8 ± 0.6	17.8 ± 0.4	19.6 ± 0.4	18.1 ± 0.4	18.4 ± 0.4	16.7 ± 0.3	17.3 ± 0.6	18.6 ± 0.4
18:1n-7	2.2 ± 0.2	1.9 ± 0.1	2.1 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	2.1 ± 0.2	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.0 ± 0.2
20:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-7	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
18:2n-6	31.5 ± 0.7	32.3 ± 0.7	31.9 ± 0.6	31.0 ± 0.7	31.6 ± 0.7	31.5 ± 0.8	31.9 ± 0.6	31.5 ± 0.6	31.2 ± 0.6	30.8 ± 0.6	31.6 ± 0.7	30.6 ± 0.7	31.7 ± 0.8	31.2 ± 0.7
18:3n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
18:3n-3	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.8 ± 0.0	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.4 ± 0.1	0.7 ± 0.0	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
20:3n-6	1.3 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	0.6 ± 0.1[c]	1.3 ± 0.0	0.9 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	1.4 ± 0.0	0.8 ± 0.1[b]	1.3 ± 0.1	0.6 ± 0.1[c]	1.3 ± 0.1	0.8 ± 0.1
18:4	0.2 ± 0.0	0.6 ± 0.5	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n-6	5.1 ± 0.1	4.7 ± 0.2	5.1 ± 0.2	4.4 ± 0.2	4.7 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	4.7 ± 0.2	4.5 ± 0.2	5.0 ± 0.2	4.6 ± 0.2	5.0 ± 0.1	4.1 ± 0.1
20:4n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:5n-3	1.9 ± 0.3	1.7 ± 0.3	1.6 ± 0.2	4.6 ± 0.3[c]	1.3 ± 0.1	1.3 ± 0.2	2.1 ± 0.3	2.9 ± 0.2	1.4 ± 0.1	2.5 ± 0.2[c]	2.0 ± 0.3	4.3 ± 0.3[c]	2.1 ± 0.3	2.8 ± 0.2
22:5n-3	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
22:6n-3	2.9 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	4.2 ± 0.2[c]	2.4 ± 0.2	2.4 ± 0.1	2.7 ± 0.2	3.3 ± 0.1	2.5 ± 0.1	3.1 ± 0.1	2.8 ± 0.2	4.2 ± 0.1[c]	2.9 ± 0.2	3.4 ± 0.1

[a]Mean values ± SEM. The differences between values before and after dietary supplementation were tested by one-factor analysis of variance and Dunnett's *t*-test for multiple comparisons of the test groups with the control group. [b]$P < 0.05$ [c]$P < 0.01$.

TABLE 4
Fatty Acid Composition in Platelets Before and After 12 wk of Supplementation with Dietary Oils (wt/wt% of total fatty acids)[a]

	Control ($n = 36$)		Cod liver oil ($n = 34$)		Olive oil ($n = 29$)		Whale oil, refined ($n = 31$)		Whale oil ($n = 36$)		Seal oil/CLO ($n = 35$)		Olive oil/CLO ($n = 35$)	
Fatty acid	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
14:0	2.0 ± 0.1	2.4 ± 0.2	2.0 ± 0.2	2.4 ± 0.2	2.1 ± 0.3	2.1 ± 0.2	2.1 ± 0.1	2.5 ± 0.2	2.0 ± 0.2	2.4 ± 0.2	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.2 ± 0.2
16:0	23.7 ± 0.3	23.6 ± 0.3	23.6 ± 0.4	23.4 ± 0.4	23.5 ± 0.5	23.0 ± 0.3	22.9 ± 0.3	23.5 ± 0.3	23.5 ± 0.4	23.9 ± 0.2	23.2 ± 0.3	23.5 ± 0.3	24.0 ± 0.6	23.1 ± 0.3
18:0	21.4 ± 0.3	21.6 ± 0.3	21.1 ± 0.2	20.4 ± 0.4	21.3 ± 0.3	21.5 ± 0.3	21.2 ± 0.2	21.2 ± 0.4	21.2 ± 0.3	21.4 ± 0.4	21.5 ± 0.2	20.7 ± 0.3	21.2 ± 0.3	21.2 ± 0.3
18:1n-9	13.7 ± 0.2	13.8 ± 0.3	13.7 ± 0.2	14.1 ± 0.3	13.0 ± 0.6	14.7 ± 0.4	13.9 ± 0.2	13.9 ± 0.5	13.9 ± 0.2	13.8 ± 0.4	13.7 ± 0.2	14.6 ± 0.3	13.6 ± 0.2	14.5 ± 0.3
18:2n-6	6.3 ± 0.2	6.2 ± 0.2	6.3 ± 0.2	5.9 ± 0.1	6.4 ± 0.3	6.3 ± 0.1	6.4 ± 0.2	6.3 ± 0.1	6.5 ± 0.1	6.0 ± 0.2	6.4 ± 0.1	5.8 ± 0.2	6.5 ± 0.2	6.0 ± 0.1
18:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
20:1	0.7 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:2	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.8 ± 0.2	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
22:0	3.3 ± 0.1	3.2 ± 0.1	3.0 ± 0.1	3.3 ± 0.1	4.4 ± 1.3	3.2 ± 0.2	3.4 ± 0.2	3.0 ± 0.1	2.9 ± 0.1	3.5 ± 0.2	3.2 ± 0.1	3.5 ± 0.2	3.1 ± 0.2	3.2 ± 0.1
20:3n-6	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	1.4 ± 0.1
20:4n-6	18.9 ± 0.5	17.1 ± 0.4	19.4 ± 0.3	15.4 ± 0.5[c]	18.6 ± 0.9	17.6 ± 0.5	19.0 ± 0.3	15.8 ± 0.4	18.9 ± 0.4	16.21 ± 0.4	18.8 ± 0.4	15.5 ± 0.4	18.3 ± 0.4	16.2 ± 0.4
20:4n-3	0.1 ± 0.0	1.1 ± 0.2	0.1 ± 0.0	1.3 ± 0.5	0.1 ± 0.1	0.9 ± 0.2	0.0 ± 0.0	0.9 ± 0.2	0.1 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	1.0 ± 0.2	0.1 ± .0.0	1.0 ± 0.2
24:0	0.8 ± 0.1	1.8 ± 0.2	1.0 ± 0.3	1.7 ± 0.1	0.9 ± 0.1	1.6 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	0.7 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	0.8 ± 0.1	1.8 ± 0.1
20:5n-3	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	2.4 ± 0.1[c]	0.9 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.8 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	1.3 ± 0.1	2.5 ± 0.1[c]	1.1 ± 0.1	1.8 ± 0.1[b]
24:1	1.0 ± 0.0	1.0 ± 0.2	0.9 ± 0.0	1.4 ± 0.4	0.7 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	1.3 ± 0.2	0.8 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	1.1 ± 0.0	0.9 ± 0.0	1.3 ± 0.2
22:5n-3	1.2 ± 0.0	1.0 ± 0.1	1.2 ± 0.0	1.4 ± 0.1[b]	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.0[b]	1.1 ± 0.0	1.2 ± 0.1
22:6n-3	2.3 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.9 ± 0.1[c]	2.4 ± 0.1	2.3 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.9 ± 0.1	2.5 ± 0.1	2.8 ± 0.2

[a]Mean values ± SEM. The differences between values before and after dietary supplementation were tested by one-factor analysis of variance and Dunnett's *t*-test for multiple comparisons of the test groups with the control group. [b]$P < 0.05$ [c]$P < 0.01$.

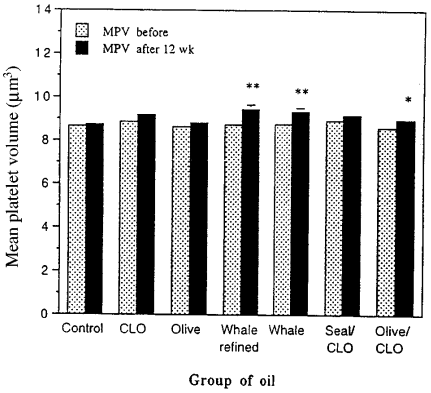

FIG. 1. Mean platelet volume (MPV) before and after 12 wk of supplementation with dietary oils. Values are means ± SEM. MPV was measured with a Coulter Counter in whole blood anticoagulated with Na_2-EDTA. MPV was significantly raised in both whale oil groups (**$P < 0.01$) and the group given a mixture of olive oil and cod liver oil (CLO) (*$P < 0.05$), tested by one-way analysis of variance and Dunnett's *t*-test for comparisons of the test groups with the control group.

istration of oils rich in these fatty acids, the levels were increased by more than 100%. Previous results showed an even stronger effect of similar oils on the levels of EPA and DHA, and in addition, whale oil induced a significant increase in these fatty acids (12). Compared to the high concentrations of n-3 PUFA in CLO and seal oil, the high content of monounsaturated fatty acids in the whale oils did not seem to influence the distribution of fatty acids in serum to the same extent. This may be due to the relatively high concentration of oleic acid (18:1n-9) already present in our regular diet.

It has been suggested that the stereochemistry of TG molecules may influence the digestion and absorption of fatty acids (26). The n-3 PUFA are located mainly in the outer positions *sn*-1 and *sn*-3 of the TG in seal and whale oil, whereas in TG from CLO the *sn*-2 position is enriched in n-3 PUFA. The present long-term study does not indicate differences in absorption of n-3 PUFA from oil of marine mammals or fish oil. Christensen *et al.* (27) demonstrated that the digestibility and lymphatic absorption in rats of n-3 PUFA from native marine oils were not significantly influenced by the localization of the n-3 PUFA within the TG. These findings, however, do not exclude possible differences in digestibility and absorption rates of n-3 PUFA from different sources. In a study in humans it was suggested that finwhale oil (with EPA mainly in positions *sn*-1/3) may provide a moderately more available source of dietary EPA than herring oil (EPA mainly in *sn*-2), as there was observed a higher average accumulation of EPA in platelet phospholipids after whale oil consumption (28). However, retroconversion from DHA (higher level in the whale oil) to EPA may have contributed to these effects (28). In addition, the short duration (10 d) of the study and the low number of subjects ($n = 3$) must be taken into account. The changes in DHA levels in serum and platelets following intake of DHA-containing oils were considerably lower than corresponding values for EPA, supporting differences in digestion and incorporation of these fatty acids, as shown by others (29).

The highly significant, positive correlation between EPA in serum and platelets indicates that the relative content of EPA in platelets is strongly dependent on the availability and the fact that EPA appears to be easily incorporated into phospholipids. Even though the serum concentration of AA (20:4n-6) was not altered significantly after intake of CLO, the increase in n-3 PUFA in platelets seemed to occur at the expense of AA. However, the fatty acid composition of phospholipids in platelets reflects the fatty acid composition of total fatty acids in serum only to some extent.

One objective of this work was to study whether the various dietary oils have any effect on platelet membrane fluidity. The oils had no observed influence on the physical properties of the platelet membrane, and thus membrane fluidity was not associated with alterations in platelet membrane fatty acid composition. This is supported by other studies on human platelets (30, 31), whereas rat platelets have shown increased membrane fluidity under the influence of a marine oil diet (32). Possibly, the modifications of the fatty acid pattern in our study were not extensive enough to alter the membrane fluidity, but this could also be due to mechanisms which are able to buffer changes in fatty acid composition and maintain the physical properties in the hydrocarbon region measured by the fluorescent probe. These findings may also be a consequence of the low sensitivity of the TMA-DPH fluorescence anisotropy technique.

In human intervention studies, the most consistent effect of n-3 PUFA on the plasma lipid profile is that they lower the level of TG in both normolipidemic and hyperlipidemic subjects (7), apparently by reducing hepatic TG synthesis and secretion of very low density lipoprotein TG (33–35). Even though our results did not show significant reductions in any of the groups, the oils with the highest relative contents of n-3 PUFA had the most pronounced reducing effects on the TG concentrations.

The overall results from several fish oil studies (7) showed that normolipidemic subjects responded to n-3 fatty acids with essentially no change in total or low density lipoprotein cholesterol and a slight rise in HDL cholesterol, which is consistent with the findings of our study. Women have a more favorable lipid pattern than men, including higher HDL cholesterol levels (36), but despite gender differences in baseline values in the present study there were no sex-related differences in serum lipids in response to the dietary oils. Lipid data from numerous fish oil studies reviewed by Harris (7) show a mean value of total cholesterol in control groups at about 4.8 mmol/L (range of means 3.4–6.2 mmol/L), and one might ask if the high level of total cholesterol (6 mmol/L) found in our

study reflects high cardiovascular risk in the population of northern Norway (37). More likely, the high cholesterol concentrations reveal the tendency of people with known moderately high cholesterol levels and a familial history of coronary heart disease to volunteer in such studies.

TxB_2 is the main oxygenated end product of the cyclooxygenase pathway in platelets and can be regarded as a parameter of platelet functionality. A nonlinear relationship has been demonstrated between the reduction of Tx production and reduction of platelet aggregation (38). It has been shown that more than 90% inhibition of Tx is necessary to obtain some inhibition of platelet aggregation (38). For practical reasons no aggregation measurements were done in the present study, but the reduced (not significantly) TxB_2 production (30–40%) observed in the CLO groups may indicate no severe impairment of the platelet aggregation caused by TxB_2. Previous studies have shown 30–65% reductions (17,39) in TxB_2 production in response to dietary marine oils, but in the study of Østerud *et al.* (17) only whale oil reduced the TxB_2 significantly. In several other *in vivo* and *in vitro* assays, dietary n-3 PUFA reduced eicosanoid synthesis significantly (40). We have used weak LPS stimulation of whole blood as a model for the reactivity of blood platelets, and we believe that this *ex vivo* system may reflect the weak stimuli for aggregation found in most *in vivo* conditions more than does measurement of maximally stimulated TxB_2 formed in serum. Thus the Tx production probably reflects the platelets' ability to be activated in this model system.

PGE_2, the other cyclooxygenase product measured, was clearly increased in all groups, including the control group during the period of 12 wk. Plasma was analyzed in bulk after each blood sampling, within 1 wk after the start of the dietary supplementation and within 3 wk after the end of the study. This difference in storage time may be the cause of the change observed. Nevertheless, when the results are adjusted for changes in the control group, the pattern is similar to the changes seen in TxB_2, with significant reductions in all CLO groups. As for TxB_2, although not significant, this may be connected to the observed reduction of 20:4n-6 in platelets in the CLO group.

For both TxB_2 and PGE_2, the measured values of the 2-series of these eicosanids may be overestimated owing to cross reactivity in the assay with the corresponding 3-series compounds. This could also possibly explain the failure to see any differences in TxB_2 and PGE_2 after intake of dietary oils.

Platelet factor 4 and β-TG are both released from the α-granules of platelets upon activation and correlate closely with the platelet aggregation score (41). In our study the β-TG concentration in plasma from LPS-stimulated whole blood was reduced in both whale oil groups (15–23%), but the changes were not statistically significant.

A reduction in platelet number associated with increased platelet volume has been observed in subjects given about 10 g of n-3 fatty acids as salmon oil per day (42). In our study the whale oils had the most pronounced effect on these platelet parameters. Large platelets are reported to be more reactive than small platelets (43) and are believed to be an independent risk factor for myocardial infarction (44). However, the reduced platelet number and slightly increased platelet size induced by fish oil supplements have been associated with reduced responsiveness to some aggregating agonists (45). It has repeatedly been observed in one individual that intake of marine oils over several months is associated with an increase in MPV and a decrease in platelet count. This is followed by a transient increase in platelet count and decrease in MPV after end of marine oil intake (Østerud, B., unpublished data); and interestingly, 4–6 wk after end of marine oil intake, there is a rise in MPV and a decrease in platelet count which resembles the platelets before intake of marine oils. Since the platelets from this person in their normal condition are large (MPV 11.0–11.5) and highly reactive, we believe that it is unlikely that the rise in MPV induced by marine oils is related to higher reactivity of platelets but may rather reflect alteration of the platelet membrane by unknown mechanisms.

Preliminary measurements do not indicate any differences between the oil groups regarding effect on the antioxidant capacity in plasma measured by enhanced chemiluminescent assay. Thus, the low levels of antioxidants present in the various oils did not seem to influence the antioxidant capacity. The supplement of PUFA may lead to enhanced oxidation of the fatty acids in plasma and possibly reduce the antioxidants buffering these reactions. However, this needs to be further investigated before any conclusions can be drawn.

In summary, EPA and DHA, both from marine fish and mammals, induced increases in the serum concentrations of the corresponding fatty acids as well as their relative contents in platelets. The absorption and incorporation of EPA were dependent on its availability. The present study indicates that none of the dietary oils induced changes in the plasma lipids, although oils containing CLO tended to induce reductions in the TG concentration. CLO, seal/CLO and olive/CLO also produced significant reductions in PGE_2 and a strong tendency toward reduction of TxB_2 generation in plasma of LPS-stimulated whole blood. β-TG, however, was not affected by any of the oils. In spite of alterations in fatty acid composition in the platelets in some of the groups, we observed no changes in platelet membrane fluidity. MPV was significantly increased and platelet counts reduced by dietary supplementation with refined and unrefined whale oils. Olive oil alone as a dietary supplement did not have significant effects on any of the parameters measured in this study. The doses of the dietary oils in this study (corresponding to 1.5 g EPA and 2.1 g DHA per day in the CLO group) were rather low compared both to other studies and to the complete diet of Greenland Inuits and Mediterranean populations. Based on the *in vivo* parameters measured (β-thromboglobulin, MPV, and platelet count), the whale oils and to some degree the combination of olive oil and CLO may have the best anticoagulant effect. These oils are also found to have antiinflammatory effects (Østerud, B., unpublished data).

The intake of fatty acids through the ordinary diet is not

adjusted for in this study. Possible relationships between the complete diet of the subjects and the parameters measured in the present study may give further explanation to some of the observations, and investigations are in progress in our group.

ACKNOWLEDGMENTS

This study was supported by the Norwegian Research Council, the Norwegian Council on Cardiovascular Diseases, the Nansen Foundation, and the Regional Authority of Northern Norway. The skillful technical assistance of Tone Jacobsen, Karin Lia, and Jan Ole Olsen is gratefully acknowledged.

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[Received September 9, 1996, and in final revised form May 21, 1997; revision accepted December 17, 1997]

COMMUNICATION

Contribution of Meat Fat to Dietary Arachidonic Acid

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ABSTRACT: Arachidonic acid (AA) in the diet can be efficiently absorbed and incorporated into tissue membranes, resulting in an increased production of thromboxane A_2 by platelets and increased *ex vivo* platelet aggregability. Results from previous studies have shown that AA is concentrated in the membrane phospholipids of lean meats. However, the concentration of AA in the visible fat portion of meats also may be significant despite being ignored in most studies. The aim of this study was to accurately quantitate the AA content of visible fat and the lean portion of beef, lamb, pork, chicken, duck, and turkey. The visible fat of meat contained a significant quantity of AA, ranging from 20 to 180 mg/100 g fat, whereas the AA content of the lean portion of meat was lower, ranging from 30 to 99 mg/100 g lean meat. Beef and lamb meats contained lower levels of AA in both the visible fat and lean portion than that from the other species. The highest level of AA in lean meat was in duck (99 mg/100 g), whereas pork fat had the highest concentration for the visible fats (180 mg/100 g). The lean portions of beef and lamb contained the higher levels of n-3 polyunsaturated fatty acids (PUFA) compared with white meats which were high in AA and low in n-3 PUFA. The present data indicate that the visible meat fat can make a contribution to dietary intake of AA, particularly for consumers with high intakes of fat from pork or poultry meat.

Lipids 33, 437–440 (1998).

The major cause of premature death in Western society is occlusive vascular disease, and the high dietary fat intake of modern societies has been implicated as one of the major dietary causes. In particular, saturated fats raise blood cholesterol levels, whereas polyunsaturated fatty acids (PUFA) lower blood cholesterol (1–3). Although, the PUFA are seen to be beneficial in this respect, there is concern that the Western diet may have an excessively high ratio of n-6/n-3 PUFA (4). This dietary imbalance is believed to cause an imbalance in the ratio of n-6/n-3 PUFA in the tissue membranes. Arachidonic acid (AA) is an essential n-6 PUFA which can be derived either from linoleic acid (18:2n-6) or directly from the diet, and it is the precursor of a multitude of vasoactive eicosanoids. It has been proposed that elevated tissue AA leads to increased production of thromboxane and leukotrienes, associated with thrombotic disease, arthritis, asthma, and psoriasis (5). Ferretti *et al.* (6) confirmed that 1.5 g/d of AA fed to 10 healthy male volunteers for 50 d led to a significant increase in metabolites of thromboxane A_2 and prostacyclin; however, these changes were not associated with increased platelet aggregation (7). Consumption of either red or white meat leads to increased levels of AA in plasma lipids (8).

We have previously calculated the AA content of the Australian diet based on the premise that all the AA in meat was found in the lean portion of the meat rather than the fat (9). In the past, most analytical studies have ignored the concentration of AA in visible fat of meat, perhaps because the level was too small to be significant when compared with the higher proportion of AA found in the lean portion of meat. The aim of this study was to examine the concentration of AA in both the visible fat and lean portion of meats commonly consumed in Australia. To achieve this, we have determined AA and other fatty acid concentrations in both visible fat and lean portions of beef, lamb, pork, chicken, duck, and turkey.

MATERIALS AND METHODS

Samples. All samples were purchased fresh from the local market and kept at −20°C for 4 d. The retail cut of meats included lamb chop, pork chop, T-bone steak, and turkey, chicken and duck drumsticks. Each type of meat was obtained from three different shops. The lean portion used was obtained from the flesh where all visible fat was trimmed off. Likewise, all the lean portion was trimmed from the fat portion, in order to avoid cross contamination. The fat of the poultry samples was obtained from underneath the skin whereas the fat from the beef and lamb was solid white visible fat. Before lipid extraction, all samples were chopped manually into fine pieces to increase the surface area of the samples exposed to solvent during lipid extraction.

Lipid analysis. The analytical procedures followed were those used previously to determine the lipid and fatty acid content of lean meat (10,11), with the following exceptions: 5-g samples of lean meat were analyzed here compared with 10-g previously, and tricosanoic acid (23:0) was used as an internal standard here compared with heptadecanoic acid previously (10). The chopped samples, approximately 1 g of visible fat and 5 g of lean portion of the meat from the six different species, were extracted with chloroform/methanol (2:1, vol/vol) containing 10 mg/L of butylated hydroxytoluene.

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Abbreviations: AA, arachidonic acid; PUFA, polyunsaturated fatty acids.

After storage of the samples overnight at 4°C, each sample was filtered, rinsed with an additional volume of extracting solvent, and partitioned against 0.2 vol of 0.9% NaCl solution until the lower phase was clear. This was evaporated under vacuum and the lipids transferred to a volumetric flask. The total lipid content was determined gravimetrically on an aliquot of this extract (10). The methyl esters of fatty acids of the total lipid extract were prepared by saponification of an exact amount of lipid (about 10 mg lipid for lean portion and 16 mg lipid for the fat portion lipid) together with 0.25 mg for the lean sample and 0.1 mg for the fat sample of tricosanoic acid (23:0) using KOH (0.68 mol/L in methanol) followed by transesterification with BF_3 in methanol (10). The 23:0 internal standard was used in preference to 17:0 because it is not present in meat samples, whereas 17:0 is a component of meat fat, particularly from ruminants (12). The AA content was determined by comparison of peak areas with that of the 23:0 internal standard. The fatty acid methyl esters were separated by capillary gas–liquid chromatography using a 50 m × 0.32 mm (i.d.) fused silica column bonded phase column (BPX70; SGE, Melbourne, Australia) with helium as carrier gas at a flow rate of 43 cm/s. The column oven was maintained at 125°C for 3 min and increased at a rate of 8°C/min to 220°C, and this temperature was maintained for the duration of the run. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters, and the results were calculated using response factors derived from chromatographing standards of known composition (Nu-Chek-Prep, Elysian, MN).

Statistics. Results are shown as mean ± SD. The differences in AA content between the lean and fat portion were tested by *t*-test, with $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

The percentage of total lipids in the visible fat portion of the meat cuts ranged from 54 to 84 g/100 g, with turkey containing the least and beef containing the most lipid among the six species. The lipid content of lean meats ranged from 1 g/100 g in turkey to 4.2 g/100 g in lamb meat (Tables 1 and 2).

Visible fat of ruminants had a lower n-6 proportion (<3%) compared with nonruminants (>11%). The lean portion of ruminant meats was lower in total PUFA (<17%) and in the proportion of n-6 PUFA (<11%) than the other meats. Lean portions from duck and turkey were particularly high in PUFA (>34%) and the n-6 fraction (>30%). In both the lean tissue and visible fat of all meats, there was a higher proportion of n-6 than n-3 PUFA. However, beef and lamb had a notably higher n-3 level in the lean tissue than the poultry and pork and a much lower n-6/n-3 ratio in lean and fat tissue than did the subcutaneous fat of poultry and pork (<2 compared with >9). These differences in n-3 PUFA levels in red and white meat are reflected in the serum phospholipid fatty acid of subjects consuming red and white meats (8). No long-chain n-3 PUFA were detected in the visible fat samples (<0.01% total fatty acids) and only trace amounts of long-chain n-6 PUFA. AA was present at levels from 0.03 to 0.3% in the fat tissue from the samples. Lean beef contained the highest proportion of n-3 PUFA (6.4%) followed by 3.7% in lean lamb and 1.4 to 3.0% in the white meats (Tables 1 and 2).

The principal fatty acid of interest in this study is the n-6 PUFA, AA. Figure 1 shows the concentration of AA in both visible fat and lean portions of the meats, which ranged from 21 to 180 mg/100 g for the visible fats and from 27 to 99 mg/100 g for the lean meats. This showed that AA was sig-

TABLE 1
Fatty Acid Composition, Total Lipid and Arachidonic Acid Content of Lean Meats

Fatty acid	Beef	Lamb	Pork	Chicken	Duck	Turkey
Total saturated	41.3 ± 0.7[a]	45.6 ± 1.4	36.2 ± 0.6	32.8 ± 0.7	35.1 ± 3.0	37.2 ± 2.3
Total monounsaturated	42.0 ± 4.0	44.0 ± 3.3	42.8 ± 4.1	46.7 ± 1.1	30.4 ± 5.8	28.0 ± 7.7
Total n-6	10.2 ± 2.0	6.6 ± 1.6	19.5 ± 3.8	18.5 ± 1.6	31.4 ± 3.1	31.6 ± 4.8
18:2n-6	5.6 ± 1.1	4.9 ± 1.3	14.4 ± 3.4	14.4 ± 1.7	18.2 ± 3.4	18.6 ± 4.4
20:2n-6	0.6 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.6 ± 0.3
20:3n-6	0.8 ± 0.2	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.2
20:4n-6	3.0 ± 0.6	1.2 ± 0.3	3.6 ± 0.3	2.5 ± 0.3	9.3 ± 1.8	9.2 ± 0.9
22:4n-6	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	1.8 ± 0.2	1.9 ± 0.3
22:5n-6	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.3	0.5 ± 0.0
Total n-3	6.4 ± 1.6	3.7 ± 0.7	1.4 ± 0.2	1.9 ± 0.1	2.9 ± 0.4	3.0 ± 0.8
18:3n-3	1.8 ± 0.5	2.0 ± 0.4	0.6 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
20:5n-3	1.7 ± 0.3	0.7 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
22:5n-3	2.5 ± 0.7	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	1.2 ± 0.2	1.2 ± 0.4
22:6n-3	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.9 ± 0.1	1.0 ± 0.3
n-6/n-3	1.6 ± 0.1	1.8 ± 0.2	13.9 ± 2.4	9.6 ± 0.4	10.7 ± 0.4	10.8 ± 1.7
Total lipid[b]	1.4 ± 0.2[b]	4.2 ± 1.0	2.0 ± 0.3	2.3 ± 0.5	1.7 ± 0.3	1.0 ± 0.1
20:4n-6[c]	28 ± 5[c]	39 ± 13	54 ± 5	43 ± 10	99 ± 38	74 ± 29

[a]Mean ± SD, $n = 3$, analyzed in duplicate; wt% of total fatty acids.
[b]g/100 g.
[c]mg/100 g.

TABLE 2
Fatty Acid Composition, Total Lipid and Arachidonic Acid Content of Lean Meats

Fatty acid	Beef	Lamb	Pork	Chicken	Duck	Turkey
Total saturated	48.7 ± 6.7[a]	52.6 ± 3.6	35.3 ± 3.5	31.1 ± 0.6	30.3 ± 0.6	33.6 ± 0.5
Total monounsaturated	41.9 ± 6.7	34.5 ± 2.5	43.8 ± 1.0	52.6 ± 2.1	54.0 ± 0.7	43.1 ± 1.3
Total n-6	1.1 ± 0.1	2.1 ± 0.1	16.8 ± 4.3	11.9 ± 2.1	12.6 ± 0.4	18.7 ± 0.7
18:2n-6	1.0 ± 0.1	1.9 ± 0.1	15.7 ± 4.2	11.6 ± 2.1	12.2 ± 0.4	18.2 ± 0.5
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-6	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.03 ± 0.01	0.04 ± 0.02	0.29 ± 0.04	0.11 ± 0.03	0.19 ± 0.01	0.28 ± 0.10
Total n-3[b]	0.6 ± 0.1	1.2 ± 0.1[b]	1.3 ± 0.3	1.0 ± 0.1[b]	0.8 ± 0.1[b]	1.2 ± 0.0[b]
18:3n-3	0.6 ± 0.1	1.1 ± 0.1	1.1 ± 0.3	1.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.0
n-6/n-3	1.9 ± 0.1	1.7 ± 0.1	12.9 ± 1.2	11.5 ± 1.2	16.7 ± 2.7	15.2 ± 0.8
Total lipid[c]	84.1 ± 1.1	81.6 ± 3.8	69.4 ± 7.0	66.4 ± 3.3	76.5 ± 9.8	54.7 ± 4.5
20:4n-6[d]	21 ± 9	31 ± 12	180 ± 8	74 ± 28	137 ± 16	102 ± 19

[a]Mean ± SD, $n = 3$, analyzed in duplicate; wt% of total fatty acids.
[b]Includes 20:5n-3.
[c]g/100 g.
[d]mg/100 g.

nificantly ($P < 0.05$) more concentrated in visible fat than in lipids of the lean portion of the meats for pork, chicken and duck, although as a percentage the AA was only a minor constituent in the visible fat (<0.3%) compared with 1.2–9.0% in the lean meat (Tables 1 and 2). The total AA concentrations in both the fat and lean portions of lamb and beef were lower than in the pork and poultry samples. The richest fat source of AA was the pork fat (180 ± 8 mg/100 g). The AA concentration of lean duck varied from 62 to 137 mg/100 g (mean ± SD; 99 ± 38), and there was also generally more variation in the fatty acid composition of lean duck meat than for the other lean meats. While we don't have an explanation for this result, it is possible that there are differences in fatty acid composition between muscle types in ducks (that is, the site on each drumstick) or that the duck samples analyzed were from animals fed diets with different proportions of n-6 PUFA. The AA concentrations from lean meat obtained in this study were in agreement with the values reported by Mann *et al.* (9).

In the present study, we found that AA concentration in the visible fat of meat cuts was greater than in the lean portion in four of six species examined. The likely reason that AA was not reported in visible fats of meat previously is because the proportion of AA (percentage of total fatty acid) is very low in visible fat of meat (0.03 to 0.3% of total fatty acids) and therefore difficult to accurately measure even using capillary gas–liquid chromatography. In a separate study, we found that more than 80% of the AA was in the triacylglycerols from similar meat fats (D. Li and A. Sinclair *et al.*, unpublished data).

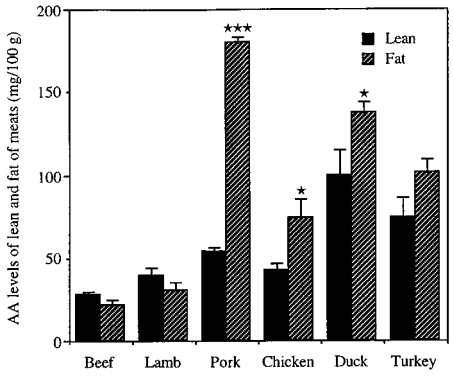

FIG. 1. Arachidonic acid (AA) levels of lean and visible fat of common Australian meats (mg/100 g). Results are shown as the mean ± SD; *$P < 0.05$, ***$P < 0.001$ (*t*-test).

Previous data from our group reported that the dietary AA intake of Australians was between 100–200 mg/d (9). This estimate was based on the AA values from raw meat and calculated assuming that AA was only found in lean meat. The present data, which indicate that visible fat contains AA, could alter the dietary intake data depending on which meat a consumer favored. In the case of beef and lamb consumers, there would be little difference between dietary AA intakes with and without fat-trimming of the meat since the AA levels are about the same in the fat and the lean. On the other hand, the dietary AA intake would be expected to be higher than previously reported for consumers who primarily ate pork, chicken, duck, and turkey. For example, a person who consumed 100 g beef which included 10% visible fat would have an AA intake of 28 mg compared with an intake of 29 mg from 100 g lean beef (without visible fat), while a person who consumed 100 g pork with 10% visible fat would have an AA intake of 67 mg compared with an intake of only 55 mg from 100 g lean pork (without visible fat). These figures are only approximate since they do not take into account the possible losses of PUFA which can occur following cooking (13) or losses due to fat dripping from meat following cooking.

In conclusion, this study shows that meat fats do contain a significant proportion of AA compared with the lean meat. Therefore, calculation of dietary intakes of AA need to take account of the AA content of both the visible and lean portion of meat.

ACKNOWLEDGMENT

This study was supported by a grant from the Meat Research Corporation of Australia.

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[Received October 29, 1997, and in final revised form February 17, 1998; revision accepted February 20, 1998]

Cycloserine-Induced Decrease of Cerebroside in Myelin

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ABSTRACT: L-Cycloserine has been shown specifically to lower brain cerebroside levels *in vivo,* but the extent to which the decrease in whole brain cerebroside content reflects lower myelin cerebroside levels is not known since a substantial portion of cerebroside is found in nonmyelin membranes. The present report demonstrates that chronically administered cycloserine lowers the proportion of cerebroside in rat brain myelin. Cycloserine-induced decrease of myelin cerebroside should provide a useful tool in investigating the role of cerebroside in maintaining myelin stability.
Lipids 33, 441–443 (1998).

Galactosylceramide (GalCer; cerebroside) is highly enriched in brain membranes as compared with other tissue membranes and constitutes a significant portion of the lipid in myelin (1). Although the specific role of GalCer in myelin has not been firmly established, the high levels suggest that GalCer significantly contributes to the organization of myelin, presumably through its interaction with other lipids and/or proteins in myelin.

Mouse mutants unable to synthesize GalCer have proven useful in answering some questions regarding the role of galactosphingolipid in myelination and myelin structure (2,3). However, these mouse models have several disadvantages as a tool to investigate the interaction of cerebroside with other myelin components; the foremost disadvantage is that sulfatide, synthesized from GalCer and a myelin component at levels ~25% of GalCer levels, is also absent in the GalCer-deficient mice, which precludes distinguishing the specific effects that the absence of GalCer has on myelin structure. An alternative experimental paradigm could be useful in overcoming disadvantages of these mouse models.

L-Cycloserine is an inhibitor of serine palmitoyltransferase (3-ketodihydrosphingosine synthase) (E.C. 2.3.1.50) (4), the first step in the synthesis of sphingolipids. Chronic, subcutaneous cycloserine injections in 16-d-old mice significantly lowered levels of whole-brain GalCer as compared to controls, although the level of other brain sphingolipids was not significantly altered (5). Because a substantial portion of the brain GalCer is present in nonmyelin membranes (1,6), the cycloserine-induced decrease in brain GalCer could reflect lower levels of GalCer in nonmyelin membranes or in myelin or in both groups of membranes. If this cycloserine treatment decreased myelin GalCer levels as observed with the level of whole brain GalCer, the experimental protocol could be used to investigate the role of myelin GalCer in myelin structure. In the present study, we asked whether chronic treatment of rats with L-cycloserine specifically decreases the concentration of myelin GalCer in a manner analogous to the cycloserine-induced decrease of whole-brain GalCer.

MATERIALS AND METHODS

Female Wistar rats, 16 d old (Charles River Laboratories, Wilmington, MA) received a daily subcutaneous injection (100 mg/kg of body weight) of L-cycloserine (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline, pH 7. Control rats received a daily injection of phosphate-buffered saline. After 7 d, rats were killed; the brain was removed; medulla oblongata, pons and cerebellum were discarded; and the remaining portion of the brain was weighed and frozen.

Myelin was isolated (7,8), and aliquots were lyophilized and used to determine total weight of myelin isolated and total myelin protein (9,10). Lipids were extracted (11) from the remaining myelin, and an aliquot of the lipid fraction was used to quantitate lipid phosphorus (12) which included glycerophospholipids and sphingomyelin. To quantitate GalCer and sulfatide, the remaining lipid fraction was subjected to alkaline methanolysis (8), and lipids in the $CHCl_3$ fraction were chromatographed on an activated silica gel thin-layer plate (Fisher Scientific, Philadelphia, PA) using $CHCl_3/CH_3OH/H_2O$ (55:27:4, by vol). GalCer and sulfatide were subsequently visualized with I_2, and silica gel areas binding these lipids were scraped from the plate. Lipids, without extraction from the silica gel, were reacted with fluorescamine (Sigma Chemical Co.) and quantitated using spectrofluorometry (13).

Data for the control and cycloserine-treated rats were obtained from six individual rats in each group and are expressed as means ± SEM. Statistical difference between cycloserine-treated and corresponding control data was determined using an unpaired Student's *t*-test.

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Abbreviation: GalCer, galactosylceramide (trivial name: cerebroside).

RESULTS AND DISCUSSION

With the notable exceptions of the medulla oblongata and pons, relatively little myelin deposition had taken place in rat brain when cycloserine treatment was initiated at 16 d of age. Since a significant amount of myelin deposition in medulla oblongata and pons would have resulted in an apparent higher level of myelin cerebroside in cycloserine-treated rats (assuming the level of cerebroside in preformed myelin is not affected by cycloserine treatment), these regions were excluded in this study. The cerebellum was not used to expedite the handling of the tissue.

Table 1 lists the effects of cycloserine treatment in 16-d-old rats injected subcutaneously with L-cycloserine for seven consecutive days. Body and brain weight were slightly, but significantly, lower (14 and 7%, respectively; $P < 0.001$) as compared to control rats. In contrast, the amount of brain myelin recovered from cycloserine-treated rats was dramatically reduced (51%; $P < 0.001$) compared to controls. A lower rate of increase in body and brain weight during the critical period of developmental myelination has been correlated with a decrease in the accumulation of brain myelin, as in the case of undernutrition; however, based on the small differences in brain and body weight between cycloserine-treated and control rats, the accumulation of brain myelin in cycloserine-treated rats, when compared to the accumulation of brain myelin in control rats, was dramatically lower than expected (14).

Compared with myelin from control rats, the total myelin lipid phosphate, i.e., phospholipid component of the myelin lipid, and the total myelin protein from cycloserine-treated rats were diminished in brain myelin. However, compared with myelin from control rats, the quantity of myelin isolated from cycloserine-treated rats was also diminished and to the same extent as the relative decrease in cycloserine-treated rats of myelin phospholipid and protein. The fact that the relative decreases of myelin phospholipid and protein parallel the relative decrease in the quantity of myelin isolated from cycloserine-treated rats suggests that the lower amount of myelin phospholipids and protein reflect the diminished myelin accumulation in cycloserine-treated rats. Nevertheless, the apparently unchanged level of total phospholipids in myelin in cycloserine-treated rats does not preclude the possibility that the levels of individual phospholipid classes are altered as a result of the altered GalCer level in myelin. Similarly, the absence of any significant change in total protein levels in brain myelin of cycloserine-treated rats compared to controls does not rule out changes in individual myelin protein levels. Myelin sulfatide levels in cycloserine-treated rats were decreased slightly but not significantly as compared to levels in control rats.

The GalCer level in brain myelin from cycloserine-treated rats was decreased significantly compared to levels in control rats, analogous to the whole-brain GalCer levels in cycloserine-treated mice (4). The partial inhibition of cerebroside synthesis could have resulted in the unexpected dramatic decrease of brain myelin deposition in cycloserine-treated rats. Baumann (15) proposed that a minimal level of specific myelin components, including GalCer, might be required for the deposition of stable myelin. Thus, it is possible that the cycloserine treatment diminished the synthesis of GalCer to a level which was insufficient for the amount of myelin deposition observed in control rats. The fact that the relative level of sulfatide in myelin was not significantly affected by cycloserine treatment, even though GalCer is the precursor to sulfatide, suggests that the regulation of myelin sulfatide levels is not necessarily linked to myelin GalCer levels.

These data demonstrate that the chronic, subcutaneous injection of 16-d-old rats with L-cycloserine, analogous to the mouse (5), results in a significant decrease in the proportion of GalCer in brain myelin. Thus, this report extends earlier observations by demonstrating that myelin GalCer is specifically reduced by cycloserine treatment. An experimental paradigm utilizing cycloserine could prove useful in delineating the role of GalCer in maintaining myelin structure through its interaction with myelin components.

TABLE 1
Effects of Cycloserine Administration on Body and Brain Weight and the Deposition and Composition of Myelin[a]

	Control	Cycloserine-treated	Decrease (%)
Body weight (g)	56.0 ± 2.2	48.2 ± 0.8[a]	14
Brain weight (g)	1.18 ± 0.02	1.10 ± 0.02[a]	7
Myelin isolated (mg/brain)	15.3 ± 0.4	7.8 ± 0.6[a]	51
Myelin lipid P (μg)	1202 ± 108	594 ± 65[b]	51
Myelin protein (μg)	2852 ± 219	1492 ± 141[b]	48
Myelin lipid P (μg)/myelin protein (μg)	0.42	0.40[c]	5
Myelin GalCer (μg)/100 μg myelin lipid P	9.39 ± 0.38	6.20 ± 0.70[a]	34
Myelin sulfatide (μg)/100 μg myelin lipid P	3.09 ± 0.22	2.91 ± 0.29[c]	6

[a]Experimental rats received a daily injection of L-cycloserine in buffer. Control rats were injected with buffer. After 7 d, rats were killed; the brain was removed; myelin was isolated; and aliquots were used to quantitate myelin weight, lipid phosphorus (glycerophospholipids and sphingomyelin), and protein. GalCer and sulfatide were isolated and quantitated from the remaining myelin. Data from control and cycloserine-treated rats (6 rats/group) are expressed as means ± SEM. Statistical difference between cycloserine-treated and corresponding control data was determined using an unpaired Student's *t*-test. [a]$P < 0.001$; [b]$P < 0.01$; [c]Not significant.

ACKNOWLEDGMENTS

This work was supported by grant NS26844 from the National Institutes of Health. The authors thank Marina Hoffman for helpful suggestions in the preparation of this manuscript.

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[Received January 27, 1997, and in final revised form January 23, 1998; revision accepted March 4, 1998]

ARTICLE

A ^{13}C Nuclear Magnetic Resonance Study of Free Fatty Acid Incorporation in Acylated Lipids in Differentiating Preadipocytes

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ABSTRACT: To understand the role of free fatty acid (FFA) incorporation in the accumulation of lipids in the adipocyte and ultimately in the development of obesity, ^{13}C nuclear magnetic resonance was used to study lipid metabolism in differentiating preadipocytes. The incorporation of ^{13}C=O-labeled FFA into cellular lipids in primary cultured rat preadipocytes and 3T3L1 preadipocytes at different stages of differentiation was monitored by the ^{13}C carbonyl chemical shift. Significant incorporation of palmitic acid into phosphatidylcholine in both the α and β acyl chain positions was found in cells at early stages of differentiation. At later differentiation stages or after extended incubation periods, most of the ^{13}C=O signals were found in the triacylglycerol (TG) molecules. Unsaturated ^{13}C=O-labeled acyl chains were detected in the TG molecules when cells were incubated with saturated ^{13}C=O-labeled FFA, indicating that intracellular dehydrogenation had occurred in the ^{13}C=O-labeled palmitoyl chain. By using ^{13}C-labeled methyl myristate as an internal intensity reference, incorporation of ^{13}C FFA into each acyl chain position of the major intracellular lipids was determined quantitatively.

Lipids 33, 449–454 (1998).

Circulating fatty acids can exist in esterified [e.g., triacylglycerols (TG) in lipoprotein particles] or unesterified forms [free fatty acids (FFA) bound to serum albumin]. In addition to acting as signal transducers and regulators of gene expression (1), FFA serve as an important energy source either for direct oxidation or for lipid synthesis and storage. Adipocytes are the major reservoir of FFA storage and release (2,3).

The applicability of ^{13}C nuclear magnetic resonance (NMR) spectroscopy for investigating adipose tissue metabolism, both *in vitro* and *in vivo*, has been demonstrated (4–7). More recently, ^{13}C NMR analysis of perfused cultured 3T3L1 adipocytes (2) documented the utility of ^{13}C=O-labeled FFA as substrates for monitoring lipid metabolism. These studies were all performed on adipose tissue or fully differentiated adipocytes, and the observed ^{13}C signals were exclusively from TG molecules.

In vivo ^{13}C NMR studies have limitations in spectral resolution and, in some cases, sensitivity. The alternative procedure of studying cell extracts by ^{13}C NMR permits optimal resolution and sensitivity for a given mass of lipid, as samples can be signal-averaged for long time periods in stable organic solutions. The latter approach is advantageous especially when the cell numbers per experiment are limited (as is often the case in studies of primary cultured cells) or when the incubation time with FFA is short (see below).

We examined incorporation of ^{13}C -labeled FFA into the lipids of differentiating preadipocytes by obtaining ^{13}C NMR spectra of cellular lipid extracts. With the high spectral resolution obtained, the incorporation of exogenous FFA into both phospholipids and TG was detected, and the specificity of the esterification reaction with respect to acyl chain position was studied. *De novo* acyl chain dehydrogenation of the TG-incorporated ^{13}C-labeled FFA both at the α and β positions was also detected. Such dehydrogenation occurred in both rat and 3T3L1 preadipocytes, but was more significant in the latter.

EXPERIMENTAL PROCEDURES

Cell preparation and differentiation. Male 3-mon-old Fischer 344 rats (Harlan Sprague-Dawley) were acclimatized for 1 wk and then were decapitated under CO_2 analgesia. The caudal portion of perirenal fat depots, which contains little brown fat (8), was removed and digested in collagenase as described previously (9). Stromal-vascular cells were isolated by centrifugation at $800 \times g$ for 10 min and were plated in basal medium with α minimum essential Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL). After 12 h, a period during which no cell replication occurs (10), the attached cells were trypsinized and replated in basal medium at a density of 4×10^4 cells/cm^2 in 25 cm^2 flasks. Replating permits separation of preadipocytes in the stromal-vascular fraction from other cell types. We have shown that 95% of cells isolated by this procedure are preadipocytes (9,11). After 1 d, the basal medium was changed, and then for another 2 d, cells were cultured in basal

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FFA, free fatty acid; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; TG, triacylglycerol.

medium by which time they were confluent. After this, cells were cultured in an enriched differentiation medium containing 10 μg/mL insulin, 10 μM glucose, 5 μL/mL Intralipid 10% (Miles Laboratory, North Chicago, IL), and 20% Nuserum (Collaborative Research, Bedford, MA), which promotes preadipocyte differentiation (9). In most experiments, cells were exposed to this medium for 2 d to achieve partial differentiation; in some experiments, cells were exposed for longer periods to increase the percentage of cells with extensive lipid accumulation before incubation with FFA.

NIH 3T3L1 cells were cultured as described previously (12). Briefly, DMEM supplemented with 10% calf serum and antibiotics was used to culture cells until confluence. The cells were then treated with DMEM containing methylisobutylxanthine (120 μg/mL), dexamethasone (0.39 μg/mL), and insulin (10 μg/mL) to induce differentiation. Two days later, methylisobutylxanthine was removed, and cells were maintained in DMEM + 10% fetal calf serum containing insulin (2.5 μg/mL) for 6 d.

Sample preparation. When cells were ready for the FFA incubation experiments, a solution containing ^{13}C=O-labeled FFA (10 mM, mole FFA/mole bovine serum albumin = 5) was added to the culture medium (final concentration of FFA = 1 mM) during a regular medium change. Cell cultures were harvested by removing the medium, washing three times with phosphate-buffered saline buffer, and scraping off the cells in methanol. After the cells were extracted by the Folch method, the organic phase containing the lipids was dried under N_2, redissolved in deuterated chloroform ($CDCl_3$), and covered with dry N_2 in the NMR tube to prevent oxidation during NMR experiments. Vacuum drying was not used to prevent the loss of low boiling-point lipids.

Chemicals. ^{13}C=O-labeled FFA (palmitic acid and myristic acid) and $CDCl_3$ were purchased from Cambridge Isotope Laboratory (Cambridge, MA). Tripalmitin was purchased from Nu-Chek-Prep (Elysian, MN). Mixed acyl chain TG (1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol and 1,3-dipalmitoyl-2-oleoyl-*rac*-glycerol, ~99%) were purchased from Sigma (St. Louis, MO). The BF_3-methanol kit (5 mL/ampule) was purchased from Supelco (Bellefonte, PA). All other organic solvents (methanol, hexane, benzene, and chloroform) were high-performance liquid chromatographic grade and purchased from Aldrich (Milwaukee, WI). ^{13}C=O-labeled methyl myristate was synthesized as follows: 5 mg of ^{13}C=O-labeled myristic acid was dissolved in 5 mL of benzene in a 25-mL test tube; 5 mL of dry methanol was added, followed by 5 mL of BF_3 in methanol (Supelco). The tube was covered with Teflon tape and a screw cap, then heated for 30 min at 100°C in an aluminum heating block. After cooling, 5 mL of deionized water was added and the methyl myristate, a long-chain methyl ester, extracted with hexane. The solvent was evaporated under a N_2 stream. The reaction yield was usually near 95%, and thin-layer chromatography (hexane/ethyl ether/acetic acid = 80:20:1) was used to check purity. There was no detectable FFA on the thin-layer chromatography plate. The methyl myristate was recrystallized in hexane at 4°C.

NMR methods. ^{13}C NMR spectra were obtained at 125 MHz on a Bruker DMX-500 spectrometer (Billerica, MA). A 5-mm triple resonance probe was used to minimize the sample volume (~0.5 mL). A 90° pulse width (12 μs), ^{1}H decoupling with the inverse-gated Waltz-16 sequence, and a pulse interval of 7.0 s were used for all spectra. All NMR experiments were performed at 12°C to minimize solvent evaporation during the data acquisition period. The center peak of solvent ($CDCl_3$) in the ^{13}C NMR spectrum was used as an internal chemical shift reference (77.0 ppm). All spectra were acquired with 8000 scans (rat cells), 4000 scans (3T3L1 cells), or 1000 scans (pure TG). Spectra were processed without linebroadening to optimize resolution. The integrated intensities had an experimental uncertainty of <5%.

To study FFA incorporation quantitatively, an internal intensity reference is needed. Ideally, this molecule would have a carbonyl signal with a resonance frequency close to, but not overlapping, the carbonyls of phosphatidylcholine (PC), TG, and other acylated common lipids or the carbonyls of FFA. For this purpose, we chose methyl myristate which has a chemical shift at 174.5 ppm in $CDCl_3$ at 12°C. However, the spin-lattice relaxation time (T_1) of the ^{13}C=O in this molecule (8.5 s) is longer than the values usually found in TG and PL (1–2 s). To achieve quantitative measurements with minimal experimental times, we first acquired a spectrum of a lipid extract with the internal integral reference using a 50-s pulse interval to achieve equilibrium intensities for every resonance; in this experiment, the integrated intensity is in direct proportion to the concentration of the nucleus. Then, we repeated the experiment with the same sample at a 7-s pulse interval. It was found that the lipid signal (per internal reference intensity) was 4.2 times higher in spectra with a 50-s pulse interval than that with a 7-s pulse interval because of attenuation of the reference signal with the longer T_1. Thus, 4.2 was used as a calibration factor to calculate the lipid quantities obtained from the integrated intensities from the ^{13}C spectrum obtained with a 7-s pulse interval.

RESULTS AND DISCUSSION

Effects of stage of differentiation on FFA incorporation. Differentiating preadipocytes take up and metabolize FFA rapidly, and the amount of lipid accumulated depends on the extent of differentiation. Cultures usually contain a mixture of cells at various stages of differentiation. Because the general features of the ^{13}C NMR spectra of lipid extracts are likely to depend on the extent of cell differentiation, cells used in this study were first examined under phase contrast microscopy and categorized into four different stages according to the extent of lipid accumulation (see Table 1). Cells at a specific stage of differentiation were then studied by ^{13}C NMR as a function of incubation time.

The ^{13}C NMR spectra of lipid extracts from rat perirenal cells at various stages of differentiation [Table 1 (rows A–C)] incubated with ^{13}C=O-labeled palmitic acid for 5 h are shown in Figure 1. Signals from naturally abundant lipids in these

TABLE 1
The Stages of Differentiation[a] of Cells as Observed by Phase Contrast Microscopy at the Time When FFA/BSA Incubation Was Initiated[b]

Sample	S1	S2	S3	S4
A	80	20	0	0
B	30	70	0	0
C	15	70	15	0
D	30	65	4.5	0.5
E	2	72	25	1

[a]Cell morphology changes with the amount of lipid accumulated. We arbitrarily divided differentiation into four stages: S1, rounded cells with faint dark stripling; S2, a dozen clear droplets seen inside cells under high-power magnification; S3, larger doubly refractive droplets, more than six droplets in each cell; S4, one to six lipid droplets visible under low power magnification.
[b]Because incubation with free fatty acid/bovine serum albumin (FFA/BSA) affects the intracellular lipid accumulation process, and hence possibly the stages of differentiation, therefore, the state of cell differentiation was determined only at the beginning of the incubation (error ±15%).

cell extracts were too weak to be detected in our spectra, and only the spectral region containing the signals from ^{13}C=O-labeled lipids is presented. [The lack of signals from ^{13}C-enriched carbons in other spectral regions also implies that there was not significant acyl chain elongation of the ^{13}C=O FFA during the incubation period (within 24 h) as discussed in a previous study (2).] Each carbonyl signal in the ^{13}C NMR spectra represents a specific carbonyl group with ^{13}C=O enrichment. By comparison with chemical shifts of known individual lipids (TG, PC, and FFA) under the same experimental conditions (not shown), the lipid mixture in the cell extract was identified as PC and TG as designated in Figure 1a. The FFA signal in $CDCl_3$, which resonates ~2–5 ppm downfield from the carbonyl signals of PC and TG, was not detected in these experiments. This finding is in agreement with previous reports that the intracellular concentrations of FFA are very low in living cells (13–15). [The chemical shift of pure FFA in dry $CDCl_3$ has been reported to be about 180 ppm (17). However, lipid extracts from tissue contain trace amounts of water and inorganic salts that are very difficult to remove. Because the chemical shifts of C=O in FFA are very sensitive to ionization conditions, these minor components might shift the C=O resonance.] The different acyl chains are designated PC(1) (α chain esterified at the *sn*-1 position), PC(2) (β chain at the *sn*-2 position), TG(1,3) (α chains at the *sn*-1 and *sn*-3 positions), and TG(2) (β chain at the *sn*-2 position). The α chains esterified at the *sn*-1 and *sn*-3 ^{13}C=O of TG with the same FFA cannot be discriminated by this technique.

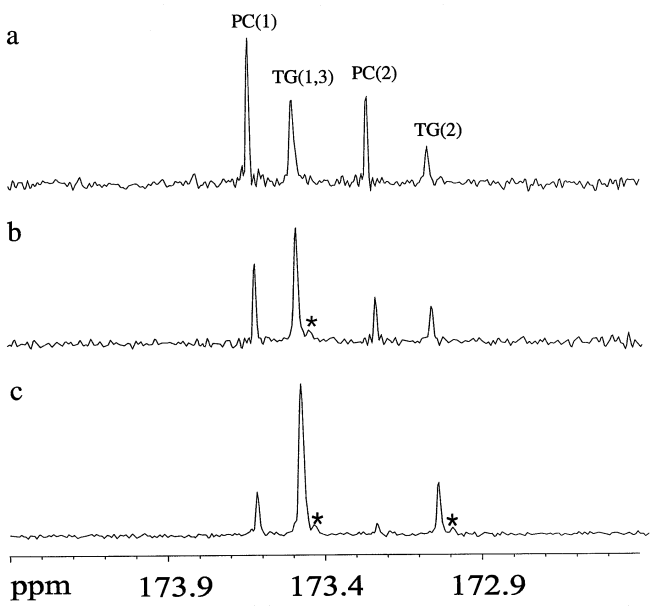

FIG. 1. ^{13}C nuclear magnetic resonance (NMR) spectra of the carbonyl region showing the distribution of ^{13}C-enriched carbonyl groups detected in extracts of differentiating rat perirenal cells after incubation with ^{13}C=O-labeled palmitic acid/bovine serum albumin for 5 h. Spectra a, b, and c correspond to different stages of differentiation as shown in Table 1, and are representative of observations from three to six repeated experiments. Peaks are designated as PC(1)/PC(2) for phosphatidylcholine at the *sn*-1/*sn*-2 (α,β) position, and TG(1,3)/TG(2) for triacylglycerol at the *sn*-1,3/*sn*-2 (α,β) positions. Peaks marked with (*) near the main peak of TG(1,2) and TG(2) are from dehydrogenated acyl chains (see Results and Discussion section).

The intensities of carbonyl signals in Figure 1 indicate that the ^{13}C=O incorporation among different lipids was not uniform, even among different acyl chain positions in the same lipid class. In most cases, when cells were incubated with ^{13}C-labeled palmitic acid, the signal intensity ratio of TG(1,3)/TG(2) was close to 2.0, demonstrating nearly equal incorporation of label among the three acyl chains in the TG molecule. On the other hand, the PC(1)/PC(2) intensity ratio was significantly higher than 1.0, demonstrating a preferential incorporation of palmitic acyl chains into the *sn*-1 carbon. Figure 1 also shows that with 5 h of incubation, cells at earlier stages of differentiation incorporated more ^{13}C=O-labeled FFA into PC than those at later stages, in which most of the ^{13}C=O carbonyl signals were detected in the TG fraction. NMR spectra were also obtained after various incubation times using cells having an initially similar stage of differentiation (Table 1, row D). Spectra of lipid extracts of cells (pooled from six animals) harvested at different time points after the FFA/bovine serum albumin incubation showed differences in incorporation of ^{13}C=O groups (Fig. 2). After incubation for 1 h, the ^{13}C=O carbonyl signals were detected in both PC and TG, with slightly more incorporation into TG (Fig. 2a). Note that this spectrum is similar to that in Figure 1b. As incubation time increased, there was a gradual increase in the TG signal intensity and a corresponding decrease in PC intensity. At 24 h, intense TG signals were seen, whereas the PC signals were nearly undetectable (Fig. 2e). These results show that the exogenous FFA is stored mainly in the TG pool, provided the incubation time is long enough, in agreement with previous results (3). The spectrum at 24 h also showed a broad, low-intensity signal slightly downfield from PC(1) peak in this spectrum (Fig. 2e). This signal may represent phospholipids other than PC, as shorter incubation times favor the incorporation of exogenous FFA into PC which turn over more rapidly than other phospholipids, but later transacylation processes may convert them to other species such as phosphatidylethanol-

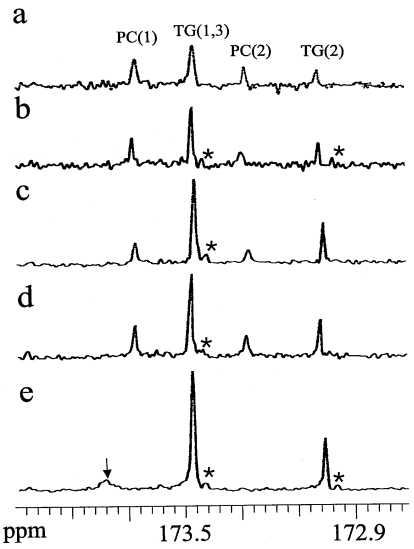

FIG. 2. The ^{13}C NMR spectra of lipid extracts of rat perirenal cells at various incubation times. The cells were at an early stage of differentiation corresponding to the data shown in Table 1 (row D). The spectra shown (traces a–e) are representative of cells incubated with ^{13}C=O-labeled palmitic acid/bovine serum albumin for (a) 1 h; (b) 3 h; (c) 7 h; (d) 11 h; and (e) 24 h, respectively. Peaks are designated as in Figure 1. See Figure 1 also for abbreviations.

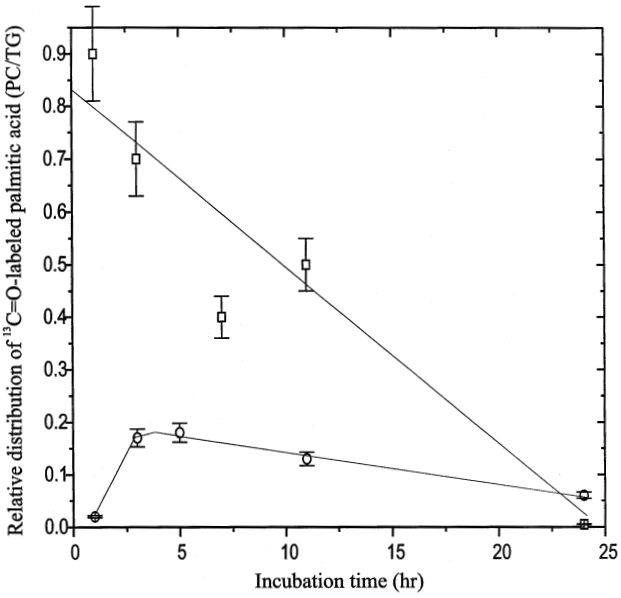

FIG. 3. The distribution of incorporated ^{13}C-enriched carbonyl in the PC fraction vs. TG fraction estimated by the integral intensity of [PC(1) + PC(2)]/[TG(1,3) + TG(2)] from NMR signals. The symbols represent results for cells in early stages of differentiation [(□), Table 1 (row D)] and a later stage of differentiation [○, Table 1 (row E), see below]. The solid lines represent the schematic trends of PC/TG distributions as the incubation time increased. See Figure 1 for abbreviations.

amine with slower turnover rates (16). Further work is required to clarify this.

The distribution of the incorporated ^{13}C=O groups between PC and TG was obtained by comparing the integral intensities of total PC signals ($\alpha + \beta$) vs. that of TG ($\alpha + \beta$) as a function of incubation time. The linear decrease of the PC/TG intensity ratio (Fig. 3, upper trace) showed that cells at early stages of differentiation tended to incorporate a higher proportion of exogenous FFA into the PC fraction (relative to TG) at early time points. Such incorporation decreased at later time points. This could indicate that the incorporated ^{13}C=O-labeled palmitoyl chain in PC tends to be replaced with unlabeled (also presumably mainly unsaturated) FFA during the deacylation/reacylation process.

In the spectra of the more differentiated cells (Fig. 1b and c) or of cells incubated for longer intervals with FFA (Fig. 2c–e), there were small signals (designated *) near the main TG(1,3) and TG(2) peaks. The intensities of these minor signals varied with the lipid composition in the medium and the extent of differentiation. The minor peaks were usually more intense in differentiating 3T3L1 cells and cannot be interpreted as naturally abundant background signals (Fig. 4a). To identify the molecules giving rise to these signals, we obtained ^{13}C NMR spectra of TG with mixed saturated and unsaturated acyl chains (Fig. 4b–d). [Although palmitoleic acid is mostly likely the product of desaturation, TG with mixed chains of palmitic and palmitoleic acids were not commercially available. Since TG containing olefinic chains at Δ9 positions have similar ^{13}C=O resonances when the acyl chains are varied from 16 to 18 (18), we used the oleoyl chain as a model for the palmitoleoyl chain.] With an oleoyl chain at the *sn*-2 position, a small upfield shift in the TG(2) was detected compared to that of tripalmitin. With oleoyl esterified at the *sn*-3 position, the TG(1,3) peak was split into two peaks of identical intensity with a lower chemical shift for the unsaturated chain (17,18). The signals representing the unsaturated acyl chains in the model systems corresponded well to the lower-frequency peaks in the cell extract (designated *). This implies that there is intracellular conversion of the saturated acyl chain of ^{13}C=O-labeled palmitoyl chains to unsaturated acyl chains. Such detection of intracellular acyl chain dehydrogenation by NMR has never been reported, probably because of limitations in signal resolution.

The more extensive dehydrogenation in 3T3L1 cells compared to primary rat preadipocytes seems to be related to substrate availability in the cell culture medium and also to how the cells obtain FFA for lipid storage. 3T3L1 cells have a high capacity for *de novo* synthesis FFA from glucose and contain 16-carbon FFA (16:0 and 16:1), whereas rat cells use predominantly exogenous FFA and contain 18-carbon (18:0 and 18:1) as the major FFA components in their TG fractions. In addition, in our experiments, 3T3L1 cells were cultured in a high-glucose medium without lipid supplementation, whereas rat preadipocytes were cultured in medium supplemented with a lipid emulsion (mostly polyunsaturated TG). Therefore, 3T3L1 cells probably have a higher tendency than rat preadipocytes to convert the TG-incorporated ^{13}C=O-labeled 16:0 to 16:1, because of the need to have unsaturated chains.

Quantitation of FFA incorporation into intracellular lipid pools by NMR. To date, quantitation of incorporation of FFA

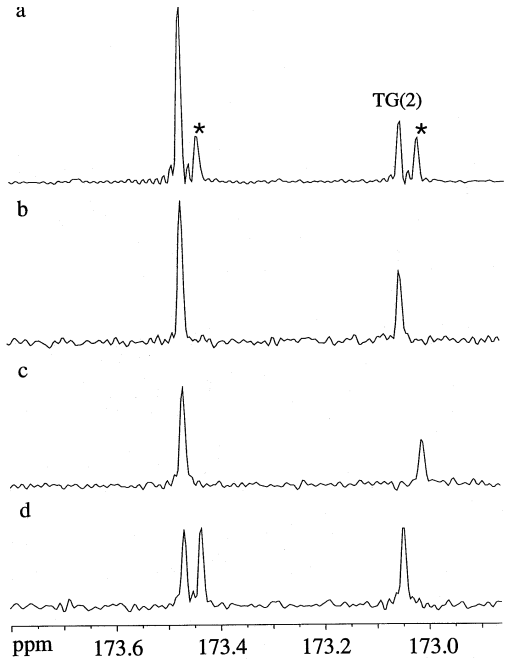

FIG. 4. The spectra of pure TG with different acyl chains compared with lipid extracts from differentiating 3T3L1 cells; (a) lipid extract from differentiating 3T3L1 cells (dominated by cells at stage 4, incubated with ^{13}C=O-labeled palmitic acid); (b) pure tripalmitin; (c) pure 1,3-*rac*-dipalmitoyl-2-oleoylglycerol; (d) pure 1,2-*rac*-dipalmitoyl-3-oleoylglycerol. Peak designation is the same as in Figure 1. Spectrum in (a) is representative of six parallel cell cultures. See Figure 1 for abbreviation.

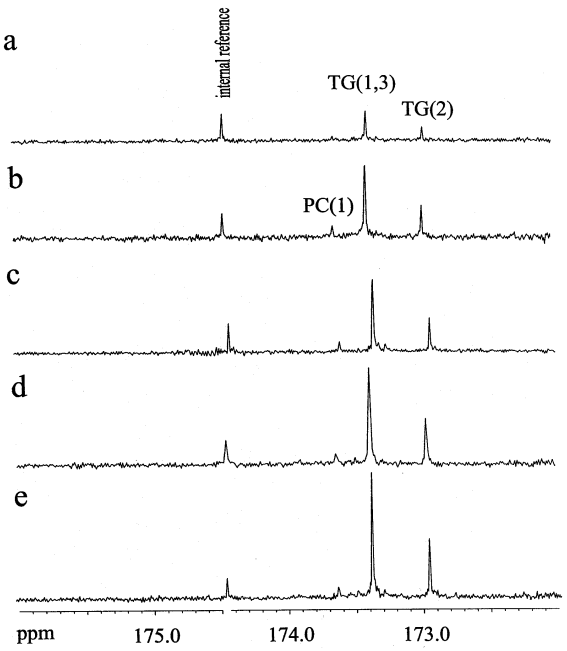

FIG. 5. ^{13}C NMR spectra of lipid extracts from differentiating rat perirenal cells at various incubation times with a ^{13}C=O-labeled methyl myristate internal reference standard. The spectra shown are representative of cells incubated for (a) 1 h; (b) 3 h; (c) 7 h; (d) 11 h; and (e) 24 h, respectively. The same amount of internal reference was added to each culture flask. To optimize signal/noise ratio, the number of culture flasks (25 cm^2) used for each spectrum was: (a, b) 3; (c) 2; (d, e) 1. Peaks are designated as in Figure 1. See Figure 1 for abbreviations.

into intracellular lipid pools has been achieved only with ^{14}C isotopically labeled molecules (3). To determine the site of labeling of a specific FFA, each presumed lipid component has to be separated by a chromatographic method before counting. ^{13}C NMR represents a method for obtaining this information without assumptions concerning the identity of the lipids involved or purification of each lipid.

Each signal in the NMR spectrum can be quantified in an absolute sense by comparison of its integrated peak intensity with that of a suitable standard, as discussed in the NMR methods section. In our experiments, 50 μg of ^{13}C=O-labeled methyl myristate (50 μL ethanol solution; 1 mg/mL) was added during the Folch extraction of highly differentiated cultures (Table 1, row E). These cells showed ^{13}C=O signals mainly in the TG(1,3) and TG(2) carbonyls. Unlike the results for the less differentiated cells which showed high PC incorporation at early incubation time points (Fig. 2), the net incorporation of ^{13}C=O FFA in the PC fraction within the 1-h incubation period was very low. Within 24 h, only a weak PC(1) signal [but not PC(2)] was detected (Fig. 5). The distribution of ^{13}C=O FFA incorporation between PC and TG was obtained by comparing the integrated intensities of the total PC signals vs. that of TG. Data are shown in Figure 3 (lower trace). There was a slight increase in the PC/TG ratio as the incubation time increased from 1 to 5 h, followed by a gradual decrease to a lower level. These results show that the incorporation of exogenous FFA into the PC and TG fractions is controlled by different kinetic mechanisms, depending on the stage of differentiation. Further investigations are needed to understand the enzymatic pathways responsible for this.

By comparing the integrated peak intensities of the lipids with that of the internal reference peak, the amount of ^{13}C=O-labeled palmitic acid in the TG ($\alpha + \beta$) was found to increase rapidly and linearly with incubation time. Within the same time period, incorporation into the PC(1) only increased slightly, and the PC(2) carbonyl was below the limit of detection (Fig. 6). At the end of the 24-h incubation, the peak intensities corresponded to ~1 μmole of ^{13}C=O-labeled palmitoyl in the lipids [TG(1,3) + TG(2) + PC(1)], which represents ~20% of the total ^{13}C=O-labeled exogenous FFA added.

Although high-resolution ^{13}C NMR spectroscopy has been applied to the study of adipose tissue previously, to our knowledge this work presents the first NMR study of differentiating preadipocytes. With the capability to probe cellular FFA metabolism at the molecular level, this NMR protocol provides a tool for evaluating the influence of environmental variables on the rate and magnitude of fat storage.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Patrice Cummins and Keith Salvatori. This work was supported by the Adipocyte Core of the Boston Obesity/Nutrition Research Center (DK46200), and grants AG/DK13925 and DK35914.

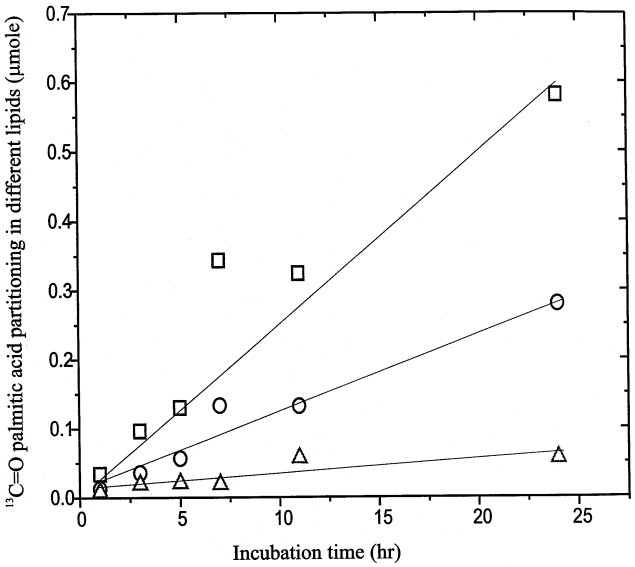

FIG. 6. The quantitative incorporation of ^{13}C=O-labeled palmitoyl chains into the TG(1,3), TG(2), and PC(1) positions, represented by □, ○, and △, respectively, as a function of incubation time. See Figure 1 for abbreviations.

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[Received November 17, 1997, and in final revised form March 18, 1998; revision accepted March 23, 1998]

Effects of Caffeine on Lipoprotein Lipase Gene Expression During the Adipocyte Differentiation Process

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ABSTRACT: In this study, the effects of caffeine on lipoprotein lipase (LPL) gene expression were investigated in the 3T3-F442A preadipocyte cell line during the adipocyte differentiation process by determining LPL enzymatic activity and its messenger RNA (mRNA) level. The results demonstrate that caffeine acts on the gene expression of LPL, an early marker of adipocyte differentiation. It has a biphasic action: it increases gene expression in terms of mRNA when it is added to preadipocytes during the early stage of differentiation, but this is accompanied by a reduction of enzymatic activity. On the other hand, when caffeine is added for long periods during differentiation and/or when it is added to mature adipocytes, it induces marked inhibition of mRNA levels, correlated with a marked reduction of secreted enzymatic activity. The inhibitory effect of caffeine on LPL mRNA level can be reproduced by theophylline, a phosphodiesterase inhibitor, and by dibutyryl cyclic AMP, a nonmetabolizable analog of cyclic AMP. However, the effect of caffeine and theophylline lasts longer than that of cyclic AMP, suggesting that a mechanism other than inhibition of cyclic AMP hydrolysis may be involved in the action of caffeine.
Lipids 33, 455–460 (1998).

Lipoprotein lipase (LPL) is an enzyme, mainly synthesized by adipose tissue and muscle, which plays a central role in the metabolism of triglyceride-rich plasma lipoproteins (1). It hydrolyzes exogenous (chylomicrons) and endogenous triglycerides (very low density lipoproteins) by generating free fatty acids and monoglycerides, using apolipoprotein CII as cofactor. A dysfunction of LPL could also be involved in the development of obesity, since this enzyme directs the distribution of dietary lipids toward adipose tissue for their subsequent storage or toward muscle where they are used as energy substrates.

The synthesis, maturation, and secretion of LPL are regulated transcriptionally or post-transcriptionally in a tissue-specific manner *via* complex mechanisms during development and in response to nutritional and hormonal variations (2,3).

Previous studies have emphasized that insulin is the hormone which predominantly regulates the activity of adipocytic LPL. Under certain physiological or pathophysiological conditions associated with an increase in the insulin level (postprandial period, obesity), an increase in LPL activity is observed in adipose tissue (4), whereas defects in insulin secretion, both in nontreated insulin-dependent diabetes and in postabsorptive and postprandial states, are correlated with a low LPL protein level (5). Many possible mechanisms of LPL regulation at posttranscriptional and posttranslational levels have been reported (6).

Adipose tissue from rats fasted overnight exhibits a decreased LPL catalytic activity (7), suggesting that this enzyme is also regulated by intracellular cyclic AMP (cAMP) content. The accumulation of cAMP has been shown to inhibit lipogenic enzymes such as glycerol phosphate dehydrogenase (8) in mouse adipocytes. Agonists known to increase cellular cAMP, such as isoproterenol (9) or epinephrine (10), induced a marked decrease of LPL enzyme activity in rat adipocytes in primary culture. However, the actual target of this regulation remains unclear, since in some cases the level of LPL mRNA has been found to be decreased (9), increased (7), or unaffected (10) depending on the model used and on the experimental conditions. Inhibition of the translation process of LPL mRNA by epinephrine has been related to the first 50 (1601–1650) nucleotides in the 3′ untranslated region of the messenger (11).

The cAMP signaling pathway can be regulated in several ways. For example, the antilipolytic action of insulin on adipocytes has been suggested to act either by stimulation of cGMP-inhibited cAMP phosphodiesterase activity (12) or by down-regulation of the β_3 adrenergic receptor (13). This might in part explain some of the action of insulin on LPL gene expression.

Under appropriate culture conditions, the 3T3-F442A preadipocyte cell line differentiates into adipocytes according to a process similar to that which occurs during the development of adipose tissue (14). Lipoprotein lipase is considered to be a specific marker of the differentiation of the 3T3-F442 preadipocyte cell line (15). This cell line is therefore an excellent model to study the action and mechanisms of effectors on LPL gene expression either during differentiation or on fully differentiated adipocytes which possess the features of highly specialized cells.

In the present study, we used caffeine, which is known to inhibit phosphodiesterase activity (16), to define the actual

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Abbreviations: CBP, CREB-binding protein; CRE, cyclic AMP (cAMP) response element; CREB, CRE-binding protein; LPL, lipoprotein lipase, PKA, protein kinase A.

role of intracellular cAMP on LPL gene expression. We showed that chronic treatment of 3T3-F442A cells by caffeine induced a marked decrease in heparin-releasable LPL activity in preadipocytes, as well as in fully differentiated adipocytes. In contrast, caffeine increased the level of LPL mRNA at an early stage of differentiation, but decreased the LPL mRNA level in mature adipocytes, suggesting that multiple targets are involved in mediating the cAMP effect.

EXPERIMENTAL PROCEDURES

Cell culture. 3T3-F442A cells were obtained through the courtesy of Dr. Howard Green (Harvard Medical School, Boston, MA) (14). Cells were seeded on tissue culture dishes (Falcon, Polylabo, Strasbourg, France) at a density of 10^3 cells/cm^2 in Dulbecco-Vogt modified Eagle's medium containing 25 mM glucose supplemented with 5% calf serum (from Gibco, Life Technologies, Cergy Pontoise, France), 5% fetal calf serum (from Dutscher, Dominique Dutscher, Brumath, France), a 0.7% penicillin/streptomycin mixture, 25 mM $NaHCO_3$ and 40 μM d-biotin from Sigma (Sigma Aldrich, Saint Quentin Fallavier, France). The medium was changed every 2 d. One day before confluence, cell cultures were washed and then cultured in Dulbecco-Vogt modified Eagle's medium containing 10% fetal calf serum and 20 nM insulin for the time indicated in the legends of the figures. Adipocyte conversion was achieved 10 to 12 d following confluence and checked by inverse phase microscopy (Nikon, Nikon France s.a., Champigny sur Marne, France). To release membrane-bound lipoprotein lipase, 100 μg/mL heparin was added to each flask, and the cells were incubated at 37°C for 40 min. The remaining cells were washed with phosphate-buffered saline at 4°C and treated with guanidium thiocyanate for RNA isolation.

RNA analysis. Total RNA was extracted using the method of Chomczynski and Sacchi (17) and total RNA (10 μg) analyzed by Northern blot as previously described (18). The blots were hybridized with a murine LPL cDNA probe labeled with [α-^{32}P]-dCTP by random priming (Amersham kit; Amersham Life Science, Les Ulis, France). In order to standardize the amount of mRNA in each line, blots were hybridized again with a 28S RNA probe labeled with [γ^{32}P]-ATP by T4 polynucleotide kinase. The intensities of the bands corresponding to LPL mRNA and the internal control, 28S rRNA, were measured by scanning densitometry of the autoradiograms. The relative amounts of LPL transcripts were quantified by calculating the ratio of the LPL-band integrated densities to those of 28S rRNA.

Lipoprotein lipase activity. The enzymatic activity was assayed by a modification of the technique of Nilson-Ehle and Ekman (19). Briefly, labeled and unlabeled trioleine (triacylglycerol emulsion), stored in benzene and chloroform, respectively, were mixed to obtain 7 mg (7.92 μmoles) of triacylglycerol with a specific activity around 10×10^6 cpm per μmole. Lysolecithin [0.3 mg (0.6 μmoles)] was then added. Solvents were removed under a nitrogen stream, followed by evaporation under reduced pressure. The emulsion was performed after adding 2.4 mL of 0.2 M Tris-HCl buffer pH 8.0 and 1.2 mL of 1% (w/v) bovine serum albumin on icebath by a Sonicator 100W model n°M72405 (Bioblock Scientific, Illkirch, France) at a setting of 3.5, which corresponds to a gram force of at least 1.5 g. Intervals of 40 s for a total of 3 min were applied. After sonication, 0.3 mL of heat-inactivated human serum and 0.3 mL of 4% bovine serum albumin in 0.2 M Tris-HCl pH 8.0 were added, and the substrate was shaken in a vortex mixer for 15 s. LPL assays were carried out in borosilicate glass tubes by mixing 100 μL of the trioleine substrate and 100 μL of the sample. The mixture was vortexed again for 15 s and agitated at 37°C for 60 min. The reaction was stopped by submerging the glass tubes into an ice bath, and the fatty acids released were isolated by a partition system of 3 mL of methanol/chloroform/heptane (1.41:1.25:1, by vol) and 1 mL of 0.1 M tetraborate buffer pH 10.5. After vigorous shaking for 15 s on a vortex mixer, the mixture was centrifuged for 15 min at $3{,}000 \times g$, and a 1-mL aliquot of the methanol/water phase was counted. Blank incubations, corrections, and calculations were performed as described by Nilson-Ehle and Ekman (19). LPL activity is expressed as milli-units (mU/mg of cellular protein), which represent the release of 1 nmol of fatty acid per min and per mg of cellular protein.

The protein content was assayed according to the method of Lowry *et al.* (20), and the triacylglycerol level was determined on lipid extracts by an enzymatic technique (Boehringer Mannheim Automated analysis kit; Boehringer Mannheim, Meylan, France). All statistical comparisons were performed using Student's test.

RESULTS

When 3T3-F442A cells were cultured in fetal calf serum in the presence of insulin, they started a process of adipocytic differentiation which included progressive accumulation of lipid droplets rich in triacylglycerol (Fig. 1). This process was slow at the beginning of differentiation from day 4 to day 7 and jumped abruptly after day 11, when the triacylglycerol level was increased sixfold. Treatment of the cells by caffeine affected neither the pattern of triacylglycerol content during the differentiation process (Fig. 1) nor the morphological changes (not shown).

The LPL gene is expressed early in the process of adipocyte differentiation. Its expression is obvious after 4 d of treatment by insulin, and secreted LPL activity was maximal after 7 d, plateaued for 11 d, and slightly decreased at 14 d (Fig. 2A). The steady-state level of LPL mRNA progressively increased from day 4 to day 11, then remained unchanged until 14 d (Fig. 2B), suggesting a progressive loss of translational efficiency of the mRNA throughout the differentiation process. When the cells were induced to differentiate in the presence of caffeine, secreted LPL activity was greatly reduced (Fig. 2A). In contrast, its mRNA increased to a greater extent in the presence of caffeine than in control cells from day 4 to day 9 and then fell abruptly at day 11 and

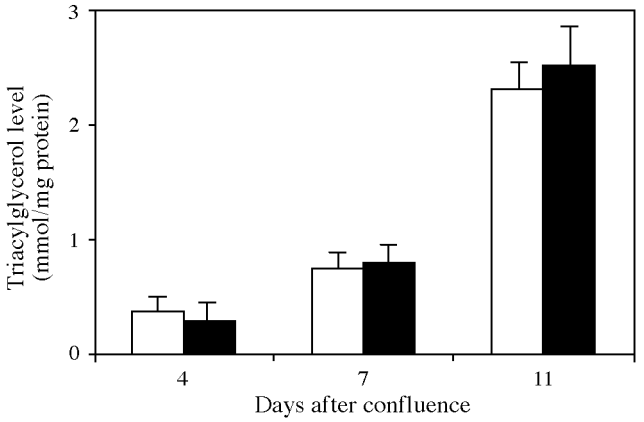

FIG. 1. Effect of caffeine on triacylglycerol content during 3T3-F442A cell differentiation. Confluent 3T3-F442A cells were induced to differentiate by 20 nM insulin on day 0. Open bars: insulin alone. Black bars: insulin plus 500 μM caffeine. Results are expressed as the mean ± SD of two independent experiments, each performed in duplicate.

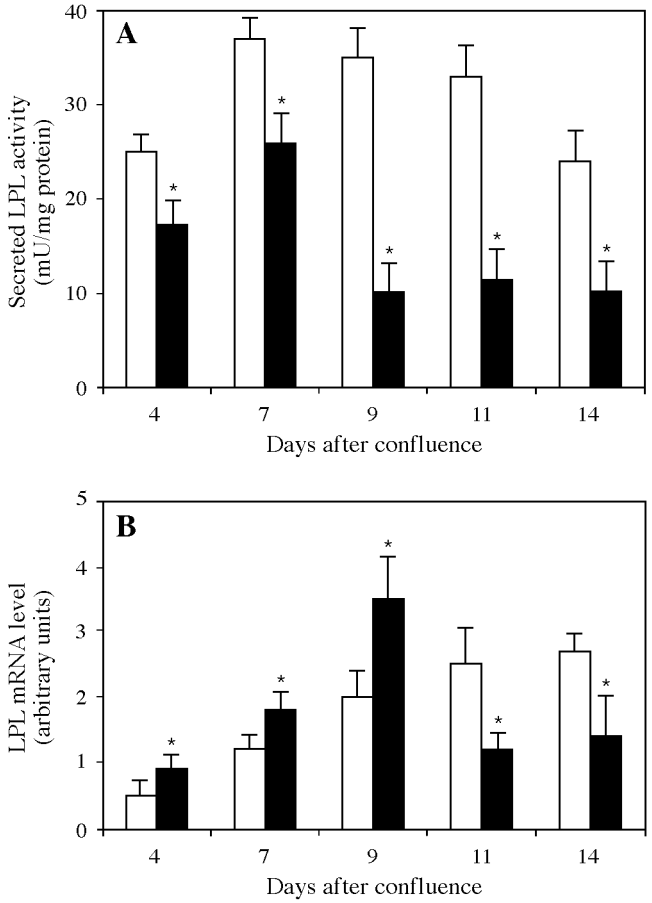

FIG. 2. Time course of the effect of caffeine on lipoprotein lipase (LPL) gene expression during 3T3-F442A cell differentiation. Confluent cells were exposed to 20 nM insulin to stimulate differentiation. Caffeine (500 μM) was added on day 0. (A) Secreted LPL activity was measured at the time indicated, as described in the Experimental Procedures section. The asterisks indicate values that differed significantly by at least $P < 0.05$ (treated cells vs. control). (B) Lipoprotein lipase mRNA level. Total mRNA was extracted and analyzed by Northern blotting as described in the Experimental Procedures section. The results are expressed as amount of LPL mRNA after correcting for the 28S rRNA signal and expressed in arbitrary units. Results are the mean ± SD of two independent experiments performed in duplicate. Open bars: insulin-treated cells. Black bars: insulin plus 500 μM caffeine-treated cells. The asterisks indicate values that differed significantly by at least $P < 0.05$ (treated cells vs. control).

remained low at day 14 (Fig. 2B). These effects of caffeine are dose-dependent, with a maximal effect from 0.5 mM caffeine (not shown).

Since caffeine did not affect either the triacylglycerol content or the morphological changes, it is unlikely that it accelerates the adipocyte differentiation process. Our observations support the idea that the caffeine effect is different at different levels of differentiation. To determine whether there was any relationship between the growth status of the culture and the action of caffeine, we determined the expression of LPL mRNA in 3T3-F442A preadipocytes and differentiated 3T3-F442A adipocytes at various times from 2 to 12 d after the onset of differentiation by insulin (Fig. 3A). At the very beginning of differentiation (days 0 to 5), caffeine exposure for 9 d stimulates induction of LPL mRNA and, in contrast, the presence of caffeine in the medium of differentiating cells inhibits the further induction of LPL mRNA by about 50% when added for 9 d at day 12. Therefore, during the differentiation process the caffeine effect is the sum of various effects on a number of important but unknown phases in adipocyte differentiation resulting in the level of LPL expression observed.

A possible cellular target of caffeine is the phosphodiesterase enzymes which hydrolyze cyclic nucleotide monophosphates. We therefore performed experiments by treating cells for 9 d with theophylline, another phosphodiesterase inhibitor (16). Theophylline exhibited the same pattern of action as caffeine (Fig. 3B). These findings suggest that the cAMP pathway is involved in the regulation of LPL gene expression. The action of caffeine and theophylline was therefore compared to that of cAMP on this regulation in fully differentiated cells (Fig. 4). When 3T3-F442A cells were incubated for 48 h with the cAMP analog, dibutyryl cAMP (db-cAMP), LPL mRNA levels decreased twofold, but this inhibition was transient, reaching baseline values within 7 d after addition of dibutyryl cAMP, in contrast with the effects of caffeine and theophylline, which remained potent after 7 d.

DISCUSSION

When confluent preadipocytes were incubated in the presence of insulin, they were induced to differentiate and accumulate triacylglycerol (14). Maximal differentiation was achieved after 11 d of insulin treatment. LPL has been shown to be one of the early genes induced during this process (15). Our results confirm this early induction, since maximal activity was found after 7 d (Fig. 1). In contrast, the cellular LPL mRNA level continued to increase after 11 d, suggesting a decrease in translational efficiency. This might be an indirect effect *via*

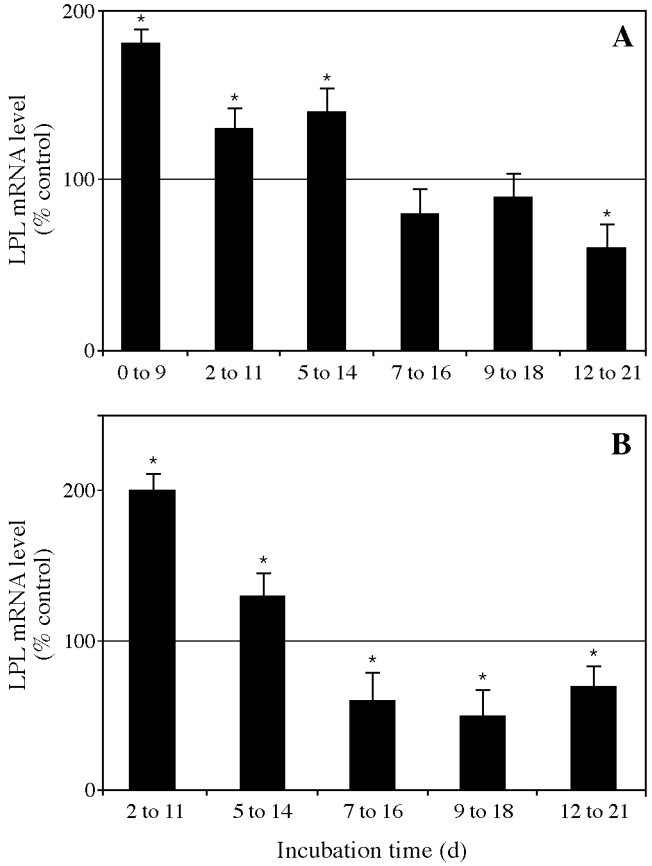

FIG. 3. Effect of chronic exposure of 3T3-F442A cells to caffeine and theophylline on adipose differentiation. 3T3-F442A cells cultured in defined medium with 20 nM insulin were exposed to caffeine (500 μM) (A) or theophylline ($7 \cdot 10^{-4}$ M) (B) for 9 d at different times after the onset of differentiation. Control cells were incubated for the same time with vehicle alone. LPL mRNA determination was performed as in Figure 2B. Results correspond to the ratio of LPL mRNA level in caffeine- or theophylline-treated cells compared to the corresponding control cells. Results are the mean ± SD of two independent experiments performed in duplicate.The asterisks indicate values that differed significantly by at least $P < 0.05$ (treated cells vs. control). For abbreviation see Figure 2.

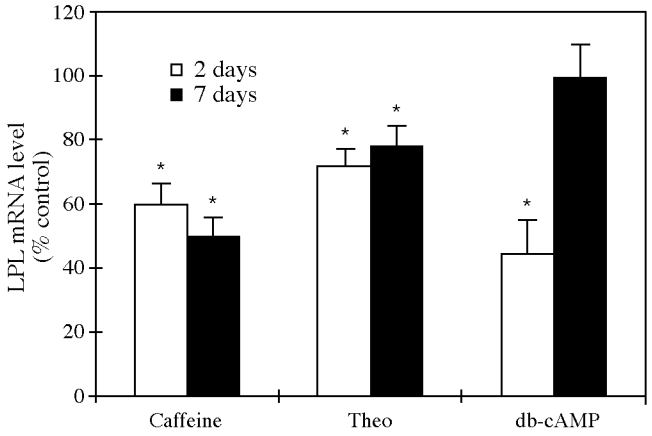

FIG. 4. Inhibitory effect of intracellular dibutyryl cyclic AMP (db-cAMP) level on steady-state of LPL mRNA level. Fully differentiated 3T3-F442A adipocytes were incubated in the presence of 500 μM caffeine, 70 μM theophylline (Theo), 10 μM db-cAMP, or vehicle alone (control) for 48 h or 7 d. LPL mRNA was determined as in Figure 2B. Data obtained by densitometric scanning were corrected for differences in RNA loading by using the 28S rRNA signal and expressed as a percentage of control. Results are the mean ± SD of two experiments in duplicate The asterisks indicate values that differed significantly by at least $P < 0.05$ (treated cells vs. control). For abbreviations see Figure 2

induction of other transactivators such as peroxysome proliferator-activated receptors or CAAT enhancer binding protein family members (21).

The addition of caffeine to the culture medium markedly decreased the secretion of LPL activity in both 3T3-F442A cells, undergoing adipocyte differentiation (Fig. 2), and in fully differentiated cells (Fig. 4). The main action of caffeine is to block cAMP degradation by inhibiting phosphodiesterase activity (16). It was therefore not surprising to observe that the caffeine effect can be mimicked by theophylline, another phosphodiesterase inhibitor, or by dibutyryl cAMP itself (Fig. 4). Our results are in agreement with the suggestion that inhibition of LPL activity induced by epinephrine is due to increased cellular cAMP (10). In fully differentiated 3T3-F442A cells, cAMP regulates the transcription of several major adipose genes, but acts solely as an antilipogenic agent since it does not elicit any loss in adipose characteristics (22). In addition, during differentiation of these cells, cAMP does not affect the morphological changes characteristic of the differentiation process (23).

There are still a number of uncertainties concerning the actual role of the cAMP/protein kinase A (PKA) pathway in LPL gene expression (10,24). In our experimental data, we demonstrated a clear biphasic effect of caffeine on LPL mRNA level, i.e., a marked increase when the drug is provided during the differentiation process and a decrease when the drug is added when adipocytes reached maturity. Since LPL activity is decreased in both conditions, this suggests that, in 3T3-F442A cells, LPL is regulated by cAMP at both transcriptional and post-transcriptional levels at least during the differentiation process. In addition, we cannot exclude that LPL mRNA turnover could be regulated. A post-transcriptional inhibition of LPL mRNA translation has been observed in epinephrine-treated 3T3-F442A cells as well as mature 3T3 L_1 cells (25), and this effect has been related to the first 50 (1601–1650) nucleotides of the 3' untranslated region of the messenger (11).

The situation concerning mature adipocytes is reminiscent of that observed in adipose tissue of rats fasted overnight, a condition of very high cellular cAMP content (7). After fasting, the majority of LPL is located in a highly inactive form in rough endoplasmic reticulum. Only mature, fully glycosylated LPL can be secreted from adipocytes (26). In addition, dimerization of LPL is essential for activity (6), and a study in 3T3-F442A cells demonstrated that LPL became a dimer after core glycosylation and transfer to the Golgi apparatus (27).

Our results suggest that caffeine stimulates LPL gene transcription at the onset of adipocyte differentiation and decreases LPL gene transcription in mature adipocytes. In addi-

tion, the inhibitory effect of caffeine might start very soon in the differentiation process since it was more potent when caffeine was added for 9 d after day 2 than when it was added for 9 d at later stages (compare Figs. 2B and 3A). Caffeine is supposed to exert its action *via* an increase in cAMP level. A variety of genes induced by cAMP share a conserved motif in their transcriptional control sequence, referred to as the cAMP response element (CRE). Such a sequence has been described in the rat LPL promoter (28), where CRE binds the CRE-binding protein CREB which is directly phosphorylated by PKA (29). Phosphorylation of CREB stimulates the transcriptional activity of several genes. This effect is mediated through the interaction with the ubiquitous P300/CBP (CREB-binding protein) coactivator family (29,30). The inhibitory effect of the cAMP/PKA pathway might occur *via* two pathways, i.e., a direct inhibitory effect of cyclic AMP response element modulator, a negative counterpart of the CREB family, or an indirect effect, buffering the P300/CBP family members which decreases the transcriptional efficiency of the genes requiring P300/CBP, such as fos/jun (31).

Other mechanisms may also be involved in the action of caffeine. Mobilization of Ca^{2+} ions from the sarcoplasmic reticulum compartment by caffeine has been described in myocardial cells (32). The actual physiological significance of the mechanisms of action of caffeine on LPL gene expression and their impact on the various levels of regulation appear quite complex and therefore require further investigation.

ACKNOWLEDGMENTS

We are grateful to Isabelle Delpit for assistance in preparing this manuscript. Financial support for this research was provided by Pierre Fabre-Santé (France).

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Tocotrienols Inhibit the Growth of Human Breast Cancer Cells Irrespective of Estrogen Receptor Status

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ABSTRACT: Potential antiproliferative effects of tocotrienols, the major vitamin E component in palm oil, were investigated on the growth of both estrogen-responsive (ER+) MCF7 human breast cancer cells and estrogen-unresponsive (ER–) MDA-MB-231 human breast cancer cells, and effects were compared with those of α-tocopherol (αT). The tocotrienol-rich fraction (TRF) of palm oil inhibited growth of MCF7 cells in both the presence and absence of estradiol with a nonlinear dose-response but such that complete suppression of growth was achieved at 8 μg/mL. MDA-MB-231 cells were also inhibited by TRF but with a linear dose-response such that 20 μg/mL TRF was needed for complete growth suppression. Separation of the TRF into individual tocotrienols revealed that all fractions could inhibit growth of both ER+ and ER– cells and of ER+ cells in both the presence and absence of estradiol. However, the γ- and δ-fractions were the most inhibitory. Complete inhibition of MCF7 cell growth was achieved at 6 μg/mL of γ-tocotrienol/δ-tocotrienol ($\gamma T_3/\delta T_3$) in the absence of estradiol and 10 μg/mL of δT_3 in the presence of estradiol, whereas complete suppression of MDA-MB-231 cell growth was not achieved even at concentrations of 10 μg/mL of δT_3. By contrast to these inhibitory effects of tocotrienols, αT had no inhibitory effect on MCF7 cell growth in either the presence or the absence of estradiol, nor on MDA-MB-231 cell growth. These results confirm studies using other sublines of human breast cancer cells and demonstrate that tocotrienols can exert direct inhibitory effects on the growth of breast cancer cells. In searching for the mechanism of inhibition, studies of the effects of TRF on estrogen-regulated pS2 gene expression in MCF7 cells showed that tocotrienols do not act *via* an estrogen receptor-mediated pathway and must therefore act differently from estrogen antagonists. Furthermore, tocotrienols did not increase levels of growth-inhibitory insulin-like growth factor binding proteins (IGFBP) in MCF7 cells, implying also a different mechanism from that proposed for retinoic acid inhibition of estrogen-responsive breast cancer cell growth. Inhibition of the growth of breast cancer cells by tocotrienols could have important clinical implications not only because tocotrienols are able to inhibit the growth of both ER+ and ER– phenotypes but also because ER+ cells could be growth-inhibited in the presence as well as in the absence of estradiol. Future clinical applications of TRF could come from potential growth suppression of ER+ breast cancer cells otherwise resistant to growth inhibition by antiestrogens and retinoic acid.

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Tocopherols and tocotrienols, collectively known as vitamin E, are lipid-soluble compounds that are often present as natural components in vegetable oils. Structurally, these compounds are similar, except that tocotrienols have an unsaturated side chain with three double bonds whereas the tocopherols have a fully saturated side chain (1). In their function as antioxidants, they are important for the protection of unsaturated lipids against peroxidation, particularly in biomembranes (2), by a mechanism resulting mainly from their ability to donate phenolic hydrogens to lipid-free radicals (3–5). Whereas the vitamin E fraction of most common vegetable oils contains mainly tocopherols, palm oil is a rich source of tocotrienols, with refined palm oil containing 133 mg α-tocopherol (αT), 130 mg α-tocotrienol (αT_3), 204 mg γ-tocotrienol (γT_3), and 45 mg δ-tocotrienol (δT_3) per kg of oil (6).

Experimental studies both *in vitro* and *in vivo* have suggested that tocotrienols may possess anticancer properties (7,8). Unlike many other fats and oils, palm oil does not enhance the yield of chemically-induced mammary tumors when fed to rats at high levels (20% w/w fat) in a semipurified diet (9–11). Evidence that this effect is related to the vitamin E fraction of the palm oil [tocotrienol-rich fraction (TRF)] was shown in our recent study (12) where chemically-induced mammary tumors were more numerous in rats fed vitamin E-free palm oil than in those animals fed palm oil containing vitamin E. Furthermore, addition of the vitamin E fraction of palm oil to a corn oil diet increased the median latency period and reduced both tumor incidence and tumor yield in a dose-dependent manner (12).

This inhibitory action of tocotrienols on breast tumor growth *in vivo* could result either from direct effects on the growth of the tumor cells themselves or from indirect sys-

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Abbreviations: DCFCS, dextran-charcoal treated fetal calf serum; ER+, estrogen receptor positive (containing estrogen receptors); ER–, estrogen receptor negative (lacking estrogen receptors); FCS, fetal calf serum; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; αT, α-tocopherol; αT_3, α-tocotrienol; γT_3, γ-tocotrienol; δT_3,δ-tocotrienol; TRF, tocotrienol-rich fraction.

temic interactions. Studies on the growth of MDA-MB-435 human breast cancer cells *in vitro* have demonstrated that the TRF of palm oil can inhibit directly the growth of these cells whereas αT did not have any such effect (13). However, the MDA-MB-435 cells lack estrogen receptors, and estrogen is known to play an important role in the growth of many breast cancer cells *in vivo* (14,15) and *in vitro* (16,17). Thus, the work presented here extends these cell culture studies to compare the effects of tocotrienols on the growth of human breast cancer cells which possess estrogen receptors and which are estrogen-responsive for growth with those cells which lack estrogen receptors.

Growth of the ER+ MCF7 human breast cancer cell line is regulated *in vitro* by estrogens (16,18) and offers a suitable model system in which to study the effects of tocotrienols. Separate sublines of MCF7 cells show different sensitivities to estrogen for growth, but the MCF7McGrath subline (19) is so dependent on estrogen for growth that it is unable to proliferate to any large extent in the absence of estrogen (16). However, recent studies have shown that growth of these cells is regulated also by complex interactions with growth factors of which one major mitogenic component would appear to be the insulin-like growth factors (IGF) (17,20). Growth regulation by the IGF system is itself complex since there are two ligands (IGFI and IGFII), two receptors (IGFIR and IGFIIR), and at least six high-affinity extracellular binding proteins (IGFBP 1-6) (21), and it would appear that estrogen can alter the expression of several of these components in breast cancer cells including IGFII (22), IGFIR (23), and IGFBP (24). Growth inhibitory effects in estrogen-responsive human breast cancer cells could thus result either from a direct antagonism of estrogen action at the estrogen receptor level (25) as found for antiestrogens such as tamoxifen and ICI 182,780 (26,27) or from interactions in growth factor pathways such as changing levels of IGFBP (28). The work presented here describes the effects of the TRF of palm oil and of individual tocotrienols on the growth of estrogen-responsive MCF7McGrath human breast cancer cells, on pS2 gene expression as a molecular marker of estrogen action (29), and on levels of secreted IGFBP.

MATERIALS AND METHODS

Materials. Plastic tissue culture dishes, growth media, and fetal calf serum (FCS) were all purchased from Gibco BRL (Paisley, Scotland). Zaponin and isoton were purchased from Coulter Electronics (Harpenden, England). Hybond membranes were bought from Amersham International (Amersham, England). 17-β-Estradiol was purchased from Steraloids (Croydon, England). The TRF and individual αT_3, γT_3, and δT_3 fractions were obtained from the Palm Oil Research Institute of Malaysia (PORIM). The αT was obtained from Sigma Chemical Company (Croydon, England) at a purity of approximately 95%.

Isolation of TRF and individual tocotrienols. Extraction of the TRF from palm oil has been described by Sundram and Gapor (30). In brief, palm oil fatty acid distillate was converted into methyl esters by esterification. The methyl esters were then removed by distillation, leaving a vitamin E concentrate. This was further concentrated by crystallization and passed through an ion-exchange column to give 60–70% pure vitamin E. Further purification was achieved by washing and then drying the concentrate followed by a second molecular distillation stage. The final purity of the vitamin E preparation, TRF, was 95–99%, its composition being (w/w) αT 32%, αT_3 25%, γT_3 29%, and δT_3 14%.

Individual tocotrienols were separated by thin-layer chromatography and further chromatographed repeatedly until a purity of about 90% (highest available) was obtained.

Culture of stock cells. The MCF7 McGrath human breast cancer cells were kindly provided by Dr. K. Osborne at passage number 390 (19). Stock cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, 10^{-8} M estradiol, and 10 μg/mL insulin in a humidified atmosphere of 10% carbon dioxide in air at 37°C. 17-β-Estradiol was dissolved in ethanol and diluted 1:10,000 in culture medium. Insulin was dissolved in 6 mM HCl and diluted 1:1,000 in culture medium. MDA-MB-231 human breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA). Stock cells were grown as for MCF7 cells but with the omission of estradiol and insulin. Cells were subcultured at weekly intervals by suspension with 0.06% trypsin/0.02% EDTA (pH 7.3).

Cell growth experiments. Cells were suspended from stock plates by treatment with phenol red-free 0.06% trypsin/0.02% EDTA (pH 7.3), added to an equal volume of phenol red-free RPMI1640 medium containing 5% dextran-charcoal treated FCS (DCFCS) (31), and counted on a hemacytometer. Cells were then added to the required volume of phenol red-free RPMI1640 medium containing 5% DCFCS at a concentration of 0.2×10^5 cells/mL and plated in monolayer in 0.5-mL aliquots into 24-well plastic tissue culture dishes. After 24 h, the medium was changed to phenol red-free RPMI1640 medium with 5% DCFCS supplemented with the appropriate concentrations of TRF, αT, individual tocotrienol fractions, with or without estradiol. Both TRF and αT were dissolved in dimethyl sulfoxide, estradiol in ethanol, and all were diluted 1:10,000 in culture medium. Control cultures contained the same volume of dimethyl sulfoxide or ethanol vehicle alone. The culture medium was changed routinely every 3–4 d.

Cell counting. Cells were washed rigorously *in situ* with phosphate-buffered saline (PBS) in order to wash off non-adherent dead cells and were then lysed in 0.5 mL 0.01 M HEPES buffer/1.5 mM $MgCl_2$ plus 2 drops of zaponin solution for 5 min. The nuclei released were counted in isoton on a Coulter counter. All cell counts were carried out in triplicate on triplicate dishes and results calculated as the mean ± standard error. *P* values were determined using Student's *t*-test for two-samples assuming unequal variance [by standard software packages Minitab and Microsoft Excel (Microsoft Corp., Redmond, WA)]. Viability of adherent cells was determined using the standard try-

pan blue dye exclusion assay technique and performing cell counts on a hemacytometer.

RNA analysis by Northern blotting. Cells were plated onto 9-cm plastic tissue culture dishes in 16-mL aliquots of phenol red-free RPMI1640 medium with 5% DCFCS. After 24 h, the medium was changed to phenol red-free RPMI1640 medium containing 5% DCFCS with or without 10^{-8} M estradiol or 8 μg/mL TRF. After a further 6 d, cells were washed *in situ* with PBS, harvested into ice-cold PBS using a rubber policeman and pelleted by centrifugation. Whole cell RNA was prepared by the guanidinium cesium chloride method (32). Aliquots of RNA (20 μg) were subjected to electrophoresis in 1.5% agarose-formaldehyde gels (32). RNA was transferred onto Hybond-N membrane and hybridized to ^{32}P-labeled cDNA probes for pS2 and 36B4 exactly as described previously (29). The pS2 DNA probe was a 300-base pair P*st*1 fragment from pS2 cDNA (29). The 36B4 DNA probe was a 220-base pair P*st*1 fragment which acted as a control since 36B4 mRNA is not regulated by estrogen (33).

Western ligand blotting of IGFBP. Cells were plated onto 3.5-cm plastic tissue culture dishes in 2.5-mL aliquots of phenol red-free RPMI1640 medium supplemented with 5% DCFCS. After 24 h, the medium was changed to phenol red-free RPMI1640 medium with 5% DCFCS and supplemented with 10^{-8} M estradiol or with 6 μg/mL of αT, αT_3, γT_3, or δT_3 as required. After a further 6 d, the medium was changed to serum-free medium: cells were washed three times with phenol red-free RPMI1640 medium and incubated in 0.5 mL of serum-free medium per dish (phenol red-free RPMI1640 medium with 15 mM HEPES buffer, 0.25% bovine serum albumin, and any supplements of estradiol or vitamin E as above) for a further 24 h. Medium conditioned by the cells was collected, cellular material removed by centrifugation, and medium stored at −70°C. Cells remaining on each dish were counted as described above.

Aliquots of the conditioned media were run on polyacrylamide gel electrophoresis, loading into each well the volume of conditioned medium equivalent to 10^5 cells. Aliquots of conditioned media were each mixed with an equal volume of gel sample buffer (26 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue), heated to 100°C for 2 min, and proteins separated by 15% polyacrylamide-SDS gel electrophoresis. Proteins were transferred onto Hybond-C extra membrane by semidry Western blotting in 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol. Western blots were hybridized to ^{125}I-IGFI as described by Hossenlopp and coworkers (34). ^{125}I-IGFI was prepared by the iodogen method (35).

RESULTS

Effect of TRF and αT on cell growth. Initial experiments were designed to determine the effect of TRF on the growth of MCF7McGrath human breast cancer cells. Figure 1A shows

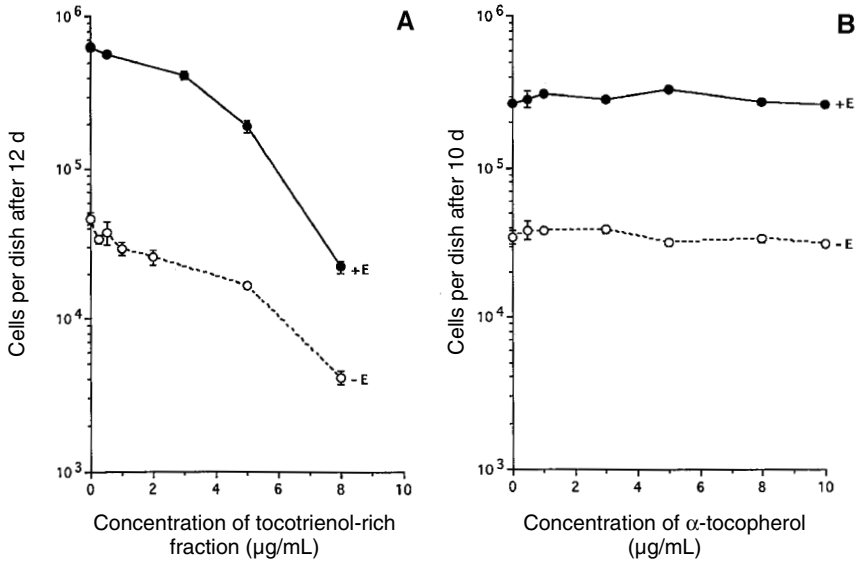

FIG. 1. Effect of the tocotrienol-rich fraction (TRF) of palm oil (A) or α-tocopherol (B) on regulation of the growth of estrogen-responsive ER+ MCF7McGrath human breast cancer cells in monolayer culture. Cells were grown for (A) 12 d or (B) 10 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% dextran-charcoal treated fetal calf serum (DCFCS) and increasing concentrations of either TRF or α-tocopherol (αT) in either the absence (open circles and dotted lines) or presence (closed circles and solid lines) of 10^{-8} M estradiol. Plating densities of the cells were (A) $1.34 \pm 0.01 \times 10^4$ cells per dish for the experiment in the absence of estradiol, and $2.41 \pm 0.08 \times 10^4$ cells per dish for the experiment in the presence of estradiol; (B) $0.43 \pm 0.02 \times 10^4$ cells per dish for the experiment in the absence of estradiol, and $0.64 \pm 0.01 \times 10^4$ cells per dish for the experiment in the presence of estradiol. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display.

that TRF inhibited the growth of these estrogen-responsive cells both in the absence and in the presence of estradiol, and in a dose-dependent manner. On its own, TRF inhibited cell growth at concentrations of 0.5 μg/mL ($P = 0.031$), 1 μg/mL ($P = 0.04$), 2 μg/mL ($P = 0.027$), 5 μg/mL ($P = 0.018$), and 8 μg/mL ($P = 0.009$). At 8 μg/mL TRF, cell numbers at 12 d fell below the plating density of the cells ($P = 0.002$). In the presence of 10^{-8} M estradiol, TRF was also able to inhibit cell growth at concentrations of 0.5 μg/mL ($P = 0.04$), 3 μg/mL ($P = 0.01$), 5 μg/mL ($P = 0.002$), and 8 μg/mL ($P = 0.005$), but cell numbers remained above plating density in all cases. In contrast to these results with TRF, Figure 1B shows that αT had no effect on growth of MCF7McGrath human breast cancer cells at any concentration tested from 0.5–10.0 μg/mL in either the absence or in the presence of 10^{-8} M estradiol (after 10 d at 10 μg/mL αT, in the absence of estradiol $P = 0.421$ and in the presence of estradiol $P = 0.535$).

These results are in line with previous studies using ER– MDA-MB-435 human breast cancer cells (13) where cell growth was similarly shown to be inhibited by TRF but not by αT. However, since these previous studies were based on different cell biological criteria, control experiments were repeated here using ER– MDA-MB-231 human breast cancer cells for direct comparison. Figure 2A shows that after 10 d, TRF inhibited cell growth at concentrations of 2 μg/mL ($P = 0.003$), 5 μg/mL ($P = 0.002$), 8 μg/mL ($P = 0.001$) and 10 μg/mL ($P = 0.0001$) but in all cases cell growth remained substantial and cell numbers well above plating density. However, at 20 μg/mL, TRF was able to inhibit MDA-MB-231 cell growth and reduce cell numbers below plating density ($P = 0.057$) (data not shown). In contrast, αT had no effect on growth of MDA-MB-231 cells at any concentration up to 10 μg/mL (Fig. 2B) or indeed even at 20 μg/mL (data not shown).

Effect of individual tocotrienols on cell growth. Further experiments studied the effects on cell growth of individual tocotrienol fractions (α-, γ-, and δ-) in order to determine which of the fractions were effective or most effective in inhibiting the cell growth. These results are shown in Figures 3 and 4. For MCF7McGrath cells, γT_3 and δT_3 were the most effective of the fractions at inhibiting cell growth in the absence of estradiol (Fig. 3) and did so in a dose-dependent manner after both 7 and 14 d in culture. Already after 7 d, γT_3 showed highly significant inhibitory effects at concentrations from 4 μg/mL ($P = 0.001$) to 6 μg/mL ($P = 0.002$). At 6 μg/mL γT_3, cell growth was completely suppressed with cell numbers even falling below the plating density ($0.130 \pm 0.086 \times 10^5$ cells on day 0; $0.057 \pm 0.002 \times 10^5$ cells on day 7; $0.021 \pm 0.076 \times 10^5$ cells on day 14). δT_3 also inhibited cell growth after 7 d at concentrations from 4 μg/mL ($P = 0.03$) to 6 μg/mL ($P = 0.007$). Again, at 6 μg/mL δT_3, cell growth was completely suppressed with cell numbers even falling below the plating density ($0.078 \pm 0.005 \times 10^5$ cells on day 0; $0.069 \pm 0.046 \times 10^5$ cells on day 7; $0.040 \pm 0.014 \times 10^5$ cells on day 14). αT_3 had no inhibitory effect on growth of MCF7McGrath cells after 7 d in culture (at 4 μg/mL $P = 0.569$; at 5 μg/mL $P = 0.482$; at 6 μg/mL $P = 0.249$) and had only a small inhibitory effect after 14 d in culture at concentrations of 5 μg/mL ($P = 0.038$) and 6 μg/mL ($P = 0.022$).

In the presence of estradiol, MCF7McGrath cell growth was inhibited most strongly by δT_3 (Fig. 3) (after 14 d, *P* val-

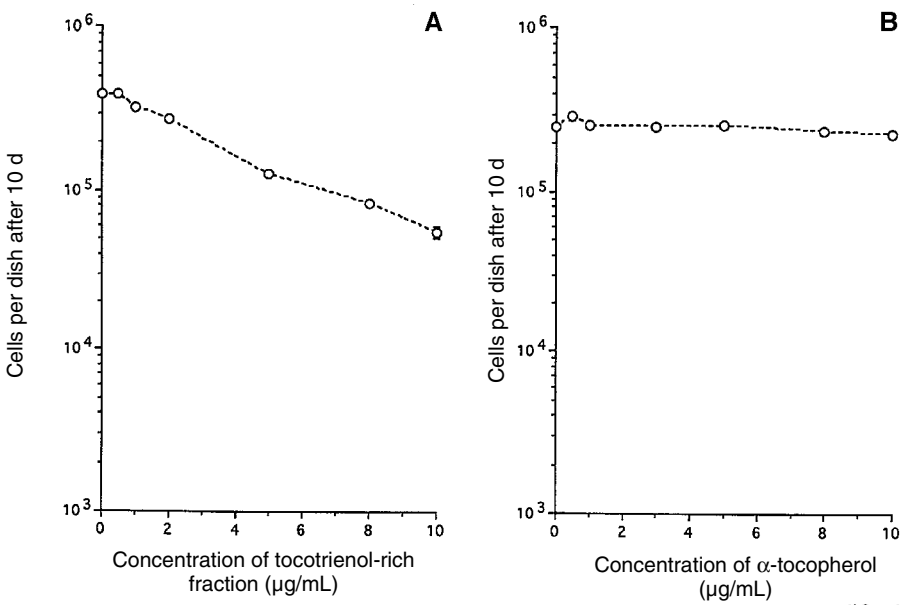

FIG. 2. Effect of the tocotrienol-rich fraction (TRF) of (A) palm oil or (B) αT on regulation of the growth of estrogen-insensitive ER– MDA-MB-231 human breast cancer cells in monolayer culture. Cells were grown for 10 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of either TRF or αT in the absence of estradiol. Cells were plated together in one experiment for A and B at a density of $1.05 \pm 0.19 \times 10^4$ cells per dish. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviations.

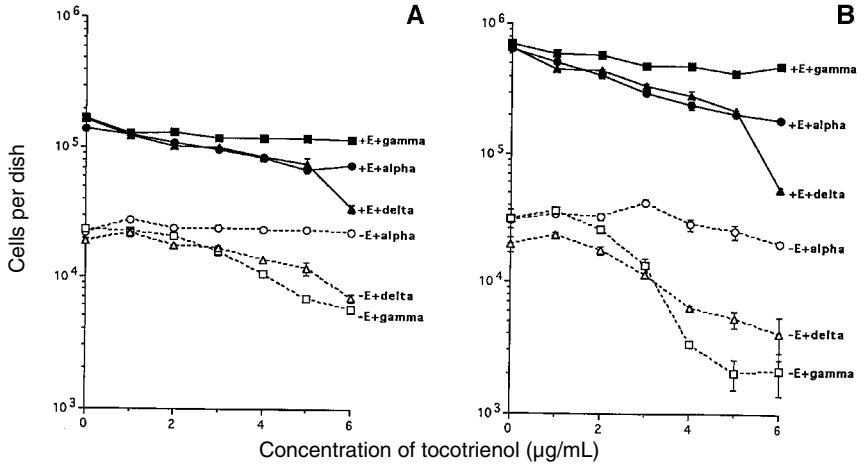

FIG. 3. Effect of individual tocotrienols on regulation of the growth of estrogen-responsive ER+ MCF7McGrath human breast cancer cells in monolayer culture. Cells were grown for (A) 7 or (B) 14 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of α-tocotrienol (αT_3) (●,○), γ-tocotrienol (γT_3) (■,□), or δ-tocotrienol (δT_3) (▲,△) in either the absence of estradiol (–E) (open symbols, dotted lines) or the presence of 10^{-8} M estradiol (+E) (closed symbols, solid lines). Cells were plated at a density of $1.06 \pm 0.08 \times 10^4$ cells per dish for the –E + αT_3 experiment, of $1.30 \pm 0.08 \times 10^4$ cells per dish for the –E + γT_3 experiment, of $0.78 \pm 0.05 \times 10^4$ cells per dish for the –E + δT_3 experiment, and of $2.44 \pm 0.21 \times 10^4$ cells per dish for the entire +E experiment with αT_3, γT_3, and δT_3. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display.

ues were 1 μg/mL $P = 0.009$; 2 μg/mL to 6 μg/mL $P < 0.001$). At a concentration of 10 μg/mL (data not shown), all three fractions could inhibit cell growth after 7 d (αT_3 by 63%, γT_3 by 32%, δT_3 by >100%) and after 14 d (αT_3 by 61%, γT_3 by 14%; δT_3 by >100%). At 10 μg/mL, δT_3 reduced cell numbers below plating density ($P = 0.008$ after 7 d; $P = 0.001$ after 14 d).

Parallel comparisons using ER– MDA-MB-231 cells showed that cell growth could be inhibited by all three tocotrienol fractions but most markedly by the δT_3 (Fig. 4). However, even at concentrations of 10 μg/mL (data not shown), cell numbers did not fall below plating density.

Cell viability. Where cell numbers in monolayer culture fell below plating density implying cell death, trypan blue vi-

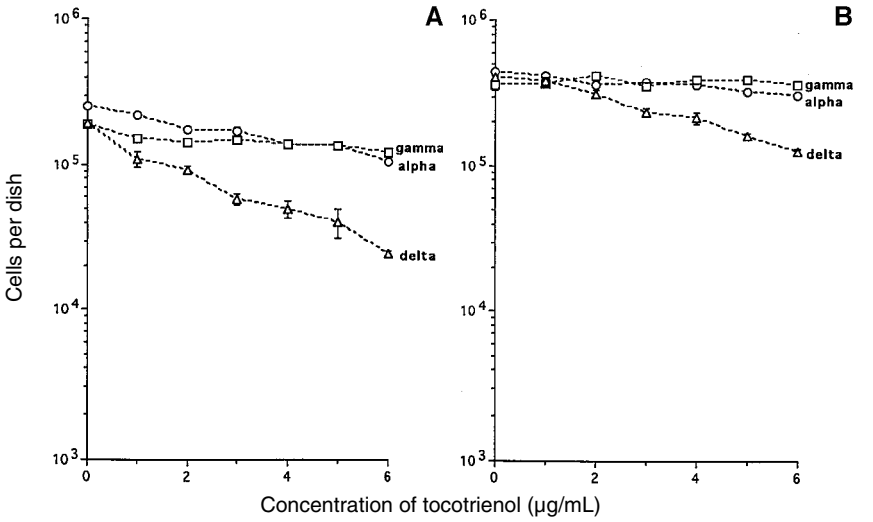

FIG. 4. Effect of individual tocotrienols on regulation of the growth of estrogen-insensitive ER– MDA-MB-231 human breast cancer cells in monolayer culture. Cells were grown for (A) 7 or (B) 14 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of αT_3 (●,○), γT_3 (■,□), or δT_3 (▲,△) in the absence of estradiol. Cells were plated together for the entire experiment at a density of $0.99 \pm 0.08 \times 10^4$ cells per dish. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviation.

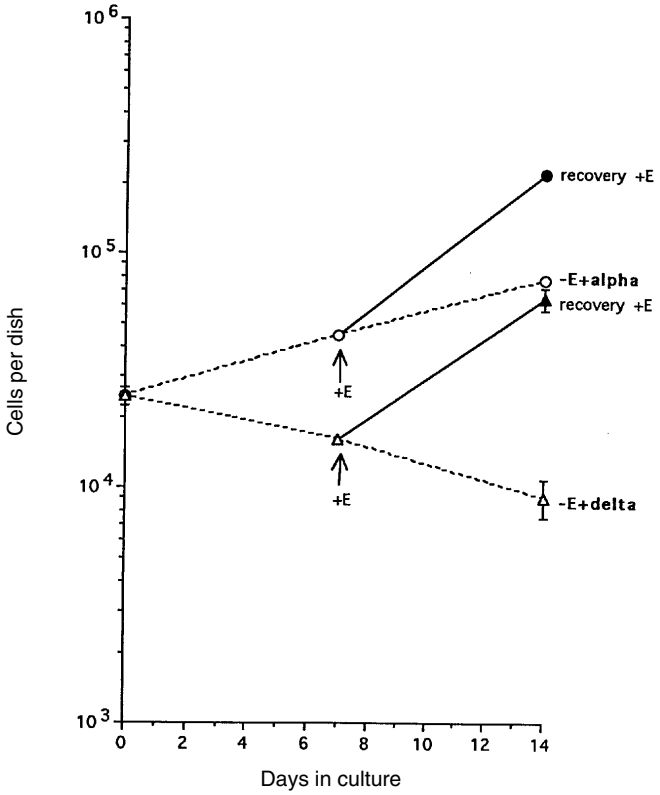

FIG. 5. Recovery of MCF7McGrath cell growth following a 7-d exposure to αT_3 or δT_3. MCF7McGrath human breast cancer cells were grown in monolayer culture for 14 d in 24-well dishes in RPMI1640 medium lacking both phenol red and estradiol but containing 5% DCFCS and either 6 μg/mL αT_3 (–E + alpha) (open circles, dotted lines) or 6 μg/mL δT_3 (–E + delta) (open triangles, dotted lines). Parallel dishes of cells were grown as above for 7 d but then for a further 7 d in RPMI1640 medium with 5% DCFCS and 10^{-8} M estradiol without tocotrienol (solid circle, solid line for 7 d with αT_3, 7 d recovery with estradiol; solid triangle, solid line for 7 d with δT_3, 7 d recovery with estradiol). Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviation.

ability counts were carried out in order to ascertain the viability of remaining adherent cells. MCF7McGrath human breast cancer cells grown without estradiol but with 10 μg/mL TRF showed a viability count of 98.8 ± 1.2% for cells remaining attached to the dish after 12 d (cf. Fig. 1A). Growth of MCF7 cells with 6 μg/mL of individual tocotrienol fractions in the absence of estradiol (cf. Fig. 3) gave viability counts after 7 d of 91.9 ± 2.4% for αT_3, 89.1 ± 1.3% for γT_3, 94.3 ± 3.1% for δT_3, and after 14 d of 78.4 ± 4.1% for αT_3, 72.0 ± 2.7% for γT_3, 73.9 ± 3.9% for δT_3. To test further for the viability of remaining adherent cells, a recovery experiment was performed in which cells were grown for 7 d with 6 μg/mL of either αT_3 or δT_3 followed by a further 7-d recovery period with estradiol. The results shown in Figure 5 demonstrate that cells exposed for 7 d to tocotrienol were subsequently still capable of regrowth in monolayer culture when tocotrienol was removed and estradiol added back.

Regulation of pS2 mRNA. In order to investigate any estrogen antagonist properties of TRF, effects of TRF were studied on pS2 mRNA levels. pS2 is an estrogen-regulated gene whose expression can be inhibited at the estrogen receptor level by antiestrogens and estrogen antagonists (29). At the concentrations tested, TRF (8 μg/mL) did not reduce the levels of pS2 mRNA either in the presence or in the absence of estradiol (Fig. 6). 36B4 mRNA is not regulated by estrogen, and levels of this mRNA are given to control for unequal loading of RNA samples. When comparisons were made of relative levels of pS2 mRNA to 36B4 mRNA, TRF was found to have no effect on pS2 mRNA levels either in the presence or in the absence of estradiol.

Regulation of IGFBP. Levels of IGFBP have also been shown to be altered by estrogen in estrogen-responsive human breast cancer cells, and this may have a functional role in growth regulation (24). Results given in Figure 7 show that IGFBP2 (32 kDa) and IGFBP4 (24 kDa) are the two major binding proteins secreted from MCF7McGrath cells, as reported previously (36). Levels of these IGFBP were not increased by γT_3 or δT_3.

DISCUSSION

The ability of the TRF of palm oil to inhibit mammary carcinogenesis in animal studies (5–12) is of potential importance for cancer prevention and treatment, but the mechanism remains unresolved even to the extent of knowing whether the action is directly on the tumor cells or indirectly *via* systemic interactions. The results of this study demonstrate that the TRF of palm oil can inhibit the growth of estrogen-responsive MCF7McGrath as well as estrogen-insensitive MDA-MB-231 human breast cancer cells *in vitro* and furthermore that MCF7 cell growth can be inhibited by TRF irrespective of the presence or absence of estradiol. These data confirm other recent studies using other sublines of ER– (13,37) and ER+ (38–40) breast cancer cells and suggest that the inhibition of tumor growth found *in vivo* could result, at least in part, from direct inhibitory effects on the growth of the cancer cells.

Comparison between growth of MCF7 and MDA cells shows that the inhibitory growth responses to tocotrienols differ in the two cell lines. Growth of MDA-MB-231 cells gave an inhibitory response which was linear with respect to increasing concentrations of tocotrienol whereas the MCF7 cell response was nonlinear. Thus, tocotrienols have greater inhibitory effects at low concentration on the MDA cells but at higher concentrations on the MCF7 cells. The 10-d growth of MDA cells (Fig. 2) could be suppressed by one doubling (3.96×10^5 to 1.98×10^5 cells/dish) by 3 μg/mL TRF, whereas 12-d growth of MCF7 cells (Fig. 1) in the presence of estradiol required 4 μg/mL TRF for suppression by one doubling (6.29×10^5 to 3.15×10^5 cells/dish). However, complete suppression of growth was achieved at lower concentration of TRF for the MCF7 cells. The growth of the MCF7 cells showed 100% growth inhibition with 8 μg/mL of TRF irrespective of the presence of estradiol, whereas the MDA cells

showed only 44% growth inhibition at this concentration, requiring 20 μg/mL TRF for 100% growth inhibition. Separation of the TRF into individual tocotrienols α-, γ- and δ- revealed that the γ- and δ-fractions were the most inhibitory to breast cancer cell growth and again showed the same differences in response in the two cell lines. The 7-d growth of MDA-MB-231 cells (Fig. 4A) could be suppressed by one doubling (1.95×10^5 to 0.98×10^5 cells/dish) by 2 μg/mL δT_3, whereas 7-d growth of MCF7 cells (Fig. 3A) in the presence of estradiol needed 5 μg/mL δT_3 for suppression by one doubling (1.66×10^5 to 0.83×10^5 cells/dish). However, complete suppression of MCF7 cell growth was achieved at concentrations of 6 μg/mL $\gamma T_3/\delta_3$ in the absence of estradiol and at 10 μg/mL δT_3 in the presence of estradiol, whereas for MDA-MB-231 cell growth complete suppression of growth was never achieved even at 10 μg/mL concentrations of the most inhibitory δT_3 component.

It is interesting that TRF (8 μg/mL) and $\gamma T_3/\delta T_3$ (6 μg/mL) could inhibit MCF7McGrath cell growth such that cell numbers were decreased below the plating density since this implies cell death. It would now be interesting therefore to determine whether tocotrienols can induce apoptotic pathways in the ER+ cells, as has already been described in breast cancer cells for hormone ablation (41), antiestrogen treatment (42,43), antagonism of IGF pathways (44), and administration of other cytotoxic agents (45,46).

Although it is often stated that αT is the most active form of vitamin E in inhibiting growth of cells (47), none of the breast cancer cells showed any sensitivity to αT in either these or other (13,39) studies. This emphasizes the importance of tocotrienols to growth inhibition of breast cancer cells. The reasons for the high sensitivity of breast cancer cells to tocotrienols are unknown but could relate either to permeability of the breast epithelial cell membrane to tocotrienols or to metabolism of tocotrienol within the breast cells (13). Alternatively, there may prove to be a pathway important specifically for breast cancer cell proliferation which is highly sensitive to tocotrienols. Inhibition of smooth muscle cell proliferation by vitamin E has been suggested to occur through alteration of protein kinase C activity (47) which could result from the antioxidant properties of vitamin E (48). Tocotrienols have been reported to affect lipid parameters, in particular linoleic acid metabolism (49), and it is known that linoleic acid plays a role in mammary epithelial cell growth and differentiation (50; unpublished personal data). Within estrogen-responsive breast cancer cells, another potential pathway of growth inhibition could be through antagonism of estrogen action, in a mechanism analogous to that characterized for growth inhibition by antiestrogens (25–27). Estrogen is known to act *via* an intracellular receptor protein which functions as a ligand-activated transcription factor (25), and it is now well established that the partial antiestrogen tamoxifen can act by interfering with transcriptional activation by the estrogen receptor and that the pure antiestrogens ICI 164,384 and ICI 182,780 can act by preventing dimerization and DNA binding of estrogen receptors (26,27). The TRF of

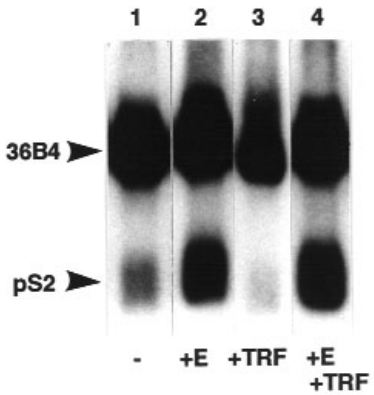

FIG. 6. Effect of the tocotrienol-rich fraction (TRF) of palm oil on expression of pS2 mRNA in MCF7McGrath human breast cancer cells. Northern blot of whole cell RNA from cells grown for 6 d in monolayer culture with 5% DCFCS alone (–) (track 1), with 10^{-8} M estradiol (+E) (track 2), with 8 μg/mL TRF (+TRF) (track 3) or with both 10^{-8} M estradiol and 8 μg/mL TRF (+E + TRF) (track 4). The blot was probed for expression of the estrogen-regulated pS2 mRNA and also a control non-estrogen-regulated 36B4 mRNA to control for any unequal RNA loadings. Positions of the mRNA are indicated by arrows. See Figure 1 for other abbreviation.

palm oil was clearly able, in the present studies, to inhibit growth of the MCF7McGrath cells in the presence of estrogen. In the absence of estrogen, TRF could also have acted to antagonize the action of residual estrogen in the cell or estrogen memory effects (51). However, TRF appeared to be unable to alter expression of the estrogen-regulated pS2 gene in MCF7McGrath cells, which is a well-established marker of estrogen receptor-mediated action (29) (Fig. 6). Furthermore, if TRF were acting on cell growth in the absence of estrogen to inhibit residual estrogen effects, it would be expected that TRF would act in a concentration-dependent manner such that lower levels of TRF would be needed to inhibit growth in the absence than in the presence of estrogen. Clearly this was not the case since inhibition of MCF7McGrath cell growth was found to require similar concentrations of TRF in the absence and in the presence of estrogen. The overall evidence would suggest therefore that tocotrienols do not act by an estrogen receptor-mediated mechanism.

An alternative mechanism for growth inhibition by TRF could relate to IGFBP (Fig. 7). Recent reports have emphasized the importance not only of estrogen but also of the insulin-like growth factors in breast cancer cell growth (20). IGFBP can influence the interaction between IGF and their receptors and so play an important role in mediating IGF regulation of breast cancer cell growth (20). It is thus possible that TRF could inhibit breast cancer cell growth by increasing production of inhibitory IGFBP in an analogous mechanism to that proposed for growth inhibition by retinoic acid in breast cancer cells (52). The growth inhibitory γT3 and δT3, however, did not appear to increase levels of IGFBP in these studies.

Inhibition of the growth of breast cancer cells by tocotrienols could have important clinical implications not only

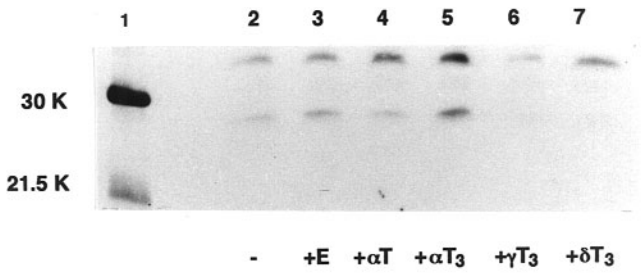

FIG. 7. Effect of individual tocotrienols on the secretion of insulin-like growth factor binding protein (IGFBP) from MCF7McGrath human breast cancer cells. Western ^{125}I-IGFI ligand blot of cells grown for 6 d in monolayer culture in RPMI1640 medium with 5% DCFCS alone (–) (track 2), with 10^{-8} M estradiol (+E) (track 3), with 6 μg/mL +αT (track 4), with 6 μg/mL αT_3 (track 5), with 6 μg/mL γT_3 (track 6), or with 6 μg/mL δT_3 (track 7). Positions of ^{14}C-labeled protein molecular weight markers are given in track 1 and sizes in kDa are indicated on the left-hand side. In tracks 2–7, the upper band corresponds to IGFBP2 (32 KDa) and the lower band to IGFBP4 (24 KDa) as reported previously (36). See Figure 1 for other abbreviation.

because tocotrienols are able to inhibit the growth of both ER+ and ER– phenotypes but also because estrogen-responsive cells could be inhibited in the presence as well as in the absence of estrogen. Unfortunately the molecular mechanism of the growth inhibition remains unknown and will need further work to elucidate. However, from a clinical perspective, it will also be of interest to determine whether breast cancer cells can develop resistance to this pathway of growth inhibition by TRF. A major problem in the therapy of breast cancer results from the ability of the tumor cells to develop resistance (14,15) to growth inhibition by estrogen withdrawal (53), antiestrogen administration (54), and retinoic acid (55). It will be very important to determine whether growth inhibition by TRF is also subject to development of resistance or not. Tocotrienols could offer an alternative clinical approach to growth suppression of breast cancer cells resistant to other regimes of therapy.

ACKNOWLEDGMENTS

This study was supported financially by the Malaysian government (KN). TRF and individual tocotrienols were all kindly supplied by PORIM. We thank Professor Pierre Chambon (Centre National de la Recherche Scientifique, Strasbourg, France) for providing the pS2 and 36B4 cDNAs. We thank Eileen Hales, Rita Gordon, Elizabeth Pontin, and John Taylor for technical assistance and research support services.

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[Received March 4, 1997, and in final revised form and accepted April 17, 1998]

Effect of Dietary Arachidonic Acid on Metabolism of Deuterated Linoleic Acid by Adult Male Subjects

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ABSTRACT: The influence of dietary supplementation with 20:4n-6 on uptake and turnover of deuterium-labeled linoleic acid (18:2n-6[d_2]) in human plasma lipids and the synthesis of desaturated and elongated n-6 fatty acids from 18:2n-6[d_2] were investigated in six adult male subjects. The subjects were fed either a high-arachidonic acid (HIAA) diet containing 1.7 g/d or a low-AA (LOAA) diet containing 0.21 g/d of AA for 50 d. Each subject was then dosed with about 3.5 g of 18:2n-6[d_2] as the triglyceride (TG) at 8:00 A.M., 12:00, and 5:00 P.M. The total 18:2n-6[d_2] fed to each subject was about 10.4 g and is approximately equal to one-half of the daily intake of 18:2n-6 in a typical U.S. male diet. Nine blood samples were drawn over a 96-h period. Methyl esters of plasma total lipid (TL), TG, phospholipid, and cholesterol ester were analyzed by gas chromatography–mass spectroscopy. Dietary 20:4n-6 supplementation did not affect uptake of 18:2n-6[d_2] in plasma lipid classes over the 4-d study period nor the estimated half-life of 24–36 h for 18:2n-6[d_2]. The percentages of major deuterium-labeled desaturation and elongation products in plasma TL, as a percentage of total deuterated fatty acids, were 1.35 and 1.34% 18:3n-6[d_2]; 0.53 and 0.50% 20:2n-6[d_2]; 1.80 and 0.92% 20:3n-6[d_2] and 3.13 and 1.51% 20:4n-6[d_2] for the LOAA and HIAA diet groups, respectively. Trace amounts (<0.1%) of the 22:4n-6[d_2] and 22:5n-6[d_2] metabolites were present. Plasma TL concentration data for both 20:3n-6[d_2] and 20:4n-6[d_2] were 48% lower ($P < 0.05$) in samples from the HIAA diet group than in samples from the LOAA diet group. For a normal adult male consuming a typical U.S. diet, the estimated accumulation in plasma TL of 20:4n-6 synthesized from 20 g/d (68 mmole) of 18:2n-6 is 677 mg/d (2.13 mmole). Dietary supplementation with 1.5 g/d of 20:4n-6 reduced accumulation of 20:4n-6 synthesized from 20 g/d of 18:2n-6 to about 326 mg/d (1.03 mmole).

Lipids 33, 471–480 (1998).

The effect of dietary arachidonic acid (AA) (20:4n-6) on linoleic acid (18:2n-6) metabolism is of interest because of their precursor–product relationship and the biological importance of these two fatty acids. Eicosanoids produced from 20:4n-6 are involved in the regulation of a variety of important physiological functions (1,2). Constriction of arterial vessels, increased platelet aggregation, and decreased bleeding times are examples of effects associated with 20:4n-6 that may have a negative impact on cardiovascular disease and strokes. There is also some concern that a diet might become prothrombotic if the n-6/n-3 balance is disrupted by a diet rich in 20:4n-6 (1,3). An early study involving humans reported that feeding 6 g/d of 20:4n-6 ethyl esters produced a potentially adverse increase in platelet sensitivity to the aggregation agent ADP (4). A recent study that used diets supplemented with 1.5 g/d of 20:4n-6 for 50 d found no effect on bleeding time or platelet aggregation (5) and a general lack of significant physiological effects due to dietary 20:4n-6 supplementation (5–8). For example, no effect was observed on immune response, apoprotein, serum triglyceride, total cholesterol, or lipoprotein levels (6,7). A large increase in the 20:4n-6 content of plasma, but not platelet, phospholipids (PL) (6) and a large increase in the eicosanoid metabolites of urine samples (8) were observed.

Metabolism of 18:2n-6 and 20:4n-6 has been studied individually in animal models, but studies that have examined the influence of dietary 20:4n-6 on the metabolism of 18:2n-6 are limited to animal and *in vitro* studies. Dietary 20:4n-6 substantially increases the 20:4n-6 content of animal tissue and human plasma PL at the expense of 18:2n-6 (6,9,10). Adding 20:4n-6 to rat liver microsome preparations did not depress desaturation *in vitro* of 18:2n-6 to 18:3n-6 (11). However, addition of 20:4n-6 to kidney cells slightly depressed 6-desaturase activity (12). Addition of 20:4n-6 to cultured cells increased conversion of 18:2n-6 to 20:4n-6 in several, but not all, cell lines (13,14). Stable isotope tracer methods have been used to show that dietary 18:3n-6, 20:3n-6, and 20:4n-6 reduced the synthesis of 20:4n-6 from 18:2n-6 in rats fed low-fat diets (15). Isotope tracer methods have not been used in humans to investigate whether dietary 20:4n-6 inhibits desaturation and elongation of 18:2n-6 or influences the uptake and disappearance of 18:2n-6 into lipid classes. The objective of this study was to investigate if supplementation of a typical U.S. diet with dietary 20:4n-6 has an influence on the synthesis, acyltransferase selectivity, uptake, and turnover of n-6[d_2] fatty acids in adult male subjects fed tracer doses of deuterium-labeled linoleic acid (18:2n-6[d_2]).

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Abbreviations: AA, arachidonic acid; CE, cholesterol ester; GC, gas chromatography; HIAA, high arachidonic acid; LCAT, lecithin:cholesterol acyltransferase; LOAA, low arachidonic acid; MS, mass spectroscopy; PL, phospholipid; TG, triglyceride; TL, total lipid; TLC, thin-layer chromatography.

TABLE 1
Plasma Lipid Concentrations and Total Deuterium-Labeled Trilinoleylglycerol Fed

Subject	Body weight	Plasma lipids[a]			18:2n-6[d_2]
		Triglyceride	Cholesterol	Total lipid	
(Code—Diet)	(kg)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/kg)[b]
HL 1 -LOAA	74.5	43.9	114	247	142
HL 3 -LOAA	67.7	41.8	138	219	158
HL 7 -LOAA	66.8	65.6	147	276	158
HL 2 -HIAA	61.8	45.4	164	321	157
HL 8 -HIAA	73.6	205.1	174	489	144
HL10 -HIAA	85.0	69.6	150	330	121

[a]mg of methyl ester/100 mL of plasma.
[b]mg 18:2n-6[d_2] fed per kg of body weight after correction for isotopic and chemical purity. Abbreviations: LOAA, low arachidonic acid; HIAA, high arachidonic acid.

EXPERIMENTAL PROCEDURES

Study design. Six Caucasian male subjects between the ages of 20 and 39 yr were housed in a metabolic ward. Medical histories, physical examinations, and clinical blood profile data indicated that the subjects were in good health and had no history of congenital ailments. The subjects' height/weight ratios, blood pressures, fasting serum cholesterol, and triglyceride concentrations were within normal ranges. Body weights, fasting serum total cholesterol, and total lipid (TL) concentrations are summarized in Table 1. Institutional ethical approval was obtained for the study protocol from the Agricultural Research Service's Human Studies Institutional Review Committee and the University of California at Davis, Human Subjects Committee. Informed consent was obtained from each subject before initiation of the study.

The subjects were provided diets that contained 1.7 g/d of 20:4n-6 [high AA (HIAA)] or 0.21 g/d of 20:4n-6 [low AA (LOAA)]. ARASCO oil (3 g/d), provided by Martek Bioscience Corp. (Columbia, MD), was added as the source of 20:4n-6 in the HIAA. An equal amount of high oleic safflower oil (3 g/d) was added to the LOAA. The subjects were fed these diets for 50 d prior to the deuterated 18:2n-6[d_2] isotope experiment. As shown in Table 2, the fatty acid compositions of the diets were similar except for 20:4n-6. The 20:4n-6 content of the total dietary fat was 0.2% (LOAA) and 2.0% (HIAA). The ARASCO oil contained 0.025% tocopherol and 0.025% ascorbyl palmitate as antioxidants. The peroxide value was 0.4 meq/kg. Analysis by gas chromatography (GC), GC–mass spectrometry (MS), and thin-layer chromatography (TLC) detected only traces of oxidation products. The fatty acid composition of the ARASCO oil (wt%) was: 0.5, 14:0; 0.2, 15:0; 11.1, 16:0; 0.5, 17:0; 10.6, 18:0; 8.7, 9*c*-18:1; 0.2, 11*c*-18:1; 6.5, 18:2n-6; 3.8, 18:3n-6; 0.9, 20:0; 0.4, 20:1n-9; 0.6, 20:2n-6; 0.6, 5,11,14-20:3; 2.5, 20:3n-6; 49.7, 20:4n-6; 1.6, 22:0; 1.2, 24:0; and 0.2%, 22:4n-6. Detailed information on the study design and diet compositions has been described previously (16).

All meals were prepared from weighed food portions and their consumption monitored. Duplicate meals were collected and analyzed for fatty acid composition. Values for total fat, protein, and carbohydrate were obtained by analysis of diet composites and were similar to calculated values based on food composition data in U.S. Department of Agriculture Handbook 8 (17). No significant changes in the subjects' weights were observed during the controlled diet period, indicating a stable energy balance.

Preparation of deuterated trilinoleylglycerol. A 100-g triglyceride (TG) sample containing *cis*-9,*cis*-12-octadecadienoic-12,13-d_2 acid (18:2n-6[d_2]) was prepared directly from *Crepis alpina* oil as described previously (18). The *C. alpina* oil contained 76% crepenynic acid, which is an "enyne" 18-carbon fatty acid with a *cis* double bond at the 9 position and a triple bond at the 12 position. The triple bond of crepenynic acid was reduced to a dideuterio-olefinic bond with Lindlar catalysis and deuterium gas. The TG product was purified by silica gel chromatography and contained 86% *cis*-9,*cis*-12-octadecadienoic acid, 3.5% *cis*,*trans*-octadecadienoic acid isomers, 5% oleic acid, 1% stearic acid, 4% palmitic acid, and 0.5% myristic acid. Residual crepenynic acid was not detected by GC or MS methods at a detection limit of 0.005%. The isotopic purity of the 18:2n-6[d_2] was 79% d_2, 4% d_1, 17% d_0.

Stable isotope study. After a 12-h overnight fast, about 5 g of TG containing 3.5 g of 18:2n-6[d_2] was mixed with 100 g of no-fat yogurt and fed to each subject at 8:00 A.M., 12:00, and 5:00 P.M. as part of their meals. The subjects were pro-

TABLE 2
Fatty Acid Composition of Diets

Fatty acid	LOAA (g/d)	HIAA (g/d)	Difference (g/d)
Saturates	21.8	22.4	–0.6
t-18:1	5.4	5.4	0.0
c-18:1	31.1	29.1	+2.0
18:2n-6	20.1	20.6	–0.5
18:3n-3	1.5	1.6	–0.1
20:4n-6	0.2	1.7	–1.5
Other	3.0	3.4	–0.4
Unknown	1.3	2.0	–0.7
Total weight	84.4	86.2	+1.8

[a]Diets: 2800 total calories; 27.1–27.8 energy percentage (en%) total fat, 15 en% protein, 57 en% carbohydrate. For abbreviations see Table 1.

vided a fat-free snack at 8:00 P.M.. The total weight of deuterated 18:2n-6[d_2] fed to each subject was about 10.4 g and replaced about 10 g of total fat containing 2 g of 18:2n-6 in the diets consumed on the day of the study. The total amount of deuterated 18:2n-6[d_2] fed per kg of body weight is given in Table 1 for each subject. A multiple dose procedure was used to maintain a relatively constant concentration of 18:2n-6[d_2] in plasma lipids over an 8-h time period in order to enhance the amount of n-6 desaturated and elongated fatty acid products synthesized from 18:2n-6[d_2]. Blood samples (*ca.* 14 mL each) were collected by venipuncture at 0, 4, 8, 12, 24, 36, 48, 72, and 96 h.

Analysis of plasma lipid fatty acids. Plasma TL were extracted with 2:1 chloroform/methanol (19). Preparative TLC was used to isolate TG, cholesterol ester (CE), and PL from plasma lipids (20,21). Known weights of triheptadecanoin, cholesteryl heptadecanoate, and diheptadecanyl-*sn*-phosphatidylcholine (Applied Science, State College, PA) were added as internal standards to the TL extract. The 17:0 internal standard data were used to calculate the concentrations (μg/mL) of deuterated and nondeuterated fatty acids in the plasma lipid classes. Methyl esters of the isolated lipid classes were prepared by heating the samples with a 5% HCl-methanol solution (22).

Plasma lipid methyl esters were analyzed by GC–MS methods. The Hewlett-Packard gas chromatograph (model 5890; Palo Alto, CA) was equipped with a Supelcowax 10 fused-silica column (30 m × 0.25 mm; Supelco Inc., Bellefonte, PA) and temperature-programmed from 165 to 265°C at 5°C/min with a 20-min final hold. The mass spectrometer used was a Hewlett-Packard model 5988A quadrupole mass spectrometer system and was operated in a positive chemical ionization mode with isobutane as the ionization reagent. The GC–MS methodology utilized selected ion monitoring of the appropriate ion masses for the fatty acid in each GC peak. The areas for each of the ion masses monitored were obtained by integration of the peaks. The specific operating conditions and computer-assisted storage and processing of the MS data have been described previously (23,24).

Weight data for isotope-labeled and nonlabeled fatty methyl ester derivatives of the plasma lipid samples were calculated from the known weight of 17:0 added as an internal standard prior to conversion of the lipid classes to their methyl esters. Response factors were determined by analysis of standard mixtures containing weighed amounts of pure fatty methyl esters purchased from Nu-Chek-Prep Inc. (Elysian, MN) and Applied Science. The accuracy of the GC–MS data was determined by adding known weights of 18:2n-9[d_2], 20:3n-6[d_2], and 20:4n-6[d_2] to TG, PL, and CE samples isolated from plasma samples drawn from other study subjects who had not been fed 18:2n-6[d_2]. The weight for each of the deuterated fatty esters added was equal to about 0.57% of the total unlabeled fatty acids in the samples. Standard deviations were based on three replicate analyses. The range for the standard deviations for the deuterated fatty acids added to TG, PL, and CE was 0.005 to 0.008%.

Statistical analysis and calculations. Data were analyzed with the SAS-PC statistical software package from Statistical Analysis System Institute, (Cary NC). A two-tailed, unpaired *t*-test was used to test for significant differences between data from subjects fed the LOAA and HIAA diets (25).

The time-course curves, obtained by plotting the deuterated fatty acid data for the nine samples collected between 0 and 96 h, were used to calculate a total area value for the concentrations (μg/mL plasma) of the deuterated fatty acids and their metabolites, as described previously (26,27). The μg/mL total area data were converted to μg/mL/g of 18:2n-6[d_2] fed and corrected for small differences in the amounts of 18:2n-6[d_2] fed to the subjects. The total area values were used to calculate the relative concentrations of 18:2n-6[d_2] and the dideuterated n-6 fatty acid products in plasma lipids. The percentage of 18:2n-6[d_2] converted to 20:4n-6[d_2] was calculated by dividing the μg/mL/g 20:4n-6[d_2] area values by the area values for 18:2n-6[d_2]. Percentage enrichment was calculated by dividing the deuterated fatty acid μg/mL data by the sum of the unlabeled plus deuterated fatty acid g/mL data.

RESULTS

Plasma lipid composition. The fatty acid compositions of plasma lipid classes from the six subjects used in this study are summarized in Table 3. The plasma data for this subset of subjects are similar to the data for the entire study group of 10 subjects (6). Plasma TG samples from the three subjects receiving the AA-supplemented diet, contained 2.1% AA compared to 1.0% AA for the LOAA diet subjects. The possibility of less conversion of 18:2n-6 to 20:4n-6 is suggested by the higher percentage of 18:2n-6 (20.9 vs. 22.5%) in the plasma TG of the HIAA diet group. As expected, the percentage of 20:4n-6 in plasma PL from the HIAA diet group was higher than for the LOAA diet subjects (15.7 vs. 11.6%). The CE fraction for samples from subjects fed the HIAA diet was higher (14.8 vs. 8.0%) in 20:4n-6 and 5.1% lower in 18:2n-6 than for CE from subjects fed the LOAA diet. The increases in 20:4n-6 percentages of PL and CE were compensated for by a decrease in 18:2n-6 and 18:1n-9 percentages.

Uptake and turnover. The percentages of 18:2n-6[d_2] in the total 18:2n-6 of plasma TG, PL, and CE samples collected over 96 h are plotted in Figure 1. The curves represent the average of data from three subjects and are nearly identical. There were no significant differences between the percentage enrichment time-course curves for individual subjects within and across diet groups. The variation for percentage enrichments in the plasma TG 4-, 8-, and 12-h samples for the LOAA and HIAA diet groups is due to the differences in absorption and clearance of chylomicron TG by individual subjects. The differences (see Table 3) between the 18:2n-6 and 20:4n-6 composition of the plasma lipid classes for subjects fed the LOAA and HIAA diets did not affect the 18:2n-6[d_2] percentage enrichment data. These results show that dietary 20:4n-6 supplementation did not influence uptake and turnover of 18:2n-6[d_2] in plasma TL, TG, PL, and CE. Max-

TABLE 3
Percentage Composition of Major Fatty Acids in Plasma Lipids from Subjects in the LOAA and HIAA Diet Groups

	LOAA diet group[a] (n = 3)				HIAA diet group (n = 3)			
Fatty acid	TG	PL	CE	TL	TG	PL	CE	TL
16:0	22.0 ±-2.6	**23.9 ± 1.0**	13.4±0.8	20.9 ± 0.5	20.0 ± 2.3	**22.0 ± 3.4**	13.1 ± 1.5	19.6 ± 0.6
16:1	4.6 ± 0.8	1.2 ± 0.2	1.9 ± 0.4	2.1 ± 0.2	3.9 ± 1.2	0.8 ± 0.1	2.0 ± 0.3	1.8 ± 0.2
18:0	2.6 ± 0.1	13.1 ± 0.9	1.1 ± 0.1	7.3 ± 0.2	4.4 ± 1.6	16.1 ± 1.0	1.1 ± 0.1	8.0 ± 1.5
18:1	44.1 ± 1.7	**17.9 ± 1.1**	20.4 ± 1.4	23.1 ± 0.4	40.5 ± 1.2	**15.0 ± 1.8**	19.1 ± 1.4	22.7 ± 2.5
18:2n-6	20.9 ± 3.8	**23.0 ± 1.2**	**51.9 ± 2.1**	**32.0 ± 1.6**	22.5 ± 0.1	**21.6 ± 1.1**	**46.8 ± 3.4**	**30.0 ± 1.6**
18:3n-6	0.4 ± 0.0	0.1 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
18:3n-3	0.7 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	1.1 ± 0.3	0.2 ± 0.0	0.4 ± 0.1	0.5 ± 0.1
20:2n-6	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.0
20:3n-6	0.2 ± 0.1	2.2 ± 0.2	0.4 ± 0.0	1.2 ± 0.1	0.3 ± 0.1	1.9 ± 0.5	0.4 ± 0.2	1.0 ± 0.2
20:4n-6	1.0 ± 0.5	**11.6 ± 1.1**	**8.0 ± 0.9**	**8.0 ± 0.7**	2.1 ± 0.1	**15.7 ± 1.9**	**14.8 ± 2.4**	**10.8 ± 2.2**
22:4n-6	0.2 ± 0.1	0.6 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.5 ± 0.2	0.8 ± 0.2	0.0 ± 0.0	0.5 ± 0.0
22:6n-3	0.1 ± 0.1	1.9 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	0.3 ± 0.3	2.1 ± 0.3	0.3 ± 0.1	1.0 ± 0.2
Other	2.9 ± 0.1	4.2 ± 1.4	1.8 ± 0.4	3.2 ± 0.4	3.7 ± 0.7	3.5 ± 0.8	1.6 ± 0.3	3.4 ± 1.0

[a]Values as %, mean, n = 3, ± standard deviation. TG, triacylglycerol; PL, phospholipid; CE, cholesterol ester; TL, total lipids. For other abbreviations see Table 1. Boldface type indicates data for fatty acids that were the most influenced by diet.

imum concentrations of 18:2n-6[d_2] occurred 12 h after the first dose and 4 h after the last dose of 18:2n-6[d_2] was fed. The half-life for 18:2n-6[d_2] in TL was between 24 and 36 h.

The average recovery of 18:2n-6[d_2] as a percentage of the total dose fed was 17% (range 10–27%). Percentage recovery was calculated from the sum of the 18:2n-6[d_2] concentrations in the 4- to 12-h plasma TL sample and a total plasma volume equal to 40 mL plasma per kg of body weight. Percentage recovery was directly related to plasma TG concentration. The lower percentage recoveries were observed for subjects with lower TG concentrations, and this relationship is likely related to plasma TG clearance rates. These percentage recovery data are only approximate because the sampling times were not frequent enough to ensure that samples containing the maximal enrichment of 18:2n-6[d_2] were collected. Variation in the percentage recovery data was not related to the 20:4n-6 content of the diet or the concentrations of 18:2n-6[d_2] and n-6[d_2] fatty acid metabolites that accumulated in plasma PL and CE.

The time-course curves shown in Figure 2 provide a comparison of percentage enrichment data and concentration (μg/mL of plasma) data for 20:4n-6[d_2] in plasma TL. The percentage enrichment of 20:4n-6[d_2] in the 36- to 96-h samples from the LOAA diet group were about 85% higher than for the same time samples from the HIAA diet group. Isotopic dilution by endogenous 20:4n-6 is responsible for the difference between the percentage enrichment and μg/mL data. The area concentrations for 20:4n-6[d_2] in total plasma of the

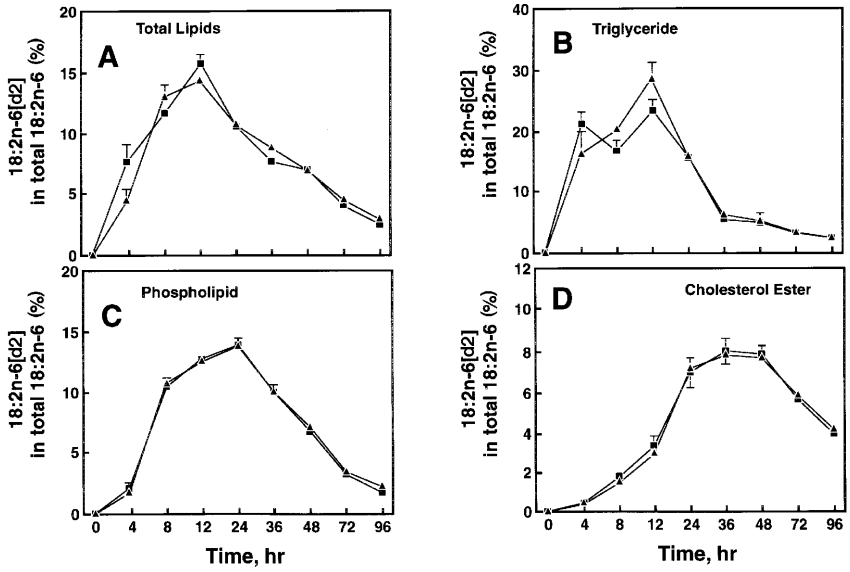

FIG. 1. Time-course curves for percentage enrichment of 18:2n-6[d_2] in plasma lipid classes of subjects from high arachidonic acid (HIAA) and low arachidonic acid (LOAA) diet groups. Each point is the average of data from three subjects. The error bars represent standard errors. Error bars are not shown when less than width of the symbols.

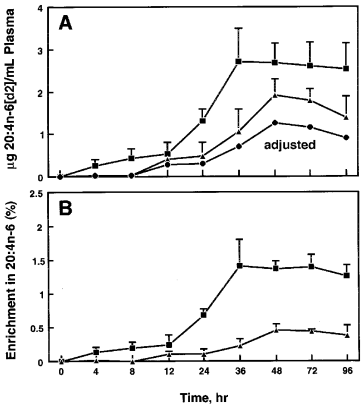

FIG. 2. Time-course curves for percentage enrichment and concentration of 20:4n-6 in plasma total lipid of subjects from LOAA (■) and HIAA (▲) diet groups Each point is the average of data from three subjects. The error bars represent standard errors. The curve labeled "adjusted" (●) represents HIAA diet group data adjusted to compensate for differences in plasma total lipid concentrations for subjects from the two diet groups. See Figure 1 for abbreviations.

LOAA diet group subjects were 35% higher than for subjects in the HIAA diet group. For the HIAA subjects, the μg/mL data underestimate the effect of the dietary 20:4n-6 supplementation on depression of 20:4n-6[d_2] accumulation because TL concentrations for the subjects in the HIAA group averaged 35% higher than for the LOAA diet group. The curve labeled "adjusted" represents the HIAA diet group data that were adjusted to compensate for differences in the plasma TL concentrations for subjects from the two diet groups. The difference in the concentration time-course curves provides a better estimate for the effect of dietary 20:4n-6 on 20:4n-6[d_2] accumulation than percentage enrichment data since the μg/mL data are independent of exogenous 20:4n-6.

The results in Figure 2 show that dietary 20:4n-6 supplementation depressed 20:4n-6[d_2] levels in plasma TL. The curve shapes for the percentage enrichment and the μg/mL plasma lipid curves are similar and show that uptake and turnover of 20:4n-6[d_2] in plasma TL were not significantly affected by dietary 20:4n-6 supplementation. The decrease in 20:4n-6[d_2] concentration between 48 and 96 h was small and indicates that there was conservation of 20:4n-6[d_2]. Maximal concentrations of 20:4n-6[d_2] occurred at 36 to 48 h after the first dose and 28 to 40 h after the last dose of 18:2n-6[d_2] was fed.

Data are summarized in Table 4 for plasma TG, PL, and CE samples that contained the maximal concentrations and maximal percentage enrichments for 18:2n-6[d_2] and its three major fatty acid metabolites. Both the percentage enrichment data in Table 4 for 18:2n-6[d_2] and the time-course data in Figure 1 were similar for subjects within the same diet group and between diet groups. The variability for the maximal concentration (μg/mL) data for all of the deuterium-labeled fatty acids was closely related to the variability in lipid class concentrations of the subjects. Overall, the maximal percentage enrichment for the 18:3n-6[d_2] was the least consistent. The 18:3n-6[d_2] percentage enrichment data for TG samples were

TABLE 4
Concentration of Deuterated Fatty Acids at Maximum Percentage Enrichment in Plasma Lipid Classes

			LOAA diet group		HIAA diet group	
Lipid class	Time[a] (h)	Fatty acid	Range	%[b]	Range	%[b]
			(μg/mL)	(avg ± SD)	(μg/mL)	(avg ± SD)
TG	12	18:2n-6[d_2]	54.5–83.6	28.7 ± 4.5	79.6–369.3	23.5 ± 2.9
	8–12	18:3n-6[d_2]	0.6–1.2	29.3 ± 4.5 **	0.5–1.1	19.8 ± 3.5 **
	12–24	20:3n-6[d_2]	0.1–0.3	9.5 ± 1.7	0.2–0.5	7.5 ± 4.5
	12–24	20:4n-6[d_2]	3.5–5.9	2.1 ± 0.6 *	0.3–6.3	0.9 ± 0.6 *
PL	24	18:2n-6[d_2]	19.0–28.6	13.8 ± 0.5	20.3–32.3	14.0 ± 0.8
	12–24	18:3n-6[d_2]	0.03–0.16	12.4 ± 4.6	0.02–0.11	13.9 ± 7.1
	36–72	20:3n-6[d_2]	0.7–1.2	5.4 ± 0.5	0.4–0.8	3.9 ± 1.7
	36–72	20:4n-6[d_2]	0.9–1.6	1.6 ± 0.2 **	0.8–1.9	0.9 ± 0.4 **
CE	24–36	18:2n-6[d_2]	4.6–19.2	8.3 ± 1.4	21.3–34.7	8.0 ± 0.9
	24–36	18:3n-6[d_2]	0.07–0.20	11.6 ± 7.9 *	0.02–0.26	3.4 ± 2.2 *
	48–72	20:3n-6[d_2]	0.08–0.18	5.5 ± 1.3	0.04–0.12	3.6 ± 2.6
	48–72	20:4n-6[d_2]	0.29–1.07	2.6 ± 1.2 *	0.20–1.20	0.8 ± 0.7 *
TL[c]	12	18:2n-6[d_2]	135–180	14.4 ± 1.6	177–405	15.6 ± 1.2
	12	18:3n-6[d_2]	1.0–2.2	15.9 ± 3.1 **	2.5–3.0	22.3 ± 2.1 **
	72	20:3n-6[d_2]	1.6–1.9	5.6 ± 0.2 **	0.8–1.3	3.3 ± 1.4 **
	72	20:4n-6[d_2]	1.8–3.9	1.6 ± 0.5 **	1.5–2.5	0.5 ± 0.1 **

[a]A range for the sample time (h) indicates that maximum concentration of the deuterium-labeled fatty acids occurred at different times.
[b]% = mean % enrichment (n = 3) ± standard deviation.** LOAA vs. HIAA data significantly different at $P < 0.05$; * significant difference at $P < 0.1$.
[c]Concentration data for TL do not equal total for TG, PL, and CE because data are for samples collected at different times. For abbreviations see Tables 1 and 3.

significantly lower (19.8 vs. 29.3%) for the HIAA diet group compared to the LOAA group subjects. Enrichment data for 20:4n-6[d_2] was significantly lower in TL, TG, PL, and CE samples from the HIAA diet group than for the LOAA group.

The major elongation and desaturation products of 18:2n-6[d_2] are 20:2n-6[d_2], 18:3n-6[d_2], 20:3n-6[d_2], and 20:4n-6[d_2]. These labeled fatty acids could be measured with an accuracy of ± 10% or better when they were present at 0.4 μg/mL or higher. The concentrations of 22:4n-6[d_2] and 22:5n-6[d_2] were generally about 10-fold lower (0.04 μg/mL), and the relative analytical error was about ±20%. The amount of 22:3n-6[d_2] in these samples was very small and could only be qualitatively measured. Dideuterated 24-carbon fatty acid products were not detected.

Acyltransferase selectivity. The distribution of n-6[d_2] fatty acid metabolites among the three major plasma lipid classes (TG, PL, and CE) is shown in Figure 3**,** where data are presented as area concentration per gram of 18:2n-6[d_2] fed (μg/mL/g 18:2n-6[d_2] fed). Note that these data do not compensate for differences between plasma lipid concentrations of individual subjects and should not be used to estimate the percentage of n-6[d_2] fatty acids synthesized from 18:2n-6[d_2]. The concentration data shown in Figure 3 are useful for determining the relative differences in the selectivity for incorporation of each n-6[d_2] fatty acid metabolite into individual plasma lipid classes.

The relative distribution patterns for the n-6[d_2] fatty acids in TG, PL, and CE are similar for subjects fed the LOAA and HIAA diets except for 20:4n-6[d_2]. These data in Figure 3 show that 18:3n-6[d_2] was selectively incorporated into TG and almost totally excluded from PL; 20:3n-6[d_2] was selectively incorporated into PL compared with TG and CE; incorporation of 20:4n-6[d_2] into PL and CE was much greater than for TG; and 20:2n-6[d_2] was selectively incorporated into TG and PL relative to CE. Comparison of the LOAA and HIAA 20:4n-6[d_2] data suggests that the lower concentration of 20:4n-6[d_2] in CE for subjects fed the HIAA diet was compensated for by a higher concentration of 20:4n-6[d_2] in TG. The selectivity of PL and CE for 20:3n-6 and 20:4n-6 is similar to the selectivity observed when subjects were fed a pulse dose of 20:3n-6[d_2] (28).

The distribution patterns for n-6[d_2] fatty acids in plasma PL were similar for the LOAA and HIAA diet groups. The CE n-6[d_2] fatty acid data for the two diet groups were also not different. These results show that dietary 20:4n-6 supplementation had little influence on the selectivity of PL and lecithin:cholesterol acyltransferase (LCAT) for these n-6 fatty acids. The patterns for the TG data for subjects from the LOAA and HIAA diet groups suggest that dietary 20:4n-6 may have inhibited incorporation of n-6[d_2] fatty acid metabolites into PL and CE.

Desaturation and elongation. The n-6[d_2] fatty acid products in plasma TL, TG, PL, and CE are plotted in Figure 4 as a percentage of the total deuterated fatty acids. The percentages were calculated from time-course curve μg/mL total area data. The percentage 18:2n-6[d_2] (not shown) was >90% of

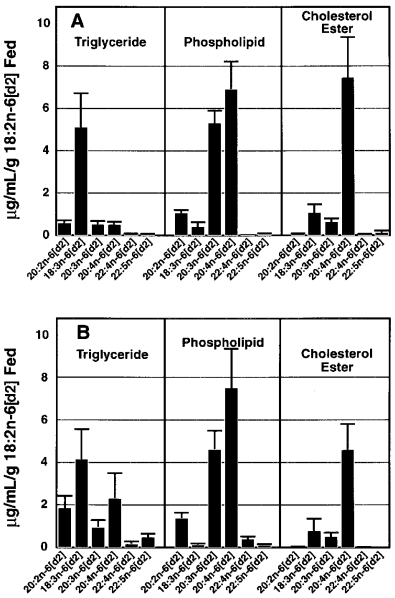

FIG. 3. Area concentrations for deuterated n-6 fatty acid metabolites in plasma triglyceride, phospholipid, and cholesterol ester of subjects fed LOAA (A) and HIAA (B) diets. Each bar is the average of data from three subjects. The error bars represent standard deviations. Data plotted are for the total areas under the time-course curves per mL of plasma per g of 18:2n-6[d_2] fed. For abbreviations see Figure 1.

the total [d_2] fatty acids in all plasma lipid classes. The percentage data in Figure 4 compensate for the differences in plasma lipid concentration between the two groups of subjects, and the metabolite data can be directly compared. Unlike the concentration data in Figure 3, these percentage data cannot be used to compare enzyme selectivity because the percentages are normalized values calculated from the total deuterated fatty acids in a specific lipid class.

The small percentage values for the n-6[d_2] fatty acids present in plasma lipids suggest that the amounts of these fatty acids synthesized over the 4-d study period were small. The sum for all the n-6[d_2] fatty acid percentages was less than 10% of the 10.4 g of 18:2n-6[d_2] fed per subject. These results show dietary 20:4n-6 supplementation depressed the percentage 18:3n-6[d_2] in TG by 65% ($P < 0.07$), 20:3n-6[d_2] in PL by 26% ($P < 0.1$), and 20:4n-6[d_2] in CE by 52% ($P < 0.14$). In plasma TL from subjects fed the HIAA diet, the percentage of 20:3n-6[d_2] was reduced by 48% ($P < 0.005$), and 20:4n-6[d_2]

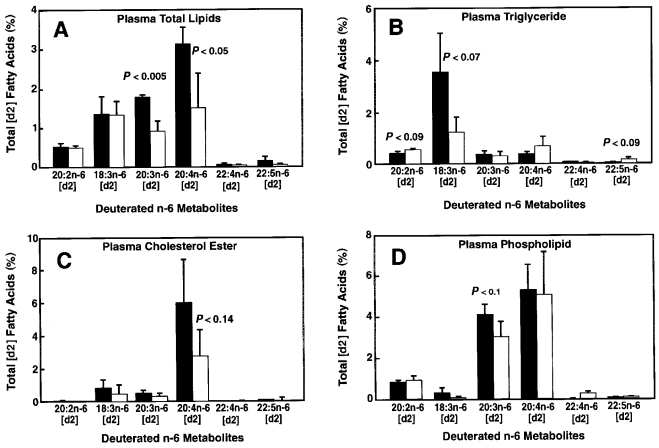

FIG. 4. Percentage of n-6 fatty acid elongation and desaturation products of 18:2n-6[d_2] in plasma lipid classes of subjects from LOAA (■) and HIAA (□) diet groups. Each bar is the average of data from three subjects. The error bars represent standard deviations. Percentage data are calculated from total-area-under-the-time-course curve concentration data. See Figure 1 for abbreviations.

was reduced by 52% ($P < 0.05\%$). The total plasma lipid data show that accumulation of 20:3n-6[d_2] in the LOAA diet group was 0.88% higher than in the HIAA diet group (1.80 vs. 0.92%) over the 4-d study period. Accumulation of 20:4n-6[d_2] was 1.62% higher in the LOAA diet group than in the HIAA group (3.13 vs. 1.51%) and represents a 52% depression of 20:3n-6 and 20:4n-6 accumulation by the 1.5 g/d of 20:4n-6 added to the HIAA diet. Thus the percentage data in Figure 4 show a 52% reduction in 20:4n-6[d_2] accumulation. This result should and does agree well with the 58% reduction in 20:4n-6[d_2] concentration calculated from the μg/mL area data in Figure 2 after adjusting the data for differences in plasma lipid concentrations.

DISCUSSION

Uptake and turnover. The curves for percentage enrichment of 18:2n-6[d_2] in the major lipid classes of plasma (Fig. 1) are evidence that the 7.5-fold (1.5 g/d) higher content of 20:4n-6 in the LOAA and HIAA diets did not measurably affect the mechanisms responsible for uptake and turnover of 18:2n-6. The curve shapes in Figure 2 for percentage enrichment and concentration of 20:4n-6[d_2] in plasma TL were similar and indicate that supplementation with 20:4n-6 may have slightly increased 20:4n-6[d_2] turnover rates. Maximal incorporation of 20:4n-6[d_2] occurred 36–48 h after the first dose of 18:2n-6[d_2] (also see Table 4). Subtraction of the 4 h required for absorption indicates that 32 to 44 h is the approximate time required for maximal accumulation of the 20:4n-6[d_2] synthesized from 18:2n-6[d_2]. The concentrations of 20:4n-6[d_2] in the 48-, 72-, and 96-h samples were not greatly different, suggesting turnover of 20:4n-6[d_2] was slow. These results from human subjects suggest that 20:4n-6 is conserved by recycling and are consistent with the results from rats fed 18:2n-6[d_2] (29).

However, when 20:4n-6[d_2] was fed to other subjects in these same diet groups, the estimated half-life for 20:4n-6[d_2] in plasma TL and PL was 36 to 44 h compared to 24–36 h for 18:2n-6[d_2] (30). Also, in the 20:4n-6[d_2] study, the percentage enrichment time-course curves for the total lipid and PL declined from a maximum at 8 and 12 h respectively, and dietary 20:4n-6 supplementation had no significant effect on turnover. The results from the 20:4n-6[d_2] study suggest that 20:4n-6 is not conserved more strongly than 18:2n-6 and that turnover of 20:4n-6 is not significantly slower than for 18:2n-6. The implication is that in the 18:2n-6[d_2] study, the synthesis of 20:4n-6[d_2] from 18:2n-6[d_2] rather than incorporation is largely responsible for the plateau in 20:4n-6[d_2] that occurred between 36 and 96 h. In effect, accumulation of 20:4n-6[d_2] was approximately equal to disappearance of 20:4n-6[d_2]. These results suggest that 20:4n-6[d_2] synthesis was regulated to replace loss of 20:4n-6[d_2].

The approximately threefold lower 20:4n-6[d_2] percentage enrichment in plasma TL from the HIAA diet group subjects was less than expected based on the ratio of 20:4n-6 in the diets. The predicted difference for percentage enrichment is about 17-fold for the following reasons. Isotopic dilution from exogenous 20:4n-6 should have reduced the percentage enrichment of 20:4n-6[d_2] for the HIAA diet group by 8.5-fold (0.21 vs. 1.7 g/d), and the concentration of 20:4n-6[d_2]

in plasma TL was about 50% lower. The lower-than-predicted difference in the percentage enrichment data suggests that accumulation of exogenous 20:4n-6 was restricted compared with accumulation of 20:4n-6[d_2] synthesized from 18:2n-6[d_2]. The threefold difference between the 20:4n-6[d_2] percentage enrichment data correlated well with the threefold difference for percentage 20:4n-6 in plasma total lipid composition data (Table 3). This correlation also suggests that accumulation of dietary 20:4n-6 was restricted in subjects from the HIAA diet group.

Acyltransferase selectivity. The n-6[d_2] fatty acid metabolite data in Figure 3 show a large difference between the concentrations of the various n-6[d_2] fatty acid metabolites incorporated into the three major plasma lipid classes. These differences are related to fatty acid chain length, number of double bonds, and double bond position. Since both 18:3n-6 and 20:3n-6 have three double bonds located at the same position relative to the terminal methyl group, chain length is the structural difference responsible for the preferential incorporation of 18:3n-6[d_2] into TG and the preferential incorporation of 20:3n-6[d_2] into PL. The concentrations of 20:3n-6[d_2] and 20:4n-6[d_2] in PL were similar. These results suggest that the additional double bond at the 5-carbon position in 20:4n-6 has little effect on PL acyltransferase selectivity. The 5-*cis* double bond is the only difference between the structure of these two fatty acids, and it is likely responsible for the preferential incorporation of 20:4n-6[d_2] into CE relative to 20:3n-6[d_2]. Exclusion of 20:2n-6[d_2] from CE is of interest because LCAT is highly selective for 18:2n-6[d_2], and the only structure difference between these two fatty acids is chain length. Comparison of the distribution pattern for 20:4n-6[d_2] in plasma TG, PL, and CE from subjects fed the LOAA and HIAA diets suggests that the transfer of 20:4n-6[d_2] to CE from PL by LCAT was partially diverted into TG.

Desaturation and elongation. The n-6[d_2] fatty acid (Table 4) precursors of 20:4n-6[d_2] are synthesized from 18:2n-6[d_2] by the following sequence: 6-desaturation → chain elongation → 5-desaturation. The maximal concentration for deuterated 18:3n-6 and 20:3n-6 in plasma lipid classes was low, which is consistent with 6-desaturase as the rate-limiting step. Qualitatively, these results with male subjects indicate that accumulation of 20:4n-6 synthesized from 18:2n-6 in humans is much lower than in rats and mice. The times for maximal incorporation (Table 4) show distinct differences for the times required to accumulate the various labeled fatty acid metabolites in TG, PL, and CE. The time required for synthesis and incorporation of 20:3n-6[d_2] and 20:4n-6[d_2] into PL and CE is approximately 36 h since maximal incorporation of 18:2n-6[d_2] occurred at 12 h and maximal incorporation of 20:n-4 occurred at 48 h.

The HIAA diet group percentage enrichment data for several n-6[d_2] fatty acid metabolites are significantly lower than data for the LOAA group. However, much of the difference is due to the greater isotopic dilution by the higher percentage of unlabeled 20:4n-6 (Table 3) in plasma lipids from the HIAA subjects rather than from reduced conversion of 18:2n-6[d_2] to n-6[d_2] metabolites. These results illustrate that percentage enrichment data must be interpreted cautiously. These single-point data do not provide a valid quantitative estimate of the amount of 18:2n-6[d_2] converted to these intermediates because the times for maximal incorporation of the individual fatty acids are different and subject-dependent. Also note that the data in Table 4 for total plasma lipid should not be compared with the sums for the individual lipid class data because the time of maximal incorporation for the various metabolites occurs at different times.

In contrast to the percentage enrichment data in Table 4 for single time-point samples, the percentage data in Figure 4 represent a weighted contribution of all samples collected over the 4-d study period. The influences of differences between plasma lipid class concentration (see Table 3) and the unlabeled fatty acid contents of the lipid classes are removed by normalizing the total deuterated fatty acid μg/mL data to a 100% basis. However, differences between percentage values still represent differences in the concentrations. Thus, conversion of the μg/mL deuterated fatty acid data to relative percentage provides a useful approach to estimate the percentage of 18:2n-6[d_2] fed that was converted to 20:4n-6[d_2] and incorporated into plasma lipids. The percentage data for 18:3n-6[d_2] and 20:2n-6[d_2] in plasma total lipids show that total accumulation of these fatty acids was nearly identical and indicate that δ-6 desaturation and elongation were not influenced by dietary 20:4n-6. These results are consistent with rat liver microsome *in vitro* results that showed exogenous 20:4n-6 and 20:5n-3 did not depress desaturation of 18:2n-6 to 18:3n-6 (11). The percentage data for PL, CE, and TL are consistent with the conclusion that the HIAA diet significantly depressed accumulation of 20:3n-6[d_2] and 20:4n-6[d_2] synthesized from 18:2n-6[d_2].

Estimate of 20:4n-6 synthesis. The following calculations assume that the 20:4n-6[d_2] incorporated into plasma TL reflects 20:4n-6[d_2] synthesis and that turnover rates for 18:2n-6 and 20:4n-6 are similar. This last assumption is supported by results from other subjects in these same diet groups that were fed 20:4n-6[d_2] (30). For the purpose of this estimate, the first step is to use the percentage of 18:2n-6[d_2] converted to 20:4n-6[d_2] over the 4-d study period to calculate the mg/d of 20:4n-6 synthesized per g of 18:2n-6. For subjects fed the LOAA diet, the percentage conversion of 18:2n-6[d_2] to 20:4n-6[d_2] was 3.13 ± 0.44% (Fig. 4) of the 18:2n-6[d_2] in total plasma lipid. A conversion of 3.13% indicates 325 mg of 20:4n-6[d_2] was synthesized from the 10.4 g of 18:2n-6[d_2] fed over the 4-d study period, or 31.3 mg of 20:4n-6[d_2] per g of 18:2n-6[d_2] fed. By extrapolation, the accumulation of 20:4n-6 synthesized from the 20 g/d of 18:2n-6 in the LOAA diet is equal to 626 mg/d. Similar calculations based on 1.51 ± 0.89% conversion of 18:2n-6[d_2] for the subjects fed the HIAA diet indicate accumulation of 302 mg/d of 20:4n-6 synthesized from 18:2n-6.

Results for subjects fed the LOAA diet suggest that accumulation of 20:4n-6 synthesized from 18:2n-6 is 2–3 times higher than the 20:4n-6 content of typical U.S. diets and that

relatively little dietary 20:4n-6 is necessary to meet requirements. These estimates based on the accumulation of 20:4n-6 synthesized from 18:2n-6 are consistent with estimates based on studies of vegetarians and the postulate that the requirement for 20:4n-6 is approximately 500 mg/d (31). The 324 mg/d difference for the amount of 20:4n-6 synthesized from 20 g/d of 18:2n-6 represents a 52% reduction in total 20:4n-6 accumulation by subjects fed the HIAA diet. This 52% reduction is similar to the 58% depression based on the 20:4n-6[d_2] area concentration data shown in Figure 2**.** These estimates for 20:4n-6 synthesis compare well with an estimate of 440 mg/d based on data from an earlier 2-d study using a single dose of deuterated 18:2n-6 (27).

Results presented here for the metabolism part of this AA study show that a relatively large increase in dietary 20:4n-6 intake had little effect on uptake and turnover, acyltransferase selectivity, and the accumulation of 18:3n-6[d_2] and 20:2n-6[d_2] synthesized from 18:2n-6[d_2]. These metabolic results are consistent with the lack of significant effects from the clinical part of this study (5–7) and with the lack of toxicity effects in rats fed about 1.2 g of 20:4n-6/d/kg body weight for 90 d (32). The only significant physiological effect observed was an increase in the urinary metabolites of thromboxane and prostaglandin I_2 when diets were supplemented with 20:4n-6 (8). The only significant metabolic effect of 20:4n-6 supplementation was the depression (about 50%) in plasma TL of the deuterated 20:3n-6 and 20:4n-6 synthesized from 18:2n-6[d_2]. This depression was less than expected based on the 8.5-fold difference in the 20:4n-6 ratio in the diets and was inversely related to the approximately twofold increase in the 20:4n-6 content of plasma PL. The inhibition of 20:4n-6 accumulation was in marked contrast with the sevenfold stimulation of 20:4n-6[d_2] synthesis from 20:3n-6[d_2] (28). Overall, these results show that moderate fluctuation in dietary 20:4n-6 intake would not be expected to have a major effect on n-6 fatty acid metabolism.

ACKNOWLEDGMENTS

Dr. Terry C. Nelsen, USDA Biometrician, for assistance with statistical procedures, and Lynne C. Copes for assistance with laboratory procedures.

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[Received June 20, 1997, and in final revised form March 9, 1998; revision accepted April 2, 1998]

Effects of the Ratio of Polyunsaturated and Monounsaturated Fatty Acid to Saturated Fatty Acid on Rat Plasma and Liver Lipid Concentrations

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ABSTRACT: The effects of dietary monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid + MUFA/saturated fatty acid (PUFA + MUFA/SFA) ratio on plasma and liver lipid concentrations were studied. In experiment I, when rats were fed with 40% fat (energy%, PUFA/SFA ratio 1.0) and 1% (w/w) cholesterol (C) diets for 21 d, a large amount of MUFA (28.1 energy%, PUFA + MUFA/SFA = 5.7) in the diet was found to increase the plasma total C, triacylglycerol (TAG), and phospholipid (PL) as compared with the low-MUFA diet (7.0 energy%, PUFA + MUFA/SFA = 1.4). The plasma very low density lipoprotein (VLDL)-C, VLDL-TAG, VLDL-PL, and low density lipoprotein (LDL)-C increased significantly in the high-MUFA diet group, but high density lipoprotein (HDL)-C did not change significantly. The high-MUFA diet resulted in greater accumulation of liver C but lesser accumulation of TAG. In experiment II, when dietary SFA was fixed at a certain level (13.2 energy%; PUFA + MUFA/SFA = 2.0), rats given a larger amount of MUFA (23.1 energy%; PUFA/MUFA = 0.2; MUFA/SFA = 1.8) showed higher plasma and liver C levels than did the low-MUFA diet (7.7 energy%; PUFA/MUFA = 2.5; MUFA/SFA = 0.6). When PUFA was fixed at a certain level (24.4 energy%), there was not a significant difference in the plasma C level between the high- and low-MUFA dietary groups (PUFA + MUFA/SFA = 4.8 and 8.4), but the higher PUFA + MUFA/SFA diet, which was high in MUFA/SFA ratio, significantly decreased the plasma HDL-C and TAG levels. However, when MUFA content was fixed at a certain level (16.4 energy%), no significant difference was observed between the two groups with different PUFA/SFA ratios of 0.2 and 4.1, but liver C level was raised in the higher PUFA/SFA diet. It appears that the PUFA/SFA ratio alone is unsuitable to predict the change of plasma C level, because a large amount of dietary MUFA may lead to an increase of plasma and liver lipids in rats. It seems that the prerequisites for keeping low plasma and liver C are (i) low MUFA/SFA ratio, (ii) high PUFA/MUFA ratio, and (iii) PUFA + MUFA/SFA ratio not to exceed 2.

Lipids 33, 481–487 (1998).

Among the dietary factors which can affect the plasma cholesterol (C) level, the amount of dietary C and the amount and the degree of saturation of fatty acids are perhaps more important than the others (1,2). A significant relationship between dietary fat, plasma lipid levels, and coronary heart disease is reported by many epidemiologists worldwide (3–7). During the last three decades many investigators have reported that polyunsaturated fatty acids (PUFA) decrease and saturated fatty acids (SFA) increase the plasma total C and low density lipoprotein (LDL)-C levels in humans (8–11) as well as in animals (12–16). However, monounsaturated fatty acids (MUFA), such as oleic acid, were once considered to have no effect on plasma C levels (17). Hence, the PUFA/SFA ratio has frequently been used in predicting the cholesterolemic effect of dietary fat. Diets with higher PUFA/SFA ratios were found to have stronger hypocholesterolemic effect than those with lower PUFA/SFA ratios (8–18).

However, since 1985, studies by Mattson and Grundy (19) and Grundy (20) and some other investigators (21–23) have reported that when MUFA-rich safflower oil or olive oil is supplemented to the diet or replaced by SFA, serum C and LDL-C levels decrease but high density lipoprotein (HDL)-C level does not show any decrease in human subjects. Therefore, it seems that MUFA may be better than PUFA in preventing or treating atherosclerosis (19–23). Sirtori *et al*. (24) reported that olive oil diet does not appear to reduce the plasma total C level in the same way as corn oil diet in high-risk patients. Becker *et al.* (25) also found that a MUFA-rich diet does not affect the plasma total C, triacylglycerol (TAG), LDL-C, and HDL-C levels when compared with an SFA-rich diet in normolipidemic men. Ginsburg *et al*. (26) concluded that a decrease in the plasma total C level in human subjects during consumption of the MUFA diet occurs in response to the removal of SFA from the diet. A meta-analysis of 27 trial studies, carried out by Mensink and Katan (27), showed that the replacement of carbohydrates by MUFA results in a negative but nonsignificant coefficient on the plasma total C and LDL-C concentrations. Perez-Jimenez *et al*. (28) studied lipoprotein concentrations in normolipidemic males consuming two high-fat diets, similar in their fatty acid composition but prepared using two different plant oils: virgin olive oil

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Abbreviations: C, cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; MUFA, monounsaturated fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol; VLDL, very low density lipoprotein.

and refined high-MUFA sunflower oil. When the two MUFA diets were compared, significantly higher concentrations of total C and LDL-C were noted with the consumption of the olive oil-rich diet.

In the present study, we emphasized the effect of the PUFA + MUFA/SFA ratio of the dietary fat, as well as the PUFA/SFA ratio, on plasma and liver lipid levels in rats. In experiment I, two oil mixtures of different fatty acid saturation, with the same PUFA/SFA ratio of 1.0 but a different PUFA + MUFA/SFA ratio of 1.4 or 5.7, were given to separate groups of rats. In experiment II, PUFA, MUFA, or SFA of the dietary fat was fixed at a certain level for each of three pairs of rat groups, respectively, and then the PUFA + MUFA/SFA ratio was changed as described in the Materials and Methods section for the three pairs of rat groups. The effects of these test diets on the plasma and liver lipid concentrations were studied.

MATERIALS AND METHODS

Animals. In experiment I, 30 male Wistar rats (Laboratory Animal Center, College of Medicine, National Taiwan University, Taipei, Taiwan), each weighing about 200 g, were used for the study. The rats were divided into two groups of 12 rats in each on the basis of their weight. On day 7, 14, and 21, four rats in each group were sacrificed. The remaining six rats were used as a control group (day 0); they were fed with commercially available rat chow (Rodent laboratory chow 5001; Purina Co., St. Louis, MO) and were sacrificed before the experiment. Rats were housed individually in stainless steel, wire-bottomed cages and were given a diet and water *ad libitum* during the different dietary periods. The food consumption per group and the individual body weights were measured every 2 d.

In experiment II, 36 male Wistar rats, each weighing about 160 g, were divided into six groups of six rats each. The test diets in this study were fed to the rats for a period of 21 d.

Diet and feeding protocol. Each group was fed the same basic, semisynthetic powdered diet which differed only in its fatty acid content. The experimental diet contained (w/w): 1% C, 4% salt mixture (AIN-76, ICN Biomedicals Inc., Costa Mesa, CA), 1% vitamin mixture (AIN76, ICN), 3% methyl cellulose, 20% casein, 51% cornstarch, and 20% fat. The different fat mixtures were prepared with various ratios of soybean oil, coconut oil, palm oil, sunflower oil, and olive oil as shown in Tables 1 and 2. Each diet supplied 15% of total energy as protein, 45% as carbohydrate, and 40% as fat. In experiment I, both diet 1 and diet 2 had PUFA/SFA ratios of 1.0, but diet 1 had a PUFA + MUFA/SFA ratio of 1.4 and diet 2 had a PUFA + MUFA/SFA ratio of 5.7 (Table 1). In experiment II, the first two diets (diet 1 and diet 2) had approximately the same amount of PUFA, but diet 1 had a PUFA + MUFA/SFA ratio of 4.8 and diet 2 had a PUFA + MUFA/SFA ratio of 8.4. The next two diets (diet 3 and diet 4) had the same MUFA level, but the PUFA + MUFA/SFA ratios were 1.0 and 7.6, respectively. The last two diets (diet 5 and diet 6) had the same SFA level, therefore, the same PUFA + MUFA/SFA ratio (2.0), but the MUFA/SFA ratios were 1.8 and 0.6, respectively (Table 2).

TABLE 1
Fatty Acid Composition of Oil Used in Test Diets in Experiment I

Fatty acids	Diet 1[a] (%)	Diet 2[b] (%)
6:0	0.1	—[c]
8:0	2.0	—
10:0	2.0	—
12:0	15.6	—
14:0	7.1	0.2
16:0	10.8	11.2
18:0	3.6	3.5
Subtotal[d]	41.2 (16.5)	14.9 (6.0)
16:1	0.1	0.6
18:1	17.3	69.7
Subtotal[d]	17.4 (7.0)	70.3 (28.1)
18:2	36.5	13.4
18:3	4.9	1.4
Subtotal[d]	41.4 (16.6)	14.8 (6.0)
PUFA/SFA ratio	1.0	1.0
MUFA/SFA ratio	0.4	4.7
PUFA/MUFA ratio	2.4	0.2
PUFA + MUFA/SFA ratio	1.4	5.7

[a]Diet 1 (low MUFA diet): 68.5% soybean oil + 31.5% coconut oil.
[b]Diet 2 (high MUFA diet): 12.3% soybean oil + 87.7% olive oil.
[c]—, not detectable.
[d]Each value in the parentheses is the energy%. PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

The pattern of fatty acids of these mixed oils (soybean oil, coconut oil, palm oil, sunflower oil, and olive oil) was analyzed by high-performance liquid chromatography (29) for verification of correct PUFA/SFA and PUFA + MUFA/SFA ratios.

Sample collection and analysis. Fasting blood samples were taken by decapitating rats on days 0, 7, 14, and 21 in experiment I or on day 21 in experiment II. The plasma (1 mg EDTA/mL) was stored at 4°C before analysis. Liver was excised, weighed, and frozen until analysis. Feces were pooled from a group during the last 3 d of the experimental period and then dried at 75°C to a constant weight, ground into a fine powder, and stored in a vacuum desiccator.

In experiment I, plasma very low density lipoprotein (VLDL), LDL, and HDL were obtained with ultracentrifugation at density 1.006, 1.063, and 1.21 g/mL, respectively. In experiment II, plasma HDL-C level was determined by a commercial kit that uses a precipitation-enzymatic method (Boehringer Mannheim Co., Meylan, France). Total plasma and liver lipids were determined by the method of sulfophospho-vanillin reaction (30). Plasma and lipoprotein C and TAG were determined by a commercial kit enzymatic method (Boehringer Mannheim Co.). Plasma and lipoprotein phospholipid (PL) were determined by using bioMérieux (Marcy-l'Etoile, France) enzyme kit.

Liver TAG was determined by the method of Soloni (31).

TABLE 2
Fatty Acid Composition (%) of Oil Used in Test Diets in Experiment II

	PUFA fixed		MUFA fixed		SFA fixed	
Fatty acids	Diet 1[a]	Diet 2[a]	Diet 3[a]	Diet 4[a]	Diet 5[a]	Diet 6[a]
6:0	—[b]	—	—	—	—	0.1
8:0	—	—	—	—	—	1.3
10:0	—	—	—	—	—	1.3
12:0	—	—	0.4	—	0.2	10.3
14:0	1.4	—	1.1	—	0.6	5.1
16:0	11.6	6.8	43.5	7.9	28.3	11.0
18:0	4.2	3.9	3.9	3.8	3.7	3.8
Subtotal[c]	17.2 (6.9)	10.7 (4.3)	48.9 (19.6)	11.7 (4.7)	32.8 (13.1)	32.9 (13.2)
16:1	0.2	—	—	0.2	0.3	0.2
18:1	22.4	28.3	40.7	40.8	57.5	19.0
Subtotal[c]	22.6 (9.0)	28.3 (11.3)	40.7 (16.3)	41.0 (16.4)	57.8 (23.1)	19.2 (7.7)
18:2	53.0	61.0	10.4	47.2	9.2	42.2
18:3	7.2	—	—	0.2	0.3	5.7
Subtotal[c]	60.2 (24.1)	61.0 (24.4)	10.4 (4.2)	47.4 (19.0)	9.5 (3.8)	47.9 (19.2)
PUFA/SFA ratio	3.5	5.7	0.2	4.1	0.3	1.5
MUFA/SFA ratio	1.3	2.6	0.8	3.5	1.8	0.6
PUFA/MUFA ratio	2.7	2.2	0.3	1.2	0.2	2.5
PUFA + MUFA/SFA ratio	4.8	8.4	1.0	7.6	2.0	2.0

[a]Diet 1: 100% soybean oil; Diet 2: 100% sunflower oil; Diet 3: 100% palm oil; Diet 4: 26% olive oil + 74% sunflower oil; Diet 5: 53% palm oil + 47% olive oil; Diet 6: 21% coconut oil + 79% soybean oil.
b—, not detectable.
[c]Each value in the parentheses is the energy%. For abbreviations see Table 1.

Liver PL was analyzed by the method of Stewart (32). Liver C was determined by the method of Abell *et al.* (33).

Feces were extracted with petroleum ether (1:10, wt/vol) and filtered. The neutral steroid content of the filtrate was determined by the method of Abell *et al.* (33). Another portion of feces was extracted with alcohol (1:5, wt/vol), and then alcohol was evaporated. The residue was suspended in petroleum ether (1:2.5, wt/vol), shaken, centrifuged, and the liquid phase was discarded. The remaining precipitate was analyzed for the acid steroid content by the method of Weber (34).

Statistical analysis. The data between two groups were compared by analysis of variance (ANOVA). Student's *t*-test was used whenever a statistically significant difference between the two groups was shown by ANOVA. All data are presented in tables and figures as mean ±SD.

RESULTS

In experiment I, the growth of rats in both groups was not different in each of the experimental periods. Figure 1 shows the plasma total C, TAG, and PL contents at the end of the feeding period. It is clear that when the PUFA/SFA ratio was fixed at 1.0, with 1% C supplement, the high PUFA + MUFA/SFA ratio (5.7, diet 2) showed significantly higher plasma total C,

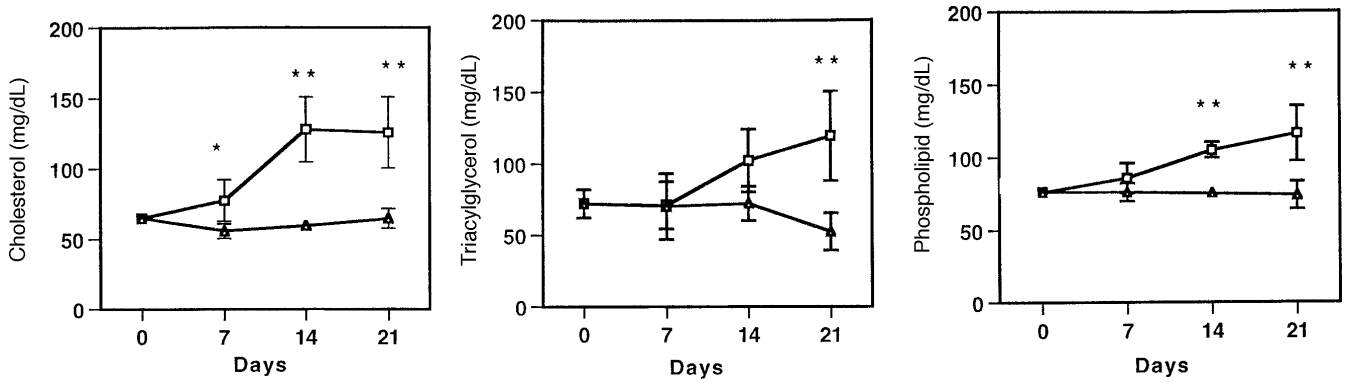

FIG. 1. Changes in the concentrations of plasma lipids during the different feeding periods in experiment I. Diet 1 (△): PUFA + MUFA/SFA = 1.4; Diet 2 (□): PUFA + MUFA/SFA = 5.7. Asterisks indicate significant difference from diet 1 period by the Student's *t*-test (*$P < 0.05$; **$P < 0.01$). Results are shown as mean ± SD ($n = 4$). PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

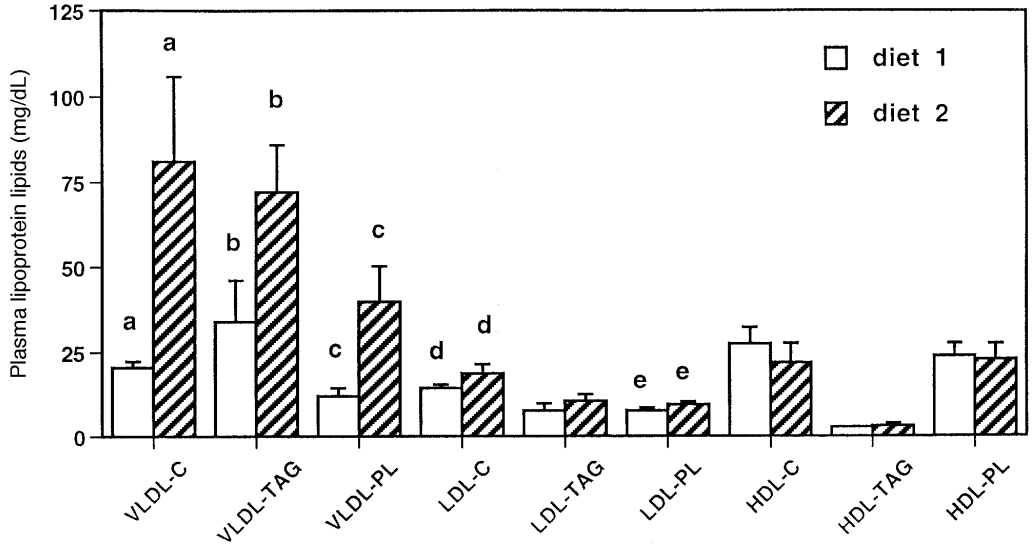

FIG. 2. Changes in the concentrations of plasma lipoprotein lipids after the 21-d feeding periods in experiment I. Diet 1: PUFA + MUFA/SFA = 1.4; Diet 2: PUFA + MUFA/SFA = 5.7. Matching letters (a–e) indicate a significant difference between mean values by Student's *t*-test (a–c, $P < 0.01$; d,e, $P < 0.05$). Each bar represents the mean ± SD ($n = 6$). Abbreviations: C, cholesterol; TAG, triacylglycerol; PL, phospholipid; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. For other abbreviations see Figure 1.

TAG, and PL than the lower PUFA + MUFA/SFA ratio (1.4, diet 1) during the last period of the experiment ($P < 0.01$). Figure 2 shows that plasma VLDL lipids were significantly higher in the high-MUFA group (diet 2) ($P < 0.01$), whereas the effect was less in HDL lipids. At the same time, high MUFA (diet 2) significantly increased the LDL-C and LDL-PL contents after the 21-d feeding periods ($P < 0.05$). In addition, as shown in Figure 3, liver C, TAG, and PL contents increased in both low- and high-MUFA diets. The high-MUFA diet resulted in greater accumulation of liver C but lesser accumulation of TAG.

In experiment II, the effects of different PUFA + MUFA/SFA, MUFA/SFA, or PUFA/MUFA ratios on the plasma lipid contents are shown in Table 3. When PUFA was fixed at a certain level, the difference in plasma total C and PL levels between the two dietary groups (PUFA + MUFA/SFA = 4.8 and 8.4) was not significant, but the higher PUFA + MUFA/SFA diet, which was high in MUFA/SFA ratio, showed a significant decrease in the plasma HDL-C and TAG. When SFA was fixed at a certain level, a high-MUFA diet (diet 5, PUFA/MUFA = 0.2) showed a significant increase in plasma total C, TAG, PL, and total lipid ($P < 0.01$) as compared with the low-MUFA diet (diet 6, PUFA/MUFA = 2.5). But when MUFA was fixed at a certain level, no significant difference was observed between the two groups with different PUFA/SFA ratios of 0.2 and 4.1 ($P > 0.05$). As shown in Table 4, when either MUFA or SFA was fixed at a certain level, rats given higher ratios of MUFA/SFA (diet 4

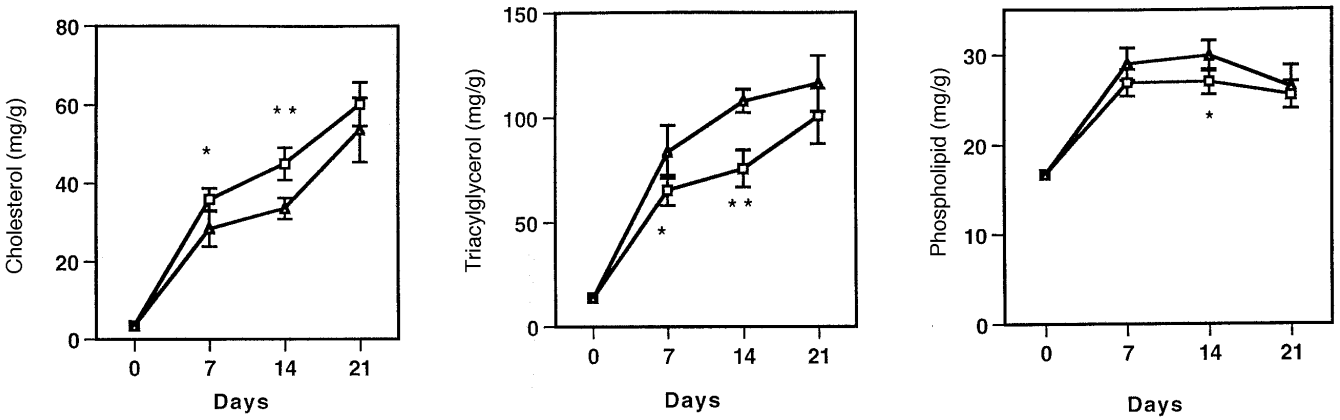

FIG. 3. Changes in concentrations of liver lipids during the different feeding periods in experiment I. Diet 1 (△): PUFA + MUFA/SFA = 1.4; Diet 2 (□): PUFA + MUFA/SFA = 5.7. Asterisks indicate significant difference from diet 1 period by the Student's *t*-test (*$P < 0.05$; **$P < 0.01$). Results are shown as mean ± SD ($n = 4$). For abbreviations see Figure 1.

TABLE 3
Effects of Difference in PUFA + MUFA/SFA, MUFA/SFA, or PUFA/MUFA Ratios on Plasma Lipids (mg/dL) in Experiment II[a]

	PUFA fixed		MUFA fixed		SFA fixed	
Plasma lipids	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Total cholesterol	150.9 ± 11.9	197.6 ± 54.1	203.3 ± 46.2	215.2 ± 85.1	338.8 ± 92.8[c]	129.5 ± 30.0[c]
HDL-cholesterol	40.5 ± 9.3[a]	29.2 ± 5.5[a]	39.3 ± 8.2	31.8 ± 5.7	32.2 ± 4.7	33.3 ± 6.2
Triacylglycerol	61.5 ± 1.1[b]	49.2 ± 8.6[b]	64.3 ± 25.2	61.4 ± 10.4	61.7 ± 11.4[d]	42.5 ± 6.8[d]
Phospholipid	94.8 ± 11.4	96.8 ± 5.3	115.1 ± 5.9	105.1 ± 14.0	133.4 ± 24.3[e]	83.7 ± 5.7[e]
Total lipid	316.3 ± 28.2	375.4 ± 83.5	396.4 ± 40.8	383.9 ± 98.5	573.0 ± 133.7[f]	252.8 ± 26.9[f]
PUFA/SFA ratio	3.5	5.7	0.2	4.1	0.3	1.5
MUFA/SFA ratio	1.3	2.6	0.8	3.5	1.8	0.6
PUFA/MUFA ratio	2.7	2.2	0.3	1.2	0.2	2.5
PUFA + MUFA/SFA ratio	4.8	8.4	1.0	7.6	2.0	2.0

[a]Diets are described in Table 2. HDL, high density lipoprotein. For other abbreviations see Table 1. Matched superscripts (a–f) indicate a significant difference between mean values (a, $P < 0.05$; b–f, $P < 0.01$) by Student's *t*-test. Results are shown as mean ± SD ($n = 6$ in all groups).

and diet 5) showed significantly higher liver C levels ($P < 0.05$). However, when PUFA was fixed at a certain level, liver lipids were not found to differ significantly in both groups which had different PUFA + MUFA/SFA ratios (4.8 and 8.4). The liver TAG level was not found to differ significantly among each dietary group in experiment II.

Fecal neutral and acidic steroids of the rats in experiment II are shown in Table 5. The results showed that when MUFA was fixed at a certain level, the high SFA group (diet 3, 19.6 energy%) excreted more neutral and acidic steroids, whereas, when PUFA or SFA was fixed at a certain level, a high-MUFA diet showed higher fecal neutral and acidic steroids.

DISCUSSION

By keeping the PUFA/SFA ratio of dietary fat at 1.0, we observed that higher dietary MUFA increased plasma total C, TAG, and PL levels (Fig. 1), as well as liver total C content (Fig. 3) in rats. The hypocholesterolemic effect of MUFA was not observed. The plasma lipids of the VLDL fraction were also increased in high-MUFA diet (Fig. 2). Even when PUFA or SFA was fixed at a certain level, rats given higher ratios of MUFA/SFA showed higher C levels (Table 3). It seems that the PUFA/SFA ratio alone is unsuitable to predict the change of plasma C level, because the MUFA tends to increase plasma C level under our experimental conditions.

In this study, we did not observe the hypocholesterolemic effect of a high-MUFA diet in rats as reported by Grundy and his associate (19,20). When PUFA/SFA was fixed at 1.0, we found that the high-MUFA diet (PUFA, 6; MUFA, 28; SFA, 6 energy%; PUFA + MUFA/SFA = 5.7) increased both plasma and liver C contents in rats as compared with the low-MUFA diet (PUFA, 17; MUFA, 7; SFA, 17 energy%; PUFA + MUFA/SFA = 1.4) in experiment I. Grundy (20) suggested that a liquid diet rich in MUFA (PUFA, 8; MUFA, 28; SFA, 4 energy%; PUFA/SFA = 2.0; PUFA + MUFA/SFA = 9.0) appeared to be at least as effective in lowering plasma C in humans as compared with the high-SFA diet (PUFA, 7; MUFA, 8; SFA, 25 energy%; PUFA/SFA = 0.28; PUFA + MUFA/SFA = 0.6). In another study, Mattson and Grundy (19) reported that, as compared with the high-SFA diet (PUFA, 4; MUFA, 16; SFA, 20 energy%; PUFA/SFA = 0.2; PUFA + MUFA/SFA = 1), the high-MUFA diet (PUFA, 7; MUFA, 29; SFA, 3 energy%; PUFA/SFA = 2.3; PUFA + MUFA/SFA = 12) lowered plasma LDL-C in normotriglyceridemic patients, but did not lower the plasma total C level in hypertriglyceridemic pa-

TABLE 4
Effects of Difference in PUFA + MUFA/SFA, MUFA/SFA, or PUFA/MUFA Ratios on Liver Lipids (mg/g) in Experiment II[a]

	PUFA fixed		MUFA fixed		SFA fixed	
Liver lipids	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Cholesterol	69.9 ± 7.6	68.4 ± 10.6	57.4 ± 8.8[a]	75.5 ± 6.7[a]	81.0 ± 5.5[c]	68.9 ± 8.2[c]
Triacylglycerol	115.5 ± 14.4	89.7 ± 24.6	93.8 ± 18.3	92.9 ± 14.2	104.9 ± 19.1	116.3 ± 25.0
Phospholipid	29.6 ± 1.7	30.1 ± 2.1	29.3 ± 1.9	28.0 ± 2.4	27.4 ± 2.8[d]	31.2 ± 1.2[d]
Total lipid	213.9 ± 17.4	199.3 ± 39.2	176.1 ± 27.8[b]	209.2 ± 22.4[b]	225.2 ± 26.0	212.5 ± 32.3
PUFA/SFA ratio	3.5	5.7	0.2	4.1	0.3	1.5
MUFA/SFA ratio	1.3	2.6	0.8	3.5	1.8	0.6
PUFA/MUFA ratio	2.7	2.2	0.3	1.2	0.2	2.5
PUFA + MUFA/SFA ratio	4.8	8.4	1.0	7.6	2.0	2.0

[a]Diets are described in Table 2. Matched superscripts (a–d) indicate a significant difference between mean values (a, $P < 0.01$; b–d, $P < 0.05$) by Student's *t*-test. Results are shown as mean ± SD ($n = 6$ in all groups). For abbreviations see Table 1.

TABLE 5
Fecal Neutral and Acidic Steroids of Rats in Experiment II

Groups	Sample number	Feces dry weight (g/d/rat)	Neutral steroids (mg/d/rat)	Acidic steroids (mg/d/rat)
PUFA fixed[a]				
Diet 1	6	1.40	46.90	2.86
Diet 2	6	1.34	53.87	5.44
MUFA fixed[a]				
Diet 3	6	1.76	57.23	7.32
Diet 4	6	1.48	38.86	2.69
SFA fixed[a]				
Diet 5	6	1.50	57.75	3.81
Diet 6	6	1.39	52.04	2.99

[a]Diet 1: PUFA + MUFA/SFA = 4.8, PUFA/MUFA = 2.7; Diet 2: PUFA + MUFA/SFA = 8.4, PUFA/MUFA = 2.2; Diet 3: PUFA + MUFA/SFA = 1.0, PUFA/MUFA = 0.3; Diet 4: PUFA + MUFA/SFA = 7.6, PUFA/MUFA = 1.2; Diet 5: MUFA/SFA = 1.8, PUFA/MUFA = 0.2; Diet 6: MUFA/SFA = 0.6, PUFA/MUFA = 2.5. For abbreviations see Table 1.

tients. It might be the effect of a low SFA content rather than the high MUFA content of the safflower oil diet (high-MUFA diet), when compared to the high-SFA diet (coconut oil or palm oil) which induced higher plasma C levels. In addition, the high-MUFA diet used by Mattson and Grundy (19) had a very high PUFA + MUFA/SFA ratio of 9 or 12, but our diets of high PUFA + MUFA/SFA ratio (5.7, 7.6, or 8.4) did not lower plasma and liver C in experiments I and II. To exclude the effect of changing the amount of PUFA, MUFA, and SFA at the same time, we fixed the amount of PUFA, MUFA, or SFA when comparing a set of two test diets (experiment II) and found that plasma and liver C levels were raised in higher MUFA/SFA diets (Tables 3 and 4). With human subjects, we reported previously that when the dietary PUFA/SFA ratio was fixed at 1.0, those subjects given a diet with a PUFA + MUFA/SFA ratio of 4.5 showed significantly higher plasma TAG, VLDL-TAG, LDL-TAG, and LDL-C levels than those subjects given a diet with a PUFA + MUFA/SFA ratio of 1.5 (35).

We have observed repeatedly in rat experiments that when the PUFA/SFA ratio was fixed at 1.0, diets rich in MUFA resulted in greater accumulation of liver C (Fig. 3). Similar results were also obtained by Peifer *et al*. (36), Beynen (37), Bulur *et al*. (38), and Spady and Dietschy (39). It is worthwhile to notice that when the amount of SFA was fixed at a certain level, the higher ratio of PUFA/MUFA (diet 6, PUFA/MUFA = 2.5, MUFA/SFA = 0.6) lowered plasma total C, TAG, and PL and liver C (Tables 3 and 4), and when the amount of MUFA was fixed at a certain level (16.4 energy%), the high-PUFA diet (diet 4, PUFA + MUFA/SFA = 7.6, PUFA/MUFA = 1.2, MUFA/SFA = 3.5) resulted in the same plasma C level but higher liver C level than did the high-SFA diet (diet 3, PUFA + MUFA/SFA = 1.0, PUFA/MUFA = 0.3, MUFA/SFA = 0.8) (this experiment was repeated twice). The reason that diet 4, which is high in PUFA, resulted in increased liver C may be its high MUFA/SFA ratio, unlike diet 6 or diet 3. It seems that the prerequisites for keeping low plasma and liver C are (i) low MUFA/SFA ratio, (ii) high PUFA/MUFA ratio, and (iii) PUFA + MUFA/SFA ratio not to exceed 2.

When the amount of PUFA was fixed at a higher level (24.4 energy%, diet 1 and diet 2), the plasma and liver lipids were less affected by changing the amounts of SFA and MUFA.

The present study indicated that a large amount of dietary MUFA supplemented with 1% C may raise plasma and liver C levels in rats, and the PUFA + MUFA/SFA ratio, if not to exceed 2, may be more suitable to predict the change of plasma lipids than the PUFA/SFA ratio.

ACKNOWLEDGMENT

This work was supported by a grant (NSC-81-0412-B039-05) from the National Science Council, Taiwan, Republic of China.

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[Received November 3, 1997, and in final revised form March 2, 1998; revision accepted March 26, 1998]

Lipid-Lowering Effects of WAY-121,898, an Inhibitor of Pancreatic Cholesteryl Ester Hydrolase

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ABSTRACT: WAY-121,898 is an inhibitor of pancreatic cholesteryl ester hydrolase (pCEH). After confirming its *in vitro* potency and relative lack of a major effect on acyl-CoA:cholesterol acyltransferase (ACAT), it was found that this compound lowers plasma cholesterol in cholesterol-fed, but not chow-fed, rats. Measures of liver cholesteryl ester content and the direct determination of cholesterol absorption (lymph–fistula model) show that inhibition of cholesterol absorption is at least one mechanism for the observed cholesterol lowering. However, WAY-121,898 was also active when administered parenterally to cholesterol-fed rats, and in cholesterol-fed hamsters cholesterol-lowering occurred with oral dosing despite no change in cholesterol absorption, suggesting other modes of action possibly relating to inhibition of liver CEH. Combination treatment in cholesterol-fed rats with the ACAT inhibitor CI-976 resulted in a greater-than-additive reduction in plasma cholesterol, implying that both pCEH and ACAT may play a role in cholesterol absorption in this species. In rabbits, WAY-121,898 prevented the rise in plasma cholesterol due to the feeding of cholesteryl ester but not in rabbits fed (free) cholesterol. In guinea pigs, the compound induced an increase in adrenal cholesteryl ester mass. Taken together, the overall profile in these animal models suggests that WAY-121,898 inhibits more than just the intestinal (lumenal) pCEH, and that the role of this enzyme in cholesterol metabolism may be different within and across species, the former depending upon the dietary cholesterol load. *Lipids 33,* 489–498 (1998).

Pancreatic, bile acid-dependent cholesteryl ester hydrolase (pCEH, E.C. 3.1.1.13) is secreted into the small intestine where it catalyzes the hydrolysis of dietary cholesteryl esters and other acylated lipids. However, several observations suggest that the role of pCEH extends beyond that of simply hydrolyzing dietary lipids. Immunolocalization of pCEH within intestinal cells has been demonstrated (1), and cultured CaCo-2 cells bind and internalize pCEH (2,3), suggesting an extralumenal site of action for the enzyme. Cholesterol absorption and/or cholesterol esterifying activity in the intestine are decreased in pancreatic fistula rats (4,5), pancreatectomized rats, (6), and in rats perfused with pancreatic fluid previously treated with antibody to pCEH (7). In addition, isolated enterocytes from common bile duct-cannulated rats exhibit a marked decrease in the ability to esterify cholesterol, which can be restored by the addition of pCEH (8). It has been proposed based on these and other experiments that pCEH, in addition to its role in lipid hydrolysis, may catalyze cholesteryl ester formation after uptake of the enzyme into the mucosal cells (1,8–11). Data support a role for pCEH in the uptake of both unesterified cholesterol (3,11) and cholesteryl ester into intestinal cells, the latter independent of ester hydrolysis (12). Thus, pCEH may be involved at several steps or sites during cholesterol absorption (i.e., cholesteryl ester hydrolysis, cholesteryl ester synthesis, uptake of unesterified and esterified cholesterol).

If the above putative functions of pCEH are correct, it suggests that pharmacologic inhibition of this enzyme might lead to inhibition of cholesterol absorption and thereby a reduction in plasma cholesterol concentration. Indeed, preliminary evidence suggests that WAY-121,898, a novel carbamate, potently and selectively inhibits pCEH *in vitro* and inhibits cholesterol absorption *in vivo* in rats, dogs, and quail (13–16). The purpose of the present study was to confirm the preliminary observations in rats (i.e., efficacy and mechanism of action), and to extend these findings by determining the activity of WAY-121,898 in other possibly more relevant animal models in which more plasma cholesterol is transported in low density lipoprotein (LDL). In addition, since rat liver can also synthesize and possibly secrete a bile salt-dependent CEH (17,18), it was of interest to compare oral and parenteral routes of administration of WAY-121,898. Finally, we determined whether co-administration with CI-976, an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) (19,20), would result in additive effects with regard to plasma cholesterol reduction. Intestinal ACAT has also been strongly implicated in the esterification of dietary cholesterol (21,22). Overall, the results suggest fundamental species differences in the response to WAY-121,898 and the possible existence of extra-intestinal sites of action. Moreover, it seems likely that both pCEH and ACAT may play a role in cholesterol absorption in rats.

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; IC_{50}, concentration required to inhibit enzyme activity 50%; LDL-C, low density lipoprotein-cholesterol; MES, 2-[*N*-morpholino] ethanesulfonic acid; pCEH, pancreatic cholesteryl ester hydrolase; SC, subcutaneous.

MATERIALS AND METHODS

Both compounds (WAY-121,898 and CI-976) were synthesized in the Department of Chemistry, Parke-Davis Pharmaceutical Division. The spectral data for WAY-121,898 (i.e., nuclear magnetic resonance, mass spectrum, CHN analysis) were consistent with the proposed structure (13). The *in vitro* inhibitory effects of WAY-121,898 on pCEH activity were assessed by using the porcine enzyme (0.0025 U/mL final concentration; Calbiochem, La Jolla, CA). The substrate was a micellized lipid suspension composed of [^{3}H]cholesteryl oleate (2 μCi/mL), 1 mM lysophosphatidylcholine, 40 mM cholate, and 0.01 mM cholesteryl oleate dispersed in a buffer of 150 mM NaCl, 1 mM EDTA and 20 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 6.0. The substrate was prepared by combining aliquots of 100 mM sodium cholate in chloroform/methanol (2:1, vol/vol), 100 mM lysophosphatidylcholine in chloroform/methanol (2:1, vol/vol), 1 mM cholesteryl oleate (in chloroform), and a volume of [^{3}H]cholesteryl oleate. Solvents were removed by evaporation under a stream of N_2. The dried lipids were resuspended in the appropriate volume of buffer to yield the desired final concentrations of lipids. The pCEH (32.7 IU/mg) was dissolved at a concentration of 0.1 mg/mL in the same buffer as that used for substrate. A more dilute lipase solution (0.15 μg/mL) was used for the assay. The assay was performed at room temperature. Five microliters of the test compound, dissolved in ethanol at a concentration 40-fold greater than the final desired test concentration, was added to individual wells of a 96-well plate. The assay wells without test compound received the same volume of ethanol alone. One hundred microliters of carboxyester lipase (0.15 μg/mL) was added to each well. The assay reaction was begun by the addition of 100 μL of substrate and stopped 10 min later by the addition of 20 μL of 1 N HCl. For quantitation of the substrate and lipolytic products, the contents of each well were transferred to glass high-performance liquid chromatography autosampler vials (12 × 32 mm) and combined with 0.6 mL of ethyl acetate/acetone (2:1, vol/vol). The vials were capped, vortexed twice, and lightly centrifuged for 5 min. The organic phase was transferred to clean autosampler vials and dried under a stream of nitrogen followed by vacuum dessication for at least 1 h. The dried extracts were dissolved in 50 μL of dioxane and then further diluted with 150 μL of isooctane. The [^{3}H]-labeled lipids were then quantitated by high-performance liquid chromatography as previously described (23). The possible effect of WAY-121,898 on microsomal ACAT activity was also determined by using the assay of Field and Salome (24) with livers from cholesterol-fed rats, as described previously (20). In these assays dimethylsulfoxide was used as the drug vehicle, and the concentration required to inhibit enzyme activity 50% (IC_{50}) was calculated (Dose-Effect Analysis with Microcomputers, Chou and Chou, BIOSOFT Software, Cambridge, United Kingdom).

Male, Sprague-Dawley rats (200–225 g, Charles River, Portage, MI) were fed either normal chow or chow supplemented with 0.5% cholic acid, 5.5% peanut oil, and varying amounts of cholesterol (0 to 1.5%). Golden Syrian hamsters (males, 90–110 g) were fed normal chow or chow containing cholesterol (0.06%) and hydrogenated coconut oil (10%) (25). Hartley guinea pigs (males, 450–500 g) were fed normal chow, and New Zealand white rabbits (males, 1.6–1.8 kg) were fed chow supplemented with 5% corn oil and either 0.5% cholesterol or 0.87% cholesteryl oleate (both containing 0.5% sterol). In all experiments, the drugs (WAY-121,898 or CI-976) were mixed into the diet to provide the indicated doses, unless otherwise stated. In one experiment a comparison was made between oral (gavage and diet-admix) and parenteral (subcutaneous, SC) drug administration. The vehicle for oral gavage was carboxymethylcellulose (CMC, 1.5%) and Tween 20 (0.2%) in water. SC dosing utilized an ethanol/PEG-200 (10:90, vol/vol) vehicle. Drugs and diets were initiated at the same time and continued for 1 wk (cholesterol-fed rats), 2 wk (chow-fed animals, cholesterol-fed hamsters), or 5 wk (cholesterol-fed rabbits). Plasma lipids and liver cholesteryl esters were measured by standard methods described previously (20).

Although liver cholesteryl ester content correlates well with measures of cholesterol absorption (26), cholesterol absorption was measured directly in rats by determining the rate of appearance of cholesterol and cholesteryl esters into mesenteric lymph after a single bolus of drug into a duodenal cannula (20). A lipid emulsion was then infused, which was prepared by dissolving 200 mg of cholesterol and 250 mg of egg phosphatidylcholine in chloroform. After removal of the chloroform by evaporation under N_2 and vacuum, 5 g safflower oil, 100 mg sodium taurocholate, and 100 mL of saline were added and the entire mixture was emulsified by sonication. The rate of infusion into the duodenum was 2.5 mL/h. In hamsters the dual-isotope method of Turley *et al.* (26) was used to measure cholesterol absorption, with the following modifications. Telezol (tiletamine/zolazepam, 40 mg/kg) was used as anesthetic instead of ether, and intravenous injections were into the jugular vein instead of the femoral. In this experiment the hamsters were fed a chow diet supplemented with 0.2% cholesterol and 10% hydrogenated coconut oil. Intralipid (Kabi Pharmacia Inc., Clayton, NC) was used as the intravenous (IV) vehicle for injection of the [^{3}H] cholesterol, and medium-chain triglyceride oil (Mead Johnson Nutritionals, Evansville, IN) was used as a vehicle to orally dose the [^{14}C] cholesterol, as described (26). Percentage cholesterol absorption was calculated from the percentages of the oral [^{14}C] and intravenous [^{3}H] doses remaining in plasma 72 h after isotope administration.

RESULTS

WAY-121,898 inhibited rat liver ACAT and porcine pCEH with IC_{50} values of 47 μM and 650 nM, respectively (Fig. 1). Thus, this compound was at least 70-fold more potent against pCEH compared to ACAT based upon these assays. With regard to the ACAT inhibitor used in this study, CI-976, we have shown that the IC_{50} for ACAT inhibition using rat liver

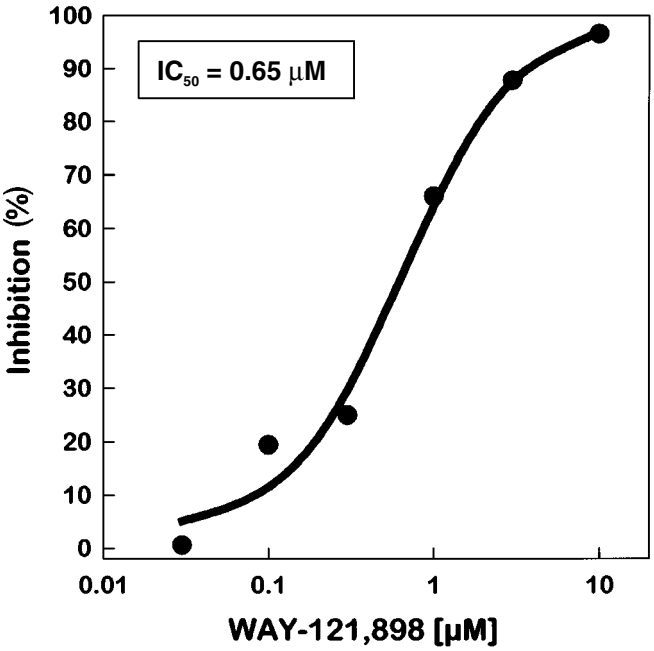

FIG. 1. Inhibition curve for WAY-121,898 against porcine pancreatic cholesteryl ester hydrolase (pCEH). Each point represents the mean of triplicate determinations. The concentration required to inhibit enyzme activity 50% (IC_{50}) is indicated.

microsomes is 180 nM, and this compound has no effect on pCEH up to 100 μM (20). CI-976 also inhibits the rabbit (20) and hamster (25,26) ACAT enzyme. Clark *et al.* (27) have also reported that WAY-121,898 not only lacks significant inhibitory activity against ACAT but also does not inhibit other serine hydrolases, including pancreatic triglyceride lipase.

In our laboratory WAY-121,898 was first evaluated in normal, chow-fed animals using diet-admix drug administration. Plasma total cholesterol concentrations were unchanged in rats at doses of 50, 100, and 200 mg/kg, but plasma triglycerides were significantly decreased (at 50 and 100 mg/kg). Liver cholesteryl esters decreased at the highest dose (Table 1). In a separate experiment (data not shown), we used oral gavage and an olive oil vehicle rather than diet-admix. Again, plasma cholesterol was unchanged, and plasma triglycerides were reduced 27, 49, and 55% at doses of 25, 50, and 100 mg/kg, respectively. Thus, in chow-fed rats, efficacy with respect to triglyceride-lowering was improved with the oil vehicle, but cholesterol-lowering was still not observed. In chow-fed hamsters, in contrast to rats, WAY-121,898 lowered plasma cholesterol by 20 and 31% at 10 and 30 mg/kg (diet-admix). Plasma triglycerides remained unchanged while liver cholesteryl esters tended to decrease in all dose groups, with significance reached at the intermediate dose of 30 mg/kg. Other modes of drug administration (gavage using different vehicles) were not evaluated in chow-fed hamsters.

Since both ACAT and pCEH have been implicated as playing a role in the absorption of exogenous (dietary) cholesterol, it was of interest to determine whether inhibitors of these enzymes would prevent diet-induced hypercholesterolemia, and furthermore, at what level of dietary cholesterol optimal efficacy would be observed. To this end, groups of rats were fed varying amounts of cholesterol and dosed orally with the drugs for 2 wk (50 mg/kg). Plasma cholesterol in controls increased steadily from about 50 mg/dL (chow alone) to almost 300 mg/dL in both experiments (from 1 to 1.5% dietary cholesterol, Table 2). Both drugs failed again to alter plasma cholesterol in the absence of added cholesterol, but they blunted the rise in plasma cholesterol with increases in dietary cholesterol load. However, with WAY-121,898, efficacy (percentage changes compared to controls) plateaued at 0.4% dietary cholesterol (about a 50% reduction), whereas with CI-976 efficacy continually increased with increasing dietary (and plasma) cholesterol up to about 70%.

Based upon the foregoing results in cholesterol-fed rats, further experiments were conducted with WAY-121,898 using 0.4% cholesterol to examine changes in the mode of drug administration and in the drug route. By using this amount of dietary cholesterol, gavage administration with an aqueous suspension vehicle was less effective than diet-admix for lowering plasma cholesterol (Fig. 2). Plasma triglyceride levels were unchanged, and gavage with an oil vehicle did not improve efficacy (data not shown). In the same experiment, plasma cholesterol was also reduced when WAY-121,898 was administered subcutaneously. In fact, at 50 mg/kg, cholesterol-lowering using the SC route (–39%) exceeded that for the oral route (–19%, –11%).

Since both CI-976 and WAY-121,898 appeared to affect diet-induced hypercholesterolemia, combination treatment using both drugs in the same animals was attempted using 0.4% dietary cholesterol. At a dose of approximately 25 mg/kg, CI-976 and WAY-121,898 reduced plasma cholesterol by 27 and 10%, respectively, but the combination resulted in a 46% reduction (Fig. 3).

TABLE 1
Effect of WAY-121,898 Plasma Lipids (mg/dL) and Liver Cholesteryl Ester Content (mg/g) in Normal, Chow-Fed Rats and Hamsters[a]

Species	Group	Dose	Plasma cholesterol	Plasma triglycerides	Liver cholesteryl esters
Rat	Controls	0	57 ± 4	135 ± 12	973 ± 121
	WAY-121,898	50	56 ± 2	115 ± 10	767 ± 45
	WAY-121,898	100	66 ± 4	91 ± 10*	672 ± 82
	WAY-121,898	200	60 ± 3	94 ± 8*	518 ± 35*
Hamster	Controls	0	146 ± 11	261 ± 22	460 ± 67
	WAY-121,898	10	127 ± 5	249 ± 15	312 ± 50
	WAY-121,898	30	125 ± 6	265 ± 19	280 ± 28*
	WAY-121,898	100	108 ± 6	227 ± 10	370 ± 41

[a]Values represent the mean ± SEM (n = 6/group); *$P < 0.05$ vs. controls.

TABLE 2
Effect of Increasing Dietary Cholesterol Load (%) on Plasma Cholesterol Levels (mg/dL) in Rats Treated with WAY 121,898 and CI-976[a]

	WAY-121,898			CI-976		
Dietary cholesterol	Control	Treated	%	Control	Treated	%
0.0	59 ± 3	55 ± 2	–7	62 ± 4	60 ± 1	–4
0.1	87 ± 5	78 ± 2	–11	93 ± 2	68 ± 4	–27
0.2	104 ± 8	75 ± 3*	–28	100 ± 7	73 ± 3	–28
0.3	122 ± 10	90 ± 5	–26	139 ± 14	73 ± 2*	–47
0.4	177 ± 13	90 ± 6*	–49	148 ± 8	82 ± 3*	–44
0.5	184 ± 20	101 ± 10*	–45	171 ± 21	83 ± 5**	–52
1.0	284 ± 25	135 ± 7**	–53	265 ± 58	83 ± 5**	–69
1.5	293 ± 38	139 ± 12**	–53	273 ± 35	79 ± 4**	–71

[a]Values represent the mean ± SEM (n = 6/group); *$P < 0.05$; **$P < 0.01$.

In order to study the mechanism of action of WAY-121,898, rats were fitted with duodenal and mesenteric lymph cannulas to measure lymphatic cholesteryl ester output during active lipid absorption. After a single oral dose of WAY-121,898 (30 mg/kg), the rise in cholesteryl ester output was blunted significantly, whereas the output of triglycerides was unaffected (Fig. 4). Lymphatic unesterified cholesterol, which was a small percentage of the total, was unaffected by drug treatment (data not shown).

It was next determined whether WAY-121,898, like CI-976 (26), is efficacious in hamsters, an animal species with more LDL-cholesterol (LDL-C) compared to the rat. After chronic drug treatment (10 d) with WAY-121,898, the animals were injected IV with [^{3}H] cholesterol and then orally gavaged with [^{14}C]cholesterol. Percentage cholesterol absorption was 45% in controls, and unaffected by WAY-121,898 at doses of 30 and 100 mg/kg (Table 3). In a second experiment, even a dose of 200 mg/kg failed to affect cholesterol absorption (data not shown). Despite this apparent lack of effect on cholesterol absorption, however, plasma cholesterol and liver cholesteryl ester concentrations were reduced in cholesterol-fed hamsters. Plasma triglycerides were unchanged.

Previously it was reported that cholesterol derived from cholesteryl ester is preferentially absorbed over free cholesterol in rabbits, and that an inhibitor of pCEH (cellulose sulfate) can inhibit cholesterol absorption in rabbits (28). To confirm these data, WAY-121,898 was dosed orally (gavage, CMC suspension) to rabbits fed chow supplemented with corn oil and either cholesterol or cholesteryl oleate for 5 wk. Plasma cholesterol increased 17-fold upon feeding cholesterol, and 15-fold with cholesteryl oleate (Fig. 5). WAY-121,898 had no effect on plasma cholesterol in cholesterol-fed rabbits, but significantly decreased plasma cholesterol by 42% in rabbits fed the cholesteryl oleate. There were no drug-

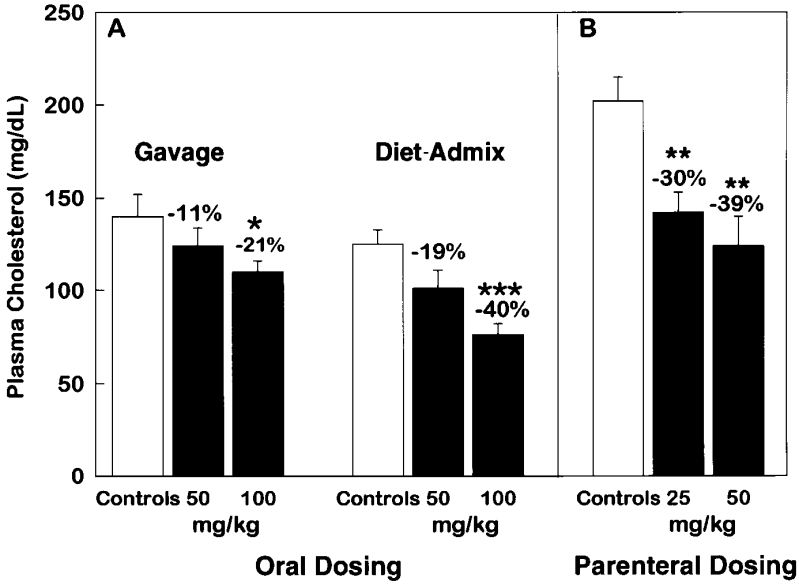

FIG. 2. Reductions in plasma cholesterol by WAY-121,898 in cholesterol-fed rats. The drug was administered either orally (A, gavage and diet-admix) or by daily subcutaneous injection (B, parenteral dosing) in rats fed chow supplemented with peanut oil, cholic acid, and cholesterol (0.4%) for 1 wk. Numbers above the drug-treated groups (closed bars) are percentage changes from control (open bars). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

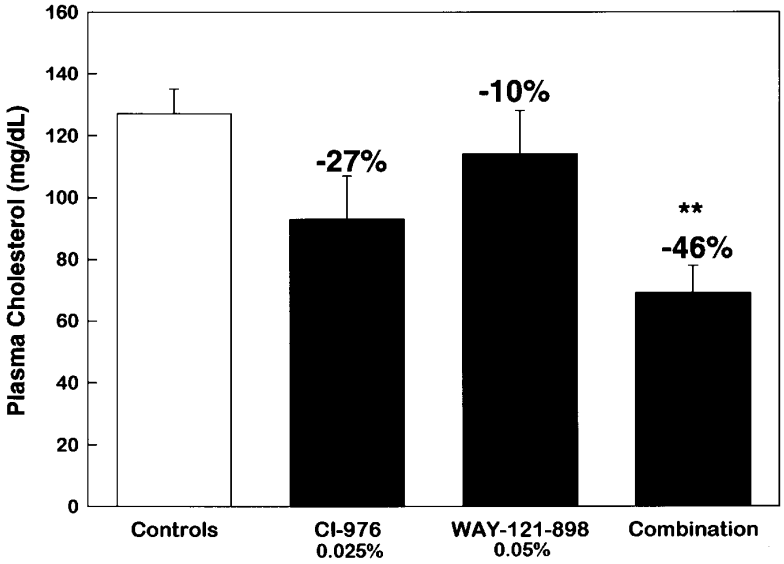

FIG. 3. Effect of CI-976 (acyl-CoA: cholesterol acyltransferase inhibitor) and WAY-121,898 (pancreatic cholesteryl ester hydrolase inhibitor) singly and in combination on plasma cholesterol in cholesterol-fed rats. The compounds were administered by diet-admix (0.025%) to rats receiving 0.04% dietary cholesterol for 1 wk. Numbers above the drug-treated groups (closed bars) are the percentage changes from control (open bars). $^{**}P < 0.01$.

induced changes in liver total, free or cholesteryl ester concentrations in any group (data not shown).

To address the specificity of WAY-121,898 and its possible extraintestinal sites of action, we evaluated the compound in guinea pigs for changes in adrenal cholesteryl ester mass. The adrenal gland contains a well-described microsomal CEH (29), and the guinea pig has been used in many studies evaluating the potential adrenal toxicity of xenobiotics (30–32). Although WAY-121,898 reduced plasma cholesterol by 16% under these conditions, it produced a significant increase in adrenal cholesteryl ester mass (Fig. 6).

DISCUSSION

The present study represents an attempt to use an *in vivo* pharmacologic approach toward understanding the possible role(s) of pCEH in cholesterol absorption. This was made possible by the disclosure of a potent and relatively selective inhibitor of pCEH, WAY-121,898 (13). In our routine screens (porcine pCEH and rat liver ACAT), this compound was approximately 70-fold more potent against pCEH compared to ACAT. We did not examine activities against pCEH from other species in the present study. Our IC_{50} value for WAY-121,898 against porcine pCEH (650 nM) is somewhat higher than that reported by the group at Wyeth-Ayerst for the bovine enzyme (200 nM) (13), yet lower than the value found by Gallo *et al.* (33) for rat pCEH (2 μM, or 24-fold higher than rat ACAT IC_{50}). *In vitro* data are lacking for inhibition of pCEH from hamster or rabbit pancreas (i.e., other species used for *in vivo* efficacy). This should be considered when interpreting the *in vivo* data. However, it has been established that there is a high degree of homology at the primary sequence level for pCEH across species (34). Moreover, WAY-121,898 clearly inhibited the absorption of cholesteryl esters in rabbits, and unlike CI-976 (25), it lowered plasma cholesterol in cholesterol-fed hamsters. These *in vivo* data suggest that the compound inhibits rabbit and hamster pCEH. Lastly, it should be noted that we did not determine the activity of WAY-121,898 against ACAT from species other than rat. It seems unlikely that WAY-121,898 would lack significant inhibitory activity against rat ACAT (27) yet be a potent inhibitor of hamster and rabbit ACAT since it did not lower plasma cholesterol in cholesterol-fed rabbits as would be expected of an ACAT inhibitor. Nonetheless, direct proof that WAY-121,898 lacks activity against rabbit or hamster ACAT would require further *in vitro* evaluation.

Besides taking advantage of the discovery of WAY-121,898, we also utilized a potent and specific ACAT inhibitor, CI-976 (19), for comparative purposes in our study. CI-976 inhibits ACAT (or ACAT "subtypes") in both endocrine and lipoprotein-producing tissues, although it is somewhat more potent in the latter (20,35). It has been shown to inhibit cholesterol absorption in cholesterol-fed rats (20) and hamsters (25,26). Based upon our observations using WAY-121,898 (pCEH inhibitor) and CI-976 (ACAT inhibitor), we have arrived at several conclusions. First, since WAY-121,898 did not lower plasma cholesterol in normal, chow-fed rats, it is tempting to conclude that pCEH is not involved in the reabsorption of biliary (endogenous) cholesterol. However, CI-976 (20) and other ACAT inhibitors (36) are also not hypocholesterolemic in chow-fed rats. Therefore, the current pharmacologic approach suggests at least three possibili-

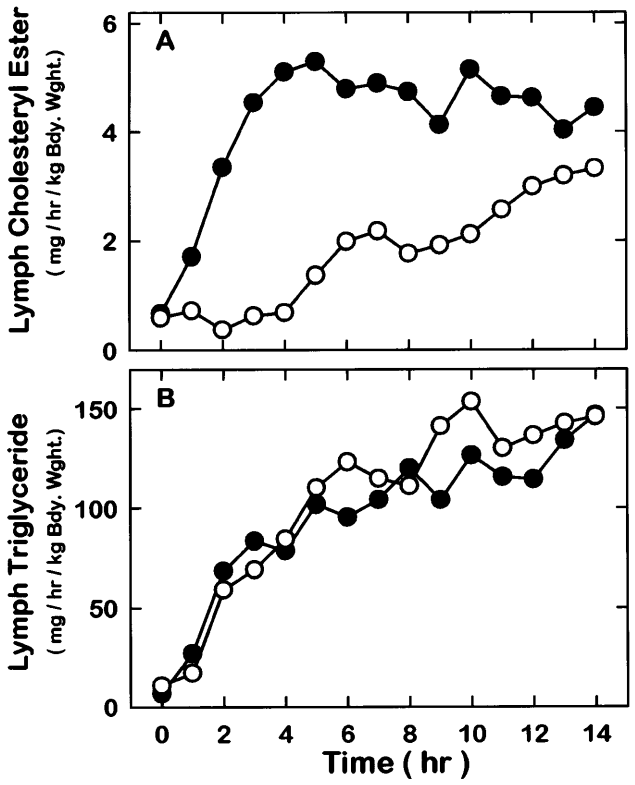

FIG. 4. Effect of a single dose of WAY-121,898 on the lymphatic output (transport) of cholesteryl esters (A) and triglycerides (B) in rats with a mesenteric lymph fistula. After collecting basal lymph for 2 h the compound (○) or vehicle alone (●) was injected directly into the duodenum *via* a cannula, and lymph was collected hourly. Each point represents the mean of two animals.

ties: (i) very little cholesterol esterification is occurring in chow-fed rats in lipoprotein-producing organs, (ii) another enzyme besides ACAT or pCEH (or ACAT subtype not sensitive to CI-976) is catalyzing intestinal esterification of biliary cholesterol, or (iii) the compounds are not potent enough to answer the question as to which enzyme is important. The fact that cholesterol absorption is normal in both pCEH knockout mice (37) and ACAT knockout mice (35) seems to support the first two possibilities. However, the disrupted gene in the ACAT knockout was originally cloned from macrophages, and more recent ACAT inhibitors with improved bioavailability lower plasma cholesterol in chow-fed rats (38). So it is possible that another ACAT-related protein or ACAT subtype exists in the intestine of these animals which catalyzes the formation of cholesteryl esters in chow-fed rats. The modest reduction (30%) in plasma triglycerides in chow-fed rats with WAY-121,898 is an unexpected finding. The lymph–fistula data would argue against an effect on triglyceride absorption, as would the report that WAY-121,898 does not inhibit pancreatic triglyceride lipase *in vitro* (27). Thus, one would have to postulate that inhibition of the bile salt-dependent CEH in the liver might alter hepatic triglyceride metabolism. Clearly, further experiments are also required to support this hypothesis.

Another finding in this study is that inhibition of pCEH by WAY-121,898 inhibits the absorption of exogenous cholesterol in rats. This was demonstrated directly using the rat lymph–fistula model, coupled with experiments in which diet-induced hypercholesterolemia was inhibited by drug treatment. These data therefore confirm the preliminary reports that WAY-121,898 is efficacious in rats fed cholesterol, and that its mechanism of action includes inhibition of cholesterol absorption (13,15,16). They are also consistent with reports of other pCEH inhibitors that inhibit cholesterol absorption in rats (39–44) and are unlikely due to a unique side effect in rats treated with WAY-121,898. However, these observations are at variance with the mouse pCEH knockout data (37), and thus suggest major species differences. Efficacy with WAY-121,898 was maximal at a dietary cholesterol load of 0.4%, but with CI-976 efficacy continued to improve with increases in dietary cholesterol. One interpretation of this observation may be that at high levels of dietary cholesterol in the rat (i.e., >0.5%) esterification may be driven more by ACAT than by pCEH, as first suggested by Heider *et al.* (45) based on rabbit experiments. At a dietary cholesterol load of 0.4%, it could be postulated that both enzymes play a role in cholesterol absorption since an additive effect was observed with WAY-121,898 and CI-976 in combination in the cholesterol-fed rat model. Interestingly, Bhat and Brockman (12) have previously suggested a role for both pCEH and ACAT in cholesterol absorption based upon experiments with rat intestinal sacs (12). Our *in vivo* rat data using both inhibitors in combination seems to support their hypothesis that pCEH catalyzes the "flip-flop" of cholesterol, *via* the ester,

TABLE 3
Efficacy of WAY-121,898 in Cholesterol-Fed Hamsters[a]

	Group		
		WAY-121,898	
Efficacy parameter	Control	30 mg/kg	100 mg/kg
Plasma cholesterol (mg/dL)	413 ± 29	308 ± 33*	291 ± 23*
Plasma triglycerides (mg/dL)	448 ± 42	399 ± 34	380 ± 47
Cholesterol absorption (%)	45.2 ± 2.1	48.4 ± 4.0	39.8 ± 4.2
Liver cholesteryl esters (mg/g)	8.7 ± 0.9	7.9 ± 0.9	5.1 ± 1.0*

[a]Values are the mean ± SEM (n = 6/group); *$P < 0.05$.

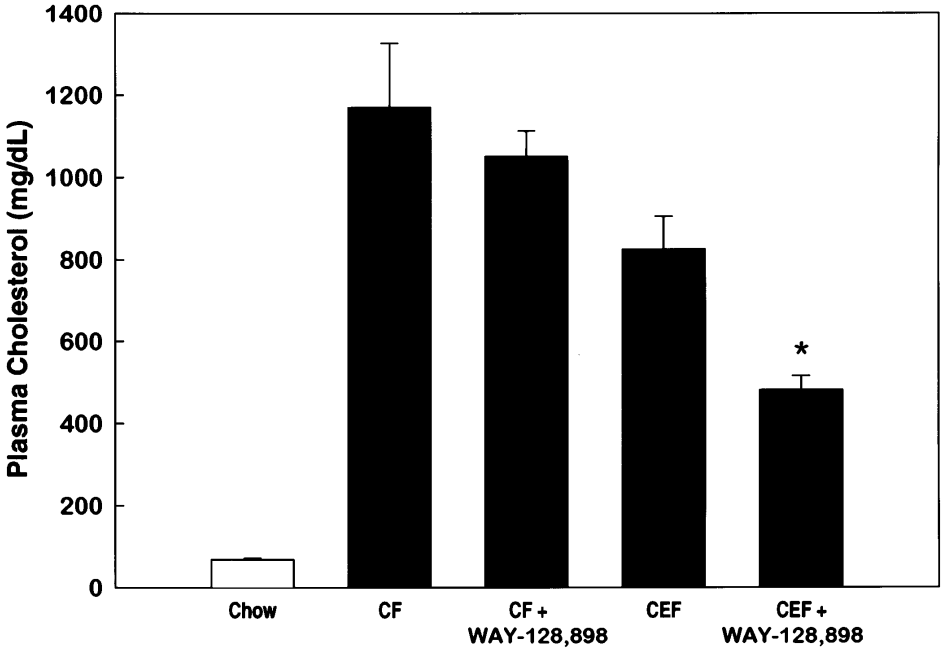

FIG. 5. Effect of WAY-121,898 on plasma cholesterol in rabbits fed either cholesterol (CF) or cholesteryl ester (CEF). *$P < 0.05$ vs. CEF controls.

across the villus membrane into the intestinal cell where ACAT catalyzes reesterification.

The effects of WAY-121,898 were distinctly different in the hamster compared to the rat. Unlike the rat, we observed cholesterol-lowering in animals on a chow diet (100 mg/kg), and the cholesterol-lowering in animals fed cholesterol was not due to inhibition of cholesterol absorption. Although CI-976 does not lower plasma cholesterol in chow-fed hamsters (25), other ACAT inhibitors are active (46), so under these conditions both ACAT and pCEH may play a role in cholesterol absorption. In cholesterol-fed hamsters, ACAT inhibitors, including CI-976, lower plasma cholesterol in part by inhibiting cholesterol absorption (25,26). Therefore, ACAT and not pCEH may mediate cholesterol esterification in the intestine of cholesterol-fed hamsters. The efficacy due to WAY-121,898 could be due to inhibition of liver CEH or CEH in plasma which has been shown to modify lipoprotein composition and structure (47). Such changes could alter lipoprotein clearance. We favor this possibility since liver cholesteryl ester concentrations were actually decreased in hamsters by WAY-121,898 rather than increased as would be expected by inhibiting liver CEH. Such a mechanism also may pertain to the efficacy we observed after parenteral dosing in cholesterol-fed rats.

In contrast to cholesterol-fed rats and hamsters, we observed no change in plasma cholesterol after WAY-121,898 treatment in cholesterol-fed rabbits, suggesting that the putative mechanisms discussed above whereby CEH might alter plasma cholesterol are not operative in this species. This lack of hypocholesterolemic activity in cholesterol-fed rabbits, which is in marked contrast to the potent effects observed with CI-976 and other ACAT inhibitors (45,48), could be due in part to the fact that rabbit liver contains no detectable CEH activity or mRNA, and intestinal CEH in rabbits is low and variable (49). Likewise, Field (22) could find no evidence for an intestinally-derived CEH in rabbit intestine. Thus, there seems to be a distinct difference between rats and rabbits with regard to responses to WAY-121,898, and not surprisingly the bulk of the data in the literature supporting a role for pCEH is largely derived from the rat. Although pCEH seems to play no role in cholesterol absorption in rabbits, WAY-121,898 did appear to inhibit the hydrolysis and subsequent absorption of dietary cholesteryl ester. Inhibition of cholesteryl ester absorption was also inhibited in pCEH knockout mice (37), and it seems reasonable to suggest that inhibition of ester hydrolysis by WAY-121,898 would likely be observed in other species as well. For example, a suicide inhibitor of pCEH inhibits cholesteryl ester absorption, or more properly, the absorption of cholesterol derived from dietary cholesteryl ester, in rats (50). Thus, it now seems indisputable that pCEH plays a primary role in the absorption of cholesteryl esters.

Although WAY-121,898 showed no adverse effects in our animal experiments, we did observe an increase in adrenal cholesteryl ester content after drug treatment in chow-fed guinea pigs. This suggests to us that the drug reached the adrenal gland to inhibit cholesteryl ester hydrolysis. Whether this would ultimately result in adrenal dysfunction is unknown, but mobilization of stored esters could theoretically be compromised with drug treatment. Despite this finding, it has been reported that this compound reduces the cholesterol

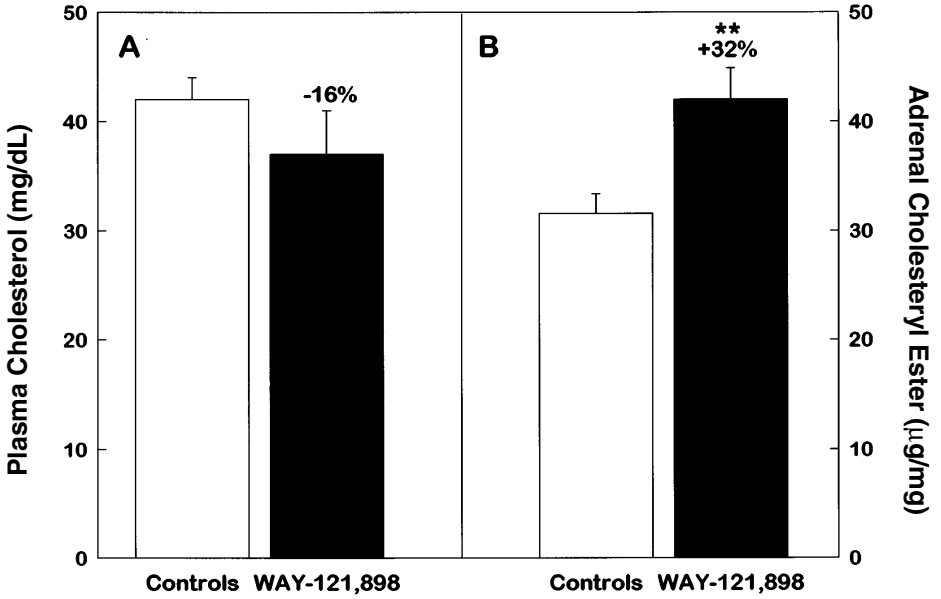

FIG. 6. Effect of WAY-121,898 (100 mg/kg) on plasma cholesterol (A) and adrenal cholesteryl esters (B) in chow-fed guinea pigs. The compound was dosed by daily (A.M.) oral gavage for 2 wk using an oleic acid vehicle. The numbers above the drug-treated groups (solid bars) are the percentage changes from controls (open bars). $^{**}P < 01$.

content, presumably cholesteryl esters, of the aorta in cholesterol-fed quail (16). Such differences between tissues may be due to differences in drug uptake or to differences between the CEH enzymes in these tissues. Regardless, these observations again underscore the variety of responses to WAY-121,898 across species, and point to the need for further clarification of the role(s) of pCEH in cholesterol metabolism.

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[Received December 3, 1997, and in final revised form and accepted April 2, 1998]

Suppression of Hypercholesterolemia in Hepatoma-Bearing Rats by Cabbage Extract and Its Component, *S*-Methyl-L-Cysteine Sulfoxide

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ABSTRACT: The effect of cabbage extract on cholesterol metabolism was studied in Donryu rats subcutaneously implanted with an ascites hepatoma cell line (AH109A). The hepatoma-bearing rats exhibited hypercholesterolemia induced by increasing cholesterogenesis in the host liver and decreasing steroid excretion into feces. The cabbage extract intake or administration reduced serum cholesterol level and enhanced fecal bile acid excretion and cholesterol 7α-hydroxylase activity, the rate-limiting enzyme of bile acid biosynthesis, in the microsomal fraction of the liver. Furthermore, *S*-methyl-L-cysteine sulfoxide, a component of cabbage, could mimic the effect of cabbage extract when orally administered. These results suggest that cabbage suppresses hypercholesterolemia responding to hepatoma growth by upregulating cholesterol catabolism and that *S*-methyl-L-cysteine sulfoxide in cabbage is one of the factors suppressing hypercholesterolemia in the hepatoma-bearing rats.
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Many dietary factors have been shown to influence serum cholesterol (Ch) levels. Rats have been used as an experimental animal in many studies on Ch metabolism. However, it has generally been necessary to use a high dose of Ch plus cholic acid (1,2) in order to induce exogenous hypercholesterolemia. Another approach in the rat has been to induce the development of solid hepatomas (3). In humans and animals, hepatoma induces abnormal serum lipid metabolism, particularly an increase in serum total Ch (T-Ch) levels (3–5). We have reported that rats subcutaneously implanted with an ascites hepatoma cell line of AH109A show a slight decrease in high density lipoprotein (HDL)-Ch and an enormous increase in the very low density lipoprotein plus low density lipoprotein (VLDL + LDL)-Ch, which result in endogenous hypercholesterolemia (6) and hypertriglyceridemia (7,8) during growth of the hepatoma. The hepatoma-induced hypercholesterolemia is shown to be induced by an increased cholesterogenesis in the host liver and a decreased steroid excretion into feces (7).

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Abbreviations: C, casein; Ch, cholesterol; Ch 7α-H, cholesterol 7α-hydroxylase; FA, fatty acids; HDL, high density lipoprotein; LDL, low density lipoprotein; NEFA, nonesterified fatty acid; SMCS, *S*-methyl-L-cysteine sulfoxide; T-Ch, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein.

In general, vegetable diets were reported to have a hypolipidemic activity (9,10), and cabbage was reported to have a positive influence on the cholesterol metabolism by decreasing an atherogenicity index [(T-Ch) − (HDL-Ch)/HDL-Ch] (11). In the present study, we examined the effect of cabbage extract treatment on endogenous hypercholesterolemia and Ch metabolism in hepatoma-bearing rats.

EXPERIMENTAL PROCEDURES

Animals and diets. Male Donryu rats (4 w of age) were obtained from NRC Haruna (Gunma, Japan). They were kept on a stock pellet diet (CE-2; CLEA Japan, Tokyo, Japan) for 4 d and on a 20% casein diet (20C) containing 5% corn oil (12) for another 7 (experiment 1) or 10 (experiment 2) d in an air-conditioned room with an 8:00 A.M. to 8:00 P.M. light cycle. Cabbage was obtained from a local market in Fuchu, Tokyo, and cabbage extract was prepared as follows. Washed cabbage was put into a juicer, centrifuged, and then filtered through a Toyo No. 2 filter paper (Advantec Toyo Co., Tokyo, Japan) to obtain cabbage extract. The extract was given to rats either by addition of its freeze-dried sample to the 20C diet at 1.3% in experiment 1, or oral administration in experiment 2. In experiment 1, rats were divided into three groups of similar body weight; two of the groups received a subcutaneous implantation of 5×10^5 AH109A cells (ascites hepatoma cell line, obtained from Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) suspended in phosphate-buffered saline to produce solid tumor in the back, as described previously (6). Rats of each of these groups were given either 20C or 20C supplemented with 1.3% freeze-dried cabbage extract. Animals of the third group received a subcutaneous sham injection of phosphate-buffered saline alone instead of AH109A as the normal control and were given the 20C. In experiment 2, rats were divided into three groups of similar body weight, and all received an implantation of AH109A. All the animals were given the 20C for another 14 d. One group was orally administered the cabbage extract (1 mL/100 g body weight/d), and another one with an aqueous solution of *S*-methyl-L-cysteine sulfoxide (SMCS, obtained from Research Organics Inc., Cleveland, OH; 25 mg/mL/100 g body weight/d). The control group was given distilled water (1 mL/100 g body weight/d) alone. All the administrations

TABLE 1
Initial Body Weight, Body Weight Gain, Food Intake, Liver and Hepatoma Weights, Hepatic Lipid Levels, and Hepatic Lipid Syntheses in Normal and Hepatoma-Bearing Rats[a]

Measurement	Normal	Hepatoma	Hepatoma + cabbage
Initial body weight (g)	121 ± 2	122 ± 3	122 ± 2
Body weight gain (g/14 d)	104 ± 4[b]	80 ± 3[c]	71 ± 6[c]
Food intake (g/14 d)	250 ± 6[b]	214 ± 5[c]	198 ± 12[c]
Liver weight (g)	10.6 ± 0.4[b]	9.5 ± 0.6[c]	7.8 ± 0.2[d]
Hepatoma weight (g)	—	23.0 ± 3.0	29.3 ± 4.0
Hepatic lipid levels (μmol/g liver)			
Triglyceride	21.3 ± 2.6[b]	10.1 ± 1.1[c]	10.1 ± 1.3[c]
Cholesterol	3.7 ± 0.0	3.4 ± 0.2	3.7 ± 0.1
Hepatic lipid syntheses (dpm × 10^4/2 h/g liver)			
Fatty acid	7.9 ± 2.1	8.0 ± 4.1	4.1 ± 1.4
Cholesterol	2.4 ± 0.5[b]	15.2 ± 3.1[c]	11.7 ± 1.7[c]

[a]Each value represents the mean ± SEM for six rats.
[b,c,d]Significantly different at $P < 0.05$ by Duncan's multiple-range test.

were conducted once a day at 9:00 A.M. for 14 d. The content of SMCS was reported to be 590 mg/100 g in cabbage (13). Thus, the dose of 25 mg/100 g body weight/d is calculated to be approximately equivalent to that of 1.3% freeze-dried cabbage extract diet intake per day or 1 mL/100 g body weight/d of the cabbage extract. The animals were deprived of their diets at 9:00 A.M. on the scheduled days but allowed free access to water until decapitation 4 h later. Blood was collected and left to clot at room temperature for 2–4 h to obtain serum. The liver and hepatoma were quickly removed, washed with cold 0.9% NaCl, blotted on filter paper, weighed, and provided for various analyses.

Lipid analyses. Total lipids were extracted according to the procedure of Folch *et al.* (14) from the liver. After aliquots of the chloroform phase had been dried, Ch (15) and TG (16) concentrations were determined as described (17). Serum lipoproteins were separated into VLDL + LDL and HDL fractions by the precipitation method (6). The total Ch of unfractionated serum (T-Ch) and HDL (HDL-Ch) was enzymatically determined with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan), and the difference between T-Ch and HDL-Ch was regarded as (VLDL + LDL)-Ch.

Hepatic lipid syntheses. Total fatty acids (FA) and Ch syntheses were measured using liver slices (100–120 mg) and 37 kBq/μmol/assay of [1-^{14}C]acetic acid (2.04 GBq/mmol; Amersham International, Buckinghamshire, United Kingdom) as described previously (7,18).

Fecal steroid excretion. Feces were collected for 2 d before sacrifice (days 12–14). Neutral sterols and bile acids were extracted according to the method of Yamanaka *et al.* (19), then enzymatically determined with commercial kits (Wako Pure Chemical Industries) as described previously (7,20).

Microsomal cholesterol 7α-hydroxylase (Ch 7α-H) activity. Liver samples weighing 2 g were homogenized in 0.1 M phosphate buffer (pH 7.4), and microsomal fractions were obtained by ultracentrifugation in a Hitachi RP83T rotor (Tokyo, Japan) at 40,000 rpm for 1 h at 4°C. Aliquots of the microsomal fractions were incubated for 40 min in 0.1 M phosphate buffer (pH 7.4) containing 111 kBq/0.2 μmol/assay of [7α-^{3}H(N)]Ch (814 GBq/mmol; NEN Research Products, Boston, MA), and the enzyme activity was evaluated by counting [^{3}H]water as described previously (21).

Statistical analysis. Data were analyzed by a one-way analysis of variance (ANOVA). When F values were significant ($P < 0.05$), differences were inspected by Duncan's multiple-range test.

RESULTS

In experiment 1, the effect of cabbage extract on the Ch metabolism was first examined in AH109A-bearing rats treated by supplementation of a freeze-dried cabbage extract to a 20C diet. As shown in Table 1, body weight gain, food intake, and liver weight were significantly reduced by bearing solid hepatoma, but the cabbage extract intake had no significant effects on these parameters and hepatoma weight (the hepatoma group vs. the hepatoma + cabbage group), except for liver weight that was decreased by the extract. The hepatic TG level was reduced in the hepatoma-bearing rats, whereas the

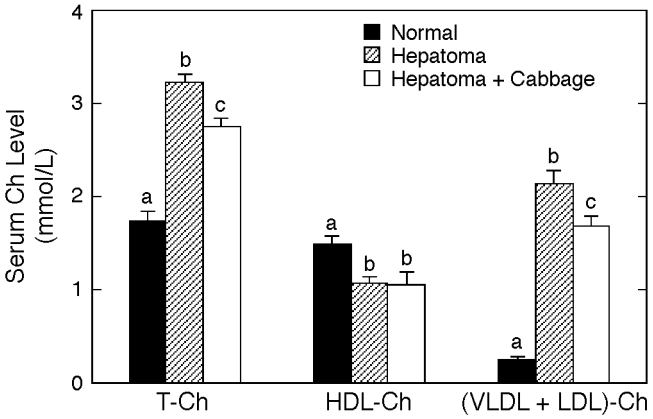

FIG. 1. Effect of cabbage extract on serum cholesterol (Ch) levels in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical bars indicate standard errors. When the F value was significant at $P < 0.05$, the means were inspected by Duncan's multiple-range test. [a,b,c]Values not sharing a common letter are significantly different. T-Ch, total cholesterol; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

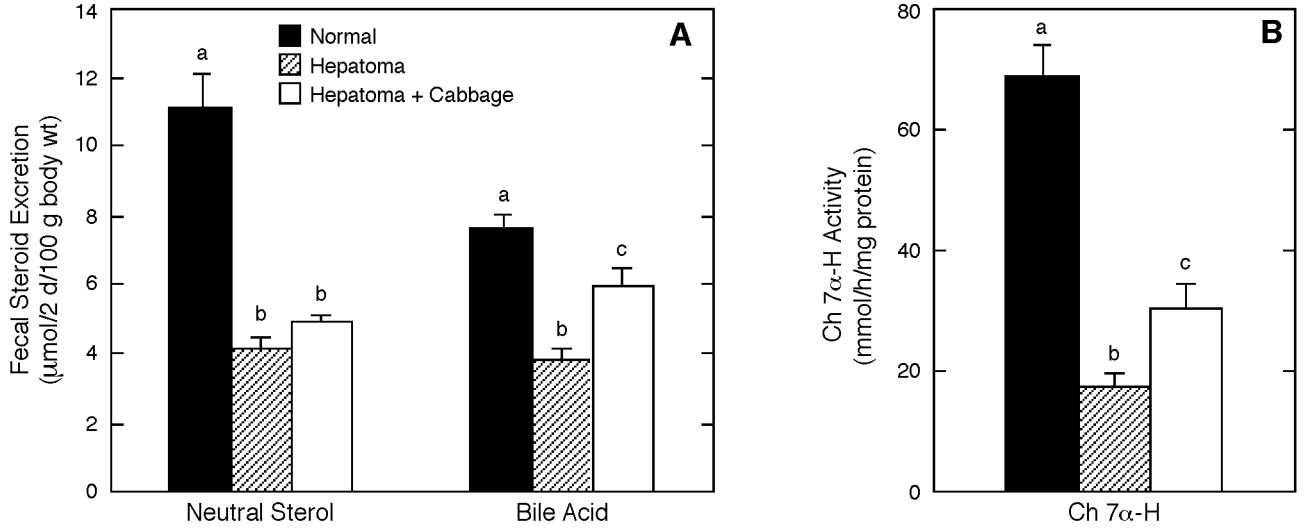

FIG. 2. Effect of cabbage extract on fecal steroid excretion (A) and microsomal cholesterol 7α-hydroxylase (Ch 7α-H) activity (B) in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical bars indicate standard errors. When the *F* value was significant at $P < 0.05$, the means were inspected by Duncan's multiple-range test. [a,b,c]Values not sharing a common letter are significantly different.

hepatic Ch was unchanged by bearing hepatoma. Cabbage extract exerted no influences on these two parameters. Changes in serum Ch levels are illustrated in Figure 1. Both T-Ch and (VLDL + LDL)-Ch levels were significantly increased by bearing hepatoma, and these elevations in the hepatoma-bearing rats were suppressed by the cabbage extract intake. The HDL-Ch level was significantly decreased in the hepatoma-bearing rats, but no change was observed between the hepatoma and the hepatoma + cabbage groups. Table 1 also shows the hepatic FA and Ch syntheses by liver slices. No significant changes were seen in FA synthesis among the three groups, while the Ch synthesis was significantly enhanced in the hepatoma-bearing rats. However, the cabbage extract intake did not affect the Ch synthesis. Fecal steroid excretion and Ch 7α-H activity in the microsomal fraction of the host liver are shown in Figure 2. The excretion of neutral sterols and bile acids into feces, as well as the Ch 7α-H activity, the rate-limiting enzyme of bile acid biosynthesis, were reduced by bearing hepatoma, and reductions in bile acid excretion and the enzyme activity were partially restored by the intake of cabbage extract.

Since SMCS, a component of cabbage, was reported to reduce the serum Ch level in Ch-loaded rats (22,23), we next investigated its effect on Ch metabolism in the hepatoma-bearing rats. In this experiment, the cabbage extract and SMCS solution were administered orally, because a slight (though not significant) reduction in food intake was observed in feeding the cabbage extract-supplemented diet in experiment 1 (as seen in Table 1). As shown in Table 2, body weight gain, food intake, liver and hepatoma weights, and hepatic Ch level were unchanged among these three groups. The hepatic TG level was significantly elevated in rats receiving SMCS compared with the other two groups. The serum Ch levels are illustrated in Figure 3. It was confirmed that the serum T-Ch and (VLDL + LDL)-Ch levels were reduced in cabbage extract-administered rats (the hepatoma + cabbage group) when compared with con-

TABLE 2
Initial Body Weight, Body Weight Gain, Food Intake, Liver and Hepatoma Weights, Hepatic Lipid Levels, and Hepatic Lipid Syntheses in Hepatoma-Bearing Rats[a]

Measurement	Hepatoma	Hepatoma + cabbage	Hepatoma + SMCS
Initial body weight (g)	184 ± 4	184 ± 4	185 ± 2
Body weight gain (g/14 d)	82 ± 6	76 ± 5	80 ± 8
Food intake (g/14 d)	246 ± 8	245 ± 7	244 ± 14
Liver weight (g)	10.7 ± 0.5	10.5 ± 0.4	10.8 ± 0.4
Hepatoma weight (g)	12.8 ± 2.5	14.0 ± 2.1	15.1 ± 3.4
Hepatic lipid levels (μmol/g liver)			
Triglyceride	13.5 ± 1.0[b]	13.4 ± 0.7[b]	19.3 ± 2.7[c]
Cholesterol	4.1 ± 0.1	3.9 ± 0.1	4.2 ± 0.1
Hepatic lipid syntheses (dpm × 10^4/2 h/g liver)			
Fatty acid	8.4 ± 1.2	9.8 ± 1.5	8.8 ± 1.2
Cholesterol	5.6 ± 1.4	5.8 ± 1.1	7.4 ± 1.2

[a]Each value represents the mean ± SEM for six rats.
[b,c]Significantly different at $P < 0.05$ by Duncan's multiple-range test. SMCS, *S*-methyl-L-cysteine sulfoxide.

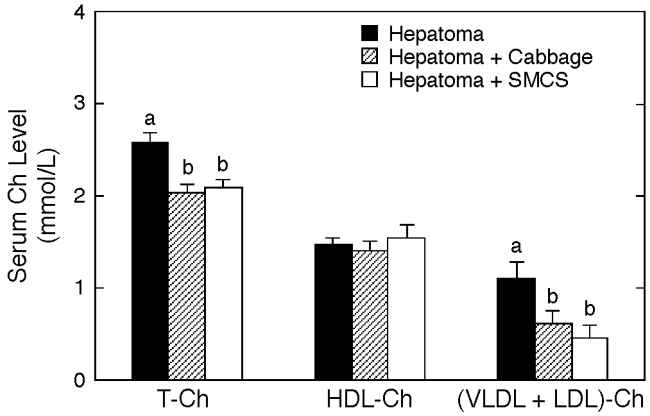

FIG. 3. Effect of cabbage extract on serum Ch levels in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical bars indicate standard errors. When the *F* value was significant at $P < 0.05$, the means were inspected by Duncan's multiple-range test. [a,b]Values not sharing a common letter are significantly different. SMCS, *S*-methyl-L-cysteine sulfoxide. See Figure 1 for other abbreviations.

trol rats (the hepatoma group), and similar reduction was observed in the SMCS-administered rats (the hepatoma + SMCS group). The hepatic FA and Ch syntheses were not affected by either the cabbage extract or SMCS administration (Table 2), while fecal bile acid excretion and Ch 7α-H activity in liver microsome were significantly enhanced in both the cabbage extract- and SMCS-administered rats (Fig. 4).

DISCUSSION

The present study investigated the effect of cabbage extract treatment on serum Ch and Ch metabolism in rats with hepatoma. The serum T-Ch level in the hepatoma-bearing state is significantly higher than that in the normal state (6–8). Results of this work show that the cabbage extract treatment significantly decreased this elevated serum T-Ch level, and that the decrease was due to significant reduction of the (VLDL + LDL)-Ch level in hepatoma-bearing rats. Treating the hepatoma-bearing rats with cabbage extract could stimulate the fecal excretion of bile acids and the microsomal activity of Ch 7α-H. The hypocholesterolemic action of cabbage extract may be due, at least in part, to a stimulation of bile acid excretion, and this stimulation may be the result of an increased Ch 7α-H activity in the liver microsomal fraction. The component(s) in cabbage which has the hypocholesterolemic activity in the hepatoma-bearing state is unknown. SMCS, which was identified by Morris and Thompson (24) as a constituent of a number of species of Cruciferae plants such as cabbage, cauliflower and broccoli, was reported to suppress hypercholesterolemia in Ch-loaded rats (22,23). So, we compared the actions of SMCS with those of cabbage extract in hepatoma-bearing rats. Results demonstrated that the effects of SMCS mimicked those of the cabbage extract in serum Ch, fecal bile acid excretion, and Ch 7α-H activity. These results suggest that SMCS is one component in cabbage which suppresses hypercholesterolemia in the hepatoma-bearing rats. Mechanism by which SMCS stimulates Ch 7α-H activity is unclear at present. SMCS has been recently reported to reduce serum Ch level in alloxan-induced diabetic rats (25). Thus, SMCS is suggested to be a strong hypocholesterolemic agent in endogenous hypercholesterolemic models as well as Ch-loaded, exogenous hypercholesterolemic ones.

In conclusion, cabbage extract has a hypocholesterolemic activity in hepatoma-bearing rats, and SMCS is one of the chemical entities in cabbage. The mechanism by which

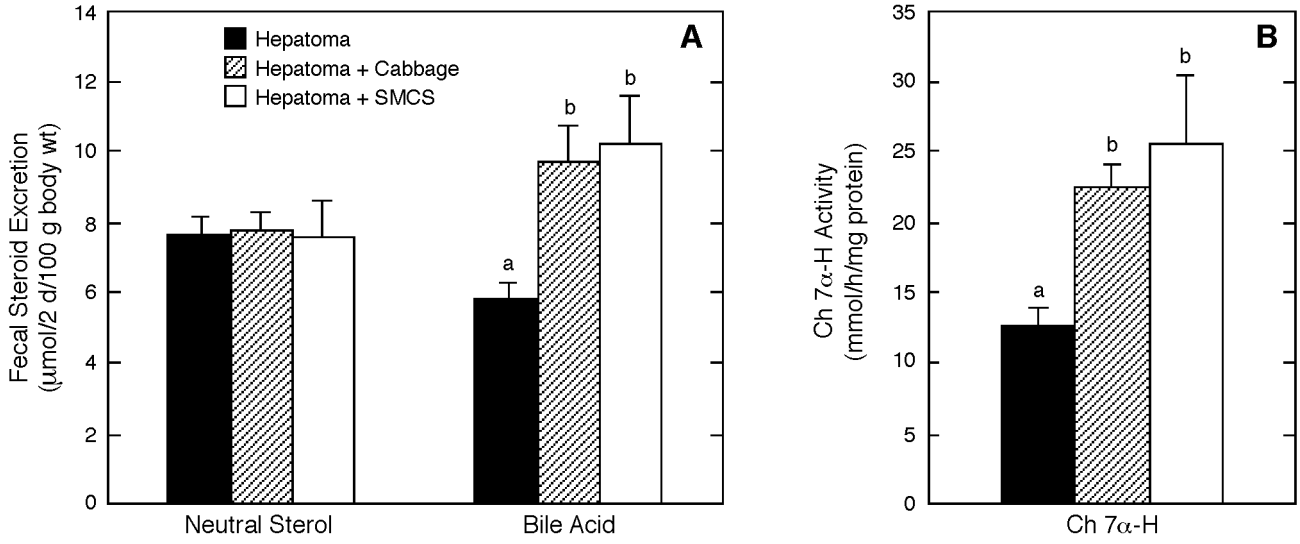

FIG. 4. Effect of cabbage extract on fecal steroid excretion (A) and microsomal Ch 7α-H activity (B) in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical bars indicate standard errors. When the *F* value was significant at $P < 0.05$, the means were inspected by Duncan's multiple-range test. [a,b]Values not sharing a common letter are significantly different. See Figures 2 and 3 for abbreviations.

SMCS stimulates Ch 7α-H activity is unknown, and further studies are needed.

ACKNOWLEDGMENTS

This work was supported by a grant from the Elizabeth Arnold Fuji Foundation and a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (No. 08660149).

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[Received July 29, 1997, and in final revised form and accepted March 23, 1998]

Effect of n-3 Fatty Acid Supplementation on Lipid Peroxidation and Protein Aggregation in Rat Erythrocyte Membranes

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ABSTRACT: Human erythrocytes in the circulation undergo dynamic oxidative damage involving membrane lipid peroxidation and protein aggregation during aging. The present study was undertaken to determine the effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation in the circulation and also the *in vitro* susceptibility of rat erythrocyte membranes to oxidative damage. Wistar male rats were fed a diet containing n-6 fatty acid-rich safflower oil or n-3 fatty acid-rich fish oil with an equal amount of vitamin E for 6 wk. n-3 Fatty acid content in erythrocyte membranes of rats fed fish oil was significantly higher than that of rats fed safflower oil. The degree of membrane lipid peroxidation and protein aggregation of rats fed fish oil was not significantly higher than that of rats fed safflower oil when the amounts of phospholipid hydroperoxides, thiobarbituric acid-reactive substances, and detergent-insoluble protein aggregates were measured. When isolated erythrocytes were oxidized under aerobic conditions in the presence of Fe(III), the degree of membrane lipid peroxidation of erythrocytes from rats fed fish oil was increased to a greater extent than that of rats fed safflower oil, whereas the degree of membrane protein aggregation of both groups was increased in a similar extent. Hence, n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation in membranes of circulating rat erythrocytes, and the supplementation increased the susceptibility of isolated erythrocytes to lipid peroxidation, but not to protein aggregation, under the aerobic conditions. If a sufficient amount of vitamin E is supplied, n-3 fatty acid supplementation may give no undesirable oxidative effects on rat erythrocytes in the circulation.
Lipids 33, 505–512 (1998).

Fish oils which contain high amounts of the n-3 fatty acids, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), have been suggested to decrease the risk of developing cardiovascular diseases and cancer (1). Epidemiological studies have shown an apparent beneficial effect of fish intake in reducing mortality from heart disease (2). On supplementation of n-3 fatty acids, arachidonic acid (20:4n-6) in phospholipids of cell membranes in various tissues of animals is replaced by these n-3 fatty acids (3,4).

Fish oils containing highly unsaturated n-3 fatty acids are readily susceptible to autoxidation under aerobic conditions (5). Glavind *et al*. (6) have reported that lipid peroxides appear in the plaque of human atheroma and that the degree of atheroma correlates with the extent of lipid peroxidation of the plaque. Since those initial observations, lipid peroxidation products have been considered to be related to various disorders in humans including atherosclerosis, diabetes, burn injury, and retinopathy (7). In relation to possible undesirable effects of fish oil intake, several studies have been done. Studies on human urine (8,9) and plasma (10–14) have shown that levels of lipid peroxidation products were slightly increased in persons eating a diet with high fish oil content. Studies on experimental animals have shown a decrease in tissue vitamin E content (15–17) and an increase in *in vitro* susceptibility to lipid peroxidation of tissues on n-3 fatty acid supplementation (15–22).

Our previous studies have shown that human erythrocytes in the circulation undergo dynamic oxidative damages including membrane lipid peroxidation (23) and protein aggregation (24) during aging. Membrane protein aggregation leads to aggregation of cell surface carbohydrate chains of band 3 glycoprotein resulting in recognition by anti-band 3 autoantibody (25). This process may be related to clearance of oxidatively damaged senescent erythrocytes by macrophages (26). It is important to elucidate whether the increased unsaturation of fatty acids in erythrocyte membranes makes them more susceptible to lipid peroxidation and protein aggregation in the circulation, and whether lipid peroxidation precedes induction of protein aggregation. The present study was undertaken to determine the effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation of rat erythrocyte membranes in the circulation, and to clarify whether protein aggregation results from lipid peroxidation.

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Abbreviations: BHT, butylated hydroxytoluene; $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; DPBS, Dulbecco's phosphate-buffered saline; DPBS(–), Ca^{2+}- and Mg^{2+}-free DPBS; HPLC, high-performance liquid chromatography; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, TBA-reactive substance.

It was found that n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation of rat erythrocyte membranes in the circulation. The supplementation increased the susceptibility of isolated erythrocytes to lipid peroxidation but produced little change in the susceptibility to protein aggregation under aerobic conditions.

MATERIALS AND METHODS

Materials. ADP was obtained from Oriental Yeast Company (Tokyo, Japan). Phosphatidylcholine (egg yolk) and phosphatidylethanolamine (egg yolk) were obtained from Nippon Fats and Oils Liposome Company (Tokyo, Japan). Phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) were prepared just before use according to the method previously described (27). Peroxide values of PCOOH and PEOOH were estimated to be 1380–1680 and 770–840 neq/mg, respectively. Cytochrome c and luminol were purchased from Sigma Chemical Company (St. Louis, MO) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Thiobarbituric acid (TBA) and Silica gel 60 for thin-layer chromatography were obtained from Merck (Darmstadt, Germany). Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate were obtained from Sigma. Octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$) was from Nikko Chemical Company (Tokyo, Japan).

Animals and diets. Sixteen 4-wk-old Wistar male rats weighing 50–70 g were supplied by Japan Laboratory Animals Inc. (Tokyo, Japan). Four animals were housed together in a stainless steel cage in a room of controlled temperature at 23 ± 1°C, humidity at 55 ± 5%, and lighting of 12 h dark–light cycle. The animals were allowed free access to food. The animals were fed a normal solid diet, Clea rodent diet CE-2 containing 4.5% soybean oil and 7.0 mg% vitamin E (Clea Japan Corp., Tokyo, Japan) during 1 wk for acclimatization. Rats were divided into two groups of eight animals each, and each group was fed a diet with safflower oil or with fish oil for 6 wk. Weights of rats after feeding were 350–450 g. A powdered diet with AIN-76 composition, in which corn oil was replaced by cornstarch, and whose vitamin E content was 4.56 mg% (Oriental Yeast Company), was stored at 4°C before use. Safflower oil and fish oil with the fatty acid composition shown in Table 1 were stored at –20°C before use. Each oil contained 32.5 mg% vitamin E. Peroxide and acid values of safflower oil were 1.1 neq/mg and 0.2, respectively, and those of fish oil were 1.2 neq/mg and 0.2, respectively. Diets for feeding were freshly prepared every day by uniformly mixing 427.5 g of the powdered AIN-76 diet, 22.5 g of each oil (5% by weight), and 62.5 mL of deionized water. The overall content of vitamin E in both diets was equal at 5.93 mg% dried solid.

Erythrocyte preparation. At the end of the feeding period, eight rats of each diet group (six for lipid and protein aggregate analysis and two for vitamin E analysis) were anesthetized with an air/diethyl ether mixture, and blood (10–12 mL) was collected over citrate-phosphate-dextrose by cardiac puncture. Blood was immediately centrifuged at 320 × *g* and 4°C for 10 min to remove plasma and buffy coats. Erythrocytes were washed four times by centrifugation at 650 × *g* and 4°C for 5 min with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) to make a cell pellet.

In vitro *oxidation of isolated rat erythrocytes*. Oxidation of erythrocytes was carried out according to the method previously described (28). Five milliliters of an erythrocyte suspension in DPBS (40% hematocrit) was mixed with an equal volume of a solution of an iron catalyst ADP/Fe(III) (a mixture of 3.4 mM ADP and 0.2 mM $FeCl_3$ in isotonic saline) and incubated under aerobic conditions at 37°C for 90 min. The cells were pelleted by centrifugation, washed twice with DPBS(–) and twice with isotonic saline to make a cell pellet.

Determination of hemoglobin. To 10 μL of the erythrocyte pellet was added 3.0 mL of water, and the lysate was centrifuged at 9,600 × *g* for 20 min. Absorbance of the supernatant at 523 nm was measured, and the hemoglobin concentration was calculated using a molecular extinction coefficient of 7,880 and a molecular weight of 64,000 (28).

Determination of vitamin E. Vitamin E contents of erythrocytes from two rats of each diet group were determined by high-performance liquid chromatography (HPLC) according to the method previously described (29). The amount of vitamin E in the sample was estimated by comparing the peak area with those of the calibration curve of the standard *dl*-α-tocopherol. Vitamin E content in erythrocytes was expressed per g hemoglobin.

Fatty acid composition of phospholipids of erythrocyte membranes. Erythrocytes were lysed and extracted with chloroform/methanol (2:1, vol/vol). The phospholipid fraction on silica gel thin-layer chromatography was methylated with 3% hydrochloric acid/methanol and analyzed by gas chromatography using an Hitachi G-5000 gas chromatograph (Tokyo, Japan) equipped with a fused-silica capillary column DB-225 (0.25 mm i.d. × 30 m) (J&W Scientific Company, Inc., Folsom, CA) and a flame-ionization detector with helium as a carrier gas.

Lipid peroxidation products in erythrocyte membranes. To 1.0 mL of the erythrocyte pellet, 1.0 mL of water was added and the mixture was left for 15 min. To the lysate, 11 mL of 2-propanol was added and the mixture was shaken occasionally for 1 h, after which 7 mL of chloroform was added to the mixture and shaken occasionally for another hour. The organic layer (18 mL) was collected by centrifugation at 1,500 × *g* for 15 min, filtered through a glass wool column, and 17 mL was isolated (30). The layer was divided into two 8-mL fractions. Both fractions were evaporated at below 30°C to dryness for determination of PEOOH, PCOOH, and TBA-reactive substances (TBARS). The amounts of lipid peroxidation products were expressed per g hemoglobin.

PEOOH and PCOOH. Contents of PCOOH and PEOOH in the lipid fraction of erythrocyte membranes were determined by a HPLC-chemiluminescence method (27,31). The lipid fraction was dissolved in 5.0 mL of chloroform/

methanol (2:1, vol/vol) and washed twice with 1 mL of 0.05 M KCl to obtain 3.5 mL of the organic layer. A 3.0-mL portion of the organic layer was evaporated to dryness at below 30°C and redissolved in 50 μL of chloroform/methanol (1:9, vol/vol). A 40-μL portion of this extract was analyzed. The amounts (neq) of PCOOH and PEOOH were determined by comparing their peak areas with a calibration curve of the peak areas of the standard solutions of PCOOH and PEOOH (0–100 neq).

TBARS. TBARS in the lipid fraction of erythrocyte membranes were determined according to the method previously described with added butylated hydroxytoluene (BHT) and in the absence and presence of EDTA (23,32). The lipid fraction of membranes was suspended in 0.9 mL of water for analysis.

Protein aggregates in erythrocyte membranes. $C_{12}E_8$-insoluble protein aggregates in erythrocyte membranes were isolated according to the method previously described (24,33). The amounts of protein in erythrocyte membranes and isolated aggregates were determined by the Lowry method (34). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of SDS-solubilized aggregates was done by the method of Laemmli (35) using 10% polyacrylamide gel under reducing conditions. The gel was stained by Coomassie brilliant blue R-250.

Statistical analysis. Data were analyzed by the Student's *t*-test.

TABLE 1
Fatty Acid Composition of Safflower Oil and Fish Oil[a]

Fatty acid	Safflower oil	Fish oil
14:0		5.8
16:0	6.9	16.4
16:1n-7	0.1	5.9
17:0		1.2
18:0	2.4	3.9
18:1n-9	12.3	15.8
18:1n-7		3.4
18:2n-6	78.0	1.3
18:3n-3	0.3	1.4
20:1n-9		1.2
18:4n-3		3.1
20:5n-3		9.6
22:1n-9		2.7
22:5n-3		2.2
22:6n-3		10.2
Unknown		15.9
Total saturated fatty acid	9.3	27.3
Total MUFA	12.4	29.0
Total n-6 PUFA	78.0	1.3
Total n-3 PUFA	0.3	26.5
Total n-6 + n-3 PUFA	78.3	27.8
UI	168	168
PI	78	114

[a]Expressed as percentages (w/w) of total fatty acids present. Abbreviations: UI, unsaturation index (sum of percentages of individual fatty acids × number of double bonds); PI, peroxidizability index (sum of percentages of individual fatty acids × number of active methylenes); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

RESULTS

Two groups of Wistar male rats were fed a diet with 5% safflower oil or 5% fish oil for 6 wk. Fatty acid compositions of safflower and fish oils are shown in Table 1. As expected, safflower oil was rich in n-6 fatty acids (n-6 total: 78.0%, and n-3 total: 0.3%) and fish oil was rich in n-3 fatty acids (n-6 total: 1.3%, and n-3 total, 26.5%). While the unsaturation index (the sum of the percentages of individual fatty acids × number of double bonds) of the fish oil was equal to that of the safflower oil, the peroxidizability index, (the sum of the percentages of individual fatty acids × number of active methylenes) of the fish oil was higher than that of safflower oil, indicating that fish oil may be more readily peroxidized. Both diets contained an equal amount (5.93 mg% dried diet) of vitamin E; the safflower oil diet had 1.5 mg vitamin E/g polyunsaturated fatty acids and the fish oil diet had 4.3 mg vitamin E/g polyunsaturated fatty acids. Erythrocytes were isolated from each of six rats of both rat groups. Fatty acid compositions of phospholipids of erythrocyte membranes of both rat groups were determined (Table 2). It was found that phospholipids of erythrocytes of rats fed safflower oil contained higher amounts (39.0%) of n-6 fatty acids than those of rats fed fish oil (13.0%), and those of rats fed fish oil contained higher amounts (22.3%) of n-3 fatty acids than those of rats fed safflower oil (1.9%). Both the unsaturation index and the peroxidizability index of the erythrocyte membrane phospholipids from rats fed a diet containing fish oil were higher than those from rats fed a diet containing safflower oil. Vitamin E content of erythrocytes was estimated to be 12% lower in two rats fed fish oil (10.3 and 10.5 μg/g hemoglobin) than in two rats fed safflower oil (11.6 and 12.0 μg/g hemoglobin).

Contents of phospholipid hydroperoxides, PCOOH and PEOOH, in erythrocyte membranes of both rat groups were determined by an HPLC-chemiluminescence method (27,31). Representative HPLC chromatograms are shown in Figure 1A. When the mean content of these phospholipid hydroperoxides of six rats of both the groups were compared (Fig. 2A), no significant differences between two groups were observed. Concentrations of PCOOH and PEOOH of both rat groups were lower than 0.7 nmol/g hemoglobin. The TBARS in erythrocyte membranes of both rat groups were estimated. TBA assay of Ohkawa's procedure at pH 3.5 was performed in the presence of BHT in order to prevent lipid peroxidation while heating under the acidic assay conditions (23,32). It has been claimed that the TBA assay in the presence of EDTA reflects exclusively malonaldehyde derivatives that liberate malonaldehyde under the assay conditions, and that the assay in the absence of EDTA reflects not only malonaldehyde derivatives but also the derivatives of alka-2,4-dienals and alk-2-enals (32,36). Representative absorption spectra of the TBA assay mixtures of erythrocyte membranes shown in Figure 3A indicate that absorption spectra from both rat groups were similar to that of standard TBA red pigment without disturbing colored pigments. When the mean values of contents of TBARS of six rats of two rat groups were compared (Fig.

TABLE 2
Fatty Acid Composition of Phospholipids of Erythrocyte Membrane of Rats Fed a Diet Containing Safflower Oil or Fish Oil[a]

Fatty acid	Safflower oil[a] Mean[b] ± SD	Fish oil[a] Mean[b] ± SD	P^c
14:0	0.2 ± 0.1	0.3 ± 0.2	0.263
15:0	0.3 ± 0.2	0.3 ± 0.0	0.758
16:0	26.2 ± 1.3	30.2 ± 1.3	0.000
16:1n-7	0.5 ± 0.1	0.9 ± 0.6	0.167
17:0	0.3 ± 0.0	0.6 ± 0.0	0.000
17:1 n-7	0.1 ± 0.0	0.2 ± 0.0	0.000
18:0	17.7 ± 1.2	13.4 ± 0.3	0.000
18:1n-9	4.8 ± 0.2	7.7 ± 0.6	0.000
18:1n-7	3.8 ± 0.2	4.2 ± 0.1	0.002
18:2n-6	7.4 ± 0.4	1.6 ± 0.1	0.000
18:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.570
20:0	0.2 ± 0.0	0.3 ± 0.1	0.279
20:1n-12	0.0 ± 0.0	0.1 ± 0.0	0.000
20:1n-9	0.1 ± 0.0	0.3 ± 0.0	0.000
20:2n-6	0.3 ± 0.1	0.1 ± 0.0	0.011
20:3n-6	0.5 ± 0.1	0.3 ± 0.2	0.186
20:4n-6	28.2 ± 0.6	10.7 ± 0.5	0.000
20:5n-3	0.0 ± 0.0	11.4 ± 0.8	0.000
22:0	1.0 ± 1.2	0.8 ± 1.6	0.529
22:1n-9	0.4 ± 0.6	0.3 ± 0.3	0.584
22:2n-6	0.1 ± 0.1	0.1 ± 0.1	0.089
22:4n-6	2.6 ± 0.2	0.1 ± 0.1	0.000
23:0	0.0 ± 0.0	0.1 ± 0.1	0.046
22:5n-3	0.4 ± 0.1	3.8 ± 0.3	0.000
22:6n-3	1.4 ± 0.1	7.1 ± 0.4	0.000
24:0	1.9 ± 0.2	2.4 ± 0.4	0.015
24:1n-9	1.5 ± 0.2	2.6 ± 0.3	0.000
Total saturated fatty acid	47.8	48.4	
Total MUFA	11.2	16.3	
Total n-6 PUFA	39.0 ± 0.8	13.0 ± 0.6	0.000
Total n-3 PUFA	1.9 ± 0.1	22.3 ± 1.3	0.000
Total n-6 + n-3 PUFA	40.8	35.3	
UI	162	183	
PI	110	131	

[a]Expressed as percentages (w/w) of total fatty acids present.
[b]Values are means for six rats.
[c]Significance of difference by Student's *t*-test between data for safflower oil- and fish oil-fed rats. For abbreviations see Table 1.

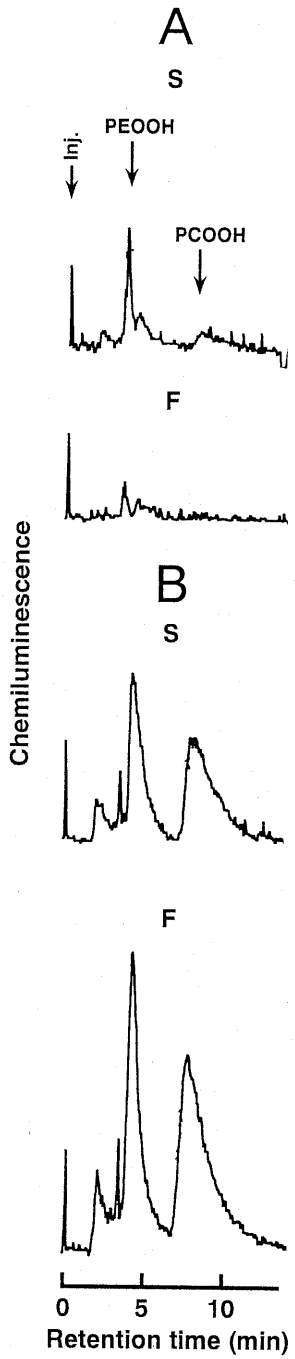

FIG. 1. Representative chromatograms of high-performance liquid chromatography-chemiluminescence assay of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing safflower oil (S) or fish oil (F); (B) membranes of erythrocytes of a rat fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min.

4A), no significant differences between the groups were observed. Contents of the substances in the presence of EDTA of both rat groups were estimated to be about 2 nmol/g hemoglobin, and those of the substances in the absence of EDTA of both rat groups were estimated to be much higher at 35 nmol/g hemoglobin. Hence, erythrocyte membranes of rats fed fish oil did not show enhanced lipid peroxidation in the circulation as compared to rats fed safflower oil.

Membrane protein aggregates insoluble in detergent $C_{12}E_8$, whose presence indicates oxidative damage to erythrocyte membranes (24,33), were obtained from erythrocytes of both rat groups. The aggregates were solubilized with SDS, and their protein contents were measured (Fig. 5A). Contents of the aggregates of erythrocytes from rats fed fish oil were similar to those from rats fed safflower oil. SDS-PAGE of the aggregates from both rat groups revealed a similar pattern (Fig. 6A). Erythrocyte membranes of rats fed fish oil did not exhibit increased oxidative protein aggregation in the circulation when compared to the membranes of rats fed safflower oil.

Erythrocytes from the two rat groups were oxidized *in vitro* at 37°C for 90 min in the presence of ADP/Fe(III) (28) under aerobic conditions. Figure 1B shows representative HPLC chromatograms of PCOOH and PEOOH, and Fig-

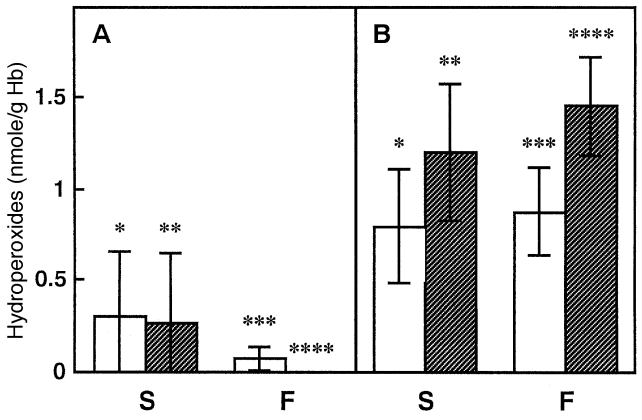

FIG. 2. Contents of PCOOH (striped bars) and PEOOH (open bars) in rat erythrocyte membranes. (A) Membranes of erythrocytes of rats fed a diet containing S or F. (B) Membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.01$; $**P < 0.02$; $***P < 0.001$; $****P < 0.001$. Hb, hemoglobin; for other abbreviations see Figure 1.

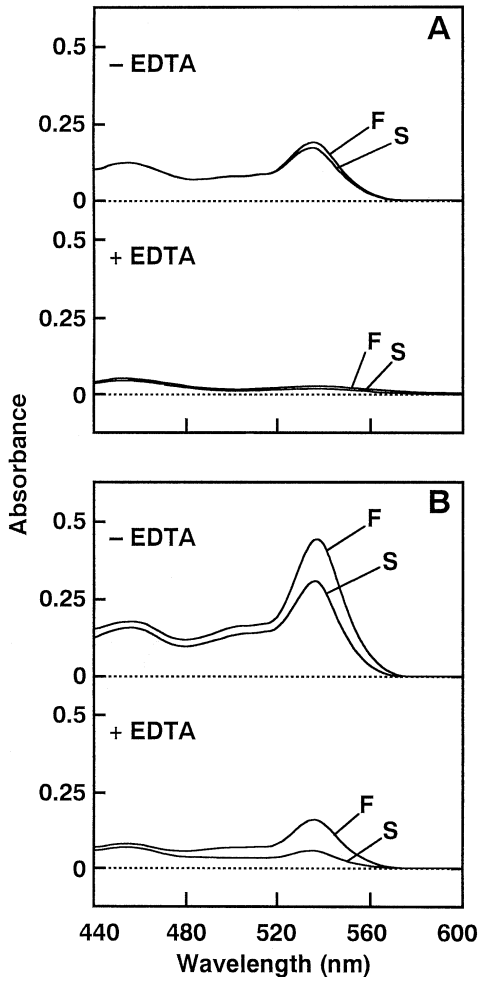

FIG. 3. Representative absorption spectra of thiobarbituric acid (TBA) reaction mixtures with and without EDTA of rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing S or F; (B) membranes of erythrocytes of a rat fed a diet containing S or F which was oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. For abbreviations see Figure 1.

ure 2B shows the concentrations of PCOOH and PEOOH from six rats of both rat groups. The hydroperoxide levels of both rat groups were extensively increased by the *in-vitro* aerobic oxidation, and the degree of the increase of the group fed fish oil was higher than that fed safflower oil. Figure 3B shows representative absorption spectra of the TBA reaction mixtures, and Figure 4B shows the mean levels of TBARS of six rats of both groups. The levels of TBARS in the presence and absence of EDTA of both groups were increased by oxidation, and the levels of the rat group fed fish oil were increased more. The TBARS of the rat group fed fish oil were increased over that of the safflower-fed group twofold in the assay without EDTA and fivefold in the assay with EDTA. The results indicate that fish oil supplementation caused enhanced *in vitro* susceptibility to lipid peroxidation of erythrocyte membranes. $C_{12}E_8$-insoluble membrane protein aggregates were obtained from erythrocytes of both rat groups oxidized *in vitro*. The amount of aggregates from both rat groups was increased by the oxidation, and the increased levels of the rat group fed fish oil was similar to those of the rat group fed safflower oil (Fig. 5B). SDS-PAGE of the aggregates from both rat groups revealed a similar pattern (Fig. 6B). Erythrocyte membrane proteins of both rat groups were similarly aggregated by the *in vitro* oxidation, and the degree of aggregation did not correspond to the degree of lipid peroxidation of membrane.

DISCUSSION

Wistar rats were fed a diet containing safflower oil or fish oil with an equal amount of vitamin E, which was carefully prepared to prevent lipid peroxidation before feeding. n-3 Fatty acid content of isolated erythrocyte membranes of rats fed fish oil was significantly higher than that of rats fed safflower

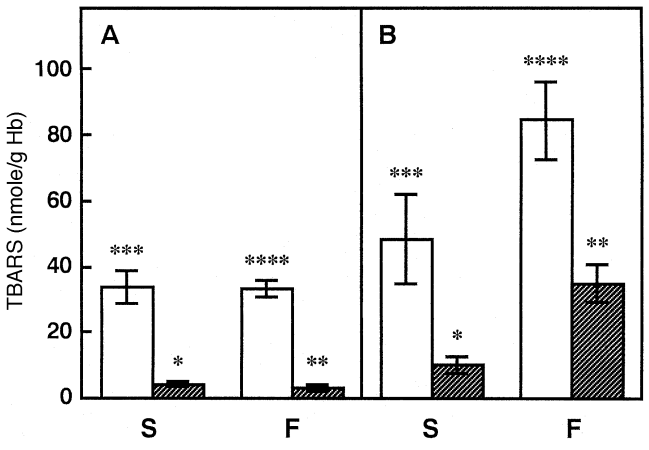

FIG. 4. Contents of TBA-reactive substances (TBARS) in the TBA assay with (striped bars) and without EDTA (open bars) of rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing S or F; (B) membranes of erythrocytes of a rat fed a diet containing S or F which was oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.001$; $**P < 0.001$; $***P < 0.02$; $****P < 0.001$. For abbreviations see Figures 1 and 3.

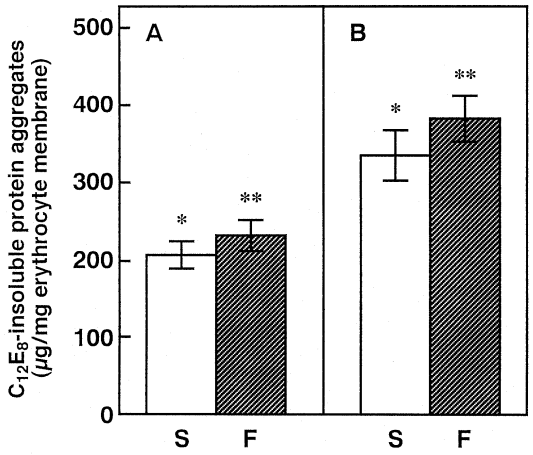

FIG. 5. Amount of detergent-insoluble protein aggregates in rat erythrocyte membranes. (A) Membranes of erythrocytes of rats fed a diet containing S or F; (B) membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.001$; $**P < 0.001$. $C_{12}E_8$, octaethyleneglycol *n*-dodecylmonoether; for other abbreviations see Figure 1.

oil. When lipid peroxidation products, phospholipid hydroperoxides, and TBARS of isolated erythrocyte membranes were determined, the degree of membrane lipid peroxidation of isolated erythrocytes with high n-3 fatty acid content was similar to that of erythrocytes with low n-3 fatty acid content. In the studies on TBARS, great attention was paid to prevent lipid peroxidation under the aerobic conditions of the assay. The assay was always conducted in the presence of antioxidant BHT (32,36), and a modified Ohkawa's method at pH 3.5 with and without EDTA (23,32) was employed. In this study, it is conceivable that water-soluble TBARS in membranes may be released into plasma while in the circulation or into the medium during the isolation of membranes, and such losses could not be determined. However, it seems unlikely that a large amount of water-soluble TBARS was released from membranes because a substantial amount of TBARS in the erythrocyte membranes oxidized *in vitro* stayed in the membranes (Figs. 3B and 4B). Vitamin E in membranes of erythrocytes with high n-3 fatty acid content was slightly decreased when compared with that of erythrocytes with low n-3 fatty acid content, indicating that vitamin E was consumed to prevent lipid peroxidation of n-3 fatty acids during the circulation, as has been suggested in earlier studies (15–22). It was found in this study that the amount of vitamin E supplemented was high enough to prevent lipid peroxidation of erythrocyte membranes in the circulation. The degree of membrane protein aggregation, as assessed by detergent $C_{12}E_8$-insoluble aggregates (24,33) and SDS-PAGE of the aggregates, was similar between erythrocytes with high and low n-3 fatty acid contents. Hence, fish oil supplementation that caused high n-3 fatty acid content in erythrocyte membranes did not cause increased lipid peroxidation and protein aggregation while in the circulation. The results indicate that n-3 fatty acid supplementation did not increase the oxidative damage of circulating rat erythrocyte membranes, which may be reasonably explained by the fact that the oxygen pressure in the rat vasculature is lower than atmospheric. A sufficient supply of vitamin E prevents oxidative damage to erythrocytes with high n-3 fatty acid content in the circulation.

The degree of membrane lipid peroxidation of erythrocytes with high n-3 fatty acid content was increased under aerobic conditions with Fe(III) ion to a higher extent than that of erythrocytes with low n-3 fatty acid content, indicating that the n-3 fatty acid supplementation increased the susceptibility of erythrocyte membranes to lipid peroxidation under aer-

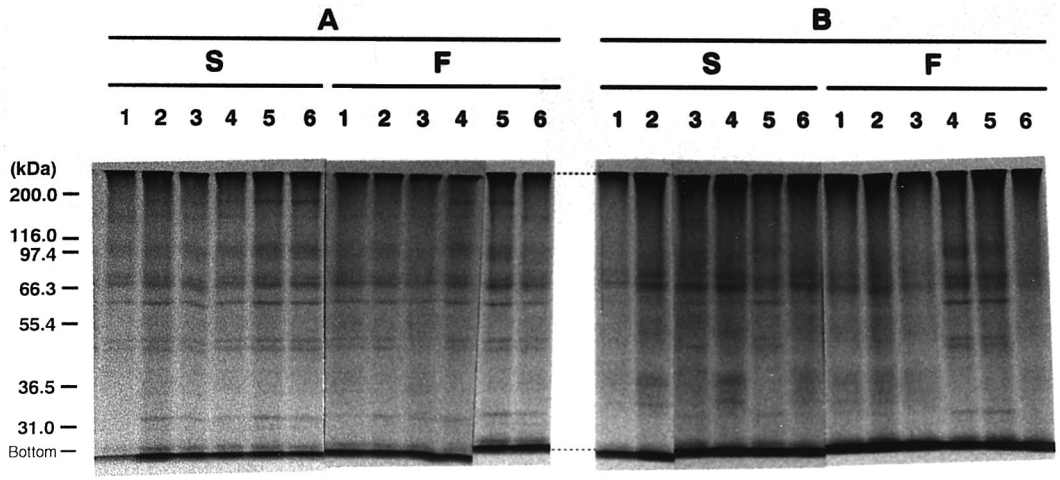

FIG. 6. SDS-polyacrylamide gel electrophoresis of detergent-insoluble protein aggregates in rat erythrocyte membrane. (A) Membranes of erythrocytes of rats fed a diet containing S or F; (B) membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. All the aggregates of six rats of each group were electrophoresed. Bands were visualized by staining with Coomassie brilliant blue R-250. Protein molecular-weight-marker (Iwaki Glass Corporation, Chiba, Japan) was used for indication of the protein positions of molecular weight. For abbreviations see Figure 1.

obic conditions. The amount of vitamin E present in membranes under these conditions may be too small to prevent oxidation of membranes with high n-3 fatty acid content. The increased susceptibility to lipid peroxidation, which was tested under the artificial aerobic conditions, does not reflect the circulation conditions of the erythrocytes *in vivo.* The degree of membrane protein aggregation of erythrocytes with high vs. low n-3 fatty acid content was increased to a similar extent by the *in vitro* oxidation, indicating that oxidative membrane protein aggregation did not correspond to lipid peroxidation, and that lipid peroxidation was not a causative factor for membrane protein aggregation.

Several studies suggesting undesirable effect of dietary n-3 fatty acid intake for humans have appeared. Measurement of human urinary and plasma levels of lipid peroxidation products in relation to increased n-3 fatty acid intake has been carried out. Daily urinary TBARS levels in humans fed a diet containing n-3 fatty acids for 40–50 d are slightly increased (8,9), but the same authors suggested the increase was caused by the lipid peroxidation products formed in food before its consumption (8). The present authors have shown in previous studies that human urinary TBARS levels of each subject eating regular Japanese food exhibited a 1.5–3-fold variation daily and 2–3-fold variation within a day (37), suggesting that the urinary TBARS level is not a good index for lipid peroxidation studies of the whole human body. Several reports show that high intakes of n-3 fatty acids led to increased human plasma TBARS and lipid hydroperoxide levels (10–14). However, in most of these studies (10,11,13) the TBA assay was carried out without addition of BHT, and TBARS could be produced during the aerobic assay procedures. Furthermore, TBARS in serum or plasma are usually very low and could not yet be identified (38). Hackett *et al*. (39) strongly argued that the TBA asssay of plasma is not reliable. By contrast, Kinsella (40) has suggested that intake of n-3 fatty acid preparations which contain vitamin E should not exert any deleterious effects *via* peroxidation when adequate levels of vitamin E are consumed (40).

Studies on experimental animals supplemented with n-3 fatty acids have shown a decrease in vitamin E content and an increased susceptibility to *in-vitro* lipid peroxidation of tissues (15–22). Supplementation of herring oil to rats increased TBARS levels in liver microsomes and susceptibility to *in vitro* lipid peroxidation (19). Supplementation of menhaden oil to rats increased TBARS levels in heart and liver and their susceptibility to *in vitro* lipid peroxidation (20). In most of these previous studies (19–21), the TBA assay was carried out without addition of any antioxidants, and there is a possibility that artifactual lipid peroxidation took place during the assay under aerobic conditions.

In conclusion, n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation in rat erythrocyte membrane and may not enhance dynamic oxidative damages of the cells. It is likely that if a sufficient amount of vitamin E is supplied, n-3 fatty acid supplementation does not bring about undesirable effects in relation to lipid peroxidation.

ACKNOWLEDGMENTS

We thank Makoto Takahashi for technical assistance in part of this work. This work was supported in part by a grant provided by the Ministry of Education, Science, Sports and Culture.

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[Received December 15, 1997, and in final revised form March 17, 1998; revision accepted March 24, 1998]

Modification of Milk Formula to Enhance Accretion of Long-Chain n-6 and n-3 Polyunsaturated Fatty Acids in Artificially Reared Infant Rats

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ABSTRACT: Artificially reared infant rats were used to determine the effects of long-chain polyunsaturated fatty acid (LCPUFA) supplementation on blood and tissue concentrations of arachidonic acid (AA) and docosahexaenoic acid (DHA). Beginning at 7 d of age, infant rats were fed for 10 d with rat milk formulas supplemented with AA at 0, 0.5 and 1.0%, or supplemented with DHA at 0, 0.5 and 1.0% of total fatty acid. The supplementation of AA increased accretion of the fatty acid in tissue and blood phospholipids with a maximum increase of 9% in brain, 15% in liver, 25% in erythrocytes, and 43% in plasma above the values of unsupplemented infant rats. Rat milk formula containing 1.0% of AA had no added benefits over that containing 0.5% of AA. The supplementation of DHA increased phospholipid DHA by a maximum of 24% in brain, 87% in liver, 54% in erythrocytes, and 360% in plasma above the unsupplemented control. The increase in tissue and blood DHA was concentration-dependent on formula fatty acid. Brain phosphatidylcholine and phosphatidylethanolamine were similarly enriched with AA and DHA by supplementation of the corresponding fatty acids. In general the observed increase of AA was accompanied by a decrease in 16:0, 18:1n-9, and/or 18:2n-6, whereas the increased DHA was associated with a reduction of 18:1n-9, 18:2n-6, and/or 20:4n-6. Clearly, infant rats were more responsive to DHA than AA supplementation, suggesting a great potential of dietary manipulation to alter tissue DHA concentrations. However, the supplementation of DHA significantly decreased tissue and blood AA/DHA ratios (wt%/wt%), whereas there was little or no change in the ratio by AA supplementation. Although the physiological implications of the levels of AA and DHA, and AA/DHA ratios achieved under the present experimental conditions are not readily known, the findings suggest that artificial rearing could provide a suitable model to investigate LCPUFA requirements using various sources of AA and DHA in rats.

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Long-chain polyunsaturated fatty acids (LCPUFA), especially arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6n-3) are found in high concentrations in neural tissues and retina (1–3). Although precise functions of LCPUFA are not completely understood, AA and DHA are currently considered essential for growth, development, and maturation of fetuses and infants (4–6). In addition, inadequate DHA level has been associated with impaired visual acuity, learning ability, and neural functions (7–11). Thus, adequate provision of LCPUFA and their precursors to ensure optimal growth and development of infants is obviously critical (6,12).

Human milk, which contains various concentrations of AA and DHA, is the preferred source of LCPUFA (13). Most infant formulas, on the other hand, are devoid of AA and DHA (13), but do contain linoleic acid (LA, 18:2n-6) and α-linolenic acid (LN, 18:3n-3), the precursors of LCPUFA (14). Preterm infants fed commercially available formulas had AA and DHA concentrations in plasma and erythrocytes that were lower than those of breast-fed counterparts (5,15,16). Similar relationships between infant formula feeding and blood LCPUFA levels were noted in full-term infants. Term infants fed conventional formulas were unable to maintain concentrations of blood AA and DHA seen in breast-fed infants during postnatal development (5,15,17–19). Coincident with the difference in plasma and erythrocyte DHA levels, the formula-fed infants had poorer visual acuity, slower information processing ability, and impaired neural functions compared to breast-fed infants (8–12). In an attempt to promote accretion of LCPUFA, DHA in the form of fish oil or LN in vegetable oil has been supplemented to infant formulas (16,18–22). Fish oil added to formula increased DHA in plasma and erythrocytes to a level comparable to that of breast feeding (16,21). Vegetable oil (e.g., soybean oil and canola oil) rich in LN also increased DHA levels in plasma and erythrocytes (10,18,22), and the extent of the increase was inversely related to LA/LN ratios in the diet (14,23). However, the maximum level of DHA achieved by dietary LN fell short of that observed in infants fed fish oil-supplemented formula or breast milk (20,23). Interestingly, the supplementation of DHA or LN in formulas has been shown to improve visual acuity and

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Abbreviations: AA, arachidonic acid; AR, artificial rearing; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LN, α-linolenic acid; LCPUFA, long-chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RMF, rat milk formula.

neural functions to various degrees (10,12,19), although such benefits were not observed in other studies (22,24,25). It should be stressed that fish oil supplementation resulted in a reduction of AA in blood which was accompanied by growth retardation of preterm infants (26). Nonetheless, when fish oil high in DHA but low in eicosapentaenoic acid (EPA, 20:5n-3) was used in formula supplementation, no such adverse effects on blood AA concentration and growth were observed (9,16).

Despite these observations there are concerns about the usefulness of fish oil to provide DHA for infants because of the potential deleterious effect of EPA (3). EPA may compete with AA for production of different series of prostaglandins and leukotrienes leading to metabolic abnormalities (27). The present study was therefore undertaken to test the use of microbial oils rich in either AA or DHA but devoid of EPA as sources of LCPUFA for infant rats. To this end, an artificial rearing (AR) model of infant rats was used throughout the study. The postnatal brain growth spurt in rats corresponds to the rapid growth of human brain during the perinatal period (28). Thus, the AR infant rats provide a useful model to study brain metabolism and development of preterm infants. One of the major advantages of the AR model is the precise control of quantity and composition of milk formula provided to each pup (29). Moreover, the model has been proven suitable for studying metabolic consequences or nutrient requirements of neonates by altering the composition and amount of milk (29–31). The results demonstrated that supplementation of AA and DHA was effective in enriching the respective fatty acids in blood and tissue phospholipids.

MATERIALS AND METHODS

Animals and artificial rearing. Pathogen-free pregnant Sprague-Dawley rats at 14 d of gestation were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in individual plastic containers. They were fed *ad libitum* a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) and had access to water at all times. For each study, newborns from four pregnant rats were delivered naturally, culled to 10 pups per dam within 12 h of birth, and were nourished by their dams until the beginning of AR. At 7 d of age, one to two weight-matched rat pups from each litter were assigned to one of three AR groups. In Study 1, the pups were fed rat milk formulas (RMF) that contained 0, 0.5, or 1.0% AA as total fatty acids by weight. In Study 2, the pups received RMF which contained 0, 0.5, or 1.0% DHA. Ten of the remaining pups comparable to those in the AR groups were assigned to one of the four dams. They were suckled by the dam throughout the lactating period and were used as models for growth rates. For artificial feeding, infant rats were anesthetized with diethyl ether for 90 s in a chamber for the purpose of permanent placement of intragastric cannulas using the nonsurgical technique of Hall (32). The cannulas were connected to plastic syringes on an infusion pump for automatic feeding for 15 min each hour (30). All pups were weighed daily, and AR care was provided, including antiseptic dressing, cleansing of the perianal area, and stimulation to eliminate (30). The daily milk intake of artificially reared rats was adjusted to match the growth rate of the mother-reared pups. The animal protocol was reviewed and approved by The Pennsylvania State University Animal Care and Use Committee.

RMF. RMF were prepared essentially according to the method of Auestad *et al*. (33). The formulas were modified from RMS-2A formula and were composed of carbohydrate, protein, fat, vitamins, and minerals that closely resembled those of rat milk (33). All formulas contained 11.8% fat (w/w) consisting of fat from milk formula base (3.4%), medium-chain triglyceride (2.0%), and corn oil (microbial oil + corn oil = 6.4%) (30). Microbial oils (Martek Bioscience, Columbia, MD) provided sources of n-6 and n-3 LCPUFA. Microbial oil A (triglyceride oil produced by *Mortierella alpina*) contained 17.7% AA, 16.3% 16:0, 15.1% 18:0, 23.4% 18:1n-9, 11.5% 18:2n-6, and small percentages of other fatty acids, but no DHA and EPA. Microbial oil B (triglyceride oil produced by *Crypthecodinium cohnii*) contained 41.9% DHA, 15.5% 14:0, 19.1% 16:0, 13.8% 18:1n-9, and small percentages of other fatty acids, but no AA and EPA. In Study 1, microbial oil A was added at the expense of corn oil to make RMF variations in AA concentrations of 0, 0.5, or 1.0%. Microbial oil B was used in Study 2 to prepare formulas containing 0, 0.5, or 1.0% DHA. All RMF were degassed with nitrogen and allocated for storage at −20°C. The formulas were rehomogenized daily before feeding.

Lipid extraction and fatty acid analysis. Infant rats were artificially fed for 10 d. At 17 d of age, the pups were anesthetized with pentobarbital (Anpro Pharmaceutical, Arcadia, CA) (5 mg/100 g body weight) for collection of blood by cardiac puncture. Livers and brains were excised, rinsed in 0.9% NaCl, blot-dried on filter paper, frozen in liquid nitrogen, and then stored at −80°C for subsequent lipid analysis. Plasma and erythrocytes were prepared by centrifugation and wash procedures described previously (34).

For analyses of fatty acids, lipid extracts were prepared from plasma, erythrocytes, brain, and liver according to the extraction procedures of Folch *et al.* (35), using chloroform/methanol (2:1, vol/vol) containing 50 μg butylated hydroxytoluene as the antioxidant. Lipid extracts were then subjected to thin-layer chromatographic separation of lipid classes using silica G plates and a hexane/ethyl ether/acetic acid (80:20:1, by vol) solvent (36). The phospholipid band remaining at the origin of the plate was scraped into an ampule for transmethylation by using 12% boron trifluoride in methanol (wt/vol) (37). The methylated fatty acids were analyzed by gas chromatography (Model 5980 Series II; Hewlett-Packard, Palo Alto, CA) equipped with an SP-2330 fused-silica capillary column (30 m × 0.25 mm i.d., 20 μm film; Supelco, Bellefonte, PA) and a flame-ionization detector. The gas chromatographic conditions were the same as those described elsewhere (38). For fatty acid analysis of phospholipid species, the lipid extracts were separated into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by thin-layer chromatography using silica G plates and a solvent mixture of chloro-

form/methanol/petroleum ether (bp 35–60°C)/acetic acid /boric acid (40:20:30:10:1.8; vol/vol/vol/vol/wt) as described in a previous study (39). The isolated individual phospholipids were transmethylated and analyzed by gas chromatography (38). Fatty acid proportions were expressed as wt% of total fatty acid. The absolute content of individual fatty acids was measured by the addition of free heptadecanoic acid as an internal standard prior to transmethylation (34).

Statistical analysis. Data are expressed as means ± SD. The significant difference in comparing three dietary treatments was analyzed by one-way analysis of variance. Tukey's pairwise comparisons (40) using Minitab Statistical software (Minitab, State College, PA) was applied to determine where the difference existed.

RESULTS

Artificially reared infant rats grew at the same rate as the mother-reared counterparts. At the onset of the artificial feeding on day 7 of life, mean body weight for three groups of infant rats fed AA- supplemented formulas were 15.5 ± 1.0 g, 16.0 ± 1.1 g, and 15.7 ± 1.0 g compared with 15.3 ± 0.8 g for mother-reared offspring. After 10 d of feeding (i.e., at 17 d of age) the mean body weights of 28.7 ± 1.7 g, 29.0 ± 0.8 g, and 29.1 ± 2.0 g were not different from 29.6 ± 1.4 g of the mother-reared infant rats with the same age. Similarly, there was no difference in body weight between a mother-reared and artificially reared infant rats before and after the feeding of DHA-supplemented formulas.

Fatty acid analyses of RMF revealed that the actual weight percentage of AA (i.e., 0, 0.46, and 0.93%) and DHA (i.e., 0, 0.51, and 0.94%) in the formulas were close to the targeted levels of 0, 0.5, and 1.0% (Table 1). There was no difference in other fatty acids among the three formulas supplemented with DHA. Similarly, the wt% of other fatty acids were maintained constant except stearic acid (18:0) in the AA-supplemented formulas. All formulas contained medium-chain fatty acids (i.e., 8:0 and 10:0), although they were lower in DHA-supplemented than AA-supplemented formulas. Overall, the unsupplemented formulas contained major fatty acids, except 18:3n-3, similar to those of rat milk from various sources compiled by Auestad *et al*. (33). The absence of 18:3n-3 in the present formulas was designed to minimize the contribution of n-3 LCPUFA by endogenous synthesis.

The first series of experiments determined the effects of AA supplementation on blood and tissue fatty acid composition of phospholipids. As shown in Table 2, AA supplemented at 0.5 and 1.0% increased AA equally in plasma by 35–43% above the level of unsupplemented formula. The increases were accompanied by a lower weight percentage of 16:0 and 18:1n-9 than that of unsupplemented. The concentrations of DHA in plasma were not altered by AA supplementation. The AA supplementation also enriched the AA level in erythrocytes by 20–25% as compared with control unsupplemented infant rats, but lowered the weight percentages of 16:0, 18:0, and 18:1n-9. The erythrocyte DHA level was higher in supplemented than unsupplemented animals.

In the brain, the weight percentage of AA in phospholipids was 9% higher in the AA-supplemented groups at either 0.5 or 1.0% AA than in the unsupplemented group (Table 3). There were slight, but significant reductions in DHA by the two levels of AA supplementation. The weight percentages of 18:2n-6, 22:4n-6, and 22:5n-6 were lower in 0.5% AA-supplemented than in unsupplemented infant rats. All other brain fatty acids remained unchanged. The weight percentages of AA in liver phospholipids of infant rats fed supplemented formulas (both at 0.5 and 1.0%) were 10–15% higher than the control (Table 3). AA supplementation had no effect on weight percentage of liver DHA or other fatty acids. Brain PE contained markedly higher concentrations of AA and DHA than PC (Table 4). AA supplementation increased AA accretion in both phospholipid species with greater percentages increases in PC (i.e., 21–25% above the control) than those in PE (i.e., 5–8% above the control). The AA supplementation did not alter weight percentages of brain DHA, 16:0, 18:0, and 18:1n-9, but lowered 18:2n-6 and increased 22:4n-6 and 22:5n-6 in PC. In PE, the increase in weight percentage of AA was accompanied by a reduction in 16:0 and 18:0 when the

TABLE 1
Fatty Acid Composition of Rat Milk Formulas Supplemented with Arachidonic or Docosahexaenoic Acid[a]

	Formula			Formula		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% DHA	0.5% DHA	1.0% DHA
8:0	8.5 ± 0.7	8.3 ± 1.2	7.8 ± 1.7	5.9 ± 1.4	5.9 ± 1.1	7.0 ± 0.8
10:0	7.3 ± 0.4	8.0 ± 1.0	7.0 ± 0.6	4.6 ± 0.3	5.0 ± 0.5	4.7 ± 0.2
12:0	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.1
14:0	3.5 ± 0.6	4.2 ± 1.3	4.0 ± 1.0	3.4 ± 1.1	3.5 ± 0.2	3.6 ± 0.5
16:0	15.8 ± 1.2	16.9 ± 2.8	16.7 ± 1.9	16.2 ± 2.0	16.1 ± 0.4	16.0 ± 1.0
18:0	3.9 ± 0.6[a]	5.7 ± 1.3[b]	5.6 ± 0.8[b]	5.3 ± 1.1	5.2 ± 0.2	5.2 ± 0.6
18:1n-9	21.5 ± 0.5	21.9 ± 1.4	22.3 ± 0.9	23.6 ± 0.3	23.1 ± 0.5	22.9 ± 0.3
18:2n-6	35.2 ± 2.2	30.9 ± 3.4	32.6 ± 3.3	38.8 ± 5.9	38.6 ± 1.1	37.4 ± 2.2
20:4n-6	—	0.46 ± 0.03[a]	0.93 ± 0.04[b]	—	—	—
22:6n-3	—	—	—	—	0.51 ± 0.01[a]	0.94 ± 0.05[b]

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each supplement with different superscript roman letters in the same row are significantly different at $P < 0.05$. AA = arachidonic acid, DHA = docosahexaenoic acid.

TABLE 2
Fatty Acids of Plasma and Erythrocyte Phospholipids in Infant Rats Fed Formulas Supplemented with AA[a]

	Plasma[a]			Erythrocyte[a]		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	28.5 ± 1.5[a]	26.8 ± 1.7[a,b]	24.9 ± 1.5[b]	32.6 ± 2.1[a]	29.4 ± 2.1[b]	29.2 ± 2.5[b]
18:0	25.2 ± 2.1	24.3 ± 1.1	24.4 ± 0.9	13.7 ± 0.2[a]	12.5 ± 1.0[b]	12.2 ± 1.3[b]
18:1n-9	9.6 ± 0.9[a]	8.2 ± 0.5[b]	8.5 ± 0.6[b]	10.9 ± 0.6[a]	9.3 ± 0.2[b]	8.4 ± 2.6[b]
18:2n-6	24.5 ± 2.8	25.3 ± 2.0	25.9 ± 2.9	9.7 ± 0.5[a,b]	10.8 ± 1.0[a]	9.4 ± 0.5[b]
20:4n-6	7.4 ± 0.7[a]	9.9 ± 0.8[b]	10.5 ± 1.4[b]	18.0 ± 1.0[a]	21.6 ± 0.9[b]	22.4 ± 1.6[b]
22:4n-6	0.3 ± 0.5	0.4 ± 0.4	0.4 ± 0.5	1.8 ± 1.5	2.9 ± 0.3	2.9 ± 0.2
22:5n-6	—	—	—	1.7 ± 0.2[a]	2.3 ± 0.3[b]	2.3 ± 0.5[b]
22:6n-3	2.3 ± 0.5	2.8 ± 0.8	2.4 ± 0.3	4.5 ± 0.4[a]	6.2 ± 1.0[b]	6.4 ± 1.1[b]
AA/DHA	3.3 ± 0.8[a]	3.7 ± 1.0[a,b]	4.4 ± 0.7[b]	4.0 ± 0.2	3.6 ± 0.5	3.6 ± 0.5

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for plasma or erythrocyte with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

formula was supplemented with 1.0% AA. The weight percentage of DHA was not affected, but the weight percentages of 22:4n-6 and 22:5n-6 were slightly increased by the AA supplementation. Consequent to the AA supplementation, AA/DHA ratios were increased in plasma and brain total phospholipids but were not changed in erythrocyte and liver total phospholipids or in brain PC and PE (Tables 2–4).

In the second series of experiments, RMF supplemented with DHA were fed to artificially reared infant rats. The supplementation of DHA markedly increased accretion of DHA in plasma phospholipids to 2.7–4.6-fold of the unsupplemented level (Table 5). The increase in DHA was accompanied by reduction of 18:0 (4–10 wt%), 18:1n-9 (13–16 wt%), and 16:0 (11 wt% only at high DHA-supplemented group). The weight percentage of plasma AA was not affected by the supplementation. Erythrocyte DHA levels were 27–54% higher in infant rats fed formula supplemented with DHA than the control (Table 5). There was a concomitant reduction in weight percentages of 18:2n-6 (5–8%) and AA (7–13%). No other fatty acids were affected by the DHA supplementation.

The supplementation of DHA also enhanced the accretion of DHA in tissue phospholipids. In the brain, weight percentages of DHA of the supplemented animals were 20–24% higher than those of unsupplemented counterparts (Table 6). Brain AA, 16:0, 18:0, and 18:2n-6 levels were not changed by DHA supplementation, but there were significant decreases in weight percentages of 18:1n-9 and 22:4n-6. Liver phospholipid DHA levels were highly enriched by the DHA supplementation (Table 6). In the supplemented groups, weight percentages of DHA were 45–81% higher than those of unsupplemented infant rats. On the other hand, the weight percentages of 18:1n-9 and 18:2n-6 were lower in the supplemented than in the unsupplemented group. There were no differences in AA, 16:0, and 18:0 among the groups. A similar trend of changes in fatty acid composition of brain PC and PE was noted. In PC, DHA supplementation increased weight percentages of DHA by 17–33% with little changes in the other fatty acids (Table 7). The enrichment of DHA in PE was more pronounced than in PC and was accompanied by slight but significant reduction in 18:1n-9, 18:2n-6, AA and 22:4n-6.

The supplementation of DHA in formulas reduced AA/DHA ratios in blood and tissues. This reduction resulted from increased DHA together with either decreased or unchanged AA. The most pronounced decrease in the ratio was seen in plasma, followed by erythrocyte phospholipids. The ratios decreased from 8:1 in the control to 3:1 and 2:1 in the supplemented groups in plasma, and from 6:1 in the control

TABLE 3
Fatty Acids of Brain and Liver Phospholipids in Infant Rats Fed Formulas Supplemented with AA[a]

	Brain			Liver		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	33.9 ± 0.9	35.6 ± 0.6	35.0 ± 1.1	20.2 ± 1.3	19.2 ± 1.7	20.8 ± 1.8
18:0	21.6 ± 0.4	22.1 ± 0.7	21.8 ± 1.0	25.8 ± 2.8	24.6 ± 1.5	26.6 ± 2.9
18:1n-9	14.4 ± 0.1[a]	15.1 ± 0.2[b]	14.7 ± 0.4[a,b]	11.2 ± 1.6	10.5 ± 1.3	11.0 ± 1.4
18:2n-6	1.3 ± 0.1[a]	1.2 ± 0.03[b]	1.3 ± 0.1[a,b]	17.9 ± 2.5	17.7 ± 2.7	15.7 ± 2.6
20:4n-6	13.8 ± 0.4[a]	15.0 ± 0.4[b]	15.1 ± 0.5[b]	13.9 ± 0.7[a]	16.0 ± 0.8[b]	15.2 ± 0.6[b]
22:4n-6	2.4 ± 0.1[a]	2.1 ± 0.1[b]	2.0 ± 0.9[a,b]	0.9 ± 0.3	1.4 ± 0.4	1.1 ± 0.4
22:5n-6	1.4 ± 0.2[a]	1.1 ± 0.1[b]	1.3 ± 0.1[a]	1.3 ± 0.8	1.1 ± 0.8	0.8 ± 0.4
22:6n-3	8.3 ± 0.4[a]	6.5 ± 0.7[b]	7.5 ± 0.6[c]	6.1 ± 0.9	7.0 ± 1.8	5.5 ± 1.3
AA/DHA	1.3 ± 0.03[a]	1.4 ± 0.1[b]	1.4 ± 0.1[b]	2.4 ± 0.4	2.4 ± 0.6	2.8 ± 1.4

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each tissue with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 4
Fatty Acids of Brain Phosphatidylcholine and Phosphatidylethanolamine in Infant Rats Fed Formulas Supplemented with AA[a]

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acid	0% AA	0.5% AA	1.0% AA	0% AA	0.5% AA	1.0% AA
16:0	61.6 ± 0.7	60.3 ± 1.2	60.4 ± 1.2	10.4 ± 0.6[a]	10.0 ± 0.6[a,b]	9.5 ± 0.5[b]
18:0	8.5 ± 0.4	8.7 ± 0.4	8.4 ± 0.3	34.9 ± 0.8[a]	33.4 ± 1.6[a]	31.8 ± 0.7[b]
18:1n-9	18.2 ± 0.5	18.4 ± 0.1	18.1 ± 0.2	9.4 ± 0.5	9.3 ± 0.5	8.9 ± 0.5
18:2n-6	1.8 ± 0.02[a]	1.3 ± 0.1[b]	1.4 ± 0.1[b]	0.7 ± 0.04	0.7 ± 0.1	0.7 ± 0.1
20:4n-6	3.9 ± 0.1[a]	4.7 ± 0.5[b]	4.8 ± 0.7[b]	17.2 ± 0.6[a]	18.2 ± 0.8[b]	18.6 ± 0.4[b]
22:4n-6	0.4 ± 0.04[a]	0.4 ± 0.1[a,b]	0.5 ± 0.1[b]	5.5 ± 0.2[a]	5.7 ± 0.2[a]	6.3 ± 0.2[b]
22:5n-6	0.2 ± 0.03[a]	0.2 ± 0.1[a,b]	0.3 ± 0.1[b]	2.3 ± 0.1[a]	2.4 ± 0.2[a]	2.9 ± 0.2[b]
22:6n-3	1.2 ± 0.1	1.4 ± 0.3	1.5 ± 0.4	17.5 ± 0.9	18.0 ± 1.1	19.1 ± 1.8
AA/DHA	3.2 ± 0.2	3.4 ± 0.5	3.4 ± 0.5	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each phospholipid with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

to 4:1 and 3:1 in the supplemented infant rats in erythrocytes (Table 5). The ratios in the liver decreased from the unsupplemented 4:1 to 3:1 and 2:1 in the supplemented groups (Table 6). There was slight but significant reduction of the ratios in brain total phospholipids, PC, and PE (Tables 6 and 7).

The increased weight percentages of AA and DHA in tissue and blood resulting from the supplementation of the corresponding AA and DHA in milk formulas led to significant changes in the absolute amounts of the fatty acids. In infant rats who received unsupplemented formula, absolute amount of total fatty acids in phospholipids of plasma was 1.03 ± 0.86 mg/mL, of erythrocytes 0.52 ± 0.05 mg/mL, of brain 9.03 ± 0.57 mg/g, and of liver 2.97 ± 0.33 mg/g. The concentrations were not altered in those fed formulas supplemented with either AA or DHA.

DISCUSSION

Long-chain polyunsaturated fatty acids, especially DHA, have been suggested as conditionally essential and should be provided to infants (41,42). Although infants are capable of desaturation and elongation of LN and LA to form DHA and AA, respectively (14,43), the synthesis may not be sufficient to meet AA and DHA requirements during the rapid phase of growth and development in infants (20,23,43). Supplementation of DHA in formula has been proven effective in enriching and maintaining DHA levels in the circulation to levels that are achieved by breast-feeding (16,19–21). More importantly, the supplementation of DHA improved visual function to a greater extent than LN supplementation in preterm infants (44,45). However, the effectiveness of LN supplementation in improving visual acuity is controversial. Makrides *et al.* (46) and Jorgensen *et al.* (11) have independently demonstrated that infants fed formulas with LN levels as high as or higher than human milk had lower visual acuity than that of breast-fed counterparts. To the contrary, most recent studies of Auestad *et al.* (22) and Innis *et al.* (24,25) showed that there was no difference in visual acuity in infants fed either breast milk or formula containing LN. Clearly, the benefits of n-6 and n-3 fatty acid supplementation require further investigation. In this context, it is pertinent to point out that supplementation of DHA from various sources has been extensively investigated in human infants (12,16,18,22), and newborn animals (30,47–49). The present study was designed to evaluate the potential use of Microbial oil A, rich in AA but devoid of DHA and EPA, and Microbial oil B, rich in DHA but devoid of AA and EPA, to enrich LCPUFA in infant rats.

The results clearly demonstrated that supplementation of

TABLE 5
Fatty Acids of Plasma and Erythrocyte Phospholipids in Infant Rats Fed Formulas Supplemented with DHA[a]

	Plasma[a]			Erythrocyte[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	22.4 ± 0.6[a]	22.2 ± 1.3[a]	19.9 ± 1.6[b]	32.4 ± 1.3	33.7 ± 1.0	33.6 ± 0.7
18:0	27.2 ± 0.6[a]	26.0 ± 0.7[b]	24.6 ± 0.7[c]	15.8 ± 0.7	15.7 ± 0.4	15.8 ± 0.5
18:1n-9	8.8 ± 0.4[a]	7.6 ± 0.8[b]	7.4 ± 0.5[b]	10.5 ± 0.4	10.5 ± 0.3	10.2 ± 0.2
18:2n-6	28.3 ± 1.1	29.0 ± 1.2	29.4 ± 2.3	13.2 ± 0.5[a]	12.1 ± 0.6[b]	12.5 ± 0.3[b]
20:4n-6	10.6 ± 0.6	10.3 ± 1.0	11.8 ± 1.1	19.4 ± 1.8[a]	18.0 ± 0.8[a]	16.9 ± 0.4[b]
22:4n-6	0.5 ± 0.1	0.5 ± 0.04	0.4 ± 0.1	1.0 ± 0.1	0.9 ± 0.03	0.8 ± 0.2
22:6n-3	1.2 ± 0.1[a]	3.3 ± 0.8[b]	5.4 ± 0.4[c]	3.4 ± 0.4[a]	4.4 ± 0.4[b]	5.3 ± 0.4[c]
AA/DHA	7.9 ± 0.7[a]	3.3 ± 0.6[b]	2.2 ± 0.2[c]	5.7 ± 0.2[a]	4.2 ± 0.3[b]	3.2 ± 0.2[c]

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each plasma or erythrocyte with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 6
Fatty Acids of Brain and Liver Phospholipids in Infant Rats Fed Formulas Supplemented with DHA[a]

	Brain[a]			Liver[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	35.2 ± 1.4	34.2 ± 0.4	35.0 ± 1.6	21.0 ± 0.9	21.8 ± 0.4	21.2 ± 1.2
18:0	25.3 ± 0.5	24.1 ± 0.9	24.4 ± 0.5	27.9 ± 0.5	27.1 ± 0.7	28.0 ± 1.2
18:1n-9	13.3 ± 0.2^{a}	12.8 ± 0.4^{b}	11.8 ± 0.2^{b}	9.0 ± 0.8^{a}	7.9 ± 0.7^{a,b}	7.5 ± 0.6^{b}
18:2n-6	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	16.8 ± 0.9a	15.4 ± 1.5^{a,b}	14.3 ± 0.7^{b}
20:4n-6	12.2 ± 0.6	13.3 ± 0.9	13.3 ± 0.6	16.0 ± 0.5	16.3 ± 1.2	17.3 ± 1.5
22:4n-6	0.2 ± 0.01^{a}	0.2 ± 0.01^{b}	0.1 ± 0.1^{b}	0.8 ± 0.01	0.7 ± 0.1	0.9 ± 0.2
22:5n-6	1.2 ± 0.03	1.2 ± 0.1	1.2 ± 0.01	0.9 ± 0.3	1.2 ± 0.4	1.1 ± 0.8
22:6n-3	8.8 ± 0.9^{a}	10.6 ± 0.7^{b}	10.9 ± 0.8^{b}	4.2 ± 0.1^{a}	6.1 ± 0.1^{b}	7.6 ± 1.0^{c}
AA/DHA	1.4 ± 0.1^{a}	1.3 ± 0.02^{b}	1.2 ± 0.1^{b}	3.9 ± 0.2^{a}	2.7 ± 0.2^{b}	2.3 ± 0.4^{b}

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each tissue with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

AA increased accretion of the fatty acid in total phospholipids of plasma, erythrocytes, brain and liver (Tables 2, 3). Similar increases in brain PE and PC were observed (Table 4). The extent of increase of AA, however, was relatively small, ranging from ≤9% for brain, ≤15% for liver, ≤25% for erythrocytes, to ≤43% for plasma above the values of the infant rats fed unsupplemented formula. Furthermore, an increase in AA content from 0.5 to 1.0% in milk formulas did not further increase tissue and blood accretion of AA. The increase of AA in most cases was accompanied by a decrease in 18:2n-6, 18:1n-9, and/or 16:0. DHA provided in milk formulas was effective in enriching the fatty acid in the circulation and tissues (Tables 5–7), and the extent of the increase above the control was greater than that of AA enrichment. Phospholipid DHA was increased up to 360% in plasma, 87% in liver, 54% in erythrocytes, and 24% in brain above that of the unsupplemented group. There was a concomitant reduction of 18:1n-9, 18:2n-6, and/or 20:4n-6 associated with these changes. Moreover, the accretion of DHA increased with increasing DHA content in milk formulas in almost all tissue and blood phospholipids. The reason for the different degree of enrichment between AA and DHA is not known. Contrary to LA and LN, both AA and DHA are not readily oxidized for energy (7). Instead, they are mostly esterified to glycerolipids (50). Therefore, the competition between β-oxidation and incorporation into complex lipids may not explain the difference. It is possible that phospholipids in various blood components and tissues are initially saturated with AA more than DHA, and the degree of saturation may determine the incorporation of LCPUFA into phospholipids. This notion is supported by the facts that weight percentages of AA are higher than weight percentages of DHA in plasma, erythrocytes, brain, and liver of unsupplemented infant rats. Thus, the data indicate that infant rats are more responsive to dietary supplementation of DHA than AA, suggesting a great potential of dietary manipulation to alter tissue DHA concentrations.

Coinciding with the disproportionate incorporation of dietary AA and DHA into tissues and blood, DHA supplementation significantly decreased the ratio of AA/DHA, whereas there was little or no change in the ratio by AA supplementation. Although the beneficial effects of DHA enrichment on retinal and neural functions have been widely recognized (7–9), the consequences of the decrease in AA/DHA ratio resulting from DHA supplementation is not known. It is reasonable to hypothesize that a decrease in the ratio may be deleterious to infant growth as has been shown by Carlson

TABLE 7
Fatty Acids of Brain Phosphatidylcholine and Phosphatidylethanolamine in Infant Rats Fed Formulas Supplemented with DHA[a]

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acid	0% DHA	0.5% DHA	1.0% DHA	0% DHA	0.5% DHA	1.0% DHA
16:0	60.2 ± 0.1^{a}	61.2 ± 0.4^{b}	59.7 ± 0.6^{b,c}	7.7 ± 0.3	7.8 ± 0.2	7.4 ± 0.4
18:0	10.0 ± 0.2	9.8 ± 0.4	9.5 ± 0.7	28.2 ± 0.7	27.6 ± 0.6	27.9 ± 1.0
18:1n-9	17.7 ± 0.3^{a}	17.3 ± 0.2^{a,b}	16.9 ± 0.6^{b}	7.9 ± 0.5^{a}	7.5 ± 1.0^{a,b}	6.6 ± 0.5^{b}
18:2n-6	1.6 ± 0.1^{a}	1.4 ± 0.01^{b}	1.6 ± 0.1^{a,b}	1.8 ± 0.5^{a}	1.7 ± 0.7^{a}	1.0 ± 0.1^{b}
20:4n-6	3.7 ± 0.1^{a}	3.4 ± 0.1^{b}	4.3 ± 0.6^{c}	24.0 ± 0.4^{a}	22.9 ± 0.4^{b}	22.5 ± 0.6^{b}
22:4n-6	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.1	6.9 ± 0.1^{a}	6.4 ± 0.3^{b}	6.4 ± 0.1^{b}
22:5n-6	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
22:6n-3	1.2 ± 0.6^{a}	1.4 ± 0.6^{b}	1.6 ± 0.6^{c}	18.7 ± 1.2^{a}	21.9 ± 1.2^{b}	24.0 ± 1.5^{c}
AA/DHA	3.1 ± 0.1^{a}	2.4 ± 0.1^{b}	2.7 ± 0.2^{b}	1.3 ± 0.1^{a}	1.1 ± 0.1^{b}	0.9 ± 0.1^{c}

[a]Values (wt%) are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each phospholipid with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

et al. (26), who found that preterm infants fed formula supplemented with fish oil had increased DHA and decreased AA levels of plasma and erythrocytes which were accompanied by poor growth. Therefore, any attempt to increase accretion of LCPUFA must take into consideration the balance between n-3 and n-6 fatty acids. Whether this can be achieved by simultaneous supplementation of AA and DHA is currently under investigation.

It is worth noting that, although supplementation of DHA consistently enriched the fatty acids in blood and tissues, the degree of enrichment of DHA in plasma and erythrocytes was markedly higher than that of brain total phospholipids, PE, and PC. If one examines the adequacy of DHA accretion in brain, the levels of fatty acid determined in plasma or erythrocytes may not serve as a completely accurate index. This notion is in agreement with a recent suggestion by Innis (41) that AA and DHA in circulating lipids are not a good index of organ LCPUFA status. Since circulating lipid, especially erythrocyte phospholipid AA and DHA, has been widely used to reflect LCPUFA status in infants (41,51,52), further studies to establish the validity for use of erythrocyte lipids are warranted.

Despite the significant increase in accretion of AA and DHA in brain phospholipids, it is not known whether the levels achieved under the present experimental conditions are adequate for growth and development since optimal concentrations of LCPUFA have not been established (7). The requirements of AA and DHA for infants, estimated from either milk fatty acid composition or infant blood lipid profile, have been suggested but none has been agreed on (7). The present study shows that an AR system using AA and DHA derived from microbial sources for supplementation may provide a useful model to establish LCPUFA requirements for infants.

ACKNOWLEDGMENT

This work was supported in part by Wyeth Nutritionals International and by Elmore Funds.

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[Received December 22, 1997, and in final revised form March 30, 1998; revision accepted April 2, 1998]

Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
Lipids 33, 521–527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al*. (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al*. (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

	Group		
Fatty acid	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at −20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 µL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 µL of the blocking solution overnight at 4°C, each well was treated with 100 µL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 µL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 µL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 µL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween 20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 µL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

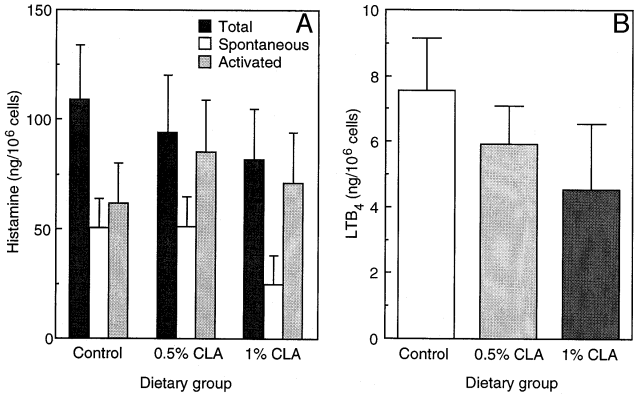

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

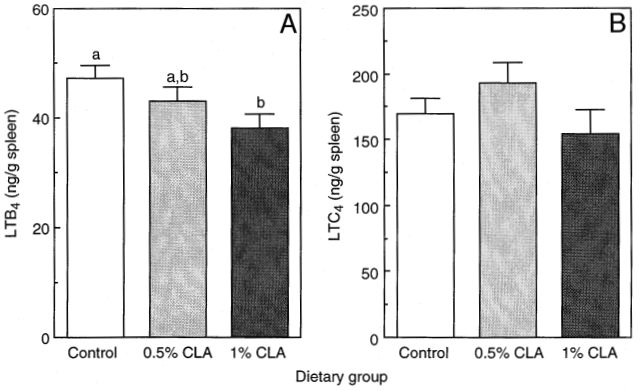

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

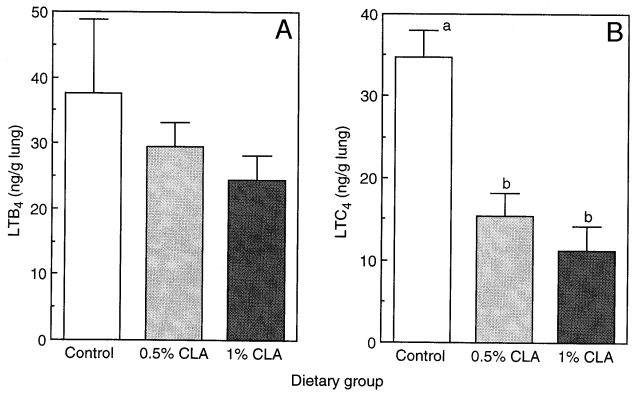

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

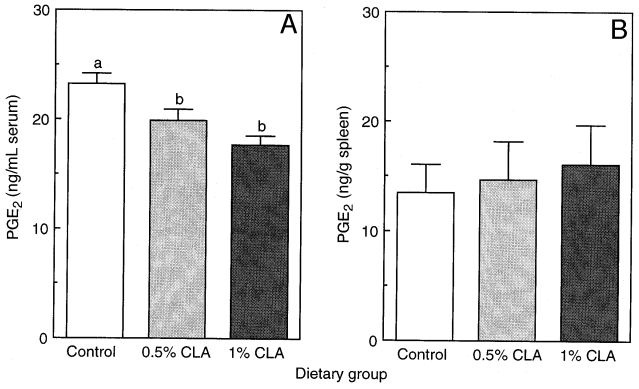

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
	(wt%)		
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9*t*,11*c*/9*c*,11*t*	n.d.	0.1	0.2
10*t*,12*c*	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9*t*,11*c*/9*c*,11*t*	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10*t*,12*c*	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

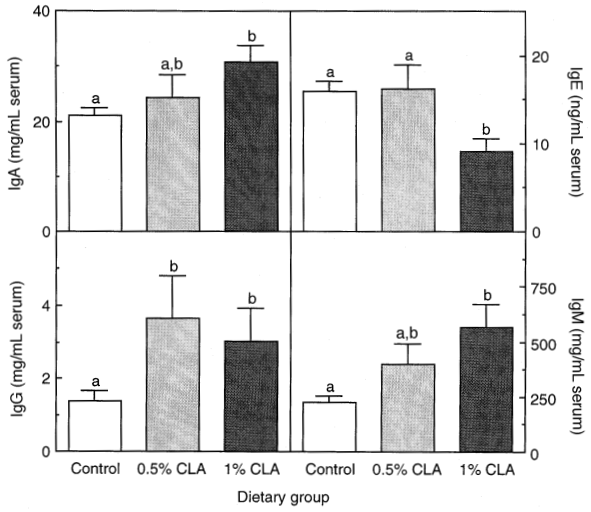

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common lettter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2. CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al*. (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

ACKNOWLEDGMENTS

The authors thank Dr. S. Samman of the Department of Human Nutrition Unit, The University of Sydney, Australia for his valuable criticism during the preparation of this manuscript. This study was supported by a Grant-in-Aid for Scientific Research B from the Ministry of Education, Culture and Science of Japan.

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[Received November 24, 1997, and in final revised form and accepted April 9, 1998]

METHOD

Simultaneous Quantitation of Ceramides and 1,2-Diacylglycerol in Tissues by Iatroscan Thin-Layer Chromatography–Flame-Ionization Detection

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ABSTRACT: Ceramides and 1,2-diacylglycerol have been demonstrated in intracellular signaling pathways. A method of simultaneous mass determination of ceramides and 1,2-diacylglycerol in tissues was developed using the Iatroscan which combines thin layer chromatography and flame ionization detection (TLC/FID) techniques. Because of relatively low amounts of these components in tissues, the fraction of nonpolar lipids, which included ceramides and glycerides, was eluted with chloroform/acetone mixture (3:1, vol/vol) through a silicic acid column to eliminate the polar phospholipids. Development of Chromarods was carried out using three solvent systems in a four-step development technique. The relationship of the peak area ratio to weight ratio compared with cholesteryl acetate added as an internal standard was linear. The amount of ceramides increased with incubation of rat heart homogenate and human erythrocyte membranes in the presence of sphingomyelinase (E.C. 3.1.4.12). The Iatroscan TLC/FID system provided a quick and reliable assessment of ceramides and 1,2-diacylglycerol.

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Ceramides and 1,2-diacylglycerol (DAG) are involved in numerous important biological processes (1–3). The activation of protein kinase C, induced by 1,2-DAG, plays an important role in transmembrane signaling systems. Ceramide generation, resulting chiefly from sphingomyelin hydrolysis, constitutes a signal transduction pathway that mediates cytokines and tumor necrosis factor-α (4,5). Ceramide production has been reported to induce NF-κB, a stress response transcription factor (6). The ultimate effects of the stimulation of the sphingomyelin pathway are the induction of cell differentiation and apoptotic death (7). Sphingolipid metabolites involving ceramides inhibit the activity of protein kinase C (8). Therefore, 1,2-DAG, a physiological endogenous activator of protein kinase C, may oppose this ceramide-related action.

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Abbreviations: 1,2-DAG, 1,2-diacylglycerol; FID, flame-ionization detection; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

The Iatroscan combines the resolution efficacy of thin-layer chromatography (TLC) with the possibility of quantification by flame-ionization detection (FID) (9). We report here the simultaneous quantitation of 1,2-DAG and ceramides from rat heart with the use of the Iatroscan in a study of the significance of the two lipids in the myocardium. The quantitation of 1,2-DAG using the TLC/FID procedure was reported by us (10) and others (11,12). For the quantitative measurement of tissue ceramides, radiolabeled sphingomyelin (13) and diglyceride kinase (14) are usually utilized. Derivatives from ceramides have been determined by high-performance liquid chromatography (HPLC) (15) and gas-liquid chromatography (16). Direct measurements of ceramides and 1,2-DAG by HPLC (17) and TLC (18) have recently been reported.

MATERIALS AND METHODS

Materials. Ceramides (type III, type IV, *N*-palmitoyl-D-sphingosine, and *N*-lignocerol-DL-dihydrosphingosine), 1,2-dioleoyl-*rac*-glycerol (diolein), triolein, oleic acid, cardiolipin from bovine heart, and sphingomyelinase (E.C. 3.1.4.12) from *Bacillus cereus* were purchased from the Sigma Chemical Co. (St. Louis, MO). Cholesteryl acetate, cholesterol, and butylated hydroxytoluene were obtained from Wako Chemical Co. (Osaka, Japan). All other chemicals were of analytical reagent grade. Male Sprague-Dawley rats weighing approximately 300 g were used in the experiments unless otherwise mentioned (Nihon SLC Inc., Hamamatsu, Japan).

Instrumentation. Sample analyses were carried out with the Iatroscan MK-5 TLC/FID analyzer (Iatron Lab. Inc., Tokyo, Japan) under the following conditions: flow rate of hydrogen, 160 mL/min; flow rate of air, 2000 mL/min; scan speed, 30 s/scan. Silica-gel, 75 μm precoated thin-layer rods (Chromarods-SIII; Iatron Inc.) were used in sets of 10. Peak areas were calculated using a potentiometric recorder (Chromatocorder 12; System Instruments, Tokyo, Japan) connected to the analog output of the Iatroscan.

Lipid extraction. Animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Heart was rapidly

excised and washed thoroughly with cold saline and then frozen immediately in liquid nitrogen and lyophilized. The lyophilized tissue samples corresponding to 12–14 mg were homogenized, in 5 mL of a chilled chloroform/methanol mixture (2:1, vol/vol) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate (0.08 mg per tube) as an internal standard, with a motor-driven glass–glass homogenizer kept in ice. The homogenate was chilled on ice 30 min and then filtered through a paper filter (Type 1; Toyo Roshi Inc., Tokyo, Japan). The filtrate was evaporated to dryness under a stream of nitrogen at 40°C. The dry residue was dissolved in 1000 µL of a chloroform/acetone mixture (3:1, vol/vol) and applied to 0.5-mL silicic acid (minus 350 mesh size; Bio-Rad, Richmond, CA) column (20 mm × 5.5 mm) equilibrated with chloroform. Ceramides, neutral lipids, and free fatty acids were eluted with 6 mL of the chloroform/acetone mixture. The elution with this solvent mixture has been reported to result in the effective resolution of ceramide components (19,20). The eluate was concentrated under a nitrogen stream at 40°C and then dissolved in 20 µL chloroform.

Chromarod development and scanning. One microliter of the lipid extracts was applied carefully to the Chromarods with a Hamilton syringe. The first development was carried out in a solvent system of chloroform/methanol/H_2O (57:12:0.6, by vol) until the solvent front had migrated approximately 2.5 cm. The rods were air-dried at room temperature for 10 min and then subjected to the second development in 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05, by vol) until the solvent front had migrated approximately 9 cm. The second development was repeated (third development). The fourth development was carried out in *n*-hexane/diethyl ether/acetic acid (98:1:1, by vol) until the solvent front had migrated approximately 11.5 cm. The Chromarods were then dried at 50°C and scanned in the Iatroscan MK-5. Each sample was analyzed with three Chromarods, and the results were averaged.

The action of sphingomyelinase. We determined the production of ceramides in the homogenate from rat heart and human erythrocyte membranes in the presence of sphingomyelinase. The fresh tissue from rat heart was homogenized with glass-Teflon™ homogenizer in the reaction buffer. Erythrocytes were washed with ice-cold phosphate-buffered saline and disrupted by freezing and thawing in lysis buffer containing 20 mM Tris-HCl, pH 7.5. The lysate was centrifuged for 30 min at 20,000 × *g* at 4°C. The resulting pellet (membrane fraction) was washed five times in lysis buffer. The heart homogenate and erythrocyte membrane preparation were incubated at 37°C in reaction buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1% Triton X-100 (final volume, 200 µL), and neutral and magnesium-dependent sphingomyelinase from *Bacillus cereus* (final 300 mU/mL). The reaction was stopped by the addition of 5 mL of the chloroform/methanol mixture (2:1, vol/vol) containing hydroxytoluene and choles-teryl acetate, and lipids were extracted as mentioned above.

RESULTS AND DISCUSSION

In the present study, most of the phospholipids including cardiolipin were excluded in the more nonpolar lipid components separated by silicic acid column chromatography; see TLC separation (Fig. 1). This polar separation on a silicic acid column was necessary to avoid overloading the Chromarods.

The four-time development procedure using three kinds of solvent systems described in the Materials and Methods section resolved a standard mixture of ceramides, 1,2-diolein, cholesteryl acetate, cholesterol, triolein, and oleic acid (Fig. 2A). Figure 2B shows an Iatroscan TLC/FID chromatogram of rat heart neutral lipids. The four kinds of ceramides, which differed in stereospecificity (D- and L-forms) and in hydroxy and nonhydroxy fatty acids, were not resolved in this system. This was determined by spiking the rat heart homogenate neutral lipid fraction with ceramide type III and type IV, palmitoyl-D-sphingosine and lignocerol-DL-dihydrosphingosine and by observing no resolution of the ceramide peak.

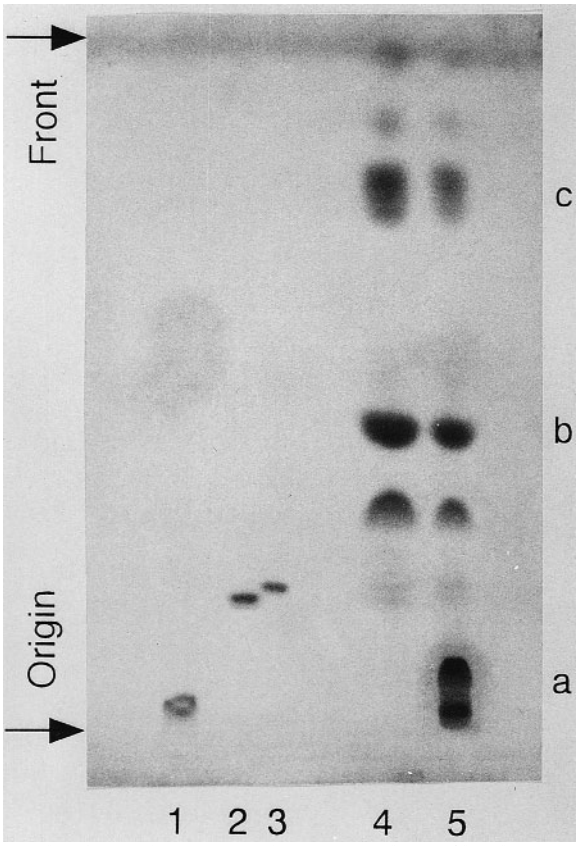

FIG. 1. Thin-layer chromatography (TLC) analysis of lipids. (1) Cardiolipin, (2) ceramides (type IV), (3) ceramides (type III), (4) nonpolar fraction separated by a silicic acid column with chloroform/acetone (3:1, vol/vol) from rat heart, (5) crude lipid extracts from rat heart. TLC was performed on silica gel plates (20 cm × 20 cm, Kieselgel 60 F_{254} from Merck, Darmstadt, Germany). The first development was in chloroform/methanol/H_2O (57:12:0.6, by vol) to 5 cm; the second development was in 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05, by vol) to 14 cm. Spots were visualized by spraying the plates with 40% sulfuric acid and heating at 150°C for 30 min. (a) Phospholipids, (b) cholesterol, (c) triglycerides.

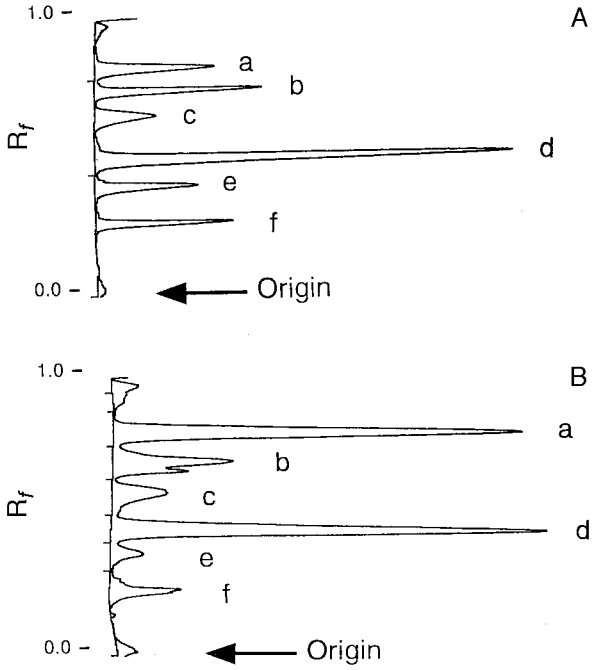

FIG. 2. (A) TLC–flame-ionization detection (FID) separation of the standard lipids. The peaks are as follows: a, cholesteryl acetate; b, triolein; c, oleic acid; d, cholesterol; e, 1,2-diolein; f, ceramides (type III). (B) TLC/FID separation of a tissue sample from a rat heart. The peaks are as follows: a, cholesteryl acetate as an internal standard; b, triglycerides; c, fatty acids; d, cholesterol; e, 1,2-DAG; f, ceramides. 1,2-DAG, 1,2-diacylglycerol; for other abbreviation see Figure 1.

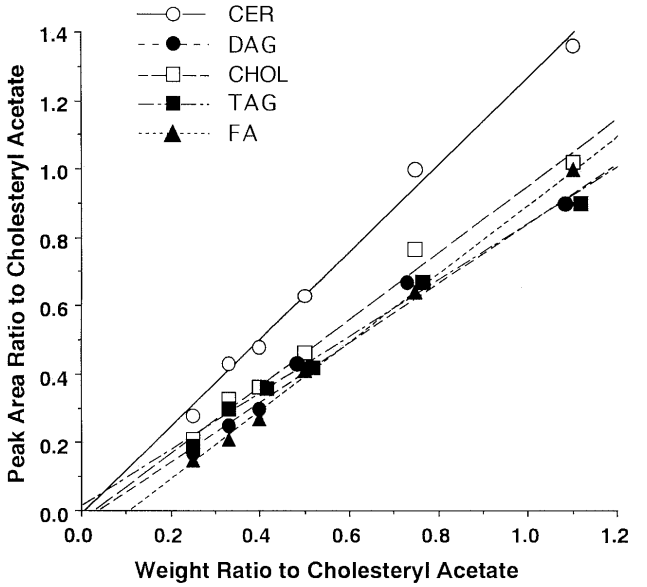

FIG. 3. Calibration curves. These lines indicate the relationship between the weight ratios and the peak area ratios of ceramides (CER), 1,2-diolein (DAG), cholesterol (CHOL), triolein (TAG), and oleic acid (FA) compared to cholesteryl acetate by the FID response. Data represent mean values from duplicate samples.

The recovery of type III ceramides, 1,2-diolein, cholesterol, and triolein by the extraction involving the silicic acid column was determined by comparing the response to cholesteryl acetate. The ratio of these compounds to cholesteryl acetate was 90.4 ± 6.9, 112.8 ± 12.9, 105.8 ± 8.7, and 110.2 ± 3.4%, respectively (n = 6, mean ± SD). The intraassay coefficient of variation was 7.7, 11.5, 8.2, and 3.1%, respectively.

Standard solutions were prepared for all the lipid classes, and the area response was calculated relative to cholesteryl acetate, chosen as the internal standard. The peak area ratios to cholesteryl acetate were compared with the weight ratios to cholesteryl acetate and are shown in Figure 3. The calculated regression equations yielded $y = 1.275x - 0.011$ and $y = 0.876x - 0.033$ for ceramides and 1,2-DAG, respectively. The relationship of the peak area ratio to weight ratio compared with cholesteryl acetate was linear.

With the Iatroscan TLC/FID procedure, it was found that the ceramides and 1,2-DAG contents in the rat hearts amounted to 0.759 ± 0.038 μg/mg dry weight and 0.384 ± 0.059 μg/mg dry weight (n = 6, mean ± SD), respectively, corresponding to 0.173 ± 0.009 and 0.0875 ± 0.0134 μg/mg wet weight, respectively. The value of 1,2-DAG was similar to our previous finding (10), and the value of ceramides was consistent with the content in rat liver (21). The ratio of ceramide content to 1,2-DAG was twice the level found in skeletal muscle in which a diglyceride kinase assay was used (22). This discrepancy may be attributed to differences in tissues or methods.

To examine the effect of sphingomyelinase on the amount of ceramides and 1,2-DAG, the heart homogenate and erythrocyte membranes were incubated in the presence of sphingomyelinase for 60 min. The homogenate of rat heart in Tris buffer contained a 10-fold amount of ceramides compared with the values of tissues frozen by liquid nitrogen even before incubation, probably due to production of ceramides in the buffer by the homogenization procedure. Incubation in the presence of sphingomyelinase produced increased ceramide levels at 30 min, but the concentration decreased at 45 and 60 min. This would suggest a further degradation of the ceramides produced and the exhaustion of sphingomyelin as a source (Fig. 4). This was consistent with the fact that sphingomyelin, which was estimated using the TLC/FID procedure (23), had almost disappeared (data not shown). Exposure to sphingomyelinase generated a large amount of ceramides in the erythrocyte membrane preparations, similar to those observed in the rat heart homogenate (Fig. 4). The ceramide levels reached a sixfold increase at 45 min. The 1,2-DAG levels also increased despite a lack of change in the cholesterol levels.

Ceramides, which were first discovered in brain tissue, have recently been identified as a putative cellular messenger (1), as has 1,2-DAG (3). The intense interest in these lipids makes a quantitative and simultaneous determination of their levels in cells and tissues desirable. Radiolabeled precursors, followed by lipid extraction and TLC separation, have been used for this purpose but have not provided quantitative data, and care must be taken regarding the choice of the labeled precursor (13). Alternatively, the mass determination of ceramides and 1,2-DAG using diglyceride kinase as described by Preiss *et al.* (14) has frequently been achieved. However, the method involves several procedures such as lipid extrac-

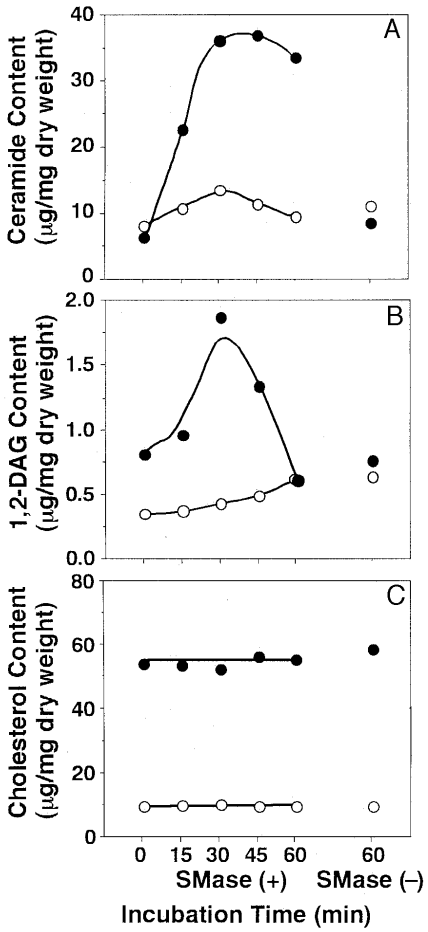

FIG. 4. Changes in ceramides (A), 1,2-DAG (B), and cholesterol (C) in homogenates from rat heart (○) and erythrocyte membranes (●) in the presence and absence of sphingomyelinase (SMase) from *Bacillus cereus* (300 mU/mL). Data represent mean values from duplicate samples. The experiment was performed twice with similar results. For abbreviation see Figure 2.

tion, labeling with radioactive phosphate by the enzyme, reextraction, separation by TLC, and quantitation of radioactivity. The Iatroscan TLC/FID system provided a quick and reliable mass assay of ceramides and 1,2-DAG in animal tissues. Although it is of interest to determine the sources of ceramides and 1,2-DAG, a mass assay is not helpful in this respect.

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[Received November 17, 1997, and in final revised form March 25, 1998; revision accepted April 17, 1998]

A Gas Chromatographic–Mass Spectrometric Method Using a PoraPLOT Column for the Detection of Hydroperoxide Lyase in *Chlorella pyrenoidosa*

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ABSTRACT: A gas chromatographic–mass spectrometric (GC–MS) method using a PoraPLOT Q column was developed for the analysis and identification of the volatile products produced by the action of hydroperoxide lyase (HPLS) upon 13-hydroperoxylinoleic or 13-hydroperoxylinolenic acids. The developed procedure required no derivatization, was not affected by the presence of water, did not require cryogenic conditions to be maintained during injection, and allowed for the quantitation of most products. An acetone powder preparation of *Chlorella pyrenoidosa* cells was triturated with borate buffer pH = 8.0, and the mixture centrifuged at 12,000 × *g*. The supernatant and pellet were assayed for HPLS activity by GC–MS analysis of the volatile products given by linoleic acid hydroperoxide. The data showed that the majority of HPLS activity resides in the pellet fraction, and that the primary volatile component was pentane, with smaller amounts of 2-(*Z*)-pentene and 1-pentene being produced. The fact that HPLS activity resides in the water-insoluble fraction of the acetone powder suggests that HPLS from *Chlorella* is a membrane-associated enzyme. This investigation also determined that a spectrophotometric assay using alcohol dehydrogenase for measuring HPLS activity was not specific, but measured enzymatic activity other than HPLS.

Lipids 33, 533–538 (1998).

Membrane-associated enzyme fatty acid hydroperoxide lyase (HPLS) cleaves linolenic or linoleic acid hydroperoxy derivatives **1** and **2**, respectively. HPLS from higher plants cleaves at the C–C bond adjacent to the hydroperoxide function that is proximal to the carboxy carbon to produce C_{12} oxo-aldehyde **3** and hexanal **4**, and hexenals **6** and **8** (Scheme 1). In crude systems the corresponding reduced hexanol **5** or hexenols **7** and **9** often are detected. HPLS derived from algae, mushroom, and grass cleaves fatty acid hydroperoxides at the C–C bond adjacent to the hydroperoxide function distal to the carboxyl carbon to form C_{13} oxo-aldehyde **10** and a C_5 fragment that varies with enzyme source (1). For example, HPLS from the microalga *Chlorella* will produce pentane **11** and 2-(*Z*)-pentene **14** (2), whereas HPLS from the blue-green microalga *Oscillatoria* and from grass produces pentanol, **12**, and 1-pentene-3-ol, **13** (3,4). Whereas HPLS in higher plants is membrane-associated, HPLS in *Chlorella* and *Oscillatoria* is reported to be water-soluble (2–4).

Although most studies involving HPLS are directed toward an understanding of lipid metabolism and the physiological role of HPLS products, there are also potential industrial uses for this enzyme. For example, the short-chain volatile products from the above reactions are major contributors to the characteristic fresh odor of many green-leaf plants and fruits that are of importance to the fragrance and flavor industry. On the other hand, the long-chain HPLS products, oxo-carboxylic acids, can be oxidized to dicarboxylic acids and used to produce polyamides similar to nylon 13,13.

Recently, we reported a procedure for immobilizing HPLS that was isolated from a partially purified aqueous extract of *Chlorella* (5). HPLS activity was assayed by a published method using **2** as substrate (6). This method is based on the ability of aldehyde **10** to serve as a substrate for yeast alcohol dehydrogenase (ADH) and thereby cause the oxidation of the ADH cofactor NADH. By following the oxidation of NADH spectrophotometrically, a crude or a partially purified enzyme extract could be assayed for HPLS activity. Doubts, however, arose about the actual presence of *Chlorella* HPLS when the expected oxo-carboxylic acid product **10** could not be identified. Accordingly, an alternative method was devised for the identification, isolation, and quantification of the volatile short-chain oxidation products formed from **2** by HPLS.

There are several prior reports concerning the analysis of short-chain HPLS oxidation products. One method measured volatile aldehydes by high-performance liquid chromatographic (HPLC) analysis of their 2,4-dinitrophenylhydrazone derivatives (7). Identification of the short-chain HPLS reaction products directly has been achieved by GC analysis of headspace volatiles, while the semivolatile products required solvent extraction (2–4,7–9). GC analysis was done with columns that separated the products based on their polarity. Accordingly, reported polar and apolar volatile products such as those expected from HPLS cleavage (Scheme 1) were poorly resolved.

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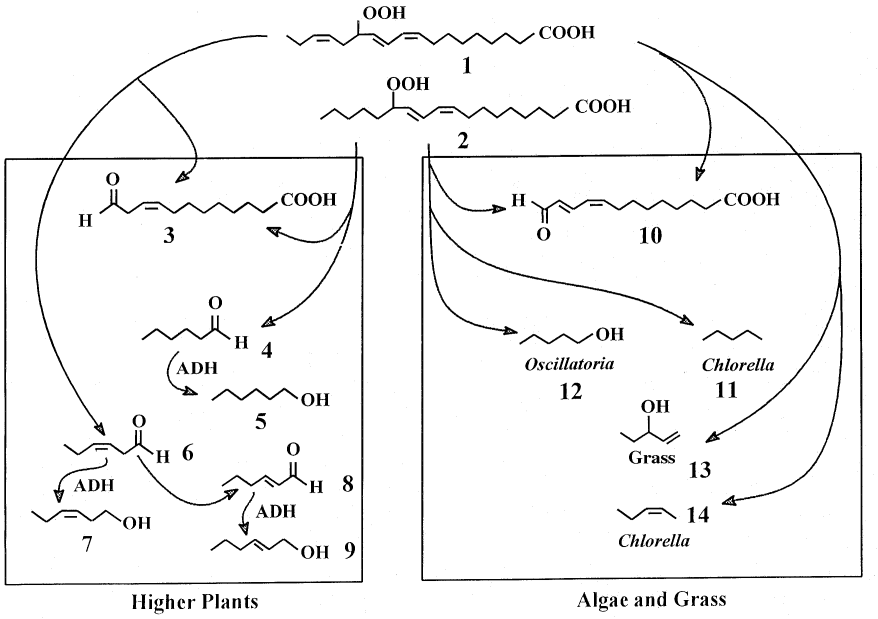
Abbreviations: ADH, alcohol dehydrogenase; GC, gas chromatograph/chromatography/chromatographic; HPLC, high-performance liquid chromatography; HPLS, hydroperoxide lyase; MS, mass spectrometer/spectrometry/spectrometric; NMR, nuclear magnetic resonance.



SCHEME 1

In this study, a GC–MS method using a porous polymer column (PoraPLOT Q) was evaluated for its ability to separate standard compounds of different polarity and volatility that are expected from the action of HPLS on hydroperoxides **1** or **2**. The main advantage of this column is that compounds are separated primarily by size, rather than polarity or boiling point. Accordingly, both polar and apolar HPLS volatile oxidation products were resolved. After determining optimal conditions for separating the expected products, the method was used to assay HPLS activity in water-soluble and insoluble protein fractions from *Chlorella pyrenoidosa*.

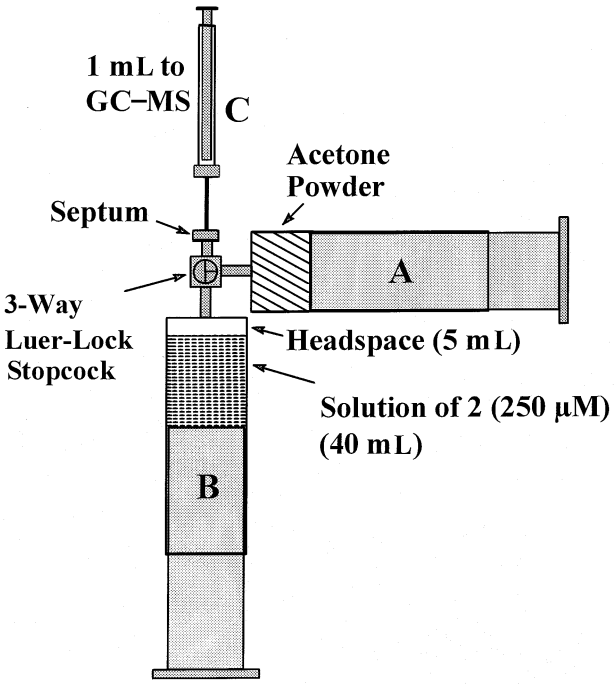
MATERIALS AND METHODS

Chemicals and materials. Soybean lipoxygenase Type 1-B, linoleic acid, ADH, and NADH were purchased from Sigma Chemical (St. Louis, MO). Water was purified to a resistance of 18 MΩ-cm using a Barnstead (Dubuque, IA) NANOpure system. Pentane, 1-pentene, pentanol, 1-hexene,1-penten-3-ol, hexanal, 2-(*E*)-hexenal, and hexanol were purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were of the highest purity available. Hydroperoxide **2** was produced as described previously (5). *Chlorella* cells were disrupted using cold acetone to give an acetone powder as reported previously (10).

Enzyme extraction and purification. Water-soluble enzymes were extracted and partially purified from the acetone powder as reported earlier (5). Further purification and molecular weight estimation of the partially purified enzyme extract was done with a Sephadex G-75 column (2.5 × 48 cm) eluted with potassium phosphate 50 mM (pH 7.0), collecting 2.8-mL fractions. The molecular weight standards (Sigma) were: aprotin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), and blue dextran (2,000 kDa).

Headspace analysis. Typically, 4 g of acetone powder obtained from *Chlorella* cells was mixed with 20 mL 0.2 M borate buffer (pH 8.0) and centrifuged at 12,000 × *g*. The supernatant was removed, and the pellet was resuspended and centrifuged (12,000 × *g*) five additional times. The pellet was divided equally, and each portion was suspended in 20 mL of buffer. One portion was boiled for 10 min to give the control preparation. The fraction to be assayed for HPLS activity was loaded into a 60-mL gas syringe and connected to a closed three-way Luer-lock stopcock (syringe A on Scheme 2). A 40-mL aliquot of an aqueous solution of **2** (250 μM) was loaded into a second 60-mL gas syringe (syringe B on Scheme 2), leaving a headspace of 5 mL. Before allowing the contents of both syringes to mix, a 10-μL aliquot of hexane in methanol (1.5 μL hexane/mL methanol) was added through the septum on the three-way stopcock (see Scheme 2) as an internal standard. The contents of syringe A were dispensed into syringe B through the stopcock, and the mixture was allowed to stand for 30 min at room temperature. Afterward, 1 mL of gas headspace was removed by syringe through the septum and manually injected into the GC–MS. After analysis, 2 mL of hexane was added to the reaction mixture for extraction of less volatile products, and 1 μL of the organic layer was injected into the GC–MS.

GC–MS of volatiles. Analysis of headspace volatiles was performed with a Hewlett-Packard (HP, San Fernando, CA) 5890 Series II Plus GC equipped with an HP 5972 Series mass detector set to scan from *m/z* 35 to *m/z* 400 at 1.2 scans per second and a PoraPLOT Q column coated with styrene-divinylbenzene polymer (25 m × 0.32 mm × 8 μm) (Chrompack, Raritan, NJ). The following oven temperature profile was used to elute the products: 50 (3 min) to 160°C (5 min)



SCHEME 2

at 15°C/min; to a final temperature of 250°C (5 min) at 15°C/min. All injections were splitless with the injector and detector set at 250°C, using He as carrier gas at 1 mL/min. For GC–MS analyses of the products in the hexane layer the oven temperature profile was: 180 (10 min) to 250°C (5 min) at 15°C/min.

*Isolation and analysis of **10***. Semipreparative purification of the C_{13}-oxo carboxylic acid methyl ester was done on a Waters (Milford, MA) LC Module 1 HPLC equipped with a Lichrosorb 5 μ Diol Column (25 × 1 cm) from Phenomenex (Torrance, CA) using a linear gradient of hexane/0.5% isopropanol to hexane/2% isopropanol (30 min). Detection of products was made with a Waters 996 Photodio Array Detector set to monitor at 276 and 233 nm and a Varex MK III Light Scattering Detector from Alltech Associates (Deerfield, IL). The fractions with absorption at 276 nm were collected and analyzed by GC–MS: column HP-5MS (30 m × 0.25 mm); 80 (5 min) to 230°C (10 min) at 10°C/min. The ^{1}H nuclear magnetic resonance (NMR) spectra of the products were obtained using a Varian (Palo Alto, CA) Unity + 400 MHz NMR instrument.

RESULTS AND DISCUSSION

GC–MS assay. One unique property of the porous polymer column used in this study is its ability to separate polar and apolar products. Separation of C_1 to C_7 compounds is accomplished with little or no influence of compound polarity; compounds are separated primarily on the basis of size. However, column elution temperatures are much higher than the boiling points of the volatiles and because of this, products in the C_3–C_5 range are trapped on the column head even at a 50°C column temperature. Accordingly, multiple injections of large sample volumes can be made without compromising compound resolution until the minimum sensitivity of the instrument is reached, approximately 10^{-10} g of sample. Figure 1

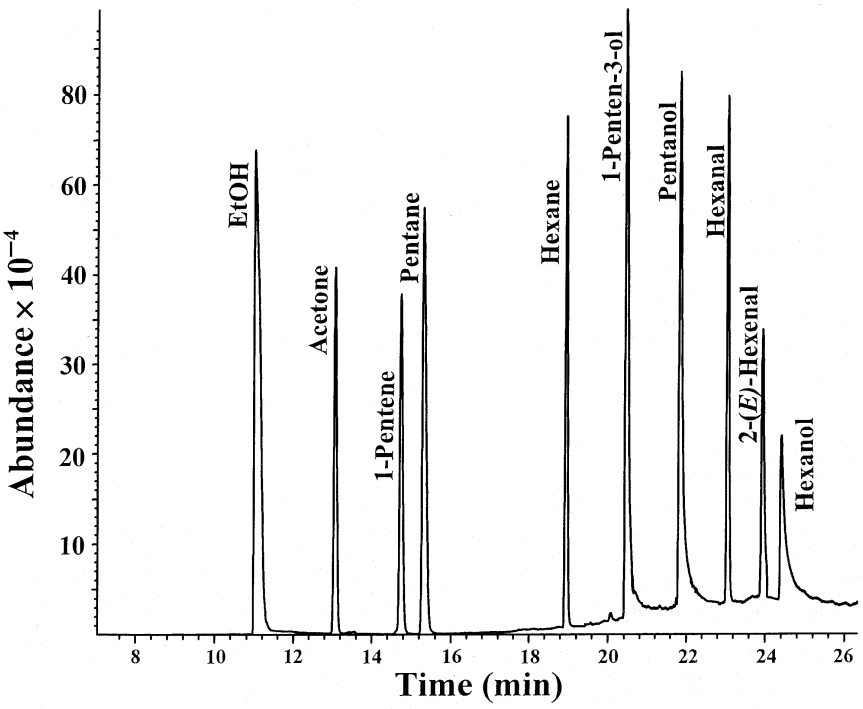


FIG. 1. Gas chromatography–mass spectrometry (GC–MS) of the headspace volatiles in a standard mixture (see the Results and Discussion section) using a PoraPLOT Q column with a temperature program described in the Materials and Methods section.

shows a chromatogram for a headspace injection of a mixture of standard compounds similar to the short-chain products expected from the oxidation of **1** or **2** with HPLS. Because the standards had different volatilities, the standards were injected by taking different amounts of headspace from three separate sample vials in order to obtain similar peak sizes. In the first vial 1 μL of acetone, ethanol, 1-pentene, pentane, and hexane were dissolved in 1 mL methanol. From the headspace of this vial was taken 100 μL for injection. The second vial contained 1 μL of pure 1-penten-3-ol, hexanal, and 2-(*E*)-hexanal, and 200 μL of the headspace was taken for injection from this vial. The third vial contained 1 μL of pure 1-pentanol and 1-hexanol, and 400 μL of the headspace was injected. Peak shape and retention times of the standards from consecutive injections of the three mixtures were identical to those obtained from a single injection of the same volume of each vial. Acetone and ethanol were included because traces were present in the acetone powder and partially purified enzyme extracts. Among the compounds injected, pentanol and hexanol were the more difficult to detect because they tend to elute as broad peaks at low concentration. For the same reason, the resolution of 2-(*E*)-hexenal and 2-(*E*)-hexenol also was poor.

Previous work (2) identified pentane as the major volatile product from the action of *Chlorella* HPLS on **2** by comparison of its retention time with a standard. In that study an active alumina column was used for GC headspace analysis. This column gave effective separation of light hydrocarbons (C_1–C_5), whereas large hydrocarbons or polar material eluted only with difficulty. Because water interacts strongly with alumina columns, precautions need to be taken with regard to the water content of samples to avoid variations in eluate retention times. In another study (3), pentanol was the only short-chain product reported from the reaction of *Oscillatoria* HPLS with **2**. In that study a Carbowax 20M column was used for the detection of pentanol. Other GC analyses of volatiles from grass and germinating soybean seedling HPLS cleavage of **2** also used polar or intermediate polarity columns (4,8). These latter columns, however, require a minimum operating temperature of at least 40°C, but at this temperature apolar short hydrocarbon fragments, such as pentane, are not retained. More recently Salch *et al.* (9) used an HP-5MS low-polarity GC column with a mass detector for the detection of pentane and 2-(*Z*)-penten-1-ol and 1-penten-3-ol. This column can elute both polar and apolar compounds, but we found that ethanol and pentane were not resolved even when cryogenic conditions were used. Also, injection of large sample amounts required cryogenic cooling to avoid peak broadening. As Figure 1 shows, a PoraPLOT column can resolve the mixture of polar and apolar C_5 and C_6 volatile products such as those expected from the interaction of HPLS with **1** or **2** with no need for subambient or cryogenic cooling when large volumes are injected. An added benefit of the column is that it is not affected by water or oxygen, which allows for direct injection of aqueous headspace samples.

By using the air-tight system described in the Materials and Methods section, shown in Scheme 2, the headspace of an active HPLS acetone powder, a heat-treated powder control, and the supernatant from a buffer wash of the active powder were assayed for HPLS activity using the PoraPLOT Q column. Injection of the headspace from the active acetone powder reaction, with hexane as internal standard, showed the presence of three peaks with retention times and mass spectra that corresponded to pentane, 2-(*Z*)-pentene, and 1-pentene (Fig. 2A and inset). Pentane yields were between 5

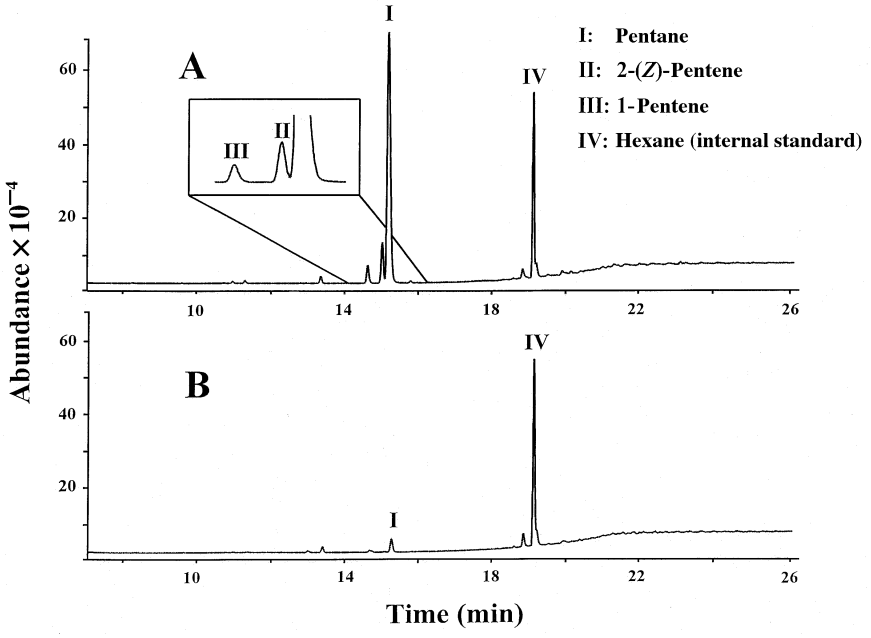


FIG. 2. (A) GC–MS of the headspace volatiles from the reaction of 40 mL of **2** (250 μM) with 2 g of hydroperoxide lyase-active acetone powder from *Chlorella pyrenoidosa*; (B) control reaction. For structure of **2** see Scheme 1; for abbreviation see Figure 1.

and 10%. Although detectable amounts of pentane also are present in the heat-treated powder control (Fig. 1B) and the buffer-washed supernatant (data not shown), the control levels of pentane were always below 3% of the amount found using the HPLS-active acetone powder. In our product analysis the presence of 2-(*Z*)-pentene could be attributed to small amounts of **1** contaminating **2**. However, the source of the 1-pentene is unclear. 2-(*Z*)-Pentene and a third unidentified product that was presumed to be 2-(*E*)-pentene were detected previously in *Chlorella* HPLS reactions when the substrate was **1** (2). Our analysis of the headspace showed that no *E*-isomer was formed.

To determine whether less volatile and more polar alcohols or aldehydes were produced, the reaction mixture was extracted with hexane, and 1 μL of the hexane phase was injected in the GC–MS. No alcohol products were detected. Although small amounts of hexanal were found in the product mixtures, this compound also was detected in the control and is presumed to be formed by nonenzymatic degradation of **2**.

Additional confirmation for the presence of the products resulting from HPLS activity in the acetone powder pellet was obtained by semipreparative HPLC isolation of the nonvolatile products of the reaction. The latter were extracted from the aqueous phase with ether and then methylated. A collected HPLC fraction with ultraviolet absorption at 276 nm gave a single peak by GC–MS with the following mass spectrum: 238 $[M]^+$; 207 $[M - CH_3O]^+$; 206 $[M - CH_3OH]^+$; 188 $[M - H_2O - CH_3OH]^+$; 178; 149; 121; 119; 109; 95; $[CH_2CH{=}CHCH{=}CHCHO]^+$; 81 $[CH{=}CHCH{=}CHCHO]^+$; 68; and 55. This spectrum corresponds to the published spectrum of **10** (2,3,9,11). ^{1}H NMR of the same fraction furnished the following chemical shifts in ppm. In parentheses are the number of protons, multiplicity, coupling constants, and carbon assignments: δ = 9.60 (1H, *d,* $J_{12,13}$ = 7.90 Hz, C13); 6.15 (1H, *dd,* $J_{11,12}$ = 15.2 Hz, C12); δ = 7.43 (1H, *ddd,* $J_{10,11}$ =11.53 Hz, $J_{9,11}$ = 0.92 Hz, C11), δ = 6.26 (1H, *ddt,* $J_{9,10}$ = 10.80 Hz, $J_{8,10}$ = 0.55 Hz, C10); δ = 5.98 (1H, *dt,* $J_{8,9}$ = 7.87 Hz, C9); δ = 3.70 (3H, *s,* ester methyl); δ = 2.30 (4H, *m*, C2,8); δ = 1.62 (2H, *m*, C3); δ = 1.45 (2H, *m*, C7); δ = 1.34 (6H, *m*, C4–6). These data match the spectrum recently reported by Salch *et al.* (9) for 13-oxo-9-(*Z*), 11-(*E*)-tridecadienoic acid.

A recent report showed that under anaerobic conditions lipoxygenase can cause the cleavage of **1** to produce **10** and a mixture of 2-(*Z*)-penten-1-ol and 1-pentene-3-ol. However, no pentane was found in this reaction (9). In our studies with *Chlorella,* pentane was the major volatile detected from the acetone powder pellet. Whether the reaction was conducted under an atmosphere of oxygen or nitrogen, no difference in the products was observed (results not shown). These data suggest that HPLS activity is the major route to the oxo-product **10** and pentane.

Spectrophotometric assay. HPLS activity has been monitored spectrophotometrically at 234 nm by following the loss of the conjugated diene chromophore of **2** or the increase at 280 nm when oxo-acid **10** is the main product. To improve the specificity of this assay a linked assay has been devised that couples ADH to HPLS. ADH reduces the aldehydes produced by HPLS to alcohols and in the process oxidizes NADH. Thus HPLS activity can be followed by the decrease in absorption at 340 nm (6). We reported earlier that a partially purified water-soluble protein fraction derived from the supernatant of *C. pyrenoidosa* and *fusca* gave the expected decrease in absorption at 234 nm and the decrease at 340 nm when **2** and ADH/NADH were present (5,7). However, a more careful examination of the ADH/NADH assay revealed that the reaction of the water-soluble protein fraction with **2** and NADH has the same kinetic profile in the presence or absence of ADH. Because only traces of the ADH/NADH-reduced alcohol derivative of **10** can be detected in organic extracts of this reaction and because the level of volatile organics is comparable to the background levels in heat-treated controls, these results indicate that the spectrophotometric assay was detecting an enzyme activity that was not HPLS. These results were obtained even after further purification of the water soluble extract from *C. pyrenoidosa* by DEAE Sepharose CL-6B columns and gel filtration, where the major protein present had a molecular weight of 46 kDa and maximal activity at pH 6.5, properties similar to those reported previously for HPLS from *C. pyrenoidosa* and *Oscillatoria* (2,3).

In conclusion, the use of GC–MS with a PoraPLOT Q column allowed the development of a method that requires no derivatization, was not affected by the presence of water, did not require cryogenic conditions to be maintained during injection, and allowed for the quantitation of most of the expected volatile products from the action of HPLS upon **1** or **2**. Moreover, the data obtained by using this method show that HPLS resides in the water-insoluble fraction obtained from *C. pyrenoidosa* acetone powder. HPLS activity in an aqueous extract of *C. pyrenoidosa* acetone powder could not be confirmed by the ADH/NADH assay, and the nature of the products arising from the degradation of **2** by enzymatic activity in the aqueous is under study.

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[Received February 9, 1998, and in final revised form March 18, 1998; revision accepted March 25, 1998]

ARTICLE

Distributions of Conjugated Linoleic Acid (CLA) Isomers in Tissue Lipid Classes of Pigs Fed a Commercial CLA Mixture Determined by Gas Chromatography and Silver Ion–High-Performance Liquid Chromatography

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ABSTRACT: Pigs were fed a commercial conjugated linoleic acid (CLA) mixture, prepared by alkali isomerization of sunflower oil, at 2% of the basal diet, from 61.5 to 106 kg live weight, and were compared to pigs fed the same basal diet with 2% added sunflower oil. The total lipids from liver, heart, inner back fat, and omental fat of pigs fed the CLA diet were analyzed for the incorporation of CLA isomers into all the tissue lipid classes. A total of 10 lipid classes were isolated by three-directional thin-layer chromatography and analyzed by gas chromatography (GC) on long capillary columns and by silver-ion high-performance liquid chromatography (Ag^+-HPLC); cholesterol was determined spectrophotometrically. Only trace amounts (<0.1%; by GC) of the 9,11-18:2 *cis/trans* and *trans,trans* isomers were observed in pigs fed the control diet. Ten and twelve CLA isomers in the diet and in pig tissue lipids were separated by GC and Ag^+- HPLC, respectively. The relative concentration of all the CLA isomers in the different lipid classes ranged from 1 to 6% of the total fatty acids. The four major *cis/trans* isomers (18.9% 11 *cis*,13 *trans*-18:2; 26.3% 10 *trans*,12 *cis*-18:2; 20.4% 9 *cis*,11 *trans*-18:2; and 16.1% 8 *trans*,10 *cis*-18:2) constituted 82% of the total CLA isomers in the dietary CLA mixture, and smaller amounts of the corresponding *cis,cis* (7.4%) and *trans,trans* (10.1%) isomers were present. The distribution of CLA isomers in inner back fat and in omental fat of the pigs was similar to that found in the diet. The liver triacylglycerols (TAG), free fatty acids (FFA), and cholesteryl esters showed a similar pattern to that found in the diet. The major liver phospholipids showed a marked increase of 9 *cis*,11 *trans*-18:2, ranging from 36 to 54%, compared to that present in the diet. However, liver diphosphatidylglycerol (DPG) showed a high incorporation of the 11 *cis*,13 *trans*-18:2 isomer (43%). All heart lipid classes, except TAG, showed a high content of 11 *cis*,13 *trans*-18:2, which was in marked contrast to results in the liver. The relative proportion of 11 *cis*,13 *trans*-18:2 ranged from 30% in the FFA to 77% in DPG. The second major isomer in all heart lipids was 9 *cis*,11 *trans*-18:2. In both liver and heart lipids the relative proportions of both 10 *trans*,12 *cis*-18:2 and 8 *trans*,10 *cis*-18:2 were significantly lower compared to that found in the diet. The FFA in liver and heart showed the highest content of *trans,trans* isomers (31 to 36%) among all the lipid classes. The preferential accumulation of the 11 *cis*,13 *trans*-18:2 into cardiac lipids, and in particular the major phospholipid in the inner mitochondrial membrane, DPG, in both heart and liver, appears unique and may be of concern. The levels of 11 *cis*,13 *trans*-18:2 naturally found in foods have not been established.
Lipids 33, 549–558 (1998).

Interest in conjugated linoleic acid (CLA) has increased in the past decade as a result of reports of several health benefits related to its consumption. CLA has been reported to protect against cancer (1–8) and atherosclerosis (9,10). In addition, CLA has been reported to decrease body fat while increasing muscle (11,12) and bone mass (13). However, commercial CLA preparations are mixtures of several positional and geometric isomers of conjugated octadecadienoic (18:2) acid. Not all the isomers in these mixtures have been resolved chromatographically, and the active isomer(s) has (have) not been identified. It has been assumed that 9 *cis*,11 *trans*-18:2 is the active isomer, because it is the major isomer present in milk, dairy products and meats (14–18). A recent epidemiological study in Finland, which appears to support this assumption,

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Abbreviations: CE, cholesteryl ester; *cis/trans*, refers to the group of CLA isomers, or a specific positional isomer, having either a *cis/trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; DPG, diphosphatidylglycerol (cardiolipin); FAME, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; GC-DD-FTIR, -direct deposition-Fourier transform infrared; GC-EIMS, -electron ionization mass spectrometry; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; TAG, triacylglycerol; TLC, thin-layer chromatography.

found a significant inverse gradient between milk intake and incidence of breast cancer in 4,697 initially cancer-free women over a 25-yr follow-up period (19).

A number of studies reported the presence of CLA isomers in human adipose tissue (20,21), bile (22), blood (15,23–25), and milk (15). CLA isomers were incorporated into both tissue total neutral lipids (5,7,26,27) and total phospholipids (1,2,5,7,26–28), as well as cancer tissue (2) of animals fed CLA diets. Only one recent study presented the content of CLA isomers in the different liver phospholipids of rats fed CLA (28). An accurate assessment of CLA content has so far been prevented, firstly, because acid-catalyzed methylation procedures, which may isomerize the conjugated *cis/trans* to *trans,trans* 18:2 isomers (29), were used in the analysis of CLA isomers (1,2,5,7,16,18,20,26,28); secondly, by the lack of chromatographic methods to separate all the individual CLA isomers. We have just reported a silver-ion high-performance liquid chromatography (Ag^+-HPLC) method which clearly separated for the first time four isomers—11 *cis*,13 *trans*-18:2, 8 *trans*,10 *cis*-18:2, 9 *cis*,11 *trans*-18:2, and 10 *trans*,12 *cis*-18:2—that were found in a commercial CLA preparation, plus the corresponding four *cis,cis* and four *trans,trans* isomers (30, and references cited therein).

In the present communication, we report for the first time the distribution of CLA isomers in the tissues of pigs fed a commercial CLA mixture at 2% of the diet. The CLA isomers were separated by both gas chromatography (GC) and Ag^+-HPLC as their fatty acid methyl esters (FAME). The identity of major CLA isomers was confirmed by GC-direct deposition (DD)-Fourier transform infrared (FTIR) spectroscopy and GC-electron ionization mass spectrometry (EIMS). Adipose lipids generally showed the same distribution of the isomers fed. Liver phospholipids showed an increase of 9 *cis*,11 *trans*-18:2 isomer, while in heart phospholipids an increase of 11 *cis*,13 *trans*-18:2 isomer was found. Diphosphatidylglycerol (DPG) in both liver and heart lipids showed a uniquely characteristic incorporation of 11 *cis*,13 *trans*-18:2.

MATERIALS AND METHODS

Animals and tissues. Pigs (Landrace boar by Landrace × Large White sow) were fed a basal diet (barley, wheat, soybean meal, and canola meal) supplemented with either 2% added sunflower oil or a CLA mixture (Natural Lipids Ltd., Hovdebygda, Norway), from 61.5 to 106 kg live weight, as described elsewhere (12). The CLA content of the commercial product was 55.4% of the total FAME, and the distribution of CLA isomers is included in Table 2 (see below). The total fat content of the diet, which included the 2% added test oil, was 3.2% on a dry matter basis. Eight pigs from each diet were selected for total lipid analysis. At time of slaughter, liver, heart, inner back fat, and omental fat were removed, and portions were immediately frozen and maintained at −70°C until analyzed.

Separation of lipid classes and preparation of FAME. Liver and heart tissues were pulverized at dry ice temperature (31), and the total lipids were extracted with chloroform/methanol (1:1, vol/vol). All the lipid classes were separated on thin-layer plates by three-directional thin-layer chromatography (TLC) using silica gel H plates (32). For each tissue, two TLC plates were used to separate 3-mg portions of total lipids each, to ensure sufficient material was available for analysis of the minor lipid classes. To each isolated lipid class (combined from the two TLC plates), a known amount of methyl heptadecanoate (17:0) was added as an internal standard to permit quantitation of the lipids. The lipid classes, in the presence of silica gel, were methylated using sodium methoxide (Supelco, Inc., Bellefonte, PA) for 15 min at 50°C to avoid isomerization of CLA isomers (29), except free fatty acids (FFA) and sphingomyelin (SM); FFA were methylated with diazomethane (33) and SM (because of *N*-acyl fatty acids) with 5% anhydrous HCl/methanol (w/w) for 1 h at 80°C (34). The resultant FAME were purified by TLC using hexane/diethyl ether/acetic acid (85:15:1, by vol) before analysis by GC and Ag^+-HPLC. Back fat and omental fat were methylated directly with sodium methoxide, and the resultant FAME were purified by TLC. Cholesterol was determined spectrophotometrically as described previously (35).

GC. The total FAME of each individual lipid class from the pig tissues investigated were analyzed by GC (model 5890; Hewlett-Packard, Palo Alto, CA), equipped with a flame-ionization detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Bridgewater, NJ) was used, and H_2 was the carrier gas at a split ratio of 1:15 (29). The column was operated at 150°C for 2 min, then temperature-programmed at 1°C/min to 200°C, followed by a second temperature program at 5°C/min to 215°C, and finally held for 20 min at 215°C; the total run time was 75 min.

HPLC. The total FAME mixtures were separated on a ChromSpher 5 Lipids (4.6 mm i.d. × 25 cm stainless steel; 5-μm particle size) silver-impregnated column (Chrompack), as described recently (30). The mobile phase was 0.1% acetonitrile in hexane, prepared fresh each day. The column was operated isocratically at room temperature for 1 h prior to the initial injection of the day. The flow rate of the mobile phase was 1.1 mL/min, and detection was by ultraviolet at 233 nm. If the HPLC column required regeneration, it was flushed with 1% acetonitrile for 4 h, followed by 1 h with 0.1% acetonitrile. The FAME of each lipid class from all eight pigs were combined for the HPLC analyses. Duplicate HPLC analyses were performed but they were identical, and therefore, no standard errors were calculated. The limited data derived in this study suggest that the response of the different CLA isomers measured at 233 nm was accurate relative to one another. There was generally good agreement in the relative distribution of CLA isomers between GC and Ag^+-HPLC results.

Derivatives of 4,4-dimethyloxazoline (DMOX). Total tissue FAME were hydrolyzed to their FFA using 1 N KOH in 95% ethanol (33). The FFA were placed into a 1-mL screw cap reaction tube with a Teflon liner, and a threefold excess

of 2-amino-2-methyl-1-propanol was added. After purging with argon, the reaction vial was heated for 0.5 h at 170°C. The DMOX derivative was then partitioned into petroleum ether as described previously (36). The reaction product was finally taken up in a minimal amount of isooctane for subsequent GC-EIMS and GC-DD-FTIR analyses.

GC-EIMS and GC-DD-FTIR. The equipment and operating conditions used for the GC-EIMS (37) and GC-DD-FTIR (30,38) are given elsewhere. For GC-EIMS, a 100-m CP-Sil 88 column was used, whereas a 50-m CP-Sil 88 column was used for the GC-DD-FTIR work.

RESULTS

Lipid class composition. The inclusion of CLA in the diet of pigs, fed from 61.5 to 106 kg live weight, did not significantly alter the liver and heart lipid class composition, except for cardiac triacylglycerol (TAG) (Table 1). Heart TAG in pigs fed CLA showed a decrease which was not significant because of the large animal-to-animal variation. Characteristic differences evident between liver and heart lipids were related to the content of phosphatidylcholine (PC), diphosphatidylglycerol (DPG), FFA, and cholesteryl ester (CE). PC, FFA, and CE were lower, and DPG was higher in heart compared to liver lipids (Table 1). There were no diet effects on the total free cholesterol and CE content in liver and heart lipids. The heart contained less free and esterified cholesterol compared to the liver (Table 1).

Separation of CLA isomers by GC. The fatty acid composition of liver, heart, inner back fat, and omental fat lipids was determined by GC using a long capillary column (100 m) with a polar liquid phase (CP-Sil 88), as demonstrated previously for milk analysis (29). The CLA region of the GC chromatogram that is bracketed between linoleic (18:2n-6) and arachidonic (20:4n-6) acids is presented (Figs. 1 and 2) in order to show the position of CLA isomers in relation to neighboring FAME present in tissue lipids. The CLA diet (Fig. 1A) and selected tissue lipid classes of pigs fed CLA (Fig. 1B–D and Fig. 2A–D) are shown. On this GC column, all the CLA isomers eluted between linolenic acid (18:3n-3) and 20:2n-6, a relatively clear region in the GC chromatogram of tissue FAME, with two exceptions. A small (<0.1%) unidentified peak marked "x" (Figs. 1A and 2A) was observed in the GC chromatograms of all lipid classes of pigs fed the control diets, and was also found in the dietary commercial CLA oil. Therefore, minor peak "x" was presumably a combination of an unknown non-CLA and CLA isomer; peak "x" was not included in Table 2. In addition, 21:0, found only in SM, coeluted with the 8 *cis*,10 *cis*-18:2 peak. The GC column was used to separate the *cis/trans* CLA isomers into four peaks in the order: 9 *cis*,11 *trans*-18:2 plus 8 *trans*,10 *cis*-18:2 (tailing peak); a minor component tentatively identified as 9 *trans*,11 *cis*-18:2; 11 *cis*,13 *trans*-18:2; and 10 *trans*,12 *cis*-18:2. The three major *cis/trans* peaks were identified by GC-DD-FTIR and GC-EIMS as their DMOX derivatives. The minor *cis/trans* peak was identified as 9 *trans*,11 *cis*-18:2 based on comparison with the 9 *cis*,11 *trans*-18:2 commercial standard mixture from Matraya Inc. (Pleasant Gap, PA), which contained trace amounts the 9 *trans*,11 *cis*-18:2 isomer (21). The *cis,cis* CLA isomers also separated into four peaks (Figs. 1 and 2). The *cis,cis* configuration was established by GC-DD-FTIR, and the molecular weight was established by GC-EIMS as the DMOX derivative. The elution order of the positional *cis,cis* CLA isomers by GC (8,10; 9,11; 10,12; and 11,13) was determined by comparison with Ag^+-HPLC results (*vide infra*) and found to be the opposite of the latter. The *trans,trans* CLA isomers separated into two peaks: 11 *trans*,13 *trans*-18:2; and 8 *trans*,10 *trans*-18:2, 9 *trans*,11 *trans*-18:2 plus 10 *trans*,12 *trans*-18:2. The *trans,trans* configuration was confirmed by GC-DD-FTIR and the molecular weight and double-bond positions by GC-EIMS.

Separation of CLA isomers by Ag^+-HPLC. The Ag^+-HPLC method, just developed (30), was applied to the separation of FAME CLA isomers in the diet (Fig. 1A') and pig tissue lipid classes (Fig. 1B'–D', and Fig. 2A'–D'). Ultraviolet detection at 233 nm selectively identified FAME with a conjugated double-bond system. Ag^+-HPLC was used to separate the *cis/trans* CLA isomers into four major peaks in the order: 11 *cis*,13 *trans*-18:2, 10 *trans*,12 *cis*-18:2, 9 *cis*,11 *trans*-18:2, and 8 *trans*,10 *cis*-18:2; see Figures 1 and 2. The structures of all four *cis/trans* CLA isomers were identified by GC-DD-FTIR and GC-EIMS as their DMOX derivatives, and by comparison with known standards (30). The *trans,trans* CLA isomers eluted first from the silver ion column. The structures of four *trans,trans* isomers were established by comparison to known and/or standard mixtures of CLA isomers (30); see Figures 1 and 2. Based on the absorption at 233 nm, there were another four unidentified peaks of FAME in this region, including the shoulder on 8 *trans*,10 *trans*-18:2. Infrared and

TABLE 1
Composition (% of total lipids) of Liver and Heart Lipid Classes of Pigs Fed Control or CLA Diets as Determined by GC[a]

Lipid class	Liver			Heart		
	Control	CLA	SD[b]	Control	CLA	SD
PC	46.9[c]	50.5	5.3	39.8	40.5	4.5
PE	24.2	23.7	1.4	22.4	24.4	3.3
PS	4.4	4.4	0.5	3.9	3.7	0.7
PI	7.5	7.3	0.9	5.8	5.8	0.7
DPG	3.2	3.2	0.6	14.6	15.3	2.0
SM	2.9	2.7	1.0	2.4	3.0	0.7
TAG	4.7	4.9	1.2	10.4	6.6	6.0
FFA	3.4	2.0	1.0	0.8	0.6	0.3
CE	1.6	1.3	0.3	<0.1	<0.1	
Cholesterol[d]	3.60	3.70	1.05	2.20	2.02	0.58

[a]CLA, conjugated linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol (cardiolipin); SM, sphingomyelin; TAG, triacylglycerol; FFA, free fatty acid; CE, cholesteryl ester; GC, gas chromatography.
[b]Pooled standard deviation (SD).
[c]Mean of six pigs/diet.
[d]Cholesterol expressed as μg/g wet weight.

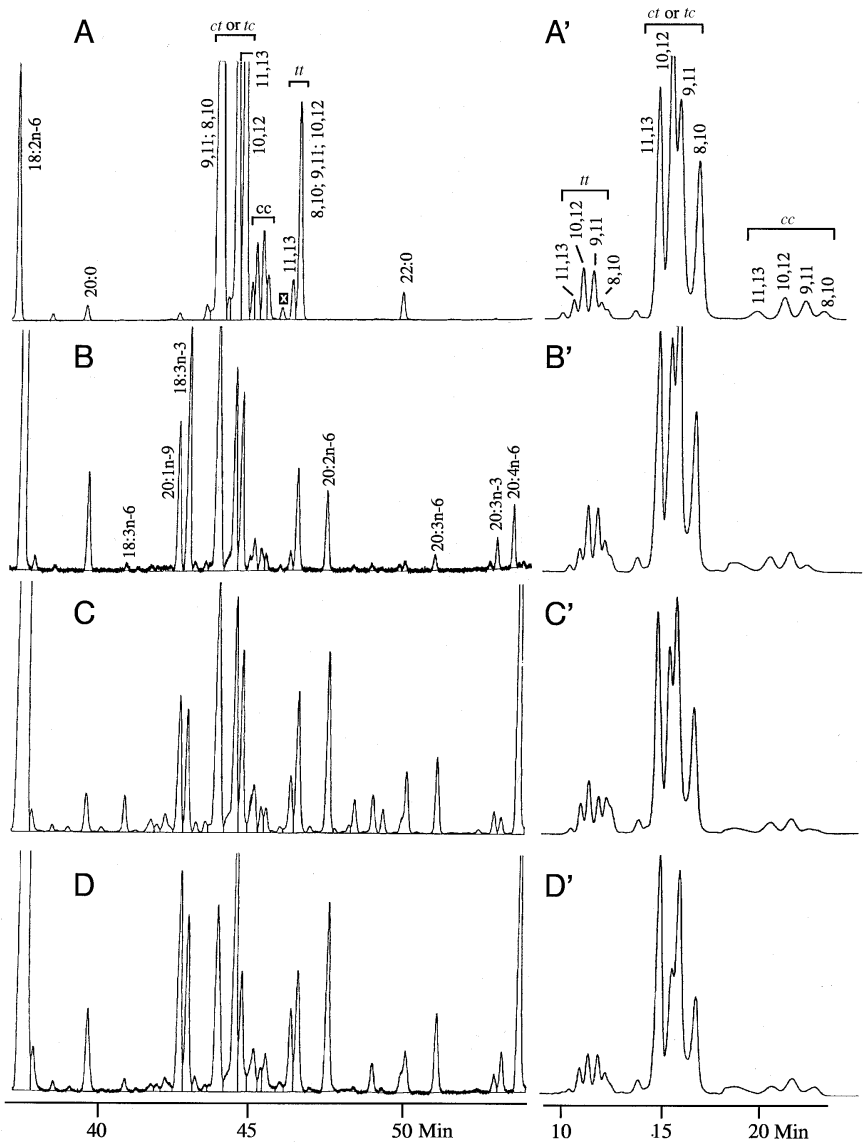

FIG. 1. Partial profiles obtained by gas chromatography (GC) (A to D) and silver-ion high-performance liquid chromatography (Ag^+-HPLC) (A' to D') of the conjugated linoleic acid (CLA) mixture fed to pigs (A,A'), and of CLA isomers found in selected tissues of pigs fed the CLA diet: omental fat (B,B'), liver triacylglycerols (TAG) (C,C'), and heart TAG (D,D'). The GC region selected was between linoleic (18:2n-6) and arachidonic (20:4n-6) acids. All known fatty acids and CLA isomers were labeled; "x" is an unknown in the GC chromatogram which was also found in the chromatograms of both the dietary CLA mixture and the tissues of pigs fed the control and CLA diets. The letters *c* and *t* refer to the *cis* and *trans* CLA isomers. The elution order of the cis,cis CLA isomers is: 8,10-, 9,11-, 10,12-, and 11,13-18:2.

mass spectral confirmation of these isomers is in progress. In the *cis,cis* region four peaks were separated which eluted in the same order as those of the *trans,trans* and *cis/trans* CLA isomers (30) (Figs. 1 and 2).

There may be slight differences in the distribution of CLA isomers between the GC and the Ag^+-HPLC chromatograms in Figures 1 and 2. There were two reasons for this difference. (i) One of eight available GC chromatograms was selected for display (Figs. 1 and 2) and may not reflect the average distribution, whereas for the Ag^+-HPLC, only a single chromatogram was available after the FAME for the same lipid class from all eight pigs on the CLA diet were combined. (ii) Other still unidentified minor components or metabolites of these complex mixtures may also be eluting in the CLA region of the Ag^+-HPLC chromatogram.

CLA content in pig tissues fed the control diet. Pigs fed the control diet showed relatively small peaks (<0.1%) corresponding in retention time to 9 *cis*,11 *trans*-18:2, 9 *trans*,11 *trans*-18:2, and unknown "x" on the GC chromatogram (chromatogram not shown). CLA isomers in tissues from control

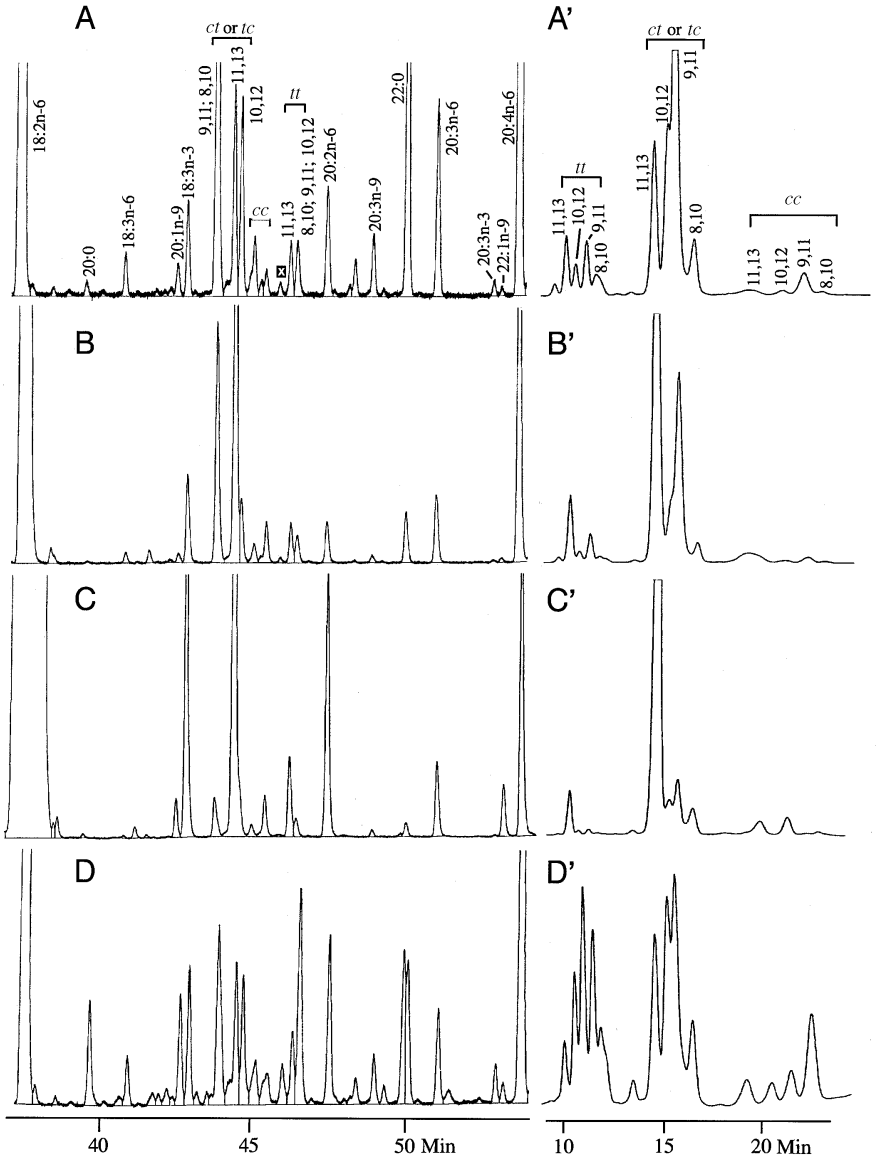

FIG. 2. Partial GC (A to D) and Ag^+-HPLC (A' to D') chromatograms of selected liver and heart lipid classes of pigs fed the CLA diet: liver phosphatidylcholine (PC) (A,A'), heart PC (B,B'), heart diphosphatidylglycerol (DPG) (C,C'), and liver free fatty acids (FFA) (D,D'). For other abbreviations see Figure 1.

animals were only confirmed by their relative retention time on GC. The concentrations of the CLA isomers in the lipids of pigs fed the control diet were too low under our experimental conditions to be detected by ultraviolet at 233 nm after Ag^+-HPLC separation.

CLA isomeric distribution in the diet and adipose tissue. The CLA preparation, included at 2% by weight in the diet, contained 81.7% *cis/trans* CLA isomers, based on Ag^+-HPLC analyses. The reported values are expressed as percentages of total CLA content. The relative concentrations of the four *cis/trans* CLA isomers were found by Ag^+-HPLC to be: 18.9% 11 *cis*,13 *trans*-18:2; 26.3% 10 *trans*,12 *cis*-18:2; 20.4% 9 *cis*,11 *trans*-18:2; and 16.1% 8 *trans*,10 *cis*-18:2. The GC results of the diet are included in Table 2. The total *trans,trans* and *cis,cis* CLA contents were 10.1 and 7.4%, respectively. The inner back fat and omental fat (Figs. 1B and 1B') showed a similar distribution of CLA isomers as that found in the dietary CLA oil.

Total CLA content in tissue lipid classes. The total CLA content in all the tissue lipid classes is shown in Table 2 (last column). The total CLA content was highest in liver TAG (6%) and lowest in liver SM (1%). Back fat showed a higher accumulation of total CLA than omental fat (Table 2). The major phospholipids, PC and PE (phosphatidylethanolamine), incorporated between 2.8 and 5.8% of total CLA. The remaining phospholipids incorporated between 1.5 and 4% of total CLA. DPG, which is a major phospholipid in inner mitochondrial membranes of liver and heart, accumulated about

TABLE 2
Relative Composition (%) Determined by GC of CLA Isomers[a] in Diet, Liver, and Heart Lipid Classes, and Inner Back Fat and Omental Fat of Pigs Fed a CLA Diet

	CLA isomers										
Tissues Diet Classes[b]	9*c*,11*t* 8*t*,10*c*	9*t*,11*c*	11*c*,13*t*	10*t*,12*c*	8*c*,10*c*	9*c*,11*c*	10*c*,12*c*	11*c*,13*c*	11*t*,13*t*	10*t*,12*t* 9*t*,11*t* 8*t*,10*t*	Total CLA (%)[c]
Diet	35.3	0.8	19.9	24.9	1.2	2.7	3.5	1.6	1.5	8.7	32.9
Liver											
TAG	33.2 ± 1.2[d]	1.6 ± 0.3	21.6 ± 1.4	16.0 ± 1.2	2.5 ± 0.6	3.8 ± 0.7	2.1 ± 0.2	2.1 ± 0.1	4.4 ± 0.3	11.7 ± 1.1	6.0 ± 0.4
PC	48.7 ± 1.4	1.6 ± 0.4	16.1 ± 1.0	15.2 ± 1.0	1.4 ± 0.2	4.3 ± 0.5	0.9 ± 0.2	1.9 ± 0.2	4.1 ± 0.5	4.8 ± 0.4	3.2 ± 0.2
PE	53.9 ± 2.7	1.4 ± 0.4	15.5 ± 1.2	9.4 ± 0.7	1.7 ± 0.2	1.8 ± 0.3	1.0 ± 0.3	1.8 ± 0.3	5.4 ± 0.8	6.4 ± 0.5	2.8 ± 0.2
PS	39.9 ± 3.0	1.1 ± 0.6	11.9 ± 1.0	9.7 ± 1.3	1.3 ± 0.9	3.4 ± 1.4	0.6 ± 0.2	1.7 ± 0.7	11.4 ± 2.5	10.2 ± 0.8	1.6 ± 0.2
PI	38.3 ± 4.2	4.0 ± 1.2	12.1 ± 1.1	8.2 ± 0.9	3.4 ± 1.0	5.1 ± 1.1	1.3 ± 0.3	2.6 ± 0.4	6.2 ± 1.0	15.0 ± 1.9	1.9 ± 0.2
DPG	17.9 ± 1.2	1.2 ± 0.9	36.9 ± 5.8	8.1 ± 2.3	1.7 ± 1.1	3.9 ± 1.1	0.9 ± 0.7	3.5 ± 1.1	6.2 ± 1.3	11.5 ± 3.4	1.5 ± 0.2
SM	25.3 ± 2.9	5.0 ± 0.8	10.4 ± 1.1	10.4 ± 1.1	—[e]	1.2 ± 0.6	1.7 ± 0.4	3.6 ± 0.7	9.8 ± 0.2	31.1 ± 3.4	1.0 ± 0.1
FFA	19.0 ± 2.7	3.4 ± 0.7	11.1 ± 1.6	14.5 ± 1.1	2.7 ± 0.7	4.3 ± 0.4	2.3 ± 0.5	3.8 ± 0.5	7.7 ± 0.8	23.1 ± 2.5	2.9 ± 0.2
CE	31.1 ± 2.2	3.3 ± 1.4	15.4 ± 1.1	13.7 ± 1.5	1.8 ± 0.9	3.8 ± 1.8	1.4 ± 0.7	2.0 ± 0.9	6.7 ± 2.4	17.9 ± 3.2	3.5 ± 0.4
Heart											
TAG	26.5 ± 3.5	2.1 ± 0.7	25.7 ± 4.3	12.1 ± 0.6	1.5 ± 0.4	4.8 ± 0.5	2.1 ± 0.1	4.0 ± 0.5	7.0 ± 1.0	12.6 ± 1.8	3.6 ± 0.4
PC	25.1 ± 2.1	0.7 ± 0.2	55.5 ± 4.3	6.2 ± 0.3	0.4 ± 0.2	1.4 ± 0.3	0.7 ± 0.1	3.7 ± 0.5	3.3 ± 0.9	2.5 ± 1.1	5.8 ± 0.4
PE	29.7 ± 2.4	0.8 ± 0.3	42.2 ± 3.8	6.8 ± 0.8	1.0 ± 0.3	1.3 ± 0.3	0.8 ± 0.2	3.0 ± 1.1	6.9 ± 1.1	5.0 ± 1.0	2.8 ± 0.2
PS	24.1 ± 4.5	0.7 ± 0.3	34.3 ± 3.1	6.5 ± 1.3	2.4 ± 1.3	3.5 ± 0.7	0.5 ± 0.2	4.6 ± 0.7	10.2 ± 0.9	8.5 ± 1.0	4.1 ± 0.5
PI	18.7 ± 2.4	1.6 ± 0.5	40.0 ± 3.6	8.3 ± 2.4	2.3 ± 0.4	3.1 ± 0.4	1.1 ± 0.3	4.7 ± 0.5	9.5 ± 2.7	9.3 ± 1.4	3.6 ± 0.3
DPG	7.2 ± 1.4	1.1 ± 0.5	65.1 ± 4.8	4.5 ± 1.0	0.1 ± 0.1	2.0 ± 0.4	0.3 ± 0.02	4.8 ± 0.4	9.6 ± 1.1	4.4 ± 2.2	1.9 ± 0.2
SM	14.8 ± 5.5	<0.05	38.3 ± 9.3	6.1 ± 2.1	—[e]	1.7 ± 0.6	<0.05	4.0 ± 1.1	15.8 ± 5.7	19.3 ± 8.0	1.8 ± 0.5
FFA	10.9 ± 1.4	2.0 ± 1.2	25.1 ± 3.0	10.7 ± 1.1	0.6 ± 0.3	4.9 ± 0.4	0.9 ± 0.5	6.8 ± 0.7	13.4 ± 1.4	17.8 ± 3.6	3.2 ± 0.2
Back fat	38.7 ± 5.2	1.9 ± 0.3	20.5 ± 3.4	17.1 ± 3.2	1.2 ± 0.3	3.1 ± 0.5	2.5 ± 0.5	1.7 ± 0.3	2.0 ± 0.4	10.7 ± 1.5	4.7 ± 0.4
Omental fat	36.1 ± 1.4	1.8 ± 0.7	20.7 ± 3.6	17.9 ± 3.2	1.1 ± 0.6	3.5 ± 0.4	2.8 ± 0.4	1.8 ± 0.4	2.1 ± 0.3	11.6 ± 1.8	2.9 ± 0.5

[a]The identification of the major CLA isomers was confirmed by spectroscopic analyses and comparison with Ag^+-HPLC. The peak between 11*c*,13*c*-18:2 and 11*t*,13*t*-18:2, labeled "x" in Figures 1A and 2A, was not included because it was also present in controls.
[b]For abbreviations see Table 1.
[c]Percentage of total CLA isomers in the total fatty acid methyl ester composition of each lipid class.
[d]Values are means ± SD (n = 8).
[e]The concentration of 8*c*,10*c*-18:2 could not be quantitated in the SM fraction using this GC column because this CLA isomer coeluted with 21:0 present in SM.

2% of total CLA. The highest relative content of *trans,trans* CLA isomers was found in the FFA fraction (Table 2); see liver FFA chromatogram (Figs. 2D and 2D').

Relative CLA isomeric distribution in liver lipids. The distribution and standard deviation (SD) data for CLA isomers determined by GC are presented in Table 2. The relative composition of CLA isomers in liver lipids is summarized graphically in Figure 3 in order to compare it to that present in the dietary CLA oil used. The sum of 9 *cis*,11 *trans*-18:2 and 8 *trans*,10 *cis*-18:2 was increased in liver PC and PE and decreased in DPG, SM, and FFA. The neutral lipids (TAG and CE) and the minor phospholipids (phosphatidylserine and phosphatidylinositol) showed no selectivity in the incorporation of these two CLA isomers compared to the diet. No further conclusions could be made on these data because these two CLA isomers were not separated by GC. To evaluate the effects of each of these two individual isomers, see the Ag^+-HPLC results (Fig. 4). On the other hand, 11 *cis*,13 *trans*-18:2 accumulated in DPG, where its relative concentration increased to 40%, compared to about 20% in the CLA oil. The 10 *trans*,12 *cis*-18:2 isomer was relatively low in all liver lipids compared to its level in the diet. The content of the four

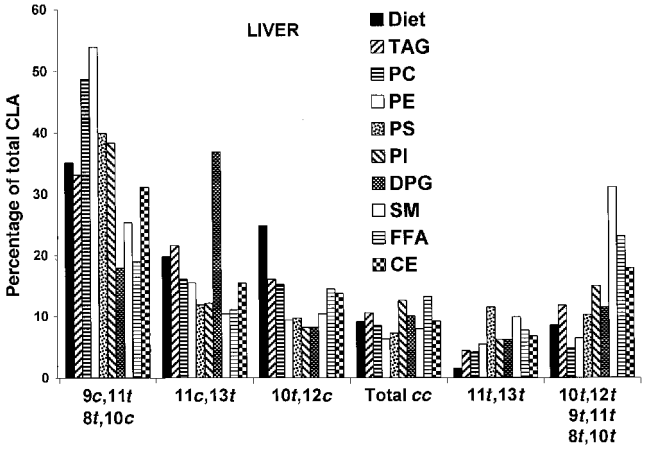

FIG. 3. Quantitative comparison of CLA isomeric distributions determined by GC for the different liver lipid classes that had been previously isolated by thin-layer chromatography (TLC). Relative amounts (*y* axis) are expressed as percentages of total CLA. The CLA isomers are shown (*x* axis) in the GC elution sequence. Total *cc* represents the total of four minor *cis,cis* CLA isomers. The distribution of the CLA isomers in the diet is included for comparison. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; CE, cholesteryl ester. For other abbreviations see Figures 1 and 2.

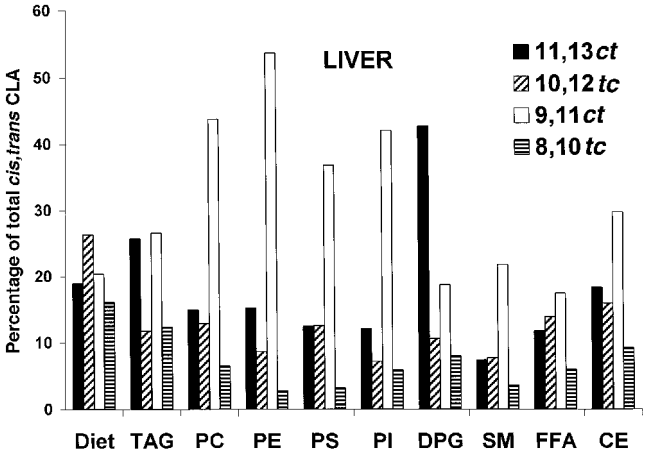

FIG. 4. Quantitative comparison of the four major *cis/trans* CLA isomers in the different liver lipid classes and the diet determined by Ag^+-HPLC. Relative amounts (*y* axis) are expressed as percentage of total *cis/trans* CLA isomers. The distribution of the *cis/trans* CLA isomers in the diet are included for comparison. For abbreviations see Figures 1–3.

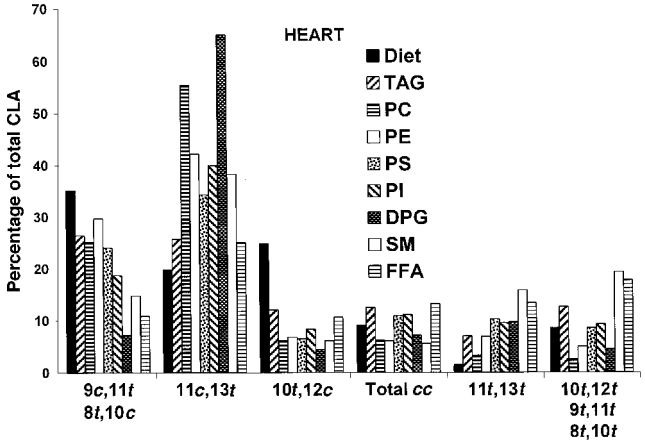

FIG. 5. Quantitative comparison of CLA isomeric distribution determined by GC for the different heart lipid classes that were previously isolated by TLC. For abbreviations see Figures 1–3.

cis,cis CLA isomers was low in liver lipids. Their total concentrations were combined for presentation in Figure 3. The *trans,trans* isomers appeared to accumulate in liver lipids: 11 *trans*,13 *trans*-18:2 into phosphatidylserine and SM, and the mixture of the three *trans,trans* CLA (10,12-, 9,11-, and 8,10-18:2) isomers into SM, FFA, and CE (Fig. 3). The increase of *trans,trans* CLA isomers in SM was due to the acid (HCl/methanol) methylation (29) required to hydrolyze the *N*-acyl fatty acids.

The distribution of the major *cis/trans* CLA isomers in all the liver lipid classes, identified by Ag^+-HPLC, is presented in Figure 4. The results indicate that 9 *cis*,11 *trans*-18:2 was the major isomer in all liver lipids, except DPG, which showed a marked increase in 11 *cis*,13 *trans*-18:2. Furthermore, most liver lipid classes showed markedly lower levels of 8 *trans*,10 *cis*-18:2, and in some lipid classes lower levels 10 *trans*, 12 *cis*-18:2 compared to the distribution found in the CLA diet.

Relative CLA isomeric distribution in heart lipids. Contrary to the liver lipids, the heart showed a high content of the 11 *cis*,13 *trans*-18:2 CLA isomer in all lipid classes, except TAG, as determined by both GC (Fig. 5) and Ag^+-HPLC (Fig. 6). The accumulation was highest in cardiac DPG, in which the relative concentration of the 11 *cis*,13 *trans*-18:2 isomer reached 77% of the total CLA (HPLC results). The second major isomer in heart lipids was 9 *cis*,11 *trans*-18:2. The heart lipids showed much lower levels of 8 *trans*,10 *cis*-18:2 and 10 *trans*,12 *cis*-18:2 compared to those found in the CLA diet (Fig. 6). The content of the *cis,cis* CLA isomers was not increased in the heart (Fig. 5), whereas the *trans,trans* CLA isomers appeared to accumulate relative to the CLA diet, especially in heart FFA (Fig. 5). Again the *trans,trans* content in the SM fractions should be viewed with caution because SM had to be methylated with HCl/methanol (29).

DISCUSSION

Improved separation of CLA isomers. By using optimal conditions, the long polar GC capillary column (100 m SP-Sil 88) separated 10 peaks attributed to CLA (Figs. 1 and 2). Some isomers coeluted, and their complete assignments were not possible, even using GC-DD-FTIR and GC-EIMS. However, identification of the CLA isomers became possible after the development of a new Ag^+-HPLC method (30). Ag^+-HPLC was used to separate *trans,trans*, *cis/trans*, and *cis,cis* CLA positional isomers found in the diet and pig tissues (Figs. 1 and 2). The CLA isomers were identified by comparison to known CLA mixtures and by GC-DD-FTIR and GC-EIMS as their DMOX derivatives (30). Some GC assignments were also confirmed by spiking the samples with known CLA standards. A systematic comparison of the relative intensities of the CLA isomers in the Ag^+-HPLC and GC chromatograms permitted the identification of the isomers resolved by GC. A good example was the one for heart lipids in which the

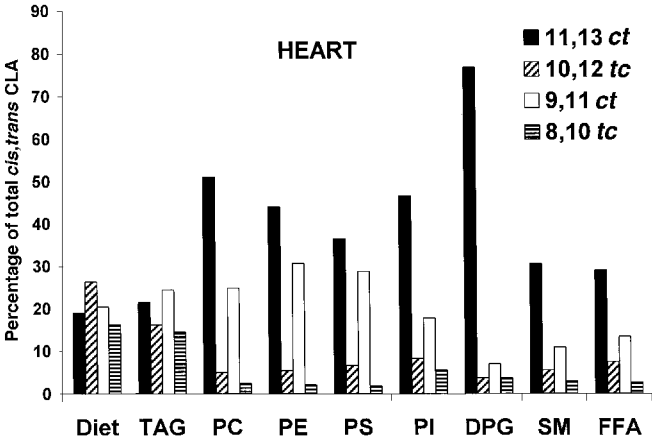

FIG. 6. Quantitative comparison of the four major *cis/trans* CLA isomers in the different heart lipid classes and the diet determined by Ag^+-HPLC. For abbreviations see Figures 1–3.

trans,trans, *cis/trans*, and *cis,cis* 11,13-18:2 were the major CLA isomers in both the Ag^+-HPLC (Fig. 2B') and GC (Fig. 2B) chromatograms.

Previous studies reporting the CLA content in animal tissues have not adequately resolved the CLA isomers, which is now possible using the Ag^+-HPLC method (30). The literature results were presented as 9 *cis*,11 *trans*-18:2 (1,2), total CLA (5,39), or total CLA in total neutral lipids and total phospholipids without (7,26) or with (27,28) some isomer identification. In one study the distribution of CLA isomers in the liver lipid classes was reported (28). Furthermore, in many studies, acid-catalyzed methylation procedures were used (1,2,5,7,13,16,18,20,26,28) to prepare FAME for GC analysis, which generally increased the content of *trans,trans* CLA isomers due to double-bond isomerization (29). That left much of the data on CLA incorporation difficult to interpret other than a general knowledge of total CLA levels. For example, Sugano *et al.* (28), who recently reported the CLA distribution in liver lipid classes of rats fed a 1% CLA preparation for 2 wk, methylated the isolated lipid classes with BF_3/methanol, which significantly increased the *trans,trans* CLA content in all lipid classes. Therefore, the reported CLA composition likely included artifacts: (i) the *trans,trans* isomers were reported as the major isomer in DPG (or cardiolipin), phosphatidylserine, phosphatidylinositol, and PE (28), and (ii) we suspect that one CLA isomer, 9 *cis*,11 *cis*-18:2, was mislabeled in liver DPG (Ref. 28, Fig. 2E). Based on our data (Fig. 2 C and C'), it is presumably 11 *cis*,13 *trans*-18:2. However, as noted earlier, the incorporation of total CLA isomers into hepatic phospholipids, which in their study ranged from 0.8 to 4.5% (28), was generally similar in magnitude to that found in pig liver phospholipids (1.0 to 3.2%, Table 2).

Distributions of CLA isomers into pig tissue lipids. This is the first report showing the accurate separation of CLA isomers found in individual tissue lipid classes from test animals fed CLA. Work is still in progress on the identification of CLA metabolites in pig tissues, and hence was not included in this publication. The total CLA content in pig tissue lipids ranged from 1 to 6%, depending on the tissue and lipid class (Table 2). Generally, the CLA content was higher in the neutral lipids such as TAG, CE, and FFA (3–6%) than in phospholipids such as SM and DPG (1–2%). However, the changes in tissue CLA incorporation appeared to have had no effect on the quantitative lipid class composition in the liver and heart of pigs fed this CLA mixture, except possibly cardiac TAG (Table 1).

Our results indicate that the relative absorption of all the CLA isomers was similar, since there was generally no difference between the distribution of CLA isomers in the commercial CLA preparation fed to pigs and in inner back fat, omental fat, liver TAG, and heart TAG. Sugano *et al.* (28) measured the lymphatic recovery and CLA isomeric composition of the lymph fluid in the rat and found that total CLA was absorbed less (~55%) than linoleic acid (70–80%). However, the composition data included a high *trans,trans* content, presumably generated during methylation with BF_3/methanol. By ignoring their *trans,trans* content in the dietary emulsion and the lymph fluid, the relative distribution of the remaining CLA isomers was similar before and after absorption, which is consistent with our results. The feeding of CLA was reported to decrease subcutaneous fat and increase lean meat (11,12). However, this physiological change was not accompanied by any change in the distribution of CLA isomers in the inner back fat (subcutaneous fat).

Liver and heart each showed a uniquely characteristic distribution of CLA isomers even though both tissues received a generally similar CLA distribution, as evidenced by their respective TAG composition. The reason for the observed differences in relative accumulation of the different CLA isomers in the tissues lipid classes is not clear. The tissue fatty acid composition is a dynamic system constantly receiving, metabolizing, oxidizing, and incorporating dietary fatty acids. The accumulation of 11 *cis*,13 *trans*-18:2 could be due to slower metabolism of this isomer or preferential incorporation. On the other hand, the low content of both 10 *trans*,12 *cis*-18:2 and 8 *trans*,10 *cis*-18:2 could be due to rapid metabolism or selective discrimination. Metabolites of CLA isomers were shown to occur in animal tissues (40,41). In fact, Sébédio *et al.* (42) found that 10,12-18:2 was metabolized to 8,12,14-20:3 and 5,8,12,14-20:4 in essential fatty acid-deficient rats. This would indicate that 10 *trans*,12 *cis*-18:2 was metabolized.

The contents of FFA in both liver and heart lipids were less than 3 and 1%, respectively (Table 1). However, in the FFA fractions, the relative concentrations of the *trans,trans* CLA isomers were more than 30% of the total CLA isomers (Fig. 2D') compared to 10% in the diet. These results suggest that the *trans,trans* CLA isomers were metabolized more slowly than the corresponding *cis/trans* CLA isomers. This result is similar to that observed when rats were fed a diet rich in erucic acid, which accumulated in the cardiac FFA fraction (31). This was partially due to the slower rate of metabolism of erucic acid compared to other fatty acids (43).

Significance of the unique distribution of CLA isomers into pig tissue lipids. The information available to date does not permit drawing a definitive conclusion regarding the favorable or adverse biological activity of any of the CLA isomers. To our knowledge, there are no studies in which individual synthetic CLA isomers have been evaluated in biological systems. The only study in which a natural extract (from fried ground beef) was used exhibited anticarcinogenic properties (44).

If 9 *cis*,11 *trans*-18:2 is assumed to be the active CLA isomer, then the incorporation/accumulation of the 11 *cis*,13 *trans*-18:2 CLA isomer into heart phospholipids, and particularly DPG in both heart and liver, could be viewed with concern. DPG is found principally in the inner mitochondrial membrane and is intrinsically involved in many of the enzymes of bioenergetics of mitochondria; see reviews (45,46). The incorporation of 11 *cis*,13 *trans*-18:2 into mitochondrial DPG could adversely affect the activity of key enzymes in mitochondrial energetics, because DPG is firmly imbedded in

many of these enzymes (45). A marked decrease in the linoleic acid content of inner mitochondrial DPG could also affect enzyme activity. In fact, Sugano *et al.* (28) showed that feeding CLA to rats produced a significant decrease of linoleic acid from 69.6 to 54.7% in liver DPG. However, we did not find a change in the content of linoleic acid in pig liver (control 75% vs. CLA 73%) or heart (control 85% vs. CLA 86%) DPG fed the CLA diet (the complete fatty acid composition will be published elsewhere). We also did not find any gross pathological abnormalities in this pig study (12). It should be noted that specific mitochondrial functions have not yet been evaluated.

The large epidemiological study conducted in Finland (19) indirectly supports the conclusion that 9 *cis*,11 *trans*-18:2 is the active isomer, because this is the major CLA isomer in milk. We have reexamined milk and dairy products using the new Ag^+-HPLC method (30) and confirmed that 9 *cis*,11 *trans*-18:2 is the major CLA isomer in milk (Sehat, N., private communication). Based on this assumption, it might be prudent to revise commercial CLA preparations to exclude the 11 *cis*,13 *trans*-18:2 CLA isomer as a major component.

On the other hand, if we assume that 9 *cis*,11 *trans*-18:2 is not the only active isomer in CLA preparations, then the incorporation of 11 *cis*,13 *trans*-18:2 into cardiac and DPG lipids (this study), and/or the desaturation and elongation of 10 *trans*,12 *cis*-18:2 in essential fatty acid-deficient rats (42), could be viewed as beneficial. Certainly many of the feeding trials, which included several CLA isomers, have also shown anticarcinogenic (1–8) and other beneficial responses (9–13). Pariza *et al.* (47) reported that 9 *cis*,11 *trans*-18:2 was metabolized to 11 *cis*,13 *trans*-20:2, and the latter exhibited similar biological activity to that of CLA isomeric mixtures. This is most interesting, since the configuration of 11 *cis*,13 *trans*-20:2 from the carboxyl group to the conjugated system is identical to that of 11 *cis*,13 *trans*-18:2.

There is a definite need for further feeding studies using pure CLA isomers to clarify these issues.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Robert C. Fouchard, Anthony Martyres, and David J.H. Kramer at Agriculture and Agri-Food Canada, Ottawa. We also thank Sigrid Windsor, Lavern Holt-Klimec, David Best, Fran Costello, Ivy Larsen, and Rhona Thacker at the Lacombe Research Center for handling and feeding the pigs and for collecting biological materials. The feeding study was supported by the Alberta Pork Producers Development Corporation, and the Agriculture and Agri-Food Research Branch Matching Investment Initiative. Contribution number S004 from the Southern Crop Protection & Food Research Center.

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[Received February 13, 1998, and in final revised form and accepted April 30, 1998]

Dietary Docosahexaenoic Acid and Immunocompetence in Young Healthy Men

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ABSTRACT: The purpose of this study was to examine the effect of dietary docosahexaenoic acid (DHA), in the absence of eicosapentaenoic acid, on human immune response (IR). A 120-d study with 11 healthy men was conducted at the Metabolic Research Unit of the Western Human Nutrition Research Center. Four subjects (control group) were fed the stabilization or basal diet (15, 30, and 55% energy from protein, fat, and carbohydrate, respectively) throughout the study; the remaining seven subjects (DHA group) were fed the basal diet for the first 30 d, followed by 6 g DHA/d for the next 90 d. DHA replaced an equivalent amount of linoleic acid; the two diets were comparable in their total fat and all other nutrients. Both diets were supplemented with 20 mg d-α-tocopherol acetate per day. Indices of IR were examined on study day 22, 30, 78, 85, 106, and 113. Addition of DHA at moderately high levels did not alter the proliferation of peripheral blood mononuclear cells cultured with phytohemagglutinin or concanavalin A, or the delayed hypersensitivity skin response. Also, additional DHA did not alter the number of T cells producing interleukin 2 (IL2), the ratio between the helper/suppressor T cells in circulation, or the serum concentrations of immunoglobulin G, C3, and interleukin 2 receptor (IL2R). DHA supplementation, however, caused a significant ($P = 0.0001$) decrease in the number of circulating white blood cells which was mainly due to a decrease in the number of circulating granulocytes. The number of lymphocytes in peripheral circulation was not affected by Dietary DHA enrichment, but the percentage of lymphocytes in white blood cells increased because of a reduction in granulocyte numbers. None of these indices was changed in the control group. Our results show that when total fat intake is low and held constant, DHA consumption does not inhibit many of the lymphocyte functions which have been reported to be inhibited by fish oil consumption.
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Both the amount and type of dietary fat modulate human immune response (IR). A reduction in total fat intake enhances several indices of IR, and the converse occurs with an increase in dietary fat (1–4). A moderate increase in the consumption of the n-6 polyunsaturated fatty acids (PUFA), linoleic acid (LA), or arachidonic acid did not lower the human IR when total fat intake was maintained constant (1,2,5). But n-6 PUFA lowered human IR when its addition to the diet caused an increase in the total fat intake (4). The addition of n-3 PUFA from fish oil or flaxseed oil also reduced several aspects of the human IR (6–19). Since total fat intake was not kept constant in most of these studies, it is difficult to determine if the decline in IR was due to the amount or the type of fat, or a combination of the two factors. Because fish oils cause a decrease in IR, their use has been explored in the management of autoimmune disorders (20–24).

Fish oils contain a variable mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), along with many other fatty acids, cholesterol, heavy metals, and chlorinated hydrocarbons. It is not known if the decline in IR caused by the fish oils is due to EPA, DHA, both, or some other factors. Purified esters of EPA lowered several aspects of the IR in humans (13,14,19). *In vitro* studies conducted with human (25) and rat lymphocytes (26) and *in vivo* studies in rats (27) showed that DHA esters/salts also inhibit immune cell functions. Until recently, purified DHA was not available for human use, and no studies regarding its effects on human IR have been done. Studies with DHA are important, because it is the major n-3 fatty acid in tissues, and the body tends to conserve it over EPA. Such studies are now possible with the availability of DHASCO^{TM} oil (a gift from Martex Corporation, Columbia, MD), which contains 40% DHA as natural triacylglycerol.

The purpose of this study was to examine the effect of a moderately high intake (6 g/d) of DHA (in the absence of EPA) on human immunocompetence, while dietary fat was held constant. To avoid increased oxidative stress from the consumption of DHA, 20 mg d-α-tocopherol acetate was added to the diets daily. We examined the effect of feeding DHASCO^{TM} oil (15 g/d) for 83 d on several indices of immune status [complete and differential blood cell count, lymphocyte phenotypic analysis and *in vitro* proliferation, serum immunoglobulin G (IgG), C3, and interleukin 2 receptor (IL2R)] in healthy men. In previous studies with dietary fish oil these indices were found to be altered.

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Abbreviations: Bq, Becquerel; CD, cluster designation; Con A, concanavalin A; CTL, cytotoxic T; DHA, docosahexaenoic acid; DTH, delayed type hypersensitivity skin response; EPA, eicosapentaenoic acid; IL, interleukin; IR, immune response; LA, linoleic acid; LTB_4, leukotriene B_4; PBMNC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acid; WBC, white blood cell.

MATERIALS AND METHODS

Subjects and study design. The study protocol was approved by the Human use committees of the University of California at Davis, and the USDA committee (Houston, TX). Twelve healthy men were selected for the study after a physical and clinical examination by a licenced physician. The study lasted for 120 d (April 1 to July 29, 1996) although no immunological tests were conducted after d 113, because of other scheduled procedures. Subjects lived at the Metabolic Research Unit of the Western Human Nutrition Research Center for the duration of the study, except when going for daily walks (2 × 2 miles) or other scheduled outings. They consumed only those foods prepared by the staff of the Metabolic Research Unit, and were under constant supervision. Subjects were divided into two groups: a control group of four was fed the stabilization or basal diet for all 120 d of the study; the remaining eight subjects (DHA group) were fed the basal diet for the first 30 d of the study and a DHA-supplemented diet for the last 90 d. One subject from the DHA group did not complete the study; hence, data from only seven subjects were evaluated to determine the effects of DHA on IR.

Both diets were intended to provide 15, 30, and 55% energy from protein, fat, and carbohydrates, respectively. As shown in Table 1, the analyzed compositions of each of the two diets were not different from one another or from the planned values. Diets composed of natural foods, except DHA and vitamin E, were fed in a 5-d rotating menu, comprising three meals and a post-dinner snack. Body weights of the subjects were maintained within 2% of their initial weights throughout the study, by adjusting their energy intake if necessary. For the DHA-supplemented group, 15 g of DHASCO^{TM} oil was incorporated by replacing an equivalent amount of safflower oil; all other foods were identical between the two diets. The DHASCO^{TM} oil, purified from an alga, contains 40% DHA as triacylglycerol. It also contains 12:0 (3.8%), 14:0 (13.5), 16:0 (11.5%), 18:0 (1.2%), 18:1n-9 (27.9), and the balance as minor components. The main effect of incorporating 15 g DHASCO^{TM} oil by replacing an equivalent amount of safflower oil was to replace 6 g linoleic acid (LA) with the same amount of DHA. The DHASCO^{TM} oil was kept in sealed containers at –20°C, and all open bottles were flushed with nitrogen before returning to the refrigerator. It was served only in cold foods such as yogurt, dips, or salads. Thus, the chances of DHA oxidation were minimized. Dietary composites from each of the 5-d menus for both diets were analyzed for macronutrients. The micronutrient content of the diets was calculated using USDA Handbook 8 (28); all nutrients were at or above the recommended dietary allowances and were not different between the two diets. Diets contained about one recommended dietary allowance of vitamin E from natural foods (calculated using values from USDA Handbook 8) and were supplemented with an additional 20 mg/d of d-α-tocopherol acetate (Bronson Pharmaceutical, St. Louis, MO).

TABLE 1
Nutrient Composition of Experimental Diets[a]

	Energy %	
Nutrient	DHA diet	Control diet
Protein	15.2 ± 1.3	14.6 ± 1.2
Carbohydrate	53.9 ± 6.0	56.4 ± 6.8
Fat, total	30.9 ± 4.3	29.0 ± 3.8
Saturated	8.9 ± 0.9	8.1 ± 0.9
Monounsaturated	9.2 ± 0.7	9.2 ± 0.8
n-6 Polyunsaturated	6.5 ± 0.8	8.5 ± 0.7
n-3 Polyunsaturated	2.8 ± 0.1	1.1 ± 0.1
Trans	1.9 ± 0.3	2.1 ± 0.3
P/S ratio	1.2	1.1
Cholesterol (mg/d)	360	360

[a]Data shown are the mean ± SD of five dietary composites from five different menu days. Polyunsaturated/saturated (P/S) ratio and cholesterol levels are calculated and not analyzed; hence, SD is not shown for these variables. DHA, docosahexaenoic acid.

Laboratory procedures. Blood samples were collected between 7: 00 and 8:00 A.M. after an overnight fast on study days 22, 30, 78, 85, 106, and 113, by antecubital venipuncture into evacuated tubes containing heparin for cell culture experiments, EDTA for blood cell count and phenotypic analysis, and no anticoagulant for preparation of sera.

Blood cell count and lymphocyte phenotypic analysis. For each blood draw, a complete and differential cell count was performed by using a Serono Baker Automated system (model 9000 diff; Allentown, PA). Phenotypic analysis for B (CD19+), T (CD3+), helper (CD3+, CD4+), suppressor (CD3+, CD8+), natural killer (CD3-,CD16+, 56+), and cytotoxic T (CTL; CD3+, CD16+, 56+) cells was done by using Becton-Dickinson FACStar flow cytometer (San Jose, CA) as previously reported (5). The numbers of lymphocytes producing IL2 were also determined with the flow cytometer, using instructions and reagents provided by Becton-Dickinson. The lymphocytes were activated by mixing 500 μL of whole blood with 500 μL RPMI-1640, containing 10 μg brefeldin A, 25 ng phorbol 12-myristate 13-acetate, and 1 μg ionomycin. An unstimulated tube containing only blood and brefeldin was used as a control. The tubes were incubated at 37°C in 5% CO_2 for 4 h. The T-cell surface antigen (CD3+) was stained for 30 min with R-phycoerythrin-Cyanin5-CD3 monoclonal antibody. The cells were incubated for 10 min in Facs Lysing Solution to lyse the red cells. The cells were then centrifuged and resuspended in Facs Permeabilizing Solution. After a 10-min incubation period, 2 mL of phosphate buffered saline was added, the cells centrifuged, and the supernatant discarded. The pellet was stained with fluorescent-conjugated intracellular IL2 monoclonal antibody and incubated for 30 min. The cells were washed once with phosphate buffered saline and resuspended in 1% paraformaldehyde. The number of IL2-producing cells was analyzed with a flow cytometer with gating on the CD3+ cells.

Proliferation of peripheral blood mononuclear cells (PBMNC). PBMNC were isolated using Histopaque-1077 as previously reported (5,6) and cultured in RPMI-1640 containing 10% autologous serum. One hundred microliters of the culture medium containing 1×10^5 PBMNC were inoculated

in each well of a 96-well flat-bottom culture plate. An additional 100 μL of the culture medium with or without the mitogens was added to each well. The mitogens used were phytohemagglutinin (PHA) and concanavalin A (Con A). Both PHA and Con A were used at three concentrations; final concentrations (mg/L) in the culture media were 2.5, 5, and 10. PBMNC were cultured for a total of 72 h; [^{3}H] thymidine, 37 K becquerel (Bq), in 50 μL, was added to each well during the last 24 h. PBMNC were collected on filter strips, and the radioactivity was determined using a Packard β-gas counter. [^{3}H] thymidine incorporation into cellular DNA (Bq/1000 cells) was used as the index of PBMNC proliferation.

Delayed type hypersensitivity (DTH) skin response and the serum IgG, C3, and IL2R. DTH response to seven recall antigens was assayed by intradermally injecting 0.1 mL of each antigen solution into the forearm. The antigens used were tuberculin purified-protein derivative (1 international test unit), mumps (4 complement-fixing test units), tetanus toxoid (1:100, vol/vol dilution of a solution containing 4 flocculation units/0.5 mL), candida (1:100, vol/vol dilution), trichophyton (1:30, vol/vol dilution), streptokinase streptase (100 KU/L), and coccidioidin (bioequivalent to U.S. reference coccidioidin 1:100, provided by the Office of Biologics, Food and Drug Administration, Washington, DC). The antigens were diluted with a diluent containing, per liter, 3 mL normal human serum and 9 g sodium chloride. Tuberculin purified-protein derivative, mumps, and tetanus toxoid were supplied by Connaught Laboratories Inc. (Swiftwater, PA). Candida (Dermatophyton O), trichophyton, and the antigen diluent were obtained from Hollister Stier (Spokane, WA). Streptokinase streptase and coccidioidin were purchased from Behringwereke A.G. (Marburg/Lahn, Germany, and Berkeley Biologicals (Berkeley, CA), respectively. Response to these antigens was determined by measuring mean induration diameters (mm) at 48 h after injections. Induration diameters with less than 4 mm were scored negative. Data are reported as the mean sum of induration diameters for all positive responses (induration score) and the number of positive responses to the seven antigens (antigen score).

Serum IgG and C3 concentrations were determined using a nephelometer as previously reported (6). Serum IL2R was quantified by using an ELISA kit purchased from Immunotech (Westbrook, ME).

Data analysis. For both dietary groups, two determinations of IR were made at the end of stabilization, middle, and end of the intervention periods (d 23, 30, 78, 85, 106, and 113). Means of the two measurements are shown in the Results section. A repeated measure of analysis of variance model was used to determine the effects of DHA on the indices of IR tested. A univariate, split-plot approach was taken using SAS PROC MIXED (29). Contrasts were constructed for comparisons among the stabilization and intervention periods, and in cases for which responses were linear, the control and DHA group slopes were compared. Changes in the parameters examined are considered significant for $P < 0.05$ unless otherwise stated.

RESULTS

The mean ± SEM for age (yr), weight (kg), and body mass index (kg/m^2) for subjects in DHA group ($n = 7$) and control group ($n = 4$) were 33.1 ± 1.8, 78.6 ± 4.2, 23.7 ± 0.8, and 33.3 ± 3.1, 74.7 ± 4.2, 23.1 ± 1.1, respectively. None of these physical characteristics was different between the two groups.

The average daily energy intake for all subjects was 11.7 MJ (2800 Kcal), and was not different between the two groups. The total fat intake was also similar between the two groups. Fatty acid composition of the two diets is shown in Table 2. Fatty acids other than DHA, linoleate, laurate, and myristate were not different between the two diets. The DHA diet contained 6.5 wt% of DHA, while the control diet contained less than 0.1% of it. Since DHA was incorporated by replacing equivalent amount of LA, the control diet contained 6.7% more LA than the DHA diet. The laurate and myristate contents of the DHA diet were approximately twice their levels in the basal diet, but they were still only 1.3 and 4.3% of the total fatty acids, respectively.

Effect of DHA on circulating white blood cells (WBC). The number of total circulating WBC, along with their differentiation into granulocytes or polymorphonuclear leukocytes (PMN), monocytes, lymphocytes, and various subsets of lymphocytes is shown in Table 3. This table shows that in the DHA group the number of circulating total WBC decreased by 10%, from the end of stabilization period (day 30) to the end of the intervention period (day 113). This decrease in WBC numbers was statistically significant ($P = 0.0001$). The decrease in WBC in the DHA group was primarily due to a decrease in the number of PMN, which were reduced by 21% ($P = 0.0001$). The decrease in the number of circulating PMN

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets[a]

FAME	DHA diet	Control diet
12:0 (laurate)	1.3 ± 0.1	0.7 ± 0.1*
14:0 (myristate)	4.3 ± 0.3	2.2 ± 0.4*
16:0 (palmitate)	16.6 ± 0.7	16.3 ± 0.7
16:1n-9	0.7 ± 0.2	0.9 ± 0.2
18:0 (stearate)	7.1 ± 0.5	7.5 ± 0.4
18:1*t*, all isomers	6.2 ± 0.5	7.0 ± 0.5
18:1n-9 (oleate)	26.6 ± 0.8	26.0 ± 0.7
18:1n-7	1.7 ± 0.1	2.0 ± 0.1
18:1n-5	1.5 ± 0.2	2.1 ± 0.4
18:2*tt* & 19:0	0.5 ± 0.1	0.6 ± 0.1
18:2n-6 (linoleate)	21.6 ± 1.2	28.3 ± 1.0*
18:3n-3 (linolenate)	2.6 ± 0.2	3.2 ± 0.1
22:0 (behenate)	0.2 ± 0.0	0.2 ± 0.0
20:5n-3 (eicosapentaenoate)	0.4 ± 0.1	0.3 ± 0.1
22:6n-5 (docosahexaenoate)	6.5 ± 0.22	>0.1 ± 0.1*
Total	98.0 ± 0.4	97.2 ± 0.3
Unknowns	2.0 ± 0.2	2.8 ± 0.1

[a]Data shown are mean ± SEM ($n = 5$). *Significantly different between two diets ($P < 0.05$). FAME, fatty acid methyl esters. See Table 1 for other abbreviation.

TABLE 3
Effect of DHA Feeding on Circulating White Blood Cells[a]

	DHA group (n = 7)		Control group (n = 4)	
Cell type	Study day 30	Study day 113	Study day 30	Study day 113
WBC[1]	5.11 ± 0.52	4.62 ± 0.44*	5.60 ± 0.51	5.53 ± 0.77
PMN[1]	2.81 ± 0.38	2.21 ± 0.28*	3.01 ± 0.44	3.08 ± 0.61
% of WBC	53.70 ± .50	47.50 ± .10*	52.35 ± 4.25	53.90 ± 3.90
Monocytes[1]	0.36 ± 0.04	0.31 ± 0.04	0.45 ± 0.04	0.46 ± 0.07
% of WBC	6.90 ± 0.71	6.44 ± 0.59	7.80 ± 0.70	7.91 ± 0.73
Lymphocytes[1]	2.00 ± 0.18	2.12 ± 0.21	2.17 ± 0.17	2.05 ± 0.15
% of WBC	39.50 ± 2.89	46.20 ± 2.85*	39.87 ± 3.91	38.15 ± 3.52
B (CD19+)[1]	0.20 ± 0.05	0.22 ± 0.05	0.22 ± 0.03	0.20 ± 0.03
T (CD3+)[1]	1.45 ± 0.14	1.59 ± 0.16	1.79 ± 0.26	1.63 ± 0.18
% producing IL2	39.15 ± 3.86	37.30 ± 1.93	44.95 ± 5.95	43.75 ± 3.45
Helper (CD3+, 4+)[1]	0.89 ± 0.08	0.94 ± 0.09	1.07 ± 0.15	0.91 ± 0.07
Suppressor (CD3+, 8+)[1]	0.55 ± 0.06	0.58 ± 0.08	0.64 ± 0.06	0.61 ± 0.07
NK (CD3-, 16+, 56+)[1]	0.25 ± 0.04	0.24 ± 0.04	0.25 ± 0.13	0.20 ± 0.07
CTL (CD3+,16+, 56+)[1]	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01

[a]WBC, white blood cells; PMN, polymorphonuclear leukocytes; CD, cluster designation; NK, natural killers; and CTL, cytotoxic T. Data shown are mean ± SEM for the number of subjects shown. Study day 30 and 113 correspond to the end of stabilization and intervention dietary periods, respectively. Although data are shown only for the end of the stabilization and intervention periods, data from study days 22,30,78,85,106, and 113 were all used to determine dietary effects using repeated measures of analysis of variance (ANOVA). Superscript(*) indicates significant change with DHA feeding (P = 0.0001), and superscript 1 indicates cell number × 10^{-9}/L blood. NK, See Table 1 for other abbreviations.

was also evident if these cells were expressed as percentage of the total WBC. The temporal changes in the number of circulating WBC and PMN for both the control and DHA groups are shown in Figure 1. This figure shows that the reduction in WBC and PMN numbers in the DHA group was evident within 50 d of DHA supplementation.

Table 3 also shows that the number of monocytes in the DHA group also decreased; however, it did not reach statistical significance. The number of total WBC, PMN, and monocytes in the control group, and that of lymphocytes in both groups remained unchanged throughout the study. The percentage of lymphocytes in the DHA group was increased (P = 0.0001) with DHA supplementation, because of the reduction in granulocytes in this group. DHA supplementation did not affect the number of B, total T, helper T, suppressor T, helper/suppressor ratio, CTL, or natural killer cells in circulation. The number of T cells producing IL2 was about 40% in both groups, and was not changed by DHA supplementation.

Effect of DHA feeding on PBMNC proliferation. Table 4 contains data regarding the proliferation of PBMNC cultured with three different concentrations of PHA and Con A. At the suboptimal concentration of PHA (2.5 mg/L), proliferation in both groups was higher at day 113, compared to the corresponding values at day 30. However this increase was not statistically significant and was not due to DHA intake. At this PHA concentration, PBMNC proliferation values at study day 78 for the DHA and control groups were 10.7 ± 1.4, and 9.2 ± 1.6, respectively, and were not different from the corresponding values at study day 30 (Table 4). PBMNC proliferation did not change at PHA concentration of 5 or 10 mg/L, or at any of the concentrations of Con A, throughout the study.

Effect of DHA on DTH skin response and serum concentrations of IgG, C3, and IL2R. Table 5 contains the data for both the groups regarding the immune indices listed above. Both the induration and antigen scores on day 113 were lower than at day 30 in both groups; however, none of these de-

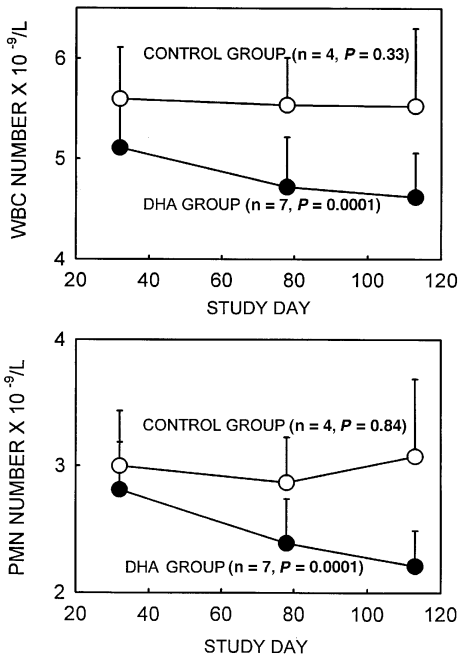

FIG. 1. Docosahexaenoic acid (DHA) feeding decreases the number of circulating white blood cells (WBC) and polymorphonuclear leukocytes (PMN). Data shown are the mean ± SEM for the number of subjects shown. The P values shown were obtained by analysis of variance.

TABLE 4
DHA Feeding Does Not Alter the Proliferation (Bq/1,000 cells) of PBMNC Cultured with Mitogens[a]

	DHA group (n = 7)		Control group (n = 4)	
Mitogen (mg/L)	Study day 30	Study day 113	Study day 30	Study day 113
PHA, 2.5	10.20 ± 0.97	13.61 ± 1.09	8.80 ± 1.00	12.56 ± 0.98
PHA, 5	13.65 ± 0.82	14.26 ± 0.83	13.37 ± 0.70	14.29 ± 1.11
PHA, 10	13.90 ± 0.92	14.47 ± 0.74	13.63 ± 0.75	15.20 ± 0.74
Con A, 2.5	6.24 ± 0.35	6.13 ± 0.45	5.55 ± 1.02	5.36 ± 0.76
Con A, 5	8.70 ± 0.43	8.52 ± 0.47	7.72 ± 0.89	7.95 ± 0.67
Con A, 10	10.08 ± 0.48	9.43 ± 0.46	9.04 ± 0.76	8.66 ± 0.95

[a]Data shown are mean ± SEM for the number of subjects shown for each group. Study days 30 and 113 correspond to the end of stabilization and intervention dietary periods, respectively. PHA, phytohemagglutinin; Con A, concanavalin A; Bq, becquerel; and PBMNC, peripheral blood mononuclear cells. None of these variables was altered by DHA supplementation as determined by repeated measures of ANOVA using data from study days 22, 30, 78, 85, 106, and 133. See Tables 1 and 3 for other abbreviations.

creases was statistically significant, and could not be attributed to DHA feeding. These changes were primarily due to a reduction in response to tetanus antigen. At day 30, six of the subjects in the DHA group tested positive for this antigen, with an induration (mean ± SEM) of 12.7 ± 2.4 mm. However, at day 113, only three of the subjects tested positive for this antigen in the DHA group, with an induration of 9.5 ± 0.5 mm. In the control group, all four subjects tested positive for tetanus on day 30, with an induration of 12.2 ± 2.4 mm. Only three subjects in the control group tested positive on day 113, with an induration of 8.0 ± 1.5 mm. It is possible that dietary enrichment or enrichment with DHA reduced the induration due to tetanus; however, because not all subjects responded to this antigen, we did not have adequate power to test the significance of the reduction in this response. Serum levels of IgG, C3, and IL2R were not affected by either diet (Table 5).

DISCUSSION

We examined if supplementation of diets with DHA triacylglycerol would lower human IR, as previously reported with fish oil supplementation. In contrast to most of the previous studies with fish oils, we maintained total fat intake constant, supplemented DHA in the absence of dietary EPA, and ensured adequate vitamin E intake by additional supplementation of d-α-tocopherol acetate at 20 mg/d. All these factors are important in evaluating the effects of n-3 PUFA on human IR.

Dietary DHA enrichment did not lower lymphocyte proliferation or the DTH skin response in the present study unlike results reported in a number of human studies in which fish oils or purified EPA were added to the diets without adjustment for the extra fat intake (8–14). There was no change in the number of lymphocytes producing IL2 or in the ratios between helper and suppressor T cells, as reported by others using EPA (8,13). Neither did DHA feeding alter the serum levels of IgG, C3, and IL2R. We believe the low and constant fat content of our experimental diets is the most likely reason why DHA did not inhibit the indices of IR as previously found by others with fish oils or EPA. It is likely that in studies with fish oils the increase in both total fat and n-3 PUFA contributed to the inhibition of IR.

Fish oils also contain EPA, which inhibits IR in humans (13,14,19), and it may be a more potent inhibitor than DHA. About 9% of the dietary DHA is retroconverted to EPA in humans (30,31). DHA supplementation raised its concentration from 1.8 to 8.1 wt% of plasma fatty acids, and that of plasma EPA from 0.38 to 3.39 wt% in the subjects included in our current study (32). Whether this amount of EPA is adequate to inhibit IR, or it is metabolized differently from the dietary

TABLE 5
DHA Feeding Does Not Alter DTH Response, and Serum Concentrations of IgG, C3, IL2R[a]

	DHA group (n = 7)		Control group (n = 4)	
	Study day30	Study day 113	Study day 30	Study day 113
Induration (mm)	39.4 ± 4.6	33.4 ± 6.4	35.1 ± 4.2	23.8 ± 1.3
Antigen Score	3.2 ± 0.4	3.0 ± 0.4	3.0 ± 0.4	2.5 ± 0.6
IgG (μmol)	86.0 ± 8.4	91.6 ± 8.4	85.1 ± 3.7	93.0 ± 8.2
C3 (μmol)	9.9 ± 0.4	9.8 ± 0.4	11.4 ± 1.5	11.8 ± 0.9
IL2R (nmol)	188.0 ± 17.1	189.3 ± 17.5	170.4 ± 15.3	195.1 ± 45.6

[a]DTH, delayed type hypersensitivity skin response; IgG, immunoglobulin G; C3, complement fraction 3; IL2R, interleukin 2 receptor. Data shown are mean ± SEM for the number of subjects shown for each group. Study days 30 and 113 correspond to the end of stabilization and intervention dietary periods, respectively. Antigen score refers to the number of antigens tested positive at 48 h after the application of 7 recall antigens. None of the variables was altered by DHA supplementation. See Table 1 for other abbreviation.

EPA is unknown. With the exception of a decrease in arachidonic acid, the plasma concentration of other fatty acids was not changed with DHA feeding (32). The modest increase in vitamin E intake in our study may also have prevented the inhibition of IR by DHA, as others (15) have shown that very high doses of α-tocopherol (200 mg/d) can overcome the inhibition of IR caused by fish oils (15 g/d). The amount of DHA in our study is comparable to the total of EPA and DHA provided from 15 g of fish oil; however, we provided only 10% of the vitamin E provided by these workers.

Which of the three factors (total fat, EPA, vitamin E) discussed above is more important cannot be answered from our study design. Regardless of the mechanisms involved, our results show that if total fat intake is maintained constant, a moderately high intake of DHA as triacylglycerol does not inhibit several indices of human IR that have previously been reported to be inhibited by fish oils, purified EPA, or flaxseed oil.

Our results do show a significant decrease in the number of circulating PMN in the subjects fed the DHA-containing diet. We are aware of only one human study with n-3 fatty acids where such effects have been reported in abstract form (33). Results from our current study regarding the number of circulating PMN contrast our recent findings with arachidonic acid feeding (5), where we found significant increase in their number in peripheral blood in the group fed arachidonic acid-containing diet. These opposing effects of DHA and arachidonic acid on the number of circulating PMN are consistent with their many other opposing effects on several physiological functions. The concept of dietary fats affecting the formation or maturation of WBC is relatively new and poorly understood. However, there are reports showing the inhibition of tumor development in animals fed diets supplemented with n-3 fatty acids (34,35). Results from a recent study by other investigators (36) showed that the number of colony-forming units–granulocyte macrophage in the bone marrow increased twofold in rats fed diets containing DHA, compared to the corresponding numbers in the rats fed a control or n-6 PUFA diet. If all of these colony-forming units attained maturation and their release into blood was proportional to their numbers in the marrow, then the number of PMN and monocytes should be higher in the blood of DHA-fed rats than those in the control or n-6 PUFA diet-fed rats. Unfortunately, these workers did not perform blood cell counts. The decrease in the number of PMN in drawn blood as seen in our study and the increase in the colony-forming units in the bone marrow of rats seem inconsistent; however, these two results become consistent if DHA blocks the migration of these cells from bone marrow to blood or increases their margination in the circulation. Of course, the differences could be simply due to species differences, but still it is important that future studies in this area monitor both colony-forming units in bone marrow and the number of granulocytes and monocytes in blood.

Colony-forming units are the precursors of both monocytes and PMN, yet we found significant decrease in the number of circulating PMN only. A close look at our data in Table 3 suggests that the number of circulating monocytes also decreased in the subjects fed the DHA-containing diet, but this decrease did not attain statistical significance. We may not be able to detect the change in the number of circulating monocytes, because of their relatively small numbers compared with PMN numbers. Results from the rat study suggest that this is the most likely explanation for our results with PMN and monocytes. However, other explanations such as the effect of DHA on PMN proliferative and storage pools, their apoptosis and egress from bone marrow could also explain our results. The number of lymphocytes, which are derived from a different progenitor cell than the PMN and monocytes, was not affected by DHA feeding. Actually, lymphocytes as a percentage of WBC increased because of the decrease in PMN count. Thus, it seems that DHA effect was specific for granulocytes or for granulocyte-monocytes.

The number of circulating PMN is influenced by a number of hormones, growth factors, toxins, and cytokines. We are not sure of the mechanisms by which DHA caused the decrease in the number of circulating PMN. We did monitor the concentrations of cortisol, T3, and T4 in the sera of our study subjects (not shown); none of these hormones changed in both groups during the study. Feeding diets containing fish oil to healthy subjects decreased leukotriene B_4 (LTB_4) production by isolated neutrophils and monocytes (12), and LTB_4 is known to enhance neutrophil chemotaxis and diapedesis (37). It is possible that increased intake of DHA reduced LTB_4 production in our study, which may have mediated the decrease in the number of circulating PMN.

It is possible that some of the effects observed in this study were due to a reduction in LA intake rather than the increase in DHA intake. The intake of LA was decreased by about 7 g/d in the high DHA diet compared to the basal diet. The subjects on the high DHA diet were still receiving more than 20 g/d LA, an amount that will flood the fatty acid metabolic acid pathways and is well above any known requirements for LA. The plasma LA content in the basal and high DHA diets fed subjects was 39 and 36%, respectively. The difference between the two groups was not statistically significant. The basal diet contained less than 50 mg DHA per day, and the high DHA diet had 6 g/d. Thus, it is unlikely that any of the changes in immune functions were due to a 20% reduction in LA intake. These were most likely the result of increase in DHA intake.

Although the PMN counts decreased by 21%, the residual counts were still within the clinically normal ranges. The subjects fed the high DHA diet did not show an increase in the rate of infections or any other health problems. The risk for infections may increase, if the subjects had a marginal PMN count prior to DHA supplementation, or if a high amount of DHA was consumed for an extended period of time. DHA may also inhibit other aspects of human IR, if it is added to diets high in total fat or low in n-6 PUFA and/or antioxidant nutrients. These questions need to be addressed in future studies. Until more information is available, people supplementing their diets with DHA should have a periodic evaluation of their immune status.

ACKNOWLEDGMENTS

The authors are appreciative of the support provided by the dietary and nursing staff of the Metabolic Research Unit in conducting this study. We also thank the volunteers who participated in the study, the Bioanalytical laboratory for sample processing, and the Martex Corporation for donating the DHASCO™ oil.

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[Received February 4, 1998, and in final revised form and accepted April 29, 1998]

The Metabolism and n-6/n-3 Ratio of Essential Fatty Acids in Rats: Effect of Dietary Arachidonic Acid and a Mixture of Sesame Lignans (sesamin and episesamin)

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ABSTRACT: In this study, we examined the effect of dietary arachidonic acid (AA) and sesame lignans on the content and n-6/n-3 ratio of polyunsaturated fatty acid (PUFA) in rat liver and the concentrations of triglyceride (TG) and ketone bodies in serum. For 4 wk, rats were fed two types of dietary oils: (i) the control oil diet groups (CO and COS): soybean oil/perilla oil = 5:1, and (ii) the AA-rich oil group (AO and AOS): AA ethyl esters/palm oil/perilla oil = 2:2:1, with (COS and AOS) or without (CO and AO) 0.5% (w/w) of sesame lignans. Dietary AA and sesame lignans significantly affected hepatic PUFA metabolism. AA content and n-6/n-3 ratio in the liver were significantly increased in the AO group, despite the dietary total of n-6 PUFA being the same in all groups, while AOS diet reduced AA content and n-6/n-3 ratio to a level similar to the CO and COS groups. These results suggest that (i) dietary AA considerably affects the hepatic profile and n-6/n-3 ratio of PUFA, and (ii) dietary sesame lignans reduce AA content and n-6/n-3 ratio in the liver. In the AO group, the concentration of acetoacetate was significantly increased, but the ratio of β-hydroxybutyrate/acetoacetate was decreased. On the other hand, the AO diet increased the concentration of TG in serum by almost twofold as compared to other groups. However, the AOS diet significantly reduced serum TG level as compared to the AO group. In addition, the AOS diet significantly increased the acetoacetate level, but reduced the β-hydroxybutyrate/acetoacetate ratio. These results suggest that dietary sesame lignans promote ketogenesis and reduce PUFA esterification into TG. This study resulted in two findings: (i) sesame lignans inhibited extreme changes of the n-6/n-3 ratio by reducing hepatic PUFA content, and (ii) the reduction of hepatic PUFA content may have occurred because of the effects of sesame lignans on PUFA degradation (oxidation) and esterification.

Lipids 33, 567–572 (1998).

Sesame lignans (sesamin and episesamin) are compounds found in sesame seeds and sesame seed oil. They have multiple biological functions such as antioxidative activity (1,2), anticarcinogenicity (2), antihypertensive effect (3,4) in rats, and alleviation of hepatic injury caused by alcohol or carbon tetrachloride (5) in mice. Sesame lignans also affect lipid metabolism, inhibit cholesterol absorption from the intestine, reduce 3-hydroxy-3-methyl-glutaryl CoA reductase activity in liver microsomes (6,7), and affect the incorporation of linoleic acid into lipid subfractions (8) in rats. They also increase the content of dihomo-γ-linolenic acid (DGLA) by the inhibition of Δ5 desaturase activity in the *Mortierella alpina* fungus and rat liver microsomes (9,10). In human studies, it has been reported that sesame lignans have hypocholesterolemic effects (11).

In our previous studies, we reported that sesame lignans inhibited Δ5 desaturation from DGLA (n-6) to arachidonic acid (AA, n-6), but not from 20:4n-3 to eicosapentaenoic acid (EPA, n-3) in cultured rat hepatocytes (12) and *in vivo* (13). We also found that sesame lignans increased the content of n-6 fatty acids, especially AA, but decreased the content of n-3 fatty acids, especially EPA, in rat liver, when EPA-rich diet was given (13). Consequently, the n-6/n-3 ratio *in vivo* was significantly reduced in the EPA-rich group but returned to almost the original level when administered with sesame lignans (13). This is because EPA and AA compete for incorporation into triglyceride (TG) and phospholipid (14), and a decrease in EPA content may lead to an increase in AA content in the liver. Both EPA and AA are polyunsaturated fatty acids (PUFA) and precursors of eicosanoids, but some functions of eicosanoids from EPA and AA are competitive (15). In addition, epidemiological and clinical studies have shown that the n-6/n-3 PUFA ratio in plasma is associated with the frequency of thrombosis occurring (16,17). Therefore we can assume that the ratio of n-6/n-3 *in vivo* is important.

AA is one of the major PUFA in cell membrane phospholipids and also the precursors of eicosanoids such as 2 series' prostaglandin and 4 series' leukotriene, which have various functions including regulation of the immune system. AA is maintained at a constant level in normal condition *in vivo*. It was recently shown that breast milk contains AA which contributes to infant development (18), and there are several studies about AA in milk formula. Moreover, additional studies reported on the effects of dietary AA in humans (19).

This study is a part of a study examining the effect of dietary sesame lignans on hepatic PUFA metabolism and the

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; HOB, β-hydroxybutyrate; PUFA, polyunsaturated fatty acid; TG, triglyceride.

n-6/n-3 ratio of essential fatty acid *in vivo*. In this report, we will focus on the effect of dietary AA and sesame lignans on fatty acid metabolism compared to that of EPA and sesame lignans which we reported in our past study (13). To investigate the effect of dietary AA and sesame lignans on PUFA metabolism in rats, the content of PUFA in the liver was determined and the n-6/n-3 ratio of PUFA was also examined. Furthermore, to study effects of dietary AA and sesame lignans on the hepatic metabolism of PUFA with respect to their oxidation and secretion as TG, the concentration levels of serum TG and ketone bodies were determined.

TABLE 1
Fatty Acid Composition of Dietary Oils[a]

	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	AA 20:4n-6
CO and COS						
Soybean oil/perilla oil = 5:1	9.53	3.44	22.6	46.3	17.2	
AO and AOS						
AA/palm oil/perilla oil = 2:2:1	18.8	2.12	18.5	6.66	12.9	40.0

[a]Values are expressed as percentage of weight. AA, arachidonic acid; CO, control oil diet; COS, CO + sesame lignans; AO, AA-rich oil diet; AOS, AO + sesame lignans.

MATERIALS AND METHODS

Materials. Sesame lignans (a mixture of sesamin and episesamin, 47:53, w/w) prepared from refined sesame seed oil and purified by the method of Fukuda *et al*. (20) were kindly donated by Suntory Ltd. (Osaka, Japan). AA ethyl esters (purity > 99%) were also donated by Suntory Ltd. Soybean oil and palm oil were provided by Ajinomoto Co., Inc. (Tokyo, Japan). Perilla oil was provided by Ohta Oil Co. (Okazaki, Japan).

Animals and diets. All experiments were approved by the Animal Experimentation Ethics Commitee of Ochanomizu University. Three-week-old male Wistar rats were purchased from Nippon Clea Co. (Tokyo, Japan). The rats were initially fed on a commercial diet (CE-2; Nippon Clea Co.) for 1 wk. The animals, weighing an average of 98 g, were then divided into four groups of six animals each. They were housed individually in an air-conditioned room at 23 ± 1°C with a 12-h light–dark cycle and were given experimental diets (15 g/d, 1–7 d; 20 g/d, 8–17 d; 25 g/d, 18–28 d) and water *ad libitum* for 4 wk. The basal diet prepared according to the standards given by the American Institute of Nutrition (AIN) (21) was provided by Eisai Co. (Tokyo, Japan), containing the following percentage of ingredients according to weight: casein, 20; glucose, 25; sucrose, 25; cornstarch, 15; filter paper, 5; AIN mineral mixture, 3.5; AIN vitamin mixture, 1; choline bitartate, 0.2; DL-methionine, 0.3. The test diets were prepared by mixing 5% (w/w) experimental oil mixtures with the basal diet. These oil mixtures were prepared: (i) the control oil diet groups (CO and COS): soybean oil/perilla oil = 5:1 and (ii) AA-rich oil groups (AO and AOS): AA ethyl esters/palm oil/perilla oil = 2:2:1. Each of the two diets was then split into two groups: with (COS and AOS) or without (CO and AO) 0.5% (w/w) of sesame lignans. The percentage of AA in AO and AOS diets is the same as that of EPA in the EPA-rich oil group in our previous study (13). The level of total n-6 PUFA is the same in all groups. The fatty acid compositions of these dietary oils are shown in Table 1.

Lipid analyses. Lipids were extracted from the livers of the rats by the method of Folch *et al*. (22). TG extracted from the liver was separated by thin-layer chromatography by the method of Skipski *et al*. (23). Margaric acid (17:0), as an internal standard, was added to the lipid extracts which was then methylated using HCl-methanol by the method mentioned in our previous paper (24) to measure the content of fatty acids in tissues. The fatty acid methyl esters were then measured by gas–liquid chromatography (Perkin-Elmer AutoSystemGC, Palo Alto, CA) on a Rascot Silliar 5CP capillary column (0.25 mm × 50 m; Nihon Chromato Works Ltd., Tokyo, Japan) as described previously (24). The TG in serum was measured by a commercial kit (Wako Pure Chemical Industries., Ltd., Osaka, Japan). Acetoacetate and β-hydroxybutyrate (HOB) in serum were measured enzymatically by another commercial kit (ketone test SANWA; Sanwa kagaku kenkyusho Co., Ltd., Nagoya, Japan).

Statistical analysis. All values are expressed as means ± SD. Significance of the difference between dietary treatments ($P < 0.05$) was determined by analysis of variance (ANOVA). Interaction between dietary AA and sesame lignans was investigated by two-way factorial ANOVA. Analyses were performed using a StatView (system 4.02) computer package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

PUFA content in the liver. Table 2 shows the effect of dietary AA and sesame lignans on PUFA contents in the liver. The AO diet significantly increased AA (20:4n-6) content, showing that a relatively large amount of external AA has been incorporated into the liver. However, the AOS diet reduced AA content to a level similar to the CO and COS groups. The COS diet increased DGLA (20:3n-6) level while the AO and AOS diets significantly increased 22:4n-6 level. The AO diet also significantly increased total n-6 PUFA content, despite dietary n-6 PUFA levels being the same in all groups. On the other hand, the AOS diet reduced total n-6 PUFA content to a level similar to the CO and COS groups. EPA (20:5n-3) content was reduced by the COS, AO, and AOS diets. EPA content in the CO, AO, and AOS groups was lower than that in the CO group. No significant differences were observed in 18:3n-3 content between the CO and AO groups, despite the difference in dietary 18:3n-3 level in the two diets. The AO and AOS diets reduced docosahexaenoic acid (22:6n-3) content. The n-6/n-3 ratio in the CO group was 2.69 (Table 1), but after dietary treatment, the ratio increased slightly to 3.78. A similar result was also obseved in the COS group. On the other hand, the n-6/n-3 ratio in the AO group was increased by over twofold, from 3.62 to 7.58 while the n-6/n-3 ratio in

TABLE 2
Effect of Dietary Arachidonic Acid and Sesame Lignans on Fatty Acid Content in the Liver[a]

	Diet			
Fatty acid	CO	COS	AO	AOS
18:2n-6	28.3 ± 7.27[a]	15.9 ± 2.16[b]	3.85 ± 0.64[c]	2.99 ± 0.24[c]
18:3n-6	0.53 ± 0.17[a]	0.16 ± 0.04[b]	0.45 ± 0.11[a]	0.10 ± 0.03[b]
20:3n-6	0.69 ± 0.14[a]	1.20 ± 0.27[b]	0.61 ± 0.17[a]	0.42 ± 0.10[a]
20:4n-6	29.8 ± 5.05[a]	32.5 ± 3.16[a]	71.7 ± 14.0[b]	42.2 ± 1.64[a]
22:4n-6	0.37 ± 0.12[a]	0.33 ± 0.06[a]	3.81 ± 1.07[b]	1.43 ± 0.21[c]
Total n-6 PUFA	59.7 ± 12.0[a]	50.1 ± 5.24[a]	82.0 ± 16.1[b]	47.6 ± 1.83[a]
18:3n-3	4.02 ± 1.29[a]	0.92 ± 0.20[b]	2.82 ± 0.73[a]	0.55 ± 0.08[b]
20:5n-3	0.84 ± 0.17[a]	0.20 ± 0.05[b]	0.47 ± 0.19[c]	0.03 ± 0.03[b]
22:5n-3	1.60 ± 0.19	1.45 ± 0.20	2.00 ± 0.50	1.83 ± 0.48
22:6n-3	9.13 ± 1.27[a]	9.08 ± 1.14[a]	5.50 ± 1.16[b]	5.68 ± 1.12[b]
Total n-3 PUFA	15.8 ± 1.79[a]	11.8 ± 1.45[b]	11.0 ± 2.18[b,c]	8.24 ± 1.52[c]
n-6/n-3	3.78 ± 0.61[a]	4.27 ± 0.33[a]	7.58 ± 1.09[b]	5.95 ± 1.24[c]

[a]Results are expressed as μmol/g tissue and are means ± SD (n = 6). The significance of differences between the dietary treatments was analyzed by analysis of variance. Mean values with different superscript letters within a given row are significantly different ($P < 0.05$). PUFA, polyunsaturated fatty acids. See Table 1 for other abbreviations.

the AOS group significantly reduced to 5.95, which is a level similar to the CO and COS groups.

Below are the results of two-way factorial ANOVA. AA content was significantly affected by both dietary AA ($P < 0.0001$) and sesame lignans ($P = 0.001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0002$). Total n-6 PUFA was also significantly affected by both dietary AA ($P = 0.0432$) and sesame lignans ($P = 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0134$). The n-6/n-3 PUFA ratio was significantly affected by dietary AA ($P < 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0157$).

PUFA composition of liver TG. Table 3 shows PUFA composition of TG fraction in the liver. The AO diet significantly increased the proportion of AA (20:4n-6). However, the AOS diet reduced the proportion of AA level. By using the two-way factorial ANOVA, the proportion of AA was significantly affected by dietary AA ($P < 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0002$).

Concentrations of serum TG. Table 4 shows the effect of dietary AA and sesame lignans on the concentration of serum TG. The AO diet significantly increased a level of TG. However, the AOS diet reduced it to a level similar to the CO and COS groups. As for the results of two-way factorial ANOVA, serum TG level was significantly affected by both dietary AA ($P = 0.0006$) and sesame lignans ($P = 0.0002$).

Concentrations of ketone bodies. Table 5 shows the effect of dietary AA and sesame lignans on the concentration of ketone bodies (acetoacetate and HOB) in blood. The COS and AOS diets slightly increased the total of ketone bodies as compared to the CO diet. The COS, AO, and AOS diets also increased acetoacetate concentration as compared to the CO diet, and an extreme increase was seen in the COS and AOS groups. On the contrary, the COS, AO, and AOS diets re-

TABLE 3
Fatty Acid Composition of Rat Liver Triglyceride[a]

	Diet			
Fatty acid	CO	COS	AO	AOS
18:2n-6	16.2 ± 2.53[a]	10.1 ± 2.77[b]	2.83 ± 0.75[c]	2.34 ± 0.35[c]
20:3n-6	n.d.	n.d.	n.d.	n.d.
20:4n-6	4.85 ± 1.55[a]	9.10 ± 2.09[a,c]	22.6 ± 4.49[b]	13.7 ± 5.14[c]
22:4n-6	n.d.[a]	n.d.[a]	0.58 ± 0.32[b]	0.47 ± 0.14[b]
18:3n-3	3.22 ± 0.85[a]	1.67 ± 0.64[b]	1.88 ± 0.29[b]	0.90 ± 0.15[c]
20:5n-3	0.39 ± 0.24	0.19 ± 0.17	0.45 ± 0.30	1.10 ± 1.89
22:5n-3	0.25 ± 0.15	0.31 ± 0.14	0.21 ± 0.06	0.26 ± 0.10
22:6n-3	0.24 ± 0.18[a]	0.47 ± 0.14[b]	0.24 ± 0.13[a]	0.29 ± 0.17[a,b]

[a]Results are expressed as percentage of weight. See Table 2 footnote. n.d., not detected. See Table 1 for abbreviations.

TABLE 4
Effect of Dietary Arachidonic Acid and Sesame Lignans on Concentration of Triglyceride in Serum[a]

Groups	Triglyceride
CO	0.99 ± 0.17[a]
COS	0.59 ± 0.15[a]
AO	1.92 ± 0.54[b]
AOS	0.92 ± 0.34[a]

[a]Results are expressed as mg/mL. See Table 2 footnote. See Table 1 for abbreviations.

duced HOB, especially in the COS and AOS groups. The ratio of HOB/acetoacetate was reduced by the COS, AO, and AOS diets, particularly by the COS and AOS diets.

Below are the results of two-way factorial ANOVA. Total of ketone bodies was significantly affected by dietary sesame lignans ($P = 0.0029$). Acetoacetate was significantly affected by dietary sesame lignans ($P < 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0274$). HOB was significantly affected by dietary sesame lignans ($P < 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P = 0.0262$). The ratio of HOB/acetoacetate was significantly affected by both dietary AA ($P < 0.0001$) and sesame lignans ($P < 0.0001$), and the interactional effect of dietary AA and sesame lignans was significant ($P < 0.0001$).

DISCUSSION

This study is a part of our investigation into the effect of dietary sesame lignans on hepatic PUFA metabolism and n-6/n-3 ratio of PUFA *in vivo*. The aim of this study was to investigate the effect of dietary AA and sesame lignans on PUFA metabolism and the ratio of n-6/n-3 PUFA in rat. In this study, we examined the effect of AA and sesame lignans on the content and n-6/n-3 ratio of PUFA in rat liver, the concentrations of TG, and ketone bodies in serum.

Dietary AA and sesame lignans significantly affected hepatic PUFA metabolism. The contents of sesamin and episesamin in the liver in the COS and AOS groups were less than 1 μg/g liver (data not shown). AA content and n-6/n-3 ratio in the liver were significantly increased in the AO group, despite the total of dietary n-6 PUFA being the same in all groups (Table 2). This result suggested that hepatic n-6/n-3 ratio was affected not only by the n-6/n-3 ratio in dietary oil but also by the PUFA profile in the diet. However, AOS diet reduced AA content and n-6/n-3 ratio to a level similar to the CO and COS groups (Table 2). These results suggest that (i) dietary AA considerably changes the hepatic PUFA profile and n-6/n-3 ratio and (ii) dietary sesame lignans reduce AA content and n-6/n-3 ratio in the liver. In addition, two-way factorial ANOVA showed significant interactional effect of dietary AA and sesame lignans on AA content and n-6/n-3 ratio in the liver. In our past study (13), we examined the dietary effects of EPA and sesame lignans. We found that (i) dietary EPA significantly increased EPA content and reduced n-6/n-3 ratio in the liver and (ii) dietary sesame lignans reduced EPA content and increased n-6/n-3 ratio. Similar results were observed in our present study. From the results of the two studies, we might consider that sesame lignans inhibit extreme changes of n-6/n-3 ratio and function to bring it close to the appropriate n-6/n-3 ratio *in vivo*. We assumed that sesame lignans maintain the appropriate n-6/n-3 ratio, not only from a decrease by n-3 fatty acid-rich diet but also from an increase by n-6 fatty acid-rich diet. This regulating effect is especially significant on the excess of EPA and AA, which are the intermediate metabolites of PUFA and precursors of eicosanoids.

We have already confirmed that sesame lignans reduce the contents of EPA in the liver (13), but have no effect on intestinal absorption of EPA and AA (25). Moreover, we can also consider that sesame lignans do not accelerate PUFA transportation into the peripheral tissues, because the peripheral tissue PUFA content was not increased by dietary sesame lignans (13,25). Consequently, it is probable that sesame lignans alter hepatic PUFA metabolism.

Free fatty acids are activated to acyl-CoA in the liver, and then (i) esterified into TG and phospholipid or (ii) changed *via* acetyl-CoA to CO_2 and H_2O by β-oxidation or (iii) changed to ketone bodies. Ketone bodies, which are the degraded products of free fatty acid in liver, are the general term for acetone, acetoacetate, and HOB. They are used as an energy source, substituting for glucose. If abnormal metabolism occurs, excess ketone bodies will be produced and released into blood and urine, finally leading to ketoacidosis. Ketone bodies in the blood are in the form of acetoacetate and HOB. Acetoacetate and HOB can be interconverted with each other through the function of HOB dehydrogenase. From our

TABLE 5
Effect of Dietary Arachidonic Acid and Sesame Lignans on Concentrations of Ketone Bodies[a]

	Diet			
	CO	COS	AO	AOS
Total ketone body	1201.2 ± 25.5[a]	1225.9 ± 1.64[b]	1212.1 ± 9.00[a,b]	1225.8 ± 1.96[b]
Acetoacetate	229.4 ± 20.7[a]	678.5 ± 176.7[b]	384.3 ± 43.6[c]	614.8 ± 116.4[b]
β-Hydroxybutyrate	982.8 ± 15.4[a]	547.4 ± 175.8[b]	827.8 ± 42.5[c]	611.0 ± 115.5[b]
β-Hydroxybutyrate/acetoacetate	4.31 ± 0.43[a]	0.92 ± 0.57[b]	2.19 ± 0.35[c]	1.06 ± 0.39[b]

[a]Results are expressed as μmol/L. See Table 2 footnote. See Table 1 for abbreviations.

former results, we can consider that sesame lignans alter plasma fatty acids by enhancing fatty acid degradation system. If sesame lignans really accelerated this system, ketogenesis rates should be promoted. In this study, we examined the concentration of ketone bodies in the rats' blood so as to investigate the relationship between dietary sesame lignans and the capacity for fatty acid degradation.

Although total ketone body concentration was slightly increased in the COS and AOS groups, the changes were small (Table 5). This might be because the rats used in this study were normal, and the total ketone bodies in blood was kept at a normal level. In the AO group, the concentration of acetoacetate was significantly increased, but HOB/acetoacetate ratio decreased (Table 5). On the other hand, the concentration of TG in serum increased by almost twofold in the AO group as compared to the other groups (Table 4). However, the AOS diet significantly reduced the serum TG level as compared to the AO group (Table 4). In addition, the AOS diet significantly increased the acetoacetate level but reduced the HOB/acetoacetate ratio (Table 5). The two-way factorial ANOVA showed significant interactional effects between dietary AA and sesame lignans which affects the levels of acetoacetate and HOB and the HOB/acetoacetate ratio. Also, AA composition of liver TG was affected by dietary sesame lignans (Table 3).

Several studies have demonstrated that fibric acid derivatives such as clofibrate and bezafibrate enhance peroxisomal β-oxidation which is responsible for the increased production of ketone bodies in rat livers (26–28). It has also been reported that dietary fenofibrate, one of the fibric acid derivatives, increased hepatic ketogenesis but decreased secretion of TG (29). Furthermore, it has also been demonstrated that the ratio of HOB/acetoacetate is decreased by clofibrate (30) and fenofibrate (29). Esterification of fatty acids into TG depends on the level of fatty acid in tissues. Moreover, this fatty acid level affects ketogenesis. Therefore, it is considered that fibric acid derivatives regulated ketogenesis and esterification of fatty acid into TG.

The results of our present study suggest that the effect of sesame lignans on the hepatic PUFA metabolism might be similar to that of fibric acid derivatives. It also suggests that (i) a large amount of AA is incorporated into the liver by dietary AA, thus stimulating the esterification into TG and slightly promoting ketogenesis, but (ii) dietary sesame lignans promote ketogenesis and reduce PUFA esterification into TG. As a result, we may conclude that sesame lignans affect hepatic metabolism of PUFA between PUFA degradation (oxidation) and esterification.

This study yields two important findings: (i) sesame lignans inhibited extreme changes of n-6/n-3 ratio by reducing hepatic PUFA content, and brought it close to the appropriate n-6/n-3 ratio *in vivo,* and (ii) the reduction of hepatic PUFA content may have occurred because sesame lignans acted at some point between PUFA degradation (oxidation) and esterification. At present, several studies are in process concerning the appropriate n-6/n-3 ratio *in vivo*. However, it is difficult to regulate the n-6/n-3 ratio by the intake of various dietary oils because by using supplements and health foods there is a risk of excessive intake of n-6 or n-3 fatty acids, such as AA or EPA. Therefore, it is valuable to find that sesame lignans have a stabilizing effect on the balance of n-6/n-3 *in vivo*.

ACKNOWLEDGMENTS

We greatly thank Suntory Ltd. and Dr. Akimoto, Institute for Biomedical Research, Suntory Ltd., Osaka, Japan, for the donation of sesame lignans and AA ethylester.

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[Received December 1, 1997, and in final revised form and accepted April 22, 1998]

Psyllium, Not Pectin or Guar Gum, Alters Lipoprotein and Biliary Bile Acid Composition and Fecal Sterol Excretion in the Hamster

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ABSTRACT: Different soluble dietary fibers known to alter cholesterol metabolism were fed to golden Syrian hamsters, and their specific impact on lipoproteins, biliary bile acid profile, and fecal sterol excretion was evaluated. Semipurified diets containing 20% fat; 0.12% cholesterol; and 8% of psyllium (PSY); high (hePE) and low (lePE) esterified pectin; or high (hvGG) and low (lvGG) viscous guar gum were fed for 5 wk. Compared to control, PSY caused a significant reduction in plasma cholesterol (2.9 ± 0.5 vs. 5.5 ± 0.5 mmol/L), whereas hePE, lePE, hvGG, or lvGG had no apparent effect on plasma lipids. Hepatic total and esterified cholesterol were substantially decreased with PSY, pectin and guar gum, whereby PSY produced the most pronounced effect. Distinctive changes existed in the bile acid profile related to the different fibers. In contrast to pectin and guar gum, PSY caused a significant increase in the cholate:chenodeoxycholate and the glycine:taurine conjugation ratio. Pectin and guar gum did not alter daily fecal neutral sterol excretion while PSY caused a 90% increase due to a higher fecal output. Daily fecal bile acid excretion and total fecal bile acid concentration were significantly increased by PSY, whereas hePE, lePE, hvGG, and lvGG revealed no or only minor effects. Taken together, the disparate hypocholesterolemic effects of PSY, pectin, and guar gum on cholesterol and bile acid metabolism in the hamster are possibly related to different physicochemical properties, e.g., viscosity and susceptibility to fermentation, affecting the fiber-mediated action in the intestine.

Lipids 33, 573–582 (1998).

Water-soluble fiber sources such as psyllium (PSY), pectin (PE), and guar gum (GG) have been shown to exert a cholesterol-lowering effect in humans and in different experimental animals (1,2). Although recent studies have attempted to elucidate the primary mechanism(s) responsible for the cholesterol-lowering effect, distinct actions of specific fibers have not been completely defined (3–9). Suggested mechanisms by which dietary soluble fibers lower cholesterol include an interruption of the enterohepatic circulation of bile acids, with an enhanced fecal excretion stimulating bile acid synthesis, a decrease in cholesterol absorption, or the production of volatile fatty acids, e.g., propionic acid in the cecum inhibiting hepatic cholesterol synthesis.

In a series of studies using the guinea pig as a model, Fernandez *et al*. (4–6) showed that the hypocholesterolemic action by different types of fiber is greatly influenced by the level of dietary cholesterol. For instance, PE had a substantial cholesterol-lowering effect with a high cholesterol diet and a more moderate effect with low dietary cholesterol. In contrast, GG was more effective with low rather than high dietary cholesterol, whereas PSY revealed similar hypocholesterolemic effects with either low- or high-cholesterol diets. Recently, Fernandez (1) has shown that different primary mechanisms specific to each type of fiber seem responsible for the observed effects of PSY, PE, or GG. Whereas PE and PSY stimulated hepatic cholesterol 7α-hydroxylase activity, the key enzyme in bile acid synthesis, GG had no effect. PE reduced cholesterol absorption independent from dietary cholesterol, GG did so only with high dietary cholesterol, whereas PSY did not affect cholesterol absorption (1). Studies with rats have shown that PSY and PE caused an increase in the bile acid pool size, altered bile acid composition, and increased cholesterol 7α-hydroxylase activity (10,11).

In previous studies with Syrian hamsters, a commonly used animal model because of similarities with human cholesterol and bile acid metabolism (12–14), we have found contrasting effects of PE, GG, and PSY when hamsters were fed a lithogenic diet high in dietary cholesterol (0.4%). Whereas PSY significantly reduced plasma, hepatic, and biliary lipids mainly by increasing the fecal excretion of bile acids, PE and GG demonstrated only minor effects in that hamster study (15).

The present study was designed to further compare the lipid-lowering effectiveness of PE, GG, and PSY using a moderate level of dietary cholesterol (0.12%) and to elucidate whether physicochemical properties such as viscosity and degree of esterification (DE) affect the hypolipidemic capacity of PE and GG. Therefore, two types of GG, a high and low

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Abbreviations: ANOVA, analysis of variance; DE, degree of esterification; EC, esterified cholesterol; FC, free cholesterol; GG, guar gum; HDL, high density lipoprotein; hePE, high-esterified pectin; HPLC, high-performance liquid chromatography; hvGG, high viscous guar gum; LDL, low density lipoprotein; lePE, low-esterified pectin; lvGG, low viscous guar gum; PE, pectin; PL, phospholipid; PSY, psyllium; TC plasma total cholesterol; TG, plasma triacylglycerols; VLDL, very low density lipoprotein.

viscous GG, and two varieties of PE differing in DE and viscosity were tested.

MATERIALS AND METHODS

***Animals*.** Male golden Syrian hamsters (SASCO, Omaha, NE) weighing 62 ± 2 g were randomly assigned to six diet groups (n = 10 per group). Hamsters were housed in groups of 3–4 per cage in a temperature-controlled environment under a 12-h light–dark cycle (lights on 1800 h) with free access to water and semipurified diets. All experimental protocols and procedures were approved by the Animal Care and Use Committee at the University of Kiel, Germany.

Diets and feeding procedures. Hamsters were fed semipurified diets containing 20% fat, 0.12% dietary cholesterol, and 10% cellulose to ensure normal bowel function and to prevent the lethal enteritis "wet tail." Diets were fed for 5 wk. The basal composition of the diets was, in g/kg dry weight: casein 200, wheat starch 288, glucose 150, cellulose 100, fat 200, mineral mix 46, vitamin mix 12, cholesterol 1.2, choline chloride 3. The dietary fat blend (in g/kg: palm stearin 88, olive oil 48, butter 40, sunflower oil 24) resembled the typical German fat intake with a polyunsaturated/saturated ratio of 0.32. The compositions of the Ausman-Hayes mineral mix (F8530 BioServ, Frenchtown, NJ) and the Hayes-Cathcart vitamin mix were detailed previously (16). In the fiber-supplemented diets, 8% supplements of the tested dietary fiber sources were added at the expense of wheat starch. The dietary fiber sources were PSY husks, two varieties of PE, a high-esterified pectin (hePE) with a DE of 69%, and a low-esterified pectin (lePE) with a DE of 34% and two types of GG, a high viscous (hvGG) and a low viscous guar gum (lvGG). The viscosities of the GG measured in 1% solutions were 6014 mPa·s for hvGG and 22 mPa·s for lvGG. The viscosities of the PE measured in 2% solutions were 71 mPa·s for hePE and 185 mPa·s for lePE. All viscosities were measured in preheated solutions (85°C for 10 min) at 25°C with increasing shear rates using a Bohlin Rheometer (Bohlin Instruments, Mühlacker, Germany). PSY was kindly supplied by Kellogg Company (Battle Creek, MI) and hePE, lePE, hvGG, and lvGG by Danisco Ingredients (Grindsted Division, Quickborn, Germany). Hamsters were given free access to food, and the actual food consumption was recorded daily. Body weights were monitored on a weekly basis.

Necropsy. After 5 wk, hamsters were housed individually in wire-bottomed cages and fasted overnight (18 h) and then exsanguinated under anesthesia using a gaseous mixture of CO_2/O_2 (50:50). Blood samples were drawn into EDTA-wetted syringes by cardiac puncture and the liver was excised, blotted, and weighed. A portion of the liver was removed and frozen for hepatic cholesterol analysis. Immediately after removal of the intact cecum, the pH of the cecal contents was measured using a spear-tip pH electrode. The total contents were then collected by gentle finger-stripping of the cecum, weighed, homogenized, and deep-frozen in liquid nitrogen. Gallbladder bile was aspirated, weighed, and analyzed for biliary lipids and bile acid composition.

Plasma lipid and lipoprotein analysis. Blood samples from six hamsters were drawn upon arrival (baseline) to establish basal plasma lipids randomly and from all hamsters (n = 10 per diet) after 5 wk. Plasma was separated immediately by centrifugation at 5,000 × g for 10 min. Plasma total cholesterol (TC) and triacylglycerol (TG) concentrations were determined by enzymatic assays (Sigma kit #352 and #336, respectively; Sigma Chemicals, Deisenhofen, Germany). Plasma lipoproteins (n = 5–6 hamsters per diet) were isolated by sequential ultracentrifugation (17) by using a L7-65 ultracentrifuge and a 50.4 Ti rotor (Beckman Instruments, Munich, Germany) and plasma pooled from two hamsters. A preservative solution (final concentration in plasma: 1 mM benzamidine, 0.04% EDTA, 0.005% gentamycin sulfate, 0.05% NaN_3) was added to protect lipoproteins from enzymatic degradation. Three fractions were isolated based on the following densities: very low density lipoprotein (VLDL) (d < 1.006 kg/L), low density lipoprotein (LDL) (1.006 < d < 1.055 kg/L), high density lipoprotein (HDL) (1.055 < d < 1.21 kg/L). With the exception of VLDL, lipoprotein fractions were dialyzed against 0.15 mol/L NaCl, 0.04% EDTA, and 0.05% NaN_3 at 4°C for 24–36 h. TC, free cholesterol (FC), TG, and phospholipid (PL) concentrations were determined using enzymatic assays (#352 for TC and #336 for TG; Sigma Chemicals and Wako Free Cholesterol C kit for FC and Wako Phospholipid B kit for PL, Wako Chemicals, Düsseldorf, Germany). Protein concentration was determined by a modification of the Lowry procedure (18). To verify the density cut-points and to check for cross-contaminations, LDL and HDL apolipoproteins were separated by gradient SDS-polyacrylamide gel electrophoresis (4–20%), and stained with Coomassie Brilliant Blue. No traces of apolipoprotein $apoB_{100}$ could be detected in the HDL fractions (data not shown).

Hepatic cholesterol analysis. Cholesterol concentrations were analyzed after extraction with chloroform/methanol following the procedure described in detail previously (19). TC was determined enzymatically (using Sigma kit #352) and FC was analyzed using high-performance liquid chromatography (HPLC). Esterified cholesterol (EC) concentrations were calculated as the difference between TC and FC.

Bile analysis. Gallbladder bile lipids were isolated using a modified Folch extraction (20). Biliary cholesterol and PL were determined enzymatically in an aliquot of the chloroform phase (Wako Free Cholesterol C kit for biliary cholesterol and Wako Phospholipid B kit for PL, Wako Chemicals). Biliary bile acids were analyzed in an aliquot of the methanol/KCl phase as taurine and glycine conjugated bile acids using an isocratic HPLC method as previously described in detail (19). Total bile acid concentration was calculated as the sum of individual bile acids (taurine and glycine conjugates of cholate, chenodeoxycholate, deoxycholate, and lithocholate) as measured by HPLC.

Determination of fecal neutral sterols and bile acids. Fecal samples were collected over a 3-d period during wk 4 from six randomly selected hamsters per diet group to measure fecal bile acid and neutral sterol excretion. Fecal sterols were

analyzed in an oven-dried fecal sample using a modification of the method of Suckling *et al.* (21) as previously detailed (19). Neutral sterols were determined by gas chromatography as described in detail previously (22). Fecal total bile acids were determined according to the micro method described by Czubayko *et al.* (23) with some minor modifications. Bile acids were methylated using methanolic-HCL (Supelco, Deisenhofen, Germany) prior to conversion to their trimethylsilyl ethers using Sylon HTP (Supelco). The trimethylsilyl derivatives were evaporated under nitrogen and resuspended in *n*-decane (Sigma Chemicals) and centrifuged for 10 min to settle the precipitates. A 1-µL sample was injected. Bile acids were separated and quantified by gas chromatography using a capillary column (DB1, 30 m, 0.25 mm i.d. and 0.25 µm film thickness; J&W Scientific, Mainz, Germany). The column was operated at 200°C hold for 1 min and then gradually increased to 220, 240, and 280°C. Injector temperature and detector were set at 280°C. Helium was used as carrier gas at a flow rate of 150 kPa. Bile acid standards included 5β-cholanic acid (internal standard), ursodeoxycholic acid, lithocholic acid, deoxycholic acid, cholic acid, chenodeoxycholic acid, hyodeoxycholic acid (all from Sigma Chemicals), and 5β-cholanic acid-3α-ol and 5β-cholanic acid-3α-7α-diol-12-one (Steraloids, Wilton, NH).

The daily cholesterol intake was calculated based on data of the daily food intake during wk 4. Daily cholesterol intake in µmol/d was as follows: control, 20.7; hePE, 22.5; lePE, 22.5; hvGG, 22.5; lvGG, 24.0; and PSY, 25.8. The apparent neutral sterol absorption was calculated as: dietary cholesterol intake per day − fecal excretion of neutral sterols (µmol/d). The total sterol balance was calculated as dietary cholesterol intake per day − [fecal excretion of neutral sterols (µmol/d) + fecal excretion of bile acids (µmol/d)].

Statistical analysis. Statistical differences were calculated using a one-way analysis of variance (ANOVA). When significant *F* ratios were found, individual means were further compared by the Bonferroni-Dunn *post-hoc* test utilizing the SuperANOVA statistical software package (Version 1.11; Abacus Concepts Inc., Berkeley, CA). Differences were considered significant at $P < 0.05$. Results were expressed as means ± SD.

RESULTS

Body weight gain and cecal weight. Food consumption, final body weights, and weight gain were not affected by the supplementation of PE and GG. Total body weight gain was significantly lower in PSY-fed hamsters compared to all other diet groups (Table 1). Weights of the cecum, the cecal wall, and the cecal content were significantly higher in PSY-fed hamsters (+211, +150, and +270%, respectively, compared to control) compared to hamsters fed the control or PE- and GG-enriched diets. Cecal pH was not significantly altered in hamsters fed the PE-, GG-, or PSY-enriched diets, suggesting no acidic conditions possibly because the hamsters were fasted for 18 h and therefore no active fermentation took place (Table 1).

Plasma lipids and lipoproteins. After 5 wk, TC concentrations were significantly lower in PSY-fed hamsters compared to all other diets (Fig. 1). TG concentrations were decreased significantly by 42% in PSY-fed hamsters compared to control (Fig. 1). No significant differences in TC and TG were observed between the control and hePE, lePE, hvGG, and lvGG diets although TC was 10% reduced by hePE. PSY significantly decreased VLDL-, LDL-, and HDL-cholesterol by 75, 47, and 38%, respectively. Whereas lePE, hvGG, and lvGG had no effect, hePE decreased VLDL-, LDL-, and HDL-cholesterol by 20, 22, and 28%, compared to control, but these reductions did not reach the level of significance (Table 2). The LDL/HDL ratio was not altered by any of the different dietary fibers.

PSY significantly affected the composition of VLDL and LDL (Table 3). In the VLDL fraction, significantly higher relative percentages of TG and lower percentages of FC and EC and PL were found. In the LDL fraction, the relative percentages of EC and PL were significantly decreased by 21 and

TABLE 1
Food Intake, Total Weight Gain, Cecal Weights, and pH of the Cecal Content in Hamsters Fed Diets Containing 8% of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	Food intake[c] (g/d)	Total body weight gain (g)	g/100 g body wt			Cecal pH
			Cecum weight	Cecal wall	Cecal content	
Control	15 ± 3[a,b]	77 ± 7[a]	0.9 ± 0.1[a]	0.38 ± 0.08[a]	0.51 ± 0.13[a]	7.13 ± 0.06[a,b]
8% Pectin						
hePE	15 ± 3[a,b]	83 ± 10[a]	1.4 ± 0.2[b]	0.56 ± 0.20[b]	0.84 ± 0.24[a]	7.12 ± 0.16[a,b]
lePE	17 ± 4[a]	86 ± 7[a]	1.2 ± 0.3[a,b]	0.44 ± 0.08[a,b]	0.76 ± 0.28[a]	7.15 ± 0.10[a,b]
8% Guar gum						
hvGG	16 ± 3[a,b]	89 ± 12[a]	1.2 ± 0.2[a,b]	0.45 ± 0.09[a,b]	0.76 ± 0.23[a]	7.15 ± 0.12[a,b]
lvGG	15 ± 3[a,b]	82 ± 11[a]	1.2 ± 0.2[a,b]	0.46 ± 0.09[a,b]	0.76 ± 0.18[a]	7.07 ± 0.06[a]
8% Psyllium	14 ± 2[b]	63 ± 10[b]	2.8 ± 0.5[c]	0.95 ± 0.16[c]	1.89 ± 0.45[b]	7.27 ± 0.16[b]

[a]Values are mean ± SD, $n = 10$ per diet; hePE, high-esterified pectin; lePE, low-esterified pectin; hvGG, high viscous guar gum; lvGG, low viscous guar gum.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way analysis of variance (ANOVA) and the Bonferroni/Dunn test.
[c]Hamsters received 25 g food (25 g wet weight = 13.9 g dry weight).

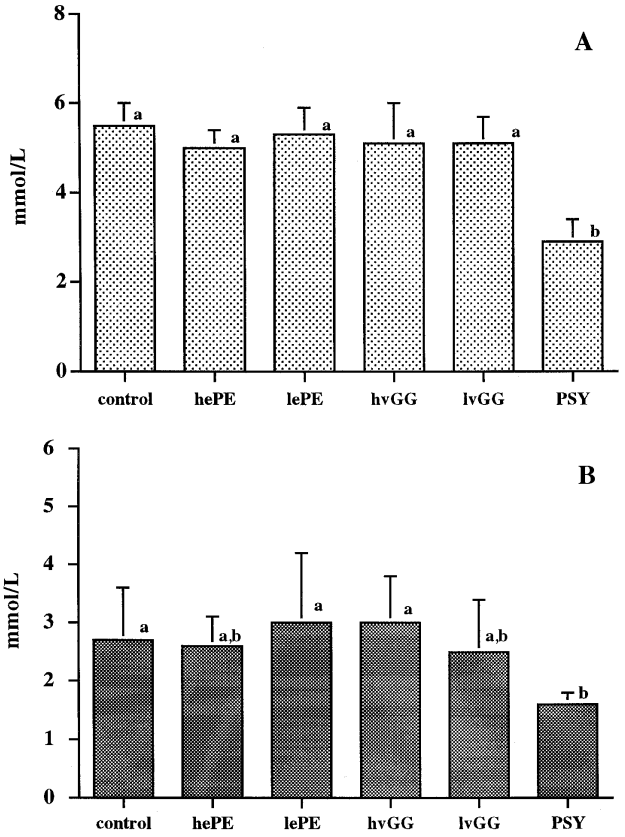

FIG. 1. Plasma (A) total cholesterol and (B) triacylglycerol concentrations of hamsters fed diets containing 8% supplements of different pectins, guar gums, or PSY. Values are mean ± SD (n = 10 per diet). Bars with different letters were significantly different ($P < 0.05$) using one-way analysis of variance (ANOVA) and the Bonferroni-Dunn test. hePE, high-esterified pectin; lePE, low-esterified pectin; hvGG, high viscous guar gum; lvGG, low visous guar gum; PSY, psyllium.

23%, respectively, while the percentages of TG and protein were significantly increased with PSY (Table 3).

Hepatic cholesterol accumulation. Liver weights of hamsters fed the PE- and GG-supplemented diets did not differ significantly compared to control. In contrast, liver weights of PSY-fed hamsters were significantly lower compared to all other diets (Table 4). A significant reduction of hepatic TC and EC occurred with hePE, lePE, hvGG, lvGG and PSY, whereby PSY caused the most pronounced effects. Whereas hePE and hvGG reduced TC by 29 and 23%, respectively, PSY caused a 89% decrease in hepatic TC. In contrast, only minor reductions of 10–20% in hepatic TC were found with lePE and lvGG. Hepatic FC concentrations did not differ between hamsters fed hePE, lePE, hvGG, and lvGG compared to control while PSY produced a significant decrease in FC compared to other diets. Whereas in livers from PSY-fed hamsters, EC accounted for only 31% of TC, 88–91% of total hepatic cholesterol was present in the form of cholesteryl esters in hamsters fed the control or PE- and GG-supplemented diets (Table 4).

Biliary lipids. The molar percentage of biliary cholesterol was significantly reduced by 57% in hamsters fed PSY (2.3 ± 0.4 mol% in controls vs. 1.0 ± 0.2 mol% with PSY). HePE and hvGG caused a modest but insignificant decrease (–17%) while lePE and lvGG had no effect (data not shown). PSY and, to a lesser extent, hvGG produced a significant increase (+7 and +3%, respectively) in the molar percentages of biliary bile acids (88.4 ± 1.1, 90.4 ± 1.8, and 94.0 ± 1.2 mol% in control, PSY-, and hvGG-fed hamsters, respectively).

Bile acid profile. Changes in the bile acid profile of gallbladder bile were distinctive for each dietary fiber (Table 5). The most evident changes were seen in the relative percentages of taurochenodeoxycholic and glycocholic acid. All dietary fibers except lvGG significantly reduced taurochenodeoxycholic acid although by a different extent, and PSY also caused a decrease in glycochenodeoxycholic acid. In fact, cheno conjugates were decreased by 68% with PSY. The relative percentage of glycocholic acid was significantly expanded from 40% in control to 55% in PSY-fed hamsters, while this bile acid was not significantly affected by hePE, lePE, hvGG, and lvGG (Table 5). As a result of the observed changes in the bile acid profile, the cholate/chenodeoxycholate ratio was dramatically increased from 2.1 ± 0.3 in control hamsters to 7.2 ± 0.8 with PSY feeding. In contrast, PE and GG had no substan-

TABLE 2
Plasma Lipoprotein Cholesterol and Triacylglycerol Concentrations of Hamsters Fed Diets Containing 8% of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	Cholesterol (mmol/L)			Triacylglycerol (mmol/L)		
	VLDL	LDL	HDL	VLDL	LDL	HDL
Control	1.07 ± 0.33[a]	0.77 ± 0.42[a]	3.70 ± 0.52[a]	2.43 ± 0.93[a,b]	0.06 ± 0.01[a]	0.12 ± 0.04[a]
8% Pectin						
hePE	0.86 ± 0.17[a]	0.60 ± 0.06[a,b]	3.40 ± 0.28[a]	2.35 ± 0.33[a,b]	0.06 ± 0.01[a]	0.11 ± 0.01[a]
lePE	1.09 ± 0.39[a]	0.78 ± 0.07[a]	3.50 ± 0.43[a]	2.71 ± 0.95[a]	0.08 ± 0.02[a]	0.11 ± 0.02[a]
8% Guar gum						
hvGG	1.03 ± 0.20[a]	0.71 ± 0.24[a,b]	3.53 ± 0.40[a]	2.75 ± 0.47[a]	0.07 ± 0.02[a]	0.11 ± 0.01[a]
lvGG	0.84 ± 0.36[a]	0.81 ± 0.19[a]	3.58 ± 0.43[a]	2.32 ± 1.03[a,b]	0.09 ± 0.01[a]	0.13 ± 0.03[a]
8% Psyllium	0.27 ± 0.03[b]	0.41 ± 0.15[b]	2.28 ± 0.26[b]	1.30 ± 0.12[b]	0.14 ± 0.05[b]	0.12 ± 0.01[a]

[a]Values are mean ± SD, n = 5–6 per diet. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. See Table 1 for other abbreviations.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.

TABLE 3
Composition of VLDL and LDL from Hamsters Fed Diets Containing 8% of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	VLDL composition (%)					LDL composition (%)				
	EC	FC	TG	PL	Protein	EC	FC	TG	PL	Protein
Control	11 ± 4[a]	7 ± 1[a]	60 ± 6[a]	14 ± 2[a]	8 ± 1[a]	34 ± 2[a]	9 ± 2	6 ± 2[a]	22 ± 2[a]	28 ± 1[a]
8% Pectin										
hePE	9 ± 1[a,b]	6 ± 1[a,b]	65 ± 2[a,b]	13 ± 1[a]	8 ± 1[a]	32 ± 3[a,b]	9 ± 2	7 ± 1[a]	21 ± 1[a,b]	30 ± 1[a,b]
lePE	9 ± 1[a]	6 ± 1[a,b]	63 ± 2[a,b]	14 ± 1[a]	8 ± 1[a,b]	32 ± 3[a,b]	9 ± 1	7 ± 2[a]	22 ± 1[a,b]	30 ± 1[a,b]
8% Guar gum										
hvGG	8 ± 1[a,b]	6 ± 1[a,b]	66 ± 2[b,c]	12 ± 1[a]	8 ± 1[a]	31 ± 1[a,b]	9 ± 1	7 ± 1[a]	23 ± 2[a]	30 ± 2[a,b]
lvGG	8 ± 1[a,b]	5 ± 1[a,b]	64 ± 2[a,b]	13 ± 2[a]	7 ± 1[a,b]	29 ± 2[b,c]	10 ± 1	7 ± 1[a]	23 ± 2[a]	30 ± 1[a,b]
8% Psyllium	5 ± 1[b]	4 ± 1[b]	72 ± 1[c]	8 ± 1[b]	9 ± 1[b]	27 ± 4[c]	8 ± 1	17 ± 2[b]	17 ± 5[b]	31 ± 2[b]

[a]Values are mean ± SD, n = 5–6 per diet; EC, esterified cholesterol; FC, free cholesterol; TG, plasma triacylglycerols; PL, phospholipids. See Tables 1 and 2 for other abbrevations.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.

tial effects on this ratio (Fig. 2). While GG and PSY decreased the percentage of taurodeoxycholic acid, glycodeoxycholic acid was significantly increased by PSY and PE but not by GG (Table 5). In control hamsters, 36% of bile acids were conjugated with taurine, while PSY, hePE, lePE, and hvGG decreased taurine conjugation. Again, PSY had the most pronounced effect leading to a reduction in taurine conjugated bile acids of more than 50%. As a result, the glycine/taurine conjugation ratio was 2.8 times higher in gallbladder bile from PSY-fed compared to control hamsters (Fig. 2). HePE, lePE, and hvGG also expanded this ratio, but this increase was not statistically significant.

TABLE 4
Liver Weight and Hepatic Cholesterol Concentrations in Hamsters Fed Diets Containing 8% Supplements of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	Liver weight (g/100 g body wt)	Total cholesterol (μmol/g)	Free cholesterol		Esterified cholesterol	
			(μmol/g)	(% TC)	(μmol/g)	(% TC)
Control	4.7 ± 0.2[a]	92.8 ± 13.2[a]	8.6 ± 1.5[a,b]	9 ± 2[a]	84.2 ± 13.4[a]	91 ± 2[a]
8% Pectin						
hePE	4.7 ± 0.3[a]	65.9 ± 9.2[b]	8.0 ± 0.4[b,c]	12 ± 2[a]	57.9 ± 9.1[b]	88 ± 2[a]
lePE	4.5 ± 0.3[a]	75.1 ± 12.7[b]	8.8 ± 0.6[a,b]	12 ± 2[a]	66.4 ± 12.7[b]	88 ± 2[a]
8% Guar gum						
hvGG	4.5 ± 0.3[a]	71.9 ± 9.8[b]	8.8 ± 0.6[a,b]	12 ± 1[a]	63.0 ± 9.4[b]	88 ± 1[a]
lvGG	4.5 ± 0.3[a]	75.7 ± 13.1[b]	9.0 ± 0.5[a]	12 ± 2[a]	66.7 ± 13.0[b]	88 ± 1[a]
8% Psyllium	3.6 ± 0.5[b]	10.5 ± 1.5[c]	7.2 ± 0.5[c]	69 ± 3[b]	3.3 ± 1.6[c]	31 ± 11[b]

[a]All values are mean ± SD, n = 10 per diet; TC, plasma total cholesterol. See Table 1 for other abbreviations.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.

TABLE 5
Bile Acid Profile of Gallbladder Bile from Hamsters Fed Diets Containing 8% Supplements of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	mol% of total bile acids					
	TCA[c]	GCA[c]	TCDCA[c]	GCDCA[c]	TDCA[c]	GDCA[c]
Control	18.7 ± 3.0[a]	39.5 ± 3.9[a]	10.8 ± 2.9[a]	18.1 ± 2.2[a,b]	5.6 ± 1.0[a]	5.9 ± 1.9[a]
8% Pectin						
hePE	16.2 ± 2.3[a,b]	44.7 ± 2.7[a]	6.5 ± 1.0[b]	17.0 ± 1.7[b]	4.8 ± 0.9[a,b]	10.0 ± 1.8[b]
lePE	15.9 ± 3.4[a,b]	44.7 ± 4.1[a]	6.6 ± 1.8[b]	16.9 ± 1.5[b]	4.7 ± 1.0[a,b,c]	10.0 ± 1.8[b]
8% Guar gum						
hvGG	16.5 ± 5.6[a,b]	43.0 ± 4.7[a]	7.8 ± 2.9[b,c]	19.6 ± 1.9[a]	3.6 ± 0.7[c]	8.8 ± 2.9[a,b]
lvGG	18.2 ± 3.1[a]	41.4 ± 5.2[a]	9.7 ± 2.2[a,c]	20.4 ± 1.5[a]	3.6 ± 0.7[c]	5.9 ± 0.5[a]
8% Psyllium	11.5 ± 3.2[b]	55.2 ± 3.4[b]	1.5 ± 0.3[d]	7.8 ± 0.7[c]	4.1 ± 0.7[b,c]	19.6 ± 4.0[c]

[a]Values are mean ± SD, n = 10 per diet.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.
[c]TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, glycodeoxycholic acid. Taurolithocholic and glycolithocholic acids were only present in traces (<1% of total bile acids). See Table 1 for other abbreviations.

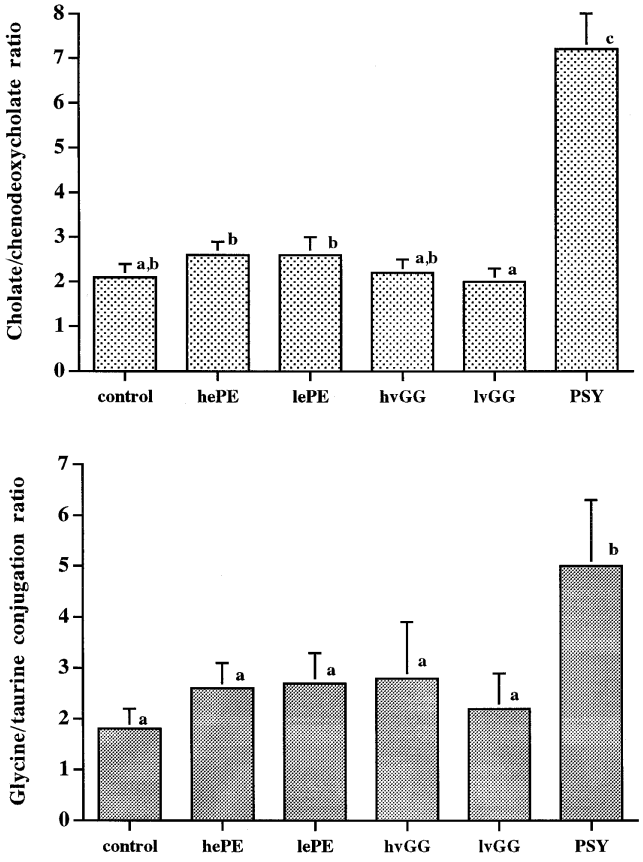

FIG. 2. Cholic acid/chenodeoxycholic acid ratio and glycine/taurine conjugation ratio in gallbladder bile of hamsters fed diets containing 8% supplements of different pectins, guar gums, or PSY. Values are mean ± SD ($n = 10$ per diet). Bars with different letters were significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni-Dunn test. See Figure 1 for abbreviations.

Fecal excretion of neutral sterols and bile acids. The daily fecal weight was significantly increased by 138% in PSY-fed hamsters compared to all other diets. In contrast, hePE, lePE, hvGG, and lvGG had only a modest effect on fecal weight (Table 6). The moisture content of the fecal samples was not different. Fecal neutral sterol excretion expressed per gram feces was not different between diet groups (Table 6). However, the daily excretion of neutral sterols was significantly higher in PSY-fed hamsters compared to all other diets owing to the higher fecal output. The bulk of neutral sterols was composed of coprostanol and cholesterol whereas other sterols were only present in small concentrations (Table 6). The fecal concentration of cholesterol was three times higher in PSY-fed hamsters compared to controls while coprostanol was decreased by 55%, suggesting a diminished intestinal breakdown of cholesterol. In contrast, PE and GG revealed no striking changes in the fecal neutral sterol profile.

Fecal total bile acid concentration (expressed per gram feces dry weight) was significantly increased in PSY-fed hamsters compared to all other diets (Table 7). PE and GG had no effect on the fecal bile acid concentration. Daily fecal bile acid excretion was more than four times higher in PSY-fed compared to control hamsters. HePE and hvGG caused a slight but not significant increase, whereas lePE and lvGG had no effect on daily fecal bile acid excretion (Table 7). Distinct changes in the excretion of individual bile acids were apparent (Table 7). There was a significant increase in deoxycholic acid with PSY while lePE, hvGG, and lvGG seemed to reduce the excretion of deoxycholic acid compared to fecal samples of control hamsters. The excretion of lithocholic acid was lower with all fiber supplements compared to control, but this effect was only significant with lePE and lvGG. Other observed differences were a greater excretion of 12-ketolithocholic acid in hamsters fed the fiber supplements compared to controls. However, owing to the large variation, these increases were not significant. In addition, substantial changes in the bile acid excretion profile existed when expressed as percentage of total bile acids. Again, PSY caused the most pronounced alterations. There was a significant decrease in the percentage of lithocholic acid from 41 ± 6% in control to 18 ± 2% in PSY-fed hamsters. In contrast, the percentage of deoxycholic acid was increased from 30 ± 5% in controls to

TABLE 6
Fecal Excretion of Neutral Sterols in Hamsters Fed Diets Containing 8% Supplements of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	Fecal weight	Total neutral sterol excretion		Distribution of neutral sterols (μmol/g)			
	(g/d)	(μmol/d)	(μmol/g)	Cholesterol	Coprostanol	Cholestanol	Other sterols[c]
Control	0.8 ± 0.1[a]	8.5 ± 1.2[a]	10.6 ± 2.1	1.5 ± 0.7[a]	7.1 ± 1.7[a]	0.33 ± 0.18	1.7 ± 0.27[a]
8% Pectin							
hePE	1.1 ± 0.3[a]	9.3 ± 3.7[a]	8.2 ± 1.6	3.6 ± 2.2[a,b]	3.5 ± 1.3[b]	0.10 ± 0.14	1.0 ± 0.26[b]
lePE	1.1 ± 0.2[a]	9.0 ± 2.4[a]	8.3 ± 1.0	1.5 ± 0.6[a]	4.9 ± 1.3[a,b]	0.35 ± 0.18	1.5 ± 0.16[a]
8% Guar gum							
hvGG	1.0 ± 0.2[a]	8.1 ± 1.8[a]	8.2 ± 1.7	2.0 ± 0.9[a]	4.2 ± 1.4[a,b]	0.35 ± 0.08	1.5 ± 0.30[a]
lvGG	1.1 ± 0.2[a]	9.0 ± 1.2[a]	8.3 ± 1.4	1.7 ± 0.7[a]	4.8 ± 1.9[a,b]	0.31 ± 0.17	1.5 ± 0.37[a]
8% Psyllium	1.9 ± 0.1[b]	16.1 ± 5.0[b]	8.4 ± 2.8	4.5 ± 1.4[b]	3.2 ± 2.8[b]	n.d.	0.82 ± 0.15[b]

[a]All values are mean ± SD, $n = 6$ per diet; n.d., not detected.
[b]Means in a column with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.
[c]Other sterols include coprostanone, cholestanone, stigmasterol, stigmastanol.

TABLE 7
Fecal Excretion of Bile Acids in Hamsters Fed Diets Containing 8% Supplements of Different Types of Pectin, Guar Gum, or Psyllium[a,b]

	Total bile acid excretion		Distribution of individual fecal bile acids (μmol/g)					
	(μmol/d)	(μmol/g)	LCA[c]	DCA[c]	CDCA[c]	Hyo-DCA[c]	UDCA[c]	keto-LCA[c]
Control	2.7 ± 0.9[a]	3.3 ± 1.3[a]	1.37 ± 0.52[a]	1.05 ± 0.55[a]	0.12 ± 0.03	0.36 ± 0.16	0.12 ± 0.04	0.33 ± 0.18
8% Pectin								
hePE	3.7 ± 1.1[a]	3.5 ± 0.7[a]	1.04 ± 0.22[a,b]	1.07 ± 0.17[a]	0.11 ± 0.13	0.43 ± 0.08	0.18 ± 0.10	0.64 ± 0.22
lePE	2.9 ± 1.0[a]	2.7 ± 0.5[a]	0.75 ± 0.13[b]	0.60 ± 0.25[a]	0.02 ± 0.05	0.38 ± 0.18	0.25 ± 0.09	0.67 ± 0.25
8% Guar gum								
hvGG	3.3 ± 0.7[a]	3.4 ± 0.7[a]	0.97 ± 0.22[a,b]	0.75 ± 0.25[a]	0.19 ± 0.04	0.54 ± 0.09	0.25 ± 0.07	0.69 ± 0.19
lvGG	2.7 ± 0.7[a]	2.5 ± 0.5[a]	0.75 ± 0.11[b]	0.52 ± 0.18[a]	0.12 ± 0.02	0.37 ± 0.11	0.13 ± 0.02	0.52 ± 0.12
8% PSY	12.1 ± 1.5[b]	6.3 ± 0.6[b]	1.12 ± 0.13[a,b]	4.12 ± 0.44[b]	0.12 ± 0.10	0.21 ± 0.15	0.19 ± 0.08	0.48 ± 0.11

[a]All values are mean ± SD, $n = 6$ per diet.
[b]In a column, means with different superscripts are significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni/Dunn test.
[c]CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; hyo-DCA, hyodeoxycholic acid (5β-cholanic acid-3α,6α-diol); 12-keto-LCA, 12-ketolithocholic acid (5β-cholanic acid-3α-ol-12-one); LCA, lithocholic acid; UDCA, ursodeoxycholic acid. See Table 1 for other abbreviations.

66 ± 6% with PSY while hePE had no effect and lePE, hvGG, and lvGG caused a slight decrease. Other changes were a significantly lower percentage of hyodeoxycholic acid with PSY and a higher percentage of 12-ketolithocholic acid with PE and GG.

Taking into account the daily cholesterol intake during wk 4, the apparent neutral sterol (cholesterol) absorption was obviously moderately depressed by PSY (Fig. 3). The calculated total sterol balance was +9.5 μmol/d in controls and was not altered by PE and GG, while a negative balance (–2.4 μmol/d) was found in hamsters fed PSY (Fig. 3).

DISCUSSION

Although the cholesterol-lowering effect of PE, GG, and PSY is well established in experimental animals and human subjects, the mechanism(s) involved remain to be elucidated (1,24). Physicochemical properties such as viscosity, fermentability, or binding capacity of bile acids have received much attention and are thought to affect the hypocholesterolemic action of soluble fibers though the supporting evidence is inconsistent. Particularly, the putative action of the fibers in the small intestine may play a major role in regulating cholesterol and bile acid metabolism by altering absorption of dietary or biliary cholesterol and reabsorption of circulating bile acids.

In the present study the effects of PE, GG, and PSY on lipoproteins, bile acid profile, and particularly on fecal sterol excretion were further investigated utilizing the hamster, which is a common model to study diet-induced hypocholesterolemic effects (12–14). In previous studies with hamsters fed 0.4% dietary cholesterol, PSY significantly lowered plasma lipids by increasing the fecal excretion of bile acids, whereas PE and GG failed to demonstrate a distinct lipid-lowering effect (15,25). To exclude that an excessive dietary cholesterol intake could possibly overwhelm possible fiber-mediated effects because the amount of dietary cholesterol clearly affects the metabolic response of different fibers (1),

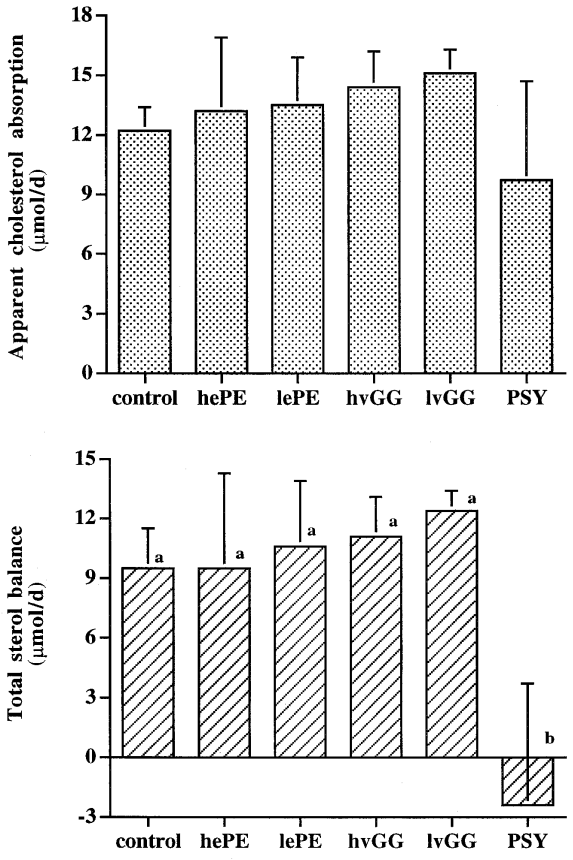

FIG. 3. Apparent cholesterol excretion (μmol/d) and total sterol balance (μmol/d) in hamsters fed diets containing 8% supplements of different pectins, guar gums, or PSY. Values are mean ± SD ($n = 6$ per diet). Bars with different letters were significantly different ($P < 0.05$) using one-way ANOVA and the Bonferroni-Dunn test. Apparent cholesterol absorption = cholesterol intake (μmol/d) – fecal excretion of neutral sterols (μmol/d). Cholesterol intake (μmol/d) was: control, 20.7; hePE, lePE and hvGG, 22.5; lvGG, 24.0 and PSY, 25.8. Total sterol balance = cholesterol intake (μmol/d) – [fecal excretion of neutral sterols (μmol/d) + fecal excretion of bile acids (μmol/d)]. See Figure 1 for abbreviations.

in the present study, diets containing a moderate level of dietary cholesterol (0.12%) were applied.

Again, 8% PSY significantly altered lipoprotein and bile acid metabolism, while the different types of PE (hePE and lePE) and GG (hvGG and lvGG) produced no substantial lipid-lowering effect. This lack of a potent hypocholesterolemic action of PE and GG could possibly be related to several aspects. First, adding these highly fermentable fibers to a diet already rich in nonfermentable cellulose (10%) could have resulted in a dilution of the expected effects of either PE or GG in the intestine, although this assumption seems rather unlikely because PSY caused a significant hypolipidemic effect with changes in lipoprotein and bile acid composition and excretion.

Second, it seems plausible that PE and GG were partially hydrolyzed, diminishing their physicochemical properties and attenuating their hypocholesterolemic potential. In fact, PE and GG are both highly fermentable and are usually almost completely degraded. Evidently, PSY is less rapidly and only partially fermented, whereas PE and possibly GG completely disappear in the human gut (28). Normally, the fermentation of soluble fibers is subject of the colonic microflora and takes place in the cecum and colon. Possibly, because of the distinctive digestive tract of the hamster (e.g., cheek pouches, pregastric and gastric pouch), a pregastric fermentation of PE and GG could have occurred (26,27). Therefore, it seems possible that GG and PE, but apparently not PSY, were already hydrolyzed and sufficiently degraded in the rumen-like forestomach. The action of PSY seems not to be impaired by pregastric fermentation, indicating that PSY, PE and GG differ in their physicochemical properties, e.g., in their fermentation capacity. These differences in the fermentative breakdown seem to relate to their hypocholesterolemic potential and could explain the disparate response on cholesterol metabolism of PSY vs. PE and GG in hamsters. However, the forestomach microflora is poorly defined, and the known bacterial populations do not hydrolyze PE or GG (29). Also, residence time of digested food in the forestomach seems rather short, reducing the effectiveness of a pregastric fermentation (30). Taken together, a pregastric fermentation of PE and GG seems speculative unless part of the colonic microflora has settled in the pregastric pouch because of the known coprophagy. To clearly prove this hypothesis, fermentation end products (short-chain fatty acids or lactic acid) need to be analyzed in the forestomach contents. Whereas PSY clearly expanded the intestinal volume (increase in the cecal content), PE and GG revealed no effect on cecal weight, further supporting the theory of a pregastric breakdown.

A third explanation for the lack of effect could be that 8% PE and GG, unlike 8% PSY, might not have been efficient enough to produce changes in lipoprotein or bile acid metabolism although studies using rats and guinea pigs have clearly demonstrated lipid-lowering effects with similar or even lower doses of PE and GG (3,5,6,31). In contrast, previous hamster studies have also shown more equivocal effects of PE and GG on cholesterol metabolism (32,33).

The lipid-lowering potential of PE and GG has been attributed to physicochemical properties such as degree of esterification and/or viscosity (27,34). In line with previous findings (34), hePE was somewhat more effective in lowering plasma cholesterol than lePE (−10 vs. −4%) even though the viscosity of hePE was lower compared with lePE (71 vs. 184 mPa·s). However, the two types of GG which differed in their viscosity (6014 and 22 mPa·s) did not reveal different effects on lipoproteins, bile acid profile, and sterol excretion. A previous hamster study also could not demonstrate a consistent effect of different viscosities on the lipid-lowering action of GG (27). Therefore, the present data do not clarify the impact of the degree of esterification or viscosity on the hypocholesterolemic effect of PE and GG.

PSY impressively reduced the plasma cholesterol and TG, which is in line with previous findings from studies with various animal models including the hamster (5,7,8,35,36). PSY not only caused a 75% reduction in plasma VLDL-cholesterol but also affected the composition of VLDL and LDL by decreasing the relative EC content and increasing the relative proportion of TG which has also been shown previously (4). These compositional changes could possibly indicate a modified production of VLDL and conversion to LDL particles, suggesting that the hypocholesterolemic effect of PSY occurs at multiple metabolic sites including the regulation of lipoprotein synthesis and catabolism as demonstrated previously (1,4).

Increased excretion of bile acids and neutral sterols due to impaired reabsorption of bile acids and diminished cholesterol absorption is thought to be the major determinant for the cholesterol-lowering action of soluble fibers. In fact, PSY accelerated bile acid removal, resulting in a 348% increase in daily bile acid excretion mainly as deoxycholic acid. Reportedly, PSY does not bind bile acids *in vitro* (35). However, our data clearly demonstrate that bile acid excretion is significantly enhanced by PSY. Although the physicochemical mechanisms involved are not fully understood, it is likely that, during the enteric gelation of PSY bile acids and preferentially taurine conjugated bile acids were entrapped. PSY also expanded daily total neutral sterol excretion (89% increase) which may indicate increased losses of endogenous cholesterol or an inhibition of cholesterol absorption. It was found that PE and GG inhibited cholesterol absorption especially in animals fed high-cholesterol diets (1) whereas PSY had no effect on cholesterol absorption (1,8,37). However, the present data suggest that cholesterol absorption was moderately inhibited by PSY. The entrapment of bile acids within the viscous matrix formed in the intestinal lumen could possibly disturb micelle formation, leading to a diminished cholesterol absorption. In humans, PSY has been shown to decrease cholesterol absorption efficiency (38). The calculated total sterol balance was markedly altered with PSY mainly owing to the increase in bile acid excretion. The positive sterol balance in control hamsters (+9.5 μmol/d) was shifted toward a negative balance in PSY-fed hamsters (−2.4 μmol/d), suggesting stimulated bile acid synthesis. Indeed, a PSY-induced up-regulation of the hepatic 7α-hydroxylase ac-

tivity, the key enzyme in bile acid synthesis, has recently been demonstrated in rats, hamsters, and guinea pigs (1,7,11). Subsequently, there is an increased demand for FC in the liver which can be attained by raising the hepatic *de novo* synthesis of cholesterol *via* stimulation of the HMG-CoA reductase activity or by stimulating the liver uptake of lipoprotein cholesterol by up-regulating the LDL-receptor activity. All of these alterations have been documented after PSY supplementation (1,7,9,11).

Another point to consider is the significant changes in the circulating bile acid profile induced by PSY and, to a minor degree, by hePE and lePE. PSY produced a predominant cholate profile with a 40% increase in glycocholate while taurochenodeoxycholate and glycochenodeoxycholate were decreased by 86 and 57%, respectively. As a result, the cholate/cheno ratio increased from 2.1 in controls to 7.2 in PSY-fed hamsters. In addition, PSY also increased the glycine/taurine conjugation ratio by impressively expanding the pool of glycine conjugated bile acids. Recirculating bile acids are known as potent inhibitors of hepatic bile acid synthesis by down-regulating cholesterol 7α-hydroxylase activity dependent on their hydrophobicity. Since chenodeoxycholate is more hydrophobic than cholate, a cheno-rich bile acid profile will inhibit bile acid synthesis more than a predominant cholate profile. The PSY-induced changes in the bile acid profile reflect a reduced hydrophobicity and an increase in hydrophilic bile acids (cholate). Moreover, PSY seems to remove selectively taurine conjugated bile acids (mostly taurocheno). As suggested, the composition of individual bile acids may be more important than the total concentration of the recirculating bile acid pool in regulating bile acid and cholesterol synthesis (39). Therefore, both the qualitative changes (decrease in chenodeoxycholate with concomitant reduction in hydrophobicity) and the quantitative changes (increased fecal bile acid excretion) in the bile acid pool produced by PSY favored a stimulation of bile acid synthesis. Taken together, these findings are consistent with the hypothesis of previous studies (11,36), demonstrating that PSY influences cholesterol and bile acid metabolism by affecting the enterohepatic circulation of the bile acid pool.

In summary, the present disparate findings further emphasize that the cholesterol-lowering action of PE, GG, and PSY results from distinct mechanisms specific to each fiber. In particular, the action of soluble fibers in the intestine, i.e., alterations of enterohepatic circulation of bile acids and changes in composition of the bile acid pool seem to play the major role in regulating cholesterol metabolism. The disparate effects of PSY, PE, and GG found in the present study are possibly related to different physicochemical properties of these fibers affecting intestinal viscosity and susceptibility to fermentation (especially in the forestomach) and therefore the fiber-mediated action in the intestine. Both increased fecal bile acid excretion and changes in bile acid profile produced by PSY seem to be the most important determinants of stimulating hepatic bile acid synthesis which is most likely the major mechanism through which PSY lowers cholesterol.

ACKNOWLEDGMENTS

The authors thank Stephan Drusch and Swantje Möller for technical assistance and Dr. Dirk Hisserich (Ratjen Backmittel, Flintbek, Germany) for supplying the basic ingredients for the diet preparations. This research project was supported by a grant from FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany), the AiF, and the Ministry of Economics (Project No. 9670).

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[Received February 3, 1998, and in final revised form and accepted April 29, 1998]

Dietary Cyanidin 3-*O*-β-D-Glucoside Increases *ex vivo* Oxidation Resistance of Serum in Rats

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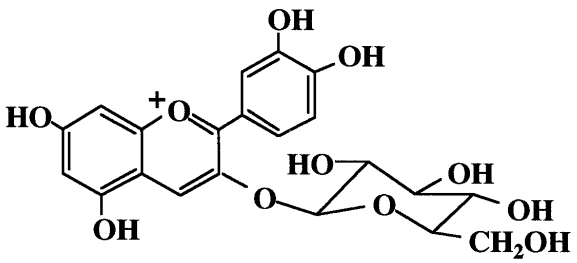
ABSTRACT: The effect of dietary cyanidin 3-*O*-β-D-glucoside (C3G), a typical anthocyanin pigment, on the generation of thiobarbituric acid reactive substances (TBARS) during serum formation *ex vivo* and susceptibility of serum to further lipid peroxidation was studied in rats. Rats were fed a diet containing C3G (2 g/kg) for 14 d. Feeding C3G resulted in a significant decrease in generation of TBARS during serum formation. The serum from the C3G-fed group showed a significantly lower susceptibility to further lipid peroxidation provoked by 2,2′-azobis(2-amidinopropane)hydrochloride or Cu^{2+} than that of the control group. No significant differences were observed in serum phospholipid, triglyceride, esterified cholesterol, and free fatty acid concentrations between the control and the C3G-fed groups. Concentrations of endogenous antioxidants remaining in the serum after blood coagulation were not affected by the C3G feeding. These results demonstrate that feeding C3G increases the *ex vivo* oxidation resistance of the serum without affecting serum endogenous antioxidant levels, and reduces the TBARS generated during serum formation without changing the concentrations of serum lipids.

Lipids 33, 583–588 (1998).

The production of active oxygen species, such as hydroxyl radicals, superoxide anion radicals, and singlet oxygen and other radicals, is believed to be involved in carcinogenesis, mutagenesis, aging, and atherosclerosis (1–6). Endogenous antioxidants are presumed to protect cells against active oxygen species (1,5–9), and there is an increasing interest in the *in vivo* protective function of natural antioxidants contained in dietary plants (10–12). We previously reported that edible beans have antioxidative activity *in vitro* (13), although these food items had not been recognized previously as having antioxidant activity. Among the beans tested, the antioxidant activity of colored pea beans (*Phaseolus vulgaris* L.) was remarkable, suggesting that pigments contained in the seed coat had a potential role as dietary antioxidants. Three anthocyanin pigments, pelargonidin 3-*O*-β-D-glucoside, cyanidin 3-*O*-β-D-glucoside (C3G) and delphinidin 3-*O*-β-D-glucoside (D3G), were isolated from red and black beans (*P. vulgaris* L.), and identified as antioxidants *in vitro* (14–16). Among the isolated pigments, C3G (Scheme 1) had significant antioxidant activity (15) and scavenging activity against hydroxyl radicals and superoxide *in vitro* (16). Therefore, it seemed possible that C3G may also act as a potent antioxidant *in vivo*.

Recently, the "French paradox," a low incidence of coronary heart disease and atherosclerosis despite eating a high-fat diet, has been suggested to relate to phenolic compounds contained in red wine. These may act as *in vivo* inhibitors of low density lipoprotein (LDL) oxidation (17–20). Red wine contains a large amount of anthocyanins, such as D3G, petunidin 3-*O*-β-D-glucoside, malvidin 3-*O*-β-D-glucoside and C3G derived from grape skin (21,22), indicating that these anthocyanin pigments may also exert an inhibitory effect upon the oxidation of LDL.

C3G is widely distributed in the human diet through crops, beans, fruits, vegetables and red wines (23), suggesting that humans can ingest considerable amounts of C3G from plant-based diets daily. In general, anthocyanin pigments are stable in acidic conditions, but are unstable and rapidly broken down in neutral conditions (24). Due to this instability at neutral pH, anthocyanins have not been recognized as antioxidants (24). However, we have recently demonstrated in *in vitro* studies that C3G has antioxidant activity (15) and the capacity to scavenge hydroxyl radicals and superoxide under physiological conditions (16). There have been no prior studies as to whether feeding C3G could improve the *in vivo* antioxidant status of animals. In addition, it has not been clarified in animals whether C3G is effectively absorbed or accumulated in tissues.



SCHEME 1

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; C3G, cyanidin 3-*O*-β-D-glucoside; D3G, delphinidin 3-*O*-β-D-glucoside; GSH, reduced glutathione; LDL, low density lipoprotein; TBARS, thiobarbituric acid reactive substance.

In the present study, we evaluated the effect of C3G, which is an anthocyanin with antioxidant activity, *ex vivo* lipid peroxidation, and oxidation resistance of liver and serum in rats.

MATERIALS AND METHODS

Chemicals. 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH), ascorbic acid, and reduced glutathione (GSH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). C3G (purity: more than 95%) was purified using the commercial food coloring material (San Red No. 5F; San-Ei Gen F.F.I., Inc., Osaka, Japan) derived from violet corn (*Zea mays* L.) using previously described high-performance liquid chromatography (25). The structure and purity were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance, fast atom bombardment mass spectra, ultraviolet–visible infrared spectra before use.

Animals and diets. Five-week-old male Wistar rats (~105 g; Japan SLC Inc., Hamamatsu, Japan) were used and housed individually in stainless-steel wire-mesh cages at 23 ± 0.3°C with a 12-h light cycle. Rats were allowed free access to water and a semipurified diet. All animals were fed the control diet containing (wt%): casein, 25; mineral mixture (AIN93G-MX) (26), 3.5; vitamin mixture (AIN93-VX) (26), 1; choline chloride, 0.2; corn oil, 5; cellulose powder, 4; and the remainder carbohydrate (sucrose/starch = 1:2) for 7 d before the experiment. C3G (2 g/kg diet) was added in place of sucrose. Rats were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University.

Experimental design and tissue preparation. Ten rats were divided into two groups and fed the control diet (control group) or the control diet containing 0.2% C3G (C3G group) for 14 d. After 14 d of feeding, the rats were killed by decapitation. After the collected blood had sat at room temperature for 5 min for coagulation, the serum was obtained by centrifugation at 1,600 × *g* for 15 min at 4°C. The separation of serum was finished within 30 min. The serum was immediately stored at −80°C until assayed. The liver was rapidly excised after being perfused with physiological saline. The liver was kept at −80°C until assayed. These conditions were standardized for all the animals.

Measurement of oxidation resistance of the serum. The serum was oxidized at 37°C by incubation with AAPH or $CuSO_4$ (27,28). The final concentrations of AAPH and $CuSO_4$ were 25 mM and 500 μM, respectively. During incubation, aliquots were taken at specific intervals, and lipid peroxidation was stopped by the addition of butylated hydroxytoluene at a final concentration of 60 μM. The degree of oxidation was immediately measured by the thiobarbituric acid reactive substances (TBARS) assay (29). The measurement of oxidation resistance of the serum was performed within 1 wk after killing the rats.

Measurement of oxidation resistance of liver homogenate. Frozen liver (0.5 g) was homogenized in 20 vol of 20 mM phosphate buffer (pH 7.4) using a Potter-Elvehejm homogenizer, and the homogenate was oxidized at 37°C by incubation with AAPH (final concentration, 5 mM). During incubation, aliquots were taken at specific intervals, and butylated hydroxytoluene was added to them at the final concentration of 60 μM. The degree of oxidation was immediately measured by TBARS assay (30). The measurement of oxidation resistance of the liver was performed within 1 wk after killing the rats.

Measurement of protein concentration of liver homogenate. The protein concentration of the liver homogenate was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Measurement of antioxidants. The α-tocopherol concentration in the serum was measured by high-performance liquid chromatography according to the method of Ueda and Igarashi (31). The ascorbic acid concentration in the serum was measured according to the method of Yamamoto *et al.* (32). The GSH concentration in the serum was measured using a commercial assay kit (GSH-400; Oxis International, Inc., Portland, OR). The serum uric acid concentration was measured using a commercial assay kit (Uric acid B-Test Wako; Wako Pure Chemical Industries, Ltd.) by the colorimetric method of Kabasakalian *et al.* (33). Serum antioxidant concentrations were measured within 1 mon after killing the rats.

Measurement of serum lipids. Serum phospholipid (34), triglyceride (35), total cholesterol (36), and free cholesterol (36) were measured by enzymatic assay with commercial assay kits (Phospholipid C-test, triglyceride G-test, Cholesterol E-test, Free cholesterol E-test, respectively; Wako Pure Chemical Industries, Ltd.). Serum esterified cholesterol concentrations were calculated by subtracting the concentration of free cholesterol from that of the total cholesterol. Serum free fatty acid was measured by a colorimetric method with a commercial assay kit (NEFA-test; Wako Pure Chemical Industries, Ltd.) (37). Serum lipid concentrations were measured within 1 mon after killing the rats.

Statistical analysis. All results were expressed as the means ± SE. Data were compared using Student's *t*-test. Probability values < 0.05 were considered significant.

RESULTS

Effect of dietary C3G on body weight gain, food intake, and liver weight. Body weight gain after 14 d, food intake, and liver weight of the rats in the C3G group were not significantly different from those in the control group (data not shown), suggesting that feeding C3G did not cause serious toxicity in rats.

Effect of dietary C3G on serum TBARS concentration and the susceptibility of the serum to lipid peroxidation. Figure 1A shows the concentration of TBARS generated during formation of serum from rats in the control and C3G groups. The serum TBARS concentration was slightly but significantly lower in the C3G group (82% of the control group) than in the control group. Figure 1B and C show the time course of

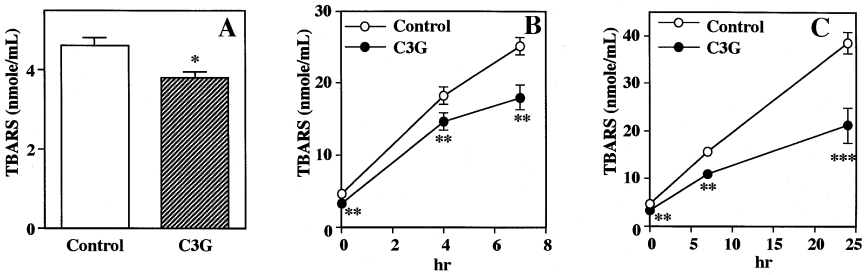

FIG. 1. Effect of cyanidin 3-*O*-β-D-glucoside (C3G) feeding on the serum thiobarbituric acid reactive substance (TBARS) concentration (A) and the susceptibility of the serum to lipid peroxidation induced by 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) (B) or $CuSO_4$ (C) in rats. The serum from the control or C3G group was incubated at 37°C with AAPH (25 mM) or $CuSO_4$ (500 μM). During the incubation at specified intervals, the TBARS concentration in the medium was measured. Values are means ± SE for five rats. *Significantly different ($P < 0.05$) from the control value. **Significantly different ($P < 0.05$) from the control value at the respective incubation times. ***Significantly different ($P < 0.01$) from the control value at the respective incubation times.

TBARS generation in serum exposed to AAPH or $CuSO_4$. The serum obtained from the C3G group showed significantly less oxidation compared with that from the control group throughout the 7-h incubation when the lipid peroxidation was induced by AAPH (Fig. 1B). In the presence of $CuSO_4$, lipid peroxidation of the serum from the C3G group was also inhibited by 67 and 52% after 7- and 24-h incubation, respectively, compared with that from the control group (Fig. 1C).

Effect of dietary C3G on serum lipid concentrations. The serum lipid concentrations in the control and C3G groups are shown in Table 1. No significant difference was observed for the phospholipid, triglyceride, esterified cholesterol, and free fatty acid concentrations between the control and the C3G groups. Total and free cholesterol concentrations in the C3G group were significantly lower than those in the control group.

Effect of dietary C3G on serum antioxidant concentrations. Serum antioxidant concentrations of the rats in the control or C3G groups are shown in Table 2. No significant differences between the sera from control and C3G groups were observed in the concentrations of α-tocopherol, GSH, ascorbic acid, and uric acid.

Effect of dietary C3G on liver protein, TBARS concentration, and the susceptibility of liver homogenate to lipid peroxidation. The liver protein concentrations in rats from the control and C3G groups were 148.6 ± 5.1, 153.1 ± 5.9 mg/g wet weight, respectively, which were not significantly different. The TBARS generated in the liver homogenates from the control or C3G groups are shown in Figure 2A. No significant difference in the TBARS concentration was observed between the control and C3G groups in the liver. Figure 2B shows the time course of TBARS generation of the liver homogenate exposed to AAPH. The susceptibility of liver to lipid peroxidation was not affected by the C3G feeding when the lipid peroxidation was induced in liver homogenate by AAPH.

DISCUSSION

Recently, the antioxidant activity of phenolic phytochemicals has been widely investigated (38–40). However, anthocyanins, which are included in the phenolic phytochemicals, are unstable in neutral conditions, and therefore we believe that anthocyanins would not have antioxidant activity. We have demonstrated in an *in vitro* study that C3G has both antioxidant activity (15) and the capacity for scavenging hydroxyl radicals and superoxide (16). C3G is one of the most prevalent anthocyanins in a plant-based daily diet, but the *in vivo* effectiveness has been uncertain. Therefore, we tried to determine whether C3G ingested by animals would act as an antioxidant *in vivo*.

In this study, the concentration of TBARS generated during serum formation was significantly lower in the C3G group than in the control group, and C3G feeding resulted in a significant resistance of the serum against further lipid peroxidation induced by AAPH or Cu^{2+}. The reduction in the serum TBARS concentration and the increase in the oxidation resistance of the serum observed in the C3G group are not due to

TABLE 1
Effect of Cyanidin 3-*O*-β-D-glucoside (C3G) Feeding on the Serum Lipids in Rats[a]

Group	Triglyceride (mg/100 mL)	Phospholipid (mg/100 mL)	Cholesterol (mg/100 mL)			Free fatty acid (meq/100 mL)
			Total	Free	Esterified	
Control	121.8 ± 6.9	276.5 ± 6.3	107.6 ± 3.9	28.0 ± 2.0	79.6 ± 4.1	0.30 ± 0.03
C3G	109.0 ± 6.9	259.8 ± 6.8	90.6 ± 2.9*	19.4 ± 1.1*	71.2 ± 2.0	0.34 ± 0.03

[a]Values are means ± SE for five rats. *Significantly different ($P < 0.01$) from the control value.

TABLE 2
Effect of C3G Feeding on the Serum Antioxidants Concentrations in Rats[a]

Group	α-Tocopherol (μg/mL)	Ascorbic acid (μg/mL)	GSH (μg/mL)	Uric acid (μg/mL)
Control	18.6 ± 0.9	5.0 ± 0.3	52.2 ± 2.3	16.0 ± 0.1
C3G	17.9 ± 0.9	5.9 ± 0.5	50.6 ± 2.4	15.0 ± 0.1

[a]Values are means ± SE for five rats. See Table 1 for abbreviation.

the decrease in serum lipids such as phospholipids, triglycerides, and free fatty acids. Frei *et al*. (27) reported that the esterified cholesterol fraction was very sensitive to oxidation induced by AAPH in human plasma. In this study, the serum concentration of esterified cholesterol was not affected at all by C3G feeding. These results indicate that dietary C3G reduces the generation of TBARS during serum formation and increases the oxidation resistance of the serum without changing the serum lipid concentrations.

The effect of red wine consumption on the susceptibility of plasma and LDL to *ex vivo* lipid peroxidation has been reported (20,41). Fuhrman (41) reported that the total polyphenols in the plasma LDL fraction were elevated fourfold after 2 wk of red wine consumption in humans, and the elevation was associated with a significant decrease in LDL susceptibility to lipid peroxidation. Red wine contains a large amount of anthocyanins such as C3G (21,22), indicating that the pigments may contribute to the inhibitory effect on oxidation of LDL. The measurement of C3G and related compounds in the serum and lipoproteins is not routinely available, but further studies are in progress to accomplish this.

The serum contains an array of antioxidants, such as ascorbic acid, α-tocopherol, uric acid, and GSH. These antioxidants decrease the rate of lipid peroxidation during the activation of platelets when blood clots to form serum. In this study, no significant differences between the control and the C3G groups were observed in the concentrations of these physiological antioxidants left in serum, suggesting that C3G feeding did not affect the concentrations of serum antioxidants in rat plasma prior to blood coagulation. Nardini *et al*. (42) reported that the α-tocopherol concentration in the plasma and lipoproteins was significantly higher in rats fed caffeic acid compared to that in the control rats, and the purported antioxidant action of caffeic acid *in vivo* may be related to both its direct antioxidant properties as well as the sparing effect on α-tocopherol. The antioxidant action of C3G, however, seems to be due to a direct antioxidant effect, since a sparing effect on endogenous antioxidants was not apparent.

In contrast to the findings in rat serum, TBARS generation during homogenation of liver concentration and the susceptibility of the liver homogenate to lipid peroxidation were not affected by C3G feeding in rats. The liver ascorbic acid, α-tocopherol, and glutathione concentrations were also not affected by C3G feeding. Because the liver contains a sufficient amount of antioxidants (for example, liver in the C3G group rats had ascorbic acid; 172.2 ± 7.1 μg/g liver, α-tocopherol; 47.6 ± 2.6 μg/g liver, and GSH; 1.8 ± 0.1 mg/g liver) and antioxidative enzymes (glutathione peroxidase, catalase, superoxide dismutase, etc.), it may be difficult to modulate the liver antioxidant capacity by C3G feeding. It is also possible that unmetabolized C3G is not present in rat liver and thus could not influence lipid peroxiation there.

Anthocyanin pigments are widely distributed in the human diet through seeds, fruits, and vegetables. Red wine also contains anthocyanins derived from grape skin. Therefore, humans ingest significant amounts of anthocyanin pigments from a plant-based diet. In the present study, we have demonstrated for the first time that oral administration of C3G is associated with antioxidant activity in rats. We have recently reported that C3G was able to react with peroxyl radicals, and then converted itself to the oxidation products, 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (25). Protocatechuic acid can also scavenge free radicals, suggesting that C3G would produce another radical scavenger by the reaction of C3G with biological radicals *in vivo*. Further studies are under way to demonstrate that these oxidation reactions of C3G occur *in vivo*, and to find evidence for the oxidized metabolites or C3G in animals.

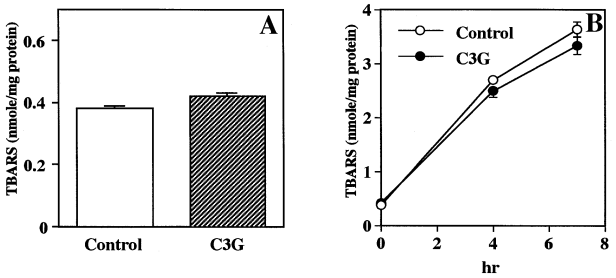

FIG. 2. Effect of cyanidin C3G feeding on the liver TBARS concentration (A) and the susceptibility of the liver homogenate to lipid peroxidation induced by AAPH (B) in rats. The liver homogenate that was obtained from the control or C3G group was incubated at 37°C with AAPH (5 mM). During the incubation at specified intervals, the TBARS concentration in the medium was measured. Values are means ± SE for five rats. See Figure 1 for abbreviations.

ACKNOWLEDGMENTS

This study was supported by a Program for Promotion of Basic Research Activities for Innovative Biosciences. The authors would like to thank Takahito Ichi, San-Ei Gen F.F.I. Inc., Toru Niwa and Tatsuya Uraji, Nikken Fine Chemicals Co., Ltd. for providing the food coloring material and purifying C3G.

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[Received January 26, 1998, and in final revised form April 15, 1998; revision accepted May 4, 1998]

Protective Effect of a Vitamin E Analog, Phosphatidylchromanol, Against Oxidative Hemolysis of Human Erythrocytes

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ABSTRACT: The protective effect of a vitamin E analog, phosphatidylchromanol [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman; PCh], against oxidative hemolysis of human erythrocytes was examined and was compared with those of vitamin E (α-tocopherol) and 2,2,5,7,8-pentamethyl-6-chromanol (PMC). These three compounds at 50 μM protected the erythrocytes from hemolysis, when erythrocyte suspension (10%, vol/vol) was incubated with a water-soluble radical generator, 2,2′-azobis(2-amidinopropane)-dihydrochloride (75 mM). When erythrocyte suspension was oxidized after pretreatment with these compounds (50 μM) for 30 min followed by washing, PCh protected about 54% of erythrocytes from the hemolysis, while α-tocopherol protected only about 16% of the cells and PMC did not show any protective effect. During preincubation, α-tocopherol, PMC, and PCh were incorporated into the cells at the concentration of 12.6, 3.7, and 16.3 nmol/mg protein, respectively. Moreover, PCh was found in the ghost membrane fraction at a 20% higher level than α-tocopherol, and no PMC was detected in this fraction. These results indicate that phosphatidyl group in PCh acts as an excellent carrier of chromanol moiety into cells as well as an anchor within membranes more efficiently than phytyl group in α-tocopherol. PMC seems to be slightly anchored within membranes because of the lack of hydrophobic side chain. The excellent antihemolytic activity of PCh is likely to be caused by its accumulation within erythrocyte membranes.
Lipids 33, 589–595 (1998).

Increasing evidence suggests that free radical-induced oxidative damages lead to various pathological events including coronary heart disease, cancer, and aging (1). In particular, lipid peroxidation in biological membranes has attracted much attention in relation to the deterioration of membrane structure and impairment of enzymatic functions (2). Much interest exists in the possibility that antioxidants reduce the risk of such degenerative diseases by inhibiting free radical-induced oxidative damage (3). Therefore, several studies have examined both natural and synthetic antioxidants for the inhibition of lipid peroxidation in membrane systems.

In phospholipid bilayers of biomembranes, the primary defense against free radical-induced oxidative damages involves vitamin E (α-tocopherol) (4,5), and the presence of specific binding sites for α-tocopherol in erythrocyte membranes was demonstrated (6,7). α-Tocopherol consists of two structural domains. One is a chromanol group, responsible for radical scavenging ability, and the other is phytyl side chain, which is required for anchoring the chromanol group within phospholipid bilayers (8). It has been accepted that the phytyl side chain of α-tocopherol facilitates the incorporation of α-tocopherol into biomembranes and arranges its chromanol group on a suitable site for scavenging chain-carrying lipid peroxyl radicals in phospholipid bilayers (9,10). Thus, the phytyl side chain of α-tocopherol has an important role in its antioxidant activity in the membranes.

In previous papers, we described a new synthetic vitamin E analog, phosphatidylchromanol [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′8′-tetramethyl-6′-hydroxychroman (PCh), Scheme 1], which contains phosphatidyl moiety as the substitute for phytyl chain (11). Its antioxidant abilities in bulk oils and phospholipid bilayers were also examined by comparing them with those of α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) (11–14). PCh is expected to act as an effectively protective reagent against oxidative cellular injury, because phosphatidyl group seems to possess high affinity for cellular phospholipid bilayers and has been actually regarded as a suitable and nontoxic carrier of pharmaceuticals into cells (15). The aim of this study is to elucidate the action of PCh as a protective reagent against oxidative damages mediated by lipid peroxidation in biomembranes.

Free radical-induced deterioration of erythrocyte membranes and resulting hemolysis serve as a good model for studying the oxidative damage in biomembranes (16–26). It

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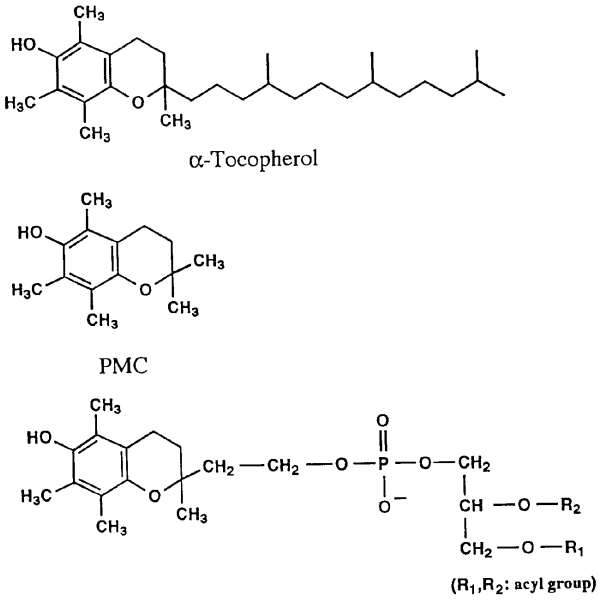
Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; PCh, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′8′-tetramethyl-6′-hydroxychroman; PL-OOH, phospholipid hydroperoxides; PMC, 2,2,5,7,8-pentamethyl-6-chromanol.



SCHEME 1

has been well studied that free radicals attack erythrocyte membranes to induce peroxidation of lipids and proteins and eventually cause hemolysis (18–23). Therefore, the oxidative hemolysis of erythrocytes induced by free radicals is a well-defined model to estimate the antioxidative action of α-tocopherol and its analogs in biomembranes. In the present study, human erythrocytes were oxidized by a water-soluble radical generator, and the protective effect of PCh on the lipid peroxidation and hemolysis was investigated and was compared with those of α-tocopherol and PMC.

MATERIALS AND METHODS

Chemicals. 2,2′-Azobis(2-amidinopropane)-dihydrochloride (AAPH) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) were obtained from Wako Pure Chemical Co. (Osaka, Japan). *dl*-α-Tocopherol and PMC were obtained from Eisai Co. (Tokyo, Japan). Probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade. PCh was synthesized enzymatically from 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman and phosphatidylcholine and purified as described previously (11).

Preparation of human blood plasma and erythrocytes. Blood was taken from healthy volunteers and then was put into tubes containing EDTA, disodium salt (1 mg/mL). Plasma and erythrocytes were separated by centrifugation at 1000 × *g* for 20 min at 4°C. Erythrocytes were washed three times with 5 vol of the phosphate buffered saline (PBS). The buffy coat was carefully removed with each wash. At the last washing, the cells were centrifuged exactly at 1000 × *g* for 20 min to obtain packed cells with a constant volume.

Assay for oxidative hemolysis. Oxidative hemolysis of erythrocytes was performed as reported by Miki *et al.* (22) with a slight modification. Washed erythrocytes (0.5 mL) were suspended in 4 vol of PBS. Antioxidants, except for PCh, were added to the cell suspension as ethanol solution. The final ethanol concentration in the suspension was less than 0.5% (vol/vol), which had no effect on erythrocytes. Since PCh was only slightly soluble in ethanol, it was dispersed in PBS with a vortex mixer for 1 min followed by ultrasonic irradiation with an ultrasonicator (Model B1210J-DTH; Branson, Tokyo, Japan) for 30 s and then added to the erythrocyte suspension. After preincubation of this suspension at 37°C for 5 min, 2.5 mL of 150 mM AAPH dissolved in PBS was added to the suspension. Oxidation was carried out in the dark at 37°C under air with continuous shaking. When erythrocytes were oxidized after pretreatment with antioxidants, washed erythrocytes (1 mL) were suspended in 9 vol of PBS. Then antioxidant was added at the concentration of 50 μM in the suspension. After preincubation of this suspension at 37°C for 30 min, erythrocytes were washed three times with 10 mL of PBS. Washed erythrocytes were oxidized by the same aforementioned procedure. In both cases, oxidation was carried out in the dark at 37°C under air with continuous shaking. At specific intervals, the reaction mixture (0.1 mL) was withdrawn, diluted with 4 vol of PBS, and centrifuged (2000 × *g* for 5 min). The absorption of the supernatant at 540 nm was measured (absorption A). Similarly, the reaction mixture (0.1 mL) was treated with 4 vol of 0.1% Triton X-100 solution to yield complete hemolysis, and the absorption of the supernatant after centrifugation was measured at 540 nm (absorption B). Percentage hemolysis was calculated from the ratio of the readings, (absorption A/absorption B) × 100.

Oxidation of ghost membranes. Ghost membranes were prepared from erythrocytes by the method of Dodge *et al.* (27), after 25 mL of erythrocyte suspension (10%, vol/vol) was preincubated with each antioxidant (50 μM) at 37°C for 30 min, followed by washing three times with PBS (25 mL). The phospholipid concentration in the ghost membrane was calculated from the phosphorus content according to the method of Bartlett (28). Ghost membranes were suspended in PBS containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) at the concentration of 1 mM. The ghost membrane suspension (0.5 mL) was preincubated at 37°C for 5 min. Oxidation was initiated by adding 80 mM AAPH (0.5 mL) dissolved in PBS containing 0.5 mM DTPA. The reaction mixture was incubated at 37°C with continuous shaking in the dark. After 4 h of incubation, the reaction mixture (0.1 mL) was withdrawn and 0.1 mL of 10 mM BHT in ethanol was added. The lipids were extracted three times by the method of Bligh and Dyer (29). The lipid portion was evaporated with nitrogen gas, and the residue was dissolved in the mixture of methanol and water (95:5, vol/vol, 0.1 mL) for high-performance liquid chromatographic (HPLC) analysis of phospholipid hydroperoxides (PL-OOH) (30). Sample (10 μL) was injected into an octylsilyl column (TSK gel OCTYL-80Ts column, 6 × 150 mm; TOSOH, Tokyo Japan) and eluted with methanol/water (92.5:7.5, vol/vol). The hydroperoxides were

detected by ultraviolet absorption at 235 nm. The concentration of PL-OOH was calculated from the standard curve of phosphatidylcholine hydroperoxides. Standard phosphatidylcholine hydroperoxides were prepared as described previously (30).

Determination of antioxidants in erythrocytes and ghost membranes. After preincubation of 15 mL of erythrocyte suspension (10%, vol/vol) with each antioxidant (50 μM) for 30 min at 37°C, erythrocytes were washed three times with PBS (15 mL) and centrifuged at 1000 × *g* for 20 min. Then a hexane/ethanol mixture (4:1, vol/vol, 4 mL) containing 25 μM BHT and water (2 mL) was added to washed erythrocytes (0.5 mL). They were mixed with a vortex mixer for 30 s and were sonicated for 1 min followed by centrifugation at 1500 × *g* for 5 min. Hexane layers obtained from repeated extraction for three times were collected and evaporated with nitrogen gas. The residue was dissolved in the mixture of acetonitrile and chloroform (6:4, vol/vol, 0.1 mL) for the analysis of α-tocopherol, or in *n*-hexane (0.1 mL) for the analysis of PMC and PCh. α-Tocopherol was quantified by HPLC on an octylsilyl column (TSK gel OCTYL 80Ts, 4.6 × 100 mm; TOSOH) with acetonitrile/water (99:1, vol/vol) as an eluent at a flow rate of 1.2 mL/min. PMC was determined by HPLC on an aminopropylsilyl column [YMC Pack A-612(NH_2), 4.6 × 100 mm; Yamamura Chemical Laboratories, Kyoto, Japan] with hexane/isopropanol (99:1, vol/vol) as an eluent at a flow rate of 1.0 mL/min. For HPLC analysis of PCh, an aminopropylsilyl silica column (Shim-Pack FLC-NH_2, 4.6 × 50 mm; Shimadzu Co., Kyoto, Japan) was used with hexane/isopropanol/10% phosphoric acid (70:30:0.5, by vol) as an eluent at a flow rate of 2.0 mL/min. In each determination, the eluent was monitored fluorometrically at an excitation wavelength of 298 nm and an emission wavelength of 325 nm using a Shimadzu RF-10A (Shimadzu Co.). The respective concentration was calculated from the standard curve of the authentic compounds.

Measurement of protein. Protein was determined by the method of Bradford (31) using bovine serum albumin as a standard.

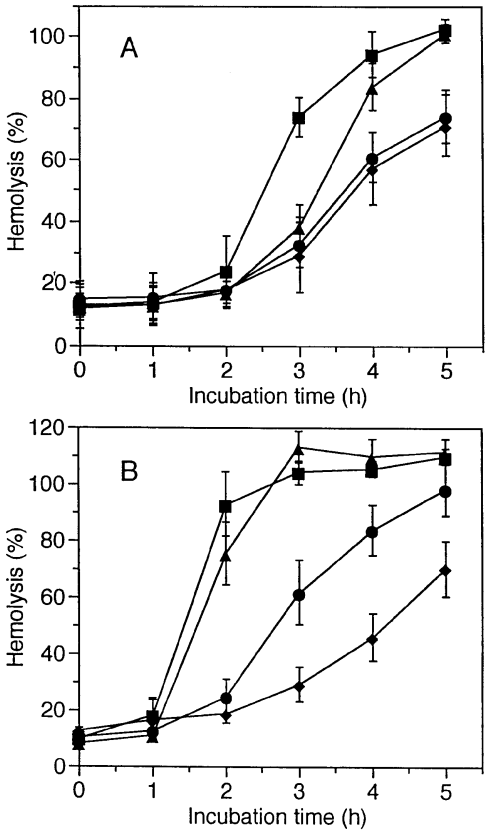


FIG. 1. Effect of coincubation (A) and preincubation (B) of erythrocyte suspension with α-tocopherol, 2,2,5,7,8-pentamethyl-6-chromanol (PMC), or 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman (PCh) on 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH)-induced oxidative hemolysis of erythrocytes. (A) Erythrocyte suspension (10%, vol/vol) in phosphate buffered saline (PBS) (pH 7.4) was incubated with AAPH (75 mM) and α-tocopherol or α-tocopherol analogs (50 μM). (B) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with α-tocopherol or α-tocopherol analog (50 μM) for 30 min followed by washing three times. After washing, the suspension was incubated with AAPH (75 mM). Each point represents the mean ± the standard deviation of three separate experiments. (■), No addition; (●), α-tocopherol; (▲), PMC; (◆), PCh.

RESULTS

Effects of antioxidants on free radical-induced hemolysis of human erythrocytes. Oxidative hemolysis was induced by a water-soluble azo compound, AAPH. Incubation of erythrocyte suspension with 75 mM AAPH at 37°C resulted in time-dependent hemolysis with an induction period (Fig. 1). In order to estimate the antihemolytic potency of α-tocopherol, PMC and PCh, erythrocyte suspensions were coincubated with each compound (50 μM) and AAPH (75 mM). α-Tocopherol and PMC were added to erythrocyte suspension as ethanol solution, while PCh was added as aqueous dispersion in PBS. These compounds retarded the occurrence of hemolysis and suppressed its percentage (Fig. 1A, coincubation experiment). α-Tocopherol and PCh exhibited nearly the same inhibitory effects and were more efficient than PMC. In a separate experiment, erythrocyte suspension was oxidized after pretreatment with each compound for 30 min, followed by washing three times to avoid the interference by unincorporated antioxidants (preincubation experiment). In this case, PMC had no protective effect (Fig. 1B). In contrast to PMC, α-tocopherol and PCh protected against hemolysis significantly even after pretreatment followed by washing. It should be noted that PCh was much more effective than α-tocopherol in suppressing the hemolysis of erythrocytes in the preincubation experiment, although little difference was observed between the two compounds in the coincubation experiment.

In addition to α-tocopherol analogs, we investigated the protective effects of BHT and probucol on oxidative hemolysis of erythrocytes in the preincubation experiment (Fig. 2). When preincubation was carried out in the absence of antioxidant, most of the erythrocytes were subjected to lysis after

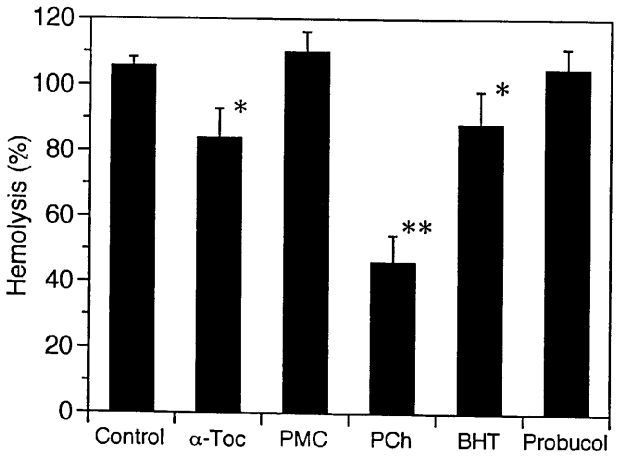


FIG. 2. Effect of preincubation of erythrocyte suspension with α-tocopherol, PMC, PCh, BHT, or probucol on AAPH-induced oxidative hemolysis of erythrocytes. Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with each antioxidant (50 μM) for 30 min followed by washing three times. After washing, the suspension was incubated with AAPH (75 mM) for 4 h. Bars represent the mean ± the standard deviation of three separate experiments. Asterisks indicate significant differences between control and antioxidant treatment: *, $P < 0.05$; **, $P < 0.01$. See Figure 1 for abbreviations.

incubation with AAPH (75 mM) for 4 h (control experiment). Probucol showed no significant protection similar to PMC, while α-tocopherol and BHT lowered the hemolysis to 83.8 ± 9.0% and 88.1 ± 9.8%, respectively. PCh exhibited a remarkable resistance to hemolysis, and the percentage of hemolysis was 46.0 ± 8.3%.

Incorporation of α-tocopherol, PMC, and PCh into human erythrocytes and their ghost membranes. In order to clarify the reason for the marked difference in antihemolytic potency among the three α-tocopherol analogs, the amounts of α-tocopherol and its analogs incorporated into erythrocytes and those accumulated within ghost membrane were measured after incubation for 30 min at 37°C (Table 1). Endogenous α-tocopherol was found at the level of 0.2 and 2.7 nmol/mg protein in erythrocytes and erythrocyte membrane fraction, respectively. The presence of endogenous α-tocopherol is likely to cause the induction period which was observed when erythrocytes without treatment of any antioxidants were exposed to AAPH (Fig. 1). When erythrocytes were incubated with α-tocopherol, PMC, or PCh at 50 μM, these compounds were incorporated into the cells at the concentration of 12.6 ± 0.7, 3.7 ± 0.2, and 16.3 ± 0.5 nmol/mg protein, respectively. In ghost membranes, PMC was not found, although α-tocopherol and PCh were found at 84.4 ± 6.2 and 101.0 ± 2.3 nmol/mg protein, respectively.

Effect of concentration of PCh in the pretreatment medium and pretreatment time on its antihemolytic activity. In order to study the antihemolytic action of PCh, the relationship between the concentration of PCh in the preincubation medium and its inhibitory effect against the hemolysis of erythrocyte was examined. The protective effect was increased with the

TABLE 1
Incorporation of α-Tocopherol Analogs into Erythrocytes[a]

	Concentration of antioxidant (nmol/mg protein)			
	Erythrocytes		Ghost membrane fraction	
Analog				
No addition		(0.19 ± 0.01)[b]		(2.68 ± 0.09)
α-Tocopherol	12.6 ± 0.7		84.4 ± 6.2	
PMC	3.7 ± 0.2	(0.21 ± 0.07)	n.d.[c]	(2.62 ± 0.10)
PCh	16.3 ± 0.5	(0.22 ± 0.03)	101.0 ± 2.3	(2.74 ± 0.13)

[a]Each value represents the mean ± the standard deviation of triplicate analyses.
[b]Values in parentheses are the concentration of endogenous α-tocopherol.
[c]Not detected. PMC, 2,2,5,7,8-pentamethyl-6-chromanol; PCh, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman.

elevation of the concentration up to 50 μM, reaching a plateau above 50 μM (Fig. 3A). The protective effect of PCh was enhanced with the increase of pretreatment time for up to 30 min, reaching a plateau for more than 30 min (Fig. 3B). Thus,

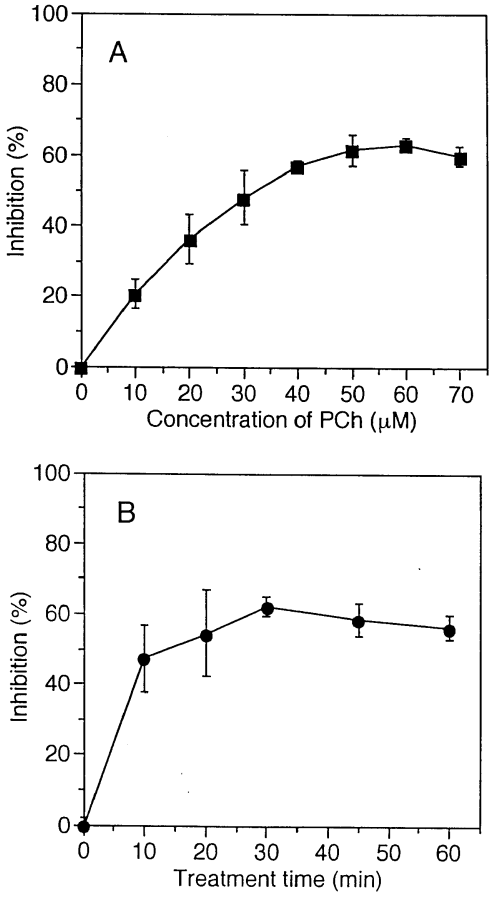


FIG. 3. Effect of the concentration (A) and the pretreatment time (B) of PCh on the protection of AAPH-induced oxidative hemolysis of erythrocytes. (A) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with PCh for 30 min followed by washing three times. (B) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was incubated with PCh (50 μM) for specific time followed by washing three times. In both cases the suspension was incubated with AAPH (75 mM) for 4 h. Each point represents the mean ± the standard deviation of three separate experiments. See Figure 1 for abbreviations.

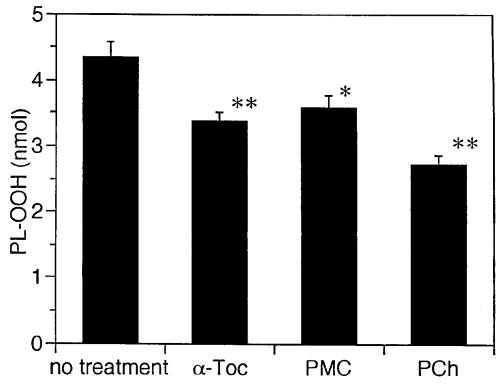


FIG. 4. AAPH-induced oxidation of α-tocopherol analog-pretreated ghost membranes. Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with α-tocopherol, PMC, or PCh (50 μM) for 30 min, followed by washing three times, and then the ghost membranes were prepared by the method of Dodge *et al.* (27). The suspension of ghost membranes containing 0.5 μmol of phospholipids was incubated with AAPH (75 mM) for 4 h. Bars represent the mean ± the standard deviation of three separate experiments. Asterisks indicate significant differences between no treatment and α-tocopherol analog treatment: *, $P < 0.05$; **, $P < 0.01$. See Figure 1 for abbreviations.

an optimal concentration and an optimal time in the pretreatment of PCh appeared in the inhibition of oxidative hemolysis.

Oxidation of ghost membrane prepared from α-tocopherol-, PMC-, or PCh-pretreated erythrocytes. The ghost membranes prepared from erythrocytes after preincubation with each compound for 30 min were subjected to the oxidation initiated by AAPH. PL-OOH were accumulated as the primary products of ghost membrane peroxidation as shown in Figure 4. A lower amount of PL-OOH was accumulated in the pretreated ghost membranes. The membranes pretreated with PCh accumulated a lower amount of PL-OOH, as compared with those pretreated with α-tocopherol and PMC. This indicates that the resistance of erythrocyte membranes to free radical-induced lipid peroxidation was enhanced by the pretreatment of erythrocytes with α-tocopherol and α-tocopherol analogs, particularly PCh.

DISCUSSION

The above results show that PCh acts as a potent protector against oxidative cell injury in human erythrocytes and that its protective effect is superior to those of α-tocopherol and PMC. α-Tocopherol, PMC, and PCh are homologs of 2,5,7,8-tetramethyl-6-chromanol with phytyl side chain, methyl group, and phosphatidyl group, at the 2-position, respectively (Scheme 1). Although their structures are identical except for the substituents at the 2-position, their antioxidant effects seem to be much different from one another in a heterogeneous system because of their inherent physical properties.

We have reported that α-tocopherol, PMC, and PCh gave almost the same peroxyl radical-scavenging activity in homogeneous solution (11). On the other hand, α-tocopherol showed the longest induction period among the three compounds in phosphatidylcholine liposomes, probably owing to their different location in phospholipid bilayers (11,13,14), in which we have proposed that chromanol moiety in PCh is located at the interface between aqueous phase and lipid bilayers (13), analogous with the polar head group of phospholipids. It is reasonable that the difference in their antioxidant activities is, at least in part, derived from location and mobility of each analog in phospholipid bilayers. However, in intact cells the incorporation and retention of antioxidant within cellular membranes are also important factors in its activity. PCh is expected to possess high affinity for cellular membranes because of its nature as phospholipid that is a major component of cellular membrane bilayers. Therefore, this study is focused on the action of PCh in cellular membranes as compared with those of α-tocopherol and PMC.

The order of antihemolytic activity of the three compounds in the preincubation experiment appeared to be correlated with their amounts incorporated into erythrocytes. The local concentrations of α-tocopherol and PCh in erythrocytes were much higher than that of PMC (Table 1), indicating that α-tocopherol and PCh are effectively incorporated into erythrocytes. PMC could hardly be retained within the ghost membrane fraction in spite of the incorporation into the cells to some extent (32,33). These results indicate that phytyl chain and phosphatidyl moiety can act as an anchor to retain a chromanol moiety within membranes. α-Tocopherol and PCh incorporated into the membranes should play a role in preventing hemolysis by protecting the oxidation of membrane lipids. In fact, we found that the ghost membranes prepared from erythrocytes pretreated with α-tocopherol analogs elevated the resistance to free radical-induced lipid peroxidation (Fig. 4).

The existence of the α-tocopherol selective uptake system in red blood cells has been reported (6,7). Kaneko *et al.* (34) demonstrated that α-tocopherol was well incorporated into cultured cell because of the high affinity of its phytyl group with cell membranes. The fact that PCh was found in a higher amount than α-tocopherol in pretreated erythrocytes and their ghost membranes indicates that the affinity of phosphatidyl group with cell membranes is higher than that of phytyl group. Shuto *et al.* (15) reported that nucleoside analogs containing a phosphatidyl residue possess a high affinity for cell membranes, resulting in the penetration into cells. Furthermore, a phosphatidyl derivative of genipin, which is a water-soluble compound having some pharmacological activities, was found to exhibit the enhanced cytotoxicity in spite of the weak cytotoxicity of genipin itself (35). Therefore, it is concluded that a phosphatidyl group in PCh acts as an excellent carrier and anchor of chromanol group in erythrocyte membranes.

After the treatment of erythrocytes with α-tocopherol and PCh for 30 min, the concentrations of α-tocopherol and PCh reached similar levels, which were 66 and 74 times in the cells, and 31 and 37 times in the ghost membranes greater than endogenous α-tocopherol concentration, respectively (Table 1). However the inhibition of AAPH-induced hemoly-

sis by the treatment with PCh was much greater than that by the treatment with α-tocopherol (Figs. 1 and 2). Thus, the marked difference in antihemolyic potency between α-tocopherol and PCh cannot be fully explained by their cellular concentrations. Free radicals generated from AAPH are known to attack erythrocyte membranes from the outside to induce lipid peroxidation in membrane phospholipids (18–23). The chromanol moiety of PCh seems to be located at the interface of the membrane and scavenges aqueous radicals rather than lipophilic radicals efficiently, while α-tocopherol mainly scavenges chain-carrying lipid peroxyl radicals in the hydrophobic region of membranes (13,14). The ability of PCh in scavenging aqueous radicals seems to be of great advantage to suppress their attacks on erythrocyte membranes and resulting in hemolysis.

Enormous amounts, as compared to endogenous α-tocopherol, of α-tocopherol and PCh incorporated into ghost (Table 1) did not result in efficient inhibition of AAPH-induced formation of PL-OOH (Fig. 4) but prevented efficient hemolysis (Fig. 1 and 2). However, it might be possible that the exogenous antioxidant prevents not only lipid peroxidation but also protein oxidation that was also observed in AAPH-induced oxidation of ghost membranes (18,22), resulting in effective prevention of hemolysis (Figs. 1 and 2).

Although PMC was incorporated into erythrocytes to some extent, it was not retained in ghost membrane fraction (Table 1). PMC can not be anchored because of the lack of long chain and may be transferred freely without orientation in the membrane (9,35,36); hence it may penetrate into cytosol easily. No inhibition on the oxidative hemolysis by PMC appears to be due to inability to be retained within the membranes. Niki *et al.* (32) reported that the mobility of PMC was quite high within and between liposomal membranes. Furthermore, they have shown that the antioxidant activity of chromanols was elevated with decreasing length of side chain in the oxidation of plasma low density lipoprotein (36). Thus, PMC, having no long side chain, can transfer easily from erythrocytes to outside of cells, resulting in less effective oxidative hemolysis of human erythrocytes.

The efficiency of antioxidants in the heterogeneous phase is determined by not only their radical-scavenging activity but also their physical properties, such as local concentration and mobility in the microenvironment (36). The present results obtained from antihemolytic activity suggest that the ability both to be incorporated into the cells and to be retained within the membranes should be involved in the factors responsible for the action of antioxidant in biomembranes. It is clear that phosphatidyl moiety makes an antioxidant molecule easier to be inserted into cellular membranes. Phosphatidyl derivatives may be promising compounds in the prevention or cure of cellular oxidative damage-related diseases.

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[Received October 24, 1997, and in final revised form May 4, 1998; revision accepted May 8, 1998]

Mechanism of Lower Oxidizability of Eicosapentaenoate Than Linoleate in Aqueous Micelles. II. Effect of Antioxidants

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ABSTRACT: We have reported that the peroxyl radicals derived from methyl eicosapentaenoate (20:5n-3) are more polar than those from methyl linoleate (18:2n-6) since the former peroxyl radicals have at least two molecules of oxygen in a molecule while the latter peroxyl radical has one. This lowers the oxidizability for 20:5n-3 in aqueous Triton X-100 micelles by enhancing the termination reaction rate for peroxyl radicals and by reducing the rate of propagation since there may be more polar peroxyl radicals derived from 20:5n-3 at the surface than within the micelle core. In this study, we measured the effect of three antioxidants, di-*tert*-butyl-4-methylphenol (BHT), 2,2,5,7,8-pentamethyl-6-chromanol (PMC) and 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox), on the oxidation of lipids in aqueous micelle. Antioxidants give a clear induction period during oxidation of 18:2n-6 initiated with a water-soluble radical initiator, and its induction length decreases in the order of BHT > PMC > Trolox. This is consistent with the proposed location of three antioxidants: being in the core of micelle, at the surface, or in aqueous phase, respectively. However, BHT does not inhibit the oxidation of 20:5n-3 efficiently, and its rate of oxidation is slower than that observed in the oxidation of 18:2n-6, supporting the idea that polar peroxyl radicals derived from 20:5n-3 are preferentially located at the surface of the micelle. Similar results were obtained when oxidation was initiated with a lipid-soluble radical initiator except antioxidants had lesser effect on the oxidation rate of 20:5n-3.
Lipids 33, 597–600 (1998).

The oxidizability of polyunsaturated fatty acids in homogeneous solution is known to be dependent on the number of bisallylic positions available for oxidation (1–4). In contrast, the oxidizability of polyunsaturated fatty acids in aqueous micelles is reported to decrease with an increasing number of bisallylic sites (5,6). We have confirmed this interesting observation by comparing the oxidation kinetics of methyl eicosapentaenoate (20:5n-3) and methyl linoleate (18:2n-6) in both homogeneous solution and aqueous micelles (7). It has been suggested that the peroxyl radicals derived from 20:5n-3 are more polar than those from 18:2n-6 since the former peroxyl radicals contain two molecules of absorbed oxygen while the latter peroxyl radicals have one (7). Therefore, we hypothesized that the polar peroxyl radicals derived from 20:5n-3 are likely to diffuse from the core to the micelle surface, and this lowers the oxidizability for 20:5n-3 in aqueous micelles by enhancing the termination reaction rate for polar peroxyl radicals derived from 20:5n-3 and by reducing the rate of propagation since there is less oxidizable 20:5n-3 at the surface than the micelle core.

To obtain additional evidence to support this hypothesis, we have measured the effects of antioxidants on the oxidation of 20:5n-3 and 18:2n-6 in aqueous micelles. We have chosen 2,6-di-*tert*-butyl-4-methylphenol (BHT), 2,2,5,7,8-pentamethyl-6-chromanol (PMC), and carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox) as antioxidants for comparison since it is reported that BHT preferentially locates in the core of micelle (8,9), PMC resides at the micelle surface (10), and Trolox remains essentially in aqueous phase (11). BHT, PMC, and Trolox can trap two molecules of peroxyl radical per molecule (12), and the rate constants for H-atom abstraction of these antioxidants by peroxyl radicals are 1.4×10^4, 3.8×10^6, and 1.1×10^6 $M^{-1}s^{-1}$, respectively (13), indicating that BHT has the lowest activity as measured in homogeneous solution.

Figure 1 shows the rate of oxygen uptake during oxidation of 2 mM 18:2n-6 (or 20:5n-3) initiated with the water-soluble radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH; 4 mM), in the presence or absence of 5 μM antioxidant (BHT, PMC, or Trolox) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 under aerobic conditions at 37°C. The micelles were prepared as follows: Appropriate amounts of 18:2n-6 (or 20:5n-3) in benzene (and, if necessary, appropriate amounts of antioxidants and/or lipid-soluble initiator) were placed in a glass flask. The solvent was removed on a water aspirator and then on a vacuum pump fol-

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Abbreviations: 18:2n-6, methyl linoleate; 20:5n-3, methyl eicosapentaenoate; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, 2,6-di-*tert*-butyl-4-methylphenol; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; Trolox, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol.

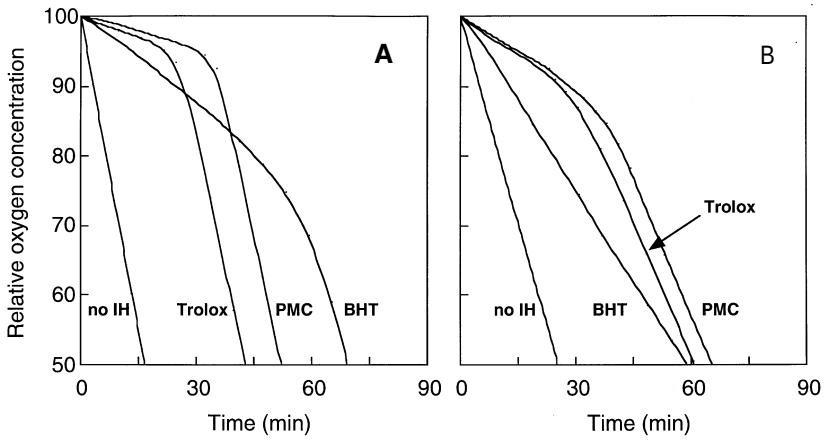

FIG. 1. Aerobic oxidation of 2 mM (A) 18:2n-6 and (B) 20:5n-3 initiated with 4 mM water-soluble 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) in the absence [no inhibitor (IH)] or presence of 5 μM antioxidants in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. Reproducibility was excellent in at least two independent measurements. PMC, 2,2,5,7,8-pentamethyl-6-chromanol; BHT, 2,6-di-*tert*-butyl-4-methylphenol; Trolox, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol.

lowed by the addition of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100. If necessary, AAPH was added to the flask. Reaction mixtures were shaken vigorously, were transferred to a reaction vessel, and oxygen concentrations within micellar suspension were monitored using an oxygen electrode.

The rate of oxygen uptake during the oxidation of 18:2n-6 in the absence of antioxidants was greater than that for 20:5n-3 under equivalent conditions (Fig. 1), and this is consistent with our previous observation (7). The addition of antioxidants to 18:2n-6 micelle retarded the rate of oxygen uptake. After the consumption of antioxidant (data not shown), the rate of oxygen uptake was similar to that observed in the absence of antioxidant; the period of inhibited oxidation is called the induction period. It is noteworthy that all antioxidants gave a clear induction period. The length of induction period produced by the addition of test antioxidants decreased in the order of BHT > PMC > Trolox. This order is consistent with the reported preferential location of the three antioxidants, BHT within the micelle core (8,9), PMC at the surface of micelle (10), and Trolox in aqueous phase (11), suggesting that Trolox trapped peroxyl radicals produced by the water-soluble radical initiator while BHT scavenged only peroxyl radicals which migrated into the micelle core. The decay of antioxidants during the induction period was measured using a reversed-phase high-performance liquid chromatograph equipped with an electrochemical detector. The results are summarized in Figure 2 where 2

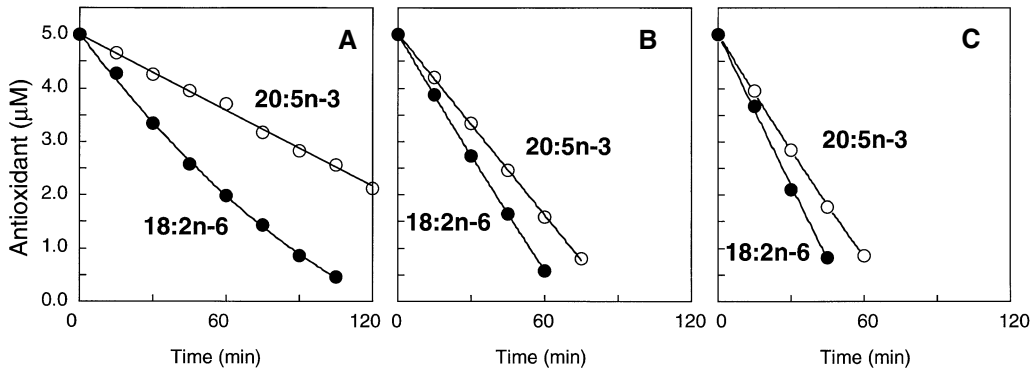

FIG. 2. Consumption of antioxidants during the aerobic oxidation of 2 mM 18:2n-6 (●) or 20:5n-3 (○) initiated with 2 mM water-soluble AAPH in the presence or absence of 5 μM antioxidants in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. (A) BHT and (B) PMC were measured by a reversed-phase high-performance liquid chromatograph equipped with an electrochemical detector (700 mV) using an octylsilyl column (5 μm, 4.6 × 150 mm; Wako, Osaka, Japan) with 50 mM $NaClO_4$ in methanol/water (4:1, vol/vol) as the mobile phase (0.8 mL/min). (C) Trolox was measured in the same system except using 50 mM $NaClO_4$ in methanol/water (1:1, vol/vol) as the mobile phase. Reproducibility was excellent in at least two independent measurements. See Figure 1 for abbreviations.

mM AAPH was used instead of 4 mM in Figure 1 to retard the relative consumption of antioxidants. The rate of disappearance of antioxidants during the oxidation of 18:2n-6 in aqueous micelle decreased in the order of BHT > PMC > Trolox. This again is consistent with the presumed distribution of antioxidants within micelles.

On the other hand, BHT did little to inhibit the oxidation of 20:5n-3 in aqueous micelles while Trolox and PMC were more efficient (Fig. 1). Thus, as expected, the rate of disappearance of BHT during the oxidation of 20:5n-3 was much smaller than that observed for 18:2n-6 oxidation. These results strongly suggest that polar peroxyl radicals derived from 20:5n-3 are not located within the micelle core where BHT resides, but rather are at the surface of the micelle which was suggested in a previous kinetic study (7).

It is important to demonstrate that the three antioxidants used in this study were of good quality. Table 1 gives the kinetic parameters of the three antioxidants measured during aerobic oxidation of 150 mM 18:2n-6 in acetonitrile initiated with 2 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), a lipid-soluble radical initiator, in the presence or absence of 20 μM antioxidants at 37°C; the induction period; the rate of oxidation during induction period; and rate of propagation measured as the rate of oxidation after the complete consumption of antioxidants. BHT, PMC, Trolox, and α-tocopherol gave essentially the same induction period as previously reported (12). Values for rate of propagation were nearly equivalent under the experimental conditions, indicating an absence of contamination. Values for rate of oxidation during induction period for PMC, Trolox, and α-tocopherol were similar and about 20 times less than that for BHT as was previously observed (13).

Figure 3 shows the rate of oxygen uptake during the aerobic oxidation of 2 mM 18:2n-6 and 20:5n-3 initiated with 2 mM AMVN, a lipid-soluble radical initiator, in the presence or absence of 2 μM antioxidant (BHT, PMC, or Trolox) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. The rate of oxygen uptake during oxidation of 18:2n-6 in the absence of antioxidant was much faster than that for 20:5n-3 at similar conditions, which is consistent with our previous data (7). BHT within the micelle core inhibited the oxidation of 18:2n-6 in aqueous micelles initiated with the lipid-soluble radical initiator but it did not efficiently suppress the oxidation of 20:5n-3 in aqueous micelles. As a consequence, the rate of BHT consumption observed in 18:2n-6 oxidation was much greater than that observed in 20:5n-3 oxidation as shown in Figure 4, where 4 μM antioxidants are used instead of 2 μM in Figure 3 to retard the relative consumption of antioxidants. In contrast, PMC located at the surface of the micelle inhibited oxidation of both 18:2n-3 and 20:5n-3 in aqueous micelles and gave a clear induction period although the inhibition of 20:5n-3 oxidation was less

TABLE 1
Aerobic Oxidation of 150 mM Methyl Linoleate in Acetonitrile Initiated with 2 mM AMVN in the Presence or Absence of 20 μM Antioxidants at 37°C[a]

Antioxidant	$t_{in}h$[b] (s)	R_{inh}[c] (nM/s)	R_p[d] (nM/s)
BHT	4320	483	711
PMC	4980	23	819
Trolox	4500	48	849
α-Tocopherol	4560	26	824
None			786

[a]Rate of chain initiation = 8.76 (nM/s).
[b]Induction period.
[c]Rate of oxidation during the induction period.
[d]Rate of propagation. BHT, 2,6-di-*tert*-butyl-4-methylphenol; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; Trolox, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile).

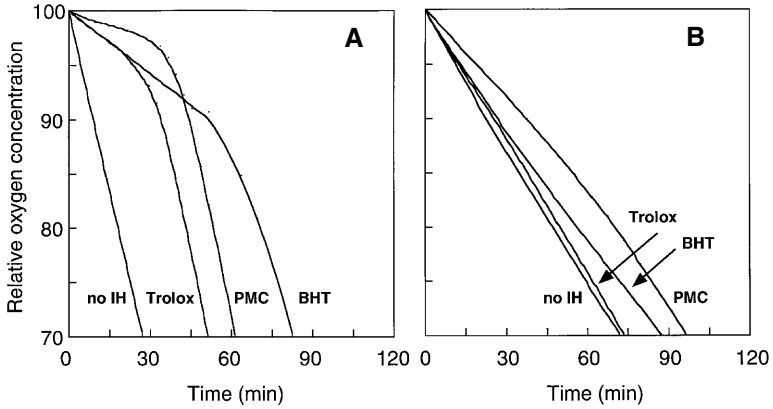

FIG. 3. Aerobic oxidation of 2 mM (A) 18:2n-6 and (B) 20:5n-3 initiated with 2 mM lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) in the absence (no IH) or presence of 2 μM antioxidants in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. Reproducibility was excellent in at least two independent measurements. See Figure 1 for abbreviations.

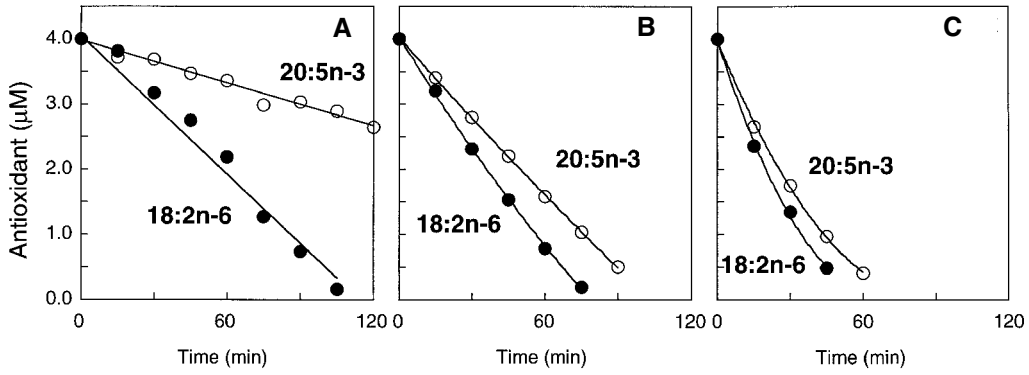

FIG. 4. Consumption of antioxidants during the aerobic oxidation of 2 mM 18:2n-6 (●) or 20:5n-3 (○) initiated with 2 mM lipid-soluble AMVN in the presence or absence of 4 μM antioxidants in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM Triton X-100 at 37°C. Antioxidants were measured as described in Figure 3. Reproducibility was excellent in at least two independent measurements. See Figure 2 for abbreviation.

clear. These results again support the view that polar peroxyl radicals formed from 20:5n-3 should relocate to the surface of the micelle rather than stay within the micelle core.

It is interesting that Trolox was consumed at a greater rate than was BHT (Fig. 4) despite that AMVN is expected to be located in the micelle. The peroxyl radicals produced from AMVN are low molecular weight compounds containing two polar function groups, cyano and peroxyl groups. Therefore, they are likely to be polar enough to migrate to the aqueous interface where Trolox is active.

In summary, we have demonstrated that the polar peroxyl radicals derived from 20:5n-3 should be located at the surface of the micelle by comparing the effects of three different antioxidants which have different site reactivities in aqueous micelles. Migration of the polar peroxyl radicals derived from 20:5n-3 to the surface of the micelle should lower the oxidizability for 20:5n-3 by enhancing the termination reaction rate for polar peroxyl radicals and by reducing the rate of propagation since there is less oxidizable 20:5n-3 at the surface than in the micelle core.

ACKNOWLEDGMENTS

We thank Dr. W.C. Dunlap for his valuable comments and Eisai Co. for the supply of PMC and α-tocopherol. This work was supported by Grants-in-Aid for Scientific Research from the Ministries of Education, Science and Culture, and Health and Welfare of Japan.

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[Received March 5, 1998, and in final revised form April 28, 1998; revision accepted April 29, 1998]

Partial Purification and Characterization of Sphingosine *N*-Acyltransferase (ceramide synthase) from Bovine Liver Mitochondrion-Rich Fraction

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ABSTRACT: Sphingosine *N*-acyltransferase (ceramide synthase, E.C. 2.3.1.24) was solubilized from bovine liver mitochondrion-rich fraction with *n*-octyl β-D-thioglucoside as the detergent and partially purified by sequential chromatography on columns of DE-32, shingosine affinity, and Sepharose CL-6B. The partially purified preparation migrated on SDS-polyacrylamide gel electrophoresis as two major protein bands of 62 and 72 kDa. The molecular mass of the enzyme estimated by gel filtration was 240–260 kDa, suggesting that the partially purified enzyme is present in a subunit form or simply has an aggregative nature. The specific activity of the final preparation for the condensation of sphingosine with stearoyl-CoA increased by 98.7-fold compared with the starting material. The optimal pH value for the ceramide synthesis was 7.5. The partially purified enzyme had an apparent K_m of 146 μM and a V_{max} of 11.1 nmol/min/mg protein for stearoyl-CoA. The K_m and V_{max} values toward sphingosine were 171 μM and 11.3 nmol/min/mg protein, respectively. Interestingly, sphinganine was also a good substrate for this enzyme, and the K_m and V_{max} values were 144 μM and 8.5 nmol/min/mg protein, respectively.

Lipids 33, 601–605 (1998).

Ceramide biosynthesis begins by the formation of sphinganine (dihydrosphingosine) from serine and palmitoyl-CoA. Addition of a fatty acyl chain to sphinganine yields dihydroceramide, which appears to precede the introduction of the 4,5-*trans*-double bond of sphingosine. These steps have been shown to take place at the cytosolic surfaces of the endoplasmic reticulum (1,2). However, there is also evidence for ceramide biosynthesis by the direct condensation of sphingosine with fatty acyl-CoA. This system has been studied with microsomes from rodent brain and liver (3–5). One of the important unresolved issues in ceramide synthesis therefore concerns whether the sphingosine and sphinganine *N*-acyltransferases both are identical and located only in microsomes. Another pathway involves the condensation of sphingosine with free fatty acid catalyzed by the reverse reaction of the lysosomal ceramidase that hydrolyzes ceramide to sphingosine and free fatty acid (6,7). Thus, the enzymic mechanism for ceramide synthesis is still poorly understood, largely because of the resistance to solubilization and the labile enzyme activity.

We became interested in the ceramide synthase activity in the mitochondrial fraction, because the enzyme activity was enriched in the mitochondria of mouse brain (4). Our recent study (8) confirmed that the specific activity (2.6 ± 0.18 U/mg protein) of mitochondrial fraction from bovine brain was 1.6 times that of the microsomal fraction. Here we report the partial purification and characterization of a sphingosine *N*-acyltransferase from bovine liver mitochondrial fraction. This study suggests that the sphingosine *N*-acyltransferase also catalyzes *N*-acylation of sphinganine and participates in ceramide synthesis in this organelle.

MATERIALS AND METHODS

Materials. The following materials were commercially obtained: D-sphingosine, DL-erythro-dihydrosphingosine (sphinganine), fatty acyl-CoA, aprotinin, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and concanavalin A (Con A) from Sigma Chemical Co. (St. Louis, MO); leupeptin and antipain from Peptide Institute (Osaka, Japan); *n*-octyl β-D-thioglucoside (OTG), phenylmethanesulfonyl fluoride (PMSF), 3,3′-diaminobenzidine tetrahydrochloride, Triton X-100, horseradish peroxidase, and celite from Wako Pure Chemical Co. (Osaka, Japan); DE-32 from Whatman (Maidstone, England); and Sepharose CL-6B from Pharmacia Biotech (Uppsala, Sweden).

Preparation of sphingosine-coated celite. Sphingosine affinity column material was prepared according to the method of Boulanger *et al.* (9). Briefly, 20 mg of sphingosine in 20 mL chloroform was mixed with 10 g dried celite, and the solvent was evaporated at 37°C under a nitrogen stream. The lipid matrix was treated with 10 vol 100 mM Tris-HCl buffer (pH 7.4)/1% glycine at room temperature overnight. The sphingosine celite was washed with cold buffer B prior to use as the column material.

Subcellular fractionation. Fresh bovine liver was obtained from a local slaughterhouse and immediately rinsed with cold

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Con A, concanavalin A; OTG, *n*-octyl β-D-thioglucoside; PMSF, phenylmethanesulfonyl fluoride.

saline. The tissue (1 kg) was cut into pieces and homogenized in 5 vol 20 mM Tris-HCl buffer (pH 7.4)/0.15 M NaCl/0.25 M sucrose (buffer A). After centrifugation at 1,000 × *g* for 10 min, the precipitate was removed, and the supernatant was centrifuged at 5,000 × *g* for 20 min. The precipitate was suspended with 5 vol buffer A, and the suspension was centrifuged again at 5,500 × *g* for 15 min. The resulting precipitate was collected and used as the mitochondrial fraction.

Solubilization and purification of ceramide synthase. All steps were performed at 4°C. (i) Solubilization of ceramide synthase: the mitochondrial pellet was suspended in 10 vol of buffer A containing 2 μg/mL antipain, 20 μg/mL aprotinin, 5 μg/mL leupeptin, 2 mM EDTA, 2 mM PMSF, and 50 mM OTG. The suspension was stirred for 2 h and then centrifuged at 10,000 × *g* for 45 min. To the precipitate was added 5 vol the same buffer, and the suspension was stirred for 1 h and centrifuged. These supernatants were combined and dialyzed against 20 mM Tris-HCl buffer (pH 7.8) for 16 h. (ii) DE-32 chromatography: the dialysate was applied to a column (5 × 20 cm) of DE-32, preequilibrated with 20 mM Tris-HCl buffer (pH 7.8). The proteins were eluted with a linear gradient of NaCl in the Tris buffer from 0 to 1.25 M at a flow rate of 42 mL/h (fraction volume, 9 mL/tube). The active fractions (tube No. 80-115) were collected and concentrated on a PM-10 membrane (Amicon Co., Beverly, MA). The concentrate was dialyzed against 20 mM Tris-HCl buffer (pH 7.4)/0.15 M NaCl (buffer B) for 16 h. (iii) Sphingosine affinity column chromatography: the dialysate was applied to a column of sphingosine-coated celite (1.6 × 10 cm), preequilibrated with buffer B. After washing the column with buffer B, the adsorbed proteins were eluted with a linear gradient of KI in buffer B from 0 to 1.5 M at a flow rate of 18 mL/h (fraction volume, 2 mL/tube). The highest enzyme activity was eluted at 0.45 M KI. The active fractions (tube No. 16-20) were collected and concentrated on a PM-10 membrane. The concentrate was dialyzed against buffer B for 16 h. (iv) Gel filtration on Sepharose CL-6B: the dialysate was applied to a column (1.6 × 40 cm) of Sepharose CL-6B, preequilibrated with buffer B containing 1 mM OTG/10% glycerol. The proteins were eluted with the same buffer at a flow rate of 13 mL/h (fraction volume, 2 mL/tube). The active fractions were collected and enzymatically characterized.

Assay of ceramide synthase. Ceramide synthase activity was determined as described previously (8). Briefly, D-sphingosine (800 nmol) in 100 μL of 20 mM Tris-HCl buffer (pH 7.4)/0.1% Tween 20 and stearoyl CoA (400 nmol) in 100 μL of 20 mM Tris-HCl buffer (pH 7.4) were placed in a 12 × 100 mm screw-cap test tube. The suspension was further diluted with the Tris buffer to a final volume of 450 μL. The enzyme reaction was started by adding 200 μL of enzyme solution plus 100 μL of liposome suspension (170–200 μg lipids). The liposomes were prepared by using the lipids derived from the mitochondrial membrane. The addition of liposome preparation increased the specific activity of solubilized ceramide synthase by 2.3-fold (8). After incubation at 37°C for 90 min, the enzyme reaction was terminated by adding 3 mL of chloroform/methanol (2:1, vol/vol). The mixture was shaken and centrifuged. A 2-mL portion of the lower layer was withdrawn and mixed with chloroform/methanol (2:1, vol/vol) and 10% (wt/vol) ammonium sulfate (1 mL each). The mixture was shaken and centrifuged. The lower layer was withdrawn and evaporated to dryness. The dried sample was allowed to react at 65°C for 15 min with benzoyl chloride and pyridine. The benzoylated ceramide was detected by high-performance liquid chromatography on a silica column with 10% 2-propanol in *n*-hexane as mobile phase.

Determination of protein. Protein concentration was determined by the method of Lowry *et al*. (10), using bovine serum albumin as reference.

Gel electrophoresis and sugar chain staining. Samples were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel under unreducing conditions (11). The separated proteins were stained with Coomassie Brilliant Blue R. For the sugar chain staining (12), proteins were transferred from the gel to a nitrocellulose membrane, using a semidry electroblot system (Sartoblot; Sartorius Co., Göttingen, Germany). The membrane was incubated at 25°C for 10 min with 2% bovine serum albumin in 50 mM Tris-HCl buffer (pH 7.4)/0.2 M NaCl and subsequently for 30 min with 50 μg/mL Con A in the Tris buffer. After washing, the membrane was further incubated for 30 min with 50 μg/mL peroxidase in the Tris buffer. After washing, peroxidase reaction was carried out at 25°C with 0.06% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in the Tris buffer.

Enzyme unit. One unit (U) was defined as the amount of enzyme which forms 1 nmol of stearoylsphingosine per min at 37°C.

RESULTS AND DISCUSSION

Solubilization of enzyme activity. Prior to purification experiments, we examined the effects of various detergents on the solubilization of ceramide synthase activity from the mitochondrial fraction (Fig. 1). Although Triton X-100 most effectively solubilized the mitochondrial proteins, it inhibited the enzyme activity in a concentration-dependent manner. CHAPS was at least a better detergent than Triton X-100 for solubilization of the mitochondrial proteins. However, the solubilized enzyme activity was very low (0.03 U/mg protein at 50 mM CHAPS). OTG was able to solubilize ceramide synthase (0.15 U/mg protein at 50 mM OTG) without solubilization of a large bulk of the mitochondrial proteins. Evidence in endoplasmic reticulum suggests that ceramide synthesis occurs on the cytoplasmic face (12). OTG might solubilize effectively the ceramide synthase that associates weakly with the outer side of the membranes. From these results, we used 50 mM OTG upon the solubilization of ceramide synthase. The buffered detergent also contained the following protease inhibitors: antipain, aprotinin, leupeptin, EDTA, and PMSF. As shown in Figure 2, these protease inhibitors greatly effected the stability of solubilized enzyme activity.

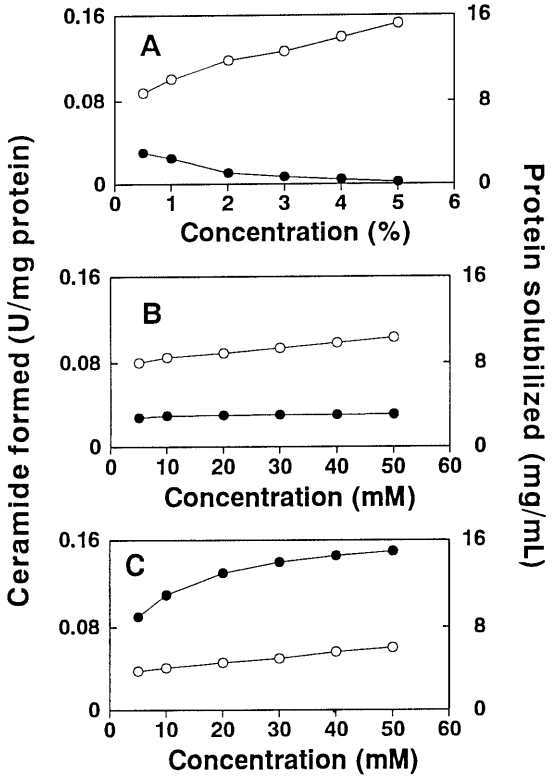

FIG. 1. Effects of various detergents on the solubilization of ceramide synthase activity from bovine liver mitochondria. Mitochondrial pellet was suspended in 10 vol buffer containing various concentrations of (A) Triton X-100, (B) CHAPS, or (C) OTG. The suspension was stirred at 4°C for 2 h, and then centrifuged at 10,000 × *g* for 45 min. The protein concentration (○) and ceramide synthase activity (●) were determined as described in the Materials and Methods section. The data represent the mean of duplicate experiments.

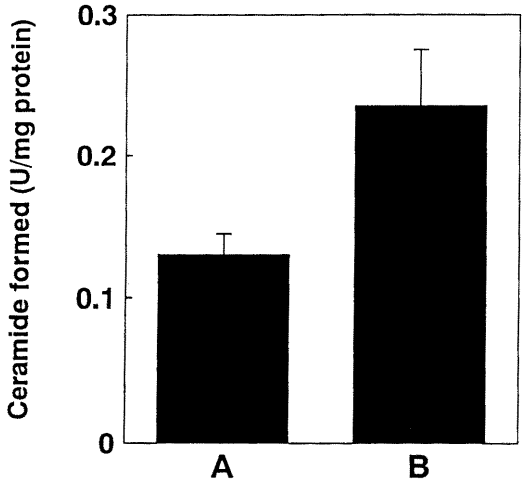

FIG. 2. Effects of protease inhibitors on the stability of the ceramide synthase activity solubilized from the mitochondrial pellet. Ceramide synthase was solubilized with (A) buffered *n*-octyl β-D-thioglucoside (OTG) alone (A) or (B) buffered OTG containing antipain, aprotinin, leupeptin, EDTA, and phenylmethanesulfonyl fluoride. After centrifugation, the enzyme activity was determined as described in the Materials and Methods section. Each bar represents the mean ± SD of triplicate experiments.

TABLE 1
Purification of Ceramide Synthase from Bovine Liver[a]

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Mitochondria	2580	387	0.15	1	100
OTG extract[b]	1526	320	0.21	1.40	82.7
DE-32[c]	165	261	1.58	10.5	67.4
Sphingosine-celite	2.81	31.8	11.3	75.3	8.22
Sepharose CL-6B[d]	1.04	15.4	14.8	98.7	3.98

[a]The starting material was 1 kg bovine liver. Enzyme activity was determined as described in the Materials and Methods section. One unit (U) is defined as 1 nmol stearoylsphingosine formed/min.
[b]Wako Pure Chemical Co. (Osaka, Japan).
[c]Whatman (Maidstone, England).
[d]Pharmacia Biotech (Uppsala, Sweden).

Enzyme purification. Table 1 summarizes the results of a typical purification of ceramide synthase from bovine liver mitochondrion-rich fraction. Buffered OTG containing the protease inhibitors solubilized the enzyme activity with yield of 82.7%. The OTG extract was next applied to a DE-32 column. At this stage, the enzyme was purified 10.5-fold from the starting mitochondria with yield of 67.4%. The enzyme fraction was subsequently bound to a sphingosine-celite column and eluted by increasing KI gradient from 0 to 1.5 M (Fig. 3). The eluate exhibited a 75.3-fold increase in the specific activity. The column was able to be used several times for the purification of this enzyme. Gel filtration on Sepharose CL-6B yielded two peaks of ceramide synthase activity (Fig. 4). One minor activity appeared as the initial pass-through fraction, while the other major activity was estimated in fractions having the molecular masses of 240–260 kDa. The latter active fraction was collected and concentrated. The final enzyme preparation did not contain the affinity column material, celite, and had a specific activity of 14.8 U/mg protein and a 98.7-fold purification. The low purification factor

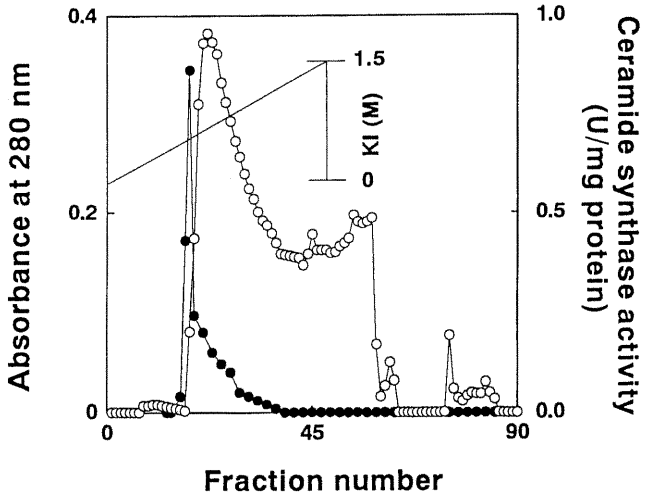

FIG. 3. Sphingosine affinity column chromatography. Column procedures were as described in the Materials and Methods section. ○, absorbance at 280 nm; ●, enzyme activity.

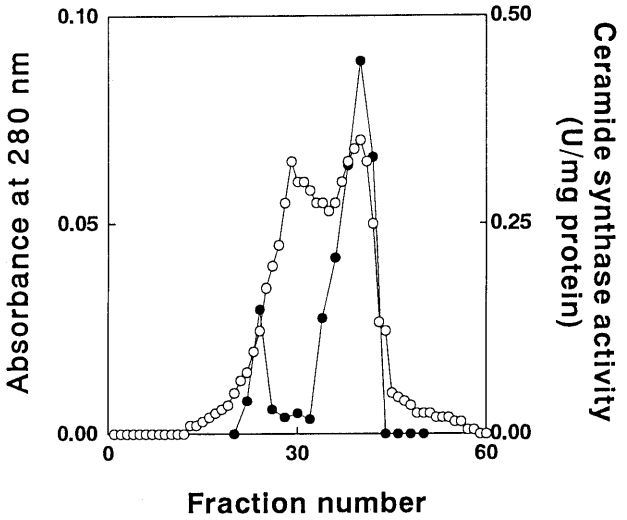

FIG. 4. Sepharose CL-6B chromatography. Column procedures were as described in the Materials and Methods section. ○, absorbance at 280 nm; ●, enzyme activity.

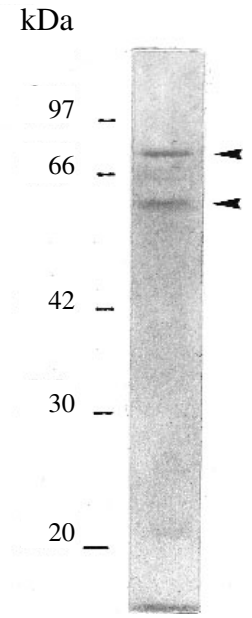

FIG. 5. Molecular mass determination of the purified enzyme on SDS-polyacrylamide gel electrophoresis. About 5 μg of the final preparation was applied on a 12% gel as described in the Materials and Methods section. Molecular mass markers: 97 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 42 kDa, aldolase; 30 kDa, carbonic anhydrase; and 20 kDa, soybean trypsin inhibitor.

may be partly caused by the labile enzyme activity. Although the ceramide synthase activity was higher in the presence of liposomes, we failed to keep the enzyme activity stable at low temperatures. An additional method is needed for stabilizing the enzyme activity.

Physicochemical properties of the partially purified enzyme. Analysis of the enzyme preparation on 12% SDS-polyacrylamide gel electrophoresis under unreducing conditions revealed two major protein bands of 62 kDa and 72 kDa (Fig. 5). To assess whether these proteins have oligosaccharide chains, they were transferred to a nitrocellulose sheet and detected by the Con A-peroxidase method (12). The two protein bands were bound to Con A and stained by peroxidase reaction. A minor band of 69 kDa was also observed (data not shown). The different molecular mass of this enzyme, determined on gel filtration (Fig. 4) and on SDS-polyacrylamide gel electrophoresis (Fig. 5), suggests that the ceramide synthase was present in the buffer solution as a subunit form, or simply in aggregated form with these proteins. Such a high-molecular-weight nature of the enzyme has been observed in gel filtration of the preparation solubilized from rat liver microsomes (5).

Substrate specificity and other enzymic properties of the partially purified enzyme. The influence of pH on the ceramide synthase activity was examined, using sphingosine and stearoyl-Co A as substrates. As shown in Figure 6, the enzyme activity was maximal at pH 7.5: more than 70% of the maximal activity was obtained over the pH range 7.0–8.0. Substrate specificity of the partially purified enzyme was also determined with various acyl (C_6–C_{22})-CoA and sphingosine or with stearoyl-CoA and sphinganine as substrates (Table 2). CoA esters having regular chain (C_{16}–C_{18}) were good substrates. Very long chain (C_{22}) or short chain (C_6–C_{12}) decreased the function as substrate. The observed substrate specificity is adequate, when considering the majority of stearoyl- and palmitoyl-chains in naturally occurring ceramides. The partially purified enzyme could form dihydroceramide with stearoyl-CoA and sphinganine as substrates. The K_m values determined for sphingosine and sphinganine were almost identical (171 and 144 μM). However, when the latter substrate was utilized, the putative dihydroceramide dehydrogenase present in the mitochondria still awaits definitive enzymological characterization. There is also some evidence for ceramide synthesis by reversal of ceramidase (6,7). The partially purified enzyme had no ceramidase activity at pH 4.5,

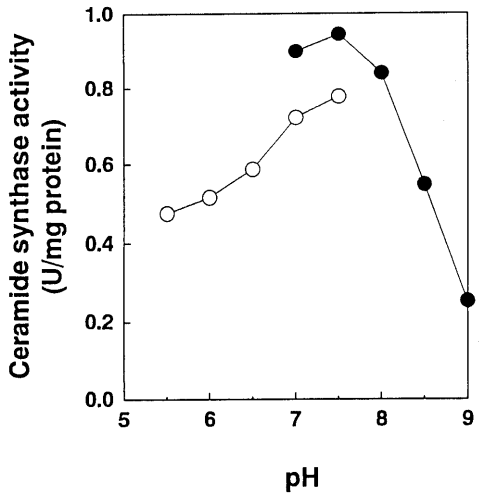

FIG. 6. Effect of pH on the purified enzyme activity. Enzyme activity was determined using stearoyl-CoA and sphingosine as substrates. Buffers used: ○, phosphate buffer; ●, Tris-HCl buffer. The data represent the mean of duplicate experiments.

TABLE 2
Substrate Specificity of the Purified Ceramide Synthase[a]

Substrate	Kinetic parameter		
	K_m (μM)	V_{max} (U)	V_{max}/K_m
Sphingosine (500 μM) plus			
Hexanoyl-Co A [$C_{6:0}$]	522	1.88	0.0036
Lauroyl-Co A [$C_{12:0}$]	358	1.75	0.0049
Palmitoyl-Co A [$C_{16:0}$]	141	7.68	0.0545
Stearoyl-Co A [$C_{18:0}$]	146	11.11	0.0761
Behenoyl-Co A [$C_{22:0}$]	299	2.53	0.0085
Oleoyl-Co A [$C_{18:1}$]	180	7.25	0.0403
Stearoyl-Co A (500 μM) plus			
Sphingosine	171	11.33	0.0663
Sphinganine	144	8.54	0.0593

[a]Enzyme activity was determined as described in the Materials and Methods section, using various concentrations (10–800 μM) of substrates. Kinetic parameters were obtained from Lineweaver-Burk plot analyses. K_m and V_{max} were calculated from the plot *x* and *y* intercepts, respectively. The data represent the mean of duplicate experiments.

when examined using stearoylsphingosine as substrate (data not shown). Thus, the partially purified enzyme may participate in the mitochondrial ceramide (dihydroceramide) synthesis. To better understand the mechanism of ceramide synthesis, purification of the mitochondrial enzyme to a homogeneous state and also the purification of ceramide synthase from the microsomal fraction are needed.

The next step following ceramide formation is the biosynthesis of sphingomyelin (13) or glucosylceramide (14) in Golgi compartments. The transportation mechanism of ceramide is not known at present. Saposin D is a candidate for the ceramide-transporting protein, because of its ability to form a complex with ceramide (15). However, saposins A–D are mainly localized in lysosomes and activate the degradation of several sphingolipids by acid hydrolases (for review, see Refs. 16 and 17). Recently, we purified a ceramide-binding protein from bovine brain cytosol fraction, whose N-terminal amino acid sequence was completely different from those of saposins A–D (Kihara, T., Soeda, S., and Shimeno, H., unpublished work). If the novel binding protein present in cytosol acts as a ceramide transporter, the pathway that ceramide is directly synthesized from sphingosine and acyl-CoA on the mitochondrial compartments and followed by the transportation to another compartment to form glycosphingolipids or to function as a lipid mediator of signal transduction (18,19) will be acceptable. We are currently examining the physiological function of the ceramide-binding protein.

ACKNOWLEDGMENT

This work was supported in part by funds from the Central Research Institute of Fukuoka University.

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[Received September 27, 1997, and in final revised form March 11, 1998; revision accepted April 20, 1998]

N-Stearoyl-phosphatidylserine: Synthesis and Role in Divalent-cation-induced Aggregation and Fusion

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ABSTRACT: *N*-Acylphosphatidylserines have been isolated from intact and injured tissues, but the participation of such acidic phospholipids in membrane aggregation and fusion has not been demonstrated. We have synthesized *N*-stearoylphosphatidylserine (NSPS) and examined divalent-cation-induced aggregation of NSPS-liposomes, which leads to membrane destabilization and fusion. The purified lipid was characterized by its chromatographic and spectroscopic (infrared and ^{1}H nuclear magnetic resonance) properties and by its chemical degradation pattern. Aggregation of unilamellar NSPS-liposomes was studied as a function of calcium and magnesium concentration. The ability of calcium and magnesium to induce vesicle aggregation is higher for phosphatidylserine (PS)-liposomes (threshold concentration 1.5 mM for calcium and 4.6 mM for magnesium) than for NSPS-liposomes (threshold concentration 2.8 mM for calcium and 6.6 mM for magnesium). The irreversibility of the aggregation reactions after adding EDTA suggests that vesicle fusion might occur in the presence of calcium and magnesium. Preliminary studies, based on mixing of both lipid and internal aqueous contents, show that fusion rather than aggregation of NSPS-liposomes occurs in the presence of calcium ions. The tendency of NSPS-liposomes to aggregate at higher cation concentrations than PS-liposomes suggests that *N*-acylation of phosphatidylserine protects the membrane against degenerative damage caused by aggregation and fusion.
Lipids 33, 607–616 (1998).

Amide-linked fatty acids occur in nature mainly in sphingolipids as part of their ceramide (*N*-acylsphingosine) backbone. In contrast, the primary amino groups of glycerophospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), are not usually *N*-acylated. Nevertheless, *N*-acylphosphatidylethanolamines (NAPE) were originally detected in wheat flour (1), and the occurrence of these phospholipids as natural constituents is now well established. Their presence has been demonstrated in microorganisms (2–4), in cotyledons of cottonseeds (5), in higher plants (6,7), in fish brain and spinal cord (8), in the granular cells from pig epidermis (9), in dog brain (10), in bovine erythrocytes (11), in degenerating baby hamster kidney cells (12), in infarcted canine myocardium (13), and in ischemic brain of immature rats after decapitation (14). Moreover, *N*-acylphosphatidylserines (NAPS) have been identified in sheep erythrocytes (15), in the central nervous system of freshwater fish (8), and in bovine brain (16). It has also been reported that NAPS accumulate in the bacterium *Rhodopseudomonas sphaeroides* when it is grown in 20 mM Tris (17–19), but Schmid *et al*. (20) established the identity of this lipid as phosphatidyl-Tris rather than NAPS.

Both NAPE and NAPS are negatively charged lipids with one more hydrophobic chain than the more common glycerophospholipids. *N*-Acyl phospholipids are found only in measurable amounts in mammalian tissues in normal physiological conditions. However, they can accumulate under conditions of degenerative change involving disintegration or digestion of membranes, although their natural occurrence never surpasses 20% of the total phospholipid content. This can occur as the result of normal metabolism, e.g., as in the granular cells of the epidermis (12,21) and, possibly, in certain populations of erythrocytes (22) or as the result of ischemic injury (8,16), infarction (13), cancer (23), or other pathological conditions (22). Since *N*-acyl derivatives may appear only after cell death, when degenerative changes begin (14), they may be detected only in small amounts, if at all, in healthy cells. Nevertheless, in some pathological conditions there are measurable amounts of such derivatives before death.

More recently, Hansen *et al*. (24,25) have shown the dose-dependent glutamate-induced formation of NAPE and *N*-acyl-ethanolamines (NAE) mediated by the *N*-methyl-D-aspartate receptor in primary cultures of mouse neocortical neurons. Because primary cultures of cerebral cortical neurons provide a sensitive and well-characterized *in vitro* model for investigation of the excitotoxic action of amino acids such as glutamate, the authors suggest that NAPE and NAE are formed during neuronal death, that these compounds may be a defense mechanism after the exposure of the neurons to glutamate, and that these compounds may be used as markers of neurotoxicity. Furthermore, the formation of NAE by injured neurons may induce apoptosis in neigh-

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Abbreviations: DPA, dipicolinic acid; FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; IR, infrared; NAE, *N*-acyl-ethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPS, *N*-acylphosphatidylserine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; NMR, nuclear magnetic resonance; NSPS, *N*-stearoyl-phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)-phosphatidylethanolamine; TLC, thin-layer chromatography.

boring neurons, which reduces the impact of a local neuronal injury within the brain.

Different biological properties have been attributed to NAPE, to NAPS, and to their alkylacyl analogs. The effect of the two *N*-acylated phospholipids on the activity of delipidated and solubilized lysosomal glucocerebrosidase, a lipid-requiring enzyme, is opposite (26,27). NAPE was reported (22) to lower serum triacylglycerols in rats made hyperlipidemic through oral administration of Triton WR-1339. 1-Alkyl-2-acyl-glycerophospho(*N*-acyl)PE show antineoplastic activity (28). 1-Alkyl- and 1-acyl-2-acetyl-glycerophospho(*N*-acetyl)PE act as inhibitors of the platelet-activating factor (1-alkyl-2-acetyl-glycerophosphocholine) (22).

It has been postulated that NAPE protect the membrane by stabilizing the lipid bilayer and by maintaining cell compartmentalization following tissue damage (22). This premise is based on data from animal systems that demonstrate the accumulation of NAPE only in degenerative conditions (13,14) and from biophysical studies of aqueous dispersions of NAPE, which indicate that these lipids stabilize a bilayer conformation where nonlamellar phases would otherwise be favored (22,29,30). Nevertheless, we have also established the influence of NAPE on the fusion properties of phospholipidic vesicles used as models of biomembranes (31).

Although *N*-acylation converts nonbilayer unsaturated PE into a bilayer lipid, the addition of Ca^{2+} can reverse the bilayer-stabilizing effect. Thus, NAPE may be involved in membrane fusion, induced by Ca^{2+}, responsible for the disruption of the sarcolemma. This hypothesis is corroborated by the following findings: (i) the discovery of a calcium-dependent acyl-transferase in membrane cells (32,33), (ii) the presence of *N*-acylated glycerophospholipids in heart cells after myocardial infarct (13), and (iii) the proposal by Post and co-workers (34,35) that cellular degradation in myocardial infarct could be the result of an increase in intracellular Ca^{2+} due to ischemia.

Even though NAPSs have been isolated from natural sources and prepared *via* semisynthetic procedures, there are no published data on the participation of such acidic phospholipids in membrane processes like permeability and fusion. The present paper deals with the synthesis and characterization of the *N*-stearoyl derivative of PS (NSPS) and examines the calcium- and magnesium-induced aggregation of NSPS-liposomes, which leads to fusion. Our results suggest that *N*-acylation of PS protects the membrane against damage by aggregation and fusion, since it eliminates the free fatty acids that destabilize membrane structure and since NSPS-liposomes have been found to aggregate at higher cation concentrations than PS-liposomes.

MATERIALS AND METHODS

Chemicals. Egg lecithin (65%; Merck A.G., Darmstadt, Germany) for synthesis was purified by aluminum oxide column chromatography and thin-layer chromatography (TLC) before use. L-Serine and stearoyl chloride, used in the synthesis of PS and NAPS respectively, and dipalmitoyl PS, used as standard for analytical TLC, were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorophore-labeled phospholipids, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)-PE (Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). $TbCl_3 \cdot 6H_2O$ and dipicolinic acid (DPA), used in the mixing assay for the aqueous content, were from Ventron (Seymour, Stockport, England) and Sigma, respectively. All other reagents were of analytical grade. Water was twice-distilled (Millipore systems), and all organic solvents were redistilled before use.

Preswollen CM-cellulose (CM 52, sodium form; Whatman, Maidstone, England), used to purify PS, was suspended in methanol and decanted several times to remove small fibers before use. Columns were packed as described by Rouser (36) for DEAE-cellulose, and were provided with a glass wool plug and some glass beads on top of the bed in order to avoid disturbance of the adsorbent during solvent changes. Before applying the lipids, methanol was removed by eluting with 3 bed volumes of chloroform/methanol (1:1, vol/vol) and 7 bed volumes of chloroform. The lipid was applied to the column dissolved in chloroform (≤50 mg lipid/mL) with a maximal amount of 5 mg lipid/mL of bed volume. The column was eluted with 3, 20, 3, 3, and 9 bed volumes of chloroform/methanol (vol/vol) 1:0, 4:1, 7:3, 3:2, and 1:1, respectively. The flow rate was 0.48 mL/min. The column was reused after washing with 10, 3, and 7 bed volumes of methanol, chloroform/methanol (1:1, vol/vol) and chloroform, respectively.

Analytical TLC was performed on layers of silica gel G (Sil G-25) purchased from Macherey-Nagel Düren (Düren, Germany). Preparative TLC was carried out on 1-mm thick layers prepared with silica gel H obtained from Merck (TLC-Kieselgel 60H, 15 μm). Samples were made visible with iodine, by spraying with ninhydrin or ammonium molybdate in H_2SO_4 or by charring.

Enzymes. Streptomyces species phospholipase D (E.C. 3.1.4.4.) Class VII was purchased from Sigma.

Synthesis of PS. PS was obtained by PC transphosphatidylation with *Streptomyces* sp. phospholipase D (37) in the presence of L-serine. Peroxide-free diethyl ether (4.32 mL) was added to 100 mg of dry PC and the mixture was sonicated (50 W for 1 min). Enzyme (70.3 U) and 2.6 g of L-serine were introduced into the reaction vessel, dissolved in 7.3 mL of 0.2 M acetate buffer pH 5.6 (40 mM $CaCl_2$), and the sonicated PC/diethyl ether solution plus 3 additional mL of diethyl ether were added. The reaction vessel was deaerated with N_2. The reaction was carried out at 30 ± 1°C under continuous stirring; the extent of the transphosphatidylation reaction was checked by TLC (Table 1, solvent systems 1 and 2) at different times. After 6 h, the reaction was stopped by the addition of 14.6 mL of 0.1 M EDTA pH 5.6 and the mixture was stirred for 10 min. Diethyl ether was removed under vacuum and PS was extracted, first with a mixture of 14.6 mL of $CHCl_3$/MeOH (2:1, vol/vol) and then with 1.46 mL of water. The lower layer was collected and further extracted three

TABLE 1
R_f Values in Thin-Layer Chromatography[a] (TLC) of *N*-Stearoyl-phosphatidylserine (NSPS) and Its Precursors in Different Solvent Systems

	Solvent system[b]						
Sample	1	2	3	4	5	6	7
PC	0.31 ± 0.03	0.46 ± 0.03	0.30 ± 0.02	—	—	—	—
PS	0.53 ± 0.07	0.14 ± 0.01	0.21 ± 0.04	0.10 ± 0.02	0.52 ± 0.02	0	0.11 ± 0.02
NSPS	—	—	0.36 ± 0.05	0.26 ± 0.06	0.89 ± 0.07	0	0.41 ± 0.03
Stearic acid	—	—	—	0.44 ± 0.05	—	0.46 ± 0.05	0.46 ± 0.05
Stearoyl chloride	—	—	—	0.39 ± 0.06	—	0.41 ± 0.06	0.37 ± 0.05

[a]Adsorbent: Silica gel G (Merck).
[b]Solvent systems: 1, chloroform/methanol/acetic acid/water (50:25:7:3, by vol); 2, chloroform/methanol/ammonium hydroxide (28%) (50:25:6); 3,chloroform/methanol/water (65:25:4); 4, chloroform/methanol/ammonium hydroxide (28%) (65:25:5); 5, chloroform/methanol/acetone/acetic acid/water (50:20:10:10:5); 6, hexane/diethyl ether/formic acid (40:10:1); 7, chloroform/methanol/ammonium hydroxide (28%) (40:10:1). The data represent the mean ± SD of at least five determinations. PC, phosphatidylcholine; PS, phosphatidylserine.

times with 14.7 mL of $CHCl_3$. The combined organic extracts were concentrated in a vacuum evaporator. PS was redissolved in $CHCl_3$ and dried by anhydrous Na_2SO_4. The product was purified by CM-cellulose column chromatography and checked by ^1H nuclear magnetic resonance (NMR) and infrared (IR). Lipid concentrations were determined by phosphorus analysis (38).

Synthesis of NSPS. NAPS have been synthesized elsewhere by acylation of bovine brain PS with fatty acid imidazolide (16) in 45% yield, after purification with TLC. The yields of their synthesis with fatty acid anhydride (18,19) were about 5% after purification by silicic acid column chromatography. The synthesis using the carbonyldiimidazole derivative of oleic acid (20) yielded 56% of NAPS after purification by TLC. Finally, NSPS has been synthesized by reacting PS with stearoyl chloride, analogous to NAPE synthesis (3), but the purification procedures and yields were not reported.

In the present study, PS was converted to NSPS following essentially the method proposed by Dawson *et al*. (6) for NAPE synthesis. Stearoyl chloride (0.157 mmol) and Et_3N (0.158 mL), used as catalyst, were added to 48 mg of PS dissolved in 19 mL of ethanol-free $CHCl_3$. The reaction mixture was deaerated under N_2 and stirred for 90 min. The reaction was stopped by addition of 5% HCl to hydrolyze the remaining stearoyl chloride. The triethylammonium chloride formed was removed by six washes in water, and the organic phase was dried by anhydrous Na_2SO_4. NSPS was purified by preparative TLC on silica gel H using first $CHCl_3$/MeOH/NH_3 (28%) (Table 1, solvent system 4) and then hexane/diethyl ether/formic acid (Table 1, solvent system 6) as eluents. The product was extracted from the adsorbent with $CHCl_3$/MeOH/H_2O (10:21:10). Chloroform and water were added to the eluate to give a final ratio of 20:21:20, and the lower layer was removed and dried. The amide bond was detected by IR and ^1H NMR. Gas–liquid chromatography (GLC) was used to confirm the incorporation of the third fatty acid chain.

IR spectra. These were obtained from a film of lipid chloroform solutions (approximately 5 mg/mL) on KCl crystals by using a Perkin-Elmer 687 IR spectrophotometer. All spectra were recorded at room temperature against a KCl crystal reference.

NMR spectra. ^1H NMR (200 MHz) spectra were recorded on a Varian XL-200 spectrometer operating in the Fourier transform mode. Samples (15–25 mg lipid/mL) were measured in 5 mm o.d. sample tubes at room temperature. Typically, 200–300 scans were averaged. $CDCl_3$ was used as solvent and for field frequency locking purposes. Chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane, which was used as the internal standard.

Fatty acids analysis. Fatty acids were converted into fatty acid methyl esters (FAME) and analyzed by GLC on a Perkin-Elmer 990 gas chromatograph equipped with a flame-ionization detector. Data were obtained with a 2 m × 2 mm i.d. column packed with 15% polymeric ethylene glycol succinate as stationary phase on Chromosorb W (100–120 mesh; Johns-Manville, Denver, CO). Helium at 30 mL/min was used as carrier gas. Injector, column, and detector temperatures were, respectively, 230, 188, and 250°C. Samples were applied as hexane solutions.

Preparation of liposomes. Liposomes were prepared by a classical method. NSPS was dissolved in $CHCl_3$, and the solvent was evaporated under a stream of nitrogen gas to deposit a lipid film on the wall of a glass test tube. Final traces of residual solvent were removed under vacuum for 12 h. Multilamellar vesicles were prepared by vortexing the dried lipid film (0.5 mg/mL) with 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 0.1 mM EDTA. Small unilamellar vesicles were prepared by sonication of the phospholipid suspensions for 20 min (with intervening periods of 30 s every 90 s) in a Braun Labsonic 2000 probe sonifier equipped with a 9.5-mm probe, operating at 48 W. During sonication the suspension was kept at 45°C under a nitrogen stream. Titanium particles and possible undispersed lipids were removed by centrifugation. It was verified by TLC that the lipid did not suffer degradation due to sonication. Before use, the liposome dispersion was passed through a 0.45 μm filter.

Assay of vesicle aggregation. Vesicle aggregation was monitored by continuous measurements of the turbidity of the liposome suspensions at 400 nm for 2 min. The optical density changes were monitored with a Hewlett-Packard 8540A ultraviolet/visible spectrophotometer equipped with a Peltier temperature control cell and continuous stirring. The NSPS concentration for the aggregation assay was 0.05 mg/mL.

Measurements were performed at 20°C before and after the addition of small aliquots of concentrated calcium and magnesium salt solutions to the liposome suspensions. In order to avoid initial bubbling problems or the formation of large vesicles, we report the increments of absorbance after 20 s of cation addition. The threshold concentrations (c_t) for the aggregation process were calculated as described by Ohki (39).

Light-scattering measurements. The size distribution of unilamellar phospholipid vesicles, with or without added divalent ions, was analyzed by dynamic light scattering using a PCS41 optics unit (Malvern Autosizer IIc) and a 5-mW He-Ne laser (Spectra Physics) at an excitation wavelength of 633 nm. The data were collected with a Malvern 7032N 72 data channel correlator, and the mean hydrodynamic diameter was calculated from a cumulant analysis of the intensity autocorrelation function. The possible reversibility of the aggregation reaction was verified by adding EDTA to the aggregated vesicles at six times the concentration of divalent ions and measuring the size again after 2 min of incubation.

Assay of vesicle fusion. The fusion of vesicles induced by calcium ions was measured by monitoring the mixing of the lipid and mixing of the internal aqueous contents using fluorescent probes.

Lipid mixing was measured, using a modification of the fluorescence resonance energy transfer assay of Struck *et al.* (40), to show the fusion of liposomal membranes. Two populations of small unilamellar NSPS vesicles were prepared: one with NBD-PE and Rh-PE at 1 and 0.5 mole%, respectively, with respect to the total lipid at the membrane (0.05 mg/mL) and the other without fluorescent probes. The fluorescence-labeled liposomes and a ninefold excess of unlabeled vesicles were mixed at 20°C to study the dilution of the fluorescent lipids by continuous monitoring of the decrease in energy transfer from NBD-PE to Rh-PE using a Kontron SFM 25 spectrofluorimeter at excitation and emission wavelengths of 440 and 536 nm, respectively. Maximal fluorescence was determined using reference liposomes containing 0.1% NBD-PE and 0.05% Rh-PE. Measurements were performed at 20°C for 2 min after the addition of small aliquots of a concentrated $CaCl_2$ solution to the liposome suspensions. Light-scattering controls were performed using unlabeled vesicles. The percentage of maximal fluorescence was calculated as:

$$\% \text{ maximal fluorescence} = \frac{\frac{F_t - S_t}{F_T - S_T} - \frac{F_0 - S_0}{F_T - S_T}}{\frac{R_t - S_t}{R_T - S_T} - \frac{F_0 - S_0}{F_T - S_T}} \times 100 \qquad [1]$$

where F_t = fluorescence intensity at time t; F_0 = fluorescence intensity at zero time; F_T = fluorescence intensity in the presence of 0.7% of Triton X-100. R and S represent the same measurements for reference liposomes and light-scattering controls, respectively (41).

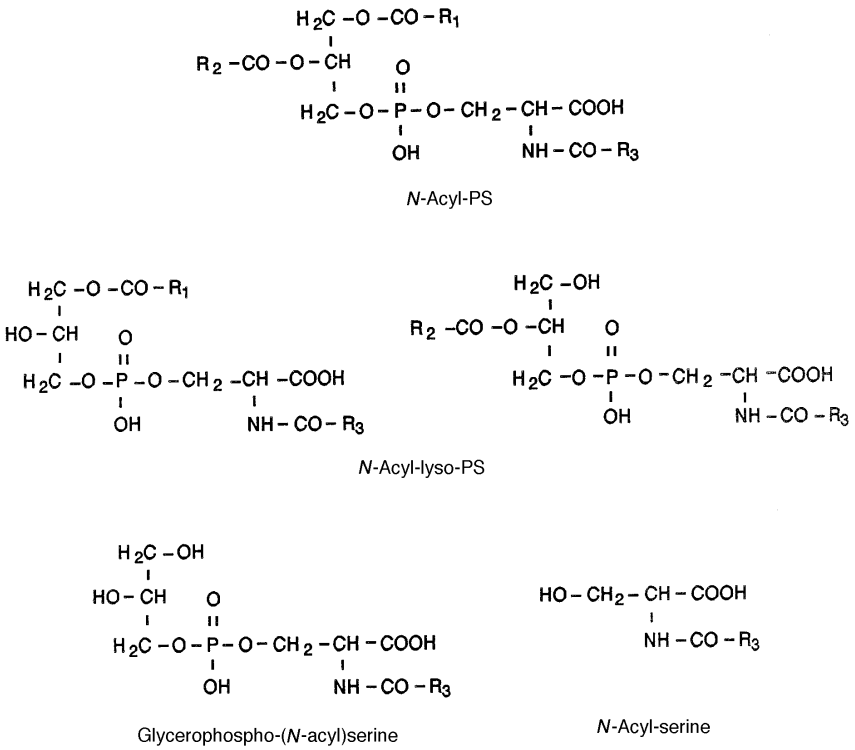
A modification of the method of Nir *et al.* (42) was used for mixing assay for the aqueous contents. Three populations of unilamellar vesicles (0.05 mg lipid/mL) were prepared in the following solutions: (i) 10 mM $TbCl_3 \cdot 6H_2O$/100 mM nitrilotriacetic acid/20 mM Tris-HCl buffer (pH 7.4), (ii) 100 mM DPA/20 mM NaCl/20 mM Tris-HCl buffer (pH 7.4) and (iii) 5 mM $TbCl_3 \cdot 6H_2O$/50 mM nitrilotriacetic acid/50 mM DPA/10 mM NaCl/20 mM Tris-HCl buffer (pH 7.4). After vesicle preparation, nonentrapped material was separated by gel filtration of 0.4 mL of the liposomal suspension on a Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) column (200 × 10 mm) previously equilibrated with 100 mM NaCl/0.75 mM EDTA/20 mM Tris-HCl buffer (pH 7.4). Liposomes were eluted by the solution used in the equilibration process, and 1.6 mL of liposomes free of nonentrapped material was collected. $TbCl_3$ and DPA vesicles were mixed in a 1:1 molar ratio at a final lipid concentration of 0.05 mg/mL for the fusion assay in a 100 mM NaCl/20 mM Tris-HCl buffer (pH 7.4). Fluorescence measures were performed at 20°C for 2 min after the addition of small aliquots of a concentrated $CaCl_2$ salt solution to the liposome suspensions in a Kontron SFM 25 spectrofluorimeter at an excitation wavelength of 276 nm. The emission fluorescence was measured at 545 nm using a 530 cut-off filter to eliminate the contribution of light scattering. The value for 100% fusion was determined using the reference liposomes prepared with solution (iii). Light-scattering controls were performed using unlabeled vesicles. The percentage of fusion was calculated in the same way as for the lipid mixing assay.

Negative stain electron microscopy. Control NSPS liposomes were prepared in 20 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 0.1 mM EDTA. To check the fusion of NSPS liposomes, an aliquot of control liposomes was incubated with Ca^{2+} (3 mM) for 2 min and then with EDTA (16 mM) for 1 h. Vesicle fusion was monitored by negative stain electron microscopy of liposome suspensions before and after the addition of calcium and EDTA. A drop of the liposome suspension was placed on a copper grid (400 mesh) for 90 s. Then buffer and liposome excess were drawn off and quickly replaced by a drop of 2% ammonium molybdate solution (pH 7.4). Finally, after 30 s the bulk of the heavy metal solution was also drawn off. Thoroughly dry grids were examined in a Hitachi MT-600 electron microscope at an acceleration of 75 kV.

RESULTS

Synthesis and characterization of NSPS. *N*-Acylserine phospholipids occur as derivatives of 1,2-diacylglycerol and exhibit the configuration indicated in Scheme 1. Sequential degradation of these phospholipids can produce *N*-acylserine lysophospholipids, glycerophospho-(*N*-acyl)serine and *N*-acylserine. Thus, the synthesis of *N*-acylserine phospholipids can be accomplished by converting PC to PS through a transphosphatidylation reaction and by acylation of serine phospholipids with fatty acyl chloride.

The conversion of PC to PS by *Streptomyces* sp. phospholipase D proceeded with 87% yield. Crude PS was found to contain the by-product phosphatidic acid and PC, and was thus purified by TLC. Purified PS was obtained in 49.5%



SCHEME 1

yield and was identified by TLC (Table 1), by IR (Fig. 1) and ^{1}H NMR (Table 2). The IR and NMR spectra were identical to those reported in the literature (15,22,43). The analysis of purified PS by GLC showed that the fatty acid composition of PS and PC was the same (Table 3). This result indicates that the enzyme does not prefer any particular molecular species of PC during the transphosphatidylation process.

Synthesis of NSPS proceeded with 82% yield. NSPS was checked by TLC (Table 1), IR (Fig. 1), NMR (Table 2), and GLC (Table 3). The increase in stearic acid for NSPS, as revealed by FAME analysis, is consistent with the molar ratio 1:2 for the excess stearic acid/total fatty acids in PS, indicating that fatty acids at the *sn*-1 and *sn*-2 positions of the NSPS are the same as in PS and that the excess stearic acid corresponds to the third acyl chain incorporated owing to the acylation process. Although the IR spectrum of NSPS was similar to that of PS, the strong absorption bands at 1660 cm^{-1} (C=O stretch of the secondary amide), 1530 cm^{-1} (NH deformation), and 3380 cm^{-1} (NH stretch) indicated the presence of an amide bond in the molecule.

NSPS was obtained in 39% yield after purification by preparative TLC on silica gel H plates, using two solvent systems as indicated in the Materials and Methods section. Crude NSPS was contaminated by a considerable amount of stearic acid, since stearoyl chloride was used in excess in the acylation reaction, and by some remaining PS. The purification step using chloroform/methanol/ammonium hydroxide (28%) (65:25:5) as solvent system eliminates all PS, but NSPS still contained small amounts of stearic acid since the spots were lengthened and there was a slight overlap. Stearic acid was

TABLE 2
Chemical Shift Values[a] of ^{1}H Nuclear Magnetic Resonance (NMR) of PC, PS, and NSPS

Class of protons	PC δ_H (ppm)[a]	PS δ_H (ppm)[a]	NSPS δ_H (ppm)[a]
–*CH*$_3$	0.89	0.89	0.89
–*CH*$_2$–	1.25	1.25	1.25
(*CH*$_3$)$_3$C–O–CO	—	—	—
–COCH$_2$*CH*$_2$–	1.61	1.61	1.61
–*CH*$_2$CH=CH*CH*$_2$–	2.01	2.01	2.01
–CO*CH*$_2$–	2.29	2.29	2.29
–(CH=CH)-*CH*$_2$–(CH=CH)–	2.80	2.80	2.80
CH–*NH*–CO–O–	2.95	—	—
–N(*CH*$_3$)$_3^+$	3.33	—	—
–*CH*$_2$N(CH$_3$)$_3^+$	3.80	—	—
PO–O*CH*$_2$–CH (N,CO)	3.85	3.80	3.80
OH–*CH*$_2$–OP	—	4.15	4.08
–*CH*$_2$O–P–O*CH*$_2$-	4.15	—	—
–*CH*$_2$OCO	4.35	4.35	4.35
–*CH*NH	—	—	4.44
–*CH*NH$_2$	—	4.45	—
–*NH*	—	—	4.83
*CH*OCO	5.23	5.24	5.24
–*CH*=*CH*–	5.34	5.34	5.34
PO*H*	—	—	—
CHCl$_3$(residual)	7.26	7.26	7.26
–NH$_3^+$	—	8.2	—
–COO*H*	—	—	—

[a]Spectra were obtained from lipids dissolved in deuteriochloroform to a final concentration of approximately 10–15 mg lipid/mL and required 200 transients. ^{1}H NMR spectra were acquired by using a Geminis-200 spectrometer operating at 200 MHz in the Fourier transform mode at room temperature. Chemical shift values are expressed in parts per million (ppm) relative to tetramethylsilane at 0 ppm. The underlined values correspond to the characteristic shifts of each lipid. For abbreviations see Table 1.

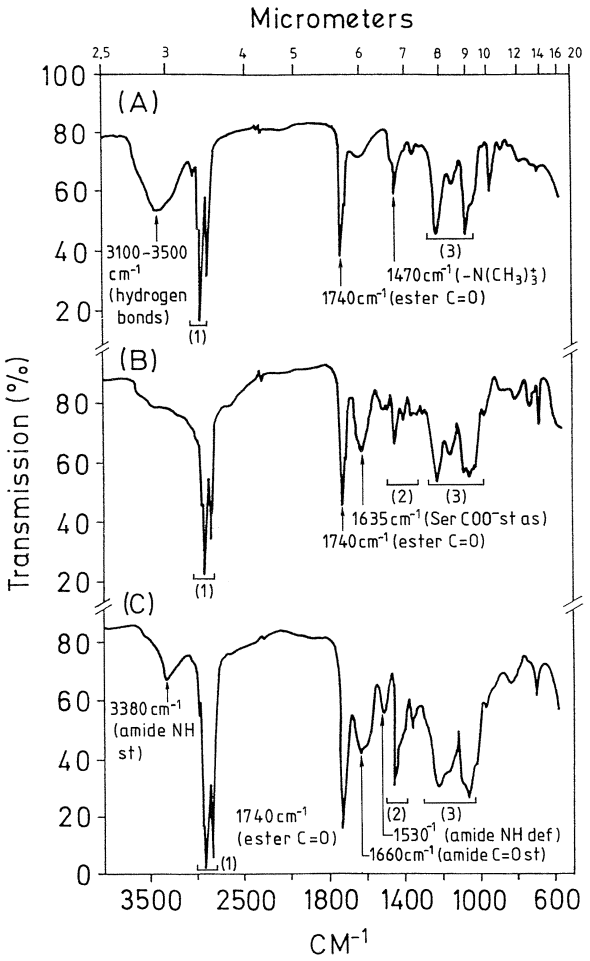


FIG. 1. Infrared spectra of phosphatidylcholine (A), phosphatidylserine (PS) (B), and *N*-stearoylphosphatidylserine (NSPS) (C). General absorption bands: (i) 2750–2950 cm^{-1} (CH stretching of CH_2); (ii) 1370–1450 cm^{-1} (CH bending of CH_3); (iii) 1090–1240 cm^{-1} [$(RO)_2(OH)P=O$ and $(RO)_2PO_2^-$ stretching].

eliminated by another TLC run using hexane/diethyl ether/formic acid (40:10:1) as eluent.

The NSPS IR spectrum was similar to that found in the literature for NAPS isolated from sheep erythrocytes (15) and bovine brain (16), or prepared by chemical acylation of palmitoyl-oleoyl-PS (20,22). Chemical shift data for the ^1H NMR spectrum of NSPS are summarized in Table 2 and are consistent with those found in the literature for *N*-oleoyl-(palmitoyloleoyl)PS (20,22).

Calcium- and magnesium-induced vesicle aggregation. Aggregation of NSPS-liposomes was studied in the presence of the divalent cations calcium and magnesium (Fig. 2). In the simplest interpretation, the initial rate of change in the optical density (increments of absorbance 20s after cation addition) of the liposome solutions measures the rate of aggregation of two vesicles. The sigmoidal plots, obtained when the turbidity changes of NSPS-liposomes 20s after cation addition were plotted vs. increasing concentrations of the two divalent ions, show clear differences in the ability of these cations to induce aggregation of NSPS-liposomes. For concentrations below 6 mM, calcium was found to be more effective than magnesium in inducing NSPS-liposomes' aggregation, but above 6 mM magnesium was more effective. The effectiveness of both cations is higher for PS-liposomes (threshold concentration 1.5 mM for calcium and 4.6 mM for magnesium) than for NSPS-liposomes (threshold concentration 2.8 mM for calcium and 6.6 mM for magnesium), suggesting that *N*-acylation of PS protects the membrane against aggregation during any degenerative process.

The calcium-threshold concentrations for aggregation of PS (1.5 mM) and NSPS (2.8 mM) are of physiological relevance since the cytosolic level of calcium in unexcited cells (typically 0.1–0.2 μM) is several orders of magnitude lower than the concentration in the extracellular medium or in the sarcoplasmic reticulum. However, this level can be raised

TABLE 3
Percentage of the Total Fatty Acids Content

Sample	Percentage of the total fatty acids[a]				
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	Others
PC	35.8 ± 0.8	12.5 ± 0.9	30.2 ± 0.2	19.1 ± 1.0	2.4 ± 0.2
PS	36.1 ± 1.1	12.1 ± 0.2	30.5 ± 0.6	18.6 ± 1.5	2.7 ± 0.2
NSPS	24.5 ± 0.1	41.2 ± 1.2	19.8 ± 2.2	12.6 ± 0.6	1.9 ± 0.1

[a]Fatty acid contents were determined by gas–liquid chromatography (GLC) of the fatty acid methyl esters produced *via* transmethylation. Data represent the mean ± SD of at least three determinations. GLC was performed on a Perkin-Elmer 990 gas chromatograph equipped with flame-ionization detector. Data were obtained with a 2 m × 2 mm i.d. column packed with 15% polymeric ethylene glycol succinate as stationary phase on Chromosorb W (100-120 mesh). Helium at 30 mL/min was used as carrier gas. Injector, column, and detector temperatures were, respectively, 230, 188, and 250°C. Samples were applied as hexane solutions. For abbreviations see Table 1.

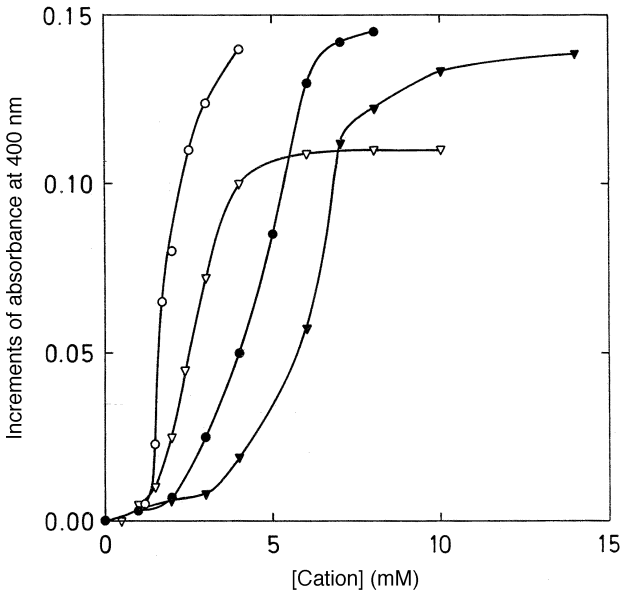


FIG. 2. Influence of calcium and magnesium concentrations on the aggregation state of NSPS-liposomes. Turbidity changes were plotted vs. $[Ca^{2+}]$ (○,▽) and $[Mg^{2+}]$ (●,▼). The phospholipid vesicle concentration was 0.05 mg/mL of 20 mM Tris-HCl buffer (pH 7.4), containing 100 mM NaCl and 0.1 mM EDTA. Measurements were performed at 20°C. (○,●) PS control, and (▽,▼) NSPS. For abbreviations see Figure 1.

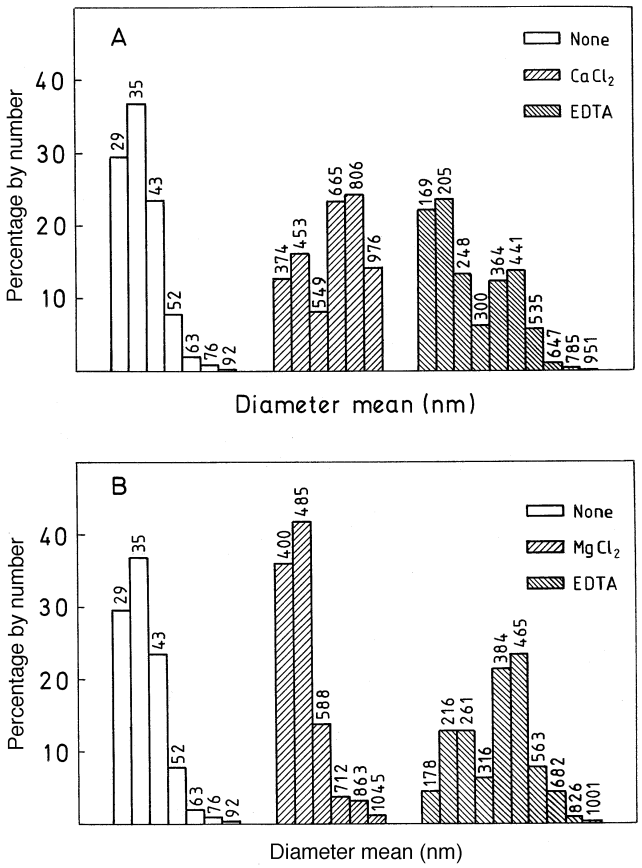


FIG. 3. Effect of calcium and magnesium on NSPS-liposome size. The state of aggregation of NSPS liposomes was monitored by photon correlation spectroscopy in the presence of 3 mM calcium (A) and 6 mM magnesium (B). Vesicle diameter was measured before and after addition of divalent cation. The reversibility of the aggregation reaction was followed by vesicle size analysis after EDTA addition, at six times the concentration of divalent ions, to the aggregated vesicles. For abbreviation see Figure 1.

abruptly by transiently opening calcium channels in the plasma membrane or an intracellular membrane, following ischemia under conditions of degenerative change involving disintegration and/or digestion of membranes. In this way, *N*-acylation of PS would delay aggregation and fusion processes due to cytosolic calcium increase.

Particle size analysis and aggregation irreversibility. The state of aggregation of unilamellar NSPS-liposomes induced by the divalent cations calcium and magnesium was also monitored by photon correlation spectroscopy. The results are summarized in Figure 3. The initial size of NSPS-liposomes prepared by sonication was in the range of 29–43 nm, but the particle size increases in the presence of both cations. After addition of EDTA to the aggregated vesicles only partial reversibility of NSPS-liposomes' aggregation occurred, since the liposomes did not return to the initial size, indicating that divalent ions induce the formation of irreversible aggregates. The irreversibility of the aggregation reactions after adding EDTA suggests that vesicle fusion might occur in the presence of either cation. Systematic aggregation and fusion studies using different NAPS liposomes are now in progress.

Calcium-induced NSPS-liposome fusion. Preliminary studies, based on mixing analysis for both lipid and internal aqueous contents (Fig. 4), show that fusion rather than aggregation of NSPS-liposomes occurs in the presence of calcium ions. Figure 4A shows the percentage of fusion of NSPS-liposomes following lipid mixing after the addition of various Ca^{2+} concentrations (1, 2, 3, and 4 mM). The increase in lipid mixing depended on calcium concentration in the medium. Mixing was inappreciable when 1 mM calcium was used, whereas the extent of lipid mixing was of 60, 75, and 90% 2 min after the addition of 2, 3, and 4 mM of calcium, respectively.

The results obtained when the mixing of the internal aqueous content was monitored are shown in Figure 4B. Aqueous mixing was also a function of calcium concentration, but at the same calcium concentration the extent of fusion measured following mixing of aqueous contents was always lower than the extent of fusion measured by lipid mixing; the mixing of aqueous contents was less than 10% with 2 mM calcium.

The increase in fusion with increasing calcium concentration in the medium correlates well with the calcium-induced aggregation shown by turbidimetric assays. The lower mixing for the internal aqueous content can be explained in two ways. First, in lipid mixing measurements there may be a contribution of fusion and semifusion processes. On the other hand, the lower internal aqueous contents mixing can be explained by a loss of fluorescence due to the leakage of encapsulated Tb-DPA complex into the EDTA-containing medium.

To confirm the fusion of NSPS-liposomes, transmission electron micrographs were obtained before and after the correlative incubation of control liposomes with 3 mM calcium and 16 mM EDTA. The increase in the size of the vesicles incubated with the divalent cation (Fig. 5) again demonstrates the calcium-induced fusion of NSPS-liposomes.

DISCUSSION

Membrane fusion is a key event in the functioning of a living organism. Thus, membrane fusion occurs in a very specific and highly controlled fashion, and specific modulators determine when and where membrane fusion occurs. Membrane fusion is an extremely complex process, and many primary and secondary factors are involved in its induction and regulation. A large number of different fusogenic lipids have been proposed to be specifically involved in membrane fusion. The presence of significant amounts of type II nonbilayer-preferring lipids in any biomembrane, and the fact that their phase preference can be triggered and membrane fusion induced by biological fusion factors, strongly suggest a central role for type II nonbilayer-preferring lipids in biomembrane fusion (44).

NAPS have been isolated from normal tissues (8,15,16); but it is not possible to rule out their formation postmortem, like NAPE (14). Although we have shown the participation of the parent compounds NAPE in membrane processes, like permeability and fusion (29,31), there are no published re-

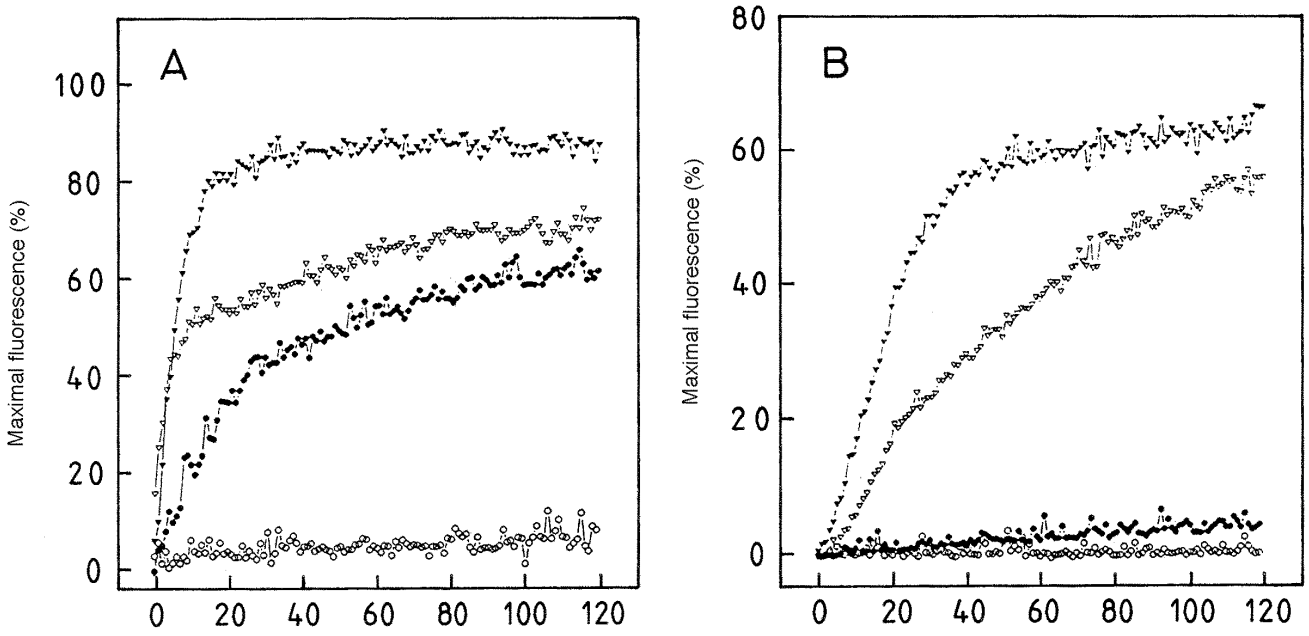


FIG. 4. Ca^{2+}-induced fusion of NSPS small unilamellar vesicles. (A) Lipid mixing; (B) internal aqueous contents mixing. Ca^{2+} [1 mM (○), 2 mM (●), 3 mM (▽), and 4 mM (▼)] was added at $t = 0$ to a vesicle suspension of 0.05 mg lipid/mL. Fluorescence measures were done at 20°C. The percentage of maximal fluorescence at 536 nm (A) and at 545 nm (B) was calculated as described in the Materials and Methods section. For abbreviation see Figure 1.

sults on the participation of NAPS in such processes. Nevertheless, the group of Casal (45) has shown differences in the phase behavior between the parent compound *N*-methylated phosphatidylserine and PS, and they have indicated that *N*-methyl-phosphatidylserine is also unusual in its interactions with metal ions, but results on this latter aspect have not been published. We have synthesized NSPS to examine divalent cation-induced aggregation of NSPS-liposomes as a previous step for membrane fusion. The conversion of egg transphosphatidylated PS to NSPS proceeded with 82% yield and, after purification by preparative TLC on silica gel H plates using two solvent systems, NSPS was obtained in 39% yield.

The purified NSPS was characterized by its chromatographic and spectroscopic (IR and ^1H NMR) properties and by chemical degradation. Although the IR spectrum of NSPS was similar to that of PS, the strong absorption bands at 1660 cm^{-1} (C=O stretch of the secondary amide), 1530 cm^{-1} (NH deformation), and 3280 cm^{-1} (NH stretch) were indicative of the presence of an amide bond in the molecule. Besides this, the NSPS IR spectrum was similar to that found in the literature for the NAPS isolated from sheep erythrocytes (15) and bovine brain (16), or prepared by chemical acylation of palmitoyl-oleoyl-PS (20,22). In addition, chemical shift data obtained from the ^1H NMR spectrum of NSPS are consistent with those found in the literature for *N*-oleoyl(palmitoyloleoyl)PS (20,22). On the other hand, the increase in stearic acid for NSPS with respect to egg transphosphatidylated PS, found by FAME analysis, agrees with the incorporation of

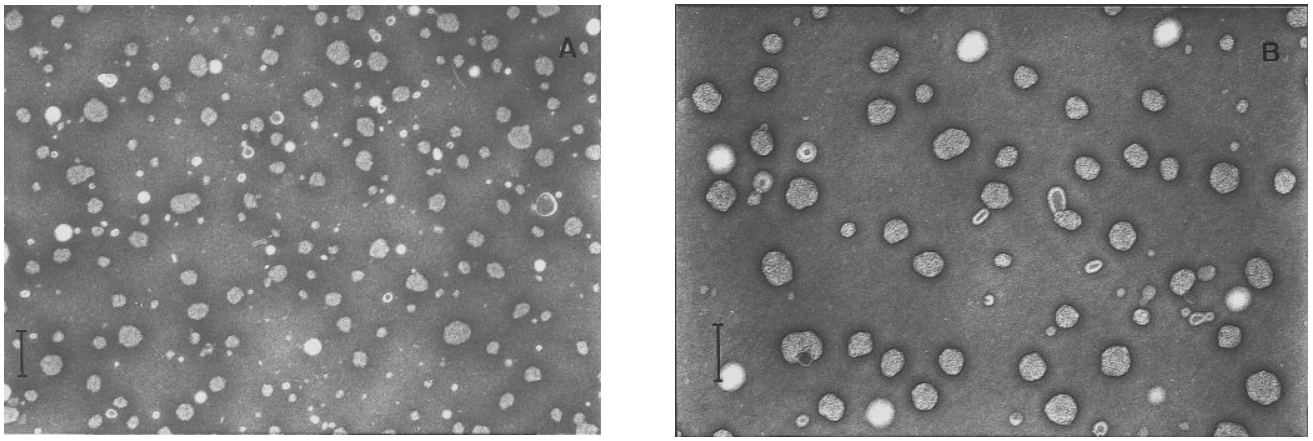


FIG. 5. Transmision electron micrographs of NSPS vesicles before (A) and after (B) their incubation with calcium (3 mM) for 2 min and then with EDTA (16 mM) for 1 h. Bar: 100 nm (A) and 400 nm (B).

this fatty acid in the third chain by the acylation process. These results indicate that the synthesized molecules are indeed NSPS.

The aggregation of NSPS-liposomes was studied in the presence of calcium and magnesium. Clear differences were found in the effectiveness of these cations in inducing aggregation of NSPS-liposomes. The threshold concentrations of these divalent ions were 2.8 mM for Ca^{2+} and 6.6 mM for Mg^{2+}; thus Mg^{2+} is much less potent than Ca^{2+} in inducing aggregation of NSPS-liposomes. The analysis of NSPS-liposomes by photon correlation spectroscopy shows a size increase in the presence of divalent cations calcium and magnesium, which agrees with the aggregation process observed turbidimetrically. The fact that the liposomes do not return to their initial size, after addition of EDTA to the aggregated vesicles, indicates that the divalent cations induce the formation of irreversible aggregates and suggests that vesicle fusion rather than mere aggregation might occur. Vesicle fusion was demonstrated by transmission electron microscopy and by the experiments showing both lipid and aqueous contents mixing when calcium cations were added to NSPS-liposomes. Nevertheless, the comparison of the results of photon correlation spectroscopy and those of lipid and internal aqueous contents mixing shows that not all aggregates led to fused vesicles. The addition of EDTA to the aggregated vesicles removes the higher aggregates, but all the remaining ones are still larger than the initial liposomes, indicating that the size of liposomes does not return to the initial value. On the other hand, the extension of fusion 2 min after calcium 3 mM addition is lower than 60%.

The calcium threshold concentration obtained by us for the aggregation of egg transphosphatidylated PS-liposomes is similar to that reported for aggregation (46) and fusion (47) in 0.1 M NaCl of unilamellar bovine brain PS vesicles. This indicates that the PS headgroup is responsible for aggregation and fusion and, thus, our PS constitutes a good tool to study the physiological consequences of PS acylation. The threshold concentrations of calcium and magnesium ions for aggregation of PS-liposomes were 1.5 mM and 4.6 mM, respectively, indicating that Ca^{2+} binds to PS more strongly than Mg^{2+}. This difference in the threshold concentrations correlates well with the results reported for the interaction of calcium and magnesium with bovine brain PS (48,49).

The effectiveness of calcium and magnesium cations in inducing vesicle aggregation and fusion is higher for PS-liposomes than for NSPS-liposomes. Since we have studied the effect of calcium and magnesium on the aggregation and fusion properties of only one type of NAPS, it may be too early to speculate on the effect of such acidic phospholipids on membrane processes. However, it is likely that *N*-acylation of PS provides a double protective effect against degerative damage of membranes through aggregation and fusion mechanisms, since *N*-acylation decreases the sensitivity of PS to calcium and magnesium ions and removes free fatty acids, which have fusogenic properties. This result is consistent with the postulated protective role for N-acyl derivatives of PE (2,29,30), since, like *N*-acyl deriv atives of PE, NSPS helps to stabilize a bilayer conformation under conditions in which fusion may be favored. Moreover, this postulated protective effect is consistent with the defense mechanism postulated by Hansen *et al*. (24,25) for the synthesis of NAPE related to the neuronal death induced by the exposure of cultured cerebral cortical neurons to glutamate.

ACKNOWLEDGMENT

This work was supported by grant no. PB94-0911-A from D.G.I.C.Y.T. (Spain).

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[Received July 24, 1997; and in final revised form March 30, 1998; revision accepted April 21, 1998]

Long-Chain Alkenes of the Haptophytes *Isochrysis galbana* and *Emiliania huxleyi*

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ABSTRACT: The major alkenes of the haptophytes *Isochrysis galbana* (strain CCAP 927/14) and *Emiliania huxleyi* (strains CCAP 920/2 and VAN 556) have been identified by nuclear magnetic resonance spectroscopy and by mass spectrometric analysis of their dimethyl disulfide adducts. The dominant alkene in *I. galbana* is (22*Z*)-1,22-hentriacontadiene, with 1,24-hentriacontadiene and 1,24-tritriacontadiene present in much lower abundance; (22*Z*)-1,22-hentriacontadiene also occurs in *E. huxleyi* (strain CCAP 920/2), together with (2*Z*,22*Z*)-2,22-hentriacontadiene (the major hydrocarbon) and (3*Z*,22*Z*)-3,22-hentriacontadiene. Minor abundances of 2,24-hentriacontadiene and 2,24-tritriacontadiene are also present in this strain. In contrast, the dominant alkene in *E. huxleyi* (strain VAN 556) is (15*E*,22*E*)-1,16,23-heptatriacontatriene with the related alkatriene 1,15,22-octatriacontatriene also present and (22*Z*)-1,22-hentriacontadiene occurring as a minor component. From structural relationships (15*E*,22*E*)-1,15,22-heptatriacontatriene is proposed to derive from the same biosynthetic pathway as that of the characteristic C_{37} alkenones which occur in both *E. huxleyi* and *I. galbana*. The C_{31} and C_{33} dienes likely derive from chain extension and decarboxylation of (*Z*)-9-octadecenoic acid or (*Z*)-7-hexadecenoic acid, using a pathway analogous to that elucidated previously in the chlorophyte *Botryococcus braunii*. Therefore, long-chain dienes and trienes, which can co-occur in haptophytes, may have distinct biosynthetic pathways.
Lipids 33, 617–625 (1998).

The Haptophyta (= Prymnesiophyta) are a distinct phylum of photosynthetic eucaryotes found throughout the oceans and in certain fresh waters (1). They constitute a high proportion of marine biomass, certain species forming enormous blooms observable from space (1). The characteristic C_{37} alkenones observed in certain species, such as *Emiliania huxleyi*, are typically amongst the most abundant lipids of Quaternary marine sediments (2), and the relative abundances of the components with different degrees of unsaturation are used as a paleo-sea surface temperature proxy (U^{k}_{37} and $U^{k'}_{37}$) (3,4). Haptophytes can also contain significant abundances of C_{31} and C_{33} alkadienes in addition to C_{37} and C_{38} alkatrienes (5–7). However, the diene components have not been reported in those marine sediments where alkenones occur in high abundance and the C_{37} and C_{38} hydrocarbons may or may not be present (2). Indeed, in a study of the Black Sea, C_{31} dienes were abundant in surface particulates but disappeared with depth and were absent from the bottom sediment. In the same sediments the alkenones were in high abundance and a C_{37} and a C_{38} triene were still present (8).

Although the positions of the double bonds in haptophyte alkenones are known and their stereochemistry has been established as *trans* (9), the long-chain alkenes have not been characterized. Therefore, as part of a wider investigation into the reasons for the differing labilities of long-chain lipids to biodegradation, we have fully elucidated the structures of the major haptophyte alkenes.

EXPERIMENTAL PROCEDURES

Culture conditions. Isochrysis galbana (CCAP 927/14) was cultured in artificial seawater medium (F/2). *Emiliania huxleyi* strains CCAP 920/2 and VAN 556 were cultured in filtered (0.6 μm) pasteurized seawater. All cultures were kept under continuous aeration and light and were harvested at stationary phase after confirmation that the cultures were axenic.

Hydrocarbon isolation. Freeze-dried cells (5–10 g) were extracted using ultrasonication in 2:1 dichloromethane/methanol, the solvent being removed by rotary evaporation. The hydrocarbon fraction was isolated from the extract using silica gel column chromatography (~5 g silica) and eluting with hexane (5 column volumes). The fraction was purified further by silica gel thin-layer chromatography (TLC; R_f = 0.95–1.0), developing with hexane.

Gas chromatography (GC). GC was carried out using a Carlo Erba 5300 instrument equipped with a polysiloxane

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Abbreviations: DMDS, dimethyl disulfide; FID, flame-ionization detection; GC, gas chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

capillary GC column (CP Sil 5CB, 50 m, 0.12 µm film, 0.32 mm i.d.; Chrompack, The Netherlands), using hydrogen as carrier gas. The temperature program was 40 (isothermal 2 min)–250°C at 12°C min^{-1}, 250–300°C at 4°C min^{-1} (isothermal 30 min). Dimethyl disulfide adducts were analyzed under similar conditions, utilizing an HT-5 GC capillary column (25 m, 0.1 µm film, 0.32 mm i.d.; SGE, Victoria, Australia); the temperature program was 40 (isothermal 2 min)–250°C at 12° min^{-1}, 250–370°C at 4° min^{-1} (isothermal 30 min). Quantitation of the alkenes was undertaken using flame-ionization detection with *n*-tricosane (99%; Sigma, Dorset, United Kingdom) as internal standard.

GC–mass spectrometry (GC–MS). GC–MS of the hydrocarbon fractions was undertaken in electron impact (EI) mode (70 eV, scan time 1 s; *m/z* 50 to 750) using a Finnigan MAT 4500 instrument and helium as carrier gas. GC conditions were as above.

Nuclear magnetic resonance (NMR) spectroscopy. NMR analyses were carried out on a Jeol GX400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C. Samples were analyzed in deuterochloroform and the resulting free induction decays were processed with the Jeol processing program SpecNMR using a Gaussian window function (GF = 0.5, BF = –0.2 for ^1H and GF = 2.0, BF = –0.3 for ^{13}C). In some cases the free induction decay was zero-filled four times to 131,072 data points and a Gaussian window applied for resolution enhancement (GF = 1.0, BF = –1.5). Spectra were referenced to the solvent (7.26 ppm for ^1H and 77.0 ppm for ^{13}C).

Formation of dimethyl disulfide (DMDS) adducts. Aliquots of total hydrocarbon fractions were adducted following the method of Carlson *et al.* (10). In brief, samples dissolved in hexane (0.2 mL) were added to DMDS (0.2 mL) and a solution of iodine in diethyl ether (6 mg in 0.1 mL). After 12 h at 40°C, sodium thiosulfate (5% aqueous; 1 mL) was added and the adducts extracted into hexane.

Catalytic hydrogenation. An aliquot of the *I. galbana* (CCAP 927/14) hydrocarbon fraction dissolved in hexane (5 mL) was hydrogenated over PtO_2 (1 mg), 2 h, at 20°C. After filtration the product was analyzed by GC and GC–MS. An aliquot was coinjected with a standard C_5 to C_{40} *n*-alkane mix (CP-6A; PolyScience, Evanston, IL).

Diimide reduction. To the hydrocarbon fraction of *E. huxleyi* (VAN 556) in diethyl ether (1 mL) was added a methanol solution of copper sulfate (0.04 mg in 0.4 mL) followed by hydrazine hydrate (0.05 mL). The reaction mixture was left open to the air and stirred at room temperature (1 h) prior to being quenched by addition of water (5 mL). The hydrocarbon products were recovered by extraction with diethyl ether (2 × 5 mL). The combined extracts were dried ($MgSO_4$) and the solvent removed by rotary evaporation. The product was purified by flash chromatography using hexane as eluant, analyzed by GC–MS, and then adducted with DMDS before analysis by GC–MS.

Standard compounds. Authentic alkene standards for NMR comparisons were obtained from either the Aldrich Chemical Company (Dorset, UK) [(*E*)-2-heptene, (*E*)-3-heptene, (*Z*)-3-heptene, (*E*)-7-tetradecene, (*Z*)-9-heneicosene, 1-heptadecene] or Lancaster Synthesis Ltd. (Lancashire, UK) [(*Z*)-2-heptene].

RESULTS AND DISCUSSION

Isochrysis galbana *(CCAP 927/14).* The hydrocarbons of *I. galbana* (CCAP 927/14) are dominated by a single component (Table 1; Fig. 1), assigned by GC–MS as a C_{31} diene (M^+ at *m/z* 432; 10% of base peak intensity). Three minor components, two C_{31} dienes and a C_{33} diene, are also present (Table 1).

The ^1H NMR spectrum of the major diene displays an intense signal at 1.25 ppm as a result of aliphatic CH_2 groups and a terminal alkyl methyl group at 0.88 ppm. Three characteristic mutually coupling vinylic resonances are observed at 5.81 ppm (1-H, *ddt*, *J* = 10.3, 17.1, and 6.6 Hz), 4.99 ppm (1-H, *ddt,* *J* = 17.1, 2.2, 1.7 Hz), and 4.92 ppm (1-H, *ddt,* *J* = 10.3, 2.2, 1.2 Hz) representing a terminal double bond, which are in accord with those recorded for the terminal double bond in 1-heptadecene (Table 2). Another set of vinylic resonances integrating for two hydrogens is observed at 5.35 ppm. The highly second-order nature of this multiplet is characteristic of a disubstituted double bond flanked by similar hydrocarbon groups representing an AA′XX′X″X‴ system. Comparison of the appearance of the chemical shift with that observed in (*Z*)-9-heneicosene and (*E*)-7-tetradecene (Fig. 2) clearly indicates that the double bond has *cis* stereochemistry (Table 3) and confirms a previous report indicating that the vinylic hydrogens in such molecules are deshielded in the *cis* relative to the *trans* isomer (11). Spectral analysis of the vinylic hydrogens in the highly second-order AA′XX′X″X‴system possessing either a *cis* or a *trans* double bond was simulated using the computer program Laocoön (12). In the case of the *cis* isomer, the splitting pattern was reproduced using $^3J_{A,A'}$ = 11.0 Hz, $^3J_{A,XX'}$ and $^3J_{A',X''X'''}$ = 6.1 Hz, $^4J_{A,X''X'''}$ and $^4J_{A',XX'}$ = –1.2 Hz and $^2J_{X,X'}$ and $^2J_{X'',X'''}$ = 12.0 Hz (sign not determined). In the case of the *trans* iso-

TABLE 1
Long-Chain Alkene Abundances in Haptophyte Algae (mg/g dry weight alga)

	31:2$\Delta^{1,22}$	31:2$\Delta^{2,22}$	31:2$\Delta^{3,22}$	31:2$\Delta^{1,24}$	31:2$\Delta^{2,24}$	33:2$\Delta^{1,24}$	33:2$\Delta^{2,24}$	37:3$\Delta^{1,15,22}$	38:3$\Delta^{1,16,23}$
Isochrysis galbana (CCAP 927/14)	1.90	trace[a]		0.10		0.02			
Emiliania huxleyi (CCAP 920/2)	0.71	2.2	0.79		0.07		0.06		
E. huxleyi (VAN 556)	0.10							4.40	0.60

[a]Trace component (<0.01 mg/g dry weight alga).

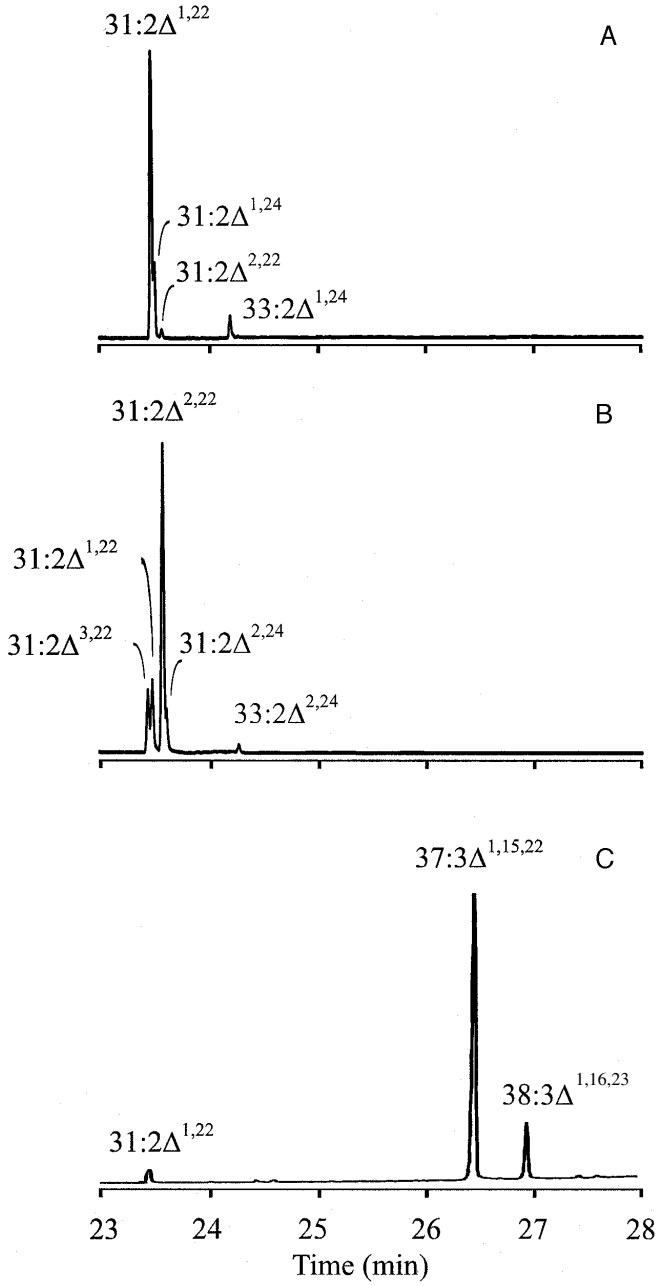


FIG. 1. Gas chromatograms of hydrocarbon fractions isolated from (A) *Isochrysis galbana* (CCAP 927/14), (B) *Emiliania huxleyi* (CCAP 920/2), (C) *E. huxleyi* (VAN 556).

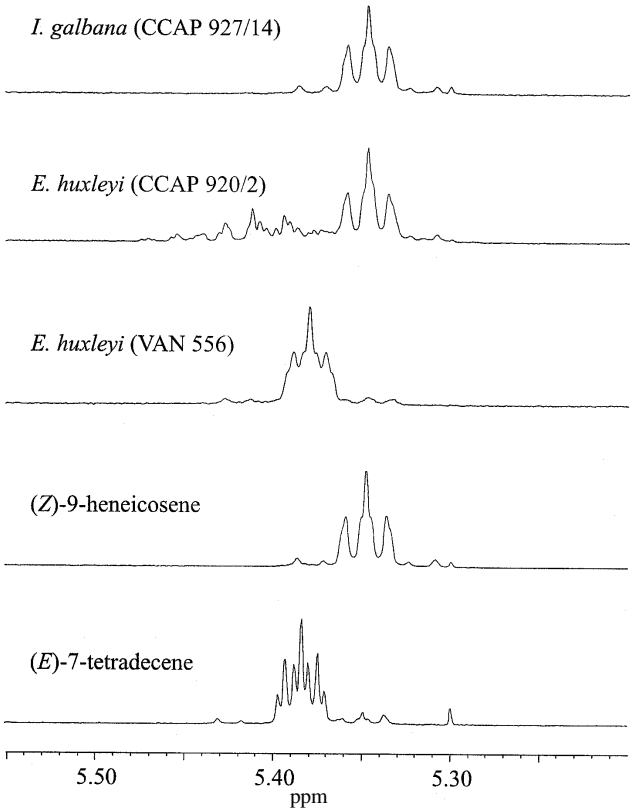


FIG. 2. Partial ^{1}H nuclear magnetic resonance (NMR) spectra comparing the mid-chain vinylic resonances in hydrocarbons from *Isochrysis galbana* and *Emiliania huxleyi* and in two alkene standards.

mer, the splitting pattern was reproduced using similar values to those for the *cis* isomer but increasing $^{3}J_{A,A'}$ to 16 Hz and $^{4}J_{A,X''X'''}$ and $^{4}J_{A',XX'}$ to −1.4 Hz. Comparison of the allylic hydrogen resonances in 1-heptadecene, (*Z*)-9-heneicosene, and (*E*)-7-tetradecene (Fig. 3) reveals differences in splitting patterns and in chemical shifts. Of particular note is a deshielding effect of the double bond in the order *trans* < *cis* < terminal (Table 4). The allylic hydrogen resonances in the *I. galbana* diene clearly comprise a composite arising from allylics next to a terminal double bond and a *cis*-substituted double bond in the middle of a long hydrocarbon chain (Table 4; Fig. 3).

Comparison of the sp^2 hybridized resonances in the ^{13}C NMR spectra of the diene with those in (*Z*)-9-heneicosene, (*E*)-7-tetradecene, and 1-heptadecene clearly confirms the presence of the terminal double bond (resonances at 114.1 and 139.3 ppm) and the *cis*-substituted double bond (resonance at 129.9 ppm) (Table 5). As observed for the midchain vinylic hydrogens in the ^{1}H NMR spectra, a *cis*-substituted double bond in such molecules also has a small but significant deshielding effect on the ^{13}C chemical shift of the mid-chain vinylic carbons.

The presence of the terminal double bond is confirmed and the position of the *cis* double bond identified as at C-22 from the mass spectrum of the DMDS adduct (Table 6, Fig. 4A, and Scheme 1, the fragmentation scheme for DMDS adducts of haptophyte alkadienes) with diagnostic ions at *m/z* 620 (M^+) and also at *m/z* 61 (A^+), 173 (D^+), 399 [$(C - 48)^+$; i.e., $C - CH_3SH$] and 465 [$(B - 94)^+$; i.e., B-DMDS] (*c.f.* reference 10) giving the structure of the major component as (22*Z*)-1,22-hentriacontadiene (Scheme 2A). The double bonds of the minor C_{31} component (Table 1) are at C-1 and C-24, characteristic ions being *m/z* 620 (M^+), 61 (A^+), 145 (D^+), 465 [$(B - 94)^+$] and 427 [$(C - 48)^+$] (Table 6). Likewise, the double bond positions in the minor C_{33} diene (Table 1) are also at C-1 and C-24, characteristic ions being *m/z* 648 (M^+), 61 (A^+), 173 (D^+), 493 [$(B - 94)^+$] and 427 [$(C - 48)^+$]

TABLE 2
Comparison of Δ1 and Δ2 Vinylic ^{1}H Nuclear Magnetic Resonances

	Chemical shift, δ (ppm), multiplicity	Coupling constants *J* (Hz)
Compound(22*Z*)-1,22-Hentriacontadiene	4.99, *ddt*, 1-H$_{trans}$ 4.92, *ddt*, 1-H$_{cis}$ 5.81, *ddt*, 2-H	$^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 17.1, $^4J_{1,3}$ = 1.7 $^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 10.3, $^4J_{1,3}$ = 1.2 $^3J_{trans}$ = 17.1, $^3J_{cis}$ = 10.3, $^3J_{2,3}$ = 6.6
(2*Z*,22*Z*)-2,22-Hentriacontadiene	5.43, *m*, 2-H[a] 5.38, *m*, 3-H[a]	$^3J_{2,3}$ = 10.8, $^4J_{2,4}$ = –1.6, $^3J_{1,2}$ = 6.1 $^3J_{2,3}$ = 10.8, $^3J_{3,4}$ = 6.8, $^4J_{1,3}$ = –1.4
(15*E*,22*E*)-1,15,22-Heptatriacontatriene	4.99, *ddt*, 1-H$_{trans}$ 4.92, *ddt*, 1-H$_{cis}$ 5.81, *ddt*, 2-H	$^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 17.1, $^4J_{1,3}$ = 1.7 $^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 10.2, $^4J_{1,3}$ = 1.2 $^3J_{trans}$ = 17.1, $^3J_{cis}$ = 10.2, $^3J_{2,3}$ = 6.6
1-Heptadecene	4.99, 1-H$_{trans}$ 4.92, 1-H$_{cis}$ 5.81, 2-H	$^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 17.1, $^4J_{1,3}$ = 1.7 $^2J_{gem}$ = 2.2, $^3J_{1,2}$ = 10.2, $^4J_{1,3}$ = 1.2 $^3J_{trans\,=\,17.1,}$ $^3J_{cis}$ = 10.2, $^3J_{2,3}$ = 6.6
(*Z*)-2-Heptene	5.43, *m*, 2-H[a] 5.38, *m*, 3-H[a]	$^3J_{2,3}$ = 10.8, $^4J_{2,4}$ = –1.6, $^3J_{1,2}$ = 6.1 $^3J_{2,3}$ = 10.8, $^3J_{3,4}$ = 6.8, $^4J_{1,3}$ = –1.4
(*E*)-2-Heptene	5.40, *m*, 2-H[a] 5.42, *m*, 3-H[a]	$^3J_{2,3}$ = 15.1, $^4J_{2,4}$ = –0.8, $^3J_{1,2}$ = 6.6 $^3J_{2,3}$ = 15.1, $^3J_{3,4}$ = 6.6, $^4J_{1,3}$ = –2.5

[a]Relative signs of coupling constants given.

(Table 6). A mass spectrum of the DMDS adduct of the smallest C_{31} diene was not recorded.

The straight-chain nature of the C_{31} dienes was confirmed by hydrogenating an aliquot of the *I. galbana* hydrocarbon fraction. GC–MS analysis of the product reveals two products, the major product being *n*-hentriacontane and the minor product, *n*-tritriacontane, as confirmed by coinjection with an *n*-alkane standard mixture.

Emiliania huxleyi *(CCAP 920/2).* The hydrocarbon composition of *E. huxleyi* (CCAP 920/2) comprises four C_{31} dienes and a single C_{33} diene (Table 1; Fig. 1).

Initial ^{1}H NMR analysis of the hydrocarbon fraction indicates aliphatic moieties with an intense resonance at 1.25 ppm (CH_2 groups) and a terminal alkyl methyl at 0.88 ppm along with the presence of a vinylic methyl group at 1.60 ppm in the major isomer and hence a C-2 double bond. This is accompanied by the characteristic second-order multiplet for the two vinylic hydrogens of a *cis*-substituted double bond (5.35 ppm) in the middle of the hydrocarbon chain as observed in the major *I. galbana* diene. Partially overlapping this multiplet is an extremely complicated series of resonances arising from the hydrogens attached to the C-2 double bond. Comparison of the chemical shifts and splitting patterns of these hydrogens and the vinylic methyl group with those in (*Z*)-2-heptene and (*E*)-2-heptene indicates that the C-2 double bond is also *cis* (Table 2). Thus, the terminal vinylic methyl group appears at 1.60 ppm in the diene, which agrees with that observed in (*Z*)-2-heptene but is significantly different from that of 1.63 ppm observed in (*E*)-2-heptene. Furthermore, the splitting pattern observed for the vinylic methyl is

TABLE 3
Comparison of Mid-chain Vinylic ^{1}H Nuclear Magnetic Resonances Including Relative Signs of Coupling Constants

Compound	Chemical shift, δ (ppm), multiplicity, *J* (Hz)
(22*Z*)-1,22-Hentriacontadiene	5.35, *m*, $^3J_{22,23}$ = 11.0, $^3J_{21,22}$ = $^3J_{23,24}$ = 6.1 $^4J_{21,23}$ = $^4J_{22,24}$ = –1.2, 22-H and 23-H
(2*Z*,22*Z*)-2,22-Hentriacontadiene	5.35, *m*, $^3J_{22,23}$ = 11.0, $^3J_{21,22}$ = $^3J_{23,24}$ = 6.1 $^4J_{21,23}$ = $^4J_{22,24}$ = –1.2, 22-H and 23-H
(15*E*,22*E*)-1,15,22-Heptatriacontatriene	5.38, *m*, $^3J_{15,16}$ = $^3J_{22,23}$ = 16.0, $^3J_{14,15}$ = $^3J_{16,17}$ = $^3J_{21,22}$ = $^3J_{23,24}$ = 6.1, $^4J_{14,16}$ = $^4J_{15,17}$ = $^4J_{21,23}$ = $^4J_{22,24}$ = –1.4, 15-H, 16-H, 22-H and 23-H
(*Z*)-9-Heneicosene	5.35, *m*, $^3J_{9,10}$ = 11.0, $^3J_{8,9}$ = $^3J_{10,11}$ = 6.1, $^4J_{8,10}$ = $^4J_{9,11}$ = –1.2, 9-H and 10-H
(*E*)-7-Tetradecene	5.38, *m*, $^3J_{7,8}$ = 16.0, $^3J_{6,7}$ = $^3J_{8,9}$ = 6.1, $^4J_{6,8}$ = $^4J_{7,9}$ = –1.4, 7-H and 8-H

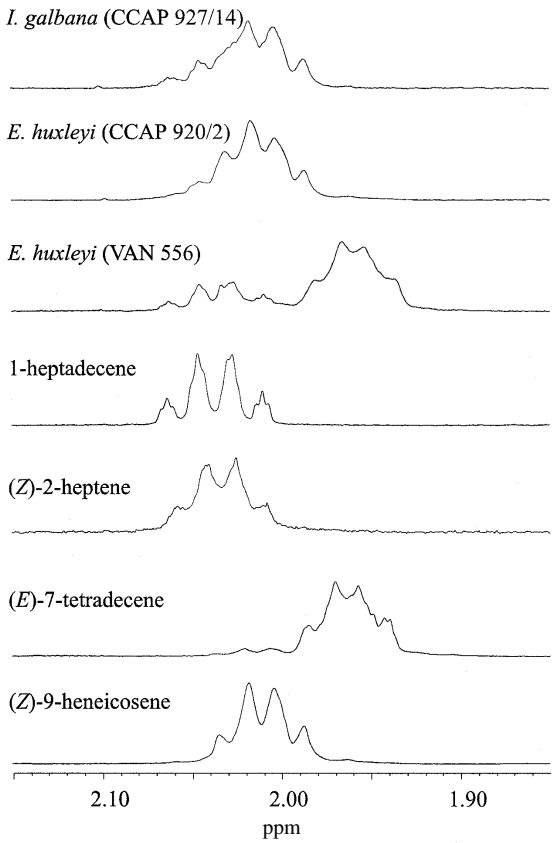


FIG. 3. Partial ^{1}H NMR spectra comparing the allylic resonances (excluding methyl groups) in haptophyte hydrocarbons and in four alkene standards. For abbreviations see Figure 2.

almost identical to that in (*Z*)-2-heptene but is completely different from that in (*E*)-2-heptene (Table 4). To interpret the complicated splitting patterns associated with the hydrogens on the C-2 double bond, we carried out decoupling experiments on both the diene and (*Z*)-2-heptene (Fig. 5). Irradiation of the vinylic methyl collapses 2-H basically into a doublet (*J ca.* 11 Hz) and 3-H into a doublet of triplets (*J ca.* 11 and 7 Hz), although for the diene the doublet of triplets is partially obscured by the midchain vinylics. In both cases a strong second-order roofing effect is observed. Similarly, irradiation of the allylic hydrogens collapses 2-H into a doublet of quartets (*J ca.* 11 and 7 Hz), and 3-H into a doublet of quartets (*J ca.* 11 and 1 Hz), both sets of multiplets again showing a strong second-order roofing effect. Simulation of the appearance of the vinylic hydrogens and vinylic methyl using the program Laocoön (12) not only confirmed the preceding interpretations but also allowed a more realistic estimation of the allylic coupling constants. Furthermore, the simulation revealed a long-range five-bond coupling from 1-H_3 to 4-H_2 (Tables 2 and 4). The appearance of the allylic hydrogens is in agreement with a composite of signals derived from the allylics next to the midchain double bond, as in (*Z*)-9-heneicosene, and those next to the C-2 double bond, as in (*Z*)-2-heptene, albeit with a slight shielding effect (Fig. 3).

In addition to those resonances associated with the major isomer, a set of multiplets associated with one of the minor components and characteristic of a terminal double bond as observed in *I. galbana*, is also clearly present.

The ^{1}H NMR data (Tables 2 and 3) indicate therefore that the major *E. huxleyi* (CCAP 920/2) diene contains two *cis*-substituted double bonds, one at C-2 and the other somewhere in the middle of the chain. One of the minor dienes possesses a terminal double bond.

These conclusions are supported by ^{13}C NMR studies. The C-2 and C-3 chemical shifts (123.6 and 130.9 ppm) in the major diene were compared with those recorded for (*Z*)-2-heptene and (*E*)-2-heptene (Table 5). Excellent agreement was obtained between the diene and (*Z*)-2-heptene, with small but significant differences in chemical shift being observed for (*E*)-2-heptene. Weaker signals attributable to a terminal double bond in one of the minor dienes are clearly observable at 114.1 and 139.3 ppm.

TABLE 4
Comparison of Allylic ^{1}H Nuclear Magnetic Resonances Including Relative Signs of Coupling Constants

Compound	Chemical shift, δ (ppm), multiplicity, *J* (Hz)
(22*Z*)-1,22-Hentriacontadiene	2.03, *m*, 3-H_2 2.01, *m*, 21-H_2 and 24-H_2
(2*Z*,22*Z*)-2,22-Hentriacontadiene	1.60, *m*, $^3J_{1,2}$ = 6.1, $^4J_{1,3}$ = –1.4, $^5J_{1,4}$ = ±1.0, 1-H_3 2.02, *m*, 3-H_2, 21-H_2 and 24-H_2
(15*E*,22*E*)-1,15,22-Heptatriacontatriene	2.03, *m*, 3-H_2 1.96, *m*, 14-H_2, 17-H_2, 21-H_2 and 24-H_2
(*Z*)-9-Heneicosene	2.01, *m*, 8-H_2 and 11-H_2
(*E*)-7-Tetradecene	1.96, *m*, 6-H_2 and 9-H_2
1-Heptadecene	2.04, *m*, 3-H_2
(*Z*)-2-Heptene	1.60, *m*, $^3J_{1,2}$ = 6.1, $^4J_{1,3}$ = –1.4, $^5J_{1,4}$ = ±1.0, 1-H_3 2.03, *m*, 4-H_2
(*E*)-2-Heptene	1.63, *m*, $^3J_{1,2}$ = 6.6, $^4J_{1,3}$ = –2.5, $^5J_{1,4}$ = ±1.0, 1-H_3 1.95, *m*, 4-H_2

TABLE 5
Vinylic ^{13}C Nuclear Magnetic Resonances

Compound	Chemical shift, δ (ppm)
(22*Z*)-1,22-Hentriacontadiene	114.1, C-1 139.3, C-2 129.9, C-22 and C-23
(2*Z*,22*Z*)-2,22-Hentriacontadiene	123.6, C-2 130.9, C-3 129.9, C-22 and C-23
(15*E*,22*E*)-1,15,22-Heptatriacontatriene	114.1, C-1 139.3, C-2 130.3 and 130.4, C-15, C-16, C-22 and C-23
1-Heptadecene	114.1, C-1 139.3, C-2
(*Z*)-9-Heneicosene	129.9, C-9 and C-10
(*E*)-7-Tetradecene	130.4, C-7 and C-8
(*Z*)-2-Heptene	123.6, C-2 130.9, C-3
(*E*)-2-Heptene	124.6, C-2 131.7, C-3

The mass spectra of the DMDS adducts indicate that the major C_{31} diene and two of the minor C_{31} isomers have a double bond at C-22, with ions at *m/z* 173 (D^+) and 399 [$(C - 48)^+$] (Table 6). The position of the second double bond in the major isomer is confirmed as C-2 from ion *m/z* 75 (A^+) and as C-1 in one of the minor C_{31} isomers from ion *m/z* 61 (A^+) (Tables 1 and 6). Thus, the major C_{31} diene in *E. huxleyi* (CCAP 920/2) is (2*Z*,22*Z*)-2,22-hentriacontadiene (Scheme 2B) and the second-most abundant is (22*Z*)-1,22-hentriacontadiene, identical to the major component in *I. galbana* (Table 1, Fig. 1 and Scheme 2A). The adduct mass spectrum of the third-most abundant C_{31} diene possesses an ion at *m/z* 89 $(A)^+$, indicating a double bond at C-3. The least abundant C_{31} diene has double bonds in the C-2 and C-24 positions, indicated by ions *m/z* 75 (A^+), 145 (D^+), 451 [$(B - 94)^+$] and 427 [$(C - 48)^+$] (Table 6), as has the C_{33} alkadiene indicated by ions *m/z* 75 (A^+) , 173 (D^+), 479 [$(B - 94)^+$] and 427 [$(C - 48)^+$] (Table 6).

Following the GC–MS studies of the DMDS adducts, we re-analyzed the ^1H NMR spectrum of the hydrocarbon fraction with a view to confirming the structure of 3,22-hentriacontadiene and determining the geometry of the C-3 double bond. Comparison of a minor triplet at 0.952 ppm (J = 7.6 Hz), representing 1-H_3, with those in (*Z*)-3-heptene and (*E*)-3-heptene, indicates *cis* stereochemistry as the result of a subtle difference in chemical shift. In (*Z*)-3-heptene this is at 0.955 (*t, J* = 7.5 Hz) and in (*E*)-3-heptene at 0.963 (*t, J* = 7.5 Hz). Therefore, this isomer is (3*Z*,22*Z*)-3,22-hentriacontadiene (Scheme 2C).

Emiliania huxleyi *(VAN 556).* The alkene distribution of *E. huxleyi* (VAN 556) is dominated by a C_{37} triene (Table 1, Fig. 1) together with lesser abundances of a C_{38} triene and a C_{31} diene (Table 1). The ^1H NMR spectrum of the triene displays an intense resonance at 1.25 ppm (CH_2 groups), a terminal alkyl methyl at 0.88 ppm, and illustrates the presence of a terminal double bond with resonances at 4.92, 4.99, and

TABLE 6
Mass-to-charge Ratio (% relative abundance) of Key Ions in Mass Spectra of Dimethyl Disulfide (DMDS) Derivatives of Alkene Isomers from Haptophyte Algae

				m/z (% abundance relative to base peak)							
Compound	x^a	y^a	z^a	A^+	D^+	$(B - 94)^+$	$(C - 48)^+$	$(C - 94)^+$	$(M - 141)^+$	M^+	Base peak
$31{:}2\Delta^{1,22}$	0	19	8	61(100)	173(68)	465(18)	399(57)	353(22)	479(6)	620(2)	61
$31{:}2\Delta^{2,22}$	1	18	8	75(70)	173(86)	451(20)	399(45)	353(40)	479(10)	620(2)	54
$31{:}2\Delta^{3,22}$	2	17	8	89(55)	173(83)	437(20)	399(20)	353(37)	479(11)	620(2)	54
$31{:}2\Delta^{1,24}$	0	21	6	61(100)	145(52)	465(17)	427(39)	381(5)	479(8)	620(1)	61
$31{:}2\Delta^{2,24}$	1	20	6	75(63)	145(45)	451(11)	427(18)	381(19)	479(4)	620(1)	54
$33{:}2\Delta^{1,24}$	0	21	8	61(100)	173(34)	493(15)	427(32)	381(29)	507(5)	648(1)	61
$33{:}2\Delta^{2,24}$	1	20	8	75(31)	173(38)	479(12)	427(17)	381(15)	507(5)	648(1)	54

[a]See Scheme 1 for interpretation.

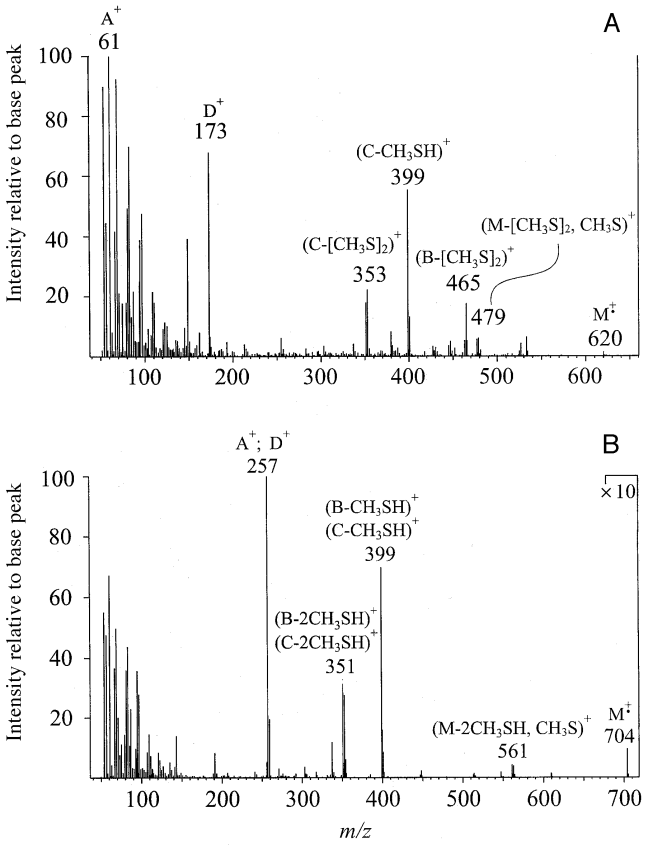
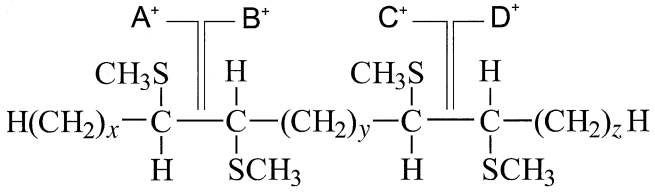


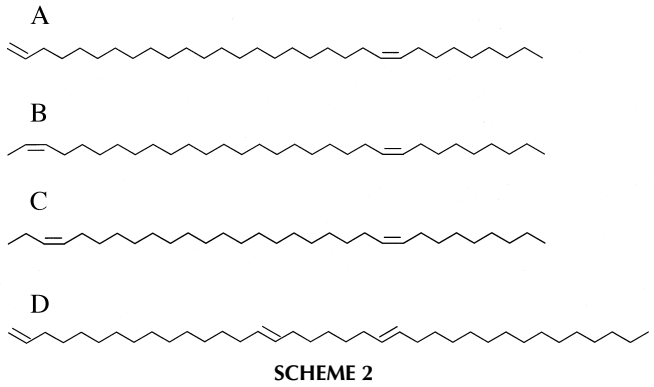
FIG. 4. Mass spectra of dimethyl disulfide (DMDS) adducts of (A) (22*Z*)-1,22-hentriacontadiene from *I. galbana* (CCAP 927/14) and (B) (15*E*,22*E*)-1,15,22-heptatriacontadiene from diimide reduction of major C_{37} triene in *E. huxleyi* (VAN 556). For abbreviations see Figure 1, Scheme 1, and Tables 6, 7.

5.81 ppm (Table 2). A second-order multiplet, representing four hydrogens and hence two double bonds in the middle of the hydrocarbon chain, is observed at 5.38 ppm. The chemical shift is in accord with a *trans* double bond (Table 3), although a clear comparison of the splitting pattern with that observed for the vinylic hydrogens in (*E*)-7-tetradecene was not immediately obvious. Zero-filling of the free induction decays and application of a window function for resolution enhancement produced a significantly improved splitting pattern comparable with that seen in (*E*)-7-tetradecene and markedly different fron that derived from (*Z*)-9-heneicosene processed in the same manner (Fig. 6).

The *trans* nature of the internal double bonds was confirmed by analyzing the chemical shifts of the allylic hydrogens. Separate multiplets were observed for the hydrogens allylic to the terminal double bond (2.03 ppm), as observed in 1-heptadecene, and for those allylic to the internal double bonds (1.96 ppm), as observed in (*E*)-7-tetradecene (Fig. 3).



SCHEME 1



SCHEME 2

^{13}C NMR chemical shifts for the sp^2 hybridized carbons (Table 5) also accord with a terminal double bond (114.1 and 139.3 ppm) and two midchain *trans* double bonds (130. 3 and 130.4 ppm).

Although the DMDS-adducted C_{37} triene can be observed by high-temperature GC–MS, the instability of the high-mass fragment ions (>*m/z* 655) did not allow the molecular ion or

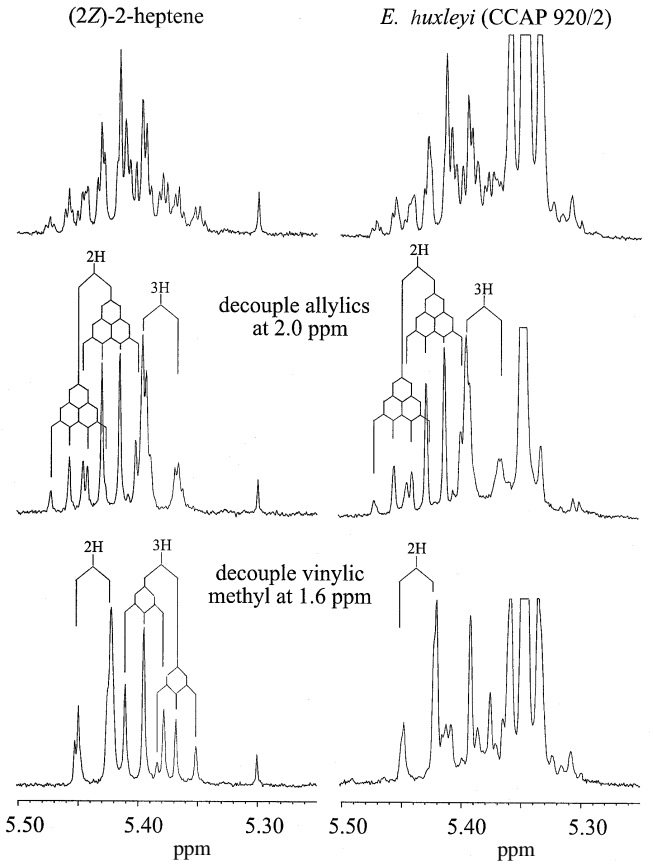


FIG. 5. Partial ^1H NMR spectra and decoupled spectra comparing the vinylic resonances in the *E. huxleyi* (CCAP 920/2) hydrocarbons with those in (*Z*)-2-heptene. For abbreviations see Figure 2.

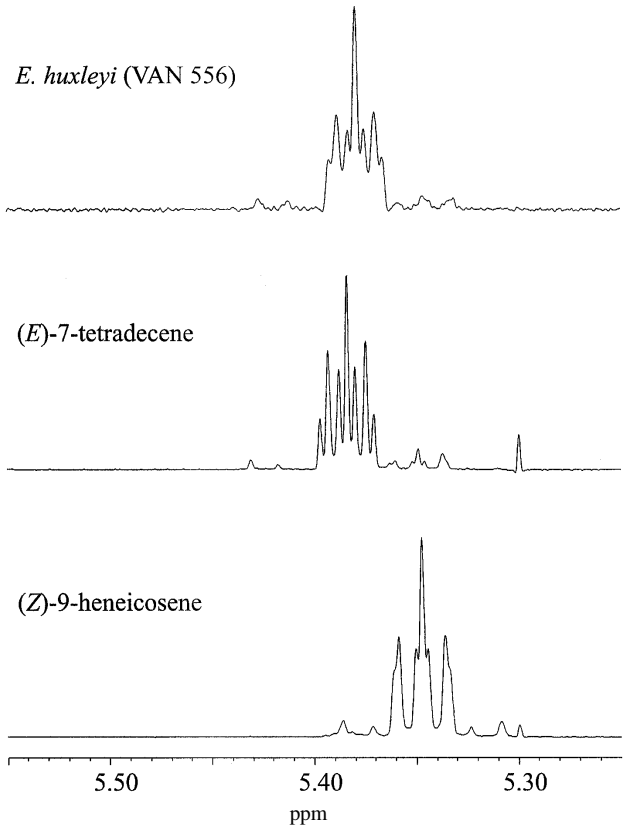


FIG. 6. Partial resolution-enhanced ^{1}H NMR spectra comparing the vinylic resonances in the *E. huxleyi* (VAN 556) hydrocarbons with those in two alkene standards. For abbreviations see Figure 2.

certain key fragment ions to be observed. Therefore, the sample was examined by desorption chemical ionization using ammonia as collision gas, giving a pseudo-molecular ion at *m/z* 814 (MNH_4^+) corresponding to a molecular weight for the DMDS-adducted C_{37} alkatriene of 796 Da.

An aliquot of the hydrocarbon fraction was reduced with diimide (13–15) in order to assign the positions of the internal double bonds, an approach used successfully in the structural elucidation of the acyclic triterpenoid botryococcene (16). Although the reduction is not entirely chemoselective, the product mixture comprises predominantly hydrocarbons derived from terminal double bond reduction. The reduced mixture was subsequently adducted with DMDS and analyzed by GC–MS. The major adducted diene has a spectrum with principal fragment ions at *m/z* 704 (M^+), 257(A^+) and (D^+), 351 [$(B - 96)^+$; i.e., $B - 2CH_3SH$] and [$(C - 96)^+$], 399 [$(B - 48)^+$] and [$(C - 48)^+$] (Table 7; Fig. 4B) indicating a C_{37} alkadiene with double bonds located symmetrically at C-15 and C-22, giving the structure of the major triene as (15*E*,22*E*)-1,15,22-heptatriacontatriene (Scheme 2D). This approach also showed the internal double bond positions of the reduced C_{38} alkatriene to be at C-15 and C-22 (Table 7). It is likely that the geometry of the double bonds in this component is *trans*, by analogy with the C_{37} alkatriene.

The minor C_{31} diene component is (22*Z*)-1,22-hentriacontadiene, assigned by retention time comparison with the component derived from *I. galbana* and from mass spectral interpretation of the fragmentation of the DMDS adduct.

Biosynthetic relationships. The *cis* geometry and the n-9 (ω9) position of unsaturation in the most abundant dienes (Table 1) is strong evidence that these lipids derive from (*Z*)-9-octadecenoic acid (oleic acid), followed by chain elongation and decarboxylation, as has been elucidated for the chlorophyte alga *Botryococcus braunii* (17). The diene isomers with n-7 (ω7) unsaturation may derive from elongation and decarboxylation of (*Z*)-7-hexadecenoic acid, though such a pathway is still to be demonstrated.

In contrast, the positions and geometry of nonterminal unsaturation in the C_{37} triene (and by analogy the C_{38} triene) correspond to those in the C_{37} (and C_{38}) haptophyte alkadienone (6), so these components likely share a common biosynthesis. The full details of such a pathway are still unknown and should be determined. However, whatever this pathway may be, it is highly probable that in haptophytes the long-chain alkatrienes and the co-occurring alkadienes have distinct biosynthetic pathways.

ACKNOWLEDGMENTS

We acknowledge the Natural Environment Research Council (NERC) for funding of the Organic Mass Spectrometry Facility at Bristol and for a research studentship for MAT. GR was funded from NERC grant GST/02/1026. We are grateful to Professor D.G. Hägele (Institut für Anorganische Chemie und Strukturchemie, Heinrich-Heine-Universität Düsseldorf, Germany) for his PC version of the program Laocoön.

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TABLE 7
Mass-to-charge Ratio (% relative abundance) of Key Ions in Mass Spectra of DMDS Derivatives of Alkenes Derived from Diimide Reduction of Haptophyte Alkatrienes

				m/z (% abundance relative to base peak)							
Compound	x^a	y^a	z^a	A^+	D^+	$(B - 96)^+$	$(C - 48)^+$	$(C - 96)^+$	$(M - 143)^+$	M^+	Base peak
$37{:}2\Delta^{15,22}$	14	5	14	257(100)	257(100)	351(31)	399(69)	351(31)	561(4)	704(1)	257
$38{:}2\Delta^{15,22}$	14	5	15	257(90)	271(82)	365(38)	399(42)	351(38)	575(5)	718(–)	61

[a]See Scheme 1 for interpretation.

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[Received November 12, 1997, and in final revised form April 15, 1998; revision accepted April 24, 1998]

Fatty Acid Composition of Bacteria Associated with the Toxic Dinoflagellate *Ostreopsis lenticularis* and with Caribbean *Palythoa* Species

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ABSTRACT: The fatty acid composition of a *Pseudomonas* sp. *(Alteromonas)* and its host, the dinoflagellate *Ostreopsis lenticularis,* vectors in ciguatera fish poisoning, has been studied. The major fatty acids in *O. lenticularis* were 16:0, 20:5n-3, and 22:6n-3, but 18:2n-6, 18:3n-3, and 18:n-3 were also identified. In contrast to other dinoflagellates, 18:5n-3 was not detected in *O. lenticularis*. Even-chain fatty acids such as 9-16:1, 11-18:1, and 13-20:1 predominated in the *Pseudomonas* sp. from *O. lenticularis,* but 16–20% of (*E*)-11-methyl-12-octadecenoic acid was also identified. The chirality of the latter was confirmed by total synthesis (28% overall yield) starting from oxacyclotridecan-2-one. The fatty acid compositions of two other *Pseudomonas* species, from the palytoxin-producing zoanthids *Palythoa mamillosa* and *P. caribdea,* were also studied and were similar to that of the *Pseudomonas* sp. from *O. lenticularis*. The possibility of using some of these fatty acids as chemotaxonomic lipids in identifying marine animals that consume toxic dinoflagellates or zoanthids is discussed.
Lipids 33, 627–632 (1998).

Ostreopsis lenticularis (Dinophyceae) Fukuyo 1981 is a toxic benthic dinoflagellate partially responsible for the production of ciguatoxins directly related to ciguatera fish poisoning (1). Of the different bacterial flora (e.g., *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, and *Vibrio*) associated with *O. lenticularis*, bacteria from the genus *Pseudomonas* (*Alteromonas)* were required for the development of toxicity in cultured *Ostreopsis* (2). Palytoxin, one of the most potent nonprotein toxins known, is produced by the zoanthids *Palythoa caribdea* and *P. mamillosa* (3,4). Palytoxin hemolytic activity has been detected in extracts of the dinoflagellates *O. lenticularis*, *Gambierdiscus toxicus*, and *Prorocentrum concavum,* as well as in bacteria associated with *O. lenticularis* grown in pure culture (5). Similarly, palytoxin hemolytic activity was detected in extracts of bacteria from the genus *Pseudomonas* isolated from the Caribbean zoanthids *P. caribdea* and *P. mamillosa* (6). There is no report in the literature on the fatty acid composition of *O. lenticularis* nor that of its most important symbiont, the unusual bacterium *Pseudomonas* sp. (2). Therefore, herein we report the fatty acid composition of both *O. lenticularis* and its associated bacterium *Pseudomonas* sp. In addition, we report the fatty acid composition of two other bacterial strains, isolated from the palytoxin producing *Palythoa* species *P. caribdea* and *P. mamillosa*, which we demonstrate share a similar fatty acid profile as the *Pseudomonas* sp. from *O. lenticularis*. This fatty acid analysis is important since the idea of using specific signature fatty acids for monitoring toxic dinoflagellate blooms, such as the ones implicated in ciguatera fish poisoning, is quite attractive.

The fatty acid composition of several toxic dinoflagellates, such as *Gymnodinium catenatum*, has been reported before (7,8). The principal fatty acids in dinoflagellates are considered to be 16:0, 20:5n-3, and 22:6n-3, followed by 14:0, 16:1n-7, and 18:2n-6. In addition, a rare 18:5n-3 fatty acid, considered by many as a characteristic fatty acid of dinoflagellates, has also been reported (7).

MATERIALS AND METHODS

General experimental procedures. Fatty acid methyl esters were analyzed using a gas chromatograph equipped with a fused silica nonpolar capillary column (30 m × 0.32 mm i.d.) containing either methyl silicone (SE-54; Supelco Inc., Bellefonte, PA) or poly(dimethylsiloxane) (SPB-1; Supelco Inc.) with He as carrier gas. Analyses were performed using the following conditions: initial temperature, 130°C; rate of increase, 3°C/min; final temperature, 260°C. Samples were also analyzed by gas chromatography–mass spectrometry (GC/MS) at 70 eV equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethylsiloxane crosslinked with 5% phenyl methylpolysiloxane. High-

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Abbreviations: ECL, equivalent chain length; ES, Erd-Schrieber medium; GC, gas chromatography; GC/MS, gas chromatography–mass spectrometry; IR, infrared; NMR, nuclear magnetic resonance.

resolution mass spectral data were recorded in a VG AutoSpec high-resolution mass spectrometer.

Culturing of O. lenticularis. *Ostreopsis lenticularis* Fukuyo 1981 was isolated from the coastal waters of southwest Puerto Rico in November of 1989 and has been kept in continuous clonal culture since that time (1). Erd-Schrieber (ES) medium was used for the maintenance of these cultures (9). Clonal cultures of *O. lenticularis* were established by carefully passing individual cells isolated from the surfaces of field-sampled macroalgae through a series of filtered seawater washes using sterile Pasteur pipettes and depression slides. After four cell washings, an individual cell was placed in a test tube with 5 mL of sterile seawater and 25% ES medium. After approximately 3 wk, the dinoflagellate cells were microscopically checked and transferred to 25 mL of sterile seawater and 50% ES media. Following an additional 3 wk, these cultures were transferred to 100 mL of seawater and 100% ES media. The process of increasing volume was then gradually continued until the desired volume for maintaining the clonal culture was reached. For maintenance purposes, these cultures were continuously transferred to fresh media after 15 to 20 d of growth. Dinoflagellates, harvested from their respective suspensions by filtration (glass fiber filters; Gelman, Fisher Scientific, Cayey, Puerto Rico), were also extracted in distilled methanol and the extracts subsequently evaluated for their toxicity in white Swiss mice using reported procedures (10).

Isolation and identification of associated bacteria. Sterile samples of the harvested dinoflagellate cell suspensions were serially diluted and streaked on Zobell agar plates for aerobic incubation at 23–25°C. Zobell blood agar was used for the growth of fastidious bacteria in aerobic and anaerobic conditions. Isolates were identified at least to generic level (11,12). Characterization was done by routine biochemical and antibiotic tests modified for marine bacteria. The API 20E system (Analytab Products, Plainview, NY) was also used for characterization, as recommended by MacDonell *et al.* (13) for marine isolates. The microorganism associated with the toxicity was identified as a *Pseudomonas* sp. (10).

Palythoa *associated bacteria.* Zoanthid species *P. caribdea* and *P. mamillosa* were sampled on shallow reef flats at La Parguera, on the southwest coast of Puerto Rico. Samples for bacteriological analyses were obtained aseptically in the field using sterile bags and gloves. Zoanthid samples were returned to the laboratory, cleaned of foreign material, rinsed with sterile artificial water (calcium and magnesium free), and homogenized. Homogenized samples were serially diluted, streaked on marine agar (Difco, Fisher Scientific) enriched with 0.1% starch (MA+), and incubated aerobically at 27°C. Colony isolation was started after 48 h of incubation and isolates stored on MA+ slants for subsequent identification. Isolated marine bacteria obtained from zoanthid homogenates were identified to genus and when possible to species using standard biochemical tests in conjunction with Gram and flagellar stains, motility, antibiotic susceptibility, and the API 20E system as described above (11–13). Both *P. caribdea* and *P. mamillosa* shared associated bacterial strains from the genus *Pseudomonas* sp. Analysis of the *Palythoa*-like hemolytic activity of extracts from both of these associated bacterial strains indicated that they produced palytoxin when grown in pure culture in the absence of their *Palythoa* hosts.

The presence of toxic, hemolytic activity in the extracts of cultured zoanthid-associated bacterial flora was detected using a hemolysis neutralization assay (14). Extracts were tested for their hemolytic activity in microtiter Elisa plates preblocked with sterile filtered 1% bovine serum albumin in a phosphate buffered saline solution. Plates inoculated with white Swiss mice red cells in the presence and absence of extracts being tested were covered and incubated at 37°C for 6 h. Results of the hemolysis assay were assessed visually.

Fatty acid isolation. The biomass thus obtained from either *O. lenticularis* (300–600 mg) and the *Pseudomonas* bacterial strains (100–120 mg) was separated by centrifugation of the cultured liquid (6000 r.p.m. for 20 min), and the lipids were extracted with chloroform/methanol (1:1, vol/vol) affording 30–50 mg of total lipids, which were methylated with 1.0 M HCl in methanol, resulting in typically 3–5 mg of fatty acid methyl esters.

Permanganate/periodate oxidation. A stock oxidant solution of sodium metaperiodate (10 mM) and $KMnO_4$ (2.5 mM) in H_2O was prepared. This solution (1 mL) together with 1 mL K_2CO_3 solution (180 mM) was added to the methyl esters (1 mg) in *t*-butanol (2 mL), and the mixture was shaken thoroughly at room temperature for 1 h. At the end of this time, the reaction mixture was acidified with one drop of concentrated H_2SO_4, and excess oxidant was destroyed with $NaHSO_3$. The reaction mixture was extracted thoroughly with ether (3×4 mL), the organic layer was dried over Na_2SO_4, and the ether removed in a stream of N_2. The acids thus obtained were methylated with 1.0 M HCl/MeOH for GC analysis.

2-Methyldodecanedioic acid dimethyl ester. GC/MS *m/z* (relative intensity) M^+ 272(0.1), 241(3), 227(1), 213(9), 199(7), 185(12), 167(6), 153(10), 126(10), 112(36), 98(34), 88(100), 83(23), 74(24), 69(36), 57(25), 55(63).

3-Methyloxacyclotridecan-2-one. To a solution of oxacyclotridecan-2-one (1.10 g, 5.6 mmol) in 10 mL of tetrahydrofuran at −78°C was added dropwise a solution of lithium diisopropylamide (LDA) (5.8 mmol) in 5 mL of tetrahydrofuran prepared from diisopropylamine (0.8 mL, 5.8 mmol) and *n*-butyllithium (1.2 mL, 5.8 mmol) in hexane. After the mixture was stirred at −78°C for 20 min, methyl iodide (0.35 mL, 5.6 mmol) was added, and the reaction mixture was stirred at −78°C for 2 h. The reaction mixture was quenched with a saturated NH_4Cl solution (20 mL), and extracted with benzene (3×30 mL). The organic phase was successively washed with a 0.1 M HCl solution (1×10 mL), a saturated $NaHCO_3$ solution (1×30 mL), a saturated NaCl solution (1×50 mL), and finally dried over Na_2SO_4. After rotoevaporation, 0.95 g (79% yield) of a colorless oil was obtained. Infrared (IR) (neat) υ_{max} 2927, 2861, 1724, 1465, 1350, 1230, 1169, 1057 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.28 (1H, *m*, H-13), 4.01 (1H, *m*, H-13), 2.51 (1H, *m*, H-3), 1.62 (4H, *m*, CH_2),

1.28–1.40 (14H, *m*, CH_2), 1.13 (3H, *d, J* = 6.9 Hz, CH_3); ^{13}C nuclear magnetic resonance (NMR) ($CDCl_3$, 75 MHz) δ 177.0 (*s*, C-2), 64.4 (*t*, C-13), 39.7 (*d*, C-3), 33.7 (*t*), 27.5 (*t*, C-12), 26.5 (*t*), 26.5 (*t*), 25.5 (*t*), 25.2 (*t*), 24.8 (*t*), 24.1 (*t*), 17.6 (*q*, CH_3); GC/MS *m/z* (relative intensity) M^+ 212(4), 194(4), 166(2), 151(3), 139(9), 138(17), 126(7), 112(17), 110(16), 98(33), 97(35), 83(61), 74(85), 69(62), 55(100); high-resolution mass spectrometry calcd for $C_{13}H_{24}O_2$ 212.1776, found 212.1769.

12-Hydroxy-2-methyldodecanal. To 3-methyloxacyclotridecan-2-one (0.56 g, 2.6 mmol), in 20 mL dry toluene at −78°C, and under N_2, 3 mL diisobutylaluminum hydride (1.0 M, 3 mmol) was slowly added over a 10-min period. The reaction mixture was stirred at −78°C for 2 h, subsequently quenched with a saturated NH_4Cl solution, and finally stirred for 1 h at room temperature. After this time the reaction mixture was filtered under vacuum, and washed with excess ether. The organic washings were evaporated *in vacuo* to give 12-hydroxy-2-methyldodecanal (0.48 g, 2.2 mmol) for an 85% yield. IR (neat) υ_{max} 3600–3100 (OH), 2940, 2856, 2724, 1728 (CHO), 1468, 1168, 1060 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.61 (1H, *d, J* = 2 Hz, CHO), 3.60 (2H, *t, J* = 6.6 Hz, H-12), 2.30 (1H, *m*, H-2), 1.67–1.51 (4H, *m*, CH_2), 1.40–1.20 (15H, *m*, CH_2), 1.07 (3H, *d, J* = 6.9 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 205.8 (*s*, C-1), 63.0 (*t*, C-12), 46.3 (*d*, C-2), 32.8 (*t*), 30.6 (*t*), 29.5 (*t*), 29.5 (*t*), 29.4 (*t*), 26.9 (*t*), 25.7 (*t*), 13.3 (*q*, CH_3); GC/MS *m/z* (relative intensity) M^+ 214(1), 172(1), 138(2), 125(1), 111(4), 97(14), 83(21), 69(31), 58(100), 55(58).

(Z)- and (E)-11-methyl-12-octadecen-1-ol. To a stirred suspension of 1-hexyltriphenylphosphonium bromide (0.41 g, 0.96 mmol) in 10 mL tetrahydrofuran was added 0.4 mL of *n*-butyllithium (2.5 M, 0.96 mmol) in hexane at 0°C over 10 min. The reaction mixture was stirred for an additional 10 min at 0°C before adding dropwise 12-hydroxy-2-methyldodecanal (0.20 g, 0.95 mmol) in 5 mL dry tetrahydrofuran. After 2 h, the reaction was quenched with a saturated NH_4Cl solution (15 mL), and the reaction mixture was extracted with ether (3 × 50 mL). The organic phase was washed with a saturated NaCl solution (1 × 20 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified through silica gel column chromatography eluting with hexane/ether (8:2, vol/vol), which finally afforded 0.18 g (69% yield) of a 9:1 ratio of (*Z*)- and (*E*)-11-methyl-12-octadecen-1-ol as a colorless oil. Spectral data for the (*Z*) isomer is presented below. IR (neat) υ_{max} 3600–3150 (OH), 2999, 2951, 2920, 2850, 1466, 1383, 1067, 725 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.29 (1H, *m, J_{cis}* = 11 Hz, H-13), 5.09 (1H, *brt*, J_{cis} = 11 Hz, H-12), 3.63 (2H, *t, J* = 6.5 Hz, H-1), 2.40 (1H, *m*, H-11), 2.00 (2H, *m*, H-14), 1.54 (2H, *m*, H-2), 1.40–1.20 (20H, *m*, CH_2), 0.90 (3H, *d, J* = 6.6 Hz, CH_3), 0.89 (3H, *brt, J* = 6.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 136.4 (*d*, C-12), 128.3 (*d*, C-13), 63.0 (*t*, C-1), 37.6 (*t*), 32.8 (*t*), 31.6 (*d*, C-11), 31.5 (*t*), 29.8 (*t*), 29.6 (*t*), 29.4 (*t*), 27.5 (*t*), 27.4 (*t*), 25.7 (*t*), 22.5 (*t*), 21.4 (*q*, C-19), 14.0 (*q*, C-18); GC/MS *m/z* (relative intensity) M^+ 282(15), 264(1), 250(1), 183(5), 165(5), 140(28), 125(28), 109(21), 97(30), 95(33), 83(61), 69(98), 67(38), 57(45), 55(100).

(Z)- and (E)-11-methyl-12-octadecenoic acid. To a solution of 11-methyl-12-octadecen-1-ol (0.11 g, 0.39 mmol) in 5 mL of dry dimethyl formamide was added a solution of pyridinium dichromate (1.56 g, 4.15 mmol) in 2 mL of dry dimethyl formamide. The reaction mixture was stirred at room temperature for 20 h, poured into 50 mL of H_2O, and extracted with ether (3 × 70 mL). The ethereal layers were combined and washed with a solution of NH_4Cl (30 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The resulting oil was purified by column chromatography eluting first with hexane/ether (8:2, vol/vol) and finally with ether to give 0.07 g (62%) of a 9:1 mixture of (*Z*)- and (*E*)-11-methyl-12-octadecenoic acid. Spectral data for the (*Z*) isomer is presented below. IR (neat) υ_{max} 3003, 2965, 2923, 2851, 1714, 1461, 1258, 1101, 1017 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.34–5.24 (1H, *m, J_{cis}* = 10.5 Hz, H-13), 5.10 (1H, *brt, J_{cis}* = 10.5 Hz, H-12), 2.40 (1H, *m*, H-11), 2.34 (2H, *t, J* = 7.5 Hz, H-2), 2.00 (2H, *m*, H-14), 1.62 (2H, *m*, H-3), 1.29–1.20 (20H, *m*, CH_2), 0.90 (3H, *d, J* = 6.6 Hz, CH_3), 0.86 (3H, *brt, J* = 6.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 178.3 (*s*, C-1), 136.3 (*d*, C-12), 128.2 (*d*, C-13), 37.4 (*t*), 33.7 (*d*, C-11), 31.5 (*t*), 31.4 (*t*), 29.7 (*t*), 29.6 (*t*), 29.5 (*t*), 29.4 (*t*), 29.3 (*t*), 29.1 (*t*), 28.9 (*t*), 27.4 (*t*), 27.3 (*t*), 24.6 (*t*), 22.4 (*q*, C-19), 13.9 (*q*, C-18); GC/MS *m/z* (relative intensity) M^+ 296(4), 264(1), 197(3), 179(6), 140(19), 125(20), 112(9), 97(24), 95(11), 83(48), 69(92), 67(22), 57(37), 55(100).

RESULTS

Fatty acid composition of O. lenticularis. The fatty acid composition of *O. lenticularis*, as well as that of its associated *Pseudomonas* sp., is presented in Table 1. Fatty acids accounted for 2–3% of the extracted biomass. As expected, the major fatty acids in *O. lenticularis* were 16:0, 20:5n-3, and 22:6n-3, which accounted for more than 50% of its fatty acid composition. Considerable amounts (23%) of the polyunsaturated fatty acids 18:2n-6, 18:3n-3 and 18:4n-3 were also identified. In contrast to other dinoflagellates (7), the fatty acid 18:5n-3 was not detected in *O. lenticularis*. More than 99% of the fatty acids from *O. lenticularis* were even-chain fatty acids.

Fatty acid composition of Pseudomonas *sp*. Even-chain monounsaturated fatty acids predominated in the *Pseudomonas* sp. from *O. lenticularis* (Table 1). For example, the biosynthetically related monounsaturated fatty acids 9-16:1, 11-18:1, and 13-20:1 accounted for more than 54% of the total fatty acids from this strain. The (*Z*)-11-octadecenoic acid was the most abundant monounsaturated fatty acid (40%), but small amounts of (*E*)-11-octadecenoic acid were also identified. These acids were characterized as methyl esters by GC/MS, and the double-bond positions were determined by dimethyl disulfide derivatization (15), as well as by $KMnO_4/NaIO_4$ oxidation.

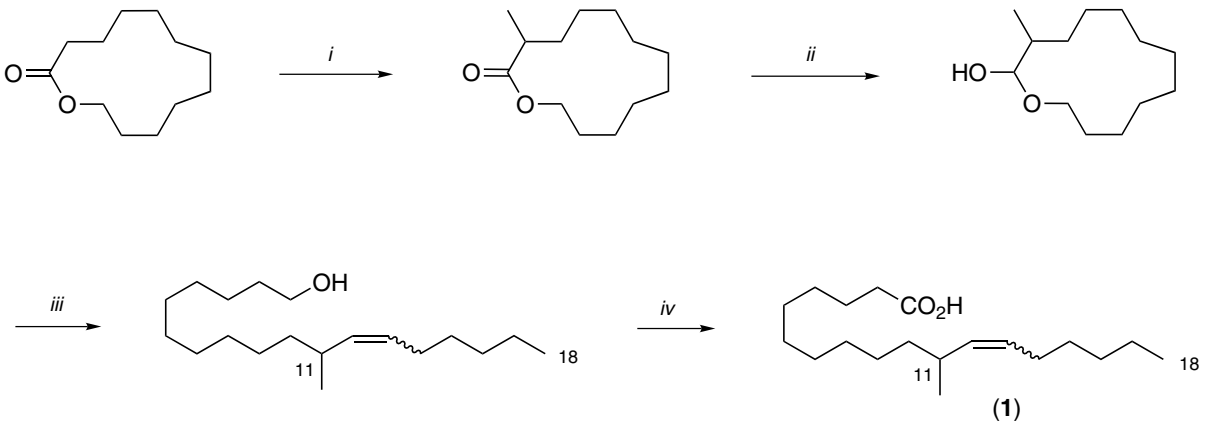
The unusual methyl-branched fatty acid 11-methyl-12-oc-

TABLE 1
The Fatty Acid Composition of the Dinoflagellate *Ostreopsis lenticularis* and Associated *Pseudomonas* Species

	Relative abundance (w/w)			
Fatty acids	*O. lenticularis*	*Pseudomonas*[a]	*Pseudomonas*[b]	*Pseudomonas*[c]
12:0	—	0.7	—	6.0
14:1	0.9	—	—	—
14:0	5.9	0.2	0.3	0.2
9-16:1	1.8	1.4	1.0	1.2
16:0	36.0	3.3	2.7	9.7
17:0	—	0.5	0.5	0.5
9,12,15-18:3	4.4	—	—	—
6,9,12,15-18:4	9.8	—	—	—
9,12-18:2	9.2	1.2	—	1.7
9-18:1	9.8	—	—	—
11-18:1	1.2	40.0	49.5	43.9
11*t*-18:1	—	0.6	0.5	4.8
18:0	2.0	18.4	18.7	4.0
11-Me-12*t*-19:1	0.4	17.4	16.6	21.9
11-19:1	—	1.0	1.3	5.5
5,8,11,14,17-20:5	10.8	—	—	—
13-20:1	—	12.9	8.0	0.5
15-20:1	—	0.2	—	—
20:0	—	1.0	0.8	—
21:1	—	1.2	—	—
4,7,10,13,16,19-22:6	7.1	—	—	—
22:0	0.4	—	—	—
24:0	0.2	—	—	—

[a]Isolated from *O. lenticularis*.
[b]Isolated from *P. mamillosa*. Traces of 13-18:1 and 19:0 were also detected.
[c]Isolated from *P. caribdea*. Traces of 11-16:1 was also detected.

tadecenoic acid (**1**, Scheme 1) was also identified in the *Pseudomonas* sp. from *O. lenticularis* by a combination of GC retention times of the methyl ester [equivalent chain length (ECl) = 18.06], MS, catalytic hydrogenation, and dimethyl disulfide derivatization as we previously described for this same acid from a *Vibrio* bacterium (16). In this work, the double-bond position at C-12 in **1** was further confirmed by $KMnO_4/NaIO_4$ oxidation followed by esterification. Methyl 11-methyl-12-octadecenoate cleaved under these conditions, and after esterification, to 2-methyldodecanedioic acid dimethyl ester and methyl hexanoate. The mass spectrum of 2-methyldodecanedioic acid dimethyl ester also confirmed the methyl substitution at C-11 in **1**, since it displayed a base peak at *m/z* 88 (McLafferty rearrangement), resulting from an ad-



Reagents and conditions: *(i)* LDA, CH_3I, THF, –78°C (79%); *(ii)* DIBAL-H, toluene, –78°C, (85%); *(iii)* $Br^-Ph_3P^+(CH_2)_5CH_3$, *n*-BuLi, THF, 0°C (69%); *(iv)* PDC, DMF, rt (62%).

SCHEME 1

ditional 2-methyl substitution in one of the α carbons of the fragmentation product.

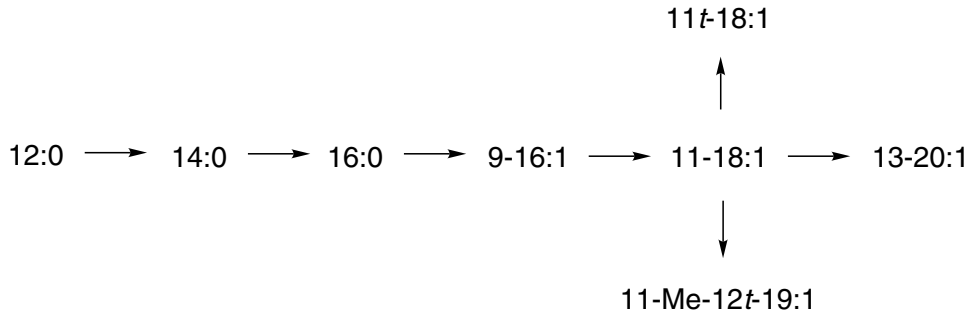
While all of the above data confirm 11-methyl-12-octadecenoic acid (**1**) as the most probable structure, the double-bond stereochemistry, i.e., *Z* or *E*, still needed to be determined. The 11-methyl-12-octadecenoic acid (**1**) was initially reported in *Byrsocarpus coccineus* seed oil (17), and more recently in *Mycobacterium fallax* (18), and in a *Vibrio alginolyticus* (16). In the aforementioned bacteria, the C-12 double-bond stereochemistry was not defined, but in *B. coccineus* it was determined as *E* (17). In order to unequivocally determine the double-bond stereochemistry in our 11-methyl-12-octadecenoic acid (**1**), as well as to establish reference ECL values for the methyl esters of the *Z* and *E* isomers of **1**, we undertook the first total synthesis of 11-methyl-12-octadecenoic acid (**1**, Scheme 1). Our synthetic scheme, which is probably the shortest possible for this compound (four steps), was planned to afford the *Z* isomer as the main product, but enough *E* isomer should be formed so as to permit careful GC/MS studies. The synthesis started with commercially available oxacyclotridecan-2-one, which was α-methylated with lithium diisopropylamide and methyl iodide in a 79% yield. Reduction of 3-methyloxacyclotridecan-2-one with diisobutylaluminum hydride in toluene at −78°C afforded, in an 85% yield, initially a lactol which opened to 12-hydroxy-2-methyldodecanal. The aldehyde was subsequently coupled, in a Wittig reaction, with 1-hexyltriphenylphosphonium bromide and *n*-butyllithium, affording (*Z*)- and (*E*)-11-methyl-12-octadecen-1-ol in a 9:1 ratio, respectively. Final oxidation with pyridinium dichromate, in dimethyl formamide, afforded a mixture of (*Z*) and (*E*)-11-methyl-12-octadecenoic acid (**1**) in a 62% yield. The methyl esters of **1** were then prepared for comparative GC studies. Careful nonpolar capillary GC coinjection of synthetic methyl (*Z*)-11-methyl-12-octadecenoate (ECL = 17.92) and methyl (*E*)-11-methyl-12-octadecenoate (ECL = 18.06) with the fatty acid methyl ester mixture from the bacterium *Pseudomonas* sp. established the natural acid as (*E*)-11-methyl-12-octadecenoic acid. It is interesting to point out that methyl (*Z*)-11-methyl-12-octadecenoate elutes before methyl octadecanoate (18:0) in nonpolar capillary GC. These retention times are important for future reference inasmuch as fatty acid methyl ester analyses are frequently used, as an aid, in bacterial identification.

Fatty acid composition of bacteria from Palythoa. The fatty acid composition of two unusual strains of *Pseudomonas* sp., isolated from the zoanthids *P. mamillosa* and *P. caribdea* is also presented in Table 1. Fatty acids from these *Pseudomonas* species also accounted for 2–3% of the extracted biomass. The fatty acid composition of *P. caribdea* has been reported (19). A striking similarity in the fatty acid composition of these two strains to the *Pseudomonas* sp. from *O. lenticularis* was observed. In the two *Pseudomonas* from *Palythoa,* the monounsaturated fatty acids 9-16:1, 11-18:1, and 13-20:1 were also particularly abundant (64–58%), while the saturated fatty acids 14:0, 16:0, and 18:0 accounted for another significant amount (14–22%) of the total fatty acid composition. The only significant difference between these two *Pseudomonas* strains is that the bacterium from *P. caribdea* had more 12:0 and less 18:0, as well as some 9,12-18:2, as compared to the *Pseudomonas* sp. from *P. mamillosa.* (Table 1). A very interesting finding is that both strains also contained (*E*)-11-methyl-12-octadecenoic acid (16–22%), which was characterized as we described above.

DISCUSSION

There are several important considerations that can be drawn from this study. One interesting conclusion is that *Pseudomonas* associated with toxin-producing marine organisms, such as *O. lenticularis* and *Palythoa*, seem to have a similar fatty acid profile, with a fatty acid biochemistry centered around (*Z*)-11-octadecenoic acid (Scheme 2). The first total synthesis of 11-methyl-12-octadecenoic acid, a key fatty acid in these bacteria, was also accomplished. GC retention times of methyl (*Z*)- and (*E*)-11-methyl-12-octadecenoate are presented as reference for further literature comparisons. The fatty acid profile of *O. lenticularis*, important vector in ciguatera fish poisoning in this area of the Caribbean, is also presented for the first time. While, in general, *O. lenticularis* has a similar fatty acid composition comparable to most toxic dinoflagellates, it is devoid of the polyunsaturated fatty acid 18:5n-3.

The (*E*)-11-methyl-12-octadecenoic acid probably originated, in these *Pseudomonas*, from 11-octadecenoic acid, *via S*-adenosylmethionine methylation, as was demonstrated before for *M. fallax* (18). Likewise, the presence of (*E*)-11-oc-



SCHEME 2

tadecenoic acid in these bacteria is probably the result of a *cis-trans* isomerization (20). Whether (*Z*)-11-octadecenoic acid or (*E*)-11-octadecenoic acid, or both, are precursors to (*E*)-11-methyl-12-octadecenoic acid is still a matter of speculation.

Some caution, however, should be taken concerning the identification of the bacterial strains associated with *O. lenticularis* and the two *Palythoa* species. The conventional microbiological techniques employed are frequently imprecise when used to identify marine bacteria. As noted in the Results section, the three strains examined in this study were found to be from the genus *Pseudomonas*. The precise identification of these strains requires further genetic analyses, and such studies are now in progress. Irrespective of this, all three strains produced toxic material when grown in pure culture and have the same signature fatty acids.

Whether we can use a simple fatty acid methyl ester analysis to verify the transmission of toxins from *O. lenticularis* to ciguatoxic fish remains to be seen. Perhaps, and even more important, is the possibility of using some of these fatty acids as biomarkers for toxicity in marine microalgae and bacteria. We have presented here a basis for such a comparison, but direct identification of some signature fatty acids, such as (*E*)-11-methyl-12-octadecenoic acid, in ciguatoxic fish still remains to be done. One particular problem is the small amounts of *O. lenticularis*, and its symbiotic bacteria, in fish. Work is in progress elucidating the fatty acids of toxin-producing bacteria in the marine environment.

ACKNOWLEDGMENTS

This work was supported by grants from the NSF-MRCE program (to NMC and TT) and the National Institutes of Health (NIH) under Grant No. 5 SO6GM08102. J.A. Restituyo thanks the NIH-MARC program for an undergraduate fellowship, while A. Emiliano thanks the University of Puerto Rico-FIPI program for a graduate fellowship.

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[Received January 29, 1998, and in final revised form and accepted April 30, 1998]

Epoxidation Reactions of Unsaturated Fatty Esters with Potassium Peroxomonosulfate

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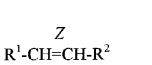
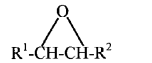
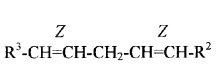
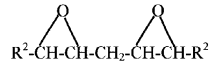
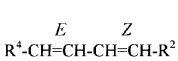
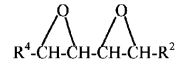
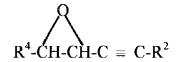
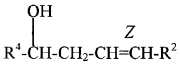
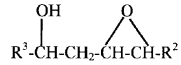
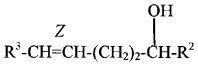
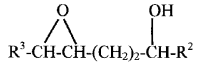
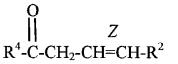
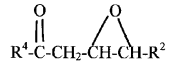
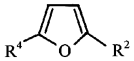
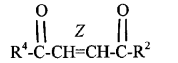
ABSTRACT: Epoxidation of the double bond in methyl oleate, octadec-11*E*-en-9-ynoate, ricinoleate (12-hydroxy-octadec-9*Z*-enoate), *iso*-ricinoleate (9-hydroxy-octadec-12*Z*-enoate), and 12-oxo-octadec-9*Z*-enoate with potassium peroxomonosulfate (oxone, 2 $KHSO_5.KHSO_4.K_2SO_4$) in the presence of trifluoroacetone or methyl pyruvate gave the corresponding monoepoxy derivatives. Reaction of Oxone® with methyl linoleate and octadeca-9*Z*,11*E*-dienoate furnished the corresponding diepoxystearate derivative. Methyl 9,12-dioxo-octadec-10*Z*-enoate was obtained when a C_{18} furanoid fatty ester (methyl 9,12-epoxy-9,11-octadecadienoate) was treated with Oxone®. The yield of these reactions was very high (85–99%), and the epoxy derivatives were readily isolated by solvent extraction. The products were identified by spectroscopic methods.
Lipids 33, 633–637 (1998).

Various oxidants, such as *m*-chloroperoxybenzoic acid (*m*-CPBA), magnesium monoperoxyphthalate hexahydrate (MMPP) and urea-hydrogen peroxide (UHP), are used to oxidize olefinic compounds to the corresponding epoxy derivatives (1–5). *m*-CPBA is the epoxidizing agent most often used in fatty acid chemistry. However, due to the shock-sensitive and explosive nature of *m*-CPBA during transportation, this reagent is now shipped in about 60–70% concentration in the presence of *m*-chlorobenzoic acid and water, which act as stabilizers. Another drawback of using *m*-CPBA for the epoxidation of unsaturated fatty esters is the need to remove *m*-chlorobenzoic acid and excess *m*-CPBA by washing with dilute sodium sulfite solution. During the washing of the reaction product mixture with sodium sulfite, the mixing of the various phases causes an emulsion to be formed, which requires long standing time for the organic layer to be clearly separated from the aqueous solution. In view of this practical problem, we decided to investigate the use of potassium peroxomonosulfate (Oxone®, DuPont Corp.; 2 $KHSO_5.KHSO_4.K_2SO_4$) as an epoxidation reagent instead. Oxone® generates dioxiranes *in situ* in the presence of ketones (such as acetone, trifluoroacetone, and methyl pyruvate). Dioxiranes are reported to be powerful epoxidation species with high reactivity toward both electron-rich and electron-deficient olefins (6,7).

In this report we present the results of the epoxidation of a number of mono- and diunsaturated fatty esters and unsaturated oxygenated fatty esters by dioxiranes generated *in situ* (Scheme 1).

Substrate	Reagents	Reaction time (h)	Product	Yield (%)
 (1)	*a*	1.5	 (2)	99
 (3)	*a*	24	 (4)	99
 (5)	*a*	1.5	 (6)	99
 (7)	*a*	1.5	 (8)	85
 (9)	*b*	1.5	 (10)	90
 (11)	*b*	1.5	 (12)	90
 (13)	*b*	0.75	 (14)	95
 (15)	*b*	4.0	 (16)	90

a: Oxone®, trifluoroacetone, $NaHCO_3$, CH_3CN

b: Oxone®, methyl pyruvate, $NaHCO_3$, CH_3CN

$R^1 = CH_3(CH_2)_7$; $R^2 = (CH_2)_7COOCH_3$; $R^3 = CH_3(CH_2)_4$; $R^4 = CH_3(CH_2)_5$

SCHEME 1

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Abbreviations: COSY, homonuclear correlation spectroscopy; IR, infrared; *m*-CPBA, *m*-chloroperoxybenzoic acid; MMPP, magnesium monoperoxyphthalate, hexahydrate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

MATERIALS AND METHODS

Thin-layer chromatography (TLC) was performed on microscope glass slides coated with silica gel G (*ca.* 0.1 mm thick), and a mixture of *n*-hexane/diethyl ether in various proportions was used as the developer. Components on the micro-plates were viewed by exposing them to iodine vapor. Column chromatographic separation was performed on silica gel (Merck, Darmstadt, Germany; type 60, 230–400 mesh ASTM) as the adsorbent and by gradient elution with a mixture of *n*-hexane/diethyl ether as the mobile phase. Infrared (IR) spectra were recorded on a Bio-Rad (Hercules, CA) FTS-7 FT-IR spectrometer. Samples were run as neat films on KBr discs. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier-transform NMR spectrometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform ($CDCl_3$) (0.2–0.3 mM) with tetramethylsilane (TMS) as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from TMS ($\delta_{TMS} = 0$ ppm).

Methyl oleate (**1**) and methyl linoleate (**3**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl octadeca-9*Z*,11*E*-dienoate (**5**) and methyl octadec-11*E*-en-9-ynoate (**7**) were synthesized according to procedures described elsewhere (8,9). Methyl ricinoleate (methyl 12-hydroxy-octadec-9*Z*-enoate, **9**) and methyl *iso*-ricinoleate (methyl 9-hydroxy-octadec-12*Z*-enoate, **11**) were isolated from *Ricinus communis* (castor) and *Wrightia tinctoria* seed oils, respectively (10). Methyl 12-oxo-octadec-9*Z*-enoate (**13**) and 2,5-disubstituted furanoid fatty ester (**15**) were synthesized as described elsewhere (11). Potassium peroxymonosulfate (Oxone®) was purchased from Aldrich Chemical Co.

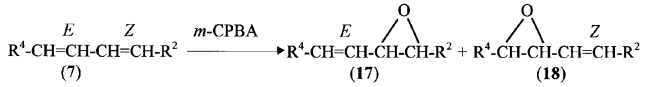
*Epoxidation of methyl oleate (**1**), methyl linoleate (**3**), methyl octadeca-9Z,11*E*-dienoate (**5**) and methyl octadec-11*E*-en-9-ynoate (**7**), as exemplified by compound **5** with potassium peroxymonosulfate in the presence of trifluoroacetone (Scheme 1).* A mixture of methyl octadeca-9*Z*,11*E*-dienoate (**5**, 0.1 g, 0.34 mmol), acetonitrile (3 mL), and aqueous disodium EDTA (Na_2EDTA) solution (2 mL, 4×10^{-4} M) was stirred for 2 min at room temperature. The reaction mixture was cooled to 0–5°C, and trifluoroacetone (0.2 mL) was added *via* a precooled syringe. Sodium hydrogencarbonate (0.13 g, 1.54 mmol) and Oxone® (0.31 g, 0.5 mmol) were then added and the reaction mixture was stirred for 1.5 h at 0–5°C. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (3×50 mL). The ethereal extract was washed with water (20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated to give methyl 9,10*Z*;11,12*E*-diepoxystearate (**6**, 0.11g, 99%). Infrared (IR) (neat): 1740 (*s*, ester C=O, str.), 1463 (*m*), 1436 (*m*), 1198 (*m*), 1172 (*m*, C-O-C), 898 (*m*, *E*-epoxy ring), and 835 (*w*, *Z*-epoxy ring) cm^{-1}; ^1H (NMR) ($CDCl_3$, δ_H) 0.88 (*t*, $J = 6.7$ Hz, 3H, C*H*$_3$), 1.26–1.63 (*m*, 22H, C*H*$_2$), 2.31 (*t*, $J = 7.5$ Hz, 3H, 2-*H*$_2$), 2.64–2.77 (*m*, 2H, 9-*H* and 12-*H*), $J_{9,10} = 4.1$ Hz), 2.87–3.03 (*m*, 2H, 10-*H* and 11-*H*, $J_{11,12} = 2.1$ Hz), and 3.66 (*s*, 3H, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, δ_C) 14.06 (C-18), 22.56 (C-17), 24.88 (C-3), 25.85/25.89 (C-14) , 26.70 (C-7), 28.07, 28.58, 29.01, 29.06, 29.08, 29.13, 29.17, 29.22, 31.58/31.64 (C-13), 31.72 (C-16), 34.05 (C-2), 51.48 (COO*C*H$_3$), 54.76, 55.42, 55.62, 55.66, 56.36, 56.60, 57.75 (C-9, C-10, C-11, C-12, epoxy carbons) and 174.30 (C-1).

*Methyl 9,10-Z-epoxystearate (**2**) from methyl oleate (**1**).* Yield: 99%; IR (neat): 1740 (*s*, ester C=O, str.), 1196 (*m*) and 1171 (*m*, C-O-C) cm^{-1}; ^1H NMR ($CDCl_3$, δ_H) 0.88 (*t*, $J = 6.5$ Hz, 3H, C*H*$_3$), 1.21–1.65 (*m*, 26H, C*H*$_2$), 2.31 (*t*, $J = 7.5$ Hz, 2H, 2-*H*$_2$), 2.88 (*m*, 2H, 9-*H* and 10-*H*), and 3.66 (*s*, 3H, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, δ_C) 14.11 (C-18), 22.69 C-17), 24.92 (C-3), 26.58 (C-12), 26.63 (C-7), 27.82 (C-8), 27.86 (C-11), 29.05 (C-4), 29.20, 29.24, 29.36, 29.56, 29.58, 31.88 (C-16) , 34.08 (C-2), 51.44 (COO*C*H$_3$), 57.19/57.24 (C-9, C-10) and 174.25 (C-1).

*Methyl 9,10-Z;12,13-Z-diepoxystearate (**4**) from methyl linoleate (**3**).* Yield: 99%; IR (neat): 1740 (*s*, ester C=O, str.), 1458 (*m*), 1437 (*m*), 1168 (*m*, C-O-C) and 890 (*m*, epoxy ring) cm^{-1}; ^1H NMR ($CDCl_3$, δ_H) 0.90 (*t*, $J = 7.0$ Hz, 3H, C*H*$_3$), 1.30–1.68 (*m*, 20H, C*H*$_2$), 1.70–1.80 (*m*, 2H, 11-*H*$_2$), 2.30 (*t*, $J = 7.5$ Hz, 2H, 2-*H*$_2$), 2.96–3.0 (*m*, 2H, 9-*H*, and 13-*H*, $J_{9,10} = 4.2$ Hz), 3.04–3.14 (*m*, 2H, 10-*H* and 12-*H*, $J_{12,13} = 4.2$ Hz) and 3.66 (COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, δ_C) 13.99 (C-18), 22.59 (C-17), 24.89 (C-3), 26.17, 26.28, 26.44 (C-7, C-15), 27.91 [C-11, confirmed by C-H homonuclear correlation spectroscopy (COSY)], 29.03, 29.17, 29.30, 31.69 (C-16), 34.03 (C-2), 51.42 (COO*C*H$_3$), 54.19, 54.33, 54.34 (C-10, C-12), 56.66, 56.70, 56.95, 56.99 (C-9, C-13) and 174.18 (C-1).

*Methyl 11,12-*E*-epoxy-octadec-9-ynoate (**8**) from methyl octadec-11*E*-en-9-ynoate (**7**).* Yield: 85%; IR (neat) 2250 (*m*, C=C), 1740 (*s*, C=O, str.) and 890 (*m*, epoxy ring) cm^{-1}; ^1H NMR ($CDCl_3$, δ_H) 0.88 (*t*, $J = 7.0$ Hz, 3H, C*H*$_3$), 1.29–1.64 (*m*, 20H, C*H*$_2$), 2.19 (*t*, $J = 6.8$ Hz, 2H, 8-*H*$_2$), 2.30 (*t*, $J = 7.4$ Hz, 2H, 2-*H*$_2$), 3.01 (*m*, 1H, 12-*H*), 3.07 (*d*, $J = 17$ Hz, 1H, 11-*H*) and 3.66 (s, 3H, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, d_C) 14.06 (C-18), 18.71 (C-8), 22.57 (C-17), 24.89 (C-3), 25.64 (C-14), 28.32, 28.62, 28.76, 28.99, 29.03, 31.72 (C-16), 34.03 (C-2), 45.73 (C-11, confirmed by C-H COSY), 51.43 (COO*C*H$_3$), 60.60 (C-12, confirmed by C-H COSY), 76.75 (C-10, confirmed by long-range C-H COSY), 84.48 (C-9, confirmed by long-range C-H COSY) and 174.18 (C-1).

*Reaction of methyl octadeca-9Z,11*E*-dienoate (**5**) with* m*-CPBA (Scheme 2).* A mixture of compound **5** (0.16 g, 0.544 mmol), dichloromethane (10 mL), *m*-CPBA (0.15 g, 0.85 mmol) was stirred for 1 h. Dichloromethane (50 mL) was then added and the reaction mixture was washed with aqueous sodium sulfite solution (5%, 3×10 mL). The organic extract was washed with water (20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated and silica column chromatographic separation (using a mixture *n*-hexane/diethyl ether, 95:5, vol/vol as eluent) of the residue gave a mixture (90% total yield) of methyl 9,10-*Z*-epoxy-octadec-11*E*-enoate (**17**) and methyl 11,12-*E*-epoxy-octadec-9*Z*-enoate (**18**). Spectral analysis was conducted on the mixture of compounds **17** and **18**. IR (neat): 1741 (*s*, C=O, str.), 1457 and 1436 cm^{-1}. ^1H-NMR ($CDCl_3$, δ_H) 0.88 (*t*, $J = 7.0$ Hz, 3H,



$R^2 = (CH_2)_7COOCH_3$; $R^4 = CH_3(CH_2)_5$

SCHEME 2

CH_3), 1.18–1.64 (*m*, 20H, CH_2), 2.07 (*q*, *J* = 6.7 Hz, 2H, 13-*H*$_2$ of **17**), 2.20 (*m*, 2H, 8-*H*$_2$ of **18**), 2.30 (*t*, *J* = 7.5 Hz, 2H, 2-*H*$_2$), 2.81 (*tt*, *J* = 27 Hz, 1H, 12-*H* of **18**), 3.04 (*m*, 1H, 9-*H* of **17** and 12-*H* of **18**), 3.34 (*m*, 10-*H* of **17** and 11-*H* of **18**), 3.66 (*s*, 3H, $COOCH_3$), 5.04 (*t*, *J* = 9.0 Hz, 1H, 10-*H* of **17**), 5.30 (*t*, *J* = 6.5 Hz, 1H, 11-*H* of **17**), 5.70 (*tt*, *J* = 7.7 Hz, 1H, 9-*H* of **18**) and 5.92 (*tt*, *J* = 6.9 Hz, 1H, 12-*H* of **17**); ^{13}C NMR ($CDCl_3$, δ_C) 14.01 (C-18), 22.54 (C-17), 24.83 (C-13), 26.22, 27.86 (C-8 of **18**), 28.71, 28.90, 28.95, 28.99, 29.04, 29.09, 29.16, 31.62/31.69 (C-16), 32.51 (C-13 of **17**), 33.98 (C-2), 51.36 ($COOCH_3$), 54.35 (C-12 of **18**), 57.12 (C-9 of **17**), 58.71(C-10 of **17**), 60.11 (C-11 of **18**), 123.96 (C-11 of **17**), 127.14 (C-10 of **18**), 136.19 (C-9 of **18**), 138.02 (C-12 of **17**) and 174.17 (C-1).

*Preparation of methyl 11,12-*E*-epoxy-octadec-9*Z*-enoate (**18**).* A mixture of methyl 11,12-*E*-epoxy-octadec-9-ynoate (1.2 g, 3.89 mmol, obtained by epoxidation of methyl octadec-11*E*-en-9-ynoate (methyl santalbate) as described elsewhere (9), Lindlar catalyst (15 mg) and ethyl acetate (10 mL) were shaken with hydrogen (90 mL of hydrogen was taken up in 4 h). The solvent was evaporated under reduced pressure, water (25 mL) was added, and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The ethereal layer was dried over sodium sulfate. The filtrate was evaporated, and column chromatographic separation of the residue using a mixture of *n*-hexane and diethyl ether (9:1, vol/vol as eluent) afforded methyl 11,12-*E*-epoxy-octadec-9*Z*-enoate (**18**, 90%). IR (neat) 1740 (*s*, C=O,str.), 1456 and 1435 cm^{-1}. ^{1}H-NMR ($CDCl_3$, δ_H) 0.88 (*t*, *J* = 6.0 Hz, 3H, CH_3), 1.20–1.64 (*m*, 20H, CH_2), 2.20 (*m*, 2H, 8-*H*$_2$), 2.31 (*t*, *J* = 7.5 Hz, 2H, 2-*H*$_2$), 2.81 (*m*, 1H, 12-*H*), 3.34 (*dd*, $J_{11,12}$ = 1.4 Hz, 1H, 11-*H*), 3.67 (*s*, 3H, $COOCH_3$), 5.04 (*t*, *J* = 9.0 Hz, 1H, 10-*H*) and 5.66 (*tt*, *J* = 7.7 Hz, 1H, 9-*H*); ^{13}C NMR ($CDCl_3$, δ_C) 14.06 (C-18), 22.57 (C-17), 24.91 (C-3), 25.91 (C-14), 27.69 (C-8), 28.98, 29.07, 29.10, 29.53, 31.75 (C-16), 32.08 (C-13), 34.07 (C-2), 51.46 ($COOCH_3$), 54.45 (C-11, confirmed by C-H COSY), 60.21 (C-12, confirmed by C-H COSY), 127.17 (C-10, confirmed by C-H COSY), 136.32 (C-9, confirmed by C-H COSY) and 174.33 (C-1).

*Reaction of Oxone® in the presence of methyl pyruvate with methyl 12-hydroxy-octadec-9Z-enoate (**9**), methyl 9-hydroxy-octadec-12Z-enoate (**11**), methyl 12-oxo-octadec-9Z-enoate (**13**) and methyl 9,12-epoxy-octadeca-9,11-dienoate (**15**), as exemplified by the epoxidation reaction of compound **13**.* A mixture of methyl 12-oxo-octadec-9*Z*-enoate (**13**, 0.2 g, 0.65 mmol), acetonitrile (6 mL), aqueous Na_2EDTA solution (4 mL, 4×10^{-4} M), and methyl pyruvate (0.66 g, 0.65 mmol) was stirred for 2 min at room temperature. Sodium hydrogencarbonate (0.26 g, 3.1 mmol) and Oxone® (0.61 g, 0.99 mmol) were added, and the reaction mixture was stirred for 45 min. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 50 mL). The organic layer was washed with water (20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated to give methyl 9,10-*Z*-epoxy-12-oxostearate (**14**, 0.2 g, 95%). IR (neat): 1740 (*s*, ester C=O, str.), 1717 (*s*, C=O str.) and 1173 cm^{-1}. ^{1}H NMR ($CDCl_3$, δ_H) 0.88 (*t*, *J* = 7.0 Hz, 3H, CH_3), 1.20–1.60 (*m*, 20H, CH_2), 2.31 (*t*, *J* = 7.4 Hz, 2H, 2-*H*$_2$), 2.47 (t, *J* = 7.3 Hz, 2H, 13-*H*$_2$), 2.60–2.66 (*dd*, *J* = 5.3 Hz, 2H, 11-*H*$_2$), 2.98 (*m*, 1H, 9-*H*), 3.28 (m, 1H, 10-*H*) and 3.66 (*s*, 3H, $COOCH_3$); ^{13}C NMR ($CDCl_3$, δ_C) 14.03 (C-18), 22.49 (C-17), 23.55 (C-14), 24.88 (C-3), 26.40 (C-7), 27.97 (C-8), 28.83, 29.00, 29.14, 29.26, 31.58 (C-16), 34.03 (C-2), 41.61 (C-11, confirmed by C-H COSY), 43.33 (C-13), 51.46 ($COOCH_3$), 52.33 (C-10), 56.49 (C-9), 174.23 (C-1) and 208.48 (C-12).

*Methyl 9,10-Z-epoxy-12-hydroxystearate (**10**) from methyl ricinoleate (**9**).* Yield: 90%; IR (neat): 3449 (*s*, OH), 1740 (*s*, C=O, str.), 1460 (*m*), 1437 (*m*), 1201 (*m*) and 1174 (*m*) cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H) 0.88 (*t*, *J* = 6.6 Hz, 3H, CH_3), 1.21–1.83 (*m*, 20H, CH_2), 2.31 (*t, J* = 7.5 Hz, 2H, 2-*H*$_2$), 2.92 (*m*, 1H, 9-*H*), 3.14 (*m*, 1H, 10-*H*), 3.66 (*s*, 3H, $COOCH_3$) and 3.87 (*m*, 1H, C*H*OH); ^{13}C NMR ($CDCl_3$, δ_C) 14.09 (C-18), 22.63 (C-17), 24.89 (C-3), 25.55/25.63 (C-14), 26.42 (C-7), 27.91 (C-8), 28.04, 29.02, 29.17, 29.30, 29.32, 31.84 (C-16), 34.05 (C-2), 34.84/35.24 (C-11), 37.46/37.82 (C-13), 51.46 ($COOCH_3$), 54.55/55.38 (C-10), 56.37/57.19 (C-9), 69.97/70.78 (C-12) and 174.29 (C-1).

*Methyl 12,13-Z-epoxy-9-hydroxystearate (**12**) from methyl* iso-*ricinoleate (**11**).* Yield: 90%; IR (neat) 3454 (*s*, OH), 1739 (*s*, C=O, str.), 1462, 1437, 1251 cm^{-1}; ^{1}H NMR ($CDCl_3$, δ_H) 0.90 (*t*, *J* = 6.9 Hz, 3H, CH_3), 1.20–1.76 (*m*, 24H, CH_2), 2.00 (O-*H*, D_2O exchangeable), 2.30 (*t, J* = 7.5 Hz, 2H, 2-*H*$_2$), 2.95 (*m*, 2H, 12-*H* and 13-*H*), 3.60 (*m*,1H, C*H*OH) and 3.66 (*s*, 3H, $COOCH_3$); ^{13}C NMR ($CDCl_3$, δ_C) 14.03 (C-18), 22.64 (C-17), 24.31 (C-11), 24.94 (C-3), 25.69/26.29 (C-7), 27.81 (C-14), 29.09, 29.25, 29.48, 31.74/31.99 (C-16), 34.11 (C-2), 37.54 (C-8, C-10), 51.49 ($COOCH_3$), 57.17/57.36 (C-12), 57.48/57.88 (C-13), 71.13/71.54 (C-9) and 174.32 (C-1) as supported by data from Tulloch and Mazurek (12).

*Methyl 9,12-dioxo-octadec-10Z-enoate (**16**) from methyl 9,12-epoxy-octadeca-9,11-dienoate (**15**).* Yield: 90%; IR (neat): 1736 (*s*, C=O, ester, str.), 1700 (*s*, C=O, oxo, str.), 1609 (*m*), 1437 (*m*) and 1395 (*m*) cm^{-1}. ^{1}H NMR ($CDCl_3$, δ_H) 0.88 (*t*, *J* = 6.8 Hz, 3H, CH_3), 1.29–1.32 (*m*, 12H, CH_2), 1.74–1.81 (*m*, 6H, CH_2), 2.29 (*t*, *J* = 7.5 Hz, 2H, 2-*H*$_2$), 2.53 (*t*, *J* = 7.1 Hz, 4H, 8-*H*$_2$ and 13-*H*$_2$), 3.66 (*s*, 3H, $COOCH_3$) and 6.31 (*s*, 2H, 10-*H* and 11-*H*); ^{13}C NMR ($CDCl_3$, δ_C) 14.04 (C-18), 22.49 (C-17), 23.39, 23.49 (C-7, C-14), 24.88 (C-3), 28.79, 28.89, 28.93, 29.03, 31.60 (C-16), 34.04 (C-2), 42.48, 42.60 (C-8, C-13), 51.44 ($COOCH_3$), 135.56, 135.79 (C-10, C-11), 174.24 (C-1), 203.03 and 203.07 (C-9 and C-12). Similar data were reported elsewhere (3).

RESULTS AND DISCUSSION

Treatment of the various unsaturated fatty esters with Oxone® in the presence of either trifluoroacetone or methyl pyruvate afforded the corresponding epoxides in very high yields as summarized in Scheme 1. The NMR spectral properties of

methyl 9,10-*Z*-epoxystearate (**2**) and those of methyl 9,10-*Z*;12,13-*Z*-diepoxystearate (**4**) from methyl oleate and methyl linoleate, respectively, were similar to those reported in the literature (2,13). From the results of the ^{13}C NMR analysis of the products, it was apparent from the shifts of the epoxy carbon nuclei that the reaction of oxone with methyl linoleate was not stereoselective. Seven signals (in the region of δ_C 54.19–56.99) instead of four were found in the spectrum for the shifts of the epoxy carbon atoms. It was possible to differentiate the signals for the C-10/C-12 epoxy carbon nuclei from those originating from the C-9/C-13 epoxy carbon atoms by the C-H COSY technique.

Epoxidation of the conjugated 9-*Z*,11-*E*-diene ester (compound **5**) with *m*-CPBA gave a mixture of mono-epoxy olefinic esters (compounds **17** and **18**, see Scheme 2) only. Compounds **17** and **18** could not be further epoxidized by *m*-CPBA. However, when the same substrate (compound **5**) was reacted with Oxone® in trifluoroacetone, both double bonds in the substrate were readily epoxidized to give the corresponding *Z,E*-epoxy derivative (**6**) in almost quantitative yield. Compound **6** comprised a mixture of stereoisomers as reflected by the seven signals arising from the shifts of the epoxy carbon atoms (C-9, C-10, C-11, and C-12) which appeared at δ_C 54.76–57.75. In an effort to identify the various carbon chemical shifts of the mixture of compounds **17** and **18**, it was necessary to synthesize one of these positional isomers. Compound **18** was prepared by semihydrogenation of methyl 11,12-*E*-epoxy-octadec-9-ynoate, which was obtained by *m*-CPBA epoxidation of methyl santalbate as reported elsewhere (14). By comparing the results of the shifts of the critical carbon atoms in the ^{13}C NMR spectrum of compound **18**, it was possible to identify many of the carbon signals arising from compounds **17** and those belonging to compound **18**.

The reactions involving methyl ricinoleate (**9**) , *iso*-ricinoleate (**11**), and methyl 12-oxo-octadec-9*Z*-enoate (**13**) with Oxone® were carried out in the presence of methyl pyruvate instead of trifluoroacetone. Reactions with Oxone® in methyl pyruvate were as effective and high yielding as those observed when conducted in the presence of trifluoroacetone. Methyl ricinoleate (**9**) reacted with Oxone® to give methyl 9,10-*Z*-epoxy-12-hydroxystearate (**10**). Of great interest was the reaction of methyl *iso*-ricinoleate (**11**) with Oxone® in methyl pyruvate. This reaction gave exclusively the corresponding epoxy-hydroxystearate derivative (**12**), which was identified by the IR absorption at 3454 cm^{-1} for the stretching vibration of the O-H group, the shifts of the epoxy protons at δ_H 2.95 (12-H and 13-H), and from the shifts of the carbon nuclei at δ_C 57.17/57.36 (C-12) and 57.48/57.88 (C-13). Unlike the reaction of methyl *iso*-ricinoleate with *m*-CPBA, the reaction with Oxone® in the presence of methyl pyruvate did not give rise to the frequently reported tetrahydrofuranyl derivative (methyl 9,12-epoxy-13-hydroxystearate). In the reaction of methyl *iso*-ricinoleate with *m*-CPBA, the hydroxy group acts as a nucleophile in an intramolecular cyclization reaction involving the dimethylene interrupted hydroxy-epoxy intermediate (1,15). The latter reaction is catalyzed by the presence of a weak acid (chlorobenzoic acid) which is found in *m*-CPBA (as a stabilizer or by-product). While in the case of Oxone® with methyl pyruvate, the presence of sodium hydrogencarbonate prevents the epoxy group from being protonated and thus preventing the cyclisation reaction to occur as observed in the case of *m*-CPBA.

Reaction of methyl 12-oxo-octadec-9*Z*-enoate (**13**) with Oxone® furnished the corresponding epoxy-oxo derivative (**14**), which was confirmed by the spectral data. By using C-H COSY technique, the shift of the C-11 carbon atom was shown to correspond to the signal at δ_C 41.61. This value was erroneously assigned in an earlier study by us when the same substrate was epoxidized with *m*-CPBA under concomitant ultrasonic irradiation (15). Ring-opening reaction of a C_{18} 2,5-disubstituted furanoid fatty ester (compound **15**) with Oxone® furnished methyl 9,12-dioxo-octadec-10*Z*-enoate (**16**) in 90% yield. The *Z*-configuration of the double bond was confirmed from the following physical data: a singlet at δ_H 6.31 (2H, 10-*H*, 11-*H*) and carbon shifts at δ_C 135.56, 135.79 (C-10, C-11).

It can be concluded from the above results that Oxone® is a stronger epoxidizing agent than *m*-CPBA. Oxone® in the presence of trifluoroacetone or methyl pyruvate converts all double bonds into the corresponding epoxides including conjugated diene systems and in the case of methyl *iso*-ricinoleate (a dimethylene interrupted hydroxy-olefin ester) epoxidation with Oxone® yields exclusively the corresponding epoxy-hydroxy derivative. Furthermore, the isolation of the epoxy derivatives from reactions involving Oxone® is a facile procedure resulting in high product yield.

ACKNOWLEDGMENTS

This work has been supported by the Lipid Research Fund, the Research Grants Committee of the University of Hong Kong, and the Research Grant Council of Hong Kong.

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[Received November 17, 1997, and in final revised form and accepted May 1, 1998]

Callyspongynes A and B: New Polyacetylenic Lipids from a Southern Australian Marine Sponge, *Callyspongia* sp.

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ABSTRACT: A *Callyspongia* sp. collected by SCUBA off Barwon Heads, Australia, has afforded two new polyacetylenic lipids, callyspongynes A and B, the structures of which were assigned by spectroscopic analysis and chemical derivatization. *Lipids 33*, 639–642 (1998).

Polyacetylenic lipids represent a class of natural product common to marine sponges, with many examples reported in recent years of this structure class varying in chain length, degrees of unsaturation, and biological activity (antimicrobial, antitumor, antifungal, and antiviral). Sponge genera known to feature polyacetylenic lipids include *Petrosia* (1–8), *Cribrochalina* (9–12), *Tetrosia* (13), *Xestospongia* (14,15), *Siphonochalina* (16), *Reniera* (17), *Haliclona* (18), and *Callyspongia* (19). This latter observation together with our recent experience in isolating novel lipids from a southern Australian *Callyspongia* sp. (20) prompted an investigation of the lipid content of another *Callyspongia* sp. in our collection. This report describes the results of a chemical analysis of a *Callyspongia* sp. collected by SCUBA at ~30 m depth off Barwon Heads (Victoria, Australia).

EXPERIMENTAL PROCEDURES

All solvents were redistilled before use. Rapid silica filtrations were carried out using Kieselgel 60 silica gel loaded into a sintered glass funnel and eluted under low vacuum. Solid phase extraction was carried out using Alltech Maxiclean silica 900 mg cartridges (Alltech Corp., Deerfield, IL) attached to luer lock syringes. High-performance liquid chromatography (HPLC) was performed on an ISCO 2350 solvent delivery system (ISCO Corp., Lincoln, NE) equipped with a rheodyne injector, Waters 401 differential refractometer and Spectra Physics 200 programmable UV/VIS wavelength detector, and recorded on either Chart V3.2 data collection package using an AD Instruments Mac Lab/2E operating on an Apple Macintosh LC; or a Data Acquisition Plotting and Analysis (DAPA) package operating on an Ipex 286PC unit under DOS 5.0. Thin-layer chromatography was carried out on Machery-Nagel Duren Alugram Sil G/UV_{254} plates and visualized by both short (254 nm)- and long (365 nm)-wavelength ultraviolet light, as well as spraying with 5% vanillin in H_2SO_4/H_2O (50% wt/vol) and heating at 100°C for 2–5 min. Chiroptical measurements $[\alpha]_D$ were obtained on a Jasco DIP-1000 digital polarimeter in a 100 mm × 2 mm cell. Infrared (IR) spectra were acquired on a Perkin-Elmer 1600 Fourier transform IR spectrophotometer. Nuclear magnetic resonance (NMR) experiments were performed on either a Varian Inova 400, Varian Unity 300, or a Varian Unity plus 400 spectrometer in the solvents indicated, with ^1H NMR referenced to residual solvent resonances and ^{13}C NMR to carbon resonances.

Electrospray ionization (ESI) (1:1 acetonitrile/water matrix) mass spectra were acquired on a Micromass Quattro II mass spectrometer at the cone voltages indicated. Electron impact ionization (EI) (70 eV) mass spectra were acquired on a JEOL ax-505H mass spectrometer. High-resolution ESI (1:1 acetonitrile/water matrix) mass measurements were recorded on a Bruker BioApex 47E FT mass spectrometer at a cone voltage of 100 kV. Gas chromatography/mass spectrometry (MS) analysis was performed on a Hewlett-Packard 5890 gas chromatograph with mass selective detector MSD HP 5970 MS and a split/splitless injector for capillary columns, using a fused-silica column, 25 m × 0.2 mm HP–5 (cross-linked 25% phenylmethyl silicone, 0.33 mm film thickness).

Collection, extraction, and isolation. A marine sponge *Callyspongia* sp. (class Demonspongia: order Haplosclerida: family Callyspongidae) (32.5 g) was collected by SCUBA (–30 m) off Barwon Heads. The sponge was diced, steeped in ethanol, and stored at –18°C until required, and a voucher specimen (registry number F77048) deposited with the Museum of Victoria. The decanted ethanol extract was concentrated *in vacuo* and partitioned into CH_2Cl_2-, methanol-, and H_2O-soluble fractions. The CH_2Cl_2-soluble fraction was further partitioned with CH_3CN, and the CH_3CN-soluble material fractionated by rapid silica filtration (10% stepwise gradient from hexane to ethyl acetate) followed by HPLC (2 mL/min elution with 15% ethyl acetate/hexane through a Phenomenex 5 μ silica 250 × 10 mm column) (Phenomenex, Torrance, CA) to yield a mixture of callyspongynes (24 mg, 0.074%) as a colorless oil. $[\alpha]_D$–3.9° (c = 0.24 $CHCl_3$); EIMS (70 eV) mass spectrum *m/z* 492 (1%, minor homolog),

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Abbreviations: EI, electron impact ionization; EIMS, electron impact mass spectrometry; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IR, infrared; MS, mass spectrometry; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetic acid; NMR, nuclear magnetic resonance.

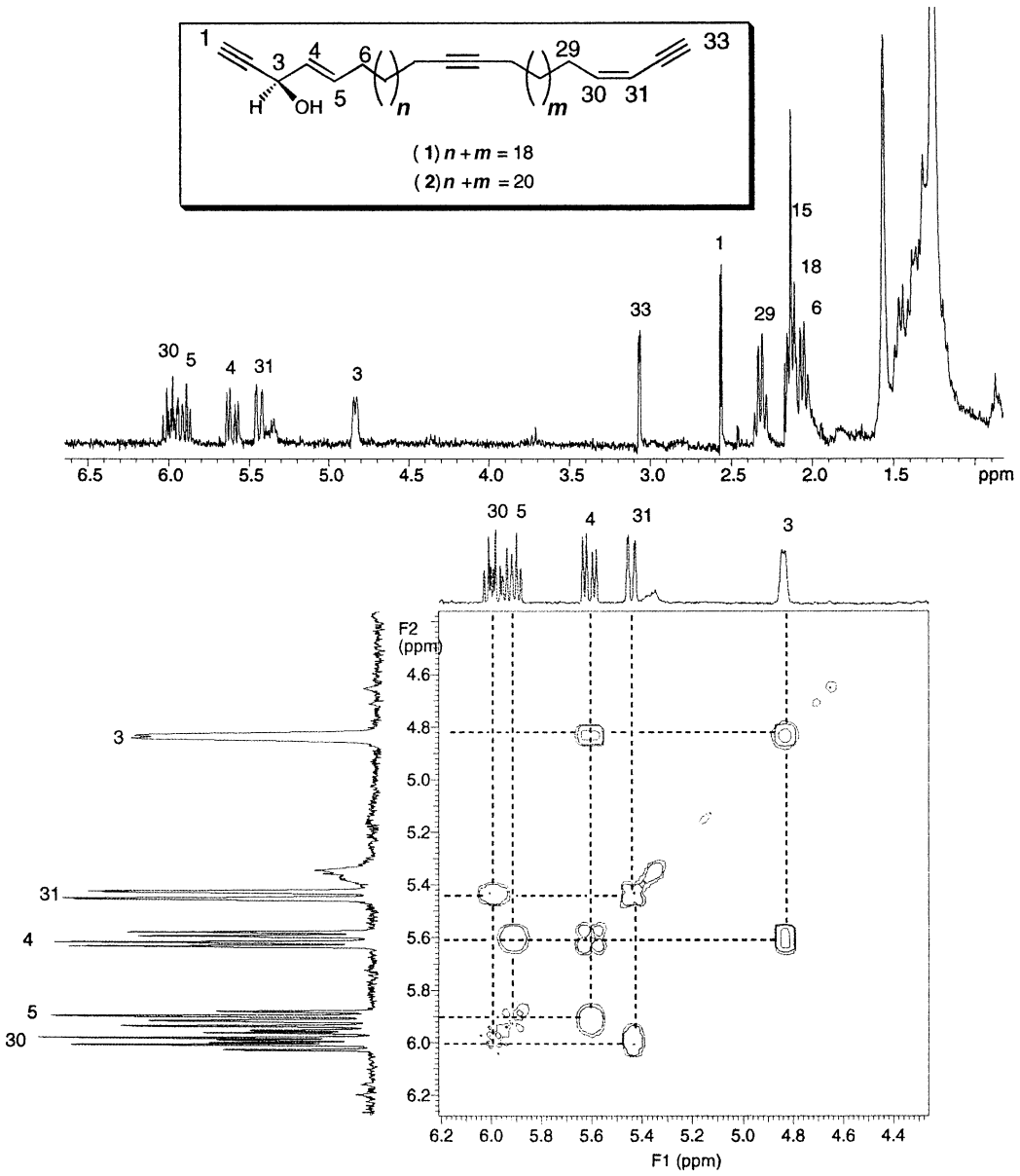

FIG. 1. ^{1}H ($CDCl_3$, 400 MHz) and an expansion of the selected homonuclear correlation spectroscopy nuclear magnetic resonance spectra of callyspongynes.

464 (4%, major homolog), 446(1), 421 (2), 381 (1), 279 (2), 252(5), 187 (10), 173 (15), 95 (56), 54 (100); high-resolution EIMS *m/z* 464.4035 ($C_{33}H_{52}O$ requires 464.4021); ESI (+) *m/z* 493 (minor homolog, M + H), 465 (major homolog, M + H); IR (film) ν_{max} 3300, 2900 cm^{-1}; ^{1}H, ^{13}C and two-dimensional NMR see Table 1.

Derivatization. A solution of dicyclohexylcarbodiimide (1.5 eq in 15 mL of dry CH_2Cl_2) was added to a solution of callyspongynes (1 eq, 9 mg in 0.5 mL of dry CH_2Cl_2) containing 4-dimethylaminopyridine (1 mg) and either (*R*)- or (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) (1.5 eq, 3.5 mg), and the solution was stirred for 16 h at 25°C. The reaction product obtained from standard aqueous workup was purified by HPLC (2 mL/min elution with 15% ethyl acetate/ hexane through a Phenomenex 5 µ silica 250 × 10 mm column) to yield the (*R*)-MTPA (6 mg, 66%) and (*S*)-MTPA (7 mg, 77%) esters, respectively. Selected Δδ(*S-R*) ^{1}H NMR shifts for the Mosher esters are shown in Figure 2.

RESULTS AND DISCUSSION

Solvent partitioning of the ethanol extract of the *Callyspongia* sp. followed by rapid silica filtration and silica HPLC yielded a mixture of callyspongynes as optically active colorless oil. Examination of the NMR data (see Fig. 1) revealed resonances consistent with a secondary alcohol [^{1}H: δ 4.84 (*dd*); ^{13}C: 62.8 (*d*) ppm], as well as a disubstituted [^{13}C: (80.2) (2*s*) ppm] and two monosubstituted acetylenes [^{1}H: δ

TABLE 1
Nuclear Magnetic Resonance ($CDCl_3$, 400 MHz) Data for Callyspongynes[a]

No.	^{13}C (ppm)	^{1}H δ, *m*, *J* (Hz)	COSY[b]	gHMBC[c] ^{1}H to ^{13}C
1	74.0	2.56, *d* 2.2	3H	C2, C3
2	83.3			
3	62.8	4.84, *dd*, 6.4, 2.2	4H	C2, C3
4	128.3	5.60, *dd*, 15.1, 6.4	3H, 5H, $7H_2$	C3, C6
5	134.7	5.90, *dt*, 15.1, 6.8	4H, $6H_2$, $7H_2$	C3, C6
6	31.9	2.06, *m*	5H,*	C4, C5,*
7–14	29.6–22.7	1.29–1.40, *m*	*	*
15	18.7	2.15, *t*, 6.8	*	*
16/17	80.2			C15, C18
18	18.7	2.14, *t*, 6.8	*	*
19–28	29.6–22.7	1.29–1.40, *m*	*	*
29	30.2	2.32, *dt*, 7.5, 7.5	30H, 31H*	C30, C31*
30	146.2	5.99, *ddt*, 10.6, 0.8, 7.5	$29H_2$, 31H, 33H	C29, C33
31	108.0	5.44, *dd*, 10.6, 1.4	$29H_2$, 30H, 33H	C29, C33
32	81.1			
33	80.3	3.06, *dd*, 1.4, 0.8	30H, 31H	C30, C31

[a]Assignments were supported by ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC), distortionless enhancement by polarization transfer (DEPT) 135° and 90° experiments. *Overlapping methylene crosspeaks.
[b]Homonuclear correlation spectroscopy.
[c]^{1}H-detected heteronuclear multiple-bond coherence.

2.56 (*d*); ^{13}C: 74.0 (*d*) and 83.3 (*s*) ppm; and ^{1}H: δ 3.06 (*dd*); ^{13}C: 80.3 (*d*) and 81.1 (*s*) ppm]. These observations were further substantiated by IR absorptions (3300 and 2900 cm^{-1}). The NMR data also established the presence of *cis* [^{1}H: δ 5.99 and δ 5.44 J_{cis} = 10.6 Hz; ^{13}C: 146.2 (*d*) and 107.2 (*d*) ppm] and *trans* [^{1}H: δ 5.60 and δ 5.90 J_{trans} = 15.1 Hz; ^{13}C: 128.0 (*d*) and 134.7 (*d*) ppm] 1,2-disubstituted double bonds. Analysis of the two-dimensional NMR data (Table 1 and Fig. 1) established the terminal structure fragments and positioned the remaining acetylenic functionality within an unbranched carbon chain. ESI MS revealed the presence of two homologs, an observation confirmed by both direct insertion and gas chromatography EI MS. High-resolution EIMS provided experimental evidence for the molecular formula of the major component callyspongyne A (**1**) ($C_{33}H_{52}O$), and by comparison defined a molecular formula for the minor homolog callyspongyne B (**2**) ($C_{35}H_{56}O$). Repeated efforts to analyze fragmentation patterns and employ tandem mass spectrometry techniques failed to unambiguously position the disubstituted acetylenic unit along the carbon chain (Fig. 2).

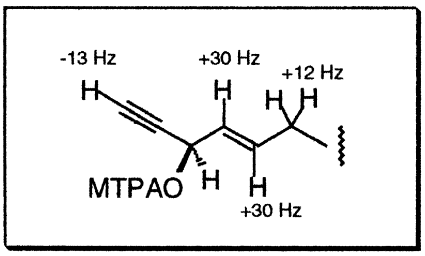

FIG. 2. ^{1}H nuclear magnetic resonance (NMR) Δδ (*S*-*R*) Mosher ester analysis. MTPA, methoxy-α-(trifluoromethyl)phenyl-acetic acid.

Application of the advanced Mosher method (21) for assignment of absolute stereochemistry required preparation of the diastereomeric MTPA esters of the callyspongyne mixture, and comparison of the resulting NMR spectra. The Δδ values as depicted in Figure 2 were diagnostic for a 3*R* absolute stereochemistry. This assignment of absolute stereochemistry to the callyspongynes when considered in conjunction with the experimentally measured -ve optical rotation was consistent with the absolute stereochemistry assigned to acetylenic lipids possessing a common C1 to C7 subunit reported from other marine sponges (1–5).

Although callyspongynes A and B (**1** and **2**) incorporate terminal acetylenic functionalities common to other polyacetylenic lipids from marine sponges, the isolated "central" acetylenic functionality is uncommon. The ecological role of such novel unsaturated lipids in marine sponges remains unknown, although in at least one instance it has been speculated that they serve as chemical defense agents to both the sponge itself and to predatory nudibranches (8).

ACKNOWLEDGMENTS

The assistance of L. Goudie in specimen collection and taxonomic classification is greatly appreciated. The Australian Research Council funded this research.

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[Received February 2, 1998, and in final revised form and accepted April 24, 1998]

ARTICLE

Comparative Effects of α- and γ-Linolenic Acids on Rat Liver Fatty Acid Oxidation

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ABSTRACT: It has been reported that both n-3 and n-6 octadecatrienoic acids can increase hepatic fatty acid oxidation activity. It remains unclear, however, whether different enzymes in fatty acid oxidation show a similar response to n-3 and n-6 octadecatrienoic acids. The activity of hepatic fatty acid oxidation enzymes in rats fed an oil mixture rich in α-linolenic acid (18:3n-3) and borage oil rich in γ-linolenic acid (18:3n-6) was therefore compared to that in rats fed an oil mixture rich in linoleic acid (18:2n-6) and a saturated fat (palm oil) in this study. Linseed oil served as the source of 18:3n-3 for the oil mixture rich in this octadecatrienoic acid and contained 30.6% 18:3n-3 but not 18:3n-6. Borage oil contained 25.7% 18:3n-6 and 4.5% 18:3n-3. Groups of seven rats each were fed diets containing 15% various fats for 15 d. The oxidation rate of palmitoyl-CoA in the peroxisomes was higher in rats fed a fat mixture rich in 18:3n-3 (3.03 nmol/min/mg protein) and borage oil (2.89 nmol/min/mg protein) than in rats fed palm oil (2.08 nmol/min/mg protein) and a fat mixture rich in 18:2n-6 (2.15 nmol/min/mg protein). The mitochondrial palmitoyl-CoA oxidation rate was highest in rats fed a fat mixture rich in 18:3n-3 (1.93 nmol/min/mg protein), but no significant differences in this parameter were seen among the other groups (1.25–1.46 nmol/min/mg protein). Compared to palm oil and fat mixtures rich in 18:2n-6, a fat mixture rich in 18:3n-3 and borage oil significantly increased the hepatic activity of carnitine palmitoyltransferase and acyl-CoA oxidase. Compared to palm oil and a fat mixture rich in 18:2n-6, a fat mixture rich in 18:3n-3, but not fats rich in 18:3n-6, significantly decreased 3-hydroxyacyl-CoA dehydrogenase activity. Compared to palm oil and a fat mixture rich in 18:2n-6, borage oil profoundly decreased mitochondrial acyl-CoA dehydrogenase activity, but a fat mixture rich in 18:3n-3 increased it. 2,4-Dienoyl-CoA reductase activity was significantly lower in rats fed palm oil than in other groups. Compared to other fats, borage oil significantly increased Δ^3,Δ^2-enoyl-CoA isomerase activity. Activity was also significantly higher in rats fed 18:2n-6 oil than in those fed palm oil. It was confirmed that both dietary 18:3n-6 and 18:3n-3 increased fatty acid oxidation activity in the liver. These two dietary octadecatrienoic acids differ considerably, however, in how they affect individual fatty acid oxidation enzymes.

Lipids 33, 647–654 (1998).

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We demonstrated previously that dietary fats rich in n-3 octadecatrienoic acid (α-linolenic acid) increased the fatty acid oxidation rate through both the mitochondrial and peroxisomal pathways in liver homogenates in rats (1,2). We also showed that dietary α-linolenic acid increased the hepatic activity of enzymes in the β-oxidation pathway, including carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase, Δ^3,Δ^2-enoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase, but unexpectedly decreased 3-hydroxyacyl-CoA dehydrogenase activity. Fish oil containing very long-chain n-3 polyunsaturated fatty acids has been amply demonstrated to increase the mitochondrial (3,4) and peroxisomal (4–7) fatty acid oxidation rate in rat liver. It is thus plausible that various n-3 polyunsaturated fatty acids can increase hepatic fatty acid oxidation in the rat. By contrast, we (1,2) previously showed that a dietary fat rich in 18:2n-6 (safflower oil) only slightly but inconsistently increased hepatic mitochondrial and peroxisomal fatty acid oxidation activity. Takada *et al*. (8), however, demonstrated that a dietary fat (of fungal origin) rich in γ-linolenic acid (18:3n-6) significantly increased the peroxisomal fatty acid oxidation rate and carnitine palmitoyltransferase activity in rat liver. Thus, it is plausible that both n-3 and n-6 octadecatrienoic acids can increase hepatic fatty acid oxidation activity. It remains unclear, however, whether different enzymes in fatty acid oxidation show a similar response to n-3 and n-6 octadecatrienoic acids. We therefore compared the effect of dietary α- and γ-linolenic acids on the activity of different hepatic fatty acid oxidation enzymes using linseed and borage oils as the sources of these linolenic acids in the rat.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitic acid was purchased from Amersham International (Bucks, United Kingdom). [1-^{14}C]Palmitoyl-CoA and nonradiolabeled palmitoyl- and linoleyl-CoA were prepared according to the method of Kawaguchi *et al*. (9). Acetyl-CoA was prepared by acylating CoA with acetic anhydride, acetoacetyl-CoA with diketene, and crotonyl-CoA with crotonic anhydride. Sorboyl-CoA, *trans*-2-octenoyl-CoA, and *trans*-3-hexenoyl-CoA were prepared using the mixed anhydride method (10). Malonyl-CoA was purchased

from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin fraction V (essential fatty acid-free) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Linseed oil rich in α-linolenic acid was purchased from Nakarai Tesque (Kyoto, Japan). Palm and safflower oils were the gift of Nisshin Oil Co. (Tokyo, Japan). Borage oil rich in γ-linolenic acid was donated by Nippon Synthetic Chemical Industry Co. (Osaka, Japan).

Animals and diets. Male Sprague-Dawley rats obtained from Charles River Japan (Kanagawa, Japan) were housed individually in animal cages in a room with controlled temperature (20–22°C), humidity (55–65%), and lighting (light on from 07:00 to 19:00 h), and fed a commercial nonpurified diet (Type NMF; Oriental Yeast Co., Tokyo, Japan). Fat content of the nonpurified diet was 5.2% and its fatty acid composition was (in wt%): 16:0, 14.8; 16:1n-7, 2.1; 18:0, 2.7; 18:1n-9, 22.2; 18:2n-6, 44.5; 18:3n-6, 0.1; 18:3n-3, 6.2; 20:4n-6, 1.1; 20:5n-3, 3.0; 22:4n-6, 0.1; 22:5n-6, 0.2; 22:5n-3, 0.4; 22:6n-3, 2.7. After 7 d of acclimatization, rats were fed purified experimental diets. The composition of the experimental diet was (in wt%): fat, 15; casein, 20; cornstarch, 15; sucrose, 43; cellulose, 2; mineral mixture (11), 3.5; vitamin mixture (11), 1; DL-methionine, 0.3; and choline bitartrate, 0.2. Animals were fed a diet containing palm oil; a fat mixture (18:2 oil) composed of palm and safflower oils (12:88, w/w); another fat mixture (18:3n-3 oil) composed of linseed, palm, and safflower oils; and tripalmitin (54:39:2:5, by wt), and borage oil for 15 d. The composition of polyunsaturated fatty acid was the sole variable, and the total polyunsaturated fatty acid content was the same in fat mixtures rich in 18:2n-6 or 18:3n-3 and a fat rich in 18:3n-6 (borage oil) (Table 1). We followed our institute's guidelines in laboratory animal care and use.

Enzyme assays. At the termination of the experimental period, rats were anesthetized lightly using diethylether and bled from the abdominal aorta, and livers were quickly excised. About 3 g each of liver was homogenized with 7 vol of 0.25 M sucrose and centrifuged at 500 × *g* for 10 min. The supernatant was recentrifuged at 9000 × *g* for 10 min to isolate mitochondria. The mitochondrial fraction was washed twice with 0.25 M sucrose containing 1 mM EDTA and 3 mM Tris-HCl (pH 7.0) and finally suspended in the same medium to give a protein concentration of 20–25 mg/mL. Mitochondrial and peroxisomal fatty acid oxidation rates were measured using a 500 × *g* supernatant fraction of liver homogenates, with [1-^{14}C]palmitoyl-CoA (0.2 mM) as a substrate, according to the method of Mannaerts *et al*. (12) as detailed elsewhere (1). The supernatant fraction of liver homogenates obtained after centrifugation at 500 × *g* for 10 min was used to measure fatty acid oxidation enzyme activity except in the case of acyl-CoA dehydrogenase (E.C. 1.3.99.3). Because acyl-CoA dehydrogenase is primarily a mitochondrial enzyme (13) and the assay using the 500 × *g* supernatant fraction as an enzyme source gave an extremely high blank value, this enzyme was assayed in an isolated mitochondrial fraction as an enzyme source. Carnitine palmitoyltransferase (E.C. 2.3.1.21) activity was measured using both the 500 × *g* supernatant and mitochondrial fractions. Carnitine palmitoyltransferase, acyl-CoA dehydrogenase, and acyl-CoA oxidase (E.C. 1.3.3.6) activities were measured according to the methods of Markwell *et al*. (14), Dommes and Kunau (15), and Hashimoto *et al*. (16) using palmitoyl-CoA as a substrate. Enoyl-CoA hydratase (E.C. 4.2.1.17) activity, using crotonyl-CoA and *trans*-2-octenoyl-CoA substrates (17), and 3-hydroxyacyl-CoA dehydrogenase (E.C. 1.1.1.35)(18) activity using acetoacetyl-CoA substrate were analyzed according to the method of Osumi and Hashimoto (17). Sorboyl-CoA for 2,4-dienoyl-CoA reductase (E.C. 1.3.1.34) (19) and *trans*-3-hexenoyl-CoA for Δ^3,Δ^2-enoyl-CoA isomerase (E.C. 5.3.3.8) (20) were used as substrates in assaying activity. The activity of succinate dehydrogenase (E.C. 1.3.99.1) (21), a marker enzyme for mitochondria, was measured in both the 500 × *g* supernatant and mitochondrial fraction to calculate mitochondrial recovery from the 500 × *g* supernatant (1). The activities of fatty acid synthetase (22), malic enzyme (E.C. 1.1.1.40) (23), glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) (24), and pyruvate kinase (E.C. 2.7.1.40) (25) were measured using a 9000 × *g* supernatant fraction of liver homogenate as an enzyme source as detailed elsewhere (1,2,26). Mitochondrial and peroxisomal oxidation rates of fatty acid were measured using nonfreeze-thawed enzyme preparations on the day rats were sacrificed. Other enzymes were assayed in enzyme preparations that had been stored at –40°C up to 10 d.

Lipid analysis. Commercial enzyme kits obtained from Wako Pure Chemical (Osaka, Japan) were used in assaying serum concentrations of cholesterol (Cholesterol C-test Wako) and free fatty acid (NEFA C-test Wako). Serum and liver lipids were extracted and purified (27). Triacylglycerol and phospholipid contents in the lipid extract were determined as described elsewhere (28,29). Cholesterol content in the liver lipid extract was analyzed enzymatically as detailed elsewhere (29). Triacylglycerol and phospholipid in the liver lipid extract were separated by thin-layer chromatography, and the fatty acid composition of these lipid molecules was determined using gas–liquid chromatography (28).

TABLE 1
Fatty Acid Composition of Dietary Fats

Fatty acids (wt%)	Palm oil	18:2n-6 Oil[a]	18:3n-3 Oil[b] (fat mixture)	Borage oil
16:0	45.3	11.6	11.6	10.7
16:1n-7	0.1	—	—	0.4
18:0	4.4	2.7	2.7	3.4
18:1n-9	39.1	16.1	16.4	16.5
18:2n-6	9.4	69.1	38.6	38.7
18:3n-6	—	—	—	25.7
18:3n-3	0.3	0.2	30.6	4.5
Total PUFA[c]	9.7	69.3	69.2	68.9

[a]Mixture of palm and safflower oils (12:88, w/w).
[b]Mixture of linseed, palm, and safflower oils, and tripalmitin (54:39:2:5, by wt).
[c]PUFA, polyunsaturated fatty acids.

Statistical analysis. The examination of significant differences of means with a pooled estimate of variance was established according to the methods of Snedecor and Cochran (30) for one-way classifications as detailed elsewhere (31).

RESULTS

There were no significant differences in average food intake and growth among the groups of rats fed different dietary fats (Table 2). Liver weight was significantly lower in rats fed 18:2n-6 oil than in those fed palm and borage oils. But no significant difference in this parameter was detected between rats fed 18:2n-6 oil and 18:3n-3 oil.

Effect of dietary α- and γ-linolenic acids on hepatic palmitoyl-CoA oxidation rate and fatty acid oxidation enzyme activity. Owing to the considerable differences detected in liver weight among groups, the effect of dietary fats on hepatic enzyme was estimated comparing both specific (nmol/min/mg protein) and total (μmol/min/100 g body weight) activity. Although only the value for specific activity is presented in each table and figure, similar results regarding the effect of α- and γ-linolenic acids on enzyme activity were confirmed when it was expressed as total activity. Compared to palm and 18:2n-6 oils, an oil mixture rich in α-linolenic acid and borage oil rich in γ-linolenic acid significantly increased specific and total activities of hepatic peroxisomal palmitoyl-CoA oxidation rate (Fig. 1), carnitine palmitoyltransferase, and acyl-CoA oxidase activity (Table 3). Compared to palm and 18:2n-6 oil, 18:3n-3 oil significantly increased specific and total activities of mitochondrial acyl-CoA dehydrogenase, whereas borage oil greatly decreased them. By contrast, dietary α-linolenic acid greatly reduced 3-hydroxyacyl-CoA dehydrogenase activity, but borage oil left it unchanged. Mitochondrial oxidation rate was significantly higher in rats fed 18:3n-3 oil than in the other groups regardless of the way the value was expressed. Compared to other fats, borage oil failed to increase the specific and total activities in the mitochondrial fatty acid oxidation rate, however. Enoyl-CoA hydratase and 3-ketoacyl-CoA thiolase activities were highest in rats fed 18:3n-3 oil, but one-way analysis of variance showed no significant difference in the specific and total enzyme activities among groups. 2,4-Dienoyl-CoA reductase activity was significantly lower in rats fed palm oil than in other groups. Compared to other fats, borage oil significantly increased Δ^3,Δ^2-enoyl-CoA isomerase activity. Activity was also significantly higher in rats fed 18:2n-6 oil than in those fed palm oil.

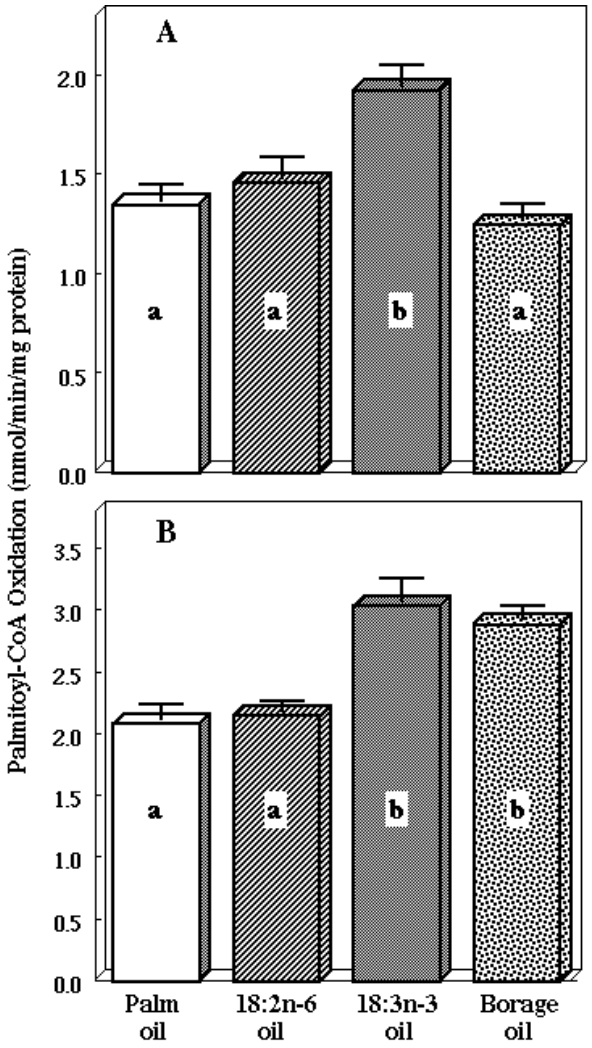

FIG. 1. Effect of α- and γ-linolenic acids on palmitoyl-CoA oxidation in rat liver homogenate. Values are means ± SE of seven determinations made on separate animals. a, b: Values with unlike letters differ significantly at $P < 0.05$. (A) Mitochondrial β-oxidation; analysis of variance (ANOVA), F value = 13.9 ($P < 0.01$). (B) Peroxisomal β-oxidation; ANOVA, F value = 11.0 ($P < 0.01$).

TABLE 2
Growth Parameters and Liver Weight of Rats Fed Various Fats

	Dietary fat[a]				ANOVA[b]	
	Palm oil	18:2n-6 Oil	18:3n-3 Oil	Borage oil	*F* value	Significance
Body weight						
Initial (g)	152 ± 3	152 ± 2	151 ± 1	151 ± 2	0.06	N.S.
Final (g)	280 ± 7	276 ± 5	280 ± 5	275 ± 5	0.216	N.S.
Growth (g/15 d)	128 ± 5	124 ± 5	128 ± 5	124 ± 3	0.242	N.S.
Food intake (g/d)	21.7 ± 1.0	20.1 ± 0.5	29.9 ± 0.7	19.6 ± 0.8	1.68	N.S.
Liver weight (g/100 g body weight)	5.32 ± 0.10[d]	4.82 ± 0.06[c]	5.08 ± 0.11[c,d]	5.29 ± 0.11[d]	5.73	$P < 0.01$

[a]Values for dietary fats are means ± SE of seven rats.
[b]ANOVA, analysis of variance; N.S., not significant. Values with unlike roman superscript letters (c,d) differ significantly at $P < 0.05$.

TABLE 3
Effects of α- and γ-Linolenic Acids on Fatty Acid Oxidation Enzyme Activities in Rat Liver

Enzymes, enzyme sources, and substrates	Dietary fat[a]				ANOVA	
	Palm oil	18:2n-6 Oil	18:3n-3 Oil	Borage oil	*F* value	Significance
	(nmol/min/mg protein)					
Carnitine palmitoyltransferase[b]						
500 × *g*	3.41 ± 0.10^{f}	3.93 ± 0.24^{f}	6.63 ± 0.52^{h}	5.13 ± 0.16^{g}	22.5	$P < 0.01$
Mitochondria	14.1 ± 0.40^{f}	15.6 ± 0.89^{f}	23.9 ± 0.55^{h}	20.3 ± 0.43^{g}	55.7	$P < 0.01$
Acyl-CoA dehydrogenase						
16:0-CoA substrate	58.6 ± 3.7^{g}	56.8 ± 2.1^{g}	70.6 ± 5.4^{h}	25.0 ± 1.0^{f}	38.3	$P < 0.01$
18:2-CoA substrate	36.5 ± 0.7^{g}	33.3 ± 2.5^{g}	49.4 ± 8.2^{h}	16.8 ± 0.7^{f}	9.58	$P < 0.01$
Acyl-CoA oxidase[b]	3.00 ± 0.13^{f}	2.89 ± 0.06^{f}	4.53 ± 0.18^{g}	4.10 ± 0.26^{g}	21.9	$P < 0.01$
Enoyl-CoA hydratase						
Crotonyl-CoA substrate	3070 ± 114	3258 ± 191	3895 ± 206	3372 ± 366	1.88	N.S.
t-2-Octenoyl-CoA substrate	1499 ± 82	1491 ± 57	1721 ± 95	1474 ± 121	1.49	N.S.
3-Hydroxyacyl-CoA dehydrogenase[c]	180 ± 17^{g}	212 ± 14^{g}	54.3 ± 3.1^{f}	192 ± 12^{g}	35.6	$P < 0.01$
3-Ketoacyl-CoA thiolase[c]	90.1 ± 5.7	87.4 ± 5.8	106 ± 13	84.4 ± 4.3	1.62	N.S.
2,4-Dienoyl-CoA reductase[d]	5.02 ± 0.36^{f}	7.02 ± 0.52^{g}	9.98 ± 1.22^{g}	8.95 ± 0.42^{g}	7.74	$P < 0.01$
Δ^3,Δ^2-Enoyl-CoA isomerase[e]	31.0 ± 1.6^{f}	39.2 ± 1.6^{g}	35.4 ± 0.7^{f,g}	43.0 ± 2.0^{h}	11.1	$P < 0.01$

[a]Values are means ± SE (7 rats/group). Carnitine palmitoyltransferase activity was measured using both 500 × *g* supernatant and mitochondrial fractions as enzyme sources. Acyl-CoA dehydrogenase activity was measured using a mitochondrial fraction as an enzyme source. Activity of other enzymes was assayed using a 500 × *g* supernatant as an enzyme source.
[b]Palmitoyl-CoA was used as substrate.
[c]Acetoacetyl-CoA was used as substrate.
[d]Sorboyl-CoA was used as substrate.
[e]*trans*-3-Hexenoyl-CoA was used as substrate. Values with unlike roman superscript letters (f–g) differ significantly at $P < 0.05$. For abbreviations see Table 2.

Effect of dietary α- and γ-linolenic acid on enzyme activity in fatty acid synthesis. Although differences were not always significant, activity of enzymes in fatty acid synthesis was higher in rats fed palm oil than in those fed various polyunsaturated fats (Fig. 2). Activity was comparable, however, among rats fed different polyunsaturated fats, with some exceptions.

Effect of dietary α- and γ-linolenic acid on serum and hepatic lipid levels. Compared to palm oil, dietary fats rich in polyunsaturated fatty acid reduced serum triacylglycerol concentration (Table 4). The extent of reduction was exaggerated in rats fed fats rich in α- or γ-linolenic acids. Although no significant difference among groups was detected, serum cholesterol concentration tended to be lower in rats fed 18:3n-3 oil and borage oil than in those fed palm and safflower oils. Serum phospholipid concentrations were comparable among rats fed polyunsaturated fats and were significantly lower than in rats fed palm oil. Serum free fatty acid concentrations were also lower in rats fed polyunsaturated fats than in those fed palm oil, and the greatest decrease was detected with borage oil. Hepatic triacylglycerol concentrations were the same among rats fed various polyunsaturated fats and were significantly lower than in rats fed palm oil. Hepatic cholesterol concentration in rats fed borage oil was the lowest among groups and was significantly less than in those fed palm and 18:2n-6 oils. Compared to palm and 18:2n-6 oils, 18:3n-3 oil also reduced this parameter, and the difference between rats fed 18:2n-6 oil became statistically significant. Hepatic phospholipid concentrations were significantly higher in rats fed 18:2n-6 and 18:3n-3 oils than in those fed palm and borage oils.

Effect of dietary α- and γ-linolenic acid on liver lipid fatty acid composition. Compared to palm oil, dietary polyunsaturated fats increased the proportion of polyunsaturated fatty acids in triacylglycerol and phospholipid (Table 5). As phospholipid is a major component of biomembranes, unsaturation of this lipid molecule should remain constant to maintain the fluidity of biological membranes under the different nutri-

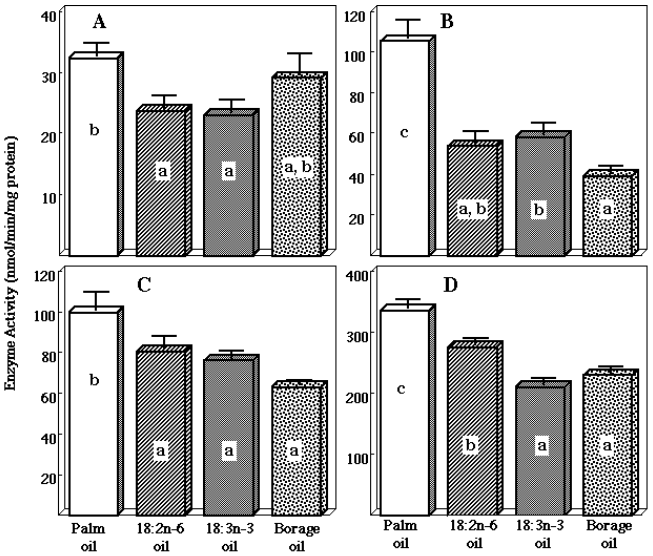

FIG. 2. Effect of α- and γ-linolenic acids on enzyme activity in fatty acid synthesis in the rat liver. Values are means ± SE determinations made on separate animals. a, b, c: Values with unlike letters differ significantly at $P < 0.05$. (A) Fatty acid synthetase activity; ANOVA, *F* value = 3.1 ($P < 0.05$). (B) Glucose 6-phosphate dehydrogenase activity; ANOVA: *F* value = 15.5 ($P < 0.01$). (C) Malic enzyme activity; ANOVA, *F* value = 5.54 ($p < 0.01$). (D) Pyruvate kinase activity; ANOVA, *F* value = 19.1 ($P < 0.01$).

TABLE 4
Effect of α- and γ-Linolenic Acids on Serum and Liver Lipid Concentrations in the Rat

	Dietary fat[a]				ANOVA[b]	
	Palm oil	18:2n-6 Oil	18:3n-3 Oil	Borage oil	*F* value	Significance
Serum lipids (μmol/dL)						
Triacylglycerol	333 ± 31^{e}	240 ± 32^{d}	164 ± 12^{c}	168 ± 10^{c}	11.5	$P < 0.01$
Cholesterol	367 ± 31	345 ± 15	298 ± 17	301 ± 29	1.98	N.S.
Phospholipid	362 ± 26^{d}	302 ± 7^{c}	303 ± 8^{c}	309 ± 19^{c}	3.10	$0.01 < P < 0.05$
Free fatty acid	224 ± 11^{e}	195 ± 18^{d,e}	162 ± 16^{c,d}	140 ± 12^{c}	6.39	$P < 0.01$
Liver lipids (μmol/g)						
Triacylglycerol	65.1 ± 11.2^{d}	34.3 ± 4.1^{c}	26.2 ± 2.8^{c}	30.6 ± 3.0^{c}	7.85	$P < 0.01$
Cholesterol	6.09 ± 0.44^{d,e}	6.19 ± 0.19^{e}	5.36 ± 0.14^{c,d}	4.90 ± 0.11^{c}	5.83	$P < 0.01$
Phospholipid	37.3 ± 1.1^{c}	41.6 ± 1.5^{d}	41.7 ± 0.7^{d}	37.9 ± 0.7^{c}	4.30	$0.01 < P < 0.05$

[a]Values are means ± SE of seven rats.
[b]For abbreviations see Table 2. Values with unlike roman superscript letters (c–e) differ significantly at $P < 0.05$.

tional conditions. Although only a limited amount of polyunsaturated fatty acids is available in the diet containing palm oil, in the present study, hepatic phospholipid but not triacylglycerol retained considerable amounts of polyunsaturated fatty acids. Therefore, the extent of the increase in the proportion of polyunsaturated fatty acids attributable to dietary polyunsaturated fats was exaggerated in the hepatic triacylglycerol fraction compared to the phospholipid fraction. Even though borage oil contained considerable amounts of γ-linolenic acid, this fatty acid represented only a minor proportion both in triacylglycerol and phospholipid. Compared to fat mixtures rich in linoleic acid, dietary fats rich in γ-linolenic acid did not increase the sum of n-6 fatty acid concentrations in these lipid fractions. A dietary fat rich in α-linolenic acid increased the proportion of n-3 fatty acid (α-linolenic acid) in triacylglycerol and eicosapentaenoic and docosahexaenoic acids in phospholipid at the expense of n-6 fatty acid.

DISCUSSION

We previously demonstrated that, compared to saturated fats (palm and coconut oils) and safflower oil, perilla and linseed oils rich in a-linolenic acid (63% α-linolenic acid for perilla oil and 56% for linseed oil) increased mitochondrial and peroxisomal fatty oxidation rates in the rat liver (1,2). We also showed that, compared to safflower and palm oils, perilla and linseed oils increased the hepatic activity of various enzymes

TABLE 5
Effect of α- and γ-Linolenic Acids on Fatty Acid Composition of Triacylglycerol and Phospholipid in Rat Liver

	Triacylglycerol						Phospholipid					
Fatty acid	Dietary fat[a]				ANOVA[b]		Dietary fat[a]				ANOVA[b]	
(wt%)	Palm oil	18:2n-6 Oil	18:3n-3 Oil	Borage oil	*F* value	Significance	Palm oil	18:2n-6 Oil	18:3n-3 Oil	Borage oil	*F* value	Significance
14:0	1.92 ± 0.24^{e}	1.05 ± 0.11^{d}	0.78 ± 0.07^{d}	0.98 ± 0.09^{d}	12.5	$P < 0.01$	0.32 ± 0.02^{f}	0.23 ± 0.01^{e}	0.18 ± 0.01^{d}	0.23 ± 0.01^{e}	35.3	$P < 0.01$
16:0	30.1 ± 0.8	25.4 ± 1.0	27.4 ± 1.5	29.4 ± 1.5	2.89	N.S.	18.3 ± 0.4^{f}	17.0 ± 0.5^{d,e}	16.5 ± 0.2^{d}	17.6 ± 0.2^{e,f}	5.92	$P < 0.01$
16:1n-7	11.0 ± 1.0^{e}	5.27 ± 0.63^{d}	4.68 ± 0.66^{d}	4.16 ± 0.57^{d}	18.1	$P < 0.01$	1.42 ± 0.11^{e}	0.55 ± 0.04^{d}	0.49 ± 0.03^{d}	0.46 ± 0.05^{d}	49.5	$P < 0.01$
18:0	3.83 ± 0.24^{e}	3.21 ± 0.10^{d}	3.99 ± 0.12^{e}	3.89 ± 0.17^{e}	4.30	$0.01 < P < 0.05$	25.9 ± 0.3^{d,e}	25.1 ± 0.3^{d}	27.0 ± 0.6^{e}	26.6 ± 0.4^{e}	3.69	$0.01 < P < 0.05$
18:1n-9	48.7 ± 0.70^{e}	28.4 ± 0.6^{d}	28.7 ± 1.4^{d}	27.4 ± 1.0^{d}	107	$P < 0.01$	9.78 ± 0.41^{e}	4.68 ± 0.11^{d}	4.08 ± 0.13^{d}	4.65 ± 0.19^{d}	122	$P < 0.01$
18:2n-6	3.78 ± 0.33^{d}	32.8 ± 0.8^{f}	23.6 ± 1.9^{e}	20.6 ± 1.3^{e}	97.9	$P < 0.01$	6.95 ± 0.17^{e}	10.8 ± 0.3^{f}	13.3 ± 0.3^{g}	5.82 ± 0.16^{d}	162	$P < 0.01$
18:3n-6	0.10 ± 0.01^{d}	0.61 ± 0.06^{e}	0.26 ± 0.02^{d,e}	3.52 ± 0.26^{f}	140	$P < 0.01$	0.11 ± 0.01^{d}	0.16 ± 0.01^{e}	0.10 ± 0.00^{d}	0.84 ± 0.03^{f}	451	$P < 0.01$
18:3n-3	0.28 ± 0.02^{d}	0.28 ± 0.01^{d}	8.34 ± 0.94^{e}	0.90 ± 0.09^{d}	70.1	$P < 0.01$	0.12 ± 0.05^{d}	0.00 ± 0.00^{d}	0.57 ± 0.06^{f}	0.23 ± 0.02^{e}	38.0	$P < 0.01$
20:3n-6	0.03 ± 0.02^{d}	0.22 ± 0.02^{e}	0.10 ± 0.02^{d,e}	1.31 ± 0.09^{f}	178	$P < 0.01$	1.07 ± 0.04^{e}	0.55 ± 0.03^{d}	1.34 ± 0.07^{f}	1.17 ± 0.02^{e}	58.6	$P < 0.01$
20:4n-6	0.14 ± 0.04^{d}	1.43 ± 0.14^{e}	0.51 ± 0.07^{d}	5.13 ± 0.45^{f}	90.1	$P < 0.01$	25.9 ± 0.8^{d}	30.1 ± 0.14^{f}	22.8 ± 0.4^{d}	31.2 ± 0.2^{f}	66.5	$P < 0.01$
20:5n-3	0.02 ± 0.00^{d}	0.03 ± 0.00^{d}	0.47 ± 0.11^{e}	0.07 ± 0.02^{d}	15.3	$P < 0.01$	0.16 ± 0.01^{d}	0.05 ± 0.00^{d}	2.34 ± 0.15^{e}	0.06 ± 0.01^{d}	225	$P < 0.01$
22:4n-6	0.07 ± 0.02^{d}	0.66 ± 0.07^{e}	0.12 ± 0.01^{d}	1.70 ± 0.17^{f}	70.4	$P < 0.01$	0.49 ± 0.02^{e}	1.22 ± 0.07^{f}	0.32 ± 0.02^{d}	1.54 ± 0.03^{g}	203	$P < 0.01$
22:5n-6	0.07 ± 0.01^{d}	0.30 ± 0.03^{e}	0.07 ± 0.01^{d}	0.70 ± 0.06^{f}	72.5	$P < 0.01$	2.13 ± 0.18^{e}	4.70 ± 0.38^{f}	0.14 ± 0.02^{d}	5.60 ± 0.19^{g}	117	$P < 0.01$
22:5n-3	0.01 ± 0.00^{d}	0.03 ± 0.01^{d}	0.52 ± 0.12^{e}	0.07 ± 0.01^{d}	16.6	$P < 0.01$	0.17 ± 0.02^{d}	0.14 ± 0.01^{d}	1.44 ± 0.09^{e}	0.20 ± 0.03^{d}	186	$P < 0.01$
22:6n-3	0.03 ± 0.01^{d}	0.29 ± 0.03^{e}	0.54 ± 0.08^{f}	0.20 ± 0.02^{e}	22.1	$P < 0.01$	7.09 ± 0.27^{f}	4.74 ± 0.34^{e}	9.42 ± 0.28^{g}	3.84 ± 0.12^{d}	90.1	$P < 0.01$
Total PUFA	4.52 ± 0.38^{d}	36.7 ± 0.9^{e}	34.5 ± 3.2^{e}	34.2 ± 2.2^{e}	60.8	$P < 0.01$	44.2 ± 0.8^{d}	52.4 ± 0.3^{f}	51.7 ± 0.5^{e,f}	50.5 ± 0.4^{e}	47.4	$P < 0.01$
n-6 Fatty acid	4.19 ± 0.37^{d}	36.0 ± 0.9^{f}	24.7 ± 2.0^{e}	33.0 ± 2.1^{f}	88.6	$P < 0.01$	36.7 ± 0.81^{d}	47.5 ± 0.4^{e}	38.0 ± 0.4^{d}	46.1 ± 0.4^{e}	111	$P < 0.01$
n-3 Fatty acid	0.33 ± 0.03^{d}	0.63 ± 0.02^{d}	9.87 ± 1.19^{e}	1.24 ± 0.09^{d}	58.6	$P < 0.01$	7.54 ± 0.28^{e}	4.93 ± 0.35^{d}	13.8 ± 0.5^{g}	4.32 ± 0.14^{d}	175	$P < 0.01$

[a]Values are means ± SE of seven rats.
[b]For abbreviations see Tables 1 and 2. Values with unlike roman superscript letters (d–g) differ significantly at $P < 0.05$. Comparisons were made between groups of rats fed different fats within fatty acid type.

in the β-oxidation pathway, including carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase, 2,4-dienoyl-CoA reductase, and Δ^3,Δ^2-enoyl-CoA isomerase, but unexpectedly decreased 3-hydroxyacyl-CoA dehydrogenase activity. A fat mixture composed of safflower and perilla oils containing α-linolenic acid at a level as low as 20% still effectively increased the mitochondrial and peroxisomal fatty acid oxidation rate and the activity of certain enzymes in the fatty acid oxidation pathway (2). This result was essentially confirmed in the present study in rats fed a fat mixture containing 30% α-linolenic acid from linseed oil.

Takada *et al*. (8) demonstrated that, compared to soybean oil, mold oil originating from a *Mucor circinelloides* and rich in n-6 octadecatrienoic acid (γ-linolenic acid) increased the peroxisomal fatty acid oxidation rate and carnitine palmitoyltransferase activity in the rat liver. The present study demonstrated that, compared to palm oil and a fat mixture rich in linoleic acid, a plant-originated oil rich in γ-linolenic acid (borage oil) also effectively increased the activity. It is therefore conceivable that dietary γ-linolenic acid, like α-linolenic acid, has a physiological activity to enhance hepatic β-oxidation in the rat liver. The present study, however, indicated that differences existed between n-3 and n-6 octadecatrienoic acids in the physiological activity affecting hepatic β-oxidation enzyme. Accordingly, dietary fat rich in α-linolenic acid enhanced both the mitochondrial and peroxisomal fatty acid oxidation rate whereas fat rich in γ-linolenic acid enhanced the peroxisomal, but not the mitochondrial, fatty acid oxidation rate. Our present study confirmed our previous finding (2) that dietary α-linolenic acid reduced 3-hydroxyacyl-CoA dehydrogenase activity in the rat liver. Borage oil rich in γ-linolenic acid, however, did not reduce this enzyme activity in the present study. In addition, α-linolenic acid enhanced mitochondrial acyl-CoA dehydrogenase activity, but borage oil profoundly reduced it.

Acyl-CoA oxidase is presumed to regulate peroxisomal fatty oxidation (13). Consistent with this assumption, an increase of peroxisomal palmitoyl-CoA oxidation by dietary fats rich in γ- and α-linolenic acids was associated with increased acyl-CoA oxidase activity in the liver homogenate. Carnitine palmitoyltransferase is regarded as a key enzyme regulating mitochondrial fatty acid oxidation (13,32). Even though dietary γ-linolenic acid, like α-linolenic acid, increased carnitine palmitoyltransferase activity, it did not increase the mitochondrial palmitoyl-CoA oxidation rate in the liver homogenate. There are two carnitine palmitoyltransferases in mitochondria, one in the outer mitochondrial membrane (carnitine palmitoyltransferase I) and the other in the inner mitochondrial membrane (carnitine palmitoyltransferase II). Freezing-thawing of mitochondria inactivates carnitine palmitoyltransferase I but not transferase II (32), meaning that the transferase activity we measured spectrophotomerically using a freeze-thawed 500 × *g* supernatant or mitochondrial fraction most likely represents transferase II activity (32). We previously demonstrated (1) that, compared to palm and safflower oils, perilla oil rich in α-linolenic acid increased both the carnitine palmitoyltransferase activity measured spectrophotomerically using freeze-thawed enzyme preparations (mitochondria and a 500 × *g* supernatant) and the activity measured radiochemically using intact mitochondria, which represents carnitine palmitoyltransferase I activity. We did not measure carnitine palmitoyltransferase I activity using intact mitochondria in our present study, so it is possible that dietary γ-linolenic acid increased carnitine palmitoyltransferase II but not I activity in the liver, thus failing to enhance the mitochondrial fatty acid oxidation rate. The determination of the transferase I activity using intact mitochondria as an enzyme source in rats fed γ-linolenic acid is therefore required to clarify this point.

Our present study confirmed the previous observation (2) that dietary α-linolenic acid decreased 3-hydroxyacyl-CoA dehydrogenase activity even though this polyunsaturated fatty acid increased both the mitochondrial and peroxisomal palmitoyl-CoA oxidation rates in liver homogenates. Several enzyme molecules possess 3-hydroxyacyl-CoA dehydrogenase activity in the rat liver (18,33,34). Some evidence (2) indicates that mitochondrial 3-hydroxyacyl-CoA dehydrogenase specific to short-chain acyl-CoA is the enzyme responsible for the reduction by dietary α-linolenic acid of 3-hydroxyacyl-CoA dehydrogenase activity in the rat liver. Dietary γ-linolenic acid did not reduce 3-hydroxyacyl-CoA dehydrogenase activity, but did decrease mitochondrial acyl-CoA dehydrogenase activity measured with palmitoyl-CoA and linoleoyl-CoA substrates in the present study. This reduced enzyme activity may account for the failure of dietary γ-linolenic acid to increase the mitochondrial β-oxidation rate in the rat liver despite the fact that this dietary fat increased carnitine palmitoyltransferase activity. Six distinct acyl-CoA dehydrogenase species differing in substrate specificity have been isolated and characterized (35,36). Four catalyze the first step of the mitochondrial β-oxidation cycle for fatty acids with different chain lengths. These correspond to short-, medium-, long-, and very long-chain acyl-CoA dehydrogenases. The remaining species are involved in oxidizing branched-chain amino acids. We used long-chain fatty acyl-CoA substrates in our present study to measure acyl-CoA dehydrogenase activity in mitochondria, so it is at least probable that dietary γ-linolenic acid reduced the activity of acyl-CoA dehydrogenase species specific to a long-chain acyl-CoA substrate. It is also possible, however, that dietary γ-linolenic acid affects the activity of both long chain acyl-CoA dehydrogenase and of other acyl-CoA dehydrogenase species in the rat liver. Acyl-CoA dehydrogenase activity will have to be measured using a variety of acyl-CoA substrates differing in chain length to clarify this point in future studies.

The degradation of unsaturated fatty acids *via* the β-oxidation pathway to yield acetyl-CoA requires the involvement of auxiliary enzymes (2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase) in addition to those required for the β-oxidation of saturated fatty acids (13,37). It is therefore rea-

sonable to expect that dietary polyunsaturated fatty acids increase the activity of these enzymes. In fact, compared to palm oil, various polyunsaturated fats significantly increased hepatic activity of these enzymes in the present study except for one occasion (Δ^3,Δ^2-enoyl-CoA isomerase in rats fed 18:3n-3 oil).

The role of the peroxisome proliferator-activated receptor (PPAR), a member of the nuclear receptor superfamily, in regulating lipid metabolism has been well demonstrated (38–40). After activation, PPAR is heterodimerized with retinoid × receptor (RXR), a member of another nuclear receptor superfamily, binds to peroxisome proliferator response element (PPRE) of a target gene, and modifies its expression. Various types of PPAR (α, γ_1, γ_2, and δ) have been identified in rodents and humans. PPARα is highly expressed in liver and may play a crucial role in regulating lipid metabolism in this tissue. So far, PPRE has been identified as the promoter of genes of various peroxisomal and mitochondrial fatty acid oxidation enzymes including acyl-CoA oxidase, peroxisomal bifunctional enzyme, medium chain acyl-CoA dehydrogenase and 3-hydroxy-3-methylglutaryl-CoA synthase, and it has been demonstrated that the expression of these genes is under the control of PPAR. Göttlicher *et al*. (41) and Keller *et al*. (42) have demonstrated that natural fatty acids are activators of PPAR to induce gene expression. These studies demonstrated that polyunsaturated fatty acids activate PPAR more than do saturated and monounsaturated fatty acids. More recent studies (43,44) indicate that fatty acids and eicosanoids are ligands of PPAR to induce its DNA binding and expression of target genes. It is therefore plausible that PPAR-mediated effects may account for dietary α- and γ-linolenic acids having different effects on only some of the hepatic fatty acid oxidation enzymes. It has been reported, however, that various polyunsaturated fatty acids including linoleic, α-linolenic, γ-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids are equally effective in activating PPAR, despite the fact that dietary linoleic, α-linolenic, and γ-linolenic acids differ considerably in how they affect hepatic fatty acid oxidation rate and fatty oxidation enzymes in our present and previous studies (1,2). Present knowledge concerning the PPAR signaling pathway therefore does not necessarily explain the data obtained with rats fed various polyunsaturated fat diets.

Evidence supports the assumption that fatty acid synthesis and oxidation rates in the liver are inversely regulated under different nutritional and pathological conditions (45–48). We previously demonstrated, however, that dietary α-linolenic acid, compared to linoleic acid, did not affect enzyme activity in the hepatic fatty acid synthetic pathway even though this octadecatrienoic acid increased mitochondrial and peroxisomal fatty acid oxidation (1,2). Compared to fat mixtures rich in linoleic acid, borage oil rich in γ-linolenic acid and a fat mixture rich in α-linolenic acid increased fatty acid oxidation activity in the liver but were essentially ineffective in modulating enzyme activity in fatty acid synthesis. It is therefore reasonable to assume that enzymes in fatty acid oxidation and synthesis are not necessarily reciprocally regulated in rats fed different fats.

In conclusion, we have shown that dietary α-linolenic acid induced hepatic β-oxidation activity in both peroxisomal and mitochondrial pathways, and dietary γ-linolenic acid in the form of borage oil increased peroxisomal but not mitochondrial β-oxidation activity. The effects of dietary α- and γ-linolenic acids on hepatic enzyme activities in the β-oxidation pathway were also found to differ considerably from one other.

ACKNOWLEDGMENT

This work was supported in part by the Bio-Renaissance Program of the Ministry of Agriculture, Forestry and Fisheries (BRP-97-I-A-7).

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[Received October 28, 1997, and in final revised form and accepted May 18, 1998]

Free Fatty Acid Fractions from Some Vegetable Oils Exhibit Reduced Survival Time-Shortening Activity in Stroke-Prone Spontaneously Hypertensive Rats

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ABSTRACT: Previously, we demonstrated that several vegetable oils that included low-erucic rapeseed oil markedly shortened the survival time (by ~40%) of stroke-prone spontaneously hypertensive (SHRSP) rats as compared with perilla oil, soybean oil, and fish oil. We considered that a factor other than fatty acids is toxic to SHRSP rats, because the survival time-shortening activity could not be accounted for by the fatty acid compositions of these oils. In fact, a free fatty acid (FFA) fraction derived from lipase-treated rapeseed oil was found to be essentially devoid of such activity. A high-oleate safflower oil/safflower oil/perilla oil mixture exhibited a survival time-shortening activity comparable to that of rapeseed oil, but the activity of this mixed oil was also reduced by lipase treatment. A partially hydrogenated soybean oil shortened the survival time by ~40%, but a FFA fraction derived from lipase-treated partially hydrogenated soybean oil shortened it by 13% compared with soybean oil. Fatty acid compositions of the rapeseed oil and a FFA fraction derived from lipase-treated rapeseed oil were similar, but those of hepatic phospholipids of rats fed the oil and FFA were slightly but significantly different. These results support the interpretation that the survival time-shortening activity exhibited by some vegetable oils is due to minor components other than fatty acids, and that an active component(s) were produced in or contaminated soybean oil during the partial hydrogenation processes.

Lipids 33, 655–661 (1998).

Perilla seed oil and fish oil rich in n-3 fatty acids have beneficial effects on animals used as models of chronic diseases of the elderly (1). Perilla oil prolonged the survival time of Donryu rats (a conventional strain) and stroke-prone spontaneously hypertensive (SHRSP) rats (a model of human stroke) by ~10 and ~15%, respectively, compared with safflower oil (2,3). The observed beneficial effects of these oils were attributed to their low n-6/n-3 ratios. Rapeseed oil (low-erucic) had been assumed to be beneficial for chronic diseases because of its relatively high oleate and α-linolenate, and low linoleate contents. Dietary rapeseed (low erucic) and olive oils were very effective for the secondary prevention of coronary heart disease (4). Unexpectedly, however, rapeseed oil shortened the survival time of SHRSP rats (by 40%) compared with soybean, safflower, perilla and fish oils (5–7).

Although an old-type rapeseed contained very high amounts of erucic acid and thyrotoxic sulfur compounds, oil made from a newly developed strain of rapeseed (double-low) contains much lower amounts of these compounds. However, Vles *et al*. (8) reported that even the double-low rapeseed oil induced myocardial necrosis in Sprague-Dawley rats. Subsequently, the incidence of myocardial necrosis was attributed to the unique fatty acid composition of double-low rapeseed oil because the incidence was positively correlated with oleic and α-linolenic acids and negatively correlated with saturated fatty acids (9). In regard to survival time-shortening activity, the positive and negative correlations observed for myocardial necrosis were not applicable because perilla oil, which prolonged the survival time of SHRSP rats, contained a similar proportion of saturated fatty acids and a higher proportion of α-linolenic acid than rapeseed oil (6).

The survival time-shortening activity was not restricted to rapeseed oil; other high-oleate vegetable oils such as high-oleate safflower, high-oleate sunflower, and olive oils were also active in this respect. A high-oleate content by itself was not responsible for the survival-time shortening activity because lard and a microbial oil containing 36-39% oleic acid were relatively safe in this animal model, and evening primrose oil containing as little as 15% oleic acid exhibited activity similar to that of the oils which reduced longevity. Therefore, the observed survival time-shortening activities of these vegetable oils could not be accounted for by their unique fatty acid compositions. We postulated the presence in these oils of a factor(s) that are toxic to SHRSP rats (6). Here, we report that FFA fractions derived from some of these oils had a lower survival time-shortening activity, which may help in identifying and developing the means to eliminate the active component(s).

MATERIALS AND METHODS

Diets and animals. A conventional basal diet containing 2.7% (w/w) oil (CE-2; Clea Japan Co., Ltd., Tokyo, Japan) was made of fish meal, soybean meal, defatted powdered milk,

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Abbreviations: FFA, free fatty acids; SHRSP, a strain of stroke-prone spontaneously hypertensive rats; SPF, specific pathogen-free; TLC, thin-layer chromatography.

wheat flour, corn, wheat bran, alfalfa meal, a vitamin mixture, and a mineral mixture. This basal diet was mixed with a vegetable oil or a derived free fatty acid (FFA) fraction in a 9:1 weight ratio. The final oil content was 12.7 wt% (29 energy %). The following vegetable oils, commercially available for human use, were used; rapeseed (low-erucic), soybean, safflower (high-linoleate), high-oleate safflower, perilla, sesame, and partially hydrogenated soybean (melting point: 30°C) oils. The source for the preparation of oleic acid ethyl ester (Wako Pure Chemical Industry, Osaka, Japan) is unclear. The fatty acid composition of the diets is shown in Table 1. Diets supplemented with these oils were kept at 4°C for less than 1 mon. Diets were replaced every 2 d to keep the peroxide values of the fed diets below 100 meq/kg.

A conventional strain of SHRSP rats, which were kindly provided by Dr. T. Suzuki (Kinki University School of Medicine, Osaka, Japan), was used in the experiment shown in Figure 1 and a specific pathogen-free (SPF) strain of SHRSP rats, which were purchased from Seack Yoshitomi Co. (Fukuoka, Japan), was used in other experiments. Blood pressure was measured every 2 wk using a photoelectric tail-cuff procedure after prewarming the rats at 35°C for 5 min in a thermostatic cage (Model UR5000; Ueda Manufacturing Co. Ltd., Tokyo, Japan). Male littermates were, as far as possible, equally distributed in different dietary groups. Rats were weaned to the conventional diet (CE-2) at 3 wk of age, and the test diets were given from 4 wk of age. The diet and 1% NaCl solution were given *ad libitum*. In the experiment shown in Figure 1, rats were kept in an air-conditioned room at 23 ± 3°C. In other experiments, rats were kept in a room specified for SPF animals; temperature and humidity were 23 ± 2°C and at 50 ± 2%, respectively.

Lipase and detergent treatment of vegetable oils. Vegetable oils and a partially hydrogenated soybean oil were dissolved at 4°C in polyvinyl alcohol solution (4.5 L) containing 1.8% polyvinyl alcohol 117 and 0.2% polyvinyl alcohol 205 (kindly provided by Kuraray Co., Ltd., Osaka, Japan). Sucrose fatty acid ester (2%) was used only for the preparation of detergent-treated hydrogenated soybean oil without added lipase (Experiment 3, Table 3). Then 0.1 M phosphate buffer, pH 8.0 (3 L), with or without 0.1% (wt/vol) Lipase AY 30 (90,000 I.U./3 g/3 L; Amano, Nagoya, Japan), was added and the mixture was stirred at room temperature or 35°C for 1 wk. Triacylglycerols in rapeseed oil, partially hydrogenated soybean oil, and a mixture of high-oleate safflower oil/safflower oil/perilla oil were hydrolyzed to FFA in 90.6, 86.3, and 91.7% of the total ester bonds, respectively. After the reaction, hexane (7.5 L) and NaCl (3 kg) were added, the hexane layer was separated, and the solvent was evaporated under reduced pressure to obtain FFA fractions or detergent-treated oils.

Lipid analysis. SHRSP rats ($n = 6$) in the rapeseed oil, soybean oil or partially hydrogenated soybean oil group were used for lipid analysis after 60 d of feeding. The SHRSP rats were anesthetized with pentobarbital (Nembutal, 50 mg/kg, i.p.; Dainabot, Osaka, Japan) after fasting for 18 h. Blood samples were collected from an abdominal vein using a syringe containing 3.8% (wt/vol) sodium citrate, and plasma was prepared by low-speed centrifugation. The thoracic aorta was excised, and the attached fat tissues were removed with the aid of a microscope. Samples were kept frozen at −80°C until lipid analysis. The total lipids were extracted from the samples according to the method of Bligh and Dyer (10), and triacylglycerols and phospholipids were separated by silica

TABLE 1
Fatty Acid Composition of the Experimental Diets[a] (% of total fatty acids)

Fatty acids[b]	Soybean oil	Rapeseed oil	Ethyl oleate/ soybean oil perilla oil[c]	Hydrogenated soybean oil (HSO)	FFA from lipase-treated rapeseed oil[d]	FFA from lipase-treated mixed oil[d,e]	FFA from lipase-treated HSO[d]	Sesame oil
14:0	0.3	0.3	0.8	0.9	0.6	0.3	0.4	0.3
16:0	12.2	7.8	9.6	13.1	10.4	9.2	11.1	10.6
16:1	0.3	0.4	0.5	1.0	0.4	0.4	0.5	0.5
18:0	3.5	2.0	2.1	5.3	2.3	2.0	6.0	4.2
*c*18:1	22.5	49.8	50.3	31.8	50.3	48.0	36.4	35.3
*t*18:1	n.d.[f]	n.d.	n.d.	14.1	n.d.	n.d.	15.6	n.d.
*tt*18:2	n.d.	n.d.	n.d.	0.9	n.d.	n.d.	1.8	n.d.
18:2n-6	51.6	27.9	26.7	27.3	27.0	27.3	22.2	44.6
18:3n-3	6.6	7.7	6.8	1.5	3.5	9.2	2.7	1.2
20:0	0.4	0.5	0.4	0.3	0.8	0.3	0.4	0.4
20:1	0.6	1.5	0.5	0.8	0.9	1.3	0.7	0.7
20:5n-3	0.6	0.6	0.7	1.3	1.1	0.6	0.6	0.6
22:0	0.4	0.3	0.4	0.6	1.3	0.3	0.5	0.3
22:1	0.2	0.3	0.4	0.4	0.4	0.3	0.2	0.4
22:6n-3	0.8	0.8	0.8	0.7	1.0	0.8	0.9	0.9

[a]Fatty acids are designated by the number of carbons; the number of double bonds, and the position of the first double bond numbered from the methyl terminus is indicated as n-3 or n-6, and *cis* or *trans* isomers are designated as *c* or *t*.
[b]Ninety grams of conventional diet that contained 2.7% oil was mixed with 10 g of oil or of free fatty acids (FFA) fraction from lipase-treated oils.
[c]Oleic acid ethyl ester/soybean oil/.perilla oil mixture was prepared by mixing oleic acid ethyl ester (57.2%), soybean oil (27.8%), and perilla oil (14.8%).
[d]See Materials and Methods section for the preparation of FFA fractions from lipase-treated oils.
[e]This mixed oil consisted of high-oleate safflower (75%), safflower (12.5%), and perilla oils (12.5%).
[f]n.d., not detected.

gel thin-layer chromatography (TLC). Petroleum ether/diethyl ether/acetic acid (80:30:1, by vol) was used as the developing solvent. Spots were located under a ultraviolet light after plates were sprayed with 0.005% (wt/vol) primuline solution (Nacalai Tesque Co., Kyoto, Japan) and the lipids were extracted from the adsorbent using Bligh and Dyer mixture. Fatty acids were analyzed as methyl esters by gas–liquid chromatography on a capillary column coated with DB-225 (0.2 mm, 30 m length; J&W Scientific, Folsom, CA), using heptadecanoic acid as an internal standard. Column temperature was programmed from 165 to 205°C at a rate of 3°C/min, then to 210°C at a rate of 1°C/min, and then to 222°C at a rate of 0.5°C/min. Injector and detector temperatures were both 250°C. The *trans*- and *cis*-18:1 contents were determined using $AgNO_3$–TLC in conjunction with gas–liquid chromatography. The $AgNO_3$-TLC analyses were performed on precoated silica gel TLC plates that had been immersed in a 10% (wt/vol) solution of $AgNO_3$ in acetonitrile, dried horizontally, and activated at 110°C for 1 h. The plates were developed with 0.75% ethanol in chloroform. The lipid bands were sprayed with primuline solution and then identified under an ultraviolet UV light. The separated fractions were scraped off the plate, and the lipids were extracted with chloroform/hexane (1:1, vol/vol) and analyzed by gas–liquid chromatography (7,11).

Statistical analysis. Data are presented as means ± SD. Statistical analysis of the survival time data was performed by log-rank and Wilcoxon signed rank method (a nonparametric method) using a computer program JMP 3.0, Statistic Made Visual (SAS Institute, Cray, NC). Statistical analysis of other data was performed by analysis of variance using Bonferroni's multiple comparison or repeated measures analysis of variance (Stat View J-4.11; Abacus Concepts Inc., Berkeley, CA).

RESULTS

Effect of oils and derived FFA fractions on survival time of SHRSP rats. Rats in all groups grew normally, and there were no differences in average body weights (data not shown) and systolic blood pressures (Fig. 1) among the rats in the rapeseed oil, soybean oil, and partially hydrogenated soybean oil groups and rats fed the FFA derived from lipase-treated rapeseed oil up to 13 wk of age. Rapeseed oil shortened the survival time of SHRSP rats by 35% compared to soybean oil (Fig. 2). In contrast, the FFA fraction derived from lipase-treated rapeseed oil did not exhibit such a survival time-shortening activity, although fatty acid compositions were not altered significantly by the lipase-treatment (Table 1). Partially hydrogenated soybean oil was as detrimental as rapeseed oil (Experiment 1, Table 2, and Fig. 2). When a triolein/soybean oil/perilla oil mixture with the same fatty acid composition as that of rapeseed oil was fed to SHRSP rats, the survival time of this mixed oil group was between those of the rapeseed oil and soybean oil groups (6). Similarly, the survival time of the oleic acid ethyl ester/soybean oil/perilla oil mixture group was between the rapeseed oil and soybean oil groups (Experiment 1, Table 1).

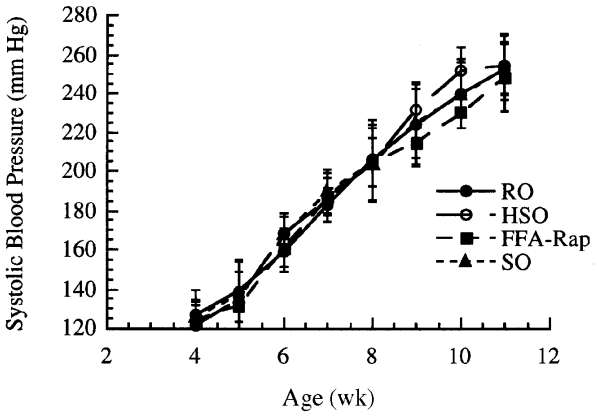

FIG. 1. Systolic blood pressure of stroke-prone spontaneously hypertensive (SHRSP) specific pathogen-free (SPF) rats under 1% NaCl loading. A diet containing 10% (w/w) rapeseed oil (RO), hydrogenated soybean oil (HSO), soybean oil (SO), or a FFA fraction derived from lipase-treated rapeseed oil (FFA-Rap) was fed to SHRSP rats from 4 wk of age. The differences among the dietary groups were not significant in the repeated measures ANOVA.

To minimize the standard deviation among experiments, we changed in subsequent experiments from using a conventional strain of SHRSP rats kept in a temperature-controlled room to SPF SHRSP rats kept in temperature, humidity, and light-controlled conditions. The difference in the mean survival times between the rapeseed oil and soybean oil groups was smaller in SPF SHRSP (Experiment 2, Table 2) than in the conventional SHRSP rats (Experiments 1, Table 2); the former had a higher blood pressure (~40 mm Hg) and appeared to be more sensitive to dietary conditions (e.g., 1%

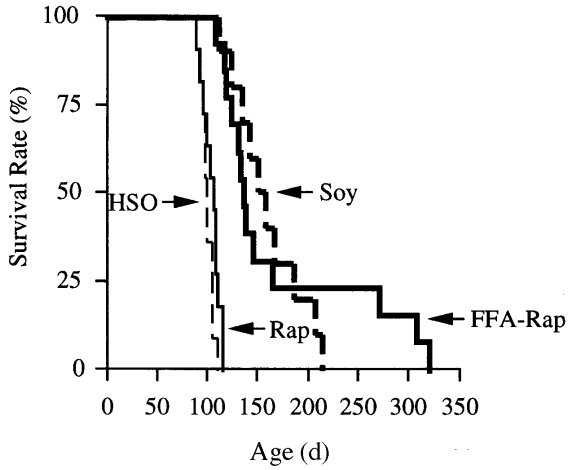

FIG. 2. Survival time-shortening activities of some vegetable oils and a free fatty acid (FFA) derived from lipase-treated rapeseed oil. Diets supplemented with different oils were fed to SHRSP rats (conventional) from 4 wk of age. NaCl solution (1%) was given *ad libitum* as drinking water. Partially hydrogenated soybean oil (HSO, $n = 11$), rapeseed oil (Rap, $n = 11$), soybean oil (Soy, $n = 10$), and a FFA derived from lipase-treated rapeseed oil (FFA-Rap, $n = 11$) were examined. Results of statistical analysis were presented in Experiment 1, Table 2. For other abbreviations see Figure 2.

TABLE 2
Survival Times of Conventional or SPF SHRSP Rats Fed Different Oils Under 1% NaCl Loading[a]

	Dietary group and number of animals				
Experiment 1 (SHRSP, conventional)	Rapeseed oil (Rap) (n = 11)	Soybean oil (Soy) (n = 10)	Hydrogenated soybean oil (HSO) (n = 11)	FFA from lipase-treated rapeseed oil (FFA-Rap) (n = 11)	Oleic acid ethyl ester/ soybean oil/perilla oil (n = 11)
Survived Day (±SD)	104 ± 3	160 ± 11	100 ± 2	145 ± 13	118 ± 6
P value by log-rank		0.0000 vs. Rap	0.1285 vs. Rap	0.0000 vs. Rap	0.0485 vs. Rap
Wilcoxon		0.0000 vs. Rap	0.2348 vs. Rap	0.0000 vs. Rap	0.0905 vs. Rap
Log-rank			0.0000 vs. Soy	0.0000 vs. Soy	0.0028 vs. Soy
Wilcoxon			0.0000 vs. Soy	0.0000 vs. Soy	0.0018 vs. Soy
Log-rank				0.0000 vs. HSO	0.0027 vs. HSO
Wilcoxon				0.0000 vs. HSO	0.0032 vs. HSO
Log-rank					0.0957 vs. FFA-Rap
Wilcoxon					0.0239 vs. FFA-Rap
Experiment 2 (SHRSP, SPF)	Rapeseed oil (Rap) (n = 11)	Soybean oil (Soy) (n = 11)	Sesame oil (Ses) (n = 11)	FFA from lipase-treated hydrogenated soybean oil (FFA-HSO) (n = 11)	FFA from lipase-treated mixed oil (n = 11)
Survived Day (±SD)	106 ± 3	130 ± 11	129 ± 8	124 ± 2	143 ± 3
P value by log-rank		0.0433 vs. Rap	0.0005 vs. Rap	0.0000 vs. Rap	0.0013 vs. Rap
Wilcoxon		0.0335 vs. Rap	0.0002 vs. Rap	0.0000 vs. Rap	0.0002 vs. Rap
Log-rank			0.6535 vs. Soy	0.7857 vs. Soy	0.7839 vs. Soy
Wilcoxon			0.1069 vs. Soy	0.0707 vs. Soy	0.0606 vs. Soy
Log-rank				0.9260 vs. Ses	0.0404 vs. Ses
Wilcoxon				0.4678 vs. Ses	0.0012 vs. Ses
Log-rank					0.0000 vs. FFA-HSO
Wilcoxon					0.0001 vs. FFA-HSO
Experiment 3 (SHRSP, SPF)	Rapeseed oil (Rap) (n = 9)	Soybean oil (Soy) (n = 9)	Detergent-treated rapeseed oil (Det-Rap) (n = 10)	Detergent-treated hydrogenated soybean oil (n = 9)	
Survived Day (±SD)	105 ± 3	121 ± 8	102 ± 2	98 ± 2	
P value by log-rank		0.0278 vs. Rap	0.0105 vs. Rap	0.0234 vs. Rap	
Wilcoxon		0.0466 vs. Rap	0.0329 vs. Rap	0.0225 vs. Rap	
Log-rank			0.0057 vs. Soy	0.0003 vs. Soy	
Wilcoxon			0.0165 vs. Soy	0.0020 vs. Soy	
Log-rank				0.2621 vs. Det-Rap	
Wilcoxon				0.5667 vs. Det-Rap	

[a]SPF, specific pathogen-free; SHRSP, stroke-prone spontaneously hypertensive rats.

NaCl loading). Despite the differences in substrains of SHRSP and in animal care conditions, a statistically significant difference was observed in the mean survival times between the soybean oil and rapeseed oil groups. Previously, we noted that a mixed oil (75% high-oleate safflower oil, 12.5% perilla oil, and 12.5% safflower oil) with a fatty acid composition similar to that of rapeseed oil shortened the survival time by 45% compared to soybean oil (6). A FFA fraction obtained by lipase treatment of the same mixture was essentially devoid of the survival time-shortening activity (Experiment 2, Table 2). The survival times of sesame oil and soybean oil groups were similar but tended to be shorter than that of rats fed the FFA fraction derived from a lipase-treated mixed oil (Experiment 2, Table 2, and Fig. 3). Previously, we noted that both hydrogenated soybean and rapeseed oils had survival time-shortening activities comparable to that of rapeseed oil (7). The FFA fraction derived from lipase-treated hydrogenated soybean oil prolonged the survival time compared with rapeseed oil. However, the survival time of rats fed the FFA fraction derived from lipase-treated hydrogenated soybean oil was significantly shorter than that of rats fed the FFA fraction derived from lipase-treated mixed oil (Experiment 2, Table 2).

The lipase treatment used for the preparation of FFA fractions involves both lipase and detergent action. The subsequent experiments (Experiment 3, Table 2) examined whether detergent treatment without added lipase removes the presumed survival time-shortening factor. Rapeseed and partially hydrogenated soybean oils were treated with detergents in the absence of lipase. The detergent treatment alone did not reduce the survival time-shortening activity of the oils (Experiment 3, Table 2).

Effect of oils and derived FFA fractions on tissue lipid composition. Lipid contents in plasma, aorta, liver, and heart were determined for the rats fed the rapeseed oil, FFA derived from lipase-treated rapeseed oil, partially hydrogenated soybean oil, or soybean oil. No significant difference was observed in the contents of plasma cholesterol, plasma triacylglycerols, plasma phospholipids, aortic cholesterol, aortic

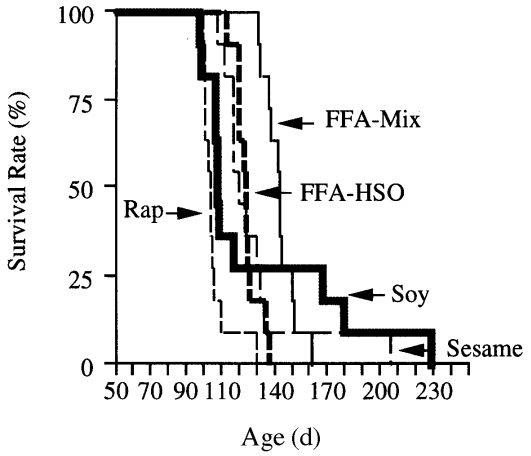

FIG. 3. Survival time-shortening activities of some vegetable oils and FFA fractions derived from lipase-treated oils. Rapeseed oil (Rap, n = 11), soybean oil (Soy, n = 11), sesame oil (Sesame, n = 11), a FFA derived from lipase-treated partially hydrogenated soybean oil (FFA-HSO, n = 11), and a FFA derived from lipase-treated mixed oil (FFA-Mix, n-11) were fed to SHRSP rats (SPF). Results of statistical analysis were presented in Experiment 2, Table 2. For other abbreviations see Figure 1.

phospholipids, liver phospholipids, liver neutral lipids, heart phospholipids, and heart neutral lipids among the four dietary groups (data not shown). The fatty acid compositions of tissue phospholipids are shown in Tables 3 and 4. *Trans* fatty acids were found in significant amounts only in the hydrogenated soybean oil group, and the compositions of other fatty acids in tissue phospholipids were affected relatively little by these diets. Interestingly, in plasma phospholipids and hepatic phospholipids, the proportion of arachidonic acid was significantly higher in the group fed the FFA derived from lipase-treated rapeseed oil than in the group fed the rapeseed oil (Tables 3 and 4). In contrast, no significant differences in fatty acid compositions among the four dietary groups were observed in the aortic phospholipids and triacylglycerols of all tissues examined (data not shown).

DISCUSSION

No survival time-shortening activity was observed in the FFA fractions from lipase-treated rapeseed oil (Fig. 1 and Experiment 1, Table 2) and mixed oil having a fatty acid composition similar to that of rapeseed oil (Experiment 2, Table 2). The chemical forms of the oils were different, e.g., triacylglycerol vs. free fatty acid, but fatty acid compositions were similar. Triacylglycerols are absorbed as 2-monoacylglycerols and FFA after pancreatic lipase hydrolysis, and the absorption rate of FFA is known to be higher than that of triacylglycerols (12). In the present experiments, no statistical differences were observed in tissue lipid contents and growth rates between the groups fed oils and those fed the FFA fractions derived from the lipase-treated oils. Our interpretation is that the presumed toxic factor(s) in these oils were inactivated by the lipase or they were hydrolyzed to a more hydrophilic compound(s) that remained in the detergent layer, or both. Lipase action was probably involved to obtain FFA fractions essentially devoid of survival time-shortening activity, because the activity was not diminished by treating oils with detergent-containing buffer without lipase.

The presumed toxic factor(s) in rapeseed oil could not be

TABLE 3
Fatty Acid Composition of Plama Phospholipids in SHRSP Rats (% of total fatty acids)

	Dietary group and number of animals			
Fatty acids	Rapeseed oil (n = 6)	FFA from lipase-treated rapeseed oil (n = 6)	Hydrogenated soybean oil (n = 6)	Soybean oil
14:0	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:0	20.9 ± 0.9	21.3 ± 0.8	20.0 ± 1.7	21.1 ± 1.3
16:1	1.1 ± 0.5	1.5 ± 0.5	1.3 ± 0.5	1.3 ± 0.4
18:0	29.3 ± 2.7[a]	28.2 ± 0.7[b]	24.4 ± 1.3[b]	29.8 ± 1.5[a]
*c*18:1	10.5 ± 1.5[a]	8.6 ± 0.5[a]	8.6 ± 2.6[b]	6.7 ± 1.4[b]
t18:1	n.d.[a]	trace	6.1 ± 0.6[a]	n.d.
18:2n-6	10.9 ± 1.4[a,b]	9.7 ± 0.5[b]	12.4 ± 1.4[a]	12.7 ± 1.6[a,b]
18:3n-6	0.2 ± 0.2	0.4 ± 0.4	0.2 ± 0.5	0.3 ± 0.2
18:3n-3	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.4	1.2 ± 0.9
20:0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2
20:3n-6	0.7 ± 0.4[b]	0.6 ± 0.1[b]	1.5 ± 0.8[a]	1.0 ± 0.5[a,b]
20:4n-6	14.9 ± 1.4[a,b]	18.4 ± 1.4[a]	13.1 ± 1.8[b]	15.3 ± 1.2[b]
22:0	0.9 ± 0.5	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1
22:4n-6	0.4 ± 0.3	0.5 ± 0.2	0.9 ± 0.1	0.7 ± 0.3
22:5n-3	0.4 ± 0.5	0.3 ± 0.3	1.0 ± 0.3	0.9 ± 0.3
22:6n-3	3.7 ± 0.9	4.1 ± 0.5	5.0 ± 1.4	3.9 ± 0.5
24:0	1.9 ± 0.4	1.7 ± 0.2	1.9 ± 0.5	1.8 ± 0.2
24:1	3.1 ± 0.8[a]	2.9 ± 0.4[a]	1.9 ± 0.5[b]	1.6 ± 0.4[b]
PL level (mg/dL)	68.6 ± 9.0	70.3 ± 4.1	68.6 ± 9.5	71.6 ± 6.9

[a]n.d., not detected; PL, phospholipids; for other abbreviations see Tables 1 and 2. Values with different roman superscripts are significantly different from each other at $P < 0.05$.

TABLE 4
Fatty Acid Composition of Hepatic Phospholipids in SHRSP Rats[a] (% of total fatty acids)

Fatty acids	Dietary group and number of animals			
	Rapeseed oil (n = 6)	FFA from lipase-treated rapeseed oil (n = 6)	Hydrogenated soybean oil (n = 6)	Soybean oil (n = 6)
14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	16.1 ± 1.2	14.9 ± 1.0	16.5 ± 0.8	16.45 ± 0.5
16:1	0.2 ± 0.1[a]	0.2 ± 0.1[a]	0.3 ± 0.0[a]	0.1 ± 0.1[b]
18:0DMA[b]	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.2 ± 0.0
18:1DMA	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
18:0	27.8 ± 1.8[a]	26.6 ± 1.1[a]	23.4 ± 0.6[b]	28.9 ± 1.5[a]
*c*18:1	9.1 ± 0.7[a]	7.9 ± 0.8[a]	8.3 ± 0.9[a]	4.7 ± 1.0[b]
*t*18:1	n.d.	n.d.	4.0 ± 0.9[a]	n.d.
18:2n-6	11.8 ± 0.4[b]	11.4 ± 0.8[b]	13.8 ± 1.2[a]	14.5 ± 1.5[a]
18:3n-6	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
18:3n-3	0.4 ± 0.1[a]	0.3 ± 0.0[a,b]	0.2 ± 0.0[c]	0.3 ± 0.0[b]
20:0	0.3 ± 0.1[a]	0.2 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]
20:1	0.3 ± 0.0[a]	0.2 ± 0.1[a,b]	n.d.	0.1 ± 0.0[b,c]
20:3n-6	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.3
20:4n-6	21.8 ± 2.8[b]	27.4 ± 0.8[a]	20.3 ± 1.3[b]	23.1 ± 1.2[b]
20:5n-3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
22:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:4n-6	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
22:5n-6	0.3 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
22:5n-3	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.2
22:6n-3	8.7 ± 0.5[a,b]	10.1 ± 0.8[a]	9.9 ± 1.5[a,b]	8.4 ± 0.5[b]
24:0	0.6 ± 0.1[b,c]	0.5 ± 0.0[c]	0.7 ± 0.0[a,b]	0.8 ± 0.1[a]
24:1	0.5 ± 0.1[a]	0.5 ± 0.1[a]	0.3 ± 0.0[b]	0.2 ± 0.1[b]
PL level (mg/g liver)	19.2 ± 0.7	21.9 ± 1.4	18.7 ± 2.2	20.4 ± 4.2

[a]Values with different roman superscripts are significantly different from each other at $P < 0.05$.

[b]DMA, dimethylacetal. For other abbreviations see Tables 1–3.

eliminated or inactivated by partial hydrogenation (7). Unexpectedly, partially hydrogenated soybean oil exhibited a survival time-shortening activity similar to rapeseed oil, suggesting that a toxic factor(s) are generated in or contaminate partially hydrogenated soybean oil during the process of production (Fig. 1 and Ref. 7). The survival time of rats fed the FFA fraction derived from lipase-treated partially hydrogenated soybean oil was significantly shorter than that of rats fed the FFA fraction derived from a mixture of high-oleate safflower oil/safflower oil/perilla oil (Table 2), suggesting that *trans* fatty acids may be partially responsible for the survival time-shortening activity. The lipase treatment was also effective for the preparation of FFA fraction from hydrogenated oils (Table 2).

Several other studies have shown or suggested the existence of minor components other than triacylglycerol in rapeseed oil. Among sulfur compounds, isothiocyanates and oxazolidinethione do not appear to be responsible for the survival time-shortening activity, because butyl isothiocyanate (8 mg/kg of soybean oil), phenethyl isothiocyanate (1 mg/kg), and allyl isothiocyanate (1 mg/kg) did not shorten the survival time at concentrations comparable to those in rapeseed oil (5). Rapeseed oil has a significantly higher phytosterol content than soybean oil (13). However, unsaponifiable compounds from rapeseed oil did not exhibit survival time-shortening activity and no significant amounts of phytosterols were found in tissue lipids (data not shown). We suggest that the toxic factor(s), if any, are different from those described above and are sensitive to lipase action.

Mean survival time of experimental animals has not been a common endpoint for the determination of the safety or nutritional adequacy of fats and oils. By using this measure, we raised a question on the safety of some of the common oils and oil products in human nutrition, although the applicability of the results to human nutrition is entirely unknown. Besides shortened survival time, we observed accelerated renal injury in SHRSP rats fed rapeseed oil and partially hydrogenated oil (Miyazaki, M., Watanabe, S., Oikawa, T., Morazumi, K., Fuzinami, T., and Okuyama, H., unpublished data). Other groups have also raised questions on the safety of some common vegetable oils using different endpoints; for example, increased mortality after injection of iron into piglets fed a rapeseed oil-supplemented, tocopherol-restricted diet (14), and reduced platelet counts in piglets fed rapeseed oil- or high-oleate vegetable oil-supplemented diets (15,16). The problem of myocardial necrosis reported in rapeseed oil-fed Sprague-Dawley rats remains unresolved (8,9). The lipase-treatment and successful preparation of FFA fraction essentially devoid of survival time-shortening activity of the original oils described in this paper may help in solving these unresolved problems raised by other laboratories.

ACKNOWLEDGMENTS

This study was supported in part by the Special Coordination Funds for Promoting Sciences and Technology of the Science and Technology Agency, Japan Government.

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[Received March 16, 1998, and in final revised form May 15, 1998; revision accepted May 18, 1998]

Docosahexaenoic Acid Does Not Affect Aggression of Normal Volunteers Under Nonstressful Conditions. A Randomized, Placebo-Controlled, Double-Blind Study

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ABSTRACT: We previously found that docosahexaenoic acid (DHA) intake prevents aggression enhancement at times of mental stress. In the present study we investigated changes in aggression under nonstressful conditions. Forty-six students of two universities took either DHA-rich fish oil capsules containing 1.5 g DHA (DHA group: 13 males and 9 females) or control oil capsules containing 97% soybean oil plus 3% of another fish oil (control group: 11 males and 13 females) for 3 mon in a double-blind fashion. At the start and end of the study they took an aggression-estimating test (P-F Study) without a stressor component. DHA (5.9 to 8.5%, $P < 0.001$) and eicosapentaenoic acid (0.7 to 1.5%, $P < 0.001$) increased in red blood cell phospholipids in the DHA group, while linoleic acid increased slightly (8.3 to 9.1%, $P < 0.002$) in the soybean oil control group. In the control group, measured aggression levels decreased from 34.8 to 29.4% ($P < 0.005$), whereas they remained stable in the DHA group (33.5 to 33.8%). The intergroup differences (–5.4 vs. 0.3%) were marginally significant ($P \leq 0.05$). Aggression levels were stable in the DHA group whether there was stressor (as previously shown) or not. This effect of DHA appears to be interesting, considering the reported association between a low intake of n-3 fatty acids and depression.
Lipids 33, 663–667 (1998).

Several epidemiological studies (1,2) have suggested that a higher intake of fish appears to be associated with a lower incidence of coronary heart disease. Dietary intervention with fatty fish (3) or α-linolenic acid (4), one of the n-3 fatty acids, has also reduced the total mortality in secondary prevention trials. In the intervention trial with fish (3), reduction in death rates was attributed to a decreased incidence of fatal arrhythmia, because fatal myocardial infarctions were significantly reduced in the fish-eating group compared with the nonfish-eating group, although the total incidence of myocardial infarction was similar between the two groups.

A recent intervention trial with n-3 long-chain fatty acids in survivors of coronary heart disease found that the standard deviation of all normal RR intervals in 24-h Holter recordings significantly increased in an n-3 fatty acid group compared with controls ingesting olive oil capsules (5). This finding is important because an increase in parasympathetic tone reflected by an increased standard deviation of RR intervals is associated with a higher ventricular fibrillation threshold and fewer episodes of ventricular fibrillation (6). Indeed, Sellmayer *et al.* (7) have successfully reduced ventricular premature complexes with fish oil in a placebo-controlled double-blind study. However, there have been few mechanistic studies on antiarrhythmic effects of fish (8,9). Taking into account that hostility may be a significant risk factor for CHD and arrhythmia (10), it is important to investigate whether fish oils might modulate hostility or aggression.

Hibbeln and Salem (11) have proposed an interesting hypothesis about n-3 fatty acids and depression. They have suggested that societies consuming large amounts of n-3 fatty acids appear to have low rates of major depression, and postulated that docosahexaenoic acid (DHA), one of the long-chain n-3 fatty acids, may reduce the development of depression. Consequently, the study of psychological effects of n-3 fatty acids has become rather important.

In our previous study (12) using university students as subjects, we showed that administration of DHA-rich fish oil prevented enhancement of aggression at times of mental stress. To investigate the effect of DHA on aggression further, we conducted a similar study under nonstressful conditions. In the present study we carefully chose the timing of psychological testing to avoid the confounding influence of any academic examinations.

SUBJECTS AND METHODS

Subjects. The purpose and plan of the present study was explained to two classes of university students to recruit volunteers; one was a fourth-year class at Toyama Medical and Pharmaceutical University and the other was a third-year class at Kogakkan University. Fifty-nine nonsmoking students of

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RBC, red blood cells.

Toyama Medical and Pharmaceutical University [21–30 yr of age (median 22), 16 males and 15 females] and Kogakkan University (20–22 yr of age, 14 males and 14 females) volunteered to enter two parallel trials. None of the subjects had participated in the previous study (12). They were in good health as determined by history and physical examinations conducted by physicians 3 mon before the trial. They did not suffer from any chronic illness including alcoholism and were not taking any medicines regularly. They were allocated to either the control or the DHA group in a double-blind manner; randomization was stratified according to site and sex (Table 1). Written informed consent was obtained from each subject, and the study was approved by the Pharmacy and Therapeutics Committee (a subcommittee of the ethics committee) of Toyama Medical and Pharmaceutical University.

Study design. The design of the present study was very similar to our previous one (12). The present study was performed in the two universities on the same protocol. All subjects were asked to maintain their body weights and physical activity levels and to consume their habitual diets during the study. They were instructed to take 10 capsules per day containing DHA-rich fish oil (for the DHA group, 1.5 g DHA/day) or control oil (the control group) for 13 wk. Three or four capsules were taken after each meal. Each capsule contained 300 mg of oil with 0.3% α-tocopherol. The fish oil used for the DHA group contained 49% DHA and 7% eicosapentaenoic acid (EPA) and was essentially odorless. The control oil was a mixture of 97% soybean oil and 3% of another fish oil (concentrated sardine oil containing 32% EPA and 16% DHA) that had been only partially deodorized. The detailed fatty acid composition of the oils is shown elsewhere (13).

At the starting point of the study and at the end (13 wk afterward), subjects were asked to come to their own universities in the early morning without having breakfast and to refrain from drinking alcoholic beverages during the previous day. Blood was taken from fasting subjects. After breakfast they were asked to take psychological tests and to complete a food frequency questionnaire for the previous 4 wk. At the end of the study they were also asked to guess what their capsules had contained. Of 22 control subjects, 10 said they were receiving control capsules, 6 said DHA capsules, and 6 were unsure. Of 20 subjects taking DHA capsules, 7 thought they were taking control capsules, 9 thought DHA, and 4 were unsure. Thus 45% of subjects in each group had correctly surmised their treatment group.

TABLE 1
The Number of Subjects at the Start and the Exclusion of Subjects[a]

	Control group				DHA group			
	Toyama		Kogakkan		Toyama		Kogakkan	
Exclusion step	M	F	M	F	M	F	M	F
Subjects at randomization	8	8	7	7	8	7	7	7
Dropouts[b]			–2	–1				–2
Significant body weight changes (|Δ| > 3 kg)			–1 (+3.5 kg)				–1 (+4 kg)	
Insufficient capsule intake (<70%)	–1			–1				–1
Decrease in red blood cell DHA (applied to DHA group only)[c]					–1	–2		
Final number	7	8	4	5	7	5	6	4

[a]The exclusion was performed before the double-blind code was broken. M, male; F, female; DHA, docosahexaenoic acid.
[b]Four of the five dropouts were due to personal reasons and the other (control male) was due to intolerance to capsules (gastrointestinal disorder).
[c]The exclusion at this step was performed without knowing the results of psychological tests after the double-blind code was broken.

The major difference between the present study and the previous one was timing. The present study started in July (during summer vacation) and ended in October in the same school year. The two check points were carefully chosen so that no important exams were scheduled within 30 d from any check points at either university.

Psychological tests. Two tests were employed. One was P-F Study, originally developed by Rosenzweig (14). We used the adult form of a Japanese version (15). This test consisted of 24 pictures illustrating frustration. Subjects were asked to look at those pictures and describe their first reactions (replies) with a couple of sentences. The frequency of appearance of their reactions to others was calculated as extraggression (%). The other test was a Japanese version (16) of Cook and Medley hostility scale (17). This test consisted of 87 questions, 50 of which were selected for measurement of hostility. Rating of these two tests was blindly done by three testers independently throughout the study. On rare occasions of discrepancy in ratings of P-F Study among the three testers, they discussed their scorings so as to reach a consensus.

Fatty acid analysis. The fatty acid composition of the total phospholipid fraction of red blood cells (RBC) was determined as described previously (18) with slight modification. Briefly, packed RBC from EDTA-anticoagulated blood were washed twice with saline. Total lipids were extracted by the method of Folch *et al.* (19). The total phospholipid fraction was separated by thin-layer chromatography, and its fatty acid composition was analyzed by gas chromatography after transmethylation (20).

Food analysis. Food intake was calculated, from two sets of food frequency questionnaires completed at the start and end of the study, with our own software. This software was a program of Kishokun (a food questionnaire program, Kan'ei-shuppan, Okayama, Japan) modified for local use.

Exclusion of subjects. According to the exclusion criteria determined prior to the trial (<70% capsule intake, and >3 kg changes in body weights), some subjects were excluded before the double-blind code was broken except for the exclusion step based on the DHA concentration of RBC (Table 1). Some subjects in the DHA group whose DHA concentration did not increase after capsule administration were excluded (Table 1). This step was performed by investigators who did not know the results of P-F Study and Cook and Medley hostility scale and applied to the DHA group only, after the code was broken and the DHA concentration of RBC was available.

Statistical analysis. Data are expressed as mean ± SD. Statview (Japanese version 4.5; Abacus Concepts, Berkeley, CA) was used for statistical analysis. A paired *t*-test was used for intragroup comparisons of the end and start of the study. Intergroup differences (the differences between intragroup differences of the two groups) were analyzed by an unpaired *t*-test. Site effect-adjusted *P*-values were calculated by two-way analysis of variance with the following two factors: sites and capsules. Baseline values of the two groups were compared by using an unpaired *t*-test. Differences with $P < 0.05$ were considered significant.

RESULTS

One subject in the control group complained of gastrointestinal disorder and dropped out before the study was completed. Subjects who completed the study complained of no serious adverse effects of capsules, and could not infer the kind of their own capsules more correctly than by chance. Excluded subjects are listed in Table 1.

Changes in the fatty acid composition of the total phospholipid fraction of RBC are shown in Table 2. In the control group, linoleic acid increased; the increase was small but statistically significant. In the DHA group, DHA and EPA increased over time, whereas arachidonic acid decreased.

Averaged daily intakes of fatty acids from food are shown in Table 3. There were no significant differences between the two groups at the start.

Changes in Cook and Medley hostility scale were not significant in either the control group (33.3 ± 11.4 to 31.9 ± 10.2%, $n = 24$) or the DHA group (38.8 ± 13.4 to 37.5 ± 16.2%, $n = 22$). This was true in both universities (data not shown).

TABLE 2
Changes (mol%) in the Fatty Acid Composition of the Total Phospholipid Fraction of Red Blood Cells (mean ± SD)[a]

	Control group ($n = 22$)[b]		DHA group ($n = 22$)	
Fatty acid	Start	End	Start	End
16:0	23.3 ± 2.5	23.2 ± 2.5	22.6 ± 2.8	22.3 ± 1.5
18:0	14.9 ± 1.0	14.9 ± 1.3	14.8 ± 1.1	14.6 ± 0.8
18:1	11.6 ± 0.8	12.0 ± 1.1	12.0 ± 1.0	12.2 ± 1.1
18:2	8.3 ± 0.7	9.0 ± 0.9[c,e]	8.5 ± 0.8	8.3 ± 0.9
20:4	9.5 ± 1.0	9.1 ± 1.9	9.4 ± 1.0	8.8 ± 0.6[c,f]
20:5	0.77 ± 0.27	0.66 ± 0.23	0.71 ± 0.19	1.35 ± 0.36[d,f]
22:6 (DHA)	4.8 ± 1.0	4.6 ± 1.1	4.8 ± 0.9	7.0 ± 1.0[d,f]

[a]Basically, there were no marked differences between the two universities, therefore data from the two universities were combined. There were no significant differences between the two groups at baseline.
[b]Two blood samples from Kogakkan University were lost during transportation.
[c]Significantly different from baseline, $P < 0.001$.
[d]Significantly different from baseline, $P < 1 \times 10^{-9}$; however, *P* was theoretically null for DHA, since subjects with decreased DHA contents were excluded beforehand.
[e]Intragroup differences were significantly different from the other group; $P < 0.001$.
[f]Intragroup differences were significantly different from the other group; $P < 0.001$. For abbreviation see Table 1.

Changes in extraggression (P-F Study) are shown in Table 4. Extraggression significantly decreased over time in the control group at both Toyama and Kogakkan, whereas it remained stable in the DHA group. The intergroup differences were significant ($P < 0.05$) by two-way analysis of variance; however, an unpaired *t*-test showed a tendency toward decreased extraggression in the control group compared with the DHA group ($P = 0.05$).

DISCUSSION

Recently, suggestions of a relationship between low n-3 fatty acid intake and increased incidence of depression (11) have been further strengthened by the findings of Adams *et al.* (21) that the ratio of RBC phospholipid arachidonic acid to EPA correlates significantly with severity of depression. Interestingly, our previous study (12) suggested an aggression-stabilizing effect of DHA administration under the stress of university examinations. This effect might prevent students from experiencing wider variances of mental reactions to stressful events and thus from mental exhaustion. This effect may also be applicable to prevention of arrhythmia. As described in the introduction, it has been suggested that the intake of n-3 fatty acids prevents life-threatening arrhythmias. Aggressive behaviors are associated with decreased parasympathetic nervous system activity, which is linked with decreased brain serotonin function (10). Also, parasympathetic tone is important for prevention of ventricular fibrillation (6). Consequently, if DHA administration stabilizes the brain serotonergic function as postulated by authors of previous studies (11,12,22), this effect may contribute to the antiarrhythmic effects of fish oils.

Extraggression in the control group was significantly lower at the end of the study. This finding was contrary to our previous study (12), in which controls exhibited increased extraggression over time. The major difference between the two studies consists in the absence of extremely important exams 7–10 d after the last psychological tests in the present study. Consequently, the presence or absence of mental stress may have contributed to the radically different result. In general, the presence of a stressor increases the degree of aggression (23,24). Actually, hostility (the Multiple Affect Adjective Checklist) and aggression (the Adjective Rating Form) scales of nondrinkers were higher than those of moderate drinkers when subjects were not provoked, whereas, after provocation, both scales increased in the two groups, and those of moderate drinkers became higher than for nondrinkers (24). Thus, it is possible that the results obtained from the psychological testing methods without stressor as performed in the present study were different from the previous results (12). The results of the present study therefore do not necessarily contradict our previous finding that DHA reduced aggression in a normal population under significant stress.

We chose identical control capsules with those used before (12) to facilitate the comparison of the two studies. In the previous study (12) the control oil did not change the fatty acid composition of the total plasma phospholipid fraction, but

TABLE 3
Averaged Daily Intakes of Lipids and Energy (mean ± SD)[a]

Item	Control group (n = 24)		DHA group (n = 22)	
	Start	End	Start	End
		g/d		
Total lipids	66.9 ± 35.7	57.3 ± 20.1	67.5 ± 25.1	55.7 ± 21.2[b]
Linoleic acid (18:2)	12.8 ± 6.8	12.9 ± 5.1	11.5 ± 4.4	10.1 ± 4.6
EPA (20:5)	0.088 ± 0.099	0.144 ± 0.098[c]	0.107 ± 0.102	0.319 ± 0.111[d]
DHA (22:6)	0.022 ± 0.024	0.043 ± 0.024[d]	0.026 ± 0.025	1.529 ± 0.028[d]
Linoleic acid/total lipids	0.20 ± 0.04	0.22 ± 0.05	0.18 ± 0.05	0.18 ± 0.04
		kJ/d		
Total energy	9.19 ± 3.92	8.34 ± 3.23	8.07 ± 1.97	7.25 ± 2.00

[a]Lipid and energy intakes were assessed by food frequency questionnaires at the start and end of the study. There were no significant differences between the two sites; therefore, data were combined. Food intake of the last 4 wk was requested at each check point. Data at the end of the study contain lipids from capsules. There were no significant differences in any data between the two groups at the start.
[b]Significantly different from baseline, $P < 0.005$.
[c]Significantly different from baseline, $P < 0.002$.
[d]Significantly different from baseline, $P < 0.001$. EPA, eicosapentaenoic acid, for other abbreviations see Table 2.

RBC fatty acids were not measured. In that study, extraggression increased in the control group under stressful conditions. However, in the present study (Table 2), linoleic acid concentrations in the RBC phospholipid fraction increased over time in the control group, but plasma fatty acids were not measured. Although the daily intake of linoleic acid did not increase in the control group even after taking capsule lipids into consideration, the ratio of the intakes of linoleic acid to total lipids tended to increase from 0.20 to 0.22 (Table 3, P = 0.08). This increment might partly explain the changes in linoleic acid in control RBC.

We did not anticipate any changes in extraggression in the control group. We carefully planned the timing of psychological testing. The study started at the beginning of summer vacation of the universities, and ended at the middle of the semester that was more than 1 mon away from any important exams. Therefore, stress levels for students appeared alike between the two check points. At least it is highly unlikely that students felt less stress at the end of the study. Then, why did measured extraggression decrease in the control group? It is possible that habituation to testing could account for such a decline, but this would not explain the failure of a drop in measured extraggression in the DHA group.

If we look at intergroup changes instead of intragroup differences, we may ask the following question: Is there any possibility that a difference in extraggression between the two groups was merely a chance finding? The intergroup differences in extraggression were not significant either in Toyama subgroup alone or in Kogakkan (Table 4). Besides, the plain sum of the two subgroups without consideration of the site effect showed only a marginally significant difference (P = 0.05) by unpaired t-test. Considering the number of comparison tests, the intergroup difference in extraggression may be a chance finding. Finally, we found at the end of our study that 45% of each treatment group had correctly identified their assignment but it is not possible to determine whether this could have further biased the testing result. Consequently, it is prudent to treat the intragroup changes in extraggression cautiously in the control group. The results of the Cook and Medley hostility scale partly support the null hypothesis about changes in extraggression.

In conclusion, the administration of DHA does not change extraggression in university students in the absence of mental stress related to examinations. Whether these findings would be applicable to patients undergoing the stress of a life-threatening disease such as myocardial infarction will be determined with further study.

TABLE 4
Changes in Extraggression After 3 mon of Capsule Administration (mean ± SD)

	Start	End	Intragroup difference
Toyama subgroup			
Control (n = 15)	33.5 ± 7.7	30.2 ± 10.6[a]	−3.3 ± 5.9
DHA (n = 12)	36.4 ± 10.7	35.1 ± 10.6	−1.3 ± 5.7
Kogakkan subgroup			
Control (n = 9)	37.0 ± 11.2	28.1 ± 13.2[a]	−8.9 ± 10.9
DHA (n = 10)	30.1 ± 13.2	32.3 ± 16.5	2.2 ± 15.3[b]
Total			
Control (n = 24)	34.8 ± 9.1	29.4 ± 11.4[c]	−5.4 ± 8.4
DHA (n = 22)	33.5 ± 12.0	33.8 ± 13.3	0.3 ± 11.0[d]

[a]Significantly different from the starting values, $P < 0.05$.
[b]Tended to be different from the corresponding values of the control group, $P = 0.09$.
[c]Significantly different from the starting values, $P < 0.005$.
[d]Significantly different ($P < 0.05$) from the control group by two-way analysis of variance but not significantly different ($P = 0.05$) by unpaired t-test. For abbreviation see Table 1.

ACKNOWLEDGMENTS

This work was partly supported by the Shorai Foundation for Science and Technology and by the Special Coordination Funds for

Promoting Science and Technology of the Science and Technology Agency of the Japanese Government. We thank Hiroko Sugimori, Michiyo Nishikawa, Mariko Obi, Tetsuchika Yoshioka, and Go Kimura for their technical assistance.

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[Received August 25, 1997; and in final revised form and accepted May 28, 1998]

Effect of the Degree of Hydrogenation of Dietary Fish Oil on the *trans* Fatty Acid Content and Enzymatic Activity of Rat Hepatic Microsomes

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ABSTRACT: The degree of fat hydrogenation and the *trans* fatty acid content of the diet affect the fatty acid composition of membranes, and the amount and the activity of some membrane enzymes. We describe the effects of four isocaloric diets containing either sunflower oil (SO, 0% *trans*), fish oil (FO, 0.5% *trans*), partially hydrogenated fish oil (PHFO, 30% *trans*), or highly hydrogenated fish oil (HHFO, 3.6% *trans*) as fat sources on the lipid composition and the *trans* fatty acid content of rat hepatic microsomes. We also describe the effect of these diets on the cytochrome P-450 content and on the aminopyrine *N*-demethylase, aniline hydroxylase, and UDP-glucuronyl transferase microsomal activities. Cytochrome P-450 content was dependent on the degree of unsaturation of the diet, being higher for the FO-containing diet and lower for the HHFO diet. Aminopyrine *N*-demethylase activity also correlated with the degree of unsaturation of the diet as did the cytochrome P-450 content did (FO > SO > PHFO > HHFO). Aniline hydroxylase activity appeared to be independent of the degree of unsaturation of the dietary fat, but correlated with the *trans* fatty acid content of the diet, which was also reflected in the *trans* content of the microsomal membranes. UDP-glucuronyl transferase activity was higher for the FO-containing diet than for the SO diet, showing intermediate values after the PHFO and HHFO diets.

Lipids 33, 669–673 (1998).

It has been proposed that several membrane functions are dependent on the lipid composition of the membrane (1,2), and that dietary fat intake can modify, to some extent, the composition and the biochemical activity of cellular membranes (3,4). The cytochrome P-450 hepatic microsomal mixed-function oxidase system (MFOS) plays a vital role in the biotransformation of a wide variety of drugs and foreign compounds and of some endogenous substances such as steroids and eicosanoids (5). The MFOS components are membrane-bound molecules, and the microsomal lipid environment, which is readily influenced by different dietary fatty acids, plays an important role in the enzyme regulation of the system (6,7).

Partially hydrogenated and highly hydrogenated vegetable and marine oils (mainly fish oil) are widely used for the preparation of margarines and shortenings for human foods and animal feed (8). Hydrogenation provides oils better stability against oxidation and facilitates mixture with other food components (9). However, this chemical process forms variable and, under some conditions, high amounts of positional and/or geometric *trans* fatty acids isomers (10). The biological effects of *trans* fatty acids have been a matter of controversy (11,12), and concerns about their consumption and biological effects arose from the "unnatural" origin of these isomeric fatty acids (13). Partially hydrogenated fish oil (PHFO) and highly hydrogenated fish oil (HHFO) are widely used in some South American and European countries for the manufacture of cheap margarines and industrial fats, and as components of diverse food items. The complex fatty acid composition of fish oils (FO) (more than 20 different n-11, n-9, n-7, n-6, and n-3 fatty acids can be identified) allows the formation of a great variety of *trans* isomers, especially when the oil is subjected to partial hydrogenation (14).

As biochemical information concerning the effects of PHFO and HHFO and the role of *trans* fatty acid isomers on the activity of MFOS components is scarce, we studied in the rat the effect of a synthetic diet containing these hydrogenated products as the fat source on the fatty acid composition of hepatic microsomal membranes, on the cytochrome P-450 content of these membranes, and on the activity of some enzymes related to MFOS. Results are compared to the effect of a vegetable oil and of a nonhydrogenated FO.

MATERIALS AND METHODS

Materials. Components of the synthetic diet were purchased at the local market. Commercial sunflower (SO), refined FO (sardine oil), PHFO, and HHFO (both prepared from sardine oil) were provided by Watts Alimentos (Santiago, Chile). Solvents and salts were purchased from Merck Chile (Santiago, Chile). Fatty acid standards (*cis* and *trans*) for gas chromatography and reagents for fatty acid methyl ester prepara-

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Abbreviations: FO, fish oil; HHFO, highly hydrogenated fish oil; MFOS, mixed-function oxidase system; MUFA, monounsaturated fatty acids; PHFO, partially hydrogenated fish oil; PUFA, polyunsaturated fatty acids; SAFA, saturated fatty acids; SO, sunflower oil.

tion and for enzyme assays were obtained from Sigma Chemicals (St. Louis, MO).

Methods. Peroxide value, the cholesterol content, the unsaponifiable matter, the free acidity, the melting point, and the iodine value of the oils were assessed according to AOAC Official methods (15). Isocaloric (16 kJ/g) synthetic diets were prepared according to Saito *et al*. (16) with some minor modifications. The total fat content was always 15.5% w/w: 0.5% w/w was provided by SO to avoid essential fatty acid deficiency, and the remaining 15% was either SO, FO, PHFO, or HHFO. Four groups ($n = 8$) of 70 day-old male Wistar rats (Biocronogen, Santiago, Chile) weighing 200–230 g and previously fed with the standard laboratory chow were separated in individual cages and fed with one of the four experimental diets for 40 d. Animals were maintained under light/dark cycles of 12 h and with water *ad libitum*. Animal weight and diet consumption were determined daily and registered. No significant changes were observed in the weight gain and in the diet consumption when the different experimental groups were compared. At the end of each experimental period, animals were sacrificed by cervical dislocation, and hepatic microsomes were prepared by ultracentrifugation according to Lake (17). Purity of microsomal preparation was determined assessing glucose-6-phosphatase activity (E.C. 3.1.3.9) according to Gierow and Jergil (18). The treatment and the managing of animals were approved by INTA's Review Board.

Microsomal lipids were extracted with methanol/chloroform (2:1, vol/vol) according to Bligh and Dyer (19). Microsomal cytochrome P-450 content was assessed as described by Omura and Sato (20). Aminopyrine *N*-demethylase cytochrome P-450 associated activity was measured through the reagent of Nash (21), and aniline hydroxylase-cytochrome P-450 associated activity was assessed through the formation of *p*-aminophenol according to Lake (17). UDP-glucuronyl transferase activity (E.C. 2.4.1.108) was assayed according to the method of Dannenberg and Zakim (22).

Total *trans* fatty acid content of the different oils used for the diets' formulations was assessed by infrared spectroscopy according to AOCS method Cd 14-61 (23). Gas chromatography of microsomal fatty acids (*cis* and *trans*) was performed in a Hewlett-Packard 5890 series II Plus gas chromatograph (Palo Alto, CA) with split injector and flame-ionization detector, and using an SP-2560 (Supelco, Inc., Bellefonte, PA) capillary column (100 m × 0.25 mm i.d.). The column temperature was raised from 140 to 240°C at 2°C/min and then was maintained at 240°C for 20 min. The injector temperature was 240°C, and the detector temperature was 260°C. Hydrogen was used as carrier gas.

Statistical analysis. Results are expressed as mean ± SD. Statistical significance of differences between mean values was assessed by analysis of variance coupled with Duncan's multiple-range test at the 5% level of significance (24).

RESULTS

Table 1 shows the chemical and physical characterization of the oils used for the diet preparation. The low peroxide value, unsaponifiable matter, and free acidity of the four oils guarantee their quality as suitable fats for the study. As the cholesterol content of the four oils was very different due to their origin and/or processing (no cholesterol for vegetable oil, high cholesterol for FO, and intermediate values for PHFO and HHFO due to hydrogenation), its content was adjusted in each oil to that of FO to avoid metabolic differences due to the sterol. The melting point of each oil correlated with the degree of hydrogenation, low for SO and FO and high for HHFO. The iodine value also agrees with the degree of hydrogenation of the oils being high for FO due to its high polyunsaturation, and low for HHFO. As expected, SO and FO showed low content of *trans* fatty acids. HHFO, as a result of the drastic reduction of its unsaturation due the strong hydrogenation, also showed a low *trans* content. However, for PHFO, 30% of the total fatty acids contained *trans* unsaturations.

Table 2 shows the *cis* and *trans* fatty acid isomers that can be identified under our experimental conditions in the microsomal membranes after the different dietary fats. For diets containing PHFO and HHFO, there were increases in the content of saturated (SAFA) and monounsaturated (MUFA) fatty acids of microsomal membranes when compared to the SO and FO diets. However, feeding rats with PHFO and HHFO reduced the content of polyunsaturated fatty acids (PUFA) of microsomes. The *trans* fatty acid content of microsomal membranes of the dietary groups fed different diets showed some differences. While membranes from rats fed SO, FO, and HHFO showed very low content of *trans* isomers, membranes from animals fed with PHFO showed a significantly higher amount of isomeric fatty acids. *Trans* fatty acids present in the PHFO-containing diet were incorporated into microsomal membranes. As the biological behavior of *trans* fatty acids has been compared to the behavior of SAFA (25), in Table 2 are also presented the SAFA + *trans* content of microsomal membranes and the ratio MUFA + PUFA/SAFA + *trans*. There was a decrease in the microsomal unsaturated

TABLE 1
Chemical and Physical Characterization of the Oils Used in the Experimental Diets

Analysis[a]	SO	FO	PHFO	HHFO
Peroxide value (meq/kg)	2.1 ± 0.2	5.8 ± 0.3	4.9 ± 0.2	1.5 ± 0.2
Cholesterol (mg/100 g)	0.0	488.1 ± 28.6	321.2 ± 21.3	254.3 ± 14.5
Unsaponifiable matter	4.7 ± 0.6	8.7 ± 1.3	6.1 ± 0.8	5.5 ± 0.4
Free acidity (μg KOH/g)	56.3 ± 5.2	110.5 ± 8.6	67.8 ± 4.8	167.8 ± 9.7
Melting point (°C)	6.0 ± 2.0	21.0 ± 3.0	29.0 ± 2.0	49.0 ± 3.0
Iodine value	112.0 ± 5.2	221.0 ± 7.6	125.5 ± 4.5	8.3 ± 0.8
Trans content (%)	n.d.	0.50 ± 0.0	30.2 ± 4.3	3.6 ± 0.5

[a]Results represent the average of eight assays ± SD; n.d.: not detected. SO, sunflower oil; FO, fish oil; PHFO, partially hydrogenated fish oil; HHFO, highly hydrogenated fish oil.

TABLE 2
Microsomal Fatty Acid Composition After the Different Oil-Containing Diets[a]

Fatty acid[b]	SO	FO	PHFO	HHFO
C14:0	0.15 ± 0.02	0.30 ± 0.07	0.25 ± 0.02	0.45 ± 0.16
C16:0	12.60 ± 0.54	15.99 ± 2.16	18.03 ± 0.47	18.66 ± 2.76
C18:0	22.28 ± 1.47	19.47 ± 1.21	24.59 ± 0.62	24.21 ± 1.41
C24:0	0.48 ± 0.11	0.25 ± 0.04	0.49 ± 0.05	0.35 ± 0.10
Total SAFA	35.51 ± 0.53	36.01 ± 0.87	43.36 ± 0.29 a,b	43.67 ± 1.10 a,b
C16:1	0.43 ± 0.20	1.02 ± 0.23	0.16 ± 0.02	2.20 ± 0.45
C18:1n-9	3.83 ± 0.28	4.50 ± 0.38	8.01 ± 0.30	10.89 ± 1.31
C18:1n-7	2.08 ± 0.14	1.65 ± 0.31	1.82 ± 0.15	3.63 ± 0.66
C24:1	0.07 ± 0.04	0.32 ± 0.08	0.21 ± 0.02	0.14 ± 0.07
Total MUFA	6.41 ± 0.16	7.49 ± 0.25	10.20 ± 0.12 a,b	16.86 ± 0.62 a,b
C18:2n-6	11.35 ± 0.67	4.59 ± 0.31	10.34 ± 0.24	6.01 ± 1.82
C18:3n-3	0.49 ± 0.11	0.27 ± 0.01	0.26 ± 0.06	0.22 ± 0.02
C18:3n-6	0.28 ± 0.09	0.05 ± 0.01	0.15 ± 0.06	0.26 ± 0.04
C20:2n-6	0.93 ± 0.31	0.41 ± 0.04	0.58 ± 0.04	0.62 ± 0.04
C20:3n-6	1.04 ± 0.23	0.72 ± 0.08	1.37 ± 0.14	1.93 ± 0.20
C20:4n-6	33.45 ± 1.59	17.76 ± 1.22	16.01 ± 0.51	17.72 ± 2.27
C20:5n-3	n.d.	9.27 ± 1.46	1.89 ± 0.03	0.92 ± 0.41
C22:4n-6	1.10 ± 0.40	0.24 ± 0.13	0.11 ± 0.09	0.44 ± 0.13
C22:5n-3	0.70 ± 0.22	2.26 ± 0.12	1.44 ± 0.18	0.48 ± 0.19
C22:6n-3	7.81 ± 1.74	19.14 ± 2.97	11.45 ± 0.48	9.54 ± 1.75
Total PUFA	57.15 ± 0.57	54.71 ± 0.63	43.60 ± 0.18 a,b	38.14 ± 0.68 a,b
C16:1n-7*t*	0.07 ± 0.05	0.11 ± 0.09	0.96 ± 0.05	0.05 ± 0.01
C18:1 n-7*t*	0.18 ± 0.10	0.33 ± 0.38	0.79 ± 0.42	0.36 ± 0.14
C18:1 n-9*t*	0.13 ± 0.12	n.d.	0.31 ± 0.14	n.d.
C20:1 n-9*t*	n.d.	n.d.	0.04 ± 0.03	n.d.
Total *trans*	0.38 ± 0.09	0.44 ± 0.23	2.10 ± 0.16 a,b	0.41 ± 0.08
SAFA + *trans*	35.89	36.45	45.46	44.08
$\frac{\text{MUFA + PUFA}}{\text{SAFA + } trans}$	1.77	1.70	1.18	1.25

[a]Expressed as percentage of methyl esters. SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See Table 1 for other abbreviations.
[b]Results represent the average of eight analyses ± SD; n.d., not detected. a, Difference from SO-containing diet; $P < 0.05$. b, Difference from FO-containing diet, $P < 0.05$.

fatty acids/SAFA ratio as a consequence of feeding animals the PHFO- and the HHFO-containing diets.

The cytochrome P-450 content of microsomal membranes (Fig. 1A) showed good correlation with the degree of unsaturation of the oils used for the diets preparation (i.e., iodine value, Table 1), a higher content of the hemoprotein corresponding to the FO-containing diet and a lower content to the HHFO diet. The cytochrome P-450 content of microsomal membranes from rats fed the SO- and PHFO-containing diets, which had relatively similar iodine values, was the same. Figure 1 also shows the activity of two cytochrome P-450 dependent membrane-bound enzymes (aminopyrine *N*-demethylase, Fig. 1B, and aniline hydroxylase, Fig. 1C), and the activity of an enzyme not associated with cytochrome P-450 (UDP-glucuronyl transferase, Fig. 1D) in response to the different oil-containing diets. Aminopyrine *N*-demethylase activity agreed, as did the cytochrome P-450 content, with the degree of unsaturation of the diet, showing a higher activity for FO and a lower activity for HHFO, and exhibiting intermediate values for SO and PHFO diets. However, when the effect of the different diets on the aniline hydroxylase activity was analyzed, different results were obtained. The activity of this enzyme showed no correlation with the degree of unsaturation of the oils, but was associated with the *trans* fatty acid composition of microsomal membranes. The most important difference in the microsomal membrane fatty acid composition was observed for the PHFO diet, i.e., the higher *trans* content and the lower MUFA + PUFA/SAFA + *trans* ratio. Aniline hydroxylase activity was significantly higher in microsomes from rats fed the PHFO diet when compared to the other oil-containing diets assayed. UDP-glucuronyl transferase activity showed significant differences when the effect of the SO diet and that of the FO diet were compared. The activity of the enzyme after the PHFO and HHFO diets showed intermediate values when compared to the activity after the SO or the FO diet, (Fig. 1D).

DISCUSSION

It has been demonstrated that variation in dietary fatty acids produces corresponding variations in the fatty acid composition of phospholipids of the microsomal membranes (26) and, thus, alters the membrane structure and fluidity, resulting in changes in the activity of MFOS enzymes (5). Our results confirmed these observations, but we also demonstrated that isomeric *trans* fatty acid content of diets is reflected in the *trans* fatty acid composition of microsomes. As communicated by Saito and Yamagushi (7), we also observed that the

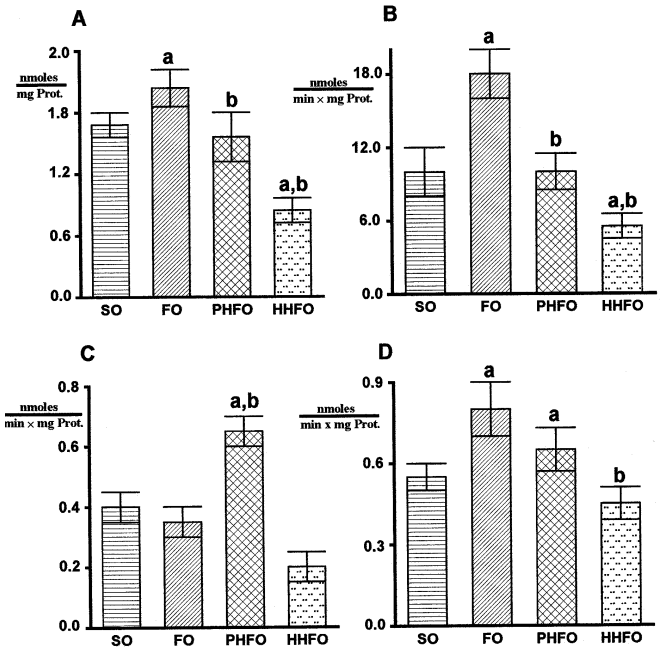

FIG. 1. Effect of sunflower oil (SO)-, fish oil (FO)-, partially hydrogenated fish oil (PHFO)-, and highly hydrogenated fish oil (HHFO)-containing diets on the cytochrome P-450 content (A), and on the aminopyrine *N*-demethylase (B), aniline hydroxylase (C), and UDP-glucuronyl transferase (D) activities of rat hepatic microsomes. Results represent the mean ± SD for $n = 8$ samples. a: difference from SO-containing diet, $P < 0.05$. b: difference from FO-containing diet, $P < 0.05$.

FO-containing diet increased the cytochrome P-450 content and the aminopyrine *N*-demethylase activity. In addition to these observations, we have also demonstrated a correlation between the degree of unsaturation of the diet and the cytochrome P-450 content, and the aminopyrine demethylase activity of microsomal membranes. Saito and Yamagushi (7) observed that the aniline hydroxylase activity of hepatic microsomes remains unchanged after feeding rats with diets containing lard, soybean oil, or sardine oil as the fat source. Diets were different in their degree of unsaturation, but all contained low amounts of *trans* fatty acids. In our experimental design, results demonstrated that this is also valid for the SO-, FO-, and HHFO-containing diets. However, we observed a high microsomal activity for aniline hydroxylase after the PHFO containing diet. If we discard the effect of the degree of unsaturation of the diet on the activity of the enzyme, which appears to be valid for the SO-, FO- and HHFO-containing diets, it can be inferred that the high *trans* content of the PHFO containing diet, which is also reflected in the higher *trans* composition of microsomes from animals fed the PHFO diet, may be affecting the aniline hydroxylase activity. Saito and Yamagushi (7) did not analyze the fatty acid composition of microsomal membranes.

On the other hand, Yang *et al.* (27) observed that a 5% FO-containing diet significantly increased the activity and the amount of UDP-glucuronyl transferase when compared to corn oil, showing correlation between the activity of the enzyme, the fat content of the diet, and the degree of unsaturation of the fat. Our results show significant differences for the UDP-glucuronyl transferase activity with the different diets assayed. The activity of the enzyme is reduced by reducing the degree of unsaturation of the diet. The activity of the enzyme with the FO diet is higher than the activity assayed after the SO diet, a result which is similar to that of Yang *et al.* (27) when comparing the effect of FO with corn oil which, such as SO, is less unsaturated than FO. However, these authors did not assay the effect of hydrogenated fats on the activity of the enzyme. According to their observation on the effect of the degree of dietary fat unsaturation on the UDP-glucuronyl transferase activity, a reduction should be expected in the activity of the enzyme after the PHFO- and the HHFO-containing diets when compared to the FO diet. Our results confirmed this assumption for the HHFO diet but not for the PHFO diet. Therefore, it can be inferred that other dietary factors and/or membrane components, different than the lipid composition of the diet and/or the fatty acid composition of the microsomal membranes, may be regulating the microsomal UDP-glucuronyl transferase activity. The total amount of sterols (cholesterol plus cholesterol-hydrogenated products) consumed by the animals may be a matter of concern. To our knowledge, the effect of partial or high hydrogenation of FO on its cholesterol content has not been studied. As diets were supplemented with cholesterol up to the amount contained in unhydrogenated FO, we cannot disregard possible effects of the cholesterol-hydrogenated products on the enzymatic parameters assayed.

ACKNOWLEDGMENTS

This research was supported by FONDECYT projects No. 2960044 (N.M.) and No. 1960988 (A.V.), and project PG-068 from University of Chile.

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[Received June 2, 1997, and in final revised form May 12, 1998; revision accepted May 15, 1998]

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

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

Lipids 33, 675–681 (1998).

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

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

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

MATERIALS AND METHODS

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

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

TABLE 1
Diet Composition (g/kg diet)

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

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

by Lamboni (23); liver mitochondria and microsomes were prepared according to Lake (24); microsomal cytochrome (cyt.) b_5 and P_{450} contents and NADPH-cyt. P_{450} reductase activity were carried out according to Lake (24); carnitine palmitoyltransferase-I (Cpt-I) activity according to McGarry *et al.* (25); glucose 6-phosphate dehydrogenase activity in the $31{,}000 \times g$ 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]

	Group		
Item	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	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

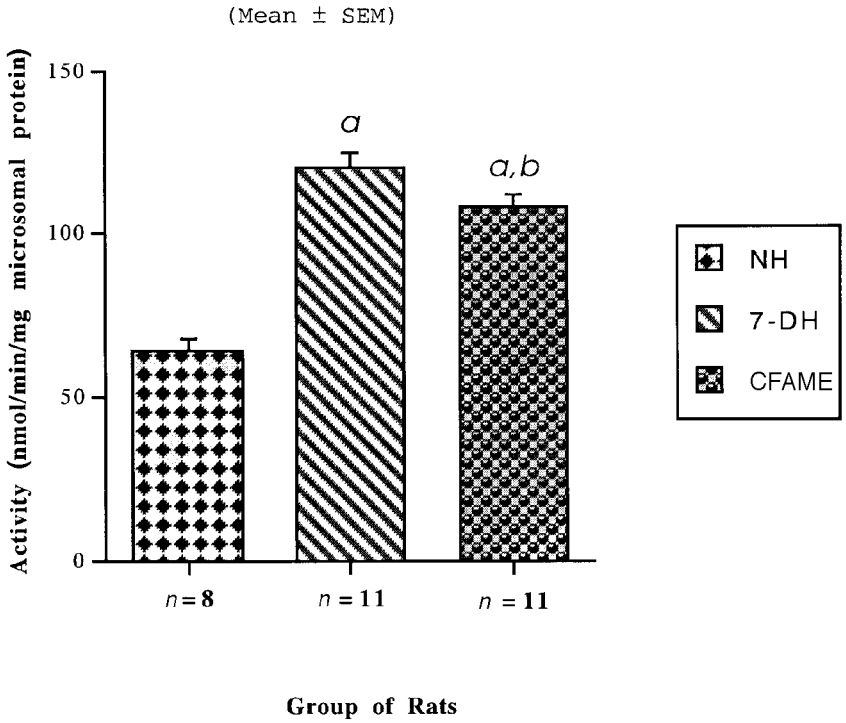

FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 7-DH, and CFAME diets. NH = group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO). 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs. CFAME = group of rats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

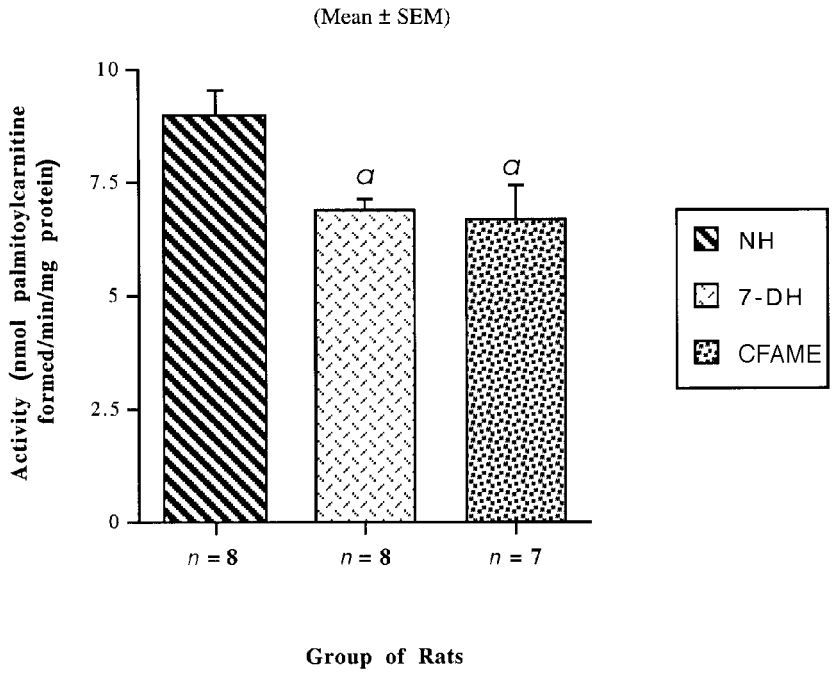

FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.01$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

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

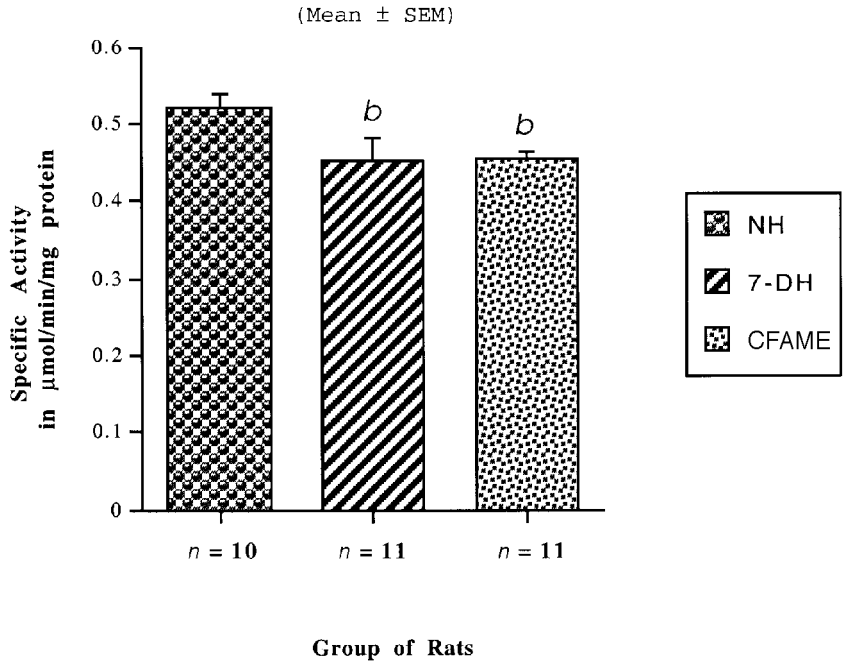

FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{b}P < 0.05$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

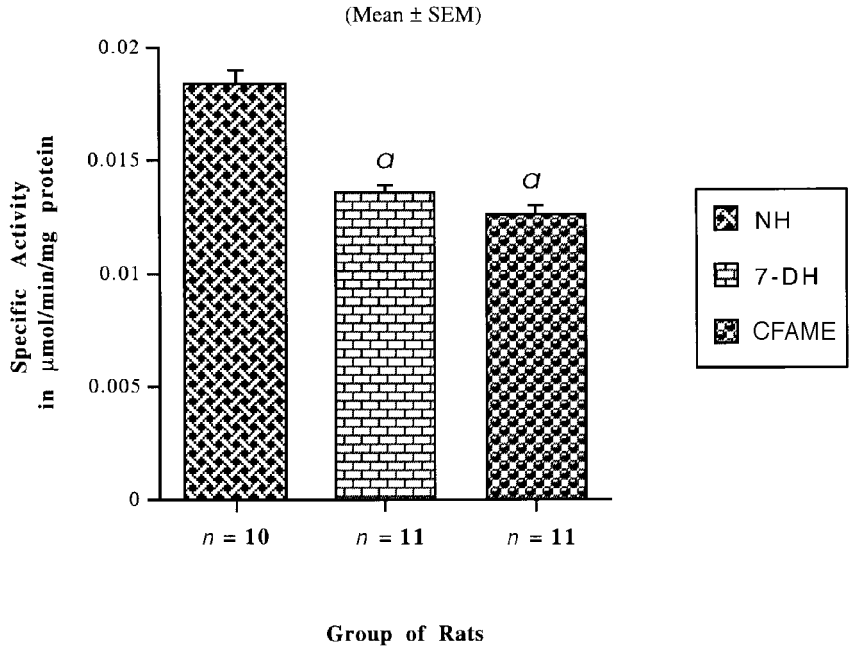

FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 7-DH, and CFAME diets. $^{a}P < 0.0001$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

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 $31{,}000 \times g$ 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).

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

Magnesium Silicate Treatment of Dietary Heated Fats: Effects on Rat Liver Enzyme Activity

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ABSTRACT: In an effort to reduce the deleterious nutritional effects of oxidation products generated in heated fats, a partially hydrogenated soybean oil (PHSBO) used 7 d (7-DH) for frying foodstuffs was obtained from a commercial deep-fat frying operation. The used fat was treated with magnesium silicate (T-7DH). Isocaloric diets containing 15% of either 7-DH or T-7-DH fats were prepared and fed to male weanling rats for 10 wk in a pair-feeding experiment and compared to control rats fed nonheated PHSBO (NH). Animals fed the 7-DH diet showed higher liver enzyme cytochrome b_5 and P_{450} activity than the T-7DH diet compared to the NH group, suggesting a positive effect of the treatment. These results suggested the presence of lower amounts of harmful compounds in the diet containing the heated used oil which had been treated with the active adsorbent.

Lipids 33, 683–687 (1998).

Fried foods taste good and appeal to most people in our twentieth-century society and are readily available through fast-food restaurants. Numerous studies state that dietary heated fats contain harmful substances (1–4) that may cause deleterious effects when fed to laboratory animals. Previously, Crampton *et al.* (5) reported that the primary cause of the lowered nutritive value of diets containing thermally polymerized linseed oil was the presence of one or more dimeric fatty acid radicals which are in some way inimical to the well-being of the animals fed such fats. Moreover, their deleterious effects could well be aggravated since they were produced at the expense of unsaturated fatty acids.

Other studies (6–8) have reported adverse biological effects of heated and oxidized fats such as enlargement or fatty necrosis of liver, retarded growth, hair loss and dermatitis when such fats were fed to rats.

Although it has been generally accepted that growth-depressing and other nutritionally harmful materials are present in the oxidized or polymeric portion of heated fats, none of the studies focused on a direct way to alleviate the deleterious effect of heated edible oils observed when such fats are fed to laboratory animals. The present study will focus on treating heated fats with the aid of an active filter aid and compare its effects on rat liver enzyme activity with those of the non-treated used oil. The heated fat, a partially hydrogenated soybean oil (PHSBO) used 7 d (7-DH) for frying foodstuffs, was obtained from a commercial restaurant deep-fat frying operation. The composition of the heated oils has previously appeared (8). The activities of the following enzymes were investigated: NADPH-cytochrome (cyt.) P450 reductase, carnitine palmitoyltransferase (CPT), isocitrate dehydrogenase (ICDH), and glucose 6-phosphate dehydrogenase

MATERIALS AND METHODS

Animals, diets, and procedures. Male weanling Sprague-Dawley rats (50–60 g) from Harlan Sprague-Dawley Inc. (Indianapolis, IN) were housed in steel wire-mesh cages and maintained on a standard chow diet for 1 wk. All animals were then assigned into three groups: a control group (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 7-DH treated one time with 10% magnesium silicate (T-7DH). Animals were assigned to cages by complete randomization (wherein cage locations are assigned at random) as described by Lamboni (8) Animals of the experimental groups were pair-fed (wherein the control animal was fed only the amount consumed by the experimental animal. There was therefore a 1-d time lag in their corresponding diets for 10 wk of the experiment). The composition of the diets fed to the rats is described in Table 1.

The following procedures and determinations were carried out: protein determination according to Lowry *et al.* (9); total liver lipid according to Folch *et al.* (10); liver glycogen according to Lo *et al.* (11) as modified by Lamboni (12); microsomal cyt. b_5 and P_{450} contents and NADPH-cyt. P_{450} reductase activity according to Lake (13); CPT-I activity according to McGarry *et al.* (14); glucose 6-phosphate dehydrogenase activity in the 31,000 × *g* supernatant fluid by the use of a Sigma Kit (Sigma Chemical Co., St. Louis, MO); ICDH activity in the liver homogenate by the use of a Sigma Kit.

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Abbreviations: CPT-1, carnitine palmitoyltransferase-I; cyt., cytochrome; 7-DH, PHSBO used 7d; T-7DH, used fat treated with magnesium silicate; NH, nonheated PHSBO; PHSBO, partially hydrogenated soybean oil.

TABLE 1
Diet Composition

	NH	7-DH	T-7DH
		(g/kg diet)	
Casein[a]	150	150	150
Dextrose anhydrous	600	600	600
Cellulose[b]	50	50	50
Vitamin mixture[a]	10	10	10
Mineral mixture[a]	40	40	40
Fat PHSBO (NH)	150	0	0
Fat 7-DH	0	150	0
Fat T-7DH	0	0	150

[a]From Harlan Teklad (Madison, WI).
[b]From Solka-Floc (James River Corporation, Berlin, NH). 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; T-7DH, group of rats fed diet containing 7-DH oil treated with magnesium silicate.

The treatment of the used heated fat was done by adding 10% magnesium silicate to the hot fat (150°C) while stirring and then filtering under vacuum through No. 42 Whatman filter paper (Maidstone, England). Gel permeation chromatography was performed to check the amount of polymers removed from the treated used oil (T-7DH) in comparison to the nontreated 7-DH oil. This assay was performed on the oils fed to animals by using a Waters (Milford, MA) 501 high-performance liquid chromatography pump, a Waters 410 differential refractometer, and a series of size-exclusion columns (two columns of Ultrastyragel 500A, 30 cm, Part # 10571, Waters; and two columns # H10 XH0066 with G1000 HXL, 30 cm; Supelco, Inc., Bellefonte, PA). Tetrahydrofuran was used as mobile phase, and the rate was 0.7 mL/min while the pressure was 1.5×10^3 psi.

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

RESULTS

Gel permeation chromatography of the oils. The analysis of the oils fed to rats showed that the nonheated (NH) PHSBO contained 100% triglycerides and no polymers. In contrast, the 7 d used oil had more polymers (about 9.9%) while the 7 d used oil which was treated with a synthetic magnesium silicate showed a lower polymer content (about 4%).

Weight gain, feed efficiency, liver weight/body weight ratio. The results in Table 2 indicate that rats fed 7-DH diet did not show any significant difference in feed efficiency among groups. Animals fed the 7-DH diet, however, lost hair and showed signs of dermatitis. The hair loss seen in the T-7DH group was evidenced by some hair deposited under individual cages of those animals.

A significant ($P < 0.05$) increase in the liver weight/body weight ratio for animals fed the 7-DH diet (Table 2) compared to rats fed the control NH diet was noted. There was no significant difference between the 7-DH and the T-7DH groups in terms of liver weight/body weight ratio.

Protein, lipid, glycogen. Animals fed the 7-DH diet showed an increased ($P < 0.0001$) liver protein content compared to those fed the NH diet (Table 2). The increase observed when animals were fed the T-7DH diet was significantly different ($P < 0.001$) from that of control rats.

After 10 wk of pair-feeding rats with their respective diets, the liver microsomal protein content significantly increased in the 7-DH rats ($P < 0.0001$) while no significant difference was observed in those fed the T-7DH diet compared to those fed the NH diet (Table 2). When the nontreated oil diet group of animals was compared to those on the treated diet, the increase observed in the former group was significantly ($P < 0.0001$) different.

The liver lipid content of animals fed the 7-DH or T-7DH diets was significantly increased ($P < 0.0001$) compared to the control group. The liver lipid content of rats pair-fed the 7-DH diet was significantly increased ($P < 0.01$) compared to those pair-fed with the treated oil diet (T-7DH).

Rats pair-fed the 7-DH diet showed that the 15% 7-DH diet induced the lowest lipid/protein ratio ($P < 0.01$) although

TABLE 2
Several Parameters of Control and Experimental Rats in a Pair-Feeding Experiment[a]

	Group		
Item	NH	7-DH	T-7DH
Feed efficiency (gram gain/gram feed)	0.232 ± 0.004	0.232 ± 0.003	0.232 ± 0.005
Liver weight/body weight ratio (mg/g)	2.75 ± 0.05	2.95 ± 0.02[b]	2.894 ± 0.06
Liver protein (mg/g)	392.65 ± 7.44	593.22 ± 17.50[a]	487.09 ± 25.84[c]
Microsomal protein (mg/g)	30.51 ± 1.01	56.47 ± 1.55[a]	30.60 ± 1.32[f]
Liver lipid (mg/g)	63.88 ± 1.69	79.66 ± 1.86[a]	72.86 ± 2.07[c,e]
Lipid/protein ratio	0.98 ± 0.02	0.81 ± 0.04[d]	0.92 ± 0.05
Liver glycogen (mg/g)	11.03 ± 0.78	6.32 ± 0.34[a]	8.81 ± 0.56[d,e]

[a]Values are means ± SEM and $n = 10$ for each group. [a]$P < 0.0001$ when compared to the control animals fed NH diet. [b]$P < 0.05$ when compared to the control group. [c]$P < 0.001$ when compared to the control animals. [d]$P < 0.01$ when compared to the control rats. [e]$P < 0.01$ when T-7DH is compared to 7-DH diet group. [f]$P < 0.0001$ when T-7DH is compared to the 7-DH diet group of rats. See Table 1 for abbreviations.

TABLE 3
Hepatic Microsomal Cytochromes Content of Control and Experimental Rats in a Pair-Feeding Experiment[a]

Item	Group		
	NH	7-DH	T-7DH
Cytochrome b_5 content (nmol/mg micr. protein)	0.46 ± 0.02	0.75 ± 0.08[a]	0.49 ± 0.03[b,c]
Cytochrome P_{450} content (nmol/mg micr. protein)	1.04 ± 0.03	1.68 ± 0.08[a]	1.15 ± 0.06[b,c]

[a]Values are means ± SEM and $n = 10$ for each group; micr. = microsomal. [a]$P < 0.0001$ when compared to the control animals fed NH diet. [b]Nonsignificant when compared to the control NH. [c]$P < 0.0001$ when T-7DH is compared to the 7-DH diet group of rats. See Table 1 for abbreviations.

there was not any significant decrease in the T-7DH group compared to the controls (Table 2).

In another pair-feeding experiment, decreased liver glycogen of animals fed the 7-DH diet was significantly different ($P < 0.0001$) from the control animals while the significance level was at $P < 0.01$ for the T-7DH group (Table 2). The liver glycogen of the T-7DH group of rats was different ($P < 0.01$) than that of the 7-DH group.

Microsomal cyt. b_5 and P_{450}. Rats pair-fed the 7-DH diet showed a highly significant increase ($P < 0.0001$) in the cyt. b_5 compared to the control group of rats fed the NH diet while the difference observed for the T-7DH group of animals was not statistically significant (Table 3). The value for the treated oil group compared to the untreated oil was significantly decreased ($P < 0.0001$).

Liver microsomal cyt. P_{450} content for the group of rats fed the 15% 7-DH diet was greatly increased ($P < 0.0001$) compared to the control group pair-fed a 15% NH diet. It was also increased ($P < 0.0001$) when compared to the T-7DH group of animals (Table 3).

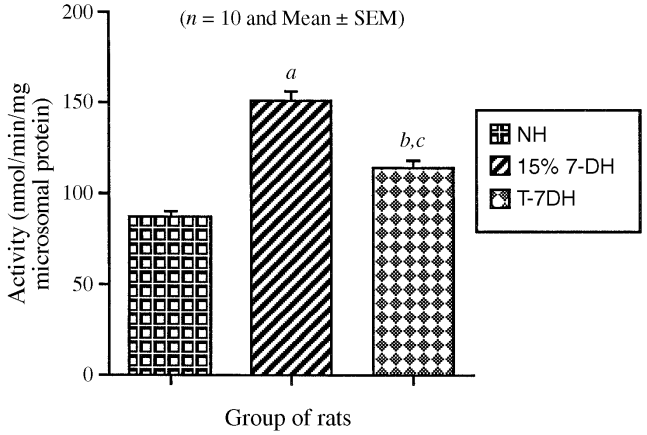

FIG. 1. NADPH-cytochrome P_{450} reductase activity in liver microsomes of rats fed NH, 7-DH, and T-7DH diets. NH = control group of rats fed diet containing nonheated partially hydrogenated soybean oil (PHSBO); 7-DH = group of rats fed diet containing PHSBO used 7 d for frying foodstuffs; T-7DH = group of rats fed diet containing 7-DH oil treated with magnesium silicate. [a]$P < 0.0001$ when compared to the control group of rats fed NH diet; [b]$P < 0.01$ when compared to NH; [c]$P < 0.0001$ when compared to 7-DH group of rats.

NADPH-cyt. P_{450} reductase. The NADPH-cyt. P_{450} reductase of rats fed the experimental diets showed an increased level of activity compared to the control group. The increase was significant at $P < 0.0001$ for the 15% 7-DH diet group of rats and at $P < 0.01$ when the used oil had been treated with magnesium silicate (Fig. 1). The activity of the 7-DH diet group of animals was higher than that of the T-7DH group ($P < 0.0001$).

CPT-I. A significant decreased activity ($P < 0.01$) of CPT-I was noted when rats were pair-fed the 7-DH diet in comparison with the control animals. Feeding the T-7DH diet to rats did not result in any statistically significant difference compared to the control group of rats (Fig. 2). The lower activity of CPT-I observed for the 7-DH diet group of rats was not significantly different from those fed the T-7DH diet.

ICDH. Decreased activity of the ICDH was observed when rats were pair fed the 15% 7-DH diet, and the results were significantly different ($P < 0.05$) from the control animals. Feeding 15% of the T-7DH diet to rats did not result in any significant difference with the control group of rats (Fig. 3). No significant difference was noted when the 7-DH diet group of rats was compared to the group of rats fed the T-7DH.

Glucose 6-phosphate dehydrogenase. The activity of D-glucose 6-phosphate:NADP-oxidoreductase measured in the liver of rats fed the 7-DH diet was depressed compared with a control group of rats fed the NH diet ($P < 0.0001$). Rats fed

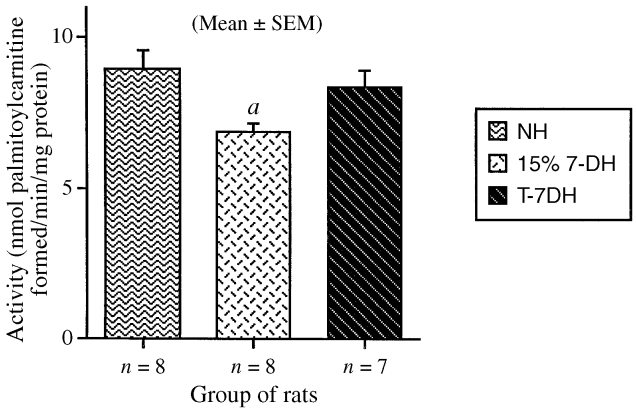

FIG. 2. Carnitine palmitoyltransferase-I activity in liver mitochondria of rats fed NH, 7-DH, and T-7DH diets. [a]$P < 0.01$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

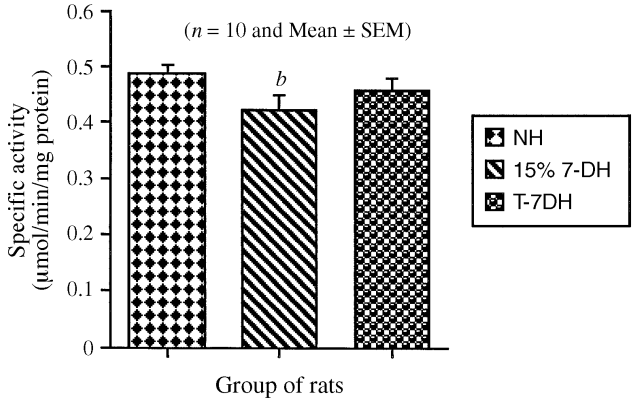

FIG. 3. Isocitrate dehydrogenase activity in liver of rats fed NH, 7-DH, and T-7DH diets. $^{b}P < 0.05$ when compared to the control group of rats fed NH diet. See Figure 1 for abbreviations.

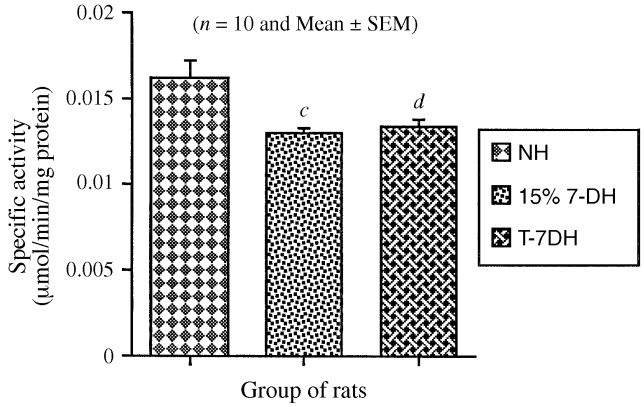

FIG. 4. Glucose 6-phosphate dehydrogenase activity in liver of rats fed NH, 7-DH, and T-7DH diets. $^{c}P < 0.001$ when compared to the control group of rats fed NH diet. $^{d}P < 0.01$ when compared to the control. See Figure 1 for abbreviations.

the T-7DH diet also had significantly depressed activity compared to controls ($P < 0.01$) (Fig. 4).

DISCUSSION

The results of the present study indicated that fats heated at deep-frying temperatures contain levels of lipid oxidation products and polymeric compounds which were decreased by treatment with a synthetic filter aid. When diets containing such treated fats were fed to rats, the results showed that such treatment decreased the deleterious nutritional effects shown by the untreated heated fats. Oils used for deep frying as well as the compounds generated in used oils caused a generalized increase in the content and activity of the liver detoxification enzymes when ingested. Significance at $P < 0.05$ for the group of rats fed the nontreated heated PHSBO (7-DH group) in comparison to the control (NH) group was noted. The nontreated oil group of rats developed enlarged livers while the treated oil group did not. Treatment of the used oil with a synthetic filter aid had a positive effect on improving the nutritional parameters of the oil when fed to rats. In addition, the nonsignificant increased liver protein (Table 2) measured for the treated oil diet group of rats compared to the control animals was highly significant when the untreated oil diet was fed. This further confirms that the deleterious effects of oxidation products and the positive effects of the treatment of the 7 d used oil decreased the amount of such oxidation products from the used oil. The increase in liver protein observed for the 7-DH diet group of rats may be a way of coping with the adverse metabolic effects of heated fats (14). Although the liver lipid content was significantly increased in both experimental groups of rats, the increase was less in the T-7DH group of animals than the group fed the 7-DH diet, suggesting again that the treatment had an effect in reducing the accumulation of lipid in the liver. The lipid/protein ratio measured in this study for all groups confirms this.

In addition to the positive effects of the treatment assigned to the used oil, the cyt. b_5 and P_{450} contents (Table 3) of the group of rats fed the T-7DH increased less than the significant increase in the 7-DH diet group of animals compared to the control NH. This further suggests that some of the components generated in the used oil had been reduced by the magnesium silicate treatment of the used oil and thus decreased its toxic effects. Therefore, less cyt. P_{450} had been formed in the liver microsomes of the T-7DH diet group of rats due to less toxic substrates leading to a less extensive detoxification process involving the enzyme NADPH-cyt. P_{450} reductase, with activity proportional to the content of cyt. P_{450} of the microsomal fraction (Fig. 1). Cyt. b_5 acts as an electron donor to cyt. P_{450} and is reduced either by NADPH-cyt. P_{450} reductase or by another microsome-bound flavoprotein NADH-cyt. b_5 reductase which is specific for NADH (15,16).

The activity of CPT-I (Fig. 2) in animals fed the treated oil diet suggests a better oxidation of long-chain fatty acids than when the used oil was not treated with synthetic magnesium silicate as indicated by the significant decreased activity of CPT-I in the 7-DH group. A possible accumulation of fatty acids into triglycerides and phospholipids for the group of rats fed the 7-DH diet could be an explanation for the fatty livers reported in the literature when such heated fats were fed to laboratory animals. The liver glycogen content of animals fed the 7-DH diet was significantly decreased (Table 2), suggesting an increased glycolytic activity with subsequent formation of more acetyl-CoA than that of the T-7DH diet group.

The statistically nonsignificant decreased activity (Fig. 3) of ICDH of animals fed the T-7DH diet compared to the control group contrasted with the statistically significant decrease in the nontreated 7-DH diet group, suggesting that there might be recovery of the impairment of the enzyme in the treated group. The treatment of used oil might be responsible for the positive effects on recovering the ICDH activity. Previously, Yoshioka *et al.* (17) reported a decreased activity of succinate dehydrogenase when rats were fed autoxidized safflower oils, and they attributed this impairment to the high carbonyl value detected in the oils.

Even though significant depressed activity of glucose 6-phosphate dehydrogenase was measured in both experimental groups of rats compared to the control group (Fig. 4), the activity in the T-7DH group was less than that of the untreated 7-DH group. This suggests less production of NADPH in the 7-DH group through the pentose phosphate pathway when it is necessary for fatty acid synthesis and for the P_{450} mixed-function oxidase system. This is supported by the increased activity of the NADPH-cyt. P_{450} reductase (Fig. 1). Reduced synthesis of fatty acid in the treated oil group of rats could explain the fact that there was no fatty liver in the T-7DH diet group of rats while it was noted in the group of rats fed the nontreated oil diet (7-DH group). The highly increased activity of NADPH-cyt. P_{450} reductase measured for the 7-DH group of rats confirms the increased utilization of NADPH to cope with the high level of toxic compounds ingested by rats when they are fed such fats. Treatment of the used oil removed a portion of the toxic components generated during the heating process of the oil by adsorption and thus alleviated the level of toxicity of the fat.

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 his Ph.D. degree at the University of Illinois (Urbana-Champaign, IL).

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[Received January 30, 1997, and in final revised form April 20, 1998; revision accepted May 8, 1998]

Protective Effects of Lemon Flavonoids on Oxidative Stress in Diabetic Rats

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ABSTRACT: The effects of lemon flavonoids, as crude flavonoids prepared from lemon juice, were investigated in diabetic rats. The oxidative stress of eriocitrin (eriodictyol 7-*O*-β-rutinoside) and hesperidin (hesperetin 7-*O*-β-rutinoside) on streptozotocin-induced diabetic rats was investigated. Diabetic rats were given a diet which contained 0.2% crude flavonoids, 0.2% eriocitrin, and 0.2% hesperidin. After the 28-d feeding period, the concentration of the thiobarbituric acid- reactive substance in the serum, liver, and kidney of diabetic rats administered crude flavonoids, eriocitrin, and hesperidin significantly decreased as compared with that of the diabetic group. The levels of 8-hydroxydeoxyguanosine, which is exchanged from deoxyguanosine owing to oxidative stress, in the urine of diabetic rats administered eriocitrin and hesperidin significantly decreased as compared with that of the diabetic rat group. Crude flavonoids, eriocitrin, and hesperidin suppressed the oxidative stress in the diabetic rats. These results demonstrated that dietary lemon flavonoids of eriocitrin and hesperidin play a role as antioxidant *in vivo*.

Lipids 33, 689–695 (1998).

Flavonoid compounds are widespread in the plant kingdom and are present in citrus fruits. Flavonoids in citrus fruits, known as bioflavonoids or vitamin P, exhibit beneficial effects on capillary permeability and fragility. These compounds have been investigated regarding their physiological function such as anti-inflammatory, anticarcinogenic, and antitumor activities (1–3). We have isolated antioxidative flavonoid glycosides from lemon fruit and identified eriocitrin (eriodictyol 7-*O*-β-rutinoside) of the flavanone glycoside (4), and 6,8-di-*C*-β-glucosyldiosmin and 6-*C*-β-glucosyldiosmin of the *C*-glucosylflavones (5). The flavonoid content in lemon fruit was analyzed; and hesperidin (hesperetin 7-*O*-β-rutinoside), also known as vitamin P, and eriocitrin were abundantly found. Eriocitrin had stronger antioxidative activity than the other citrus flavonoid compounds and was abundantly present in lemon and lime fruits, although hesperidin is widely distributed the among citrus fruits (6).

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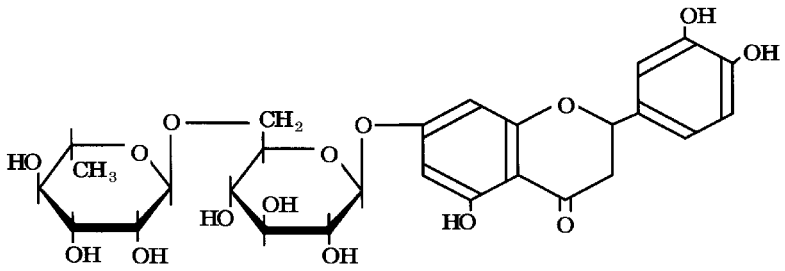
Abbreviations: CAT, catalase; CF, crude flavonoids; GSH, reduced gluthathione; GSH-Px, glutathione peroxidase; GST, glutathione *S*-transferase; 8-OHdG, 8-hydroxydeoxyguanosine; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substances.

Much attention has been focused on the involvement of oxidative stress of active oxygen and free radicals in aging and disease. The active oxygen species have been proposed as the attacking agents on polyunsaturated fatty acids in cell membranes. Lipid peroxidation is suspected to be strongly associated with aging and carcinogenesis (7). Antioxidants in food are expected to prevent diseases caused by oxidative stress (8,9). It is important to determine how antioxidants in food are metabolized *in vivo* and how antioxidant metabolites function *in vivo*. We had reported the metabolic pathway of eriocitrin by human intestinal bacteria for the exploration of antioxidative mechanism of eriocitrin *in vivo* (10). Oxidative modification of various proteins and lipids in plasma is found in diabetes (11). Diabetes mellitus is associated with increased lipid peroxidation, which may contribute to long-term tissue damage and increased oxidative stress as assessed by plasma hydroperoxides. Oxidative stress is an early state in the disease pathology and may contribute to the development of complications (12). Investigation of the pathophysiology of the secondary complications of diabetes is increasingly focusing on the role of oxidative stress in its initiation and progression (13). There is now ample evidence supporting the involvement of reactive oxygen species in this disease.

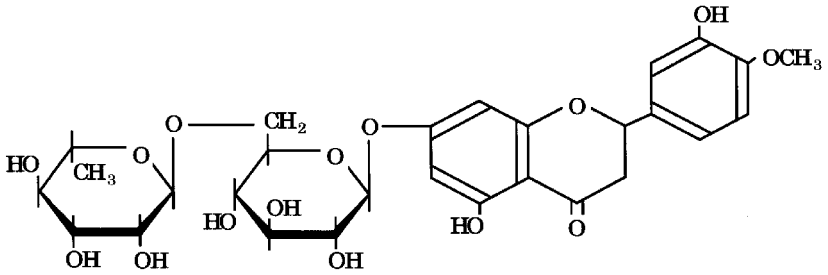
In this study, we examined whether dietary lemon flavonoids suppress oxidative stress *in vivo*, and determined whether these compounds are useful in the prevention of diabetic complications caused by oxidative stress. Oxidative stress was induced in rats using streptozocin (STZ), which is toxic to β-cells and is widely used for the induction of experimental diabetes mellitus (14). The protective effects of the lemon flavonoids eriocitrin and hesperidin, as well as crude flavonoids (CF) prepared from lemon juice, on oxidative stress were then determined.

EXPERIMENTAL PROCEDURES

Materials and chemicals. CF were prepared from lemon juice (4). This was applied to a Cosmosil 75C 18-OPN ODS column (Nacalai Tesque, Inc., Kyoto, Japan), which was washed with water and successively eluted with 100% methanol. The eluate was concentrated under reduced pressure and lyophilized. The lyophilized powder containing CF was examined for flavonoid glycosides content by high-performance



eriocitrin (eriodictyol 7-*O*-β-rutinoside)



hesperidin (hesperetin 7-*O*-β-rutinoside)

SCHEME 1

liquid chromatography according to the method of Miyake *et al.* (6). The CF (1.00 g) contained eriocitrin (385 mg), hesperidin (182 mg), 6,8-di-*C*-β-glucosyldiosmin (72.2 mg), narirutin (25.0 mg), and diosmin (22.0 mg). Eriocitrin was prepared from lemon peel extract (4), and the purity was greater than 99.0% as determined by high-performance liquid chromatography. Hesperidin was obtained as a reagent-grade chemical from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chemical structures of eriocitrin and hesperidin are shown in Scheme 1.

Animals and diets. Five-week-old male Wistar strain rats (Japan SLC, Ltd., Hamamatsu, Japan) were individually housed in stainless-steel cages with screen bottoms. The animals were kept under controlled conditions with a 12-h light/dark cycle (0800–2000 light) and at 22–24°C. All rats were fed commercial CE-2 pellets (Crea Japan, Ltd., Tokyo, Japan) and water *ad libitum*. Diabetes was induced in ether-anesthetized rats by the administration of STZ (60 mg/kg) as a freshly prepared solution (50 mg/mL) in saline *via* intraperitoneal injection. Diabetic rats were fed on CE-2 pellets and water *ad libitum* for 48 h after the administration of STZ. The increase of glucose content in urine of diabetic rats was checked by a commercial glucose kit (TES-Tape, Shionogi Pharmaceutical Company, Ltd., Tokyo, Japan). Three additional groups of diabetic rats were fed water *ad libitum* and CE-2 pellets containing CF, eriocitrin, or hesperidin for 28 d.

This experiment was carried out with five groups of five rats. The control group was the nondiabetic rat group (C), which had not received STZ. Diabetic rats were randomly assigned to four groups of five animals: a diabetic group fed CE-2 pellets (D); a diabetic group fed CE-2 pellets containing 0.2% CF (S+CF); a diabetic group fed CE-2 pellets containing 0.2% eriocitrin (D+ERI); and a diabetic group fed CE-2 pellets containing 0.2% hesperidin (D+HES). The feeds were prepared by mixing CE-2 pellets with the powder of CF, eriocitrin, and hesperidin. The food intake (g) of individual rats was measured daily. Body weight of the rats was measured weekly.

Tissue collection and processing. After 27 d on diet, rats were placed in individual metabolic cages in order to collect urine for 24 h. The collected urine was used to determine 8-hydroxydeoxyguanosine (8-OHdG).

After feeding the rats for 27 d, they were fasted for 10 h. On day 28 the rats were then anesthetized with ether, blood was collected into tubes by cardiac puncture, and the livers and kidneys were removed and stored at −80°C. Liver and kidney tissues were homogenized twice in 10 vol of 50 mM sodium phosphate buffer (pH 7.4) at 4°C for 15 s. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 1,900 × *g* for 5 min. The supernatant was used for various measurements. Blood was centrifuged at 1,900 × *g* for 10 min to obtain serum.

The serum, the supernatant of homogenized liver and kidney, and the urine of rats were stored at −80°C and processed for biochemical measurements within 2 wk.

Measurement of thiobarbituric acid-reactive substances (TBARS). The serum TBARS concentration was determined by the method of Yagi (15) and is expressed as nmol of malondialdehyde per mL of blood. The liver and kidney TBARS concentrations were measured by the method of Uchiyama

and Mihara (16) using a homogenate prepared by homogenizing 1 g of frozen rat liver and kidney (a section from the main lobe) with 9 mL of 1.15% KCl.

Determination of 8-OHdG. Rat urine was centrifuged at 1,900 × *g* and the precipitate was removed. The content of 8-OHdG in the urine was measured using the 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for Control Aging, Fukuroi City, Japan) (17,18). Creatinine content in the urine was measured using commercial kits (Wako Pure Chemical Industries, Ltd.) (19).

Measurement of antioxidative enzyme activities and glutathione. The supernatants of homogenized liver and kidney were analyzed for their antioxidant enzyme activities and content of glutathione. Superoxide dismutase (SOD) activity was measured by the xanthine/xanthine oxidase/nitro-blue tetrazolium system (20), and the catalase (CAT) activity was measured by a spectrophotometric method, following a decrease in absorbance at 240 nm and 25°C, due to hydrogen peroxide decomposition (21). Glutathione peroxidase (GSH-Px) activity was measured using the procedures of Lawrence and Burk (22) with *t*-butyl hydroperoxide as the substrate, following the decrease in absorbance of NADPH at 340 nm. Glutathione *S*-transferase (GST) activity was assayed by measuring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (23). The content of GSH was measured by an enzymatic method (24). Protein content was measured using a commercial protein assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Lipid analyses. Total cholesterol and triglyceride in the serum were enzymatically measured using commercial kits (cholesterol E-test and triglyceride E-test, respectively; Wako Pure Chemical Industries, Ltd.).

Statistical analyses. All values were expressed as mean ± S.E. ($n = 5$). The data for each of the five groups were statistically analyzed by Duncan's multiple-range test, and significant differences in the means were $P < 0.05$.

RESULTS AND DISCUSSION

General features of diabetic animals. As shown in Figure 1, the growth rate of the STZ-induced diabetic rat group (D) was slower than that of the nondiabetic rat group (C), as indicated by the smaller body weight increase during the 28-d period. The growth curves of the diabetic rat groups administered lemon flavonoids as CF, eriocitrin, and hesperidin (D+CF, D+ERI, and D+HES) also showed a slower growth rate than that of the nondiabetic rat group (C). The average food intake of the diabetic rat groups (D, D+CF, D+ERI, and D+HES) in 1 d was greater than that of the nondiabetic rat group (C). As shown in Table 1, the volume of urine and the glucose level in serum and urine of the diabetic rat groups (D, D+CF, D+ERI, and D+HES) were higher than those for the nondiabetic rat group (C). The decrease in body weight gain, the increase in food intake, the high volume of urine, and the high glucose levels in blood and urine for the STZ-induced diabetic rats are typical symptoms of diabetes (25). There was no improvement in these symptoms for the diabetic rats after the administration of lemon flavonoids such as the CF, eriocitrin, and hesperidin.

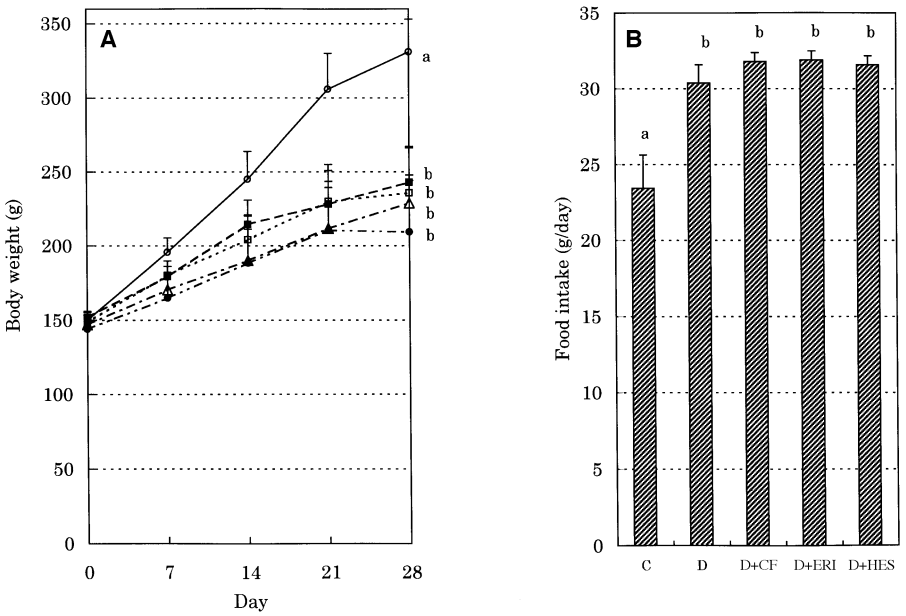


FIG. 1. Effect of dietary lemon flavonoids on body weight gain (A) and food intake (B) of diabetic rats. Diet groups (A) were: nondiabetic group (○; C), diabetic group (●, D), diabetic group administered crude flavonoids (△; D+CF), diabetic group administered eriocitrin (□; D+ERI), diabetic group administered hesperidin (■, D+HES). Values are means ± SEM of five rats per group. Lines or bars that do not share a common lowercase letter are significantly different at $P < 0.05$.

TABLE 1
Effect of Lemon Flavonoids on Liver, Kidney, Serum, and Urine of Diabetic Rats[a]

	Rat groups				
	C	D	D+CF	D+ERI	D+HES
Liver weight (% body weight)	3.95 ± 0.18[a]	5.30 ± 0.50[b]	5.42 ± 0.18[b]	5.19 ± 0.33[b]	5.08 ± 0.47[b]
Kidney weight (% body weight)	0.68 ± 0.02[a]	1.29 ± 0.13[b]	1.16 ± 0.05[b,c]	1.09 ± 0.03[c]	1.14 ± 0.10[b,c]
In serum (mL/100 mL)					
Glucose	170.6 ± 10.8[a]	532.9 ± 60.5[b]	609.3 ± 75.4[b]	521.1 ± 46.2[b]	559.7 ± 75.4[b]
Total cholesterol	42.6 ± 5.29[a]	81.8 ± 25.7[b]	77.6 ± 14.3[b]	74.8 ± 7.03[b]	79.7 ± 15.5[b]
Triglyceride	36.1 ± 3.96[a]	247 ± 77.3[b]	202 ± 45.7[b]	142 ± 71.7[b]	143 ± 34.7[b]
In urine					
Glucose (mL/100 mL)	0.07 ± 0.03[a]	10.92 ± 1.40[b]	9.81 ± 1.64[b]	11.09 ± 1.37[b]	9.47 ± 1.07[b]
Volume (mL/24 h)	10.6 ± 2.4[a]	136.7 ± 17.5[b]	151.0 ± 24.3[b]	114.3 ± 57.3[b]	115.0 ± 20.6[b]

[a]Values are means ± SEM of five rats per group. Values within the same row that do not share a common superscript roman letter are significantly different at $P < 0.05$. Abbreviations: C, nondiabetic group; D, diabetic group; D+CF, diabetic group administered crude flavonoids, (CF); D+ERI, diabetic group administered eriocitrin; D+HES, diabetic group administered hesperidin.

The liver and kidney weights (% body weight) for the diabetic rat group (D) were higher than those of the nondiabetic rat group (C) because of the hypertrophy of the liver and kidney caused by the STZ induction (Table 1). These are typical symptoms of diabetic rats (25). The kidney weights for the diabetic rat groups administered CF (D+CF) and hesperidin (D+HES) were not different from those of the diabetic rat group (D). However, kidney weights for rats fed eriocitrin (D+ERI) were significantly lower than for the diabetic rat group (D) ($P < 0.05$). The eriocitrin of lemon flavonoids may play an important role for improvement of the hypertrophy of the kidney in the diabetic rats. With respect to the liver weights, there were no differences among the diabetic rat groups. The levels of triglyceride and total cholesterol in the serum of the diabetic rats had been reported to be greatly elevated (25). The level of triglyceride in the serum of the diabetic rat groups administered lemon flavonoids (D+ERI and D+HES) was lower than in the diabetic rat group (D) but there was no significant difference ($P < 0.05$). Hesperidin decreases the triglyceride in the blood of rats fed fat (26). Eriocitrin and hesperidin may suppress the triglyceride in the blood of diabetic rats. There was no difference among the diabetic rat groups in the level of total cholesterol in serum.

TBARS levels of liver, kidney, and serum. The changes in the TBARS of the liver, kidney, and serum of diabetic rats after the administration of the lemon flavonoids are summarized in Figure 2. There was a significant increase in the TBARS of the liver, kidney, and serum in the diabetic rat group (D) compared to the control group. However, the TBARS levels of the liver, kidney, and serum in the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) were significantly decreased compared to the TBARS of the diabetic rat group (D). The increase of malondialdehyde, caused by lipid peroxidation, in tissue and blood of STZ-induced diabetic rats has been previously reported (13,27). The increased levels of TBARS, the reactive substance of malondialdehyde and TBA, resulted in increased levels of oxygen free radicals which attacked the polyunsaturated fatty acids in cell membranes and caused lipid peroxidation. STZ can also give rise to oxygen free radicals because of the increase in blood glucose levels in diabetes (14). An amino-carbonyl reaction, the so-called Maillard reaction, occurs *in vivo* as well as *in vitro* and is associated with the chronic complications of diabetes mellitus and aging in human beings (28). In particular, long-lived proteins such as lens crystallins, collagens, and hemoglobin may react with reducing sugars thus undergoing dehydration, rearrangement, cleavage, and polymerization reactions to produce advanced glycation end products (13,28). Lemon flavonoids of the CF, eriocitrin, and hesperidin affected the suppression of lipid oxidation in the liver, kidney, and serum. We postulated that these flavonoids play a role as free radical scavengers *in vivo* and prevent the development of complications associated with diabetes.

Determination of 8-OHdG in rat urine. Lipid peroxidation may play an important role in carcinogenesis, and there is speculation that oxidative damage can occur in DNA during the peroxidative breakdown of the membranes' polyunsaturated fatty acids (8). It is reported that mutation in mitochondrial DNA is caused by oxygen radicals and hydroxy radicals that oxidize 2′-deoxyguanosine to 8-OHdG (29). In STZ-induced diabetic rats, STZ stimulated H_2O_2 generation and caused DNA fragmentation (30). Since 8-OHdG has continued to serve as a good biomarker for the estimation of oxidative damage in DNA, we determined 8-OHdG in the urine of diabetic rats using the monoclonal antibody against 8-OHdG (17,18). The levels of total 8-OHdG over 24 h in the urine of diabetic rats and of 8-OHdG relative to creatinine content in the urine are shown in Figure 3. There was a significant increase of 8-OHdG in the urine of the diabetic rat group (D) compared to the control group. However, the level of 8-OHdG in the urine of the diabetic rat groups administered eriocitrin (D+ERI) and hesperidin (D+HES) significantly decreased compared to 8-OHdG of the diabetic rat group (D). The diabetic rat group administered the lemon CF (D+CF) did not have a significant decrease, but showed a tendency to decrease. The effects on total 8-OHdG for 24 h and 8-OHdG relative to creatinine content were almost the same. CF, eriocitrin, and hesperidin suppressed the generation 8-OHdG in the urine of the diabetic rats. This result suggests that the di-

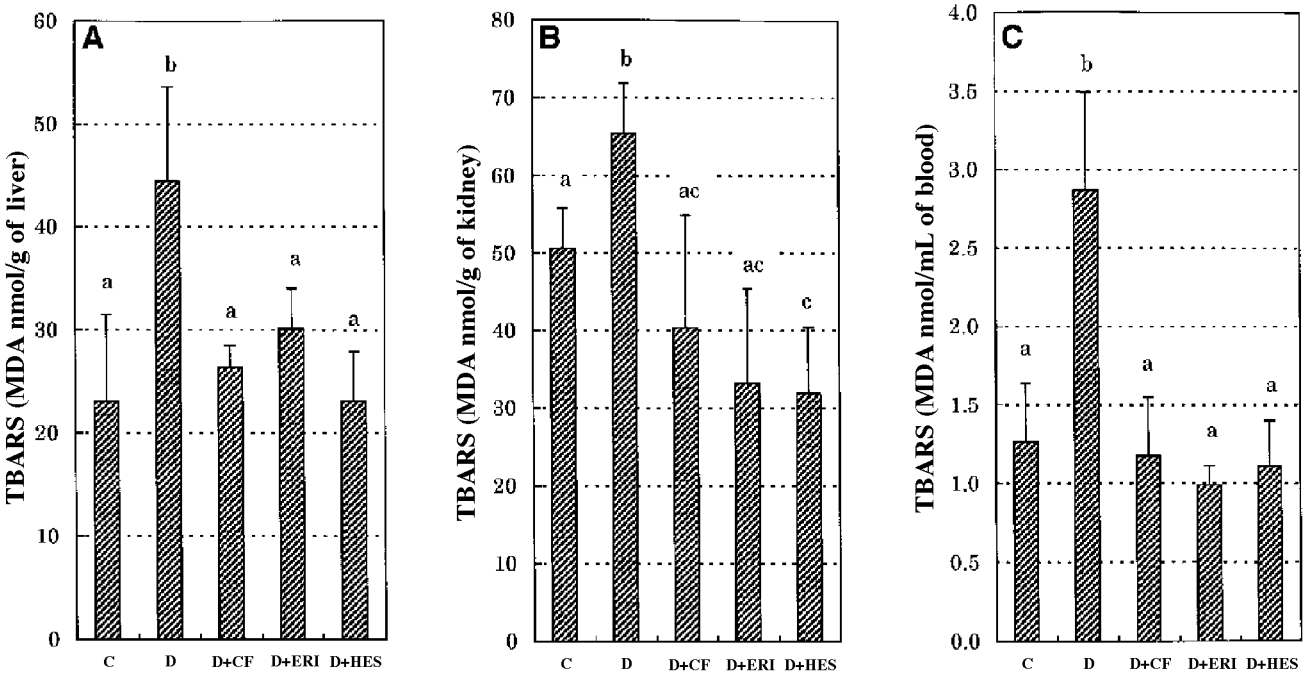


FIG. 2. Effect of lemon flavonoids on liver (A), kidney (B), and serum (C) thiobarbituric acid-reactive substances (TBARS) of diabetic rats, measured in terms of malondialdehyde. Values are means ±SEM of five rats per group. Values within the same panel that do not share a common lowercase letter are significantly different at $P < 0.05$. MDA, malondialdehyde; for abbreviations see Figure 2.

etary lemon flavonoids eriocitrin and hesperidin play a role in scavenging the free radicals generated by oxidative stress *in vivo*.

Influence of antioxidative enzyme activities. A considerable body of clinical and experimental evidence suggests the involvement of free radical-mediated oxidative processes in the pathogenesis of diabetic complications (13). The increase in the production of free radicals can result from the hyperglycemia-induced enhancement in glucose autoxidation, pro-

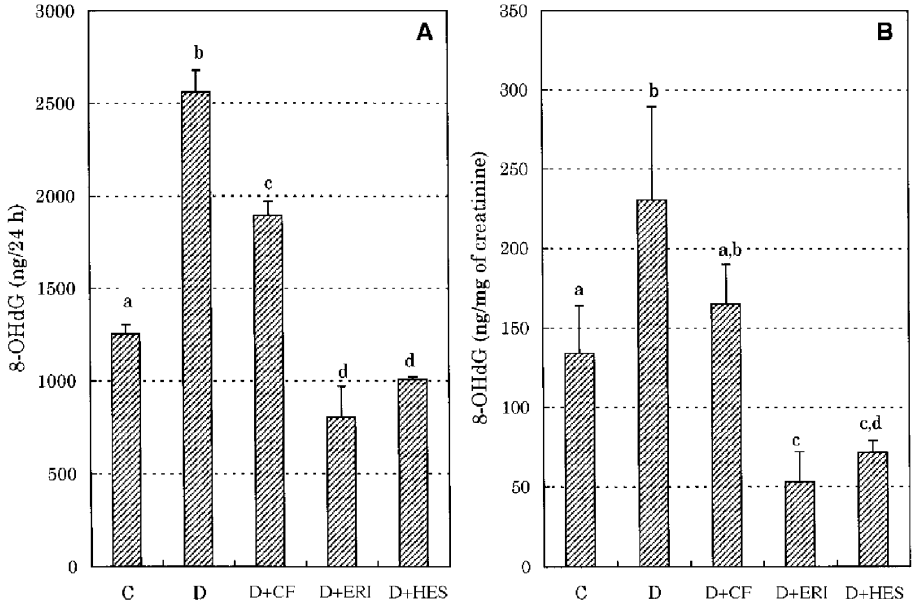


FIG. 3. Effect of lemon flavonoids on the total 8-hydroxydeoxyguanosine (8-OHdG) levels in a 24-h period (ng/24 h) (A) and on the content of 8-OHdG relative to the content of creatinine (ng/mg of creatinine) (B) in the urine of diabetic rats. Values are means ± SEM of five rats per group. Values within the same panel that do not share a common lowercase letter are significantly different at $P < 0.05$. For other abbreviations see Figure 1.

TABLE 2
Effect of Lemon Flavonoids on the Antioxidative Enzyme Activities (U/mg protein) of Diabetic Rats[a]

	Rat groups				
	C	D	D+CF	D+ERI	D+HES
Activity in liver					
GSH-Px	111.6 ± 12.2[a]	89.2 ± 5.8[b]	141.2 ± 10.3[c]	155.6 ± 10.9[c]	142.0 ± 19.1
GST	9.88 ± 1.15[a]	8.75 ± 0.93[a]	9.93 ± 1.27[a]	9.09 ± 1.33[a]	8.71 ± 0.94[a]
SOD	1.29 ± 0.11[a]	1.18 ± 0.02[a]	1.13 ± 0.10[a]	1.17 ± 0.16[a]	1.22 ± 0.15[a]
CAT	331 ± 14.1[a]	226 ± 19.4[b]	319 ± 23.9[ac]	355 ± 36.8[a]	264 ± 44.8[b,c]
Activity in kidney					
GSH-Px	87.4 ± 23.6[a]	93.0 ± 19.5[a]	137.6 ± 11.2[b]	132.2 ± 17.2[b]	133.8 ± 17.2[b]
GST	2.02 ± 0.11[a]	2.88 ± 0.29[b,c]	2.97 ± 0.28[b]	2.50 ± 0.19[c]	2.48 ± 0.40[a,b,c]
SOD	2.17 ± 0.09[a]	2.24 ± 0.10[a]	2.21 ± 0.20[a]	2.06 ± 0.14[a]	2.12 ± 0.09[a]
CAT	109 ± 17.0[a]	119 ± 20.3[a]	123 ± 24.3[a]	114 ± 23.4[a]	100 ± 25.5[a]
Content of GSH in liver[b]	7.51 ± 0.62[a]	4.89 ± 1.42[b]	15.63 ± 5.64[c]	15.56 ± 3.66[c]	11.51 ± 3.61[c]

[a]Values are means ± SEM of five rats per group. Values within the same row that do not share a common superscript roman letter are significantly different at $P < 0.05$. CAT, catalase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GST, glutathione *S*-transferase; SOD, superoxide dismutase. For other abbreviations see Table 1.
[b]mM/mg of protein.

tein glycation, and subsequent oxidative degradation of glycated protein (28). The glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species (31). It consists of GSH and an array of functionally related enzymes. If the diabetic state is associated with a generalized increase in tissue oxidative stress, it may be reflected in changes of the tissue glutathione antioxidant system. The activities of the antioxidative enzymes SOD, CAT, GST, and GSH-Px in diabetic rats fed CF, eriocitrin, and hesperidin are shown in Table 2. The activities of SOD and GST in the liver and that of SOD and CAT in the kidney were not different among the rat groups. The activity of GSH-Px in the liver and kidney and of GST in kidney for the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) increased when compared with the non-diabetic rat group (C) and the diabetic rat group (D). GSH-Px and GST work together with GSH during the decomposition of hydrogen peroxide or other organic hydroperoxides (31). The lemon flavonoids eriocitrin and hesperidin may function to increase the concentration of these enzymes as well as the antioxidative activity *in vivo*. The activities of CAT and the content of GSH in the liver for the diabetic rat group (D) decreased when compared with the nondiabetic rat group (C). However, the activities for the diabetic rat groups administered lemon flavonoids (D+CF, D+ERI, and D+HES) were higher than for the diabetic rats (D). The content of GSH consumed by oxidative stress in the diabetic rats suggests that administration of the lemon flavonoids afforded an improvement.

CF in lemon fruit contained many compounds such as flavonoids and phenolic compounds. Eriocitrin and hesperidin, present in CF of lemon, had a strong suppressing effect on oxidative stress as evidenced by decreased levels of TBARS and 8-OHdG in tissues of diabetic rats. These results suggest that dietary lemon flavonoids eriocitrin and hesperidin may play a role in preventing the development of diabetes and be useful in preventing diseases caused by oxidative stress.

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[Received February 27, 1998, and in final revised form and accepted May 18, 1998]

Distribution of α- and γ-Tocopherols in Atlantic Salmon *(Salmo salar)* Tissues[1]

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ABSTRACT: Groups of Atlantic salmon parr (mean initial weight 9.5 g) were fed three diets, the first containing no tocopherol supplement, the others supplemented with either all-*rac*-α-tocopherol (A-T) or RRR-γ-tocopherol (G-T). Tocopherol concentrations in the liver, serum, testes, kidney, brain, gill, muscle, and perivisceral fat were measured after 36 wk. Despite a higher dietary intake of G-T, compared to A-T, deposition of γ-tocopherol (γT) was less efficient than of α-tocopherol (αT) in most tissues except in the perivisceral fat, an adipose tissue. In fish fed the G-T diet, the γT/αT ratio was highest in the perivisceral fat and lowest in the liver, indicating that the liver is the most discriminatory organ for retaining αT as compared to γT, and the perivisceral fat is more suitable for the storage of γT. A negative correlation ($P < 0.01$) was observed between the γT/αT ratio and the corresponding tissue phospholipid content, suggesting that γT is less efficiently deposited compared to αT in the phospholipid-rich membranes which are presumed to be the functional site for lipid antioxidants *in vivo*. During restricted intake of αT, the liver and muscle exhibited the greatest reduction of this tocopherol among the tissues analyzed. The presence of minimal αT in the muscle from fish fed the tocopherol-unsupplemented diet led to greater susceptibility to lipid peroxidation after frozen storage than was the case for muscle containing higher concentrations of either αT or γT. However, both αT and γT were effective stabilizers of salmon muscle lipids during frozen storage.

Lipids 33, 697–704 (1998).

Tocopherols are powerful *in vivo* antioxidants which are intimately associated with lipids of cell membranes and are postulated to exert wide-ranging effects on aging, cancer, arthritis, platelet aggregation, and prostaglandin synthesis (1,2). Natural plant tocopherols include alpha (αT), beta (βT), gamma (γT), and delta (δT) forms, but αT is widely believed to be the most biologically efficacious form of vitamin E in animals. In humans consuming diets abundant in both αT and γT, tissues are preferentially enriched with αT rather than γT (3), and the reasons for the discrepancy of incorporation in animal tissues of these two most abundant plant tocopherols have been actively researched. In studies on rats, intestinal absorption rates and tissue uptake after absorption for the two tocopherols were reportedly similar, but γT disappeared faster than αT from the tissues after 24 h through a once-obscure mechanism (4,5). In normal human subjects, αT and γT are similarly absorbed at the intestinal level, yet mechanisms involving the action of a hepatic αT-binding protein result in the reduction of plasma γT concentration by 24 h (6). However, while the existence of the αT-binding protein in the hepatocyte (7,8) may explain the greater prevalence of circulating αT, it does not fully account for why γT, once it has reached the tissues, is not retained to the same degree as αT. Behrens and Madere (9) inferred that although γT is able to reach the different organ tissues in the rat, it is unable to bind to membrane structures with as high an affinity as αT, suggesting that molecular specificity at the site of antioxidant activity in the tissues may well be one of the crucial factors.

The hypothesized functional site of tocopherols in the cell lies within the lipid components of cell membranes (10). The phytyl chain facilitates incorporation and retention of tocopherols in membranes, with the hydroxy group optimally positioned for scavenging free radicals (11). Recently, fluorescence spectroscopy techniques provided evidence that αT is wholly embedded within the phospholipid-rich membrane bilayer with the chromanol head oriented toward the polar interfacial region (10,12). In highly active biological cells, αT concentrates in the membranous components where it inhibits peroxidative damage to the unsaturated lipids within the membrane bilayer (13). Cell membranes are complex structures, and specificity for incorporation of different forms of related compounds is no doubt rigorously controlled. It is therefore provocative to speculate that biodiscrimination for αT and γT may exist at the level of incorporation within cell membrane lipids rather than merely from the total tissue lipid pool. Marine fish phospholipids are characterized by a high (35–45%) amount of long-chain polyunsaturated fatty acids, particularly docosahexaenoic acid (14). Although αT is the

[1]Presented in part at the Annual Meeting of the American Oil Chemists' Society, San Antonio, Texas, May 1995.

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Abbreviations: A-T, all-*rac*-α-tocopherol-supplemented diet; G-T, RRR-γ-tocopherol-supplemented diet; 5-NS, 5-doxylstearate; 16-NS, 16-doxylstearate; TBARS, 2-thiobarbituric acid-reactive substances; no-T, non-tocopherol-supplemented diet; αT, α-tocopherol; γT, γ-tocopherol.

major tocopherol found in lipids of marine fish (15), our recent data verified that γT can be deposited along with αT in Atlantic salmon (*Salmo salar*) muscle lipids (16,17). A feasible approach to gaining insight into this matter of membrane distribution of tocopherols is to relate the proportions of the γT to the αT to the tissue phospholipid content in different salmon organs. We presumed that if discrimination between incorporation of these tocopherols in the membrane lipid bilayer does not occur, the γT/αT ratio would remain similar in various tissues despite varying phospholipid contents.

Diets for cultured Atlantic salmon are usually supplemented with tocopherol, solely in the αT form. Other than *in vivo* antioxidant-related functions, membrane-bound αT reduces postmortem lipid oxidation in fish products by stabilizing membrane lipids (16,18,19). In rainbow trout, adequate tocopherol intake improved the sensory quality of the fillets (20), thus affecting the quality, and ultimately the marketability, of the fish product.

The objectives of this experiment were to compare the deposition of dietary all-*rac*-α-tocopherol and RRR-γ-tocopherol in various tissues of Atlantic salmon in relation to tissue phospholipid content, and to examine the pattern of tocopherol depletion in tissues during restricted intake of these two tocopherols. Another objective was to determine if dietary γT deposited in the muscle functions similarly to αT in providing oxidative stability to the muscle after frozen storage at –40°C for 6 mon.

TABLE 1
Composition of the Test Diets[a]

Feed ingredient	Amount (w/w%)
Casein	32.5
Gelatin	4.0
Herring meal	12.0
Krill meal	3.0
Dextrin	11.0
Starch[b]	11.5
Celufil	3.7
Amino acid mix[c]	0.7
Vitamin mixture[d]	2.5
Mineral mixture[e]	5.0
Herring oil[f]	14.0

[a]Tocopherol contents by analysis were 6 mg αT/kg for the nontocopherol-supplemented diet (no-T), 36 mg αT/kg for the all-*rac*-α-tocopherol-supplemented diet (A-T), and 15 mg αT/kg + 78 mg γT/kg for the RRR-γ-tocopherol-supplemented diet (G-T).
[b]Contains 8% cornstarch and 3.5% pregelatinized starch.
[c]Supplied 0.5% DL-methionine and 0.2% L-phenylalanine.
[d]Supplied vitamins at the following levels (IU or mg/kg diet): vitamin A, 8000 IU; vitamin D_3, 3000 IU; vitamin K, 40; thiamin, 50; riboflavin, 70; *d*-calcium pantothenate, 200; *d*-biotin, 1.5; folic acid, 20; vitamin B_{12}, 0.15; niacin, 300; pyridoxine, 30; inositol, 500; ascorbic acid, 50; choline chloride, 5000.
[e]Supplied minerals at the following levels (mg element/kg diet): manganese, 40; iron, 50; copper, 15; zinc, 100; magnesium, 398; iodine, 5; cobalt, 15; sodium, 985; fluorine, 4.5; phosphorus, 2900; potassium, 2500.
[f]To the weighed herring oil (14 w/w% of the diet), 0.015% of tocopherol supplement was added to the A-T diet (DL-α-tocopherol) and G-T diet (D-γ-tocopherol) prior to incorporation of the oil with the dry ingredients. In the no-T diet, Celufil (US Biochemicals, Cleveland, OH) replaced tocopherol.

MATERIALS AND METHODS

Fish and diets. Atlantic salmon parr (9.5 g average initial weight) were randomly distributed into tanks (50 fish/tank) containing 200 L fresh water maintained at 2 L/min flow rate and an average temperature of 16 ± 2°C. The photoperiod was automatically set at 12 h of light. Feeding to satiety during the lighted period was done by hand twice daily except on weekends when fish were fed only once per day.

The formulations of the three semipurified diets were the same except for tocopherol supplementation as shown in Table 1. All-*rac*-α-tocopherol was obtained from US Biochemical Corporation (Cleveland, OH) and RRR-γ-tocopherol from Sigma (St. Louis, MO). Test diets were steam-pelleted in a laboratory pellet mill (California Pellet Mill, San Francisco, CA) and stored in sealed plastic containers at –35°C. Feed rations sufficient for about 7 d were thawed at one time. The test diets had similar moisture (8%), protein (43%), and lipid (15%) contents.

After 36 wk on the experimental diets, six fish were randomly selected from each treatment, stunned with a blow to the head, and then weighed. Blood was collected using heparinized vials (Becton-Dickinson, Rutherford, NJ) and centrifuged for 5 min to separate the serum for collection. The liver, brain, gill, kidney, and portions of the white muscle and perivisceral fat (an adipose tissue surrounding the gastrointestinal tract) were then rapidly excised and weighed. Precocious maturation was observed in more than 90% of the males sampled at 36 wk, and testes from these mature individuals were also collected for lipid and tocopherol analyses. Since individual organ weights were occasionally too small for tocopherol analysis, tissue samples from two individuals in the same treatment were combined for each replicate analysis.

Lipid extraction and tocopherol analyses. Immediately after excision and weighing, the tissues were transferred to a test tube containing 18 mL hexane/isopropanol (3:2, vol/vol), homogenized, and extracted for total lipids following the method of Hara and Radin (21).

Total lipid extracts were quantitatively dissolved in hexane and tocopherol contents of the diets, and tissues were determined by high-performance liquid chromatography with fluorescence detection following the AOCS method (22). The high-performance liquid chromatography system consisted of a Partisil 5 μm column (11 cm × 4.7 mm; Whatman, Clifton, NJ) equipped with a guard column, a Waters (Milford, MA) U6K Injector, a Waters 6000A Solvent Delivery System, a Waters 420 AC Fluorescence Detector with 280 excitation and 338 nm emission filters, and a Perkin-Elmer (Norwalk, CT) LCI-100 Laboratory Computing Integrator. The isocratic mobile phase was hexane/isopropanol (99.5:0.5, vol/vol) at 1.5 mL/min flow rate. Peaks were identified by comparing retention times with those of authentic αT and γT standards or by coinjecting the tocopherol standard with the lipid sample. Quantitation was achieved by reading sample peak areas against linear regression lines plotted for standard solutions of the tocopherols.

Analysis of lipid composition. The percentage composition of tissue lipids from fish fed the supplemented diet (G-T) was determined using a TH-10 Mark III TLC/FID Iatroscan (thin-layer chromatography with flame-ionization detection; Iatron Laboratories, Tokyo; Canadian Agent Scientific Products and Equipment, Rexdale, Ontario, Canada) attached to an SP-4200 computing integrator. The lipid sample was spotted onto silica gel-coated quartz rods (Chromarods SIII) and developed in a solvent system consisting of hexane/chloroform/isopropanol/formic acid (80:14:1:0.2, by vol) for 50 min. The analytical conditions for quantitation of lipid classes followed the method of Parrish and Ackman (23). Standards used for identification and quantitation of lipid class peaks in the sample were tripalmitin, cholesterol, 1,2-dipalmitoyl-3-phosphatidylcholine, cholesteryl palmitate (Serdary Research Laboratories, London, Ontario, Canada), and palmitic acid (Nu-Chek-Prep, Elysian, MN). Standard curves for each lipid type were plotted by chromatographing known quantities of the standards in the same solvent system and on the same rods as those used for tissue lipid samples.

Oxidative susceptibility of the muscle. The oxidative susceptibility of the muscle was determined by the 2-thiobarbituric acid-reactive substances (TBARS) test according to Woyewoda *et al.* (24) using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO) to make up the malonaldehyde standard solution. Intact muscle samples from six fish were stored for 6 mon at −40°C prior to the TBARS test. At the end of 6 mon, tocopherol levels in the muscle samples were not remeasured since drastic decreases in tocopherols were not expected under this storage condition. Ackman and Timmins (25) reported that no dramatic decreases in αT content of smoked salmon fillets occurred within 12 wk of storage at −10°C. Since the storage temperature in this new experiment was lower and the muscles were not exposed to treatments (e.g., brining and smoking) which would potentially degrade tocopherols, the content of the muscles should not significantly differ from the values determined prior to storage.

Statistical analysis. Differences in means of tissue lipid content and TBARS values were determined by one-way analysis of variance and Tukey's test, and the relationship between tissue tocopherol and phospholipid content was determined by regression analysis using Systat 5.2 (Evanston, IL).

RESULTS AND DISCUSSION

No unexplained fish mortalities or gross pathological symptoms were observed during the feeding trial. After 36 wk, the average weight of fish was 85 g, and no remarkable differences in specific growth rate were observed between dietary treatments.

Table 2 shows the lipid contents of various tissues of Atlantic salmon after 36 wk on the experimental diets, and Table 3 shows that lipid class composition of the tissues from the fish fed the G-T diet. There were no significant differences in lipid content of the tissues of fish from the different dietary treatments except in the perivisceral fat. Fish used in this experiment were judged lean at sacrifice, considering the low lipid content of white muscle tissue. Salmon do not deposit much fat in fresh water but do so after smolting into salt water when dietary fat can be raised to 27–29% of the diet, resulting in over 16% fat in market fish.

TABLE 2
Lipid Contents[a] (%) of Tissues, Wet Weight, in Atlantic Salmon Fed no-T, A-T, or G-T Diets

	Diet group		
Tissue	no-T	A-T	G-T
Brain	7.8 ± 0.2	7.9 ± 0.3	7.3 ± 0.1
Gill	14.8 ± 1.3	14.4 ± 0.5	13.4 ± 0.3
Kidney	6.3 ± 0.6	6.2 ± 0.5	7.0 ± 0.5
Liver	3.0 ± 0.4	3.9 ± 0.9	2.9 ± 0.2
Muscle	1.7 ± 0.1	1.5 ± 0	1.6 ± 0.0
Perivisceral fat	92.1 ± 0.2[b]	95.5 ± 0.4[c]	92.7 ± 1.0[b,c]
Serum (mg/mL)	18.4 ± 0.9	18.2 ± 1.3	18.9 ± 1.8
Testes	2.1 ± 0.3	1.9 ± 0.3	2.1 ± 0.8

[a]The no-T and G-T diets contained some α-tocopherol derived from the dietary ingredients. Mean ± SEM followed by different superscripts (b,c) within one row are significantly different ($P < 0.05$), $n = 2$ or 3; each n is a pooled sample from two fish. See Table 1 for abbreviations.

Phospholipids, sterols, and triacylglycerols were the major components in all tissues. The perivisceral fat contained small amounts of phospholipids and extremely high levels of triacylglycerols, a characteristic of its function as major tissue depot for lipid reserves. The gill lipids exhibited a large proportion of triacylglycerol probably originating from fatty supportive tissues of the gill arch.

Comparative tissue distribution of αT and γT. Despite a 100% higher dietary concentration of γT compared to αT (Table 1), the tissue γT content of the G-T_{fed} fish were lower than αT in tissues from the all-*rac*-α-tocopherol supplemented diet (A-T)-fed fish in the liver, serum, kidney and testes, whereas no difference was seen in the brain tissue (Fig. 1). Muscle and gill γT contents were slightly higher than those of αT although this difference was less pronounced than the proportions of the dietary tocopherol concentrations. Only the perivisceral fat γT concentration greatly exceeded (almost

TABLE 3
Composition of Lipids (%) from Tissues of Atlantic Salmon Fed the γ-Tocopherol Diet[a]

	Lipid class		
Tissue	Triacylglycerol	Sterol	Phospholipid
Brain[b]	2.4 ± 0.5	19.4 ± 0.3	77.2 ± 0.2
Gill	90.8 ± 1.5	1.7 ± 0.4	8.4 ± 1.1
Kidney	61.9 ± 2.8	4.8 ± 0.2	33.3 ± 2.7
Liver	18.6 ± 4.0	6.6 ± 0.9	74.8 ± 3.2
Muscle	35.1 ± 2.1	3.7 ± 0.7	62.2 ± 2.0
Perivisceral fat	98.7 ± 0.2	0.4 ± 0	0.9 ± 0.2
Serum	39.2 ± 1.9[c]	8.3 ± 0.1	52.5 ± 2.0
Testes	7.5 ± 1.3	17.7 ± 0.2	74.8 ± 1.2

[a]Values are mean ± SD of 2–3 lipid extracts.
[b]The brain contained 1.3% free fatty acids, a value higher than in other samples.
[c]Includes both sterol esters and triacylglycerols.

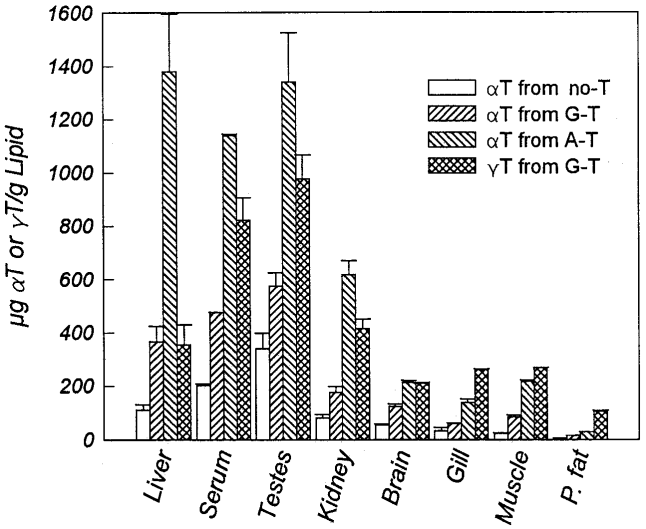

FIG. 1. Concentrations (μg/g lipid) of tocopherols in selected tissues of fish fed no supplemental tocopherol (No-T), or supplemented with α-tocopherol (A-T), or supplemented with γ-tocopherol containing a small proportion of alpha (G-T), after 36 wk. P. fat, perivisceral fat.

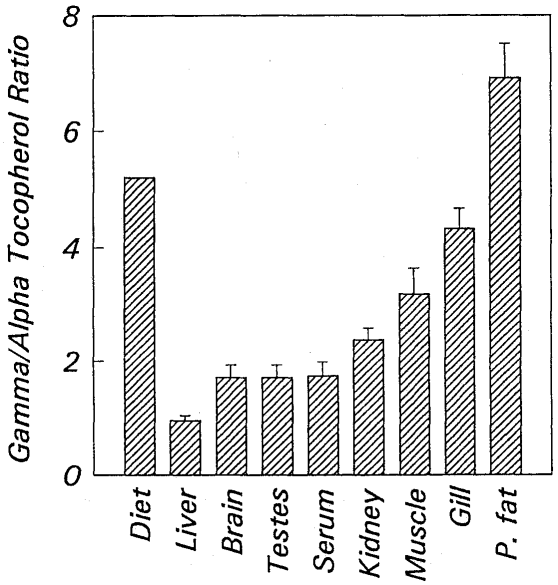

FIG. 2. Ratio of γT/αT in the G-T diet and in selected tissues of the fish fed the G-T diet for 36 wk. See Figure 1 for abbreviations.

400%) that of αT in the corresponding tissue of the (A-T)-fed fish. It is thus apparent that γT is preferentially stored in the adipose tissue, and its accumulation in the total lipids of critical tissues in Atlantic salmon is inferior to that of αT. This difference in tissue distribution of αT and γT is interesting because previous reports have shown that adipose tissue tocopherol constitutes little more than an inactive reserve of this compound. In humans, more than 90% of adipose tissue tocopherols is largely dissolved in the lipid droplet within the cell rather than in the surrounding membrane and therefore unavailable for immediate use (26). Furthermore, adipose αT is not rapidly released back into the bloodstream after αT supplementation in humans is halted (27). In guinea pigs, Machlin *et al.* (28) also found that adipose tissue tocopherol is not readily available for maintaining plasma tocopherol levels and preventing signs of vitamin E deficiency, thereby having no immediate nutritional value and simply representing a tissue depot with slow turnover rate. In a similar context, it is likely that γT stored in the adipose tissue of Atlantic salmon is biochemically inactive as opposed to αT found in the critical tissues.

In fish fed the G-T diet containing primarily γT and minimal αT, different tissues had varying affinities for the two tocopherols (Fig. 1). The γT/αT ratio (Fig. 2) was lowest in the liver, highest in the perivisceral fat, and the other tissues exhibited intermediate values. These results emphasized the importance of the liver in selecting αT over γT in Atlantic salmon. Similarly the fact that the γT/αT ratio was higher in the perivisceral fat than in the diet indicated preferential storage of γT in the depot organ as opposed to αT which distributed largely within critical tissues. The perivisceral fat is mostly triacylglycerol that would derive both fatty acids and tocopherols from the circulating chylomicrons (8). The gills are also rich in triacylglycerols and they have the next-highest ratio (Fig. 2). Other body sites may acquire their lipids from very low density lipoprotein after the discrimination process screens out and excretes tocopherols other than alpha. The relationship between the γT/αT ratio and tissue phospholipid content showed a trend of decreasing ratio with increasing tissue phospholipid content (Fig. 3). A greater accumulation of αT compared to γT in phospholipid-rich tissues suggests that preference for αT over γT in Atlantic salmon tissues appears to be governed at the level of incorporation in the lipid bilayer of membrane structures. The difference in their incorporation at sites close to the phospholipids in cell membranes may impact heavily on their *in vivo* antioxidant activities.

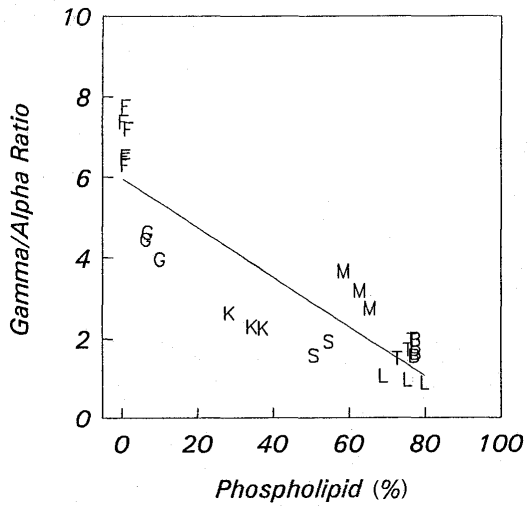

FIG. 3. Correlation between γT/αT ratio and phospholipid contents (%) from tissues of fish fed the G-T diet for 36 wk (r^2 = 0.77; n = 26; P < 0.01); B (brain), G (gill), F (perivisceral fat), K (kidney), L (liver), M (muscle), S (serum), T (testes). See Figure 1 for other abbreviations.

Erickson *et al*. (29) found that the αT concentration in milk was at least three times higher in the lipid of the fat globule membrane than in the lipid inside the fat globule. During lipid oxidation, tocopherol associated with the membrane was lost more rapidly than that found inside the fat globule, implying its greater role in determining the oxidative stability of milk. Correspondingly, the distribution of αT within the lipid bilayer of cell membranes is now well established. Krishnamurthy and Bieri (30) and Gruger and Tappel (13) were some of the early investigators who observed that tocopherols in active biological cells concentrate largely in phospholipid-rich cellular regions, e.g., mitochondria and microsomal fractions. Buttris and Diplock (31) also verified that the inner and outer mitochondrial and microsomal membrane sections were the major tocopherol-containing sites, implying the specific localization of tocopherols in membrane regions particularly liable to oxidative attack. In 1989, Gomez-Fernandez *et al.* (12) used fluorescence spectroscopy techniques to determine the position of tocopherol within phospholipid vesicles based on tocopherol's intrinsic fluorescence and the quenching action of membrane probes, e.g., 5-doxylstearate (5-NS), 16-doxylstearate (16-NS), and acrylamide. Acrylamide, a water-soluble quencher with very low capacity to penetrate the phospholipid bilayer was inefficient in quenching αT located in the fluid membrane. On the other hand, 5-NS and 16-NS, probes known to be located at a graded series of depths from the surface of the bilayer, were both effective, 5-NS more than 16-NS, in quenching the fluorescence of αT. It is thus clear that αT lies embedded within the phospholipid bilayer with its chromanol head located near the polar region of the membrane. Fukuzawa *et al.* (10) further clarified that no interaction takes place between the hydroxyl group in the chromanol head of αT and the polar interfacial region, indicating that the hydrophobic chromanol head is poorly exposed at the surface. No analogous studies were done for γT. Since γT has one methyl group less than αT in the chromanol head, it is highly probable that molecular discrimination of incorporation in membrane lipid bilayers exists between the two tocopherols due to their different steric structures in the chromanol region. The presence of more methyl substituents in the chromanol region should increase lipophilic properties, thereby making αT the more soluble tocopherol in lipid substrates (32). Interestingly, a recent report showed α-tocotrienol as a more potent scavenger of peroxyl radicals in liposomes compared to αT, presumably due to a difference in vitamin E-membrane interactions conferred by the hydrocarbon tails (33). Unsaturation in the tail of αT gives rise to an altered three-dimensional molecular structure in α-tocotrienol which may profoundly affect the orientation and organization of membrane phospholipids and result in different antioxidant potencies between the two vitamin forms (33).

The scatter of the points in Figure 3 strongly suggests the involvement of other mechanisms of biodiscrimination between γT and αT which take place at the cellular level in response to the diverse metabolic functions of various tissues. For instance, Ackman and Cormier (15) found that metabolically active dark muscle in cod was richer in αT than the white muscle tissue, since confirmed for Atlantic salmon (34). In this new experiment, the liver and testes exhibited similar proportions of tissue phospholipids, but the γT/αT ratios differed by about 75% (Fig. 2). Also, the muscle showed a higher γT/αT ratio (*ca*. 3) than would have been expected from the relatively high phospholipid content, possibly due to γT deposition in neutral lipid of adipocytes attached to connective tissue (35,36), as well as the redirection of essential αT from muscle phospholipids into phospholipids of other and more critical tissues. Hamre and Lie (34) suggest that each organ may have unique combinations of mechanisms by which they take up lipids and tocopherols from plasma.

As already pointed out (7,8), another discriminatory mechanism relates to the existence of an αT-specific factor, which is reportedly present in the rat, but not yet studied in fish. Catignani and Bieri (37) were the first to report the presence of a tocopherol-binding protein in rat liver which was specific for, but not necessarily limited to, αT since α-tocotrienol and γT also exhibited minimal competitive effects. The protein, a 30 kDa αT-binding protein, exists uniquely to the hepatocyte in the rat, and regulates plasma tocopherol concentration by preferentially incorporating the αT into nascent very low density lipoproteins (8). Another αT-binding protein of molecular mass 14.2 kDa was also recently identified and suspected to be responsible for the intracellular transport and distribution of αT in all tissues including the liver (38). Although the existence of a 30 kDa αT-binding protein in fish liver has not yet been verified, our data suggest that an analogous protein exists in Atlantic salmon liver since this organ appeared to be the most biased tissue for accumulating αT.

The interrelationships between tissue phospholipids, αT-specific protein factor, or other mechanisms which collectively result in biodiscrimination between αT and γT distribution in Atlantic salmon tissues require further investigation. Nevertheless, the greater accumulation of γT in adipose tissue compared to αT, and the negative correlation obtained between γT/αT and tissue phospholipid content suggest that inferior structural compatibility of γT in the phospholipid-rich membrane bilayer, compared to that of αT, may partly explain the distribution pattern of the two tocopherols in Atlantic salmon tissues.

Tissue αT distribution. The tissue lipid tocopherol content from all fish groups after 36 wk on the experimental diets is shown in Figure 1. The nontocopherol supplemented diet (no-T)-fed fish showed the lowest amounts of αT in lipids of all tissues examined, while the (G-T)-fed fish exhibited only somewhat higher tissue αT content. The (A-T)-fed fish exhibited the highest αT content in all tissues analyzed.

To establish which of the tissues of Atlantic salmon were prone to lose tocopherol during restricted intake of this compound, the ratios of tocopherol content of the (A-T)- and (no-T)-fed fish (calculated as [αT content of A-T]/[αT content of no-T]) were determined for each of the tissues examined (Fig. 4). These ratios reflect the magnitude of tocopherol loss in the tissues of the no-T-fed fish. After a period of low

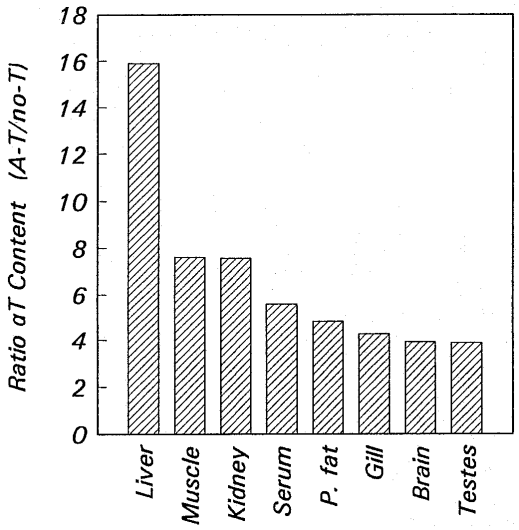

FIG. 4. Ratios of tissue αT concentrations in tissues of the (A-T)- and (no-T)-fed fish after 36 wk. See Figure 1 for abbreviations.

tocopherol intake and almost 900% weight gain, the (no-T)-fed fish still retained appreciable quantities of tocopherol in certain tissues such as the testes. The brain is another tissue that tenaciously retained tocopherol during the period of low tocopherol intake in Atlantic salmon (Fig. 4). Clearly, the different tissues of Atlantic salmon have variable ability for retention and deposition of tocopherols during a period of restricted intake of this compound. The liver showed the greatest disparities, indicating its function as a major depot organ for tocopherol which is likely mobilized during periods of deprivation (Fig. 4), a result similar to that obtained by Cowey *et al.* (39) in rainbow trout. Although the muscle showed moderate loss of tocopherol (Fig. 4), it accounts for at least 50% of the fish weight, and therefore lost greater actual amounts of tocopherol compared to the liver. Cowey *et al.* (40) suggested the possibility that tocopherol is translocated from the muscle of rainbow trout to other more critical organs during the deprivation periods. This also applies to Atlantic salmon in this experiment where drastic decreases in liver and muscle tocopherols probably reflect transport into the developing testes from the precocious males, since the testes exhibited the highest concentration of tocopherol among the tissues of the (no-T)-fed fish, and the least margin of difference in tocopherol concentration among the tissues of the (no-T)- and (A-T)-fed fish (Fig. 4). Lie *et al.* (41) reported a similar scenario for female Atlantic salmon where a net transport of tocopherol from peripheral tissues to the gonads occurred during vitellogenesis, thus protecting the reproductive organ from critical tocopherol deficiency status at the expense of the other organs.

The pattern of tissue tocopherol depletion observed in Atlantic salmon follows that generally observed for other animal models. In rats, significant amounts of tocopherol loss from the liver occurred when dietary intake of this compound was minimal (42). In contrast, tocopherol stores in the nervous system tissues and brains of rats and dogs declined slowly during restricted intake (43,44). Bieri (42) speculated that the kinetics of tissue tocopherol depletion in rats may be affected by the respective amounts of labile and bound tocopherol present in the particular organ.

Oxidative susceptibility of the muscle. In Atlantic salmon, the liver and muscle exhibited drastic decreases in tocopherol concentrations during restricted intake of this compound, implying mobilization of their tocopherol stores toward other and more critical tissues. As the muscle constitutes the consumable portion of the fish, tocopherol loss from this organ may adversely affect the postmortem quality of the resultant fish flesh product. Salmon farming is an international and intensively competitive industry where quality has only comparatively recently superseded maximal growth as a major concern (45,46).

Sigurgisladottir *et al.* (16) reported that with experimental Atlantic salmon fillets containing more γT than αT, TBARS values were lower after a forced oxidation test than for fillets containing only a smaller amount of αT. In this new experiment, the presence of minimal αT in the muscle from fish fed the no-T diet exposed the tissue to significantly greater susceptibility to lipid peroxidation after frozen storage at –40°C for 6 mon than was the case for muscle containing higher concentrations of either αT or γT (Fig. 5). Muscle samples from the G-T treatment exhibited tissue αT levels that were closer to the no-T rather than to those from the A-T treatments, yet the muscle TBARS values were significantly better than that of the no-T treatment and corresponded more closely with those of the A-T treatments. Thus, the significant drop in oxidative susceptibility of the G-T muscle samples compared to the control was most likely due to the presence of γT. The results of this experiment confirm the reports of Sigurgisladottir *et al.* (16,17) that γT improves the oxidative stability of Atlantic salmon muscle.

Frozen storage of natural fish muscle does not halt natural biochemical processes such as loss of αT content and of its protective effect on lipids. There are, however, seasonal effects, probably linked to sexual maturity or food availability, that can at times lead to very rapid loss of αT and consequent rancidity even in a lean species such as sole (47). Our view that storage of muscle samples for 6 mon at –40°C would not

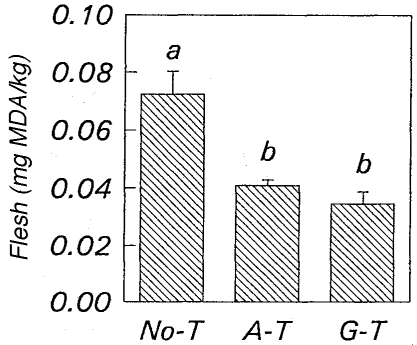

FIG. 5. Thiobarbituric acid-reactive substances in salmon fillets from the different dietary treatments expressed as mg malonaldehyde (MDA)/kg flesh. See Figure 1 for abbreviations.

lead to an excessive reduction in αT or γT may have been optimistic. A recent study of artificially fed caged Atlantic mackerel *Scomber scombrus* (48) mentioned a storage study of frozen salmon muscle (−30°C) that did show an increase in TBARS over 6 mon (details not available). Unfortunately the authors of this study on mackerel enriched in γT appear to have carried out the storage on homogenized muscle samples, thus maximizing exposure to oxygen in this notoriously oxidation-sensitive species (49). Our earlier frozen storage study (25) on smoked mackerel and salmon was carried out on intact muscle tissue, as was the procedure here. The composition of farmed salmon muscle differs in one important aspect from the muscle composition of our experimental fish. Farmed salmon muscle usually contains two pigments, astaxanthin and canthaxanthin (50). The former is the basis of the natural color of wild Atlantic salmon. Like carotene itself, such carotenoids can protect highly unsaturated fatty acids against oxidation, and carotene operates effectively in conjunction with αT (51).

The protective effects of dietary αT on maintaining the oxidative quality of fish flesh products have already been demonstrated for species such as rainbow trout and channel catfish (18,20). O'Keefe and Noble (18) reported reduced oxidative rancidity of frozen stored (−10°C) channel catfish fed 100 mg αT/kg diet or higher concentrations, and suggested that longer storage periods may require higher concentrations of dietary tocopherol (up to 400 mg/kg diet) to maintain acceptable fillet quality. The critical dietary αT levels which afford oxidative stability to the resultant fillets may relate to the quality of dietary lipids, since a diet containing a high n-3 polyunsaturated fatty acids content and a low tocopherol content resulted in rancid flavor development in Atlantic salmon fillets (19).

Most of the above-published results dealt with αT as the dietary ingredient. Except for Sigurgisladottir *et al.* (16,17) and Hamre and Lie (34), there appear to be no previous reports on the deposition of other tocopherols in salmon muscle, particularly of γT and its potential role in preserving the oxidative quality of fish-food products. This study confirms that dietary intakes of either αT or γT by Atlantic salmon were effective in maintaining the oxidative stability of the muscle tissue. Moreover, the low level of αT in the diet of the (G-T)-fed fish appeared to be adequate for the health of growing fish of this size. The no-T diet contained only half as much αT, derived from the marine animal products in the diet, and the fish fed this diet showed no adverse effects despite the inclusion of highly unsaturated fatty acids in the diet.

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[Received July 29, 1996, and in final revised form and accepted May 27, 1998]

Cholesterol Oxidation in Meat from Chickens Fed α-Tocopherol- and β-Carotene-Supplemented Diets with Different Unsaturation Grades

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ABSTRACT: The production of B-ring and side-chain oxysterols was evaluated in meat from chickens fed diets differing by the kind of oil or fat added. The effect of supplementary levels of natural antioxidants, as α-tocopherol and β-carotene, on the meat cholesterol oxidative stability was also studied. Lard, sunflower and olive oil were used as dietary fat. Raw and cooked meats were analyzed for oxysterols, and cholesterol was also quantified. Oxysterol analyses were carried out by combining the use of solid-phase extraction, thin-layer chromatography, capillary gas chromatography, and capillary gas chromatography–mass spectrometry. Oxysterols were detected within the 0.1–0.5 μg/g range in raw meat. Cooking increased the oxysterol content of the meat, and levels as high as 5 μg/g muscle tissue were observed. B-Ring oxysterols were mainly produced: the α- and the β-epoxycholesterols, the 7α- and 7β-hydroxycholesterols, and the 7-ketocholesterol. The results showed that the meat from the chickens fed the olive oil-based diet containing α-tocopherol at 200 mg/kg of diet presented the best cholesterol oxidative stability. A positive effect could not be found for dietary β-carotene administered at levels of 15 and 50 mg/kg of diet. Furthermore, a significant decrease in the tissue cholesterol content was observed with the olive and the sunflower oil-based diets.

Lipids 33, 705–713 (1998).

Food oxidation affects the quality and the safety of the human diet by generating compounds having biological activities that could adversely affect health (1–3). Unsaturated lipids are particularly susceptible to oxidation, and the mechanism has been thoroughly studied and well documented in the literature (4). Lipid oxidation is responsible for quality deterioration of muscle foods and results in the formation of lipid hydroperoxides which decompose into secondary products such as aldehydes, alcohols, ketones, and short-chain carboxylic acids (5–7). Cholesterol is also prone to oxidation, producing a wide variety of cholesterol oxides, termed oxysterols (8). Cholesterol oxidation may proceed by the same mechanisms described for lipid oxidation or can be initiated by free radicals generated during lipid oxidation (8).

Oxidized lipids and cholesterol oxides are implicated in the enhancement of atherosclerosis (9,10). Oxysterols are well absorbed from the diet and are transferred into cholesterol-rich lipoproteins such as very low density lipoproteins, low density lipoproteins (LDL), and chylomicrons (11,12). Oxysterols have been detected in LDL and in atheromatous plaque (13,14). They can modify cell membrane fluidity and permeability and inhibit cholesterol biosynthesis by suppressing hydroxy methyl glutaryl-CoA reductase activity. They also are cytotoxic, immunosuppressant and affect the production of prostacyclin by endothelial cells, cause apoptosis, and inhibit cellular cholesterol efflux (15–17). All these deleterious effects support the hypothesis that oxysterols are involved in atherosclerosis (9,15).

A variety of cholesterol oxides have been detected in cholesterol-rich foods, such as spray-dried egg powder, meat and meat products (18,19). Storage, cooking, and processing procedures increase the oxysterol level in foods as a result of increased cholesterol and lipid oxidation (18,19). One way to increase the oxidative stability of lipids and cholesterol in foods is to increase the amounts of natural antioxidants such as α-tocopherol (vitamin E) or β-carotene in the diet. Feeding diets supplemented with α-tocopherol to animals like chickens, cows, and pigs resulted in vitamin accumulation in the animal muscle (20,21) and better oxidative stability under prooxidative conditions such as storage and cooking (20,21). Meat from pigs and calves fed such diets had reduced cholesterol oxidation when stored or cooked (22,23).

Few reports about antioxidant effects of β-carotene-supplemented diets have been published. The results were not so convincing as those reported for α-tocopherol, and a lack of protective action of β-carotene has been reported (24). In the present work, broiler chickens were fed one of four diets supplemented with various amounts of α-tocopherol and β-carotene. Lard (rich in saturated fatty acids), sunflower oil (rich in 18:2n-6 and 18:3n-3), and olive oil (rich in 18:1n-9)

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Abbreviations: cGC, capillary gas chromatography; α-epoxide, 5,6α-epoxy-5α-cholestan-3β-ol; β-epoxide, 5,6β-epoxy-5β-cholestan-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β-7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 20α-hydroxycholesterol, cholest-5-en-3β,20-diol; 25-hydroxycholesterol, cholest-5-en-3β,25-diol; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one; LDL, low density lipoprotein; triol, 5α-cholestane-3β,5,6β-triol.

were used as dietary fat. The study was focused on cholesterol oxidation and levels in raw and cooked broiler meat and evaluated the extent to which these parameters were affected by the dietary natural antioxidants and the unsaturation of the dietary fat. Interest in this meat arises from the increasing production of poultry in the United States, Asia, and Europe (25). The results of this study relate directly to the quality and the safety of poultry meat.

MATERIALS AND METHODS

Animals. Four experiments were carried out. For each experiment, 280 eight-day-old female broiler chicks of the Ross strain were used. They were placed in 24 flat-deck cages of 1 square meter each. The chicks were raised according to routine practices in terms of light and temperature. Except for the first experiment, each experiment lasted 6 wk and consisted of three dietary treatments, differing by the fat type included in the basal diet and by the amount of antioxidant supplemented (Table 1). At the end of each experiment, the chickens were slaughtered and legs were separated, vacuum packed, and stored at −20ºC until needed for processing. Analyses were performed within 2 mon. Thighs were thawed and processed for cholesterol and oxysterol analyses. The composition of the basal diet was maize (50.56%), soybean meal (39.58%), fat (6.00%), calcium carbonate (1.00%), dicalcium phosphate (2.00%), salt (0.40%), DL-methionine (0.16%), vitamins and minerals (0.40%).

Meat cooking. Broiler thighs were cooked in polyethylene bags. Samples were placed in a water bath at 85ºC until the inside temperature reached 80ºC (50 min). Samples were immediately processed for cholesterol and cholesterol oxides analyses after cooking.

Cholesterol oxides determination. Raw and cooked meat from the four experiments were analyzed for the following cholesterol oxides: cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (α-epoxycholesterol), 5,6β-epoxy-5β-cholestan-3β-ol (β-epoxycholesterol), cholest-5-en-3β,20-diol (20α-hydroxycholesterol), 5α-cholestane-3β,5,6β-triol (triol), and cholest-5-en-3β,25-diol (25-hydroxycholesterol) using a method developed by García-Regueiro and Maraschiello (26). 19-Hydroxycholesterol (5-cholestene-3β-19-diol) (50 µg), used as the internal standard, and butylated hydroxytoluene (BHT) (0.002%) were added to 20 g of meat prior to extraction. Lipid extraction was carried out according to the procedure of Folch *et al*. (27). The total extract (~1 g) was successively passed through silica and Florisil® columns (Varian Associates, Palo Alto, CA) of 10 g. Thin-layer chromatography permitted further purification of cholesterol oxides and their separation from cholesterol. The detection, identification, and quantification of oxysterols as trimethylsilyl ether derivatives were performed by using capillary gas chromatography (cGC) in the solvent-venting mode with flame ionization detection as described earlier (26). A fused-silica open tubular capillary column (30 m × 0.25 mm i.d.) coated with 5% phenylmethylsilicone and with a film depth of 0.25 µm (J&W Scientific, Folsom, CA) was used. An initial column temperature of 220°C was programmed at a rate of 5°C/min to 310ºC, which was held for 25 min (26). Peaks were identified by using relative retention times (Table 2). Oxysterols detected by cGC were confirmed by using cGC–mass spectrometry in the single-ion monitoring mode (26). Table 2 lists the major abundant ions characterizing trimethylsilylether-oxysterol derivatives and the characteristic ions chosen for selected ion monitoring.

Check on the production of artifactual cholesterol oxides. To 20 g of meat sample was added 20 mg of cholesterol, which was nearly twice the cholesterol content initially present. This sample and another of 20 mg of cholesterol alone were subjected to the entire procedure, with 50 µg of 19-hydroxycholesterol being added as internal standard to both samples prior to extraction. This experiment was to determine artifactual oxysterol production during sample processing.

Cholesterol determination. Fat (50 mg) extracted from meat by the procedure of Folch *et al.* (27) was saponified for cholesterol determination as described earlier (28). The cGC procedure was modified; the column is described in the preceding paragraph. Helium was the carrier gas at a head pressure of 2.0 bar. The initial column temperature of 240ºC was programmed at a rate of 12.5ºC/min to 310ºC, which was held for 4 min. Solvent-venting injection was used (28). Cholesterol was determined in raw and cooked meat with 5α-cholestane as the internal standard.

Statistics. Within each dietary group and for each experi-

TABLE 1
Experiment Descriptions

Dietary treatment	Experiment I	Experiment II	Experiment III	Experiment IV
Fat	Lard	Lard	Sunflower oil	Olive oil
Basal	Basal	Basal	Basal	Basal
Basal + α-tocopherol	—	Basal + 200 mg α-tocopheryl acetate/kg of diet	Basal + 200 mg α-tocopheryl acetate/kg of diet	Basal + 200 mg α-tocopheryl acetate/kg of diet
Basal + β-carotene	Basal + 15 mg/kg β-carotene	Basal + 50 mg β-carotene/kg of diet	Basal + 15 mg β-carotene/kg of diet	Basal + 15 mg β-carotene/kg of diet

TABLE 2
Relative Retention Times[a] Used for Identification of Oxysterols by Capillary Gas Chromatography and Major Abundant Ions Characterizing Trimethylsilyl Ether Cholesterol Oxides

Oxysterols	RRT	Mass spectral ions[b] (*m/z*)			
7α-Hydroxycholesterol	0.938	**546**	**458**	457	**456**
19-Hydroxycholesterol1	1	**366**	354	353	352
7β-Hydroxycholesterol	1.026	546	458	**457**	456
β-Epoxycholesterol	1.044	**474**	459	445	**384**
α-Epoxycholesterol	1.058	474	459	384	366
20α-Hydroxycholesterol	1.089	462	**461**	281	**201**
Triol	1.130	546	456	**404**	**403**
25-Hydroxycholesterol	1.174	546	456	327	**131**
7-Ketocholesterol	1.181	514	**472**	**367**	131

[a]Relative retention time (RRT) = (retention time of solute)/(retention time of 19-hydroxy cholesterol).
[b]Ions chosen for identity confirmation by mass spectrometry are bold and underlined.
[c]Capillary gas chromatographic retention time of 19-hydroxycholesterol was 18.09 ± 0.27 min (mean ± relative standard deviation), calculated from seven consecutive manual injections. Abbreviations: 7α-Hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; β-epoxycholesterol, 5,6β-epoxy-5β-cholestan-3β-ol; α-epoxycholesterol, 5,6α-epoxy-5α-cholestan-3β-ol; 20α-hydroxycholesterol, cholest-5-en-3β,20-diol; triol, 5α-cholestane-3β,5,6β-triol; 25-hydroxycholesterol, cholest-5-en-3β,25-diol; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one.

ment, each determination was carried out on independent samples of raw and cooked meat. A least-squares analysis using the GLM (General Linear Models) from SAS (29) was carried out including the dietary treatment and the nature of the sample (raw or cooked) as fixed effects. Correlation analysis were performed with the CORR procedure from SAS (29).

RESULTS

The thigh samples from the four experiments were analyzed for B-ring (7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol, cholestanetriol, 7-ketocholesterol) and side-chain cholesterol oxides (20α-hydroxycholesterol, 25-hydroxycholesterol) and cholesterol itself.

Cholesterol oxides in raw broiler meat. Tables 3 to 6 show the cholesterol oxide content in raw meat for the four experiments and their respective three dietary treatments (basal, basal plus α-tocopherol, basal plus β-carotene). Cholesterol oxides resulting from the B-ring oxidation of cholesterol were the main products, i.e., 7-hydroxyisomers, epoxyisomers and 7-ketocholesterol. Total oxysterol concentrations within the range of 0.1 μg/g muscle tissue were quantified. Cholesterol oxides were not detected in all samples, as shown in Table 7. Low concentrations (<0.1 μg/g) of 20α-hydroxycholesterol were detected in a limited number of raw-meat samples from the sunflower oil experiment. Triol and 25-hydroxycholesterol were not detected (Tables 3–7). There were no significant differences in total oxysterol concentrations in raw meat between each dietary treatment in spite of an obvious decrease shown by the dietary group supplemented with α-tocopherol within each experiment (Tables 3–6). The groups fed α-tocopherol also showed a decrease in the frequency of the oxysterol occurrence (Table 7). When the results obtained from all the control groups were compared, the muscle from the experiment with sunflower oil contained significantly higher amounts of oxysterols than the muscle from experiments with lard and sunflower oil (Table 8). This was also observed for the groups fed α-tocopherol. The groups supplemented with β-carotene had no significant differences between them (Table 8). In conclusion, total amounts of oxysterols reported were lower than 0.5 μg/g, and the ratio of total amount of oxysterol to cholesterol ranged from 0.003 to 0.10% (Table 8).

Cholesterol oxides in cooked broiler meat. When cooked and raw meats were analyzed and compared, a marked increase in the cholesterol oxides content was noted for each experiment and for each dietary treatment (Tables 3–6). The production of B-ring cholesterol oxides contributed to the overall oxysterol increase. This result is reflected in Table 9

TABLE 3
Experiment I (lard diet with or without β-carotene): Cholesterol Oxides in Raw and Cooked Broiler Meat[a]

	Raw meat[b]		Cooked meat[b]		
	Basal	Basal + β-carotene	Basal	Basal + β-carotene	SE
7α-Hydroxycholesterol	<0.01	0.02	0.45	0.45	0.06
7β-Hydroxycholesterol	0.01	0.05	0.76	0.78	0.10
β-Epoxycholesterol	n.d.	0.03	0.74	0.62	0.07
α-Epoxycholesterol	n.d.	n.d.	0.11	0.20	0.04
20α-Hydroxycholesterol	n.d.	n.d.	n.d.	n.d.	—
Triol	n.d.	n.d.	n.d.	0.05	0.02
25-Hydroxycholesterol	n.d.	n.d.	n.d.	n.d.	—
7-Ketocholesterol	<0.01	0.01	0.54	0.79	0.09
Total oxysterols	0.02[a]	0.11[a]	2.58[a]	2.86[a]	0.29

[a]Broiler chickens were fed a diet containing lard. Basal: broilers fed the basal diet only. Basal + β-carotene: broilers fed the basal diet supplemented with 15 mg β-carotene/kg of diet. For each dietary treatment $n = 6$ for raw and cooked meat.
[b]Results are expressed as least square means (LS means) values in μg/g tissue for oxysterols. SE, standard error of the LS means; n.d., not detected. For other abbreviations, see Table 2. Superscript roman letter (a) indicates significant difference ($P < 0.05$).

TABLE 4
Experiment II (lard diet with or without antioxidants): Cholesterol Oxides in Raw and Cooked Broiler Meat[a]

	Raw meat[b]				Cooked meat[b]			
	Basal	Basal + β-carotene	Basal + α-tocopherol	SE	Basal	Basal + β-carotene	Basal + α-tocopherol	SE
7α-Hydroxycholesterol	0.09	0.06	0.12	0.15	0.78	0.43	0.79	0.19
7β-Hydroxycholesterol	0.06	n.d.	0.05	0.11	1.07	0.12	0.46	0.14
β-Epoxycholesterol	0.07	0.09	0.08	0.07	0.62	0.21	0.27	0.09
α-Epoxycholesterol	0.03	0.02	0.02	0.03	0.22	0.06	0.13	0.04
20α-Hydroxycholesterol	n.d.	n.d.	n.d.	—	0.05	n.d.	0.03	0.02
Triol	n.d.	n.d.	n.d.	—	0.06	n.d.	0.02	0.02
25-Hydroxycholesterol	n.d.	n.d.	n.d.	—	n.d.	n.d.	n.d.	—
7-Ketocholesterol	0.13	n.d.	0.11	0.11	0.89	0.15	0.59	0.14
Total oxysterols	0.37[a]	0.17[a]	0.37[a]	0.37	3.70[a]	0.96[b]	2.52[a]	0.45

[a]Broiler chickens were fed a diet containing lard. Basal: broilers only fed the basal diet. Basal + α-tocopherol: broilers fed the basal diet supplemented with 200 mg α-tocopherol/kg of diet. Basal + β-carotene: broilers fed the basal diet supplemented with 50 mg β-carotene/kg of diet. For each dietary treatment $n = 12$ for raw and $n = 8$ for cooked meat.
[b]See footnote *b* in Table 3 for statistical treatment and abbreviations. Different superscript roman letters (a,b) indicate significant differences ($P < 0.05$).

which presents the sum of the α- and β-epoxycholesterol, the sum of the 7α- and 7β-hydroxycholesterol, and the sum of the three C7-oxysterols for raw and cooked meat. Triol was detected in some cooked samples at mean values lower than 0.1 μg/g (Tables 3, 4, 6, and 7). Some cholesterol oxides were detected in all of the samples analyzed, particularly in the experiment with sunflower oil (Table 7). In some cases, the 20α-hydroxycholesterol and the 25-hydroxycholesterol were detected but only in a couple of samples from the groups fed the basal diet and those supplemented with β-carotene (Tables 4, 5, and 7). The experiment with sunflower oil generally yielded the highest values for total oxysterol content (4.62 for the basal group and 4.63 μg/g for the β-carotene-supplemented group; see Table 5). In the experiments with lard and sunflower oil, the dietary groups supplemented with α-tocopherol had significantly less cholesterol oxidation (Tables 4,5). The groups fed the basal diet and the groups supplemented with β-carotene did not differ significantly (Tables 3–6). No significant differences were detected between the dietary treatments with olive oil. In summary, cooked meat had an increased ratio of oxysterol to cholesterol when compared with raw meat. When the experiments were compared, the diet supplemented with sunflower oil yielded significantly higher total amounts of oxysterols than the three other experiments (Table 8). Dietary α-tocopherol reduced the total oxysterols and the ratio of total oxysterol to cholesterol (Table 8).

Oxidation ratios. In raw meat, the 7α-isomer was the predominant alcohol and the β-epoxide was the predominant epoxide (Tables 3–6). In cooked meat, the β-epoxide and the 7β-hydroxycholesterol were the predominant isomers as shown in Table 9. 7α-Hydroxycholesterol predominated only in a few cases (Table 9).

The production of artifactual oxysterols. As described earlier, our procedure permits the detection of oxysterol concentrations at the ng/g level (26). No oxysterol peaks were detected at the respective relative retention times when cholesterol-spiked samples were analyzed by cGC. The absence of artifactual oxysterols was confirmed by cGC–mass spectrometry in the single-ion monitoring mode, meaning that artifactual oxysterols, if present, could be generated at levels below the sensitivity range of the procedure and would minimally

TABLE 5
Experiment III (sunflower oil with or without antioxidants): Cholesterol Oxides in Raw and Cooked Broiler Meat[a]

	Raw meat[b]			Cooked meat[b]			
	Basal	Basal + β-carotene	Basal + α-tocopherol	Basal	Basal + β-carotene	Basal + α-tocopherol	SE
7α-Hydroxycholesterol	0.21	0.14	0.18	0.87	0.50	1.41	0.18
7β-Hydroxycholesterol	0.11	0.01	n.d.	1.50	0.83	1.08	0.19
β-Epoxycholesterol	0.07	0.01	n.d.	0.84	0.50	0.72	0.11
α-Epoxycholesterol	0.05	0.01	n.d.	0.39	0.21	0.51	0.05
20α-Hydroxycholesterol	0.01	0.01	0.05	n.d.	n.d.	0.14	0.04
Triol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—
25-Hydroxycholesterol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—
7-Ketocholesterol	0.09	0.03	0.05	1.01	0.55	0.89	0.12
Total oxysterols	0.53[a]	0.22[a]	0.27[a]	4.62[a]	2.58[b]	4.63[a]	0.48

[a]Broiler chickens were fed a diet containing sunflower oil. Basal, Basal + α-tocopherol: see Table 4, footnote *a*. Basal + β-carotene: broilers were fed the basal diet supplemented with 15 mg β-carotene/kg of diet. For each dietary treatment $n = 8$ for raw and cooked meat.
[b]See footnote *b* in Table 3 for statistical treatment and abbreviations. Different superscript roman letters (a,b) indicate significant differences ($P < 0.05$).

TABLE 6
Experiment IV (olive oil with or without antioxidants): Cholesterol Oxides in Raw and Cooked Broiler Meat[a]

	Raw meat[b]			Cooked meat[b]			
	Basal	Basal + β-carotene	Basal + α-tocopherol	Basal	Basal + β-carotene	Basal + α-tocopherol	SE
7α-Hydroxycholesterol	0.10	0.04	0.08	0.55	0.14	0.56	0.16
7β-Hydroxycholesterol	0.02	n.d.	0.04	0.35	0.36	0.59	0.14
β-Epoxycholesterol	0.03	0.01	0.15	0.31	0.32	0.61	0.12
α-Epoxycholesterol	n.d.	n.d.	0.05	0.03	0.07	0.17	0.04
20α-Hydroxycholesterol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—
Triol	n.d.	n.d.	n.d.	0.03	n.d.	n.d.	0.01
25-Hydroxycholesterol	n.d.	n.d.	n.d.	0.01	n.d.	n.d.	0.005
7-Ketocholesterol	0.02	n.d.	0.01	0.54	0.29	0.55	0.14
Total oxysterols	0.12[a]	0.05[a]	0.32[a]	1.82[a]	1.18[a]	2.47[a]	0.50

[a]Broiler chickens were fed a diet containing olive oil. Basal, Basal + α-tocopherol, Basal + β-carotene: for details of these experiments, see footnote *a* in Table 5.
[b]See footnote *b* in Table 3 for statistical treatment and abbreviations. Superscript roman letter (a) indicates significant difference ($P < 0.05$).

contribute to the overestimation of results. Furthermore, the amounts of cholesterol oxides obtained in raw meat agreed with those reported by Zubillaga and Maerker (30). Artifact production was minimized by taking strict precautions such as using butylated hydroxytoluene (0.002%) during the extraction procedure, avoiding elevated temperatures (>40°C) during sample handling, and storing at low temperatures for very short periods. The Folch extracts were stored at –40°C for less than 48 h. After the solid-phase extraction step, the purified extract is unstable and more susceptible to oxidation due to the loss of BHT during the solid-phase extraction. The purified extract obtained after this extraction was stored overnight at –40°C.

Cholesterol in raw and cooked meat. The cholesterol levels in raw and cooked meat are listed in the Table 8. Within each experiment, cholesterol concentrations in raw meat did not significantly differ between dietary treatments. On the other hand, raw meat in the experiments with sunflower and olive oil gave a significantly reduced cholesterol content when compared with the experiment using lard (Table 8). Values ranging from 63.31 to 65.20 mg/100 g of muscle tissue and from 60.99 to 70.83 mg/100 g of muscle tissue were measured for the two experiments with lard. Cholesterol values from 55.00 to 57.48 mg/100 g of muscle tissue and from 49.10 to 51.10 mg/100 g of muscle tissue were measured for the experiments with sunflower and olive oils, respectively.

TABLE 7
Frequency of Oxysterol Occurrence Within Each Experiment and for Each Dietary Treatment[a]

	Experiment II[b]			Experiment III[b]			Experiment IV[b]		
	Basal	Basal + α-tocopherol	Basal + β-carotene	Basal	Basal + α-tocopherol	Basal + β-carotene	Basal	Basal + α-tocopherol	Basal + β-carotene
	Raw meat								
7α-Hydroxycholesterol	0.50	0.33	0.42	0.63	0.50	0.75	0.25	0.25	0.38
7β-Hydroxycholesterol	0.17	0.00	0.25	0.63	0.13	0.13	0.25	0.00	0.25
β-Epoxycholesterol	0.42	0.42	0.42	0.50	0.13	0.00	0.25	0.13	0.63
α-Epoxycholesterol	0.25	0.17	0.17	0.38	0.13	0.00	0.00	0.00	0.25
20α-Hydroxycholesterol	0.00	0.00	0.00	0.13	0.13	0.25	0.00	0.00	0.00
Triol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25-Hydroxycholesterol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7-Ketocholesterol	0.33	0.00	0.33	0.75	0.25	0.38	0.25	0.00	0.13
	Cooked meat								
7α-Hydroxycholesterol	0.75	0.75	0.88	1.00	1.00	1.00	0.75	0.50	0.88
7β-Hydroxycholesterol	0.88	0.38	0.75	1.00	1.00	1.00	0.75	0.75	0.75
β-Epoxycholesterol	0.88	0.50	0.75	0.88	0.88	0.88	0.75	0.75	1.00
α-Epoxycholesterol	0.75	0.17	0.38	1.00	1.00	1.00	0.25	0.38	0.50
20α-Hydroxycholesterol	0.17	0.00	0.25	0.00	0.00	0.25	0.00	0.00	0.00
Triol	0.08	0.00	0.13	0.00	0.00	0.00	0.13	0.00	0.00
25-Hydroxycholesterol	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00
7-Ketocholesterol	0.88	0.17	0.75	1.00	1.00	1.00	1.00	0.75	1.00

[a]Frequency was calculated as follows: (number of samples wherein the given oxysterol was detected)/(total number of samples).
[b]For experimental parameters and abbreviations, see Tables 2–6.

TABLE 8
Effect of Fat Quality on the Total Amount of Oxysterol, the Ratio of Total Amount of Oxysterol to Cholesterol, and Cholesterol Levels[a]

	Groups only fed the basal diet				Groups fed basal diet plus α-tocopherol				Groups fed basal diet plus β-carotene			
	Exp. I[b]	Exp. II	Exp. III	Exp. IV	Exp. II	Exp. III	Exp. IV	Exp. I	Exp. II	Exp. III	Exp. IV
Total oxysterols (raw meat)	0.02 ± 0.16^{a}	0.37 ± 0.12^{a,b}	0.53 ± 0.14^{b}	0.12 ± 0.14^{a}	0.17 ± 0.05^{a,b}	0.22 ± 0.06^{b}	0.05 ± 0.06^{a}	0.11 ± 14^{a}	0.37 ± 0.10^{a}	0.27 ± 0.12^{a}	0.32 ± 0.12^{a}
Total oxysterols (cooked meat)	2.58 ± 0.80^{a}	3.70 ± .0.69^{a,b}	4.62 ± 0.69^{b}	1.82 ± 0.69^{a}	0.96 ± 0.53^{a}	2.58 ± 0.53^{b}	1.18 ± 0.53^{a}	2.86 ± 0.82^{a,b}	2.52 ± 0.75^{a}	4.63 ± 0.75^{b}	2.47 ± 0.75^{a}
Cholesterol (raw meat)	65.20 ± 3.06^{a}	60.99 ± 2.16^{a}	55.88 ± 2.65^{b}	51.10 ± 2.65^{b}	64.25 ± 2.54^{a}	55.00 ± 3.11^{b}	49.10 ± 3.11^{b}	63.31 ± 4.27^{a,c}	70.83 ± 3.01^{a}	57.48 ± 3.70^{b,c}	49.96 ± 4.01^{b}
Cholesterol (cooked meat)	92.50 ± 4.03	93.21 ± 3.49	79.16 ± 3.49	77.48 ± 3.49	91.94 ± 3.96	86.51 ± 3.96	79.60 ± 3.96	91.27 ± 3.63	93.27 ± 3.14	86.98 ± 3.14	74.01 ± 3.14
Ox/chol (raw meat)	0.003 ± 0.03	0.06 ± 0.02	0.10 ± 0.03	0.02 ± 0.03	0.03 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.06 ± 0.02
Ox/chol (cooked meat)	0.28 ± 0.09	0.39 ± 0.08	0.59 ± 0.08	0.24 ± 0.08	0.11 ± 0.07	0.30 ± 0.07	0.17 ± 0.07	0.32 ± 0.10	0.28 ± 0.09	0.54 ± 0.09	0.35 ± 0.09

[a]Results (LS mean ± SE) are expressed in mg/100 g tissue for cholesterol and in µg/g for total oxysterols. Ox/chol, total oxysterols/cholesterol, expressed in percentages.
[b]For experimental parameters and abbreviations, see Tables 2–6. Different superscript roman letters (a,b,c) indicate significant differences between columns ($P < 0.05$).

These results showed that dietary unsaturated oils decreased tissue cholesterol levels. The increase in the cholesterol content of cooked meat could be explained by the loss of moisture (Table 8, lines 3 and 4; Ref. 18).

DISCUSSION

Oxidation can occur in the A and B cholesterol rings and also in the cholesterol side chain (8). In this study, B-ring cholesterol oxides, i.e., the 7α- and 7β-hydroxycholesterol, the 7-ketocholesterol, and the α- and the β-epoxide, were the main ones detected in raw and cooked meat. The presence of these oxysterols in raw meat at low concentrations and low frequency might result from mild lipid oxidation occurring during the period between slaughter and the vacuum packaging of thigh samples. Monahan *et al*. (22) also observed minimal cholesterol oxidation in raw pork stored at −20ºC. Lipid oxidation was reported not to be totally inhibited at −20ºC, and the storage of chicken thigh meat at −20ºC during 150 d minimally increased thiobarbituric acid values (22,31). The oxidation process principally affects the polyunsaturated fatty acids of the muscle membrane phospholipids (32). In raw meat, initiation can be caused by generation of hydroxyl radicals (·OH) or a myoglobin-derived radical (5,6,33). This latter compound is the major catalyst of lipid oxidation in raw chicken meat (31). Autoxidation of oxymyoglobin occurs at −20ºC (34). The generation of alkoxy and peroxy radicals from lipid hydroperoxides in the presence of ferrous and ferric ions can lead to the formation of 7α- and 7β-cholesterol hydroperoxides (35,36). The C7-oxysterols detected in raw meat could be the result of the thermal decomposition of cholesterol hydroperoxides in the gas chromatograph system (37). The predominance of 7α-hydroxycholesterol in raw meat is not consistent with radical-mediated cholesterol oxidation, suggesting another origin. The predominance of 7α-hydroxycholesterol over its β-isomer was reported earlier (8,38). Cooking increases lipid oxidation, which leads to the formation of characteristic flavors (7). This study showed that cooking increased the concentration of oxysterols by a factor of 10 compared with raw meat. B-Ring cholesterol oxides were produced in essentially all the samples (Table 7). An increase in cholesterol oxide production is likely the consequence of accelerated lipid oxidation (23). At high temperatures, the 7α- and the 7β-cholesterol hydroperoxides resulting from radical attack on cholesterol decompose into their respective alcohols and, by dehydration, yield the 7-ketocholesterol. Recently, Nielsen *et al.* (39) showed that 7-ketocholesterol can also be produced by free radical-induced dehydrogenation of the 7-alcohols. The 7α-cholesterol hydroperoxide epimerizes to the quasi-equatorial substituent at high temperatures (40). Epimerization of the 7α-hydroxycholesterol to the 7β–derivative is also possible (8). The predominance of the 7β-isomer observed in the majority of the cases (Table 9) accounts for radical-mediated cholesterol oxidation (8). Cholesterol epoxidation was marked by β-stereoselectivity (Table 9). The increased epoxidation could be ex-

TABLE 9
Ratios and Sums of Cholesterol Oxides (epoxides and C7-oxysterols)[a]

	Experiment I[b]		Experiment II[b]			Experiment III[b]			Experiment IV[b]		
	Basal	Basal + β-carotene	Basal	Basal + α-tocopherol	Basal + β-carotene	Basal	Basal + α-tocopherol	Basal + β-carotene	Basal	Basal + α-tocopherol	Basal + β-carotene
Sums											
Epoxides (raw meat)	—	0.03	0.10	0.11	0.10	0.12	0.02	0.05	0.03	0.01	0.15
Epoxides (cooked meat)	0.85	0.82	0.84	0.27	0.40	1.23	0.71	1.23	0.34	0.39	0.78
7 Hydroxy + keto (raw meat)	0.02	0.08	0.15	0.06	0.17	0.32	0.15	0.18	0.12	0.04	0.12
7 Hydroxy + keto (cooked meat)	1.75	2.02	2.74	0.70	1.84	3.38	1.88	3.38	1.44	0.79	1.70
Ratios											
β/α-Epoxides (cooked meat)	6.7	3.1	2.8	3.5	2.1	2.1	2.4	1.4	10.3	4.6	3.6
7β/7α (cooked meat)	1.7	1.7	1.4	0.3	0.6	1.7	1.7	0.8	0.6	2.6	1.1

[a]7 Hydroxy: 7-hydroxyisomers; keto, 7-ketocholesterol; 7α, 7α-hydroxycholesterol; 7β, 7β-hydroxycholesterol. Results expressed in μg/g muscle tissue.
[b]For experimental parameters and abbreviations, see Tables 2–6.

plained by exposing cholesterol to increasing amounts of H_2O_2 and lipid hydroperoxides, as evidenced by α/β epoxide ratios ranging from 1:3 to 1:10 and favoring the β-isomer (Table 9 and Refs. 35,41,42). β-Epoxycholesterol is more labile than its α isomer, and that could account for some reduced ratios at high temperature and acidic pH (Table 8) (42,43). Sevanian and McLeod (35) suggested that the attack on cholesterol by peroxyl radical, originated from the polyunsaturated fatty acids of phospholipids, involves preferentially the β-face (35). Moreover, the cholesterol hydroperoxides are cholesterol-epoxidizing agents (44). The hydration of both epoxides gives the triol which was detected in only a few samples in this study (8).

Diet influences the composition of the muscle membrane fatty acids (20). Lin *et al.* (20) reported that neutral lipids are more affected than polar ones and that the ratio of unsaturated to saturated membrane fatty acids increased with dietary fat unsaturation. Cholesterol oxidation increases with the unsaturation of liposomal membrane fatty acids (35). Different dietary fat unsaturation had no effect on cholesterol oxidative stability in raw meat (Table 8). On the other hand, in cooked meat the highest production of oxysterols in the sunflower oil-based diet is probably caused by an higher proportion of polyunsaturated fatty acids in the muscle membrane. The lower unsaturation of lard and olive oil could account for less cholesterol oxidation observed in meat from those groups (Table 8). Lin *et al.* (20) reported that meat from broiler chickens fed olive oil had a better oxidative stability during refrigerated and frozen storage. This was related to the accumulation in muscle tissue of oleic acid, which is less susceptible to oxidation.

Although differences in the effects of natural antioxidants were not significant in raw meat, the groups supplemented with α-tocopherol showed reduced amounts of cholesterol oxides in cooked meat, suggesting a protective effect (Table 8 and Refs. 20,21). Supplemental α-tocopherol increased the lipid oxidative stability and, as a consequence, reduced the production of oxysterols during cooking. In the sunflower oil-based diet, in spite of the beneficial action of the α-tocopherol, the production of cholesterol oxides remained high during cooking and was comparable to the levels detected for the control groups on the lard- and olive oil-based diets (Table 8). The high unsaturation level of the sunflower oil diet reduced the antioxidative action of the α-tocopherol administered at 200 mg/kg of diet. Besides a low extent of unsaturation, olive oil also contains polyphenols that are potent antioxidants (45). The possible presence of these molecules in muscle tissues could have contributed to the better cholesterol oxidative stability observed for the three dietary groups fed the olive oil (Tables 6 and 8).

In raw meat, the groups supplemented with β-carotene showed similar and sometimes higher oxysterols values when compared with their respective control group, indicating no protective action against cholesterol oxidation or possibly a prooxidant effect (Tables 3–6). Increasing the levels of supplemental β-carotene also did not influence the total oxysterol levels in cooked meat (Table 8). King *et al.* (24) also observed the lack of effect of supplemental β-carotene and found that it was not as well absorbed as α-tocopherol and acted as a prooxidant at 25 mg/kg of diet, as indicated by thiobarbituric acid-reactive substance values in nonheated meat, but not in heated meat. No studies on the effects of dietary β-carotene on cholesterol oxidation have been reported in the literature. Another finding from this study is that the unsaturation grade of the diet significantly decreases the cholesterol levels in the muscle tissues, the lowest levels being achieved with the olive oil-based diet (Table 8). The administration of α-tocopherol and β-carotene did not influence the cholesterol levels within each dietary group.

In conclusion, the present study demonstrated that cooking increased the content of B-ring oxysterols in broiler meat to concentrations as high as 5 μg/g. Cholesterol oxidation was greatly influenced by dietary fat unsaturation. A decrease in the cholesterol content of muscle tissue was also observed when the broilers were fed diets containing unsaturated fats. The dietary administration of α-tocopherol at 200 mg/kg of

diet increased the cholesterol oxidative stability, whereas β-carotene at 15 and 50 mg/kg of diet did not manifest any protective effect. Oxysterols have been shown to be absorbed from the diet and accumulate in LDL and could contribute to the atherogenic properties of oxidized LDL (9–11,46). In the United States, studies have estimated a mean intake of 20 g of poultry meat per day and a consumption of 216 g of cooked red meat per day for heavy meat consumers (47,48). The present work shows that a meat less likely to generate oxysterols during cooking was obtained by the combined use of dietary olive oil and α-tocopherol in poultry feed. Decreased dietary ingestion of oxysterols may reduce their accumulation in human LDL and limit their possible pathological action on the arterial wall (49).

ACKNOWLEDGMENTS

This work was carried out within the framework of the European concerted action DIET-OX (AIR project No. PL 921577): Dietary Treatment and Oxidative Stability of Muscles and Meat Products. Nutritive Value, Sensory Quality and Safety. C. Maraschiello was holder of an EC AIR grant (AIR1-CT-94-5025). We are grateful to Drs. Pere Gou and Luis Guerrero for their advice on the statistical analysis.

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[Received August 11, 1997; and in final revised form and accepted May 18, 1998]

Effects of α- and γ-Tocopherols on the Autooxidation of Purified Sunflower Triacylglycerols

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ABSTRACT: The antioxidant effects of α- and γ-tocopherols were evaluated in a model system based on the autooxidation of purified sunflower oil (p-SFO) triacylglycerols at 55°C for 7 d. Both tocopherols were found to cause more than 90% reduction in peroxide value when present at concentrations >20 ppm. α-Tocopherol was a better antioxidant than γ-tocopherol at concentrations ≤40 ppm but a worse antioxidant at concentrations >200 ppm. Neither α- nor γ-tocopherol showed a prooxidant effect at concentrations as high as 2000 ppm. The amount of tocopherols consumed during the course of oxidation was positively correlated to the initial concentration of tocopherols, and the correlation was stronger for α- than for γ-tocopherol. This correlation suggested that, besides reactions with peroxyl radicals, destruction of tocopherols may be attributed to unknown side reactions. Addition of $FeSO_4$, as a prooxidant, caused a 12% increase in the peroxide value of p-SFO in the absence of tocopherols. When tocopherols were added together with $FeSO_4$, some increase in peroxide value was observed for samples containing 200, 600 or 1000 ppm of α- but not γ-tocopherol. The addition of $FeSO_4$, however, caused an increase in the amount of α- and γ-tocopherols destroyed and led to stronger positive correlations between the amount of tocopherols destroyed during oxidation and initial concentration of tocopherols. No synergistic or antagonistic interaction was observed when α- and γ-tocopherols were added together to autooxidizing p-SFO.
Lipids 33, 715–722 (1998).

Lipid oxidation is one of the most detrimental quality parameters for lipids rich in polyunsaturated fatty acids (PUFA). Vegetable oils have different susceptibilities to oxidative degradation due to differences in fatty acid composition and in their contents of known and unknown antioxidant/prooxidant components. α- and γ-Tocopherols are the major natural antioxidants in most vegetable oils acting, *via* numerous biochemical and biophysical mechanisms, as efficient scavengers of active oxygen species and free radicals (1). Although it is well accepted that tocopherols act as antioxidants mainly by inhibiting free radical propagation *via* donation of phenolic hydrogens to peroxyl radicals, the absolute and relative antioxidant potency of the different tocopherols varies depending on temperature, composition of lipids, concentration of tocols used, and experimental setup (1–3).

The chemical structures of α-tocopherol (5,7,8-trimethyl tocol) and γ-tocopherol (7,8-dimethyl tocol) support a hydrogen-donating power in the order α > γ (1,4), but a reversed order (γ > α) of antioxidant activity was obtained when the two tocopherols were compared in various lipid systems (2,5–11). The relatively low potency of α-tocopherol, compared to γ-tocopherol, was attributed to possible participation of its tocopheroxyl radical in a number of propagative reactions (reviewed in Ref. 1). We tested the effects of α- and γ-tocopherols (at concentrations of 0–2000 ppm) on the development of peroxides in purified sunflower oil (SFO) and rapeseed oil (RSO) (containing no tocopherols, no iron or copper, and no pigments) after incubation at 55°C for 1–7 d, but neither tocopherol showed any prooxidant effect even at the very high concentrations used (12). These experiments suggested that α-tocopherol is not a prooxidant *per se* but may act as a prooxidant synergist (or co-prooxidant) when present at high concentrations together with known prooxidants such as transition metal ions, lipid peroxides, or other oxidizing agents.

In this paper, we report results from an investigation on the antioxidant activities of α- and γ-tocopherols against peroxidation of purified sunflower oil (p-SFO) triacylglycerols. p-SFO, free from anti- and prooxidant species that would influence the results of tocopherol action, was used because it is a rich source of the highly oxidizing linoleic (*cis*-9, *cis*-12-octadecadienoic) acid and was prepared by alumina column chromatography of commercial SFO. The effects of 1–2000 ppm of α- and γ-tocopherols on the stability of p-SFO were investigated using peroxide value (PV) measurements. Selected α- and γ-tocopherol concentrations (200, 600, and 1000 ppm) were used to study the effects of inclusion of ferrous sulfate on the antioxidant performance of the two tocols. Differences in antioxidant potency between individually added α- and γ-tocopherols and their mixtures were also investigated.

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Abbreviations: BHT, butylated hydroxytoluene; HOSO, high-oleic sunflower oil; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusive chromatography; PF, protection factor; p-HOSO, purified high-oleic sunflower oil; p-RSO, purified rapeseed oil; p-SFO, purified sunflower oil; PUFA, polyunsaturated fatty acids; PV, peroxide value; RSO, rapeseed oil; SFO, sunflower oil; TO•, tocopheroxyl radical.

MATERIALS AND METHODS

Materials and reagents. A commercial sample of SFO was used for the study of effects of tocopherols. A sample of high-oleic sunflower oil (HOSO), obtained as a gift from Dr. Maha Misbah (Unilever Research, Vlaardingen, The Netherlands), and a commercial sample of RSO were used, together with SFO, for an intercomparison study on the effects of oil purification on its stability. The fatty acid composition, calculated iodine values, and α- and γ-tocopherol levels of these oils are described in Table 1. Alumina used for column chromatography (neutral Al_2O_3, 70–120 mesh, Art. 1077) and tocopherol standards (isomer kit, Art. 15496) were purchased from Merck (Darmstadt, Germany). According to the manufacturer, the tocopherols were from the *d*-form (RRR) and were 95% pure [by high-performance liquid chromatography (HPLC)]. Sea sand and ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were from Sigma Chemical Co. (St. Louis, MO). All other solvents and chemicals, of reagent grade or better quality, were obtained from local suppliers and were used without further purification.

Oil purification. SFO, HOSO, and RSO were purified from antioxidants and from trace metals and other prooxidants *via* adsorption chromatography to yield p-SFO, purified HOSO (p-HOSO), and purified RSO (p-RSO) triacylglycerol fractions. A glass column (40 × 2.5 cm i.d.), plugged with glass wool, was packed with 250 g of alumina (activated at 100°C for 8 h and then at 200°C for 12 h) suspended in *n*-hexane, capped with sea sand, and conditioned by prewashing with 200 mL of *n*-hexane. The oil (100 mL) was dissolved in an equal volume of hexane and passed through the column, which was then washed with 200 mL of *n*-hexane. The chromatographic column was wrapped with aluminium foil to prevent light-induced oxidations during the purification process, and triacylglycerols were collected in an aluminum foil-wrapped flask. Analysis of the purified oils by thin-layer chromatography (Merck precoated silica gel 60 thin-layer chromatographic plates, 0.25 mm layer thickness and chloroform/diethyl ether; 90:10, vol/vol) showed that they are composed mainly of triacylglycerols together with minor amounts of sterol esters (results not shown). The purified oils were found to contain undetectable amounts of tocopherols (HPLC, <0.5 ppm), peroxides (FeSCN colorimetric, <0.6 mequiv O_2/kg oil) and iron and copper (atomic absorption spectroscopy, <0.01 and <0.001 ppm, respectively). The method is especially easy and fast (1 h) and gives a good recovery (*ca.* 80%) of triacylglycerols that are practically free of all minor components except for sterol esters.

TABLE 1
Fatty Acid Composition, Calculated Iodine Values, and Tocopherol Levels of the Oils Used in This Study

	Sunflower oil (SFO)	Rapeseed oil (RSO)	High-oleic sunflower oil (HOSO)
Fatty acids (relative wt%)[a]			
Palmitic (16:0)	6.4	5.2	4.0
Stearic (18:0)	4.1	2.0	2.3
Oleic (18:1)	25.2	60.8	81.5
Linoleic (18:2)	62.7	22.4	10.1
Linolenic (18:3)	0.1	6.9	0.1
Others[b]	1.5	2.7	2.0
Calculated iodine value[c]	130.5	109.1	87.8
Tocopherol levels (ppm)[d]			
α-Tocopherol	526	224	445
γ-Tocopherol	7	341	20

[a]Fatty acids were analyzed as methyl ester derivatives by gas chromatography: column, NB-351 (25 m × 0.32 mm i.d., 0.2 mm film, Nordion Ltd., Helsinki, Finland), column temperature: 160°C (2 min), 4°C/min, 240°C (5 min); injector temperature: 240°C; and flame ionization detector temperature: 260°C.
[b]Other minor fatty acids include: 14:0, 16:1, 20:0, 20:1, 22:0, 22:1, 24:0, and 24:1.
[c]Iodine values were calculated according to the following AOCS (13) formula: iodine value = % 18:1 × 0.860 + % 18:2 × 1.732 + % 18:3 × 2.616.
[d]Tocopherols were analyzed by high-performance liquid chromatography as explained in the Materials and Methods section.

Autooxidation experiments and stability measurements. Different oxidation experiments were performed. First the stabilities of samples of SFO, HOSO, RSO and their purified triacylglycerol fractions (p-SFO, p-HOSO and p-RSO, respectively) were compared. Thereafter, p-SFO was chosen for the following experiments: (i) studies of antioxidant effects of α- and γ-tocopherols at 1, 2, 4, 7, 20, 40, 70, 100, 200, 400, 700, 1000, 1500, and 2000 ppm levels, (ii) the effects of inclusion of 50 ppm of ferrous ion on the antioxidant performance of α- and γ-tocopherols used at 200, 600, and 1000 ppm levels, and (iii) the differences in the antioxidant effect between individual tocopherols and of mixtures thereof. Ferrous sulfate was added to the purified oils as solution in ethanol, and ethanol was later removed by evaporation under vacuum. Tocopherols were added to the oil-containing vials (*vide infra*) as solutions in hexane, and hexane was evaporated under a stream of nitrogen.

Portions of oil samples (*ca.* 1 g) were oxidized in 6-mL borosilicate glass vials (40 × 22 mm; Chromacol Ltd., London, United Kingdom) at 55°C in a thermostated oven in the dark. Vials were taken from the oven and cooled in a dessicator before samples were taken for analysis. Experiments were performed twice, and each sample was incubated and analyzed in duplicate. The stability of the oxidized samples was evaluated by measuring PV by a modified ferric thiocyanate method (14). The protection factor (PF) was calculated for different α- and γ-tocopherol concentrations as follows:

$$PF = (PV_{control} - PV_{sample})/PV_{control} \quad [1]$$

Analysis of residual tocopherols. The amount of residual α- and γ-tocopherols in thermooxidized oils was quantified by HPLC analysis on a Hibar pre-packed LiChrosorb NH_2 column (25 cm × 4 mm i.d., particle size 5 mm; Merck). The mobile phase was *n*-heptane/methyl *tert*-butyl ether/tetrahydrofuran/methanol (79:20:1:0.1, by vol) at a flow rate of 1.0 mL/min. The system consisted of an HPLC pump (Pharmacia

LKB Biotechnology, Uppsala, Sweden), a 10-μL injection loop, and a Merck F-1050 fluorescence spectrophotometer. The peaks were detected at an excitation wavelength of 295 nm and an emission wavelength of 320 nm. Peaks were recorded and integrated using the JCL 6000 chromatography data system (Jones Chromatography, Mid-Glamorgan, United Kingdom) and the amounts of α- and γ-tocopherols were quantified against references used as external standards.

Analysis of the polymerized materials. To study the effects of α- and γ-tocopherols on polymerization of the oils after removal from the oven, the vials were allowed to stay in a dark cupboard for 4 wk. Then dimers and polymers were analyzed by high-performance size exclusion chromatography (HPSEC) essentially as described by Hopia *et al.* (15). Specifically, portions of the oxidized oils were dissolved in tetrahydrofuran to a concentration of *ca.* 20 mg/mL and analyzed in an HPSEC system consisting of an HPLC pump (Pharmacia LKB Biotechnology), a Rheodyne injector fitted with a 20-μL sample loop (Cotati, CA), and a Waters refractive index detector (Waters Associates, Milford, MA) thermostated to 35°C. The separation was performed on two (100- and 50-Å) columns (PLGEL 30 × 0.8 cm i.d.; Polymers Laboratories Inc., Amherst, MA) connected in series. The mobile phase was HPLC-grade tetrahydrofuran (Merck) stabilized with 0.025% butylated hydroxytoluene (BHT) and was used at a flow rate of 0.8 mL/min. Peaks were recorded and integrated using the JCL 6000 chromatography data system, and quantitation of each lipid class was based on peak areas assuming equal detector response.

Statistical analysis. Differences between different treatments were evaluated statistically by an analysis of variance procedure, the general linear model supported by the statistical analysis system (16). Statements in the text referred to as significant apply to a 5% significance level.

RESULTS AND DISCUSSION

The model system. To understand certain mechanistic parts of the lipid oxidation process, one should use simple predictive model systems. Under specified conditions, these models should be able to provide the user with a reasonable prediction of a specific problem. Important factors to be considered and perhaps controlled include: (i) relative levels and chemical nature of substrates, anti- and prooxidants, synergists and physicochemical modifiers (17), (ii) temperature, which acts directly by affecting rates of different reactions or indirectly by affecting relative solubility of various reactants, and (iii) oxygen tension as determined by surface-to-volume ratio and by solubility, which is influenced by temperature and viscosity of the reaction mixture.

Purified triacylglycerols are the most valid models to study oxidation of vegetable oils (18). Purified triacylglycerol fractions must be prepared from vegetable oils to provide the natural complexity of fatty acid distribution and to the eliminate anti- and prooxidant species normally present in the oils. Apart from having a similar fatty acid composition, purified triacylglycerols also exhibit a viscosity approximately similar to that of their oils of origin. The other important factors to be considered in autooxidation models are the reaction temperature and the dimensions of the reaction vessel. A temperature of 55°C was used to minimize thermal decomposition of hydroperoxides (19). The borosilicate glass vials used as reaction vessels enabled fair oxidation of control oils and were easy to use owing to their small size and their disposable nature.

Before the start of the actual experiments, the oxidative stabilities of p-SFO, p-RSO and p-HOSO triacylglycerols were compared to those of their unpurified precursors. Results on PV measurements after incubation of the six oils at 55°C for 7 d (Fig. 1) indicated that both the fatty acid composition (Table 1) and the nonglyceride components are important for oil stability. The importance of the degree of fatty acid unsaturation in the stability of these oils is obvious when comparing p-SFO, p-RSO, and p-HOSO triacylglycerols. The great difference in stability between the nonpurified oils and their purified fractions emphasizes the importance of nontriacylglycerol constituents as natural oxidation inhibitors in vegetable oils. Tocopherols are generally accepted as the major natural antioxidants in plant oils although other phenolic compounds, which may be more potent as antioxidants, are present in some oils, e.g., olive (20) and sesame (21).

p-SFO was chosen as a model substrate for this study for two main reasons: (i) SFO is one of the important sources of dietary linoleic acid, and results to be gained on its stabilization may find some nutritional applications; and (ii) owing to its higher iodine value (Table 1), p-SFO is much more susceptible to oxidation than p-RSO and p-HOSO (Fig. 1) and may therefore serve as a potential illustrative substrate for studies on structure–activity relationships among tocopherols.

Effects of α- and γ-tocopherols on peroxidation of p-SFO. Figure 2 shows a plot of the PF against the concentration (1–2000 ppm) of α- and γ-tocopherols in p-SFO. Results indicate that both α- and γ-tocopherols acted as very efficient antioxidants and almost completely inhibited the peroxida-

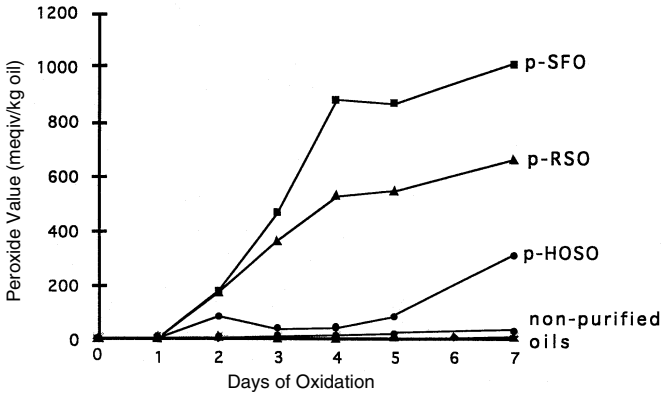

FIG. 1. The oxidative stability of purified (p-SFO, p-RSO, and p-HOSO) and nonpurified (SFO, RSO, and HOSO) sunflower, rapeseed and high-oleic sunflower oils, respectively. (For details on purification, see the Materials and Methods section.)

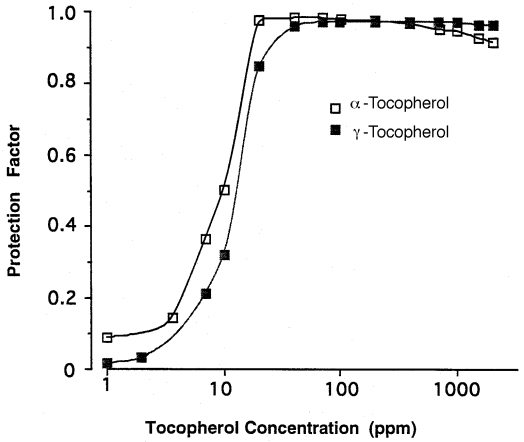

FIG. 2. Effects of adding α- and γ-tocopherols on the stability of p-SFO. Oil samples containing 1–2000 ppm of α- or γ-tocopherol were incubated in the dark at 55°C for 7 d. Peroxide values were measured, and a protection factor was calculated as explained in the Materials and Methods section. α-Tocopherol is significantly more effective than γ-tocopherol in the concentration range 1–20 ppm, and γ-tocopherol is significantly more effective than α-tocopherol in the concentration range 1000-–2000 ppm ($P < 0.05$). For abbreviation see Figure 1.

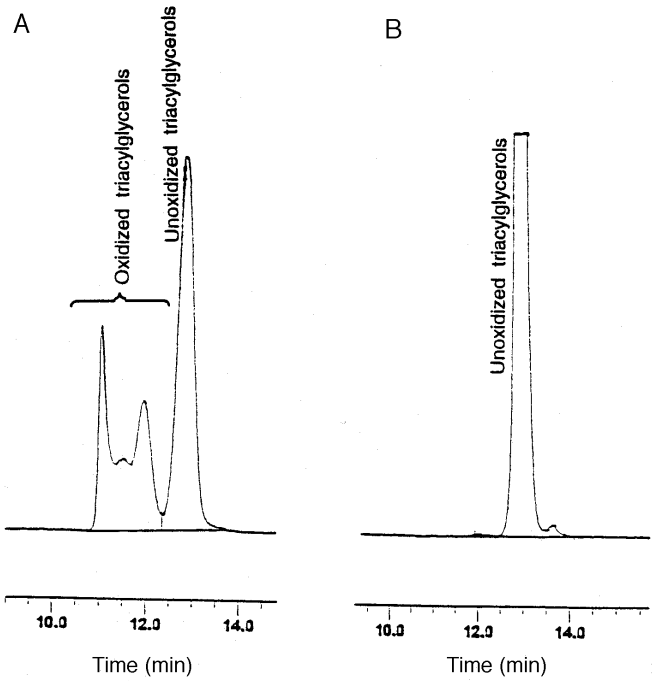

FIG. 3. Typical parts of high-performance size exclusion chromatograms showing formation of polymers, dimers, and oligomers in (A) control samples of p-SFO and (B) samples of p-SFO containing 500, 1000, or 2000 ppm of either α- or γ-tocopherols, upon incubation at 55°C for 7 d and then at room temperature for 4 wk. For abbreviation see Figure 1.

tion of p-SFO during the 7 d of oxidation when present at concentrations above 20 ppm (PF ≈ 1.00). These results are in accordance with previous findings that concentrations as low as 11 ppm of γ-tocopherol in p-RSO were enough to cause a marked inhibition of peroxide and secondary product formation (18). Further details on the differences between α- and γ-tocopherols can be summerized as follows: (i) α-tocopherol was significantly more effective than γ-tocopherol at low concentrations (1–20 ppm), (ii) both tocopherols were equally effective within the concentration range 40–200 ppm, and (iii) γ-tocopherol was sightly more effective than α-tocopherol at concentrations greater than 200 ppm and the difference became significant at concentrations higher than 1000 ppm.

The most interesting finding from this experiment is, however, the absence of any prooxidant effect for either α- or γ-tocopherols even at the very high concentrations used. Several investigators demonstrated that tocopherols (particularly α-tocopherol) act as prooxidants when present in high concentrations in autooxidizing lipids (e.g., 9, 22–24). Huang *et al.* (22), using stripped corn oil as substrate, and Terao and Matsushita (23), using methyl linoleate as substrate, described the prooxidant effect they observed for high concentrations of α-tocopherol as an "initial" prooxidant effect that was not observed at later stages of oxidation. To check if a prooxidant effect becomes evident after a longer period of oxidation, samples containing 500, 1000, and 2000 ppm tocopherols were stored in a dark cupboard for 4 wk after the 7 d of incubation in the oven. Analysis of dimers and polymers by HPSEC showed no signs of polymerization in samples containing either α- or γ-tocopherols, although the controls contained *ca.* 55% total dimers and polymers (Fig. 3). In conclusion, both α- and γ-tocopherols were very efficient inhibitors of PUFA peroxidation in the dark at 55°C for 7 d and afterward at room temperature for 4 wk, even when present at very high concentrations. That this conclusion is in contradiction with previous reports (9,22–24) may be attributed to differences in experimental conditions, interpretation of results, and possibly the absence of a standardized definition of a prooxidant.

Antioxidant effects of α- and γ-tocopherols in absence and presence of ferrous ions. The role of transition metal ions (such as as iron, copper, cobalt, nickel, manganese, etc.) in catalyzing peroxidation reactions in fats and oils is well documented (25–27) despite lack of precise knowledge of the mechanism(s) involved. In this experiment, PV of p-SFO controls containing 50 ppm of ferrous were *ca.* 12% higher than those of controls without this salt (PV ≈ 1200 mequiv/kg oil).

Figure 4A shows the PV of samples of p-SFO containing 200, 600, and 1000 ppm of α- and γ-tocopherols upon oxidation for 7 d in the presence or absence of 50 ppm of ferrous ions. γ-Tocopherol was slightly less effective as an antioxidant than α-tocopherol at 200 ppm but was significantly more effective at higher concentrations, in agreement with results from the previous experiment (Fig. 2). In addition, PV for samples containing α-tocopherol were significantly higher, in the order 200 < 600 < 1000 ppm, whereas differences in PV between samples containing 200, 600, or 1000 ppm of γ-tocopherol were minor. The presence of iron caused a significant enhancement of the perooxidation of p-SFO containing 600 or 1000 ppm of α-tocopherol and a very minor increase in the PV of p-SFO containing γ-tocopherol. The presence of ferrous sulfate significantly enhanced the rates of losses of both tocopherols (Fig. 4B), and the losses were much greater

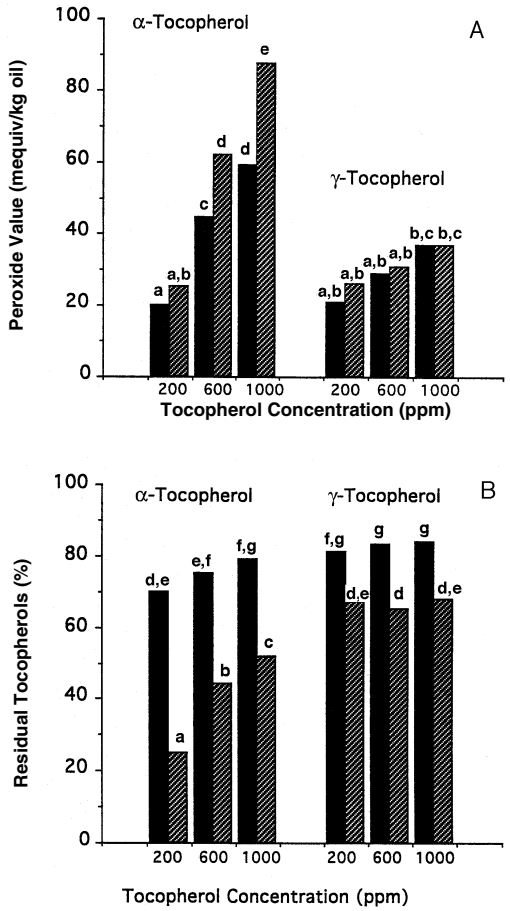

FIG. 4. Effects of concentration of added α- or γ-tocopherols (200, 600, or 1000 ppm) on (A) the peroxidation of p-SFO and (B) the consumption of tocopherols in the absence (solid bar) and presence (hatched bar) of of 50 ppm of ferrous ions as $FeSO_4 \cdot 7H_2O$. Bars marked by different letters (a–g) are significantly different ($P < 0.05$).

for α- than for γ-tocopherol. Data presented in Figure 5 clearly demonstrate that the amounts of tocopherols consumed have high positive correlations with the initial concentrations of tocopherols added. The correlations for both α- and γ-tocopherols were much higher in the presence of ferrous ($y = 29.86 + 0.470\ x$, $R^2 = 0.981$; and $y = 2.22 + 0.326\ x$, $R^2 = 0.998$ for α- and γ-tocopherols, respectively) than in its absence ($y = 11.61 + 0.211\ x$, $R^2 = 0.981$; and $y = 6.85 + 0.157\ x$, $R^2 = 0.991$ for α- and γ-tocopherols, respectively). Despite these iron-induced differences in peroxide formation and tocopherol consumption, both α- and γ-tocopherols were still very efficient antioxidants even when present at 1000 ppm in the presence of 50 ppm of ferrous ions.

The difference in antioxidant activity between α- and γ-tocopherols can be explained by considering the theory of "Antioxidant Strength and Efficiency" as explained and used by Marinova and Yanishlieva (28). The finding that α-tocopherol was consumed more quickly than γ-tocopherol (Fig. 4B) is in agreement with difference in redox potentials (+0.273, and +0.348 V, respectively, Ref. 29) which implies that α-tocopherol is a stronger hydrogen-donor and is more vulnerable to oxidation than γ-tocopherol. Hence, α-tocopherol can participate easily in side reactions (reactions other than those with peroxyl radicals) which might have negative effects on the lipid peroxidation rate at certain time points. Participation of α-tocopherol in side reactions makes it a less efficient antioxidant than its γ-homolog. The high positive correlations between the initial amount of tocopherols in the oxidized oils and the rates of tocopherol consumption (Fig. 5) support the assumption that tocopherols are consumed not only in antioxidation reactions but also in first-order side reactions, the rates of which are much greater for α- than for γ-tocopherols. Data showing a decrease in the effectiveness of α-tocopherol with

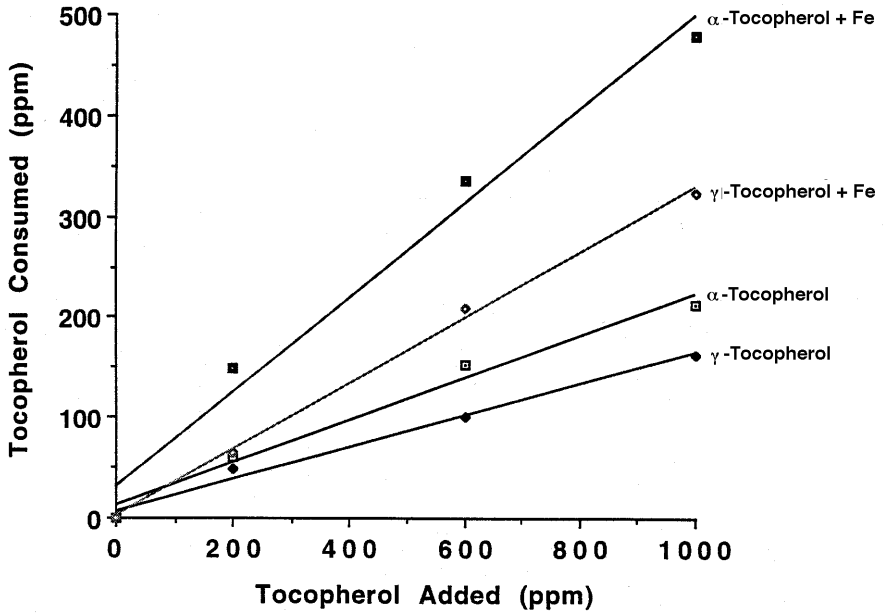

FIG. 5. Correlation between the concentration of added α- or γ-tocopherols (200, 600, or 1000 ppm) and the amount of tocopherols consumed in the absence and presence of ferrous sulfate heptahydrate.

increasing concentrations due to side reactions were also obtained and discussed by Yanishlieva and Marinova (30).

An evaluation of the importance of side reactions for α- and γ-tocopherol antioxidant activity can be achieved by considering the lipid oxidation reactions in samples containing or lacking these antioxidants (see Scheme 1, a theoretical representation of major oxidation pathways in systems composed of PUFA and tocopherols). When high tocopherol concentrations are still available, reactions given by Equations 2 and 9 will dominate over those given by Equations 3 and 5, resulting in inhibition of PUFA oxidation. The slight decrease in antioxidant efficiency observed for high α-tocopherol concentrations (1000–2000 ppm) is not yet explainable. It was suggested (see literature review in Ref. 1) that this effect is due to reactions 7 and 8 of tocopheroxyl radicals (TO·) with unperoxidized lipids and their hydroperoxides, respectively. Studies on the kinetics of the abstraction of hydrogen by the TO· from fatty acids or from lipid hydroperoxides showed that these reactions have significantly very low rates (in the order of some 10^{-1} $M^{-1}s^{-1}$) compared to reaction of α-tocopherol with peroxyl radicals (in the order of some 10^5–10^6 $M^{-1}s^{-1}$) and TO· coupling reactions (Eq. 13 of Scheme 1) (see literature review in Ref. 1). The availability of residual tocopherols at these points of oxidation provides further evidence that the previously mentioned mechanism cannot explain the decrease in antioxidant activity at high concentrations of α-tocopherol. This interpretation implies that a net prooxidant effect on the oxidation of PUFA will not be observed until all the tocopherol is consumed (Eqs. 8 and 10 of Scheme 1). The high positive correlation between tocopherol consumption and initial tocopherol concentration (Fig. 5) may be attributed to self-oxidation and formation of some prooxidant species by reaction with oxygen (10). It has been reported by Doba *et al.* (31) that oxygen reacts very slowly with TO·, and they suggested that oxygen has no effect of α-TO· decay. However, the relevance of the reaction of α-TO· radical with oxygen in our model cannot be evaluated and requires further investigations.

The relative importance of different tocopherol-consuming reactions seem to be affected markedly by their structure and concentration, degree of fatty acid unsaturation, availability of oxygen and its diffusion kinetics, temperature and presence of other antioxidants or prooxidants (e.g., metal salts). Although the presence of $FeSO_4$ did not cause the tocopherols to show any prooxidant effect in our model system, its inclusion enhanced the rate of peroxidation in samples containing higher concentrations of α-tocopherol and led to a very rapid consumption of both α- and γ-tocopherols. Many reviews (32–36) were published on the prooxidant effects of iron and other transition metal traces on PUFA peroxidation, but interactions between metals and tocopherols are not yet precisely understood. According to these reviews, low-valency-state metals are more effective prooxidants owing to either or both of following mechanisms: (i) they can generate, *via* formation of metal-oxygen transition complexes, active oxygen species (e.g., $O_2{\cdot}^-$ and $HO_2{\cdot}$):

$$O_2 + M^{n+} \rightarrow [M^{(n+1)+} \text{----} O_2) \rightarrow O_2{\cdot}^- + M^{(n+1)+} \quad [15]$$

(ii) they can generate radicals *via* degradation of hydroperoxides by Fenton and Fenton-like reactions:

$$AOOH + M^{n+} \rightarrow AO{\cdot} + OH^- + M^{(n+1)+} \quad [16]$$

$$LOOH + M^{n+} \rightarrow LO{\cdot} + OH^- + M^{(n+1)+} \quad [17]$$

It was previously suggested that free radical species generated by these reactions can destroy the tocopherols:

$$M^{n+1} + O_2{\cdot}^- + TOH \rightarrow TO{\cdot} + HO_2{\cdot} + M^{n+} \quad [18]$$

Moreover, tocopherols and TO· are able to reduce metal ions to lower state valences which will recycle reactions 15–17 (37–39):

$$TOH + M^{(n+1)+} \rightarrow TO{\cdot} + H^+ + Mn^{n+} \quad [19]$$

$$TO{\cdot} + M^{(n+1)+} \rightarrow TO^+ + Mn^{n+} \quad [20]$$

Reactions 19 and 20 may be responsible, at least in part, for the enhanced tocopherol destruction observed in this study. A comparison of the prooxidant effects of $FeSO_4$ and hemoglobin showed that organic forms of iron may be more active than inorganic forms as prooxidants (40). The prooxidant effects of trace metal ions are determined by their electronic configuration, valency state, and type of associated ligands (27). Thus, the form in which trace metal ions occur in vegetable oils and the mechanism by which they propagate oils' oxidation in the presence and absence of tocopherols and other minor constituents are important areas for further research.

Mixed vs. individual addition of α- and γ-tocopherols. Because vegetable oils contain a mixture of natural α- and γ-to-

Initiation	TOH + initiator → TO·	[2]
	LH + initiator → L·	[3]
Propagation	L· + O_2 → LOO·	[4]
	LOO· + LH → LOOH + L·	[5]
	TO· + O_2 → unknown products	[6]
	TO· + LH → TOH + L·	[7]
	TO· + LOOH → TOH + L·	[8]
Antioxidation	LOO· + TOH → LOOH + TO·	[9]
	LOO· + TO· → TO.OOL	[10]
Chain branching	LOOH → LO· + ·OH	[11]
	2 LOOH → LOO· + H_2O + LO·	[12]
Termination	TO· + TO· → TO.TO	[13]
	LO_2· + L· → LOOL	[14]

Abbreviations: TOH = tocopherol molecule, TO· = tocopheroxyl radical, LH = unsaturated fatty acid, L· = fatty acid alkyl radical, LOO· = fatty acid peroxyl radical, LOOH = fatty acid hydroperoxide, TO.OOL = tocopheroxy-fatty acid peroxyl adduct, LO· = fatty acid alkoxyl radical, OH·= hydroxyl radical, TO.TO = tocopherol dimer, LOOL = difatty acid peroxide.

SCHEME 1

copherols, one of the aims of this study was to investigate whether α- and γ-tocopherols will synergize or antagonize each other's effect when present in mixtures. The effects of mixing α- and γ-tocopherols on the stability of p-SFO were tested in a Latin-square design using combinations of 100, 300, and 500 ppm levels of each tocopherol (Table 2). Similar to the results obtained in the previous experiments, addition of α- and γ-tocopherols in mixtures to p-SFO triacylglycerols significantly inhibited their rate of peroxidation (PV = 16.4–57.4 mequiv O_2/kg oil, Table 2) compared to the controls (PV = 1004 mequiv O_2/kg oil). Figure 6A compares the protective effects of mixtures containing 100, 300, or 500 ppm concentrations of each tocol with those containing 200, 600, or 1000 ppm levels of either tocol alone. The mixture containing 100 ppm of α-tocopherol together with 100 ppm of γ-tocopherol was also more effective than 200 ppm of either α- or γ-tocopherol alone. The stability of mixtures containing 300 or 500 ppm of each tocol was intermediate between those containing γ-tocopherol alone or α-tocopherol alone at 600 or 1000 ppm levels, respectively. These results (Fig. 6A) also show that the mixture containing 100 ppm of α-tocopherol and 100 ppm of γ-tocopherol had the highest antioxidant activity among all other mixtures, and that higher concentrations of the mixed tocopherols are less effective in inhibiting peroxide formation than mixtures containing lower concentrations of mixed tocopherols. This finding suggests possible synergism between α- and γ-tocopherols when they are present at relatively low concentrations. Buttner (41) showed quite elegantly that antioxidants with large differences in redox potentials (e.g., α-tocopherol and ascorbate) show a pecking order, or hierarchy, for free radical reactions. Omura (42) showed that the BHT and butylated hydroxyanisole pair act synergistically because BHT can regenerate butylated hydroxyanisole from its radicals. Determination of residual tocopherols (Fig. 6B) showed that, although α-tocopherol was consumed more quickly than γ-tocopherol, γ-tocopherol co-oxidized with its α-homolog and that complete consumption of α-tocopherol was not a prerequiste for the oxidation of γ-tocopherol. Percentage losses of α-tocopherol were slightly higher in mixtures than in samples containing this tocol alone, and an opposite trend was observed for γ-tocopherol (Fig. 6B). This effect was, however, not enough to qualify α-tocopherol to act as a strong regenerator of its γ-homolog. Obviously, the hierarchy in the case of α- and γ-tocopherols is not significant to allow the type of synergism that was observed for BHT and BHA.

TABLE 2
Effect of Mixing Tocopherols on Peroxide Value[a] of p-SFO After 7 d of Oxidation

γ-Tocopherol concentration (ppm)	α-Tocopherol concentration (ppm)		
	100	300	500
100	16.4	30.4	38.1
300	28.1	35.4	51.1
500	28.2	46.8	57.4

[a]Expressed in milliequivalents O_2/kg oil. For abbreviation see Table 1.

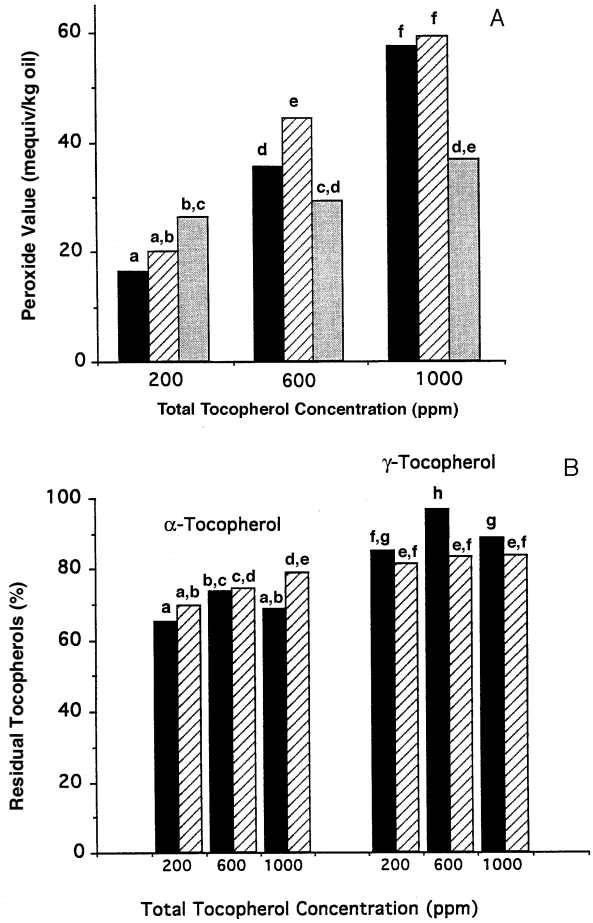

FIG. 6. Comparison between the effects of (A) mixed (solid bar) vs. individually added α-tocopherol (hatched bar) or γ-tocopherols (shaded bar) on peroxidation of p-SFO triacylglycerols; (B) mixed (solid bar) vs. individually added α- or γ-tocopherol (hatched bar) on residual tocopherols during the peroxidation of p-SFO. Bars marked by different letters (a–g) are significantly different ($P < 0.05$).

In conclusion the work conducted in this study revealed that γ-tocopherol is a more efficient antioxidant than α-tocopherol possibly because of the latter's ability to participate in reactions other than those with peroxyl radicals, especially in the presence of trace metal ions. It was further found that α- and γ-tocopherols may synergize each other when present in concentrations less than 200 ppm but not when present at higher concentrations. Interestingly, no prooxidant effect was observed for α-tocopherol at all concentrations tested (1–2000 ppm) and in the presence of ferrous ions. Work is in progress to study the differences between α- and γ-tocopherols in influencing the molecular nature of hydroperoxides and their degradation products using methyl linoleate model system.

ACKNOWLEDGMENTS

Maria Dolores Fuster thanks the Spanish Ministry of Education and Science (Madrid) for a 3-mon scholarship to conduct this research in Uppsala. From Unilever Research Laboratorium (Vlaardingen, The Netherlands), we thank Dr. Maha Misbah for a kind gift of HOSO

and Dr. C. W. van Oosten for suggesting alumina column chromatography for the preparation of purified natural triacylglycerols.

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[Received February 6, 1998, and in revised form May 11, 1998; revision accepted May 15, 1998]

Tanacetum (*Chrysanthemum*) *corymbosum* Seed Oil—A Rich Source of a Novel Conjugated Acetylenic Acid

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ABSTRACT: A new conjugated *trans,trans*-diunsaturated acetylenic acid (17%), found for the first time in nature as a main component of the seed oil of *Tanacetum (Chrysanthemum) corymbosum*, was shown to be octadeca-8*t*,10*t*-dien-12-ynoic acid. Another already known naturally occurring acetylenic acid, crepenynic acid (10%), was found also in the seed oil. The structure of this new unusual fatty acid was confirmed by chromatographic (thin-layer chromatography, gas chromatography, high-performance liquid chromatography) and spectroscopic (infrared, ultraviolet, mass spectrometry) methods by using different chemical derivatizations (deuteration, preparation of picolinyl ester, dimethyloxazoline, and maleic anhydride adduct).
Lipids 33, 723–727 (1998).

Seed oils of a large number of species of Asteraceae have been studied previously and found to contain a range of unusual fatty acids, e.g., Δ3 *trans* (1), Δ5 *cis* (2), Δ6 *cis* (3), conjugated (4,5), acetylenic (6,7), epoxy (8,9) and hydroxy (10) acids, and these have been reviewed elsewhere (11–14). *Tanacetum (Chrysanthemum)*, a genus of the Asteraceae, is less studied with respect to the fatty acid composition of the seed oils. Useful representatives of the genus *Chrysanthemum* are known. *Chrysanthemum parthenium* (feverfew) is an ornamental medicinal herb, believed to be antiseptic and used as an antidote, aperient, carminative, sedative, and vermifuge for hysteria and parturition. *Chrysanthemum cinerarifolium* (pyrethrum or Dalmatian insect flower) is cultivated for the dried inflorescences, which are used in the manufacture of insecticides and parasiticides (15). *Chrysanthemum coronarium* (16) has been reported to contain epoxy (vernolic and coronaric) acids as seed oil components.

In continuation of our exploration of the potential of less common oil seeds, fatty acids from seeds of *Tanacetum corymbosum*, an ornamental plant growing in Germany, were investigated by means of chromatographic and spectroscopic methods. Two unusual fatty acids were identified.

EXPERIMENTAL PROCEDURES

Seeds. Seeds of *T. (Chrysanthemum) corymbosum* were a generous gift of the Botanic Gardens (University of Münster, Münster, Germany).

Extraction of seed oil. The ground seeds were extracted at room temperature by grinding with a mortar and pestle in petroleum ether (boiling range, 40–60°C) containing 0.001% butylated hydroxytoluene (BHT).

Preparation of fatty acid methyl esters (FAME). Mixed FAME were prepared by transesterification of the seed oil with 2.0 M sodium methoxide in methanol (17).

Deuteration. Methyl esters of unsaturated fatty acids were subjected to deuteration with deuterium gas and Wilkinson's catalyst (18,19). The methyl ester (up to 2 mg) and Wilkinson's catalyst (5 mg) in dioxane (1 mL) were degassed with helium in a tube fitted with a Teflon-lined septum. The vessel was purged with 5 vol of deuterium with constant stirring and then was left with an atmosphere of deuterium at 60°C for 2 h. The solvent was removed in a stream of nitrogen, and the deuterated ester was obtained by adsorption chromatography on a small column of Florisil™ (0.5 g; Sigma Chemical Co., Poole, United Kingdom), eluted with hexane/acetone (96:4, vol/vol). It was converted to the picolinyl ester for analysis by gas chromatography–mass spectrometry (GC–MS).

Preparation of picolinyl esters. The free acids (1 mg), prepared by hydrolysis with 0.1 M ethanolic potassium hydroxide, were converted to the acid chlorides by reaction with oxalyl chloride (0.5 mL) at ambient temperature overnight. Excess reagent was removed in a stream of nitrogen, and the product reacted immediately with a solution (0.5 mL) of 3-hydroxymethylpyridine in dichloromethane [20 mg/mL; stored over beads of molecular sieve—type 4A (Fisher Scientific, Loughborough, United Kingdom)]. After 1 h at ambient temperature, the solvent was evaporated, the product taken up in isohexane (5 mL), and washed with water (2 × 2 mL). The hexane solution was dried over anhydrous sodium sulfate; then the solvent was evaporated. The product was dis-

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Abbreviations: BHT, butylated hydroxytoluene; DMOX, 4,4-*N,N*-dimethyloxazoline; ECL, equivalent chain length; FAME, fatty acid methyl ester; FTIR, Fourier transform infrared spectroscopy; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet spectroscopy.

solved in isohexane containing BHT (50 ppm) for analysis by GC–MS.

Preparation of dimethyloxazoline (DMOX) derivatives. DMOX derivatives were prepared by heating the free acid with 2-amino-2-methyl-1-propanol at 180°C for 2 h (20).

Preparation of maleic anhydride adduct. The mixed FAME (14 mg) of *T. corymbosum* were added to maleic anhydride (20 mg) in toluene and refluxed under nitrogen for 2 h. The solvent was removed by vacuum evaporation, and the residue was dissolved in *n*-hexane, washed with distilled water, and dried with anhydrous sodium sulfate (21).

Thin-layer chromatography (TLC). To obtain additional information on unusual components in oils, analytical TLC of oils and of FAME was carried out on a 0.25-mm layer of Silica gel G (Merck, Darmstadt, Germany), using a solvent system of *n*-hexane/diethyl ether [70:30, vol/vol (oil) or 80:20, vol/vol (FAME)]. Spots were visualized by spraying with phosphomolybdic acid (5% in ethanol) and heating at 100°C.

Capillary gas–liquid chromatography (GLC). Capillary GLC analysis of FAME was carried out using a Perkin-Elmer 8310 Gas Chromatograph equipped with a flame-ionization detector and a 30 m × 0.25 mm DB 23 capillary column (J&W Scientific, Folsom, CA) under the following conditions. The oven temperature was programmed to increase from 160 to 230°C at 2°C/min, and held at this point for 5 min before cooling down again to 160°C. The split/splitless injector temperature was 250°C, and the flame-ionization detector temperature was 270°C. Hydrogen was the carrier gas at a flow rate of 1.0 mL/min (head pressure 180 kPa H_2). Split injection was used with a split ratio of 50:1. Components were quantified by electronic integration. Identification of FAME was confirmed by comparison of equivalent chain lengths (ECL) with those of standards or of FAME from previously known seed oils.

GC–MS. The derivatives were submitted to GC–MS with a Hewlett-Packard 5890 Series II plus gas chromatograph (Palo Alto, CA) attached to an HP model 5989 MS Engine. The latter was used in the electron impact mode at 70 eV with a source temperature of 250°C. The GC was fitted with on-column injection. For picolinyl ester and DMOX derivatives, a capillary column of fused silica coated with Supelcowax 10^{TM} (25 m × 0.25 mm, 0.25 μm film; Supelco UK, Poole, United Kingdom) was used. After holding the temperature at 80°C for 3 min, the oven temperature was increased by temperature-programming at 20°C/min to 180°C, then at 2°C/min to 280°C, where it was held for 15 min. Helium was the carrier gas at a constant flow rate of 1 mL/min, maintained by electronic pressure control.

High-performance liquid chromatography (HPLC). An HPLC instrument was used that consisted of a Philips (Cambridge, United Kingdom) PU 4100 Gradient Pumping System, Philips PU 4026 Refractive Index Detector, Rheodyne 7125 Injector, and Gilson 201 Fraction Collector. Preparative separations of FAME (100–200 μg in 10 μL) were carried out on two Shandon Hypersil C_{18} columns (250 × 4 mm, 5 micron particles; Phase Separations Ltd., Macclesfield, United Kingdom) connected in series at ambient temperature. Methanol/water (90:10, vol/vol) at a flow rate of 1.0 mL/min was used as the mobile phase (22).

Ultraviolet (UV) absorption spectroscopy. UV spectra were determined from solutions of the seed oil in *n*-hexane, using a Philips UNICAM PU 8720 UV/VIS Scanning Spectrophotometer, with a focusing attachment, to confirm the presence of any conjugated fatty acids.

Fourier transform infrared spectroscopy (FTIR). FTIR spectra were determined from oil films on sodium chloride cells using a Perkin-Elmer PARAGON 1000 FTIR spectrometer (Beaconsfield, United Kingdom) with a focusing attachment to check the presence of any unusual (*trans* unsaturated or oxygenated) fatty acids.

RESULTS AND DISCUSSION

Extraction of the seeds of *T. corymbosum* with petroleum ether yielded 16.7% oil. TLC of the seed oil and of the mixed FAME did not reveal any oxygenated fatty acids. The fatty acid composition of the seed oil and the chromatographic sep-

TABLE 1
Fatty Acid Composition and GLC Separation Characteristics of Fatty Acid Methyl Esters in the Seed Oil of *Tanacetum corymbosum* (Asteraceae)

Fatty acid	GLC area (%)	RRT[a]	ECL on DB 23
16:0	4.2	1.0000	16.00
17:0	0.3	1.2618	16.9
18:0	1.6	1.5818	18.00
18:1Δ9*c*	4.5	1.6691	18.22
18:1Δ11*c*	0.6	1.6952	18.29
18:2Δ9*c*,12*c*	58.3	1.8836	18.76
18:3Δ9*c*,12*c*,15*c*	0.2	2.0964	19.30
20:0	0.3	2.3691	20.00
18:2Δ9*c*,12a	10.1	2.4636	20.20
18:3Δ8*t*,10*t*,12*a*	17.8	4.1164	23.62
Others	2.1		

[a]RRT, relative retention time; GLC, gas–liquid chromatography; ECL, equivalent chain length; DB23 (J&W Scientific, Folsom, CA); *c, cis; t, trans; a,* acetylene.

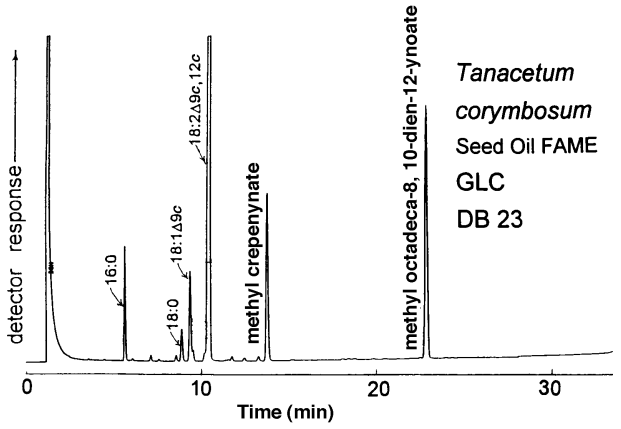

FIG. 1. Gas–liquid chromatogram (GLC) of total fatty acids of *Tanacetum corymbosum* seed oil separated on a DB 23 column (J&W Scientific, Folsom, CA) as their methyl esters.

aration characteristics on a DB 23 capillary column are presented in Table 1.

As can be seen from Table 1, GLC analysis of the methyl esters of mixed fatty acids of *T. corymbosum* seed oil revealed two unusual components along with more common fatty acids. The first unusual component (10.1%) had an ECL value of 20.20 on the DB 23 column. The second fatty ester (17.8%) had an ECL of 23.63. GLC separation of the FAME of *T. corymbosum* seed oil is illustrated in Figure 1. Tentative identification of the proposed methyl crepenynate in *T. corymbosum* seed oil was based on the appearance of a single peak, following coinjection into GLC with *Crepis sibirica* seed oil FAME, known to contain crepenynic acid, and this was subsequently confirmed by GC–MS.

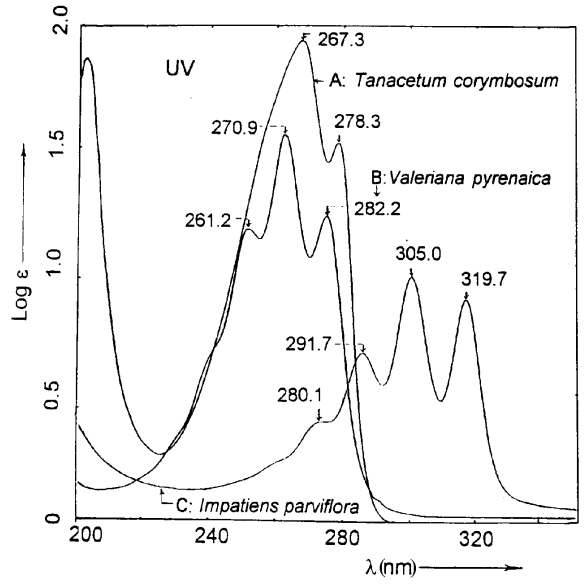

FIG. 2. Ultraviolet (UV) spectra of the seed oils. A: *T. corymbosum* (λ_{max} = 267.3; 278.3 nm), B: *Valeriana pyrenaica* (λ_{max} = 261.2; 270.9; 282.2 nm) and C: *Impatiens parviflora* (λ_{max} = 280.1; 291.7; 305.0; 319.7 nm). See Figure 1 for other abbreviation.

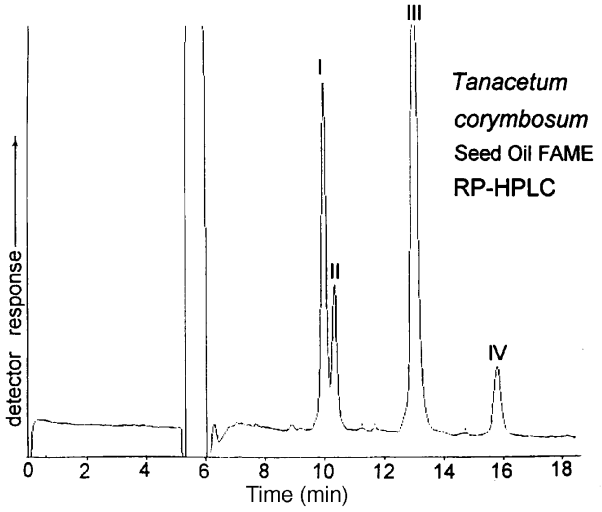

FIG. 3. Separation of the mixed fatty acid methyl esters of *T. corymbosum* seed oil by reversed-phase high-performance liquid chromatography (RP-HPLC) on two columns of Shandon Hypersil C_{18} (Phase Separations Ltd., Macclesfield, United Kingdom) connected in series. I–IV, Fractions I–IV.

The FTIR spectrum of the *T. corymbosum* seed oil exhibited characteristically strong *trans* absorption at 983.3 cm^{-1}, which is indicative of a conjugated *trans* double-bond group (23,24). The UV spectrum of the oil showed two maxima at 267.3 and 278.3 nm which were also characteristic of a conjugated *trans,trans* diunsaturated acetylenic acid (5,25,26), in comparison with those of *Valeriana pyrenaica* and *Impatiens parviflora* seed oils known to contain α-eleostearic and α-parinaric acids, respectively (Fig. 2).

The presence of the conjugated *trans,trans* unsaturation in the unknown ester was further indicated by the fact that the GLC peak of this compound only decreased strongly (more than 50%) after reaction of the mixed FAME of *T. corymbosum* seed oil with maleic anhydride, indicating that the unknown fatty acid had formed a maleic anhydride adduct by the Diels-Alder reaction. The UV spectrum of the compound after reaction with maleic anhydride was changed markedly, giving two new strong maxima at 241.1 and 248.1 nm.

Preparative separation of mixed methyl esters by reversed-phase HPLC gave four fractions (Fig. 3), and the composition of each was determined by GLC on a DB 23 column. The unknown ester was enriched in Fraction I with a content of 91% (Table 2). Fraction II consisted mainly of methyl crepenynate (66.1%) in addition to the unknown ester (25.9%). Fraction III contained only methyl linoleate. The main constituents of Fraction IV were methyl oleate (77.3%) and *cis*-vaccenate (9.6%). The unknown ester from Fraction I was deuterated for study by GC–MS.

The structures of the two unusual fatty acids were determined by MS of various derivatives. The mass spectrum of the methyl ester of the component believed to be crepenynate gave a molecular ion at *m/z* = 292 and several abundant fragments closely resembling those published for this fatty acid. The mass spectra of methyl esters did not provide a fragmen-

TABLE 2
Fatty Acid Composition of Main Components of Individual Fractions of *T. corymbosum* FAME Resulting from Preparative HPLC Separation[a]

Fraction number of HPLC	Fatty acids	GLC area (%)
Fraction I	18:0	1.2
	18:1Δ9*c*	1.6
	18:3Δ8*t*,10*t*,12*a*	91.3
Fraction II	18:0	3.4
	18:1Δ9*c*	1.1
	18:2Δ9*c*,12*a*	66.1
	18:3Δ8*t*,10*t*,12*a*	25.9
Fraction III	18:2Δ9*c*,12*c*	100
Fraction IV	16:0	1.7
	18:1Δ9*c*	77.3
	18:1Δ11*c*	9.6

[a]HPLC, high-performance liquid chromatography; FAME, fatty acid methyl ester; see Table 1 for other abbreviations.

tation pattern suitable for characterization of structure because of the ionization of double and triple bonds, resulting in their migration (27). However, the picolinyl ester and DMOX derivatives of this fatty acid had spectra identical to that of authentic crepenynate with ions diagnostic for the positions of the double and triple bonds (28).

The second methyl ester gave a molecular ion at *m/z* = 290. The mass spectra of the picolinyl ester and DMOX derivatives of this acid are illustrated in Figure 4A and 4B. The former has the expected molecular ion at *m/z* = 367. There is a gap of 76 amu between *m/z* = 220 and 296, equivalent to C_6H_4, but this does not indicate precisely where each double and triple bond is located, other than between carbons 8 to 13. The mass spectrum of the DMOX derivative has the expected molecular ion at *m/z* = 329, and it is evident that the conjugated system lies somewhere between carbons 7 and 13, but the exact position cannot be fixed definitively. Indeed, it could be argued from the gap of 12 amu between *m/z* = 168 and 180 that the first double bond is in position 7. It seems possible that the high temperature required to prepare the DMOX derivative may have caused isomerization.

As none of the derivatives of the unknown gave mass spectra which were interpretable unequivocally in terms of the positions of the double and triple bonds, the methyl ester was subjected to deuteration with Wilkinson's catalyst and converted to the picolinyl ester for analysis by MS. The spectrum is illustrated in Figure 4C. The molecular ion is at *m/z* = 383 as expected. A series follows of ions 14 amu apart, representing cleavage at successive methylene groups to *m/z* = 312. The next ion is at *m/z* = 296, a loss of 16 amu or CD_2, and there is a similar gap to *m/z* = 280. The next series of ions at *m/z* = 265, 250, 235, and 220 are 15 amu apart, equivalent to a loss of CHD. The remaining ions are 14 amu apart. The ions 15 amu apart illustrate where single bonds were present in the original molecule, i.e., positions 8 and 10, while those 16 amu apart locate the triple bond, i.e., position 12.

Together with the spectroscopic evidence, the data confirmed that the unknown was octadeca-8*t*,10*t*-dien-12-ynoic acid, which has not been reported in nature. It seems plausible that crepenynic acid (octadeca-9*c*-en-12-ynoate), which was also identified in the oil, is an intermediate in the biosynthesis of the new acid, but little appears to be known of the biosynthesis of conjugated fatty acids.

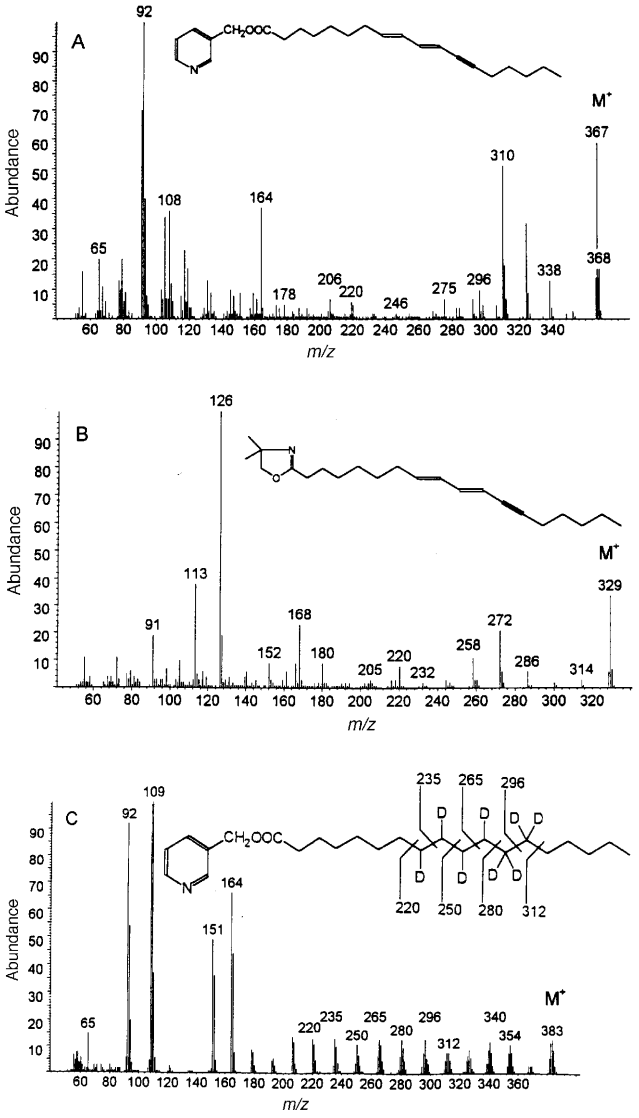

FIG. 4. The mass spectrum of different derivatives of octadeca-8,10-dien-12-ynoic acid of *T. corymbosum* seed oil. A: the picolinyl ester, B: the 4,4-*N,N*-dimethyloxazoline derivative, C: the picolinyl ester of deuterated octadeca-8,10-dien-12-ynoic acid. See Figure 1 for other abbreviation.

ACKNOWLEDGMENTS

N. Tsevegsuren thanks the Royal Society, London, for a research fellowship, during which this work was carried out. The authors are indebted to H. Vogt, H. Brockmann, and H. Voss from the Botanical Garden (University of Münster, Münster, Germany) for the gift of seed samples and to P.R. Keen and P. Dewsbury of Department of Animal and Plant Sciences (University of Sheffield, United Kingdom) for their assistance in GLC and HPLC separations. The authors wish to thank Prof. E. Haslam, R. Hansen, and W. Simon from De-

partment of Chemistry, University of Sheffield, for providing GC–MS (methyl esters), IR and UV spectra; Honorary Research Professor F.D. Gunstone and Dr. G. Dobson from Scottish Crop Research Institute (Invergowrie, Dundee, Scotland) for giving useful advice; and G.W. Robertson for providing the mass spectra. The Scottish Office Agriculture, Environment and Fisheries Department provided funding for WWC.

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[Received February 3, 1998, and in final revised form and accepted May 20, 1998]

Fullerene Lipids: Synthesis of Dialkyl 1,2-[6,6]-Methano-[60]-fullerene Dicarboxylate Derivatives

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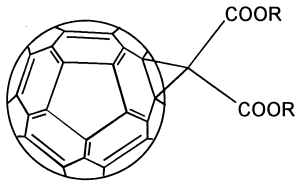
ABSTRACT: Reaction of C_{60} fullerene with dialkyl bromomalonate (where the alkyl groups consist of short-, medium-, and long-saturated chains or unsaturated long chains) in the presence of sodium hydride gives [6,6]-bridged mono-adducts of methanofullerene. The spectroscopic properties of such fullerenoid lipids are reported.

Lipids 33, 729–732 (1998).

The reactivity and chemical derivatization of fullerenes have drawn keen and continuous attention after discovery of a method for bulk production (1). A large range of functionalized fullerenes has been prepared for material and pharmaceutical applications (2–6). For instance, methanofullerene derivatives bearing the salts of carboxylic acids possess inhibitory activity against human immunodeficiency virus protease (7,8). The biological activity of water-soluble fullerenes has also been explored, with results showing structural dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including human immunodeficiency virus-protease inhibition (9). However, limited attention has been given to the functionalization of fullerene with groups containing long-chain ester functions. The synthesis of a C_{60} fullerene containing three fatty acids was described by Murakami *et al*. (10), who studied the multibilayer structure and the electronic properties of this compound. Bingel (11) reported the synthesis of diethyl methanofullerene dicarboxylate by Michael addition of a stabilized α-halocarbanion to fullerenes followed by intramolecular subsitution of the halogen.

In this paper we describe the synthesis of various dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives containing saturated and unsaturated alkyl groups (compounds **1**–**14**), which are novel lipid molecules with an extraordinary large nonpolar spherical carbon cage unit (the buckminsterfullerene system) and two ester functions with protruding alkyl chains of varying chain lengths (saturated and unsaturated) (Scheme 1). The synthesis of compound **7**, containing a C_{18} alkyl chain, was recently reported (12).



(1–14)

1, R = CH_2CH_3

2, R = $(CH_2)_3CH_3$

3, R = $(CH_2)_5CH_3$

4, R = $(CH_2)_7CH_3$

5, R = $(CH_2)_8CH_3$

6, R = $(CH_2)_9CH_3$

7, R = $(CH_2)_{17}CH_3$

8, R = $(CH_2)_9CH{=}CH_2$

9, R = $(CH_2)_5CH{=}CH(CH_2)_{10}CH_3$

10, R = $(CH_2)_8CH{=}CH(CH_2)_7CH_3$

11, R = $(CH_2)_8CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

12, R = $(CH_2)_5C{\equiv}C(CH_2)_{10}CH_3$

13, R = $(CH_2)_8C{\equiv}C(CH_2)_7CH_3$

14, R = $(CH_2)_{12}C{\equiv}C(CH_2)_7CH_3$

SCHEME 1

MATERIALS AND METHODS

Infrared (IR) spectra were recorded on a Bio-Rad (Richmond, CA) FTS-7 FT-IR spectrometer. Samples were run as mulls or neat films on KBr discs. Ultraviolet spectra were recorded on a Hewlett-Packard Diode Array Spectrophotometer, model 8452A (Hewlett-Packard, Palo Alto, CA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform ($CDCl_3$) with tetramethylsilane as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from tetramethylsilane ($\delta_{TMS} = 0$ ppm). Mass spectral analyses were carried out on Finnigan MAT-LCQ [atmospheric pressure chemical ionization (APCI)] (Finnigan Corp., San Jose, CA). Microanalyses were performed by Butterworth Laboratories Ltd. (Middlesex, United Kingdom).

Bromomalonic acid was prepared from malonic acid by the procedure described by Yankwich and Stivers (13). Diethyl bromomalonate was prepared from diethyl malonate as described elsewhere (14). *n*-Butanol, *n*-hexanol, *n*-octanol, *n*-nonanol, *n*-decanol, *n*-octadecanol, 10-undecen-1-ol, methyl

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Abbreviations: APCI, atmospheric pressure chemical ionization; IR, infrared; NMR, nuclear magnetic resonance.

oleate, and linoleate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Octadec-6-enoic acid and erucic acid [22:1(13*c*)] were isolated from carrot and rapeseed oil, respectively. Octadec-6-ynoic, octadec-9-ynoic, and docos-13-ynoic acids were prepared by bromination–dehydrobromination reaction of the corresponding olefinic esters as described elsewhere (15). The unsaturated and long-chain alcohols were prepared by lithium aluminum hydride reduction from the corresponding fatty acids or methyl esters. C_{60} fullerene was purchased from Lancaster Synthesis Ltd. (Eastgate, Lancashire LA3 3DY, United Kingdom) or from Materials and Electrochemical Research Corp. (Tucson, AZ).

General method for the preparation of dialkyl bromomalonate intermediates as exemplified by the synthesis of distearyl bromomalonate. A solution of dicyclohexylcarbodiimide (0.47 g, 2.3 mmol) in dichloromethane (10 mL) was added to a mixture of bromomalonic acid (0.2 g, 1.1 mmol), 1-octadecanol (0.53 g, 2.0 mmol), and dichloromethane (10 mL) over a period of 1 h at room temperature. The reaction mixture was stirred for 16 h and then filtered. The precipitate collected on the filter paper was washed with *n*-hexane (20 mL). The combined organic solution was evaporated under reduced pressure. The residue was chromatographed on a silica gel (30 g) column using a mixture of *n*-hexane/diethyl ether (95:5, vol/vol) to give distearyl bromomalonate (0.5 g, 59%).

*General method for the preparation of dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives as exemplified by the synthesis of distearyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**7**).* A mixture of distearyl bromomalonate (0.28 g, 0.4 mmol), sodium hydride (0.98 g, 2.1 mmol), and toluene (10 mL) was stirred at room temperature for 10 min. A solution of C_{60} fullerene (150 mg, 0.21 mmol) in toluene (110 mL) was added, and the reaction mixture was stirred for 16 h at room temperature. The color of the reaction mixture changed from purple to wine red. Water (50 mL) was added and the reaction mixture was extracted with chloroform (2×30 mL). The combined organic extract was washed with water (30 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated under reduced pressure. The residue was stirred with diethyl ether (50 mL), and precipitate (unreacted C_{60} fullerene, 41 mg) was filtered. The filtrate was evaporated under reduced pressure and the residue was dissolved in toluene (3 mL). The toluene solution was loaded onto a silica gel (30 g) column in *n*-hexane. The column was eluted with a mixture of toluene/*n*-hexane 1:9 vol/vol (100 mL) to remove traces of C_{60} fullerene (purple band). The polar wine-red colored product was subsequently eluted with a mixture of diethyl ether/*n*-hexane (0.5:99.5, vol/vol) (500 mL) to give distearyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (104 mg, 69% yield based on reacted fullerene).

*Selected spectroscopic and mass spectral data of the synthesized compounds **1**, **2**, **7**, **11**, and **13**. (i) Diethyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**1**)*: λ_{max} (CH_2Cl_2) 252, 318 nm; IR (mull, cm^{-1}): 2983, 2929, 2855, 1748, 1463, 1442, 1429, 1296, 1265, 1238, 1096, 528; ^1H NMR ($CDCl_3$, δ_H, J/Hz) 1.49 (*t*, *J* = 7.1, 6H, CH_3), 4.57 (*q*, *J* = 7.1, 4H); ^{13}C NMR ($CDCl_3$, δ_C) 14.25 (CH_3), 53.43 (methano bridge), 63.42 ($COOCH_2$), 71.60 (C_{60}-sp^3 C), 139.04, 140.95, 141.92, 142.22, 142.99, 143.02, 143.08, 143.89, 144.62, 144.69, 144.89, 145.19, 145.26, 145.24 (C_{60}-sp^2 C), 163.61 (C=O); mass spectral analysis (APCI): *m/z* 879.1 (M^+); microanalysis: found C, 90.76, H, 1.42 ($C_{67}H_{10}O_4$, calcd. C, 91.57, H, 1.15).

*(ii) Dibutyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**2**)*: λ_{max} (CH_2Cl_2) 254, 286, 326 nm; IR (mull, cm^{-1}): 2962, 2929, 2868, 1745, 1462, 1429, 1266, 1233, 1206, 1186, 1177, 1063, 755, 527; ^1H NMR ($CDCl_3$, δ_H, J/Hz) 1.01 (*t, J* = 7.5, 6H, CH_3), 1.48–1.58 (*m*, 4H, CH_2), 1.79–1.88 (*m*, 4H, CH_2), 4.50 (*t, J* = 6.6, 4H, $COOCH_2$-); ^{13}C NMR ($CDCl_3$, δ_C) 13.69 (CH_3), 19.16 (CH_2CH_3), 30.54 ($CH_2CH_2CH_3$), 52.36 (methano bridge), 67.19 ($COOCH_2$), 71.65 (C_{60}-sp^3 C), 139.01, 140.95, 141.91, 142.21, 142.89, 143.01, 143.08, 143.88, 144.61, 144.68, 144.88, 145.18, 145.26, 145.35 (C_{60}-sp^2 C), 163.71 (C=O); mass spectral analysis (APCI): *m/z* 935.1 (M^+); microanalysis: found C, 91.22, H, 1.93 ($C_{71}H_{18}O_4$, calcd. C, 90.17, H, 2.14).

*(iii) Distearyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**7**)*: λ_{max} (CH_2Cl_2) 254, 288, 326 nm; IR (neat, cm^{-1}): 2969, 2922, 2851, 1746, 1463, 1428, 1266, 1233, 1205, 755, 526; ^1H NMR ($CDCl_3$, δ_H, J/Hz) 0.88 (*t, J* = 6.7, 6H, CH_3), 1.25-1.59 (*m*, 60H, CH_2), 1.84 (quintet, *J* = 7 Hz, 4H, $COOCH_2CH_2$), 4.49 (*t, J* = 6.5, 4H, $COOCH_2$); ^{13}C NMR ($CDCl_3$, δ_C) 14.15 (CH_3), 22.72, 26.03, 28.62, 29.27, 29.39, 29.65, 29.70, 29.75, 31.95 (CH_2), 52.47 (methano bridge), 67.50 ($COOCH_2$), 71.70 (C_{60}-sp^3 C), 139.00, 140.96, 141.93, 142.22, 143.00, 143.02, 143.09, 143.89, 144.61, 144.70, 144.89, 145.19, 145.27, 145.40 (C_{60}-sp^2 C), 163.73 (C=O); mass spectral analysis (APCI): *m/z* 1327.5 (M^+); microanalysis: found C, 86.91, H, 4.64 ($C_{99}H_{74}O_4$, calcd. C, 89.56, H, 5.62).

*(iv) Dilinoleyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**11**)*: λ_{max} (CH_2Cl_2) 258, 280, 326 nm; IR (cm^{-1}): 3007, 2925, 2853, 1748, 1558, 1507, 1540, 1232, 1092, 754, 526; ^1H NMR ($CDCl_3$, δ_H, J/Hz) 0.89 (*t, J* = 6.8, 6H, CH_3), 1.29–1.37 (*m*, 64H, CH_2), 1.84 (quintet, *J* = 7 Hz, 4H, CH_2), 2.01–2.08 (*m*, 8H, CH_2), 2.77 (*t, J* = 6.0, 4H, CH_2), 4.49 (*t, J* = 6.6, 4H, $COOCH_2$), 5.34–5.37 (*m*, 8H, olefin); ^{13}C NMR ($CDCl_3$, δ_C) 14.11 (CH_3), 22.59, 25.66, 26.00, 27.23, 28.60, 29.23, 29.28, 29.36, 29.55, 29.70, 31.54 (CH_2), 52.44 (methano bridge), 67.45 ($COOCH_2$), 71.68 (C_{60}-sp^3 C), 127.90, 128.08, 130.02, 130.23 (CH=CH of linoleyl), 138.99, 140.94, 141.91, 142.20, 142.99, 143.01, 143.09, 143.88, 144.60, 144.65, 144.69, 144.88, 145.18, 145.26, 145.37 (C_{60}-sp^2 C), 163.69 (C=O); mass spectral analysis (APCI): *m/z* 1319.3 (M^+); microanalysis: found C, 90.40, H, 5.18 ($C_{99}H_{66}O_4$, calcd. C, 90.11, H, 5.04).

*(v) Di-9-octadecynyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate (**13**)*: λ_{max} (CH_2Cl_2) 254, 294, 324 nm; IR (cm^{-1}): 2953, 2927, 1746, 1463, 1428, 1266, 1233, 1205, 1186, 1114, 711, 526; ^1H NMR ($CDCl_3$, δ_H, J/Hz) 0.88 (*t, J* = 6.7, 6H, CH_3), 1.27–1.49 (*m*, 44H, CH_2), 1.84 (quintet, *J* = 7 Hz, 4H, CH_2), 2.13 (*t, J* = 7.9, 8H, C≡C-CH_2), 4.49 (*t, J* = 6.6, 4H,

$COOCH_2$); ^{13}C NMR ($CDCl_3$, δ_C) 14.15 (CH_3), 18.77, 18.79 ($C\equiv C\text{-}CH_2$), 22.68, 25.91, 28.59, 28.90, 29.00, 29.15, 29.19, 29.24, 31.86 (CH_2), 52.42 (methano bridge), 67.43 ($COOCH_2$), 71.66 (C_{60}-sp^3 C), 80.08, 80.37 (C≡C acetylene), 138.98, 140.94, 141.90, 142.20, 142.98, 143.00, 143.08, 143.87, 144.59, 144.64, 144.67, 144.87, 145.16, 145.25, 145.36 (C_{60}-sp^2 C), 163.67 (C=O), mass spectral analysis (APCI): *m/z* 1319.5 (M^+); microanalysis: found C, 88.87, H, 4.99 ($C_{99}H_{66}O_4$, calcd. C, 90.11, H, 5.04).

DISCUSSION

The cyclopropanation of C_{60} fullerene by reaction with bromomalonates in the presence of an auxiliary base is a reliable method of fullerene functionalization. This approach allows the exclusive formation of [6.6]-bridged adducts to be formed. By controlling the reaction time and the amount of bromomalonate, mono adducts can be obtained. However, it is also possible to form higher adducts (bis up to hexakis) with a stereochemically defined addition pattern using template activation with 9,10-dimethylanthracene (11,16–19).

In the synthesis of the dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives (compounds **1–14**), the procedure described by Bingel (11) was adopted with a slight modification. Instead of treating dialkyl malonates with bromine to form the dialkyl bromomalonates, it was essential to prepare bromomalonic acid first and then esterify the latter with saturated or unsaturated fatty alcohols to yield the corresponding dialkyl bromomalonates (61% average yield). This approach ensured that the alkyl chains containing unsaturated centers would not be brominated when unsaturated dialkyl malonates were used as substrates.

C_{60} fullerene was then treated with the respective dialkyl bromomalonates in the presence of sodium hydride at room temperature in toluene to give the requisite dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives (35–69% yield, based on reacted C_{60} fullerene). While our work was in progress, a recent publication described a similar reaction for the preparation of compounds **1** and **7** by the direct treatment of C_{60} fullerene with malonate in the presence of CBr_4 and diazabicyclo[5.4.0]undec-7-ene (12).

The diethyl (**1**), dibutyl (**2**), dihexyl (**3**), and dinonyl (**5**) 1,2-[6,6]-methano-[60]-fullerene dicarboxylates are dark-brown solids with very high melting points (>240, 210–211, 177–179, and 153–159°C, respectively). The dioctyl and the longer chain saturated and unsaturated dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate analogs (compounds **4**, **6–14**) are viscous liquids which cannot be crystallized from organic solvents. C_{60} fullerene is very insoluble in common organic solvents such as acetone, diethyl ether and tetrahydrofuran, but the dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylates are readily soluble in diethyl ether and chlorinated solvents. Such a difference in the solubility between C_{60} fullerene and the functionalized fullerene molecule facilitated the isolation and purification of the requisite products from the reaction mixture. It was necessary to remove as much of the unreacted C_{60} fullerene as possible prior to purification of the requisite product by column chromatography. The mass spectral analysis (APCI technique) gave the exact molecular mass unit (M^+) for the various products. However, we were unable to recrystallize the products isolated by column chromatography before submission to microanalysis. Some of the results from the microanalysis were slightly off the mark, which reflected the possible presence of some impurities (unreacted C_{60} fullerene).

The ^1H and ^{13}C NMR spectral analyses of these compounds (**1–14**) were conducted in solutions of deuteriochloroform. The proton shift of the methylene group adjacent to the carboxylate function appeared in the region of δ_H 4.49–4.57. The shifts of the remaining protons of the alkyl chain were characteristic and comparable to those observed in the alkyl chain of fatty esters or fatty alcohols.

The ^{13}C NMR spectral analysis of the products showed 14–15 signals for the sp^2 carbon nuclei of the fullerene cage (region of δ_C 138–145). The signal at δ_C 71 (2 carbon equivalent, sp^3 carbons) was due to the shifts of the carbon atoms at the junction of the [6,6]-fused rings of the fullerene and to which the cyclopropane system is attached. The shift of the carbon of the methano bridge of the cyclopropane ring appeared at about δ_C 52 . The presence of the carboxylate groups was evident from the signals at δ_C 163. The shifts of the remaining carbon atoms (methylenic, olefinic, and acetylenic) were found in their respective chemical shift regions. From these results we are able to show that long- and short-chain, saturated and unsaturated dialkyl malonate derivatives of C_{60} fullerene can be successfully synthesized.

ACKNOWLEDGMENTS

This work has been supported by the Lipid Research Fund and the Committee on Research and Conference Grants of The University of Hong Kong.

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[Received December 18, 1997, and in final revised form and accepted May 12, 1998]

Production of Antiserum for Sensitive Enzyme-Linked Immunosorbent Assay of 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic Acid by Chemiluminescence

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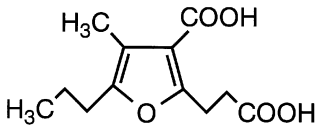
ABSTRACT: To obtain a specific antiserum for use in enzyme-linked immunosorbent assay (ELISA) of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), we prepared a hapten-carrier conjugate in which the CMPF hapten was linked to a carrier protein through the 5-(1-hydrazopropyl) group. The antisera raised against this antigen in guinea pigs had excellent specificity for CMPF, showing little cross-reactivity with closely related compounds and no significant cross-reactivities with other furan compounds. The results indicated that a specific antiserum to CMPF could be produced by an antigen whose CMPF moiety is linked to a carrier protein through a position remote from the inherent functional groups. A standard curve of CMPF by ELISA using a chemiluminescence system showed a high sensitivity and a linearity in the range of 5–100 ng/mL.
Lipids 33, 733–736 (1998).

The furan fatty acids first shown in fish by Glass *et al.* (1,2) were later detected in extremely small quantities in a variety of animals (3,4), and, to a lesser extent, in plant sources (5) and marine bacteria (6). In human feeding experiments, the correlation of furan fatty acid intake with urofuranic acid excretion (7) suggested that the furan fatty acids might be degraded to urofuranic acids (4). Binding of many acidic drugs and hormones to serum albumin is decreased in patients with renal failure (8–11). A number of endogenous ligand substances in uremic serum inhibit the binding of drugs to serum proteins (12–16). In particular, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) (Scheme 1) in an endogenous, ligand is tightly bound to serum protein (17,18) and, therefore, the concentration of CMPF in uremic plasma is not significantly decreased by hemodialysis (19). Recently, it was suggested that CMPF inhibits transport of thyroxine into hepatocytes in uremia (20), inhibits the active uptake of thyroxine into anterior pituitary cells (21), and enhances drug inhibition of thyroxine binding in serum (22). Analysis of CMPF has previously been performed by gas chromatographic (23), and gas chromatographic–mass spectrometric techniques (24), as well as by high-performance liquid chromatography (19) but these methods require sample preparation and can be inconvenient for routine screening of many samples. Therefore, we have tried to develop a sensitive enzyme-linked immunosorbent assay (ELISA) for CMPF.



SCHEME 1

In the present study, we prepared CMPF–protein conjugate as an immunogen used for the production of a specific antiserum and an assay antigen, toward the development of a sensitive ELISA for CMPF by chemiluminescence system .

EXPERIMENTAL PROCEDURES

Reagents. 2,2-Dimethyl-1,3-dioxane-4,6-dione was purchased from Kodak Company (Rochester, NY). Bovine serum albumin (BSA) Fr. VI and ovalbumin (OVA) were from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 G and 60 HF_{254} were supplied by Merck (Darmstadt, Germany). Anti-guinea pig IgG antibody-alkaline phosphatase conjugate was obtained from Chemicon International Inc. (Temecula, CA) and Lumiphos 530 {containing 4-methyl-4(3-phosphatephenyl)spiro[1,2-dioxetane-3,2′-adamantane] disodium salt (3.3×10^{-4} M), magnesium chloride (8.0×10^{-4} M), cetyltrimethylammonium bromide (1.13×10^{-3} M), and fluorescein surfactant (5.6×10^{-5} M) in 2-amino-2-methyl-1-propanol buffer (0.75 M, pH 9.6)} from Wako Pure Chemicals (Osaka, Japan). Other general reagents of analytical

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Abbreviations: BSA, bovine serum albumin; CMF, 3-carboxy-4-methyl-2-furanpropanoic acid; CMEF, 3-carboxy-4-methyl-5-(1-propenyl)-2-furanpropanoic acid; CMFM, 3-carboxy-4-methyl-5-(1-oxopropyl)-2-furanpropanoic acid methyl ester; CMHF, 3-carboxy-4-methyl-5-(1-hydroxypropyl)-2-furanpropanoic acid; CMOF, 3-carboxy-4-methyl-5-(1-oxopropyl)-2-furanpropanoic acid; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; CMPM, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid methyl ester; CMZF, 3-carboxy-4-methyl-5-(1-hydrazopropyl)-2-furanpropanoic acid; ELISA, enzyme-linked immunosorbent assay; IR, infra red; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, 0.1 M sodium phosphate-buffered saline (pH7.2); UV, ultraviolet.

grade were obtained from Nakarai Tesque (Kyoto, Japan). CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid methyl ester (CMPM), 3-carboxy-4-methyl-5-(1-hydroxypropyl)-2-furanpropanoic acid (CMHF), 3-carboxy-4-methyl-5-(1-propenyl)-2-furanpropanoic acid (CMEF), 3-carboxy-4-methyl-5-(1-oxopropyl)-2-furanpropanoic acid methyl ester (CMFM), and 3-carboxy-4-methyl-2-furanpropanoic acid (CMF) used for the examination of cross-reactivities of anti-CMPF antiserum were prepared according to the methods of Pfordt *et al*. (25) in our laboratory.

Spectroscopic analyses of furan propanoic acids. Ultraviolet (UV) spectra of methanol solutions were taken on a Hitachi U3200 spectrophotometer (Tokyo, Japan) and infrared (IR) spectra on Shimadzu spectrometer IR-406 (Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL JNMF X-100 (Tokyo, Japan).

*Synthesis of hapten: 3-Carboxy-4-methyl-5-(1-oxopropyl)-2-furan propanoic acid, CMOF (**2**).* To a solution of CMFM (**1**; 500 mg) in methanol (10 mL) was added 1 M sodium hydroxide (5.0 mL), and the mixture was allowed to stand at room temperature for 12 h. The reaction mixture was concentrated to 3.0 mL; to this was added 2 M hydrochloric acid (5.0 mL), and the resulting solution was extracted with chloroform (3 × 20 mL), washed with water (25 mL), dried over anhydrous sodium sulfate, and then the resulting solution was evaporated to dryness under reduced pressure. The residue was purified by preparative thin-layer chromatography on silica gel 60 HF_{254} plates using chloroform/methanol (5:1, vol/vol) as a solvent. The product (R_f = 0.1) was eluted with chloroform/methanol/water (20:10:1, by vol) to give CMOF (**2,** 300 mg) as a colorless amorphous substance. UV (λ_{max} 275 nm). ^{1}H NMR (acetone-d_6) δ: 2.78 (2H, *m*, CH_2CH_2COOH), 3.36 (2H, *m*, CH_2CH_2COOH). IR ν_{max} cm^{-1}: 1710 (aliphatic COOH), 1690 (olefinic COOH).

*3-Carboxy-4-methyl-5-(1-hydrazopropyl)-2-furanpropanoic acid, CMZF (**3**).* To a solution of CMOF (**2**, 100 mg) in methanol (3.0 mL) was added hydrazine hydride (100 mg) and this was allowed to stand at room temperature for 2 h. The resulting solution was evaporated to dryness under reduced pressure, the residue was purified by preparative thin-layer chromatography on silica gel 60 HF_{254} to give CMZF (**3**, 40 mg) as an oily product. UV (λ_{max} 278 nm). ^{1}H NMR (dimethylsulfoxide-d_6) δ:1.92 (3H, s ,4-CH_3), 2.55 (2H, m, CH_2CH_2COOH), 3.55 (2H, m, CH_2CH_2COOH). IR ν_{max} cm^{-1}: 1710 (aliphatic COOH), 1690 (olefinic COOH).

Preparation of antigens: *CMZF–BSA conjugate (**4a**).* To a solution of BSA (50 mg) in 0.2 M phosphate buffer (pH 6.8, 5.0 mL) were added CMZF (**3**, 30 mg) in methanol (0.5 mL) and 2 wt% aqueous glutaraldehyde (0.5 mL), and the reaction mixture was stirred at 4°C overnight.

The resulting solution was dialyzed against water (3 × 2 L) and then lyophilized to give CMZF-BSA conjugate (**4a**, 38 mg). Measurement of UV absorbance at 285 nm in aqueous solution of the hapten, BSA, and the conjugate revealed that 23 moles of CMZF (**3**) were coupled to each mole of BSA.

*CMZF–OVA conjugate (**4b**).* To a solution of OVA (5 mg) in 0.1 M phosphate buffer (pH 6.8, 1.0 mL) were added CMZF (**3**, 3 mg) in methanol (0.15 mL) and 2 wt% aqueous glutaraldehyde (50 μL). The procedure that followed was carried out as described above to obtain a solution of CMZF-OVA conjugate (**4b**, 4 mg).

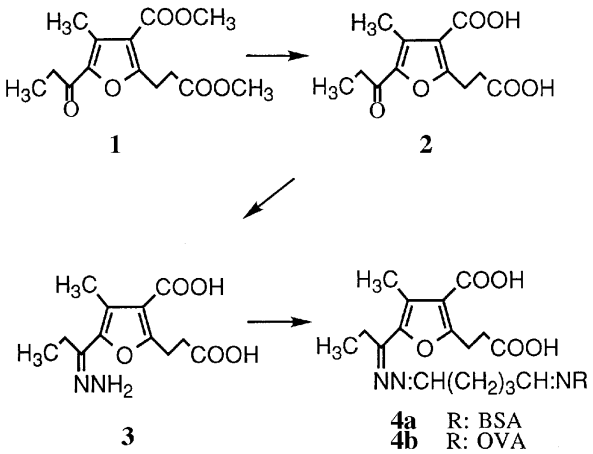
Immunization. Three male guinea pigs were used for immunization. CMZF–BSA conjugate (0.5 mg) was dissolved in sterile isotonic saline (0.25 mL) and emulsified with Freund's complete adjuvant (0.25 mL). This emulsion was injected at multiple sites over the scapulae and in the back. This procedure was repeated at intervals of 2 wk for 2 mon and then once a month. The guinea pigs were bled 10 d after the booster injection. The sera were separated by centrifugation at 800 × *g* for 20 min and stored at −80°C.

ELISA. Fifty microliters of CMZF–OVA conjugate (1 μg/mL) in 0.1 M sodium phosphate-buffered saline (pH 7.2) (PBS) was added to each well of a 96-well microtiter plate (Sumilon MS-K, Sumitomo Bakelite Co., Tokyo, Japan) and incubated at room temperature for 3 h. Unbound conjugate was removed from the plate with four washes of PBS containing 0.5 wt% Tween 20 (PBS/Tween). To block the unoccupied solid phase to minimize nonspecific binding, 0.1 wt% casein solution in PBS (PBS/casein, 0.22 mL) was added, and the mixture was incubated at room temperature for 2 h. The wells were washed with PBS/Tween (4 × 0.22 mL) and 50 μL of a 2000-fold diluted antiserum with PBS/casein was incubated with a standard solution of CMPF (0–200 ng/mL) in PBS (50 μL) at 4°C overnight. The solution was removed and the wells were washed as before. Anti-guinea pig IgG antibody-alkaline phosphatase (1:5000 dilution, 50 μL) was added to each well, and the plate was incubated at room temperature for 1 h. Unbound antibody–enzyme conjugate in solution was removed, and each well was washed as before. Lumiphos 530 (60 μL) was added to each well, and the light counts were measured by a Luminous CT-9000D microplate reader (Dia-itoron Co., Ltd.).

Specificity. The cross-reactivities were examined by calculating the ratio of the quantity of CMPF to the quantity of related compounds corresponding to 50% inhibition of solid phase antigen binding to the antiserum.

RESULTS AND DISCUSSION

To prepare an immunogen for the production of a specific antiserum to CMPF, we synthesized a CMPF hapten which could be coupled to a carrier protein without affecting the functional groups of CMPF, which are important antigen determinants in the CMPF molecule. The starting material, 2,2-dimethyl-1,3-dioxane-4,6-dione, was transformed into a methyl ester (**1**) in four steps (25). Next, as indicated in Scheme 2, upon treatment with sodium hydroxide, CMFM (**1**) was hydrolyzed to furnish the dicarboxylic acid (**2**). Condensation of CMOF (**2**) with hydrazine produced the hydrazone derivative (**3**). Linking of CMZF (**3**) to BSA and OVA was accomplished using glutaraldehyde as a cross-linking reagent. We have therefore successfully prepared CMZF-protein con-



SCHEME 2

jugates (**4a** and **4b**) with intact functional groups in CMPF. The UV spectra of two hapten–protein conjugates and two proteins demonstrate that a sufficient number of hapten molecules were attached to each protein.

Three immunized guinea pigs yielded antisera exhibiting binding activity to CMPF. High binding activity of CMPF was present in all antisera 5 mon after the primary immunization. Titers were determined by measuring the ability of diluted antiserum to bind 50% of solid phase antigen. The typical standard curve of CMPF was constructed with a 2000-fold diluted antiserum with PBS/casein using a solid phase antigen. Chemiluminescence intensity was measured while the concentrations of competing CMPF were varied from 0–200 ng/mL. The binding inhibition curve for quantitation was obtained at 5–100 ng/mL as shown in Figure 1. The detection limit of CMPF by ELISA using chemiluminescence system was 10-fold higher than that by ELISA using colorimetric system (data not shown).

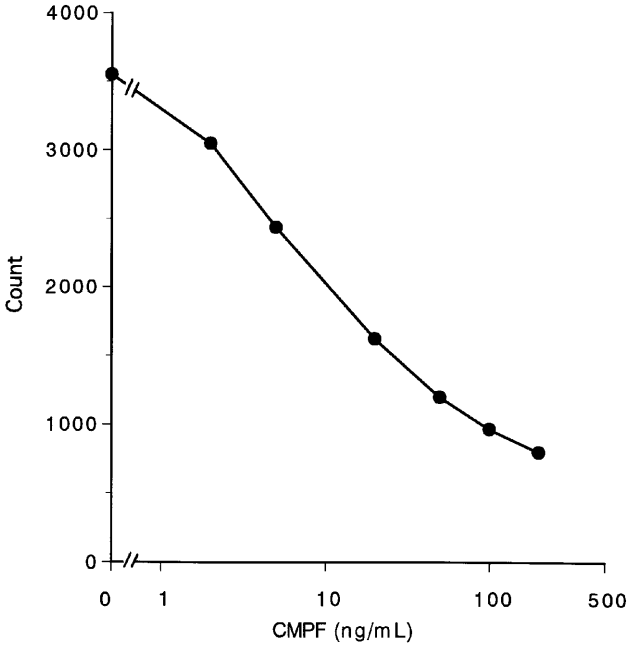


FIG. 1. Typical standard curve of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) by enzyme-linked immunosorbent assay using chemiluminescence system.

TABLE 1
Cross-reactivity (%) of Anti-CMPF Antiserum with Compounds Related to CMPF[a]

Compound	Cross-reactivity
CMPF	100
CMEF	31.2
CMHF	15.2
CMF	8.7
CMFM	<0.01
CMPM	<0.01
Furfuryl acetate	<0.01
Tetrahydrofurfuryl acrylate	<0.01
3-Indoleacetic acid	<0.01
2-Furanoic acid	<0.01
2-Methyl furan	<0.01

[a]CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; CMEF, 3-carboxy-4-methyl-5-(1-propenyl)-2-furanpropanoic acid; CMHF, 3-carboxy-4-methyl-5-(1-hydroxypropyl)-2-furanpropanoic acid; CMF, 3-carboxy-4-methyl-2-furanpropanoic acid; CMFM, 3-carboxy-4-methyl-5-(1-oxopropyl) 2-furanpropanoic acid methyl ester; CMPM, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid methyl ester.

The cross-reactivity of antiserum to CMPF analogs was calculated by the method of Abraham (26). Table 1 indicates that the antiserum is highly specific for CMPF. The antiserum raised against the CMZF–BSA conjugate exhibited 31.2% of cross-reactivity with CMEF and 15.2% of cross-reactivity with CMHF. The cross-reactivities of CMEF and CMHF were due to the close similarity of the structures of these compounds to CMPF by the use of the 1-oxopropyl group for coupling to carrier protein. The antiserum showed low cross-reactivity with CMF (8.7%), indicating that the antiserum also recognizes the 5-side chain. Furthermore, the antiserum exhibited no significant cross-reactivities with CMFM (<0.01%) and other furan compounds (<0.01%). The antibody particularly recognizes functional groups of CMPF remote from the 1-oxopropyl group which is linked to the carrier. The antiserum strongly discriminates CMPM from CMPF. It is generally accepted that conjugation of an antigenic carrier through positions on a hapten distal to their functional groups would elicit antibodies of greater specificity than those raised by conjugation through the carboxyl acids as the inherent functional group in CMPF. Our results demonstrated that the antiserum was highly specific to CMPF with minimal cross-reactivity to the closely related urofuran compounds.

The utilization of this sensitive ELISA for determination of CMPF in biological fluids will be the subject of a future communication.

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[Received April 24, 1998; and in revised form and accepted May 18, 1998]

9-Hydroxy-traumatin, A New Metabolite of the Lipoxygenase Pathway

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ABSTRACT: 9-Hydroxy-traumatin, 9-hydroxy-12-oxo-10*E*-dodecenoic acid, was isolated as a product of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid as catalyzed by enzyme preparations of both soybean and alfalfa seedlings. This suggested that 9*Z*-traumatin, 12-oxo-9*Z*-dodecenoic acid, was being converted into 9-hydroxy-traumatin in an analogous manner to the previously identified enzymic conversion of 3*Z*-nonenal and 3*Z*-hexenal into 4-hydroxy-2*E*-nonenal and 4-hydroxy-2*E*-hexenal, respectively. Other metabolites of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid were similar for both soybean and alfalfa seedling preparations, and they are briefly described.
Lipids 33, 745–749 (1998).

A plant wound-healing hormone, traumatic acid (2*E*-dodecenedioic acid), was identified almost 50 years ago (1). It is certain that this compound arises from 12-oxo-9*Z*-dodecenoic acid (9*Z*-traumatin) through the hydroperoxide lyase chain cleavage of either 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPODE) or 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid into a six-carbon aldehyde and 9*Z*-traumatin followed by aldehyde oxidation and double-bond rearrangement of the latter [see reviews on the hydroperoxide lyase pathway (2–4)]. More recently, the wound-healing properties of 12-oxo-10*E*-dodecenoic acid (10*E*-traumatin) were reported (5).

This author is unaware of any report of an in-chain hydroxylated form of 9*Z*- or 10*E*-traumatin as a product of the lipoxygenase pathway. In this work, 9-hydroxy-12-oxo-10*E*-dodecenoic acid (9-hydroxy-traumatin) was identified as a product of 13*S*-HPODE metabolism by both alfalfa and soybean seedlings.

MATERIALS AND METHODS

Enzyme preparations and reaction conditions. Two grams of seeds, either soybeans (*Glycine max* cv Williams) or alfalfa (*Medicago sativa*), were germinated on moistened Kimpac (Kimberly-Clark, Corp., Neenah, WI) for 3 d at room temperature. The washed seedlings were Polytron-homogenized for 30 s in 20 mL 50 mM Na phosphate buffer (pH 7.0) with or without 0.5% Triton X-100. The homogenates were centrifuged at 8,000 × *g* for 15 min. The floating oleosome layer was removed by aspiration. A final concentration of 1 mM 13*S*-HPODE served as a substrate and was added as a 4% solution in methanol. Although the supernatant solution was used "as is" in some experiments, the yield of 9-hydroxy-traumatin was very low under these conditions. The yield improved after the enzyme solution was diluted 10-fold with 50 mM phosphate buffer (pH 7.0), and all results presented here utilized the 10-fold diluted supernatant solutions (total reaction volume of 20 mL). After addition of 13*S*-HPODE, the reaction was permitted to proceed for 20 min at 25°C with magnetic stirring. A larger-scale experiment (150 mg 13*S*-HPODE substrate) was completed essentially as a scaled-up version of the smaller incubations. That is, a 10-fold diluted supernatant prepared from soybean seedlings was incubated with 1 mM 13*S*-HPODE in 50 mM phosphate buffer (pH 7.0) for 20 min at 25°C with magnetic stirring (total reaction volume of 480 mL). All reactions were terminated by adjusting the pH to 4.0 with 1 M oxalic acid, and extracting with a 3-fold volume of $CHCl_3$/MeOH (2:1, vol/vol). The preparation of 13*S*-HPODE was described previously (6).

Preparation of derivatives and thin-layer chromatography (TLC) isolation. The extracted products were methyl-esterified by a short exposure (*ca.* 15 s) to CH_2N_2 in diethyl ether/MeOH (9:1, vol/vol). A short exposure to CH_2N_2 was important to minimize side reactions often observed with aldehydes [see review (6)].

For direct analysis of the total reaction mixture by gas chromatography–mass spectrometry (GC–MS), the solvent was removed from the methyl esters and trimethylsilyloxy ethers (OTMSi) were prepared as described (6). In certain experiments the product methyl esters (equivalent to 1 mg of 13*S*-HPODE originally added as substrate) were first treated with $SnCl_2$ to reduce residual 13*S*-HPODE to the corresponding hydroxyl derivative, and this reduction was followed by the preparation of OTMSi derivatives. For reduction, a quantity of 3.8 mg $SnCl_2 \cdot 2H_2O$ (Aldrich Chemical, St. Louis, MO) in 0.64 mL pyridine/MeOH (1:1, vol/vol) was added for a 1 h reaction time. Pyridine was an essential ingredient in the re-

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Abbreviations: EIMS, Electron impact-mass spectrum; GC–MS, gas chromatography–mass spectrometry; ^{1}H NMR, proton nuclear magnetic resonance spectrometry; 13*S*-HPODE, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 9-hydroxy-traumatin, 9-hydroxy-12-oxo-10*E*-dodecenoic acid; OTMSi, trimethylsilyloxy ether; TLC, thin-layer chromatography; traumatic acid, 2*E*-dodecendioic acid; 9*Z*-traumatin, 12-oxo-9*Z*-dodecenoic acid; 10*E*-traumatin, 12-oxo-10*E*-dodecenoic acid.

duction in order to minimize acid-catalyzed reactions caused by $SnCl_2$. Reduction products were extracted by adding a 3-fold volume of $CHCl_3/H_2O$ (2:1, vol/vol) followed by washing the $CHCl_3$ layer twice with H_2O.

For purification of methyl 9-hydroxy-traumatin, the technique of "chemical" chromatography was utilized. First, the methyl-esterified product mixture was separated by TLC using Silica Gel 60 F_{254} precoated plates from Merck (20 cm × 20 cm × 0.25 mm) and diethyl ether/hexane (7:3, vol/vol) as developing solvent. GC–MS monitoring revealed that the methyl 9-hydroxy-traumatin migrated as a partially pure substance between $R_f = 0.2$ and 0.4. The silica scraped from this portion of the plate was extracted with ethyl acetate, and the solvent was evaporated with a stream of N_2. Next, the dried sample was silylated with OTMSi reagent, and then separated by TLC using hexane/diethyl ether (7:3, vol/vol). Methyl 9-OTMSi-traumatin was detected as an ultraviolet-absorbing band at $R_f = 0.38$, and this derivative was extracted from the scrapings by ethyl acetate giving pure methyl 9-OTMSi-traumatin as assessed by GC–MS and proton nuclear magnetic resonance (^{1}H NMR).

Spectral methods. GC–MS was accomplished with a Hewlett-Packard Model 5890 (Palo Alto, CA) gas chromatograph interfaced with a Model 5971 mass selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5MS cross-linked 5% phenyl methyl silicone, 0.25 mm × 30 m, film thickness 0.25 µm. The methyl-esterified–OTMSi derivatives were separated by temperature programming from 160 to 260°C at a rate of 5°C/min then held at 260°C for 10 min (He flow rate = 0.67 mL/min).

Methyl 9-OTMSi-traumatin (in $CDCl_3$ solution) was examined by ^{1}H NMR utilizing a Bruker model ARX-400 spectrometer (400 MHz) (Karlsruhe, Germany).

RESULTS AND DISCUSSION

When either a soybean or alfalfa supernatant preparation was used "as is" to catalyze the transformation of 13*S*-HPODE, only a trace amount of 9-hydroxy-traumatin (methyl ester, 9-OTMSi ether) was observed by GC–MS. However, after a 10-fold dilution of the seedling extracts, methyl 9-OTMSi-traumatin was readily analyzed. One of two possibilities for the reduced amount of 9-hydroxy-traumatin produced by the undiluted preparation was the facile reaction of 9-hydroxy-traumatin with protein sulfhydryl and amino groups in the relatively concentrated enzyme solution. Another was the possibility of artifact production from the relatively larger amount of unreacted 13*S*-HPODE extracted from the diluted reaction mixture. To eliminate this latter possibility, residual 13*S*-HPODE was reduced in a portion of the product mixture with $SnCl_2$ in MeOH/pyridine (pyridine was included to suppress acid-catalyzed reactions), and this mixture was compared with the unreduced product (Fig. 1). There was little difference in the relative abundance of 9-hydroxy-traumatin in the two samples (based on ion abundance); the slightly smaller amount in the reduced sample could easily be attributed to additional sample handling. As seen in Figure 1, the principal products observed as arising from free-radical decomposition of 13*S*-HPODE were isomeric 13-oxo-9,11-tridecadienoic acid and 13-oxo-9,11-octadecadienoic acid. Authentic metabolites, not due to free-radical decomposition of 13*S*-HPODE, were also tentatively identified by their electron impact mass spectra (EIMS) compared with the literature. Most of these metabolites have been described in soybean before (7,11,12,16,17–20), with the exception of 9-hydroxy-traumatin and one isomeric epoxyhydroxyoctadecenoic acid. However, the latter compound, 11,12-epoxy-13-hydroxy-9-

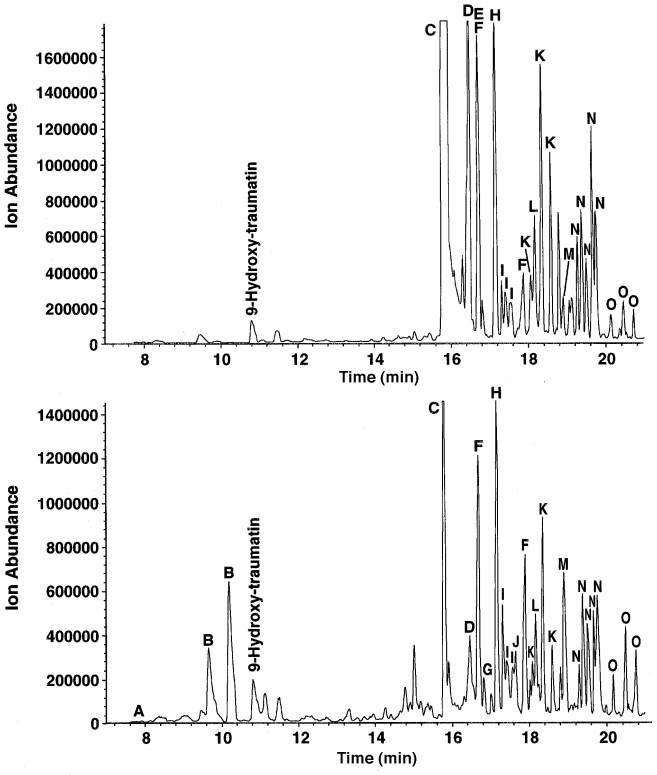

FIG. 1. Gas chromatography–mass spectrometry (GC–MS) analysis of 9-hydroxy-traumatin and other metabolites of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13S-HPODE) (as methyl esters/trimethylsilyloxy ethers) as produced by a soybean seedling preparation (10-fold diluted supernatant). Top: residual hydroperoxides reduced by $SnCl_2$ before analysis; bottom: unreacted 13*S*-HPODE was not reduced before analysis. Tentative identification was from literature mass spectra as follows (as methyl esters/trimethylsilyloxy ethers): A, traumatin (7); B, 13-oxo-9,11-tridecadienoic acid (9*Z*,11*E* and 9*E*,11*E* isomers) (8); 9-hydroxy-traumatin (this report); C, 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (9); D, 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9); E, 13-hydroxy-9*E*,11*E*-octadecadienoic acid [spectrum the same as C (9)]; F, 13-oxo-9,11-octadecadienoic acid (9*Z*,11*E* or 9*E*,11*E*) (10); G, 9-oxo-10,12-octadecadienoic acid (10); H, 12,13-epoxy-11-hydroxy-9-octadecenoic acid (11); I, overlapping peaks of isomeric H and 9,10-epoxy-11-hydroxy-12-octadecenoic acid (11,12); J, 11,12-epoxy-13-hydroxy-9-octadecenoic acid (13); K, isomeric 9,10-epoxy-13-hydroxy-11-octadecenoic acid (14); L, 12,13-epoxy-9-hydroxy-10-octadecenoic acid (15); M, 12,13-epoxy-9-oxo-10-octadecenoic acid and 9,10-epoxy-13-oxo-11-octadecenoic acid (Gardner, H.W., unpublished data); N, isomeric 9,10,13-trihydroxy-11-octadecenoic acid, 9,12,13-trihydroxy-10-octadecenoic acid, and 11,12,13-trihydroxy-9-octadecenoic acid (16); O, isomeric dihydroxyoxooctadecenoic acid (16).

octadecenoic acid, has been identified as a product of *Vicia faba* peroxygenase/epoxygenase (21). Isomeric oxodihydroxyoctadecenoic acids and trihydroxyoctadecenoic acids are presumed to arise from epoxide hydrolase (22) action on epoxyoxooctadecenoic acids and epoxyhydroxyoctadecenoic acids, respectively. The positional isomers of epoxyhydroxyoctadecenoic acid and epoxyoxooctadecenoic acids observed by their mass spectra indicated a mixed reaction mechanism of: (i) alkoxyl radical rearrangement (23) (e.g., the 13-hydroperoxide gives rise to 12,13-epoxides) and (ii) peroxygenase oxidation of double bonds (19,21) of either hydroxyoctadecadienoic acid or oxooctadecadienoic acid (e.g., the 13-hydroperoxide affords 11,12-epoxides and/or 9,10-epoxides from epoxidation of the 9- and 11-double bonds). The epoxyoxooctadecenoic acids were ascertained to be both 12,13- and 9,10-epoxides by treatment with BF_3–MeOH followed by GC–MS of their methoxy-OTMSi derivatives as described previously (23). The products observed as a result of 13*S*-HPODE metabolism by alfalfa seedling extracts were remarkably similar to soybean, the only observable difference being the relative abundance of metabolites (data not shown). No evidence could be found for the previously reported γ-ketol (24). Also not detected in the product mixtures of either soybean or alfalfa seedlings were ketols from allene oxide synthase-catalyzed reactions. It is known from previous work that expression of allene oxide synthase in soybean seedlings is comparatively low (25).

When control extracts were incubated without added 13*S*-HPODE, it was interesting to find traces of 9-hydroxy-traumatin present. Based on the abundance of the principal fragment ion (*m/z* 157), the controls had at least 12-fold less 9-hydroxy-traumatin present at the expected GC retention time (Fig. 2). In one control 9-hydroxy-traumatin was not detected (data not shown). According to the literature (e.g., Ref. 7), the activity of hydroperoxide lyase, the enzyme that cleaves 13*S*-HPODE into 9*Z*-traumatin and hexanal, is improved by detergent. However, when seedlings were homogenized with 0.5% Triton X-100 in buffer, the production of 9-hydroxy-traumatin was not significantly increased, compared to the preparation without detergent (data not shown).

9-Hydroxy-traumatin was easily isolated by a two-step TLC procedure affording about 0.3 mg from a mixture obtained from a reaction using 150 mg 13*S*-HPODE catalyzed by a soybean seedling preparation. The first step involved TLC isolation of the methyl-esterified mixture, including 9-hydroxy-traumatin (methyl ester), using diethyl ether/hexane (7:3, vol/vol). After derivatization by OTMSi reagent, the chromatographic properties of methyl 9-OTMSi-traumatin were changed considerably from the other components of the mixture, permitting facile TLC isolation by hexane/diethyl ether (7:3, vol/vol). Since it is highly unlikely that the chromatographic properties of coeluting compounds would be identical after a specific derivatization, we have found this method of "chemical chromatography" to be useful. Depending on the functional groups present on the compound of interest, a variety of other reagents, such as $NaBH_4$ reduction, chromic acid oxidation etc., has been utilized in other research.

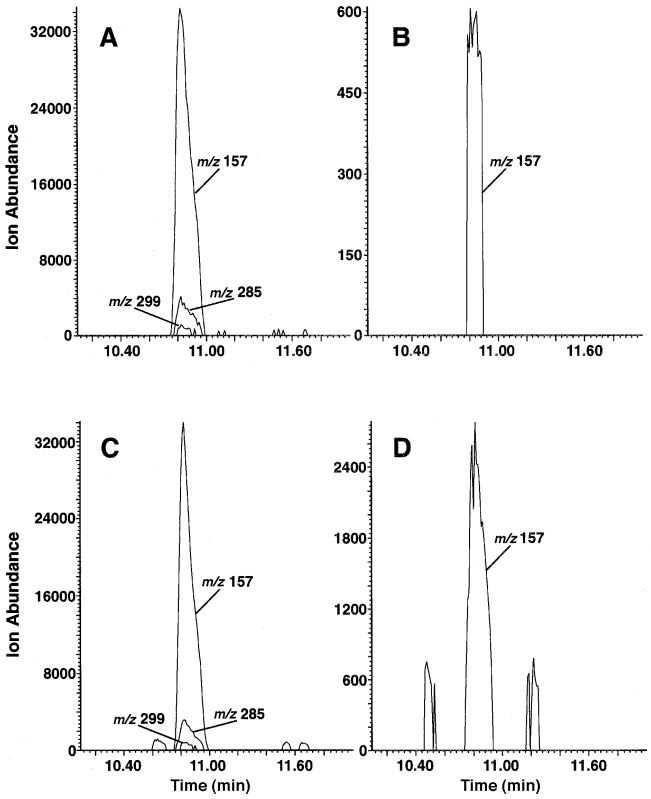

FIG. 2. GC–MS ion chromatograms (*m/z* 157 [M – $(CH_2)_7COOMe]^+$, 285 [M – CHO]$^+$, and 299 [M – Me]$^+$) showing the elution of 9-hydroxy-traumatin (as methyl ester/trimethylsilyloxy ether) with ion abundances illustrated. All treatments utilized supernatant preparations diluted 10-fold with buffer. Panel A, soybean seedling preparation treated with 1 mM 13*S*-HPODE; panel B, soybean supernatant preparation control without 13*S*-HPODE; panel C, alfalfa seedling preparation treated with 1 mM 13*S*-HPODE; panel D, alfalfa supernatant preparation control without 13*S*-HPODE. For abbreviations see Figure 1.

The methyl 9-OTMSi-traumatin isolated by "chemical chromatography" was examined by ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.109 (9H, *m*, H-9-OTMSi), 1.30 and 1.60 (12H, *m*, H-3 to H-8), 2.29 (2H, *t*, H-2), 3.66 (3H, *s*, H-OMe), 4.35 (1H, *dt*, H-9), 6.25 (1H, *ddd*, $J_{9,11}$ = 1.6 Hz, $J_{11,12}$ = 8 Hz, $J_{10,11}$ = 15.5 Hz, H-11), 6.78 (1H, *dd*, $J_{9,10}$ = 4.5 Hz, $J_{10,11}$ = 15.5 Hz, H-10), 9.56 (1H, *d*, $J_{11,12}$ = 8 Hz, H-12). The coupling, $J_{10,11}$ = 15.5 Hz, proved that the double bond was 10*E*.

The methyl ester/OTMSi derivative of 9-hydroxy-traumatin gave the following EIMS *m/z* (rel. int.): 314 [M]$^+$ (0.3), 299 [M – Me]$^+$ (2), 285 [M – CHO]$^+$ (10), 270 [M – CHO –Me]$^+$ (2), 267 [M – MeOH – Me]$^+$ (2), 230 (4), 157 [M –$(CH_2)_7COOMe]^+$ (100), 129 (17), 73 [trimethylsilyl]$^+$ (91). The OTMSi derivative was subjected to $NaBH_4$ reduction in MeOH followed by treatment with OTMSi reagent affording the bis OTMSi derivative with the following EIMS *m/z* (rel. int.): 373 [M – Me]$^+$ (0.7), 357 [M – MeO]$^+$ (2), 298 [M –TMSiOH]$^+$ (2), 285 [M – $CH_2OTMSi]^+$ (12), 259 [M –CH=$CHCH_2OTMSi]^+$ (3), 231 [M – $(CH_2)_7COOMe]^+$ (89), 147 (23), 73 [trimethylsilyl]$^+$

(100). The preceding $NaBH_4$-reduction product was further treated with H_2 and Pd on $CaCO_3$ catalyst in MeOH for 1 h. The hydrogenated product was treated with OTMSi reagent and subjected to EIMS *m/z* (rel. int.): 359 $[M - MeO]^+$ (0.7), 333 $[M - MeOH - Me]^+$ (2), 259 $[M - (CH_2)_3OTMSi]^+$ (56), 233 $[M - (CH_2)_7COOMe]^+$ (14), 143 $[M - (CH_2)_7COOMe - TMSiOH]^+$ (100), 73 $[trimethylsilyl]^+$ (68). In summary, the ^1H NMR data combined with GC–MS of various derivatives left little doubt that the original compound was 9-hydroxy-traumatin.

Although the pathway of 9-hydroxy-traumatin was not directly demonstrated, it is likely that 9*Z*-traumatin, the hydroperoxide lyase-catalyzed product, is the precursor. Conclusive proof must await the direct testing of 9*Z*-traumatin as a substrate. Because 9*Z*-traumatin readily isomerizes in most plant extracts into 10*E*-traumatin, it would be desirable to develop a synthetic method to produce the substrate of interest.

The oxidation is proposed to occur in a manner analogous to the conversion of 3*Z*-nonenal and 3*Z*-hexenal into 4-hydroxy-2*E*-nonenal and 4-hydroxy-2*E*-hexenal, respectively (Fig. 3). In the conversion of 3*Z*-nonenal to 4-hydroxy-2*E*-nonenal, the first step is oxygenation of 3*Z*-nonenal to 4-hydroperoxy-2*E*-nonenal by lipoxygenase (and possibly 3*Z*-alkenal oxygenase) (26,27). Although the oxidation of 3*Z*-nonenal is an n-6 oxidation typical of the reactions completed at alkaline pH values by soybean lipoxygenase-1 with its normal polyunsaturated fatty acid substrates, at neutral pH values soybean lipoxygenase-2 and -3, as well as lipoxygenase-1, are capable of using the carboxylic end of the molecule as a signal to oxygenate at C-9 (4). The second step involves a dual pathway, whereby, the 4-hydroperoxide is reduced to the 4-hydroxide by hydroperoxide-dependent peroxygenase. In the process of reduction, this enzyme also utilizes the oxidation potential of the hydroperoxide to oxidize 3*Z*-nonenal to 3,4-epoxynonanal, which undergoes rearrangement into a second molecule of 4-hydroxy-2*E*-nonenal (28). Examination of the stereochemistry of the 4-hydroxyl of 4-hydroxy-2*E*-nonenal showed that the product was nearly racemic (26,28), probably because of the dual nature of the biosynthetic pathway. However, it was shown that the first product, 4*S*-hydroperoxy-2*E*-nonenal, could be formed in fairly high chiral purity by oxidizing 3*Z*-nonenal with purified soybean lipoxygenase isozymes (26). The stereoconfiguration of the 9-hydroxyl of 9-hydroxy-traumatin was not examined in this study because it was not expected to be instructive considering the crude enzyme preparations utilized. Such preparations would

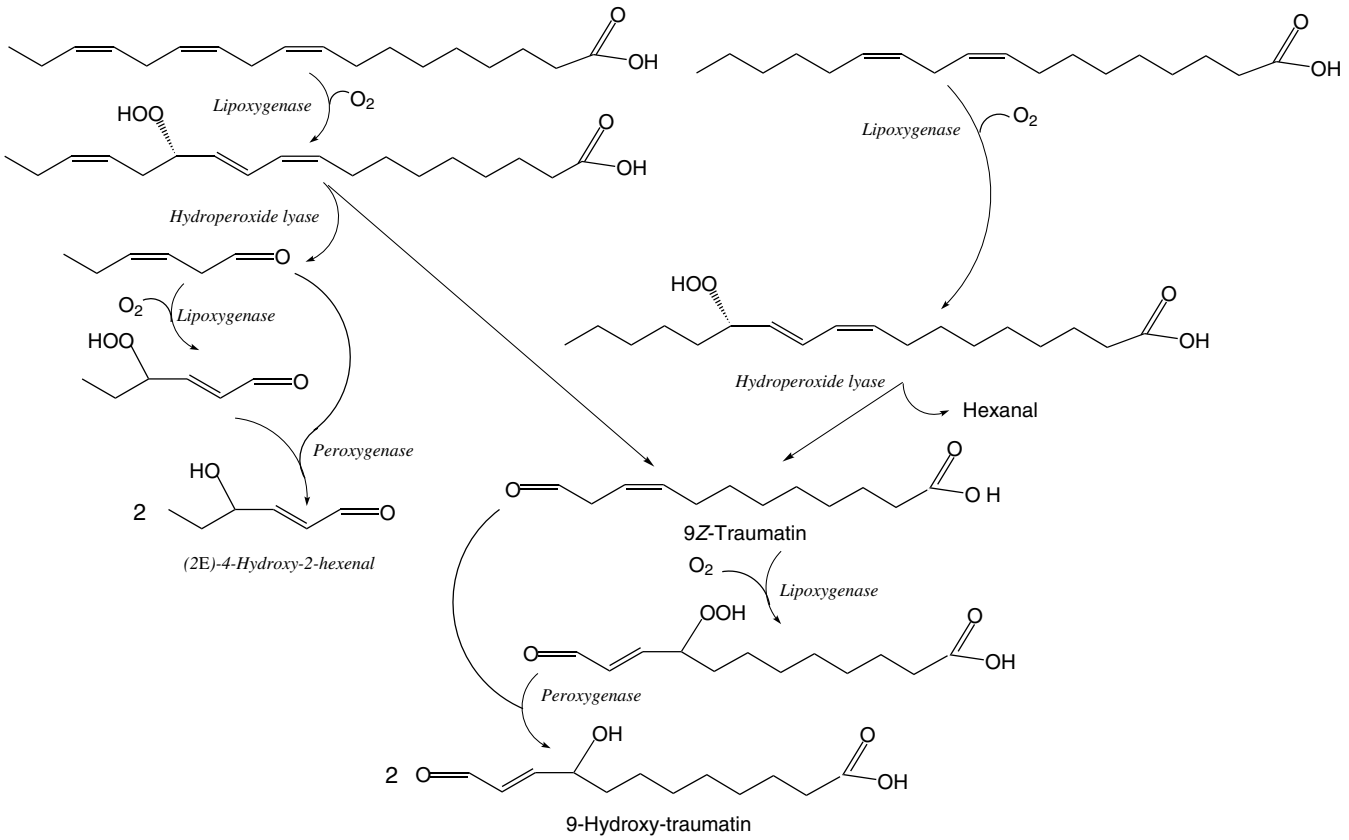

FIG. 3. Proposed reaction scheme of 9-hydroxy-traumatin biosynthesis from 9*Z*-traumatin, compared with known pathways in the formation of analogous hydroxylated aldehydes (not shown is the formation of 4-hydroxy-2*E*-nonenal from 3*Z*-nonenal originating from hydroperoxide lyase cleavage of the 9-hydroperoxide of linoleic acid). Peroxygenase performs a dual function of reducing the hydroperoxides formed from 9*Z*-traumatin, 3*Z*-hexenal or 3*Z*-nonenal, as well as epoxidizing a second molecule of 9*Z*-traumatin, 3*Z*-hexenal or 3*Z*-nonenal affording 9,10-epoxy-12-oxododecanoic acid, 3,4-epoxyhexanal or 3,4-epoxynonanal, respectively, which subsequently rearrange into a second molecule of hydroxylated product (28).

be expected to be composed of enzymes catalyzing the dual pathway, that is, oxygenase(s) and hydroperoxide-dependent peroxygenase.

The question of physiological significance is most interesting. Certainly, the oxidation product of 10*E*-traumatin, traumatic acid, as well as 10*E*-traumatin itself, have plant wound-healing properties. Currently, the physiological effect of 9-hydroxy-traumatin is unknown, but this compound should be a reactive molecule similar to its analog, 4-hydroxy-2-nonenal, which is known to have multiple effects on organisms, including responses known to be indicative of lipid signals (29–31).

ACKNOWLEDGMENTS

The author thanks M.J. Grove for diligent technical support and D. Weisleder for collection of ^{1}H NMR data.

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[Received June 4, 1998, and in final revised form and accepted July 14, 1998]

Rate Constants for Quenching Singlet Oxygen and Activities for Inhibiting Lipid Peroxidation of Carotenoids and α-Tocopherol in Liposomes

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ABSTRACT: The $^1O_2$ quenching rate constants (k_Q) of α-tocopherol (α-Toc) and carotenoids such as β-carotene, astaxanthin, canthaxanthin, and lycopene in liposomes were determined in light of the localization of their active sites in membranes and the micropolarity of the membrane regions, and compared with those in ethanol solution. The activities of α-Toc and carotenoids in inhibiting $^1O_2$-dependent lipid peroxidation (reciprocal of the concentration required for 50% inhibition of lipid peroxidation: $[IC_{50}]^{-1}$) were also measured in liposomes and ethanol solution and compared with their k_Q values. The k_Q and $[IC_{50}]^{-1}$ values were also compared in two photosensitizing systems containing Rose bengal (RB) and pyrenedodecanoic acid (PDA), respectively, which generate $^1O_2$ at different sites in membranes. The k_Q values of α-Toc were 2.9×10^8 $M^{-1}s^{-1}$ in ethanol solution and 1.4×10^7 $M^{-1}s^{-1}$ (RB system) or 2.5×10^6 $M^{-1}s^{-1}$ (PDA system) in liposomes. The relative $[IC_{50}]^{-1}$ value of α-Toc in liposomes was also five times higher in the RB system than in the PDA-system. In consideration of the local concentration of the OH-group of α-Toc in membranes, the k_Q value of α-Toc in liposomes was recalculated as 3.3×10^6 $M^{-1}s^{-1}$ in both the RB and PDA systems. The k_Q values of all the carotenoids tested in two photosensitizing systems were almost the same. The k_Q value of α-Toc in liposomes was 88 times less than in ethanol solution, but those of carotenoids in liposomes were 600–1200 times less than those in ethanol solution. The $[IC_{50}]^{-1}$ value of α-Toc in liposomes was 19 times less than that in ethanol solution, whereas those of carotenoids in liposomes were 60–170 times less those in ethanol solution. There were no great differences (less than twice) in the k_Q and $[IC_{50}]^{-1}$ values of any carotenoids. The k_Q values of all carotenoids were 40–80 times higher than that of α-Toc in ethanol solution but only six times higher that of α-Toc in liposomes. The $[IC_{50}]^{-1}$ values of carotenoid were also higher than that of α-Toc in ethanol solution than in liposomes, and these correlated well with the k_Q values.

Lipids 33, 751–756 (1998).

α-Tocopherol (α-Toc) and carotenoids have recently been implicated in the prevention of or protection against serious human health disorders such as cancer and heart disease. These effects have been attributed in part to their antioxidant properties.

Singlet oxygen ($^1O_2$) has aroused much interest as a biological oxidant. It is generated in a variety of biological systems and by photosensitization by the absorption of light. Many studies have shown that α-Toc and carotenoids scavenge $^1O_2$, but in most of these studies a homogeneous solution such as ethanol (EtOH), not membranes, has been used (1–6). It is important to know the antioxidant properties and activities of the compounds on $^1O_2$ in membranes, because these compounds are located in membranes in biological systems. Recently, we reported the kinetics and dynamics of $^1O_2$ scavenging by α-Toc in liposomes (7,8). Here we examined the $^1O_2$-scavenging rate constants (k_Q values) of carotenoids such as β-carotene (β-Car), astaxanthin, canthaxanthin, and lycopene in model membranes using phosphatidylcholine liposomes and compared them with that of α-Toc. $^1O_2$ was site specifically generated in liposomes by photosensitization with two photosensitizers; water-soluble Rose bengal (RB), which generates $^1O_2$ at the membrane surface, and lipid soluble pyrenedodecanoic acid (PDA), which becomes located in an inner region of the membranes where $^1O_2$ is generated. We also measured the inhibitory activities of carotenoids and α-Toc on $^1O_2$-dependent peroxidation of egg yolk phosphatidylcholine in liposomes and EtOH solution and compared the results with their k_Q values. Their antioxidant dynamics and kinetics in membranes were interpreted based on our results with respect to the membrane localizations of the antioxidants and the local concentrations and mobilities of their active moieties in the membranes.

MATERIALS AND METHODS

Materials. α-Toc was a gift from Eisai Co. (Tokyo, Japan). Astaxanthin and canthaxanthin were kindly provided by

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Abbreviations: BHT, butylhydroxytoluene; β-Car, β-carotene; DMPC, dimyristoylphosphatidylcholine; DPBF, 1,3-diphenyl-isobenzofuran; egg PC, egg phosphatidylcholine; EP, endoperoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IC_{50}, concentration at which 50% inhibition occurs; k_Q, rate constant for quenching $^1O_2$; $^1O_2$, singlet oxygen; PC-OOH, phosphatidylchline hydroperoxide; PDA, 12-(1-pyrene) dodecanoic acid; RB, Rose bengal; SA, stearylamine; α-Toc, α-tocopherol.

Hoffman-La Roche, Basel, Switzerland. β-Car was purchased from Sigma Chemical Co. (St. Louis, MO). Lycopene was supplied by Kagome Co. (Tochigi, Japan). Butylhydroxytoluene (BHT), 12-(1-pyrene)-dodecanoic acid (PDA), stearylamine (SA), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Dimyristoylphosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (egg PC) were products of Nippon Oil and Fats Co. (Tokyo, Japan). The fatty acid composition (mol%) of egg PC was 33.1 palmitate, 1.2 palmitoleate, 11.6 stearate, 30.0 oleate, 15.5 linoleate, 3.3 arachidonate, and 5.3 docosahexaenoate. Diethylenetriaminepentaacetic acid (DTPA) was from Nakalai Tesque Co. (Kyoto, Japan). RB was from Kanto Chemicals (Tokyo, Japan) and 1,3-diphenyl-isobenzofuran (DPBF) was from Tokyo Chemical Co., Tokyo. All other reagents were of analytical grade.

Preparation of liposomes and photosensitized oxidation. Liposomes were prepared as described previously (7). For studies on PDA-sensitized photooxidation, a solution of α-Toc, carotenoids, DMPC or egg PC in chloroform with or without SA, and PDA was evaporated with a stream of nitrogen under reduced pressure. For measurement of the rate constant of $^1O_2$ scavenging, a solution of DPBF in EtOH was further added to the resulting thin film, which was then re-evaporated. The thin film was dispersed in 10 mM HEPES buffer (pH 7.0) containing 0.5 mM DTPA in a vortex mixer and ultrasonicated in a Bransonic bath (Yamato, Tokyo, Japan) at 40°C for 2 min (positively charged liposomes) or 20 min (neutrally charged liposomes). For studies on RB-sensitized photooxidation, a solution of RB in HEPES buffer (pH 7.0) was added to the dispersed sample before sonication. The liposome suspension was placed at a distance of 4 cm from a 85-W halogen-tungsten lamp (Toshiba JD 100W 110V 85WN-E) and photoirradiated at 1564 lumen at 37°C.

Calculation of the $^1O_2$ scavenging rate constants (k_Q) *of carotenoids and α-Toc.* The k_Q values were determined by the method of Young *et al.* (9) by measuring the inhibition by carotenoids or α-Toc of photooxidation of DPBF, a specific $^1O_2$ trap. To exclude the participation of the lipid radical chain reaction, we used DMPC liposomes which are in a liquid crystalline state at 37°C. $^1O_2$ was generated at the membrane surface using RB in SA-DMPC liposomes, because negatively charged RB interacts at the membrane surface of these positively charged liposomes (8). PDA generated $^1O_2$ in the inner region of the membrane irrespective of the membrane charge (7). The calculated values of k_Q in DMPC liposomes were revised taking account of the local concentrations of reactants, all of which were assumed to be present in membranes.

Quantitation of hydroperoxides of egg PC (PC-OOH). The rate of lipid peroxidation was measured by quantitating PC-OOH formation, which was followed by HPLC with a ultraviolet-detector at 234 nm (10). After incubation, 20 μL of the reaction mixture was subjected to HPLC in a Hitachi L-6000 apparatus. A reversed-phase column (YMC-Pack A-212 C8; Yamamura Chemical Lab., 6 × 150 mm) was used and developed with methanol/H_2O/triethylamine (95:5:0.01, by vol) at a rate of 1 mL/min.

RESULTS AND DISCUSSION

Values for the rate constants (k_Q) of α-Toc and carotenoids for scavenging $^1O_2$ in DMPC liposomes and EtOH solution were measured by the technique of Young *et al.* (9) using Equations 1 and 2:

$$S_0/S_{\alpha\text{-}T} = 1 + (k_Q/k_d)\,[\alpha\text{-Toc}] \quad [1]$$

$$S_0/S_{Car} = 1 + (k_Q/k_d)\,[Car] \quad [2]$$

where S_0 and $S_{\alpha\text{-T}}$ or S_{Car} denote the slopes of first-order plots of disappearance of DPBF in the absence and presence of α-Toc or carotenoids. [Car] refers to the concentration of all carotenoids. The rate of deactivation of $^1O_2$ (k_d) is the rate constant for natural decay of $^1O_2$ to $^3O_2$. The k_d value of $^1O_2$ in liposomes has not yet been determined. We used the k_d values in EtOH solution (8.3×10^4 s^{-1}) (11) and *tert*-BuOH (3.0×10^4 s^{-1}) (12) for the RB system and PDA system, respectively, because $^1O_2$ is generated at a polar membrane surface in the RB system and a hydrophobic membrane inner region in the PDA system. The micropolarity of the environment around the OH-groups of α-Toc in liposomes close to the membrane surface was inferred to be equal to that in EtOH (13), in which the dielectric constant is higher than that in *tert*-BuOH. Table 1 shows the k_Q values for carotenoids and α-Toc in EtOH solution and liposomes. β-Car, astaxanthin, and canthaxanthin showed similar k_Q values. The k_Q of lycopene obtained in the photoirradiation systems (data not shown) was about 10 times less than that reported in the system using endoperoxides (EP) such as 3,3′-(1,4-naphthylidene)dipropionate (4), which is known to generate only $^1O_2$. Lycopene would be unstable on exposure to light. The k_Q values of carotenoids in liposomes in the RB system were recalculated using the k_d value in *tert*-BuOH, and were almost the same as those in the PDA system (Table 1). The active site of β-Car, which is at the center of the conjugated polyene chain, is reported to be localized in the hydrophobic region (14,15) where $^1O_2$ is also highly localized irrespective of its generation site because of its higher solubility in the hydrophobic region than in the polar region (16), resulting in similar k_Q values of carotenoids calculated using the k_d value in *tert*-BuOH in either photosensitizing system.

The k_Q values in liposome membranes were revised taking account of the concentrations of reactants in membranes. Their concentrations in liposome membranes were 242 (SA-DMPC liposomes) and 266 (DMPC liposomes) times those in the bulk phase, as calculated from the volume of membranes assuming that the volume of one DMPC molecule is 1253 Å^3 (17). The revised k_Q values are shown in Table 1.

The inhibitory effects of carotenoids and α-Toc on $^1O_2$-dependent lipid peroxidation in EtOH solution and liposomes were compared. The rate of PC-OOH formation was higher

TABLE 1
Rate Constants (k_Q) of α-Toc, Carotenoids and BHT for Scavenging $^1O_2$ Generated with RB, PDA, and EP in EtOH Solution and Liposomes[a]

	k_Q ($M^{-1}s^{-1}$)										
	EtOH				Liposomes						
					RB			PDA		Average[h]	EP
	RB	PDA	Average (RB + PDA)/2	EP Reported[b,c]	Apparent[d]	Apparent[e]	Rev.[f]	Apparent[e]	Rev.[g]	(RB + PDA)/2	Estimate[j]
α-Toc	3.6×10^8	2.1×10^8	2.9×10^8	3.0×10^{8b}	3.4×10^9		1.4×10^{7d}	6.6×10^8	2.5×10^6	3.3×10^{6i}	3.5×10^6
β-Car	1.3×10^{10}	1.0×10^{10}	1.2×10^{10}	1.4×10^{10b}	14.4×10^9	5.2×10^9	2.1×10^{7e}	4.5×10^9	1.7×10^7	1.9×10^7	2.3×10^7
Canthaxanthin	1.3×10^{10}	1.4×10^{10}	1.4×10^{10}	2.1×10^{10b}	12.5×10^9	4.5×10^9	1.8×10^{7e}	4.5×10^9	1.7×10^7	1.8×10^7	2.7×10^7
Astaxanthin	2.4×10^{10}	2.1×10^{10}	2.3×10^{10}	2.4×10^{10b}	12.7×10^9	4.6×10^9	1.9×10^{7e}	5.1×10^9	1.9×10^7	1.9×10^7	2.0×10^7
BHT	8.2×10^6			3.4×10^{6c}	9.7×10^7	3.5×10^7	1.4×10^{5e}				5.9×10^4

[a]The concentrations of carotenoids, α-Toc, and BHT were 2.5–15 μM, 25–100 μM, and 0.1–3 mM, respectively. The concentrations of DPBF, RB, and PDA were 250, 5, and 250 μM, respectively. The concentrations of other reactants in the liposome systems were 5 mM DMPC with or without 0.5 mM SA, and 10 mM HEPES buffer (pH 7.0) containing 0.5 mM DTPA. The EtOH solution and liposomes were photoirradiated for ~30 s, in RB system and ~8 min in PDA system at 37°C.
[b]Reference 4.
[c]Reference 5.
[d]Values calculated using the k_d value in EtOH (8.3×10^4 s^{-1}).
[e]Values calculated using the k_d value in *tert*-BuOH (3.0×10^4 s^{-1}).
[f]Values in liposomes revised considering that the local concentrations of reactants were 242 times higher than in the bulk phase in the RB system (SA-DMPC liposomes).
[g]Values in liposomes revised considering that the local concentrations of reactants were 266 times higher than in the bulk phase in the PDA system (DMPC liposomes).
[h]Averaged values of revised values for RB and PDA except the value of α-Toc.
[i]k_Q of α-Toc = k_Q (RB) × 4/34 × 100/50 or k_Q (PDA) × 20/34 × 100/50.
[j]k_Q (liposomes: EP) = k_Q (EtOH: EP) × k_Q (liposomes: Average)/ k_Q (EtOH: Averaged). Abbreviations: α-Toc, α-tocopherol; BHT, butyl hydroxytoluene; RB, Rose bengal; PDA, 12-(1-pyrene) dodecanoic acid; EP, endoperoxide; β-car, β-carotene; DPBF, 1,3-diphenyl-isobenzofuran; DMPC, dimyristoyl-phosphatidylcholine; SA, stearylamine; HEPES, *N*-2-hydroxyethylpiperazone-*N*′-2-ethanesulfonic acid; DTPA, diethylene triaminepentaacetic acid; k_Q, rate constant for quenching $^1O_2$.

in liposomes than in EtOH solution (data not shown) as reported previously (7). α-Toc and carotenoids inhibited the formation of egg PC-OOH by photoirradiation in a concentration dependent manner. Their concentrations for 50% inhibition of the initial rate of lipid peroxidation (IC_{50}) were measured in EtOH solution and liposomes. Table 2 shows the activities of the compounds for inhibiting $^1O_2$-dependent lipid peroxidation as reciprocals of IC_{50} value: $[IC_{50}]^{-1}$. Higher $[IC_{50}]^{-1}$ values indicate higher antioxidant activity. β-Car, astaxanthin, and canthaxanthin showed similar $[IC_{50}]^{-1}$ values. The order of lipid peroxidation-inhibiting activities was: all carotenoids examined > α-Toc > BHT in both EtOH solution and liposomes. These values were well correlated with the order of their k_Q values. The $[IC_{50}]^{-1}$ value of lycopene (data not shown) was about 10-fold less than those of other carotenoids, like its k_Q value, possibly because of its lability to light.

TABLE 2
Activities of α-Toc, Carotenoids, and BHT for Inhibiting Lipid Peroxidation Induced by $^1O_2$ Generated with RB and PDA in EtOH Solution and Liposomes[a]

	$[IC_{50}]^{-1}$ (10^3 M^{-1})				
	EtOH	Liposomes			
	RB system	RB system		PDA system	
		Apparent	Rev.[b]	Apparent	Rev.[c]
α-Toc	13.5	172	0.71	196	0.74
β-Car	83.3	333	1.38	1477	5.6
Canthaxanthin	236	333	1.38	2933	11.0
Astaxanthin	238	476	1.97	2960	11.1
BHT	0.063	4.5	0.019	6.9	0.026

[a]The concentrations of carotenoids, α-Toc, and BHT were 0.1–50 μM, 0.5–200 μM, and 0.025–50 mM, respectively. The concentrations of RB and PDA were 5 and 250 μM, respectively. The concentrations of other reactants in the liposome systems were 5 mM PC with or without 0.5 mM SA, and 10 mM HEPES buffer (pH 7.0) containing 0.5 mM DTPA. The EtOH solution and liposomes were photoirradiated for ~60 and ~6 min, respectively, in RB system, and ~180 min in PDA system at 37°C.
[b]Revised values in SA-egg liposomes calculated assuming that the concentration of antioxidants in the membrane phase was 242 times that in the bulk water phase.
[c]Revised values in egg liposomes calculated assuming that the concentration of antioxidants in the membrane phase was 266 times that in the bulk water phase. egg PC, egg phosphatidylcholine; IC_{50}, concentration at which 50% inhibition occurs; for other abbreviations see Table 1.

TABLE 3
Ratios of the k_Q Values and the Relative Activities for Inhibiting Lipid Peroxidation in the RB System to Those in the PDA System in EtOH Solution and Liposomes

	RB system/PDA system		
	Ratio of k_Q value		Ratio of activity for inhibiting lipid peroxidation[a]
	EtOH	Liposomes	Liposomes
α-Toc	1.7	5.6	5.4
β-Car	1.3	1.2	1.4
Canthaxanthin	0.93	1.1	0.71
Astaxanthin	1.1	1.0	1.0

[a]Activity relative to that of astaxanthin: $[IC_{50}]^{-1}/[IC_{50}]^{-1}$(astaxanthin). For abbreviations see Tables 1 and 2.

Table 3 shows the ratios of the k_Q values in the RB system to those in the PDA system for α-Toc and carotenoids and their relative activities for inhibiting lipid peroxidation in EtOH solution and liposomes. There was little difference in the k_Q values in the RB system and PDA system in EtOH solution. But in liposomes, the k_Q value for α-Toc in the RB system was about five times greater than that in the PDA system, although the k_Q values for carotenoids were almost the same in the two photosensitizing systems. The relative activity of α-Toc in inhibiting lipid peroxidation in liposomes in the RB system was also five times greater than that in the PDA system but there was no appreciable difference in the activities of carotenoids for inhibiting lipid peroxidation in the RB system and PDA system. These results were well correlated with results on the k_Q values in the RB and PDA systems. The OH-groups of α-Toc are mainly localized in the region close to the membrane surface (18–22), so their concentration in this region, where $^1O_2$ is generated by RB, is higher than their concentration postulated by assuming that they are uniformly distributed in the membrane. This would explain the relatively higher k_Q value in the RB system (1.4×10^7 $M^{-1}s^{-1}$). On the contrary, PDA generates $^1O_2$ in the hydrophobic region where the concentration of the OH-groups of α-Toc is lower than that postulated as described above, resulting in relatively lower k_Q values in the PDA system ($2.5 \times 10^6 M^{-1}s^{-1}$). Takahashi *et al.* (19) reported that α-Toc scavenges lipid radicals close to the membrane surface more effectively than those in deep regions of the bilayer membrane and also effectively scavenges radicals attacking from outside the membranes. On the contrary, the quenching sites of carotenoids are localized in the hydrophobic region (14,15) where the concentration of $^1O_2$ is also high irrespective of its generation site, because $^1O_2$ is more soluble in the hydrophobic region than in the polar region (16). Thus, the k_Q values of all carotenoids were similar irrespective of the photosensitizer.

The k_Q value of α-Toc in liposomes was recalculated as 3.3×10^6 $M^{-1}s^{-1}$ in both the RB and PDA systems from Equation 3 in consideration of the local concentrations of the OH-groups of α-Toc in membranes, which were assumed to be about 0, 50, and 50% in the polar zone (10 Å), hydrogen belt (4 Å), and hydrophobic core (20 Å) of DMPC liposomes, respectively (18–22).

$$S_0/S_{\alpha\text{-T}} = 1 + (k_Q/k_d)\ [\text{OH-group of } \alpha\text{-Toc}] \quad [3]$$

where $[\text{-OH group of } \alpha\text{-Toc}]_{RB} = [\alpha\text{-Toc}] \cdot [(10 + 4 + 20)/4] \cdot [50/100]$ and $[\text{-OH group of } \alpha\text{-Toc}]_{PDA} = [\alpha\text{-Toc}] \cdot [(10 + 4 + 20)/20] \cdot [50/100]$. The recalculated k_Q values for α-Toc may be similar in the RB and PDA systems, because the OH-groups of α-Toc in the region closed to the membrane surface are supposed to have higher reactivity but lower mobility than those in the inner membrane region from consideration of the findings (i) that the k_Q value of α-Toc in homogeneous solution is higher in solvents with higher dielectric constants (7,18) and (ii) that the chromanol moiety of α-Toc is near the membrane surface and its mobility is restricted, possibly by hydrogen bonding of its OH-groups with ester carbonyl moieties of membrane PC (18, 23).

The revised k_Q values in liposomes for α-Toc and carotenoids were 88 and 600–1200 times lower, respectively, than those in EtOH solution (Table 4). The lower k_Q values in liposomes than in EtOH solution may have been due to the lower diffusion rates of reactants in membranes than in EtOH solution and to their lateral diffusion in membranes but their three-dimensional diffusion in EtOH solution.

The k_Q values of carotenoids were about 40–80 times that of α-Toc in EtOH solution, but only six times that of α-Toc in liposomes (Table 4). The mobilities of carotenoids would be suppressed more than that of α-Toc in membranes, resulting in greater decreases in their k_Q values than that of α-Toc in liposomes than in EtOH solution. The $[IC_{50}]^{-1}$ values for carotenoids were also decreased more than that for α-Toc in liposomes than in EtOH solution (Table 5), consistent with results on their k_Q values. The membrane structure may inhibit the interaction of $^1O_2$ with carotenoids more strongly than with α-Toc and BHT, resulting in greater reduction in k_Q and $[IC_{50}]^{-1}$ values for carotenoids than for α-Toc and BHT in the membrane system.

The order of the ratios of $[IC_{50}]^{-1}$ values in liposomes to those in EtOH solution, that is, $[IC_{50}]^{-1}$ (liposomes)/$[IC_{50}]^{-1}$ (EtOH), was BHT (radical scavenger) > α-Toc (radical scavenger and $^1O_2$ quencher) > carotenoids ($^1O_2$ quencher) (Table 5). Some radical-type active oxygens generated by the minor reaction (Type I action) of RB photoirradiation would participate in the progress of lipid peroxidation more in liposomes

TABLE 4
Ratios of k_Q Values in Liposomes to Those in EtOH Solution and Those of Carotenoids to Those of α-Toc

	Ratio of k_Q value		
	Liposomes/EtOH	Carotenoid/α-Toc	
		EtOH	Liposomes
α-Toc	1/88[a,b]	1[b]	1.0[a]
β-Car	1/632[b]	41[b]	5.8[b]
Canthaxanthin	1/778[b]	48[b]	5.6[b]
Astaxanthin	1/1211[b]	79[b]	5.8[b]
BHT	1/58[b]		

[a]k_Q of α-Toc in liposomes: 3.3×10^6 $M^{-1}s^{-1}$.
[b]Average k_Q values in the RB and PDA systems. For abbreviations see Tables 1 and 2.

TABLE 5
Ratios of the $[IC_{50}]^{-1}$ Values of Carotenoids to Those of α-Toc and Those in Liposomes to Those in EtOH Solution[a]

	Ratio of $[IC_{50}]^{-1}$ Value (RB system)		
	Liposomes/EtOH	Carotenoid/α-Toc	
		EtOH	Liposomes
α-Toc	1/19	1.0	1.0
β-Car	1/60	6.2	1.9
Canthaxanthin	1/171	17.1	1.9
Astaxanthin	1/121	17.6	2.8
BHT	1/3.3		

[a]For abbreviations see Tables 1 and 2.

than in EtOH solution, because PC molecules are ordered and packed in membranes that are suitable for progression of the radical chain reaction.

Since photoirradiation systems are reported to generate small amounts of active oxygens other than $^1O_2$, we attempted to estimate the liposomal k_Q values of α-Toc and carotenoids in the EP system, which generates only $^1O_2$. The k_Q values for α-Toc and carotenoids have been measured by the EP method in EtOH solution (4,5) but not in liposomes because EP is heat-labile and so cannot be used in the liposome system. We estimated the liposomal k_Q values in the EP system using the reported k_Q value in EtOH solution in the EP system (4,5) from Equation 4,

$$k_Q\ (\text{liposome: EP}) = k_Q(\text{EtOH: EP}) \times \frac{k_Q\ (\text{liposome: Average})}{k_Q\ (\text{EtOH: Average})} \quad [4]$$

where k_Q (EtOH: EP) is the reported k_Q value in EtOH solution in the EP system (4,5), and k_Q (liposome: Average) and k_Q (EtOH: Average) are the averaged k_Q values obtained by photoirradiation with RB and PDA in liposomes and EtOH solution, respectively, except that we took the k_Q (liposome: Average) for α-Toc as $3.3 \times 10^6\ M^{-1}s^{-1}$(Table1). The estimated k_Q values in liposomes in the EP system shown in Table 1 were about 90 (α-Toc) and 600–1200 (carotenoids) times less than those in EtOH solution.

The k_Q values for quenching $^1O_2$ in membranes of α-Toc and carotenoids and their activities for inhibiting $^1O_2$-dependent lipid peroxidation obtained in this study reflect their antioxidant properties in biological systems better than those in homogeneous solution. Considerations of the antioxidant properties and functions of α-Toc and carotenoids suggest that their physicochemical behaviors in membranes may be particularly important.

We show for the first time that the following factors are important in influencing the k_Q and $[IC_{50}]^{-1}$ values of α-Toc and carotenoids in membranes: (i) the concentrations of antioxidants, which are concentrated in membranes, (ii) the membrane localizations of active groups of antioxidants and their local concentrations in membranes, (iii) the solubility and generation site of $^1O_2$ in membranes, depending on the localization of photosensitizers in the surface or inner region, and (iv) the mobility of antioxidants in membranes, which is lower than that in EtOH solution.

ACKNOWLEDGMENTS

The authors thank Dr. Akira Shibata of Faculty of the Pharmaceutical Sciences, Tokushima University, for useful discussion. This study was supported by a special funding from Tokushima University.

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[Received February 3, 1998, and in final revised form June 10, 1998; revision accepted June 17, 1998]

Dietary Oxidized Cholesterol Modulates Cholesterol Metabolism and Linoleic Acid Desaturation in Rats Fed High-Cholesterol Diets

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ABSTRACT: The interactive effect of high dietary levels of oxidized cholesterol on exogenous cholesterol and linoleic acid metabolism was examined in male 4-wk-old Sprague-Dawley rats given high-cholesterol diets. The rats were pair-fed purified diets free of or containing either 0.5% cholesterol alone or both 0.5% cholesterol and 0.5% oxidized cholesterol mixture (containing 93% oxidized cholesterol) for 3 wk. Hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity was reduced in rats given cholesterol alone or both cholesterol and oxidized cholesterol. However, hepatic cholesterol 7α-hydroxylase activity was lowered only when rats were given both cholesterol and oxidized cholesterol, although dietary cholesterol increased this activity. Reflecting this effect, acidic steroid excretion was lowest among the groups of rats given cholesterol and oxidized cholesterol. On the other hand, the activity of hepatic Δ6 desaturase, a key enzyme in the metabolism of linoleic acid to arachidonic acid, was increased in rats given both cholesterol and oxidized cholesterol, although dietary cholesterol alone lowered its activity. As a result, the Δ6 desaturation index, 20:3n-6 + 20:4n-6/18:2n-6, in liver and serum phospholipids tended to be higher in the group fed both cholesterol and oxidized cholesterol than in the one fed cholesterol alone. Thus, dietary oxidized cholesterol significantly modulated exogenous cholesterol metabolism and promoted linoleic acid desaturation even when it was given at high levels together with a high cholesterol diet.

Lipids 33, 757–764 (1998).

Recently, it has been shown that endogenous oxidized cholest-5-en-3β-ol (cholesterol derivatives) are formed during oxidative modification of low density lipoprotein (1–3). Some of these may be sources of oxidized cholesterol in the walls of arteries, because oxidized low density lipoprotein is taken up by macrophages *via* scavenger receptors. Many oxidized cholesterol derivatives are biologically deleterious agents potentially influencing vital physiological and biochemical processes. There is evidence from *in vivo* studies that oxidized cholesterol exhibits toxic actions (4). On the other hand, cholesterol in various common foods is also susceptible to reactions with oxygen under mild conditions, yielding a wide range of oxidation products in high levels during processing and storing of foods. In fact, high levels of oxidized cholesterol have been detected in various processed foods such as egg (total level of oxidized cholesterol, 1.3–4151 μg/g), dairy (0.19–1037 μg/g), meat (0.6–4878 μg/g), marine fish (5.2–287 μg/g) and miscellaneous processed (1.1–147.8 μg/g) products (5). Therefore, oxidized cholesterol in tissues could be partly exogenously absorbed from the diet. Peng *et al.* (6) assumed that the rate of absorption of oxidized cholesterols is not significantly different from that of cholesterol. We also observed that approximately 30% of oxidized cholesterol was absorbed into lymph when oxidized cholesterol was orally administered in rats (7). In addition, numerous studies showed that oxidized sterols influence specific key enzymes in cholesterol and fatty acid metabolism in *in vitro* bioassay (4). On the other hand, we observed dietary oxidized cholesterol disturbed growth and modulated cholesterol and fatty acid metabolism in *in vivo* studies (8,9).

Thus, dietary oxidized cholesterol appears to specifically modify various parameters of lipid metabolism, although dietary cholesterol also affects cholesterol and linoleic acid metabolism. Notwithstanding the significance of deleterious activities of oxidized cholesterol derivatives, only limited information concerning the interactive effect of dietary oxidized cholesterol on exogenous cholesterol and linoleic acid metabolism *in vivo* is available, particularly during consumption of large amounts of it. Therefore, we sought to know how high dietary levels of oxidized cholesterol modulate exogenous cholesterol and linoleic acid metabolism in rats fed high-cholesterol diet, because commonly consumed foods contain oxidized cholesterol along with unoxidized cholesterol.

MATERIALS AND METHODS

Chemicals. 3-Hydroxy-3-methyl[1-^{14}C]glutaryl-coenzyme A (HMG-CoA; 55 mCi) was purchased from Amersham Inter-

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Abbreviations: Chol, 0.5% cholesterol added; cholesterol, cholest-5-en-3β-ol; Chol-free, cholesterol-free; Chol + OxChol, both 0.5% cholesterol and 0.5% oxidized cholesterol-added; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 7β-hydroxycholesterol, cholest-5-en-3β, 7β-diol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TBARS, thiobarbituric acid-reactive substances.

national, plc, (Buckinghamshire, United Kingdom). [1-^{14}C]Linoleic acid (53 mCi/mmol) and [4-^{14}C] cholesterol (51 mCi/mmol) were produced by New England Nuclear, (Boston, MA), and purified by thin-layer chromatography as described previously (9) prior to use.

Animals and diets. Twenty-four male Sprague-Dawley rats (4-wk old), purchased from Seiwa Experimental Animals (Fukuoka, Japan) and housed individually in stainless steel mesh cages, were given a nonpurified diet (type NMF; Oriental Yeast Co., Tokyo, Japan) and acclimated for 4 d in an air-conditioned room with controlled temperature and reversed light and dark cycle (20–23°C and lights on 1700 to 0500). Rats were then divided into three groups of six rats each; one group was fed a cholesterol-free diet (Chol-free group), the second was fed a diet with 0.5% added cholesterol alone, (Chol group) and the third was fed a diet with a mixture of both 0.5% cholesterol and 0.5% oxidized cholesterol mixture (Chol + Ox Chol group). Diets were prepared according to the recommendations of the American Institute of Nutrition (10), and contained by weight percentage: casein, 20; safflower oil (Rinoru Oil Co., Nagoya, Japan), 5; mineral mixture (AIN76), 3.5; vitamin mixture (AIN76), 1.0; choline bitartrate, 0.2; DL-methionine, 0.3; cellulose, 5.0; cornstarch, 15; sodium cholate, 0.25; and sucrose to 100. Cholesterol (0.5%) or both cholesterol (0.5%) and oxidized cholesterol mixture (0.5%) were added at the expense of sucrose. An oxidized cholesterol mixture was prepared from pure cholesterol by heating as described previously (9). The preparation contained by weight percentage: cholesterol, 8.2; cholest-5-en-3β,7α-diol, 5.5; cholest-5-en-3β,7β-diol, 18.2; 5,6α-epoxy-5α-cholestan-3β-ol, 2.9; 5,6β-epoxy-5β-cholestan-3β-ol, 9.0; 5α-cholestan-3β,5,6β-triol, 5.7; 3β-hydroxycholest-5-en-7-one, 22.0; cholest-5-en-3β,25-diol, 3.8; and unknown oxidized sterols, 23.5. Experimental diets were prepared weekly, packed in a pouch containing an oxygen adsorbent and stored at 4°C to avoid peroxidation. Diets were changed every day, and body weight and food intake were recorded daily. The Chol-free and Chol groups were given diets at the level consumed by the Chol + Ox Chol group because dietary oxidized cholesterol caused a significant reduction in food intake. After 3 wk, rats were killed by decapitation at 1000 for the measurement of liver enzyme activities and serum and liver lipids. The liver was excised immediately, frozen with liquid nitrogen, and kept at −80°C until analyzed. Blood serum was prepared by centrifugation after allowing blood to clot at room temperature. Feces were collected for 2 d, beginning 10 d before killing, and lyophilized.

Analyses. The activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and Δ6 desaturase of liver microsomes were measured according to the procedure reported previously (9). The concentration of microsomal protein was determined by the method of Lowry *et al*. (11). Total liver and serum lipids were extracted by method of Folch *et al*. (12) and analyzed for the concentrations of cholesterol, triglyceride, and phospholipids (13). Serum high density lipoprotein (HDL)-cholesterol was measured using a commercially available kit (HDL C-test, Wako Pure Chemicals, Osaka, Japan). Liver and blood lipoperoxides were also analyzed by the methods of Yagi (14) and Ohkawa *et al.* (15) as thiobarbituric acid-reactive substances (TBARS), although complete lipoperoxide values were not always shown by this method. The fatty acid compositions of liver and serum phospholipids were analyzed as their methyl esters by gas–liquid chromatography (Shimadzu gas chromatograph GC-8APF, Kyoto, Japan) on 10% Silar 10C column (3 mm × 2 m) after phosphatidylcholine (PC) and phosphatidylethanoleamine (PE) were separated by thin-layer chromatography (16) and derivatized. The concentration and composition of acidic steroids excreted in feces were also analyzed by gas–liquid chromatography using a 3% AN-600 column (3 mm × 2 m) as described previously (17). The concentrations of oxidized cholesterol in liver, serum, and feces were quantified by capillary gas–liquid chromatography (Shimadzu gas chromatograph GC-14A) using ULBON HR-1 column (0.25 mm × 50 m, liquid phase thickness, 25 μm; Shinwa Chemical Industries, Ltd., Kyoto, Japan) under the conditions described previously (18).

Statistical analysis. Data were expressed as mean ± SE of six animals. The exact nature of the differences ($P < 0.05$) among the groups was determined by Duncan's new multiple-range test (19).

RESULTS

Growth parameters and liver weight. No statistically significant differences among groups were observed in body weight gain according to the type of diet (Table 1). However, the relative liver weight at the time of killing was significantly higher in the Chol group than in the Chol-free or Chol + Ox Chol group.

Concentrations of liver and serum lipids. The concentration of liver cholesterol was significantly lower in the Chol + Ox Chol group than in the Chol group, although its level was significantly higher than that of Chol-free group (Table 2). The liver triglyceride level in the Chol + Ox Chol group significantly decreased compared to the Chol group, and its level tended to be lower than for the Chol-free group. Contrary to these effects, the concentration of liver phospholipids was significantly higher in the Chol + Ox Chol group than in the Chol group, although its level was comparable to that of Chol-free group. Reflecting such modulation, the cholesterol/phospholipid ratio in the Chol + Ox Chol group (1.85 ± 0.13) was significantly lower than in the Chol group (3.78 ± 0.19), although the ratio was significantly higher than for the Chol-free group (0.34 ± 0.07).

On the other hand, the diet containing both cholesterol and oxidized cholesterol, as compared to cholesterol alone, significantly lowered the concentration of serum cholesterol (Table 3). Moreover, the concentration of serum HDL-cholesterol was significantly lowered in both the Chol and the Chol + Ox Chol groups compared with the Chol-free group, and its level tended to be lower in the Chol + Ox Chol group

TABLE 1
Effects of Dietary Oxidized Cholesterol on Growth Parameters in the Rats Given High-Cholesterol Diet[a]

Groups	Initial body wt (g)	Weight gain (g/10 d)	Food intake (g/d)	Liver weight (g/100 g body weight)
Chol-free	113 ± 1	158 ± 8	18.1 ± 0.6	5.41 ± 0.21[b]
Chol	113 ± 1	171 ± 2	18.4 ± 0.2	6.93 ± 0.09[c]
Chol + Ox Chol	113 ± 1	162 ± 1	17.9 ± 0.3	5.80 ± 0.07[b]

[a]Data are presented as mean ± SE for six rats in each group.
[b,c]Values without a common superscript letter are significantly different at $P < 0.05$. Abbreviations: Chol-free, cholesterol-free diet; Chol, 0.5% cholesterol diet; Chol + Ox Chol, both 0.5% cholesterol and 0.5% oxidized cholesterol diet.

TABLE 2
Effect of Dietary Oxidized Cholesterol on Liver Lipid Concentrations in the Rats Given High-Cholesterol Diets[a]

Groups	Total cholesterol (mg/g)	Triglyceride (mg/g)	Phospholipid (mg/g)
Chol-free	5.68 ± 0.81[b]	68.2 ± 10.6[b,c]	16.8 ± 0.4[c]
Chol	57.8 ± 2.1[d]	76.6 ± 11.3[c]	15.3 ± 0.4[b]
Chol + Ox Chol	32.3 ± 2.2[c]	43.9 ± 3.8[b]	17.5 ± 0.2[c]

[a]Data are presented as mean ± SE for six rats in each group.
[b–d]Values without a common superscript letter are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 3
Effect of Dietary Oxidized Cholesterol on Serum Lipid Concentrations in the Rats Given High-Cholesterol Diets[a]

Groups	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	Triglyceride (mg/dL)	Phospholipid (mg/dL)
Chol-free	97.7 ± 5.2[c]	62.5 ± 1.5[d]	154 ± 21	111 ± 8
Chol	194 ± 12[d]	47.2 ± 4.1[b]	146 ± 25	92.6 ± 12.2
Chol + Ox Chol	142 ± 14[d]	39.1 ± 2.6[b]	126 ± 5	84.2 ± 10.1

[a]Data are presented as mean ± SE for six rats in each group.
[b–d]Values without a common superscript letter are significantly different at $P < 0.05$. For abbreviations see Table 1.

than the Chol group. However, the HDL-cholesterol/total cholesterol ratio was same for the Chol + Ox Chol and the Chol groups (Chol-free, 64 ± 3; Chol, 24 ± 3; Chol + Ox Chol 28 ± 2%). Although a significant difference was not observed in the concentrations of serum triglyceride and phospholipids, the concentrations in the Chol + Ox Chol group tended to be lower than in both the Chol-free and Chol groups.

Key enzyme activities of liver microsomes. Figure 1 shows the specific activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and Δ6 desaturase of liver microsomes. The HMG-CoA reductase activities in both the Chol and Chol + Ox Chol groups were significantly lower than that in Chol-free group; however, there was no difference between the Chol and the Chol + Ox Chol groups, although the activity tended to be lower in the Chol + Ox Chol group than in the Chol group. The cholesterol 7α-hydroxylase activity was significantly higher in both the Chol and the Chol + Ox Chol groups than in the Chol-free group; however, the activity in Chol + Ox Chol group was significantly lower than in the Chol group. On the other hand, the Δ6 desaturase activity in the Chol or the Chol + Ox Chol group was significantly decreased compared to the Chol-free group; however, the activity in the Chol + Ox Chol group was significantly higher than in the Chol group.

Fatty acid profiles of liver. Table 4 shows the fatty acid compositions of liver phospholipids. Dietary cholesterol significantly increased the proportion of palmitoleic, oleic, dihomo-γ-linolenic, and linoleic acids and decreased those of stearic acid in PC and PE. Arachidonic acid in PE rather increased while decreasing in PC; docosahexaenoic acid in PE was not significantly altered while decreasing in PC. Reflecting the modulation in the fatty acid composition, the ratio of Δ6 desaturation index (20:3n-6 + 20:4 n-6)/18:2 n-6 was sig-

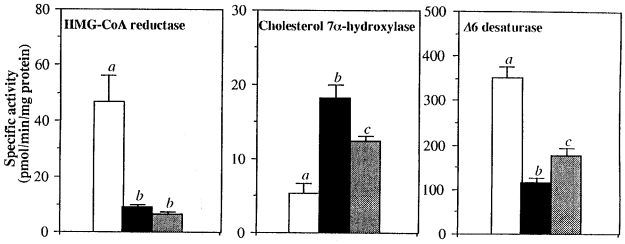

FIG. 1. Effect of dietary oxidized cholesterol on the activities of key enzymes in cholesterol metabolism and linoleic acid desaturation in rats given high-cholesterol diets. Values are presented as mean ± SE for six rats in each group. Values without the same letter are significantly different at $P < 0.05$. Open bars: cholesterol-free dietary group; solid bars, 0.5% cholesterol-added grouip; grey bar, both 0.5% cholesterol and 0.5% oxidized cholesterol mixture-added group.

TABLE 4
Fatty Acid Compositions of Liver and Serum Lipids[a]

Groups	Fatty acids (wt%)								Ratio
	16:0	16:1	18:0	18:1	18:2n-6	20:3n-6	20:4n-6	22:6n-3	(20:3 + 20:4)/18:2
Liver									
Phosphatidylcholine									
Chol-free	21.4 ± 0.7^{c}	3.4 ± 0.4^{b}	11.4 ± 1.2^{c}	8.1 ± 0.3^{b}	11.8 ± 0.4^{b}	0.6 ± 0.0^{b}	34.6 ± 0.9^{c}	5.3 ± 0.6^{c}	3.0 ± 0.1^{d}
Chol	19.9 ± 0.4^{c}	5.7 ± 0.3^{d}	8.2 ± 0.3^{b}	12.9 ± 0.6^{d}	18.5 ± 0.9^{d}	2.7 ± 0.3^{d}	28.6 ± 1.4^{b}	1.6 ± 0.2^{b}	1.7±0.2^{b}
Chol + Ox Chol	18.0 ± 0.3^{b}	4.6 ± 0.3^{c}	10.7 ± 1.1^{bc}	9.9 ± 0.4^{c}	15.7 ± 0.6^{c}	1.6 ± 0.2^{c}	34.0 ± 0.8^{c}	2.9 ± 0.2^{c}	2.3 ± 0.1^{c}
Phosphatidylethanolamine									
Chol-free	20.9 ± 0.7^{c}	0.3 ± 0.0^{b}	14.2 ± 1.0^{c}	6.0 ± 0.4^{b}	3.8 ± 0.5^{b}	0.4 ± 0.0^{b}	36.6 ± 1.0^{b}	14.1 ± 0.6^{d}	10.3 ± 0.9^{c}
Chol	17.6 ± 0.4^{b}	2.2 ± 0.2^{d}	11.1 ± 1.1^{b}	10.8 ± 0.8^{c}	7.7 ± 0.4^{d}	1.3 ± 0.2^{c}	40.9 ± 0.9^{c}	4.5 ± 0.5^{b}	5.6 ± 0.3^{b}
Chol + Ox Chol	18.2 ± 0.7^{b}	1.3 ± 0.1^{c}	13.4 ± 1.3^{c}	9.5 ± 0.9^{c}	5.6 ± 0.6^{c}	0.9 ± 0.2^{c}	39.3 ± 1.8^{bc}	8.2 ± 0.5^{c}	7.6 ± 1.0^{b}
Serum									
Phosphatidylcholine									
Chol-free	24.6 ± 1.7	1.1 ± 0.2^{b}	15.4 ± 1.2^{c}	8.6 ± 1.1^{b}	16.5 ± 1.5^{b}	0.7 ± 0.0^{b}	24.4 ± 3.4	0.2 ± 0.1	1.7 ± 0.3
Chol	22.3 ± 1.1	2.5 ± 0.2^{c}	11.6 ± 1.0^{b}	13.3 ± 1.2^{c}	26.5 ± 0.9^{d}	2.6 ± 0.4^{d}	21.7 ± 2.1	0.2 ± 0.1	1.1 ± 0.1
Chol + Ox Chol	21.7 ± 1.1	2.8 ± 0.3^{c}	10.7 ± 0.9^{b}	10.9 ± 1.3^{bc}	20.7 ± 1.5^{c}	1.3 ± 0.2^{c}	26.6 ± 3.7	0.1 ± 0.0	1.4 ± 0.3

[a]Data are presented as mean ± SE for six rats in each group.
[b–d]Values without a common superscript letter are significantly different at $P < 0.05$. For abbreviations see Table 1.

nificantly decreased in the Chol group compared to the Chol-free group. Contrary to this observations, the reduction of the ratio was moderate when the rats were given both cholesterol and oxidized cholesterol because the effect on the proportions of these fatty acids was small. Therefore, the index was higher in the Chol + Ox Chol group than in the Chol group, particularly in PC. A similar tendency was also observed in liver PE and serum PC fatty acid compositions.

Contents of oxidized cholesterols in liver and serum. Table 5 shows the concentration of major oxidized cholesterol derivatives in the liver and serum. Dietary oxidized cholesterol elevated cholesterol derivative levels in liver. Cholest-5-en-3β, 7α-diol; 5,6α-epoxy-5α-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol; 5α-cholestan-3β,5,6β-triol; and 3β-hydroxycholest-5-en-7-one were detected only in the Chol + Ox Chol group, whereas cholest-5-en-3β,7β-diol was detected

TABLE 5
Effect of Dietary Cholesterol on Sterol Composition of Liver in the Rats Given High-Cholesterol Diets[a]

Component	Group		
	Chol-free	Chol	Chol + Ox Chol
Liver		(μg/g)	
Cholesterol	7,405 ± 828^{c}	56,555 ± 609^{e}	2,147 ± 1,930^{d}
7α-Hydroxycholesterol	n.d^{f}	n.d	48.1 ± 6.1
7β-Hydroxycholesterol	59.7 ± 5.4^{c}	151 ± 48.7^{c}	432 ± 33.1^{d}
5β-Epoxycholesterol	n.d.	n.d.	106 ± 32.9
5α-Epoxycholesterol	n.d.	n.d.	60.0 ± 12.3
Cholestanetriol	n.d.	n.d.	98.1 ± 21.8
7-Ketocholesterol	n.d.	n.d.	42.2 ± 11.3
Unknown steroids[b]	87.2 ± 16.7^{c}	75.4 ± 33.9^{c}	796 ± 157^{d}
Serum		(mg/dL)	
Cholesterol	113 ± 5.5^{c}	198 ± 11.5^{d}	163 ± 19.2^{d}
7α-Hydroxycholesterol	n.d.	n.d.	0.5 ± 0.1
7β-Hydroxycholesterol	1.0 ± 0.1^{c}	0.9 ± 0.2^{c}	2.8 ± 0.4^{d}
5β-Epoxycholesterol	n.d.	n.d.	2.7 ± 0.5
5α-Epoxycholesterol	n.d.	n.d.	1.1 ± 0.2
Cholestanetriol	n.d.	n.d.	1.5 ± 0.4
7-Ketocholesterol	n.d.	n.d.	1.8 ± 0.6
Unknown steroids[b]	1.8 ± 0.2^{c}	0.6 ± 0.2^{c}	12.7 ± 1.3^{d}

[a]Data are presented as means ± SE for six rats in each group. Cholesterol, cholest-5-en-3β-ol; 7α-hydroxychol-esterol, cholest-5-en-3β, 7-α-diol; 7β-hydroxy-cholesterol, cholest-5-en-3β, 7β-diol; 5β-epoxycholesterol, 5,6β-epoxy-5β-cholestan-3β-ol; 5α-epoxycholesterol, 5,6α-epoxy-5α-cholestan-3β-ol; cholestanetriol, 5α-cholestan-3β,5,6β-triol; 7 keto-cholesterol, 3β-hydroxycholest-5-en-7-one.
[b]Composed of more than 20 compounds. Mainly plant sterols in the non cholesterol and cholesterol-added groups, whereas oxidized cholesterols in addition to plant sterols in the oxidized cholesterol group.
[c–e]Values without a common superscript letter are significantly different at $P < 0.05$.
[f]Not detected. For other abbreviations see Table 1.

TABLE 6
Effect of Dietary Oxidized Cholesterol on Fecal Acidic and Neutral Steroid Excretion in the Rats Given High-Cholesterol Diets[a]

	Group		
	Chol-free	Chol	Chol + Ox Chol
Dried fecal weight (g/d)	1.47 ± 0.09	1.50 ± 0.05	1.41 ± 0.07
Neutral steroids (mg/d)			
Coprostanol	0.07 ± 0.02^{d}	0.45 ± 0.06^{d,e}	0.87 ± 0.24^{e}
Cholesterol	3.87 ± 0.16^{d}	33.40 ± 2.11^{e}	62.69 ± 4.94^{f}
Unknown steroids[b]	3.21 ± 0.17^{d}	6.27 ± 0.29^{e}	21.62 ± 1.67^{f}
Total neutral steroids	7.15 ± 0.16^{d}	40.11 ± 2.32^{e}	85.17 ± 6.65^{f}
Acidic steroids (mg/d)			
Lithocholic	0.28 ± 0.10^{d}	0.41 ± 0.12^{e}	0.89 ± 0.09^{f}
Deoxycholic	6.92 ± 0.61	6.32 ± 1.22	5.52 ± 0.54
Chenodeoxycholic	0.50 ± 0.15^{d}	0.70 ± 0.09^{f}	0.66 ± 0.16^{e}
Hyodeoxycholic + ursodeoxycholic	0.95 ± 0.16^{d}	1.41 ± 0.25^{d,e}	1.99 ± 0.21^{e}
Cholic	8.82 ± 0.63^{d,e}	11.48 ± 0.74^{e}	5.04 ± 0.48^{d}
α-Muricholic	2.51 ± 0.15^{d}	4.39 ± 0.49^{e}	1.22 ± 0.07^{d}
β-Muricholic	1.98 ± 0.13^{d}	2.02 ± 0.07^{e}	1.90 ± 0.09^{d}
Unknowns[c]	2.82 ± 0.80^{e}	1.48 ± 0.24^{d}	2.29 ± 0.46^{d}
Total acidic steroids	23.04 ± 0.80^{d}	26.82 ± 1.84^{e}	17.68 ± 0.9^{d}
Deoxycholic/Cholic	0.82 ± 0.11^{d,e}	0.57 ± 0.10^{d}	1.11 ± 0.21^{e}
Lithocholic/Chenodeoxycholic	0.48 ± 0.24^{d}	0.56 ± 0.13^{d}	1.15 ± 0.16^{e}
Total steroids (mg/d)	30.2 ± 0.8^{d}	66.9 ± 2.5^{e}	102.8 ± 7.4^{f}

[a]Data are presented as mean ± SE for six rats in each group.
[b]Composed of more than 20 components including oxidized cholesterols and plant sterols and their metabolites.
[c]Composed of more than five unknown components.
[d–f]Values without a common superscript letter are significantly different at $P < 0.05$. For abbreviations see Table 1.

even in the rats given no oxidized cholesterol. In addition, the concentration of cholest-5-en-3β,7β-diol in the Chol group was higher than that in the Chol-free group. The same changes were also observed in serum, although the level of cholest-5-en-3β,7β-diol was same level between the Chol-free and the Chol groups.

Fecal steroid excretion. Table 6 summarizes between groups data for composition of fecal steroids. Although the weight of dried feces was comparable between groups, the fecal excretion of neutral steroids in the Chol + Ox Chol group was the highest among the three groups. The excretion of cholesterol and unknown neutral steroids was significantly higher in the Chol + Ox Chol group than the Chol group. The concentration of coprostanol in feces of the Chol + Ox Chol group was higher than other two groups.

In contrast, dietary oxidized cholesterol significantly lowered the excretion of acidic sterols, the value in the Chol + Ox Chol group being lower than in both the Chol and Chol-free groups. The composition of acidic sterols was also modified by dietary oxidized cholesterol, and the ratio of secondary to primary bile acids (deoxycholic acid/cholic acid and lithocholic acid/chenodeoxycholic acid) in the Chol + Ox Chol group was significantly increased compared to that observed in the rats fed the cholesterol-only supplement.

TBARS value of blood and liver. Figure 2 shows the concentrations of blood and liver lipoperoxides measured as TBARS. The TBARS values of blood and liver significantly increased after feeding the diet containing both cholesterol and oxidized cholesterol compared to either the cholesterol-free diet, or that which cholesterol alone was added. The value of the Chol group was lower than that of the Chol-free group although this difference was not statistically significant.

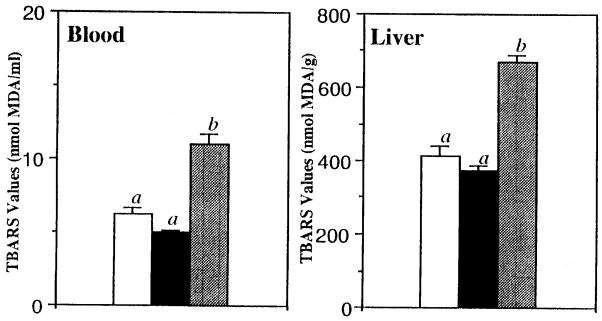

FIG. 2. Effect of dietary oxidized cholesterol on blood and liver thiobarbituric acid-reactive substances (TBARS) values in rats given high cholesterol diets. Values are presented as mean ± SE for six rats in each group. Values without the same letter are significantly different at $P < 0.05$. MDA, malondialdehyde. For abbreviations see Figure 1.

DISCUSSION

Our previous observations indicated that dietary oxidized cholesterol modulates some parameters of lipid components and metabolism accompanying aging (8,9). The present study also showed that high dietary levels of oxidized cholesterol disturbed exogenous cholesterol metabolism and modified various parameters of lipid metabolism when rats were fed high-cholesterol diets.

Relative liver weight in the Chol + Ox Chol group was significantly lower compared to the Chol group, although food intake and weight gain of rats were not different due to the paired-feeding protocol. In addition, the color of the livers of rats fed oxidized cholesterol (dark-red) was different from that of the rats of other groups (Chol-free group, bright-red; Chol group, whitish-red). We previously observed that dietary oxidized cholesterol increased hepatic glutamic pyruvic transaminase levels (9). Thus, dietary oxidized cholesterol may exert deleterious effects against hepatic function, as various oxidized cholesterol derivatives exhibit toxic action in bioassay (4). However, more detailed studies on this toxic effect of dietary oxidized cholesterol on hepatic function are required because whether the *in vitro* toxic property of oxidized cholesterol is the same or similar to the effect *in vivo* has not yet been determined.

The concentration of liver cholesterol in the Chol + Ox Chol group was significantly decreased compared to the Chol group. Moreover, the same tendency was shown in serum cholesterol level. These depressive effects on cholesterol of liver and serum may be due to the large excretion of cholesterol in feces. In fact, we observed that oxidized cholesterol inhibited the absorption of cholesterol from the intestines in a previous study (20). In addition, the reduction of sterol biosynthesis may also affect the concentration of liver cholesterol, as reduction of the hepatic HMG-CoA reductase activity was observed when rats were fed diets containing either both cholesterol and oxidized cholesterol or cholesterol alone. Various oxidized cholesterol derivatives have been shown to be potent inhibitors of sterol biosynthesis *in vitro* (21,22). In the limited information about *in vivo* studies, Erikson *et al*. (23) observed that HMG-CoA reductase activity was inhibited by perfusion of the liver with cholest-5-en-3β, 25diol. Vargas *et al.* (24) also reported that hepatic HMG-CoA reductase activity in laying hens was suppressed by feeding an oxidized cholesterol mixture. These observations were consistent with the present results.

Despite dietary cholesterol increasing cholesterol 7α-hydroxylase activity, this enzyme was inhibited by dietary oxidized cholesterol, although this activity may partly be lowered by the promotion of cholesterol excretion in feces. The hydroxylase activity has also been shown to be inhibited by oxidized cholesterol in an other *in vivo* study (25). Therefore, this inhibitory action may be partly caused by the competitive inhibition of cholesterol binding to liver microsomal cytochrome P-450 by oxidized sterols as suggested by Böstrom (25). The decrease of the hydroxylase activity by dietary oxidized cholesterol may be associated with the reduction in fecal acidic steroids excretion. The change in composition of fecal acidic steroids also indicates a differential effect of oxidized cholesterol on enzymes involved in bile acid conversion as reported previously (26,27). Moreover, oxidized cholesterol can be converted to unusual acidic *steroids* by intestinal bacteria, yielding oxidized cholesterol derivatives with increased toxicity. In fact, there was an increase in the ratio of secondary to primary bile acids in the rats fed oxidized cholesterol. This modulation might be a factor in colon cancer because deoxycholic and lithocholic acids are known to have potent promoter properties (28,29).

On the other hand, the hepatic Δ6 desaturase activity was significantly higher in the Chol + Ox Chol group than in the Chol group, although the Δ6 desaturase activity was suppressed by dietary cholesterol as reported previously (30). These results were consistent with the observation of Hochgraf *et al*. (31), although they gave oxidized linoleic acid to rats. As a result, the desaturation index of linoleic acid expressed as the ratio of (20:3n-6 + 20:4n-6/18:2n-6) in the Chol + Ox Chol group tended to increase in liver PC, PE, and serum PC compared to the Chol group. This modulation may be caused by increased fluidity of hepatic microsomes accompanying a decreased cholesterol/phospholipid ratio as Hochgraf *et al*. reported (31).

Thus, oxidized cholesterol modulated key enzymes in the metabolism of cholesterol as well as polyunsaturated fatty acids in liver microsomes. Unesterified oxidized cholesterol derivatives may exert various deleterious actions on hepatic lipid metabolism shortly after being incorporated into the cells as demonstrated by Brown and Goldstein (32), although it is generally believed that cells acquire exogenous cholesterol by two mechanisms, diffusional transfer (33) and receptor-mediated endocytosis (32).

Dietary oxidized cholesterol influenced the levels of triglyceride and phospholipid in the liver and serum. The same tendency was observed in plasma but not in liver of chicks given oxidized cholesterol (34). On the other hand, the concentration of liver cholesterol ester was lower in the rats given both cholesterol and oxidized cholesterol than in those given cholesterol alone, although these data were not shown. Some *in vitro* studies (35,36) found that oxidized cholesterol derivatives stimulated the fatty acyl-CoA:cholesterol acyltransferase activity in cultured cells and isolated microsomes. However, this did not occur in microsomes from bovine adrenal cortex and murine macrophage-like cells (37,38). The apparent inhibition of acyl-CoA:cholesterol acyltransferase activity by dietary oxidized cholesterol may be observed in this experimental condition because the enzyme in our experiment was saturated with sterol substrate as Smith and Johnson suggested (4), although our result was consistent with the latter.

The TBARS value of blood and liver was higher in Chol + Ox Chol group than in the other two groups. Although the interaction of oxidized cholesterol derivatives such as hydroperoxycholesterols with the TBA reagent cannot be excluded, it is possible that oxidized sterols stimulate the formation of free radicals in the tissues leading to reaction with coexisting fatty acids to form TBARS.

In conclusion, the present results demonstrated diverse effects of a high dietary level of oxidized cholesterol on exogenous cholesterol and linoleic acid desaturation when rats were fed a high-cholesterol diet. Emanuel *et al*. (39) reported that oxidized cholesterol derived from foodstuffs elevated oxidized cholesterol levels in human plasma. These oxidized cholesterol derivatives may have deleterious effects on lipid

metabolism, as observed in the present study. Our experimental conditions may not necessarily reflect usual diets because the level of oxidized cholesterol in the experimental diet was very high compared to a natural diet. Therefore, further studies are needed to confirm the current results under more physiological conditions. However, these observations help elucidate how high dietary levels of oxidized cholesterol modulate exogenous cholesterol metabolism and linoleic acid desaturation when high-consuming a cholesterol diet.

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[Received January 5, 1998, and in final revised form May 6, 1998; revision accepted June 19, 1998]

The Cholesterol-Lowering Effect of Guar Gum in Rats Is Not Accompanied by an Interruption of Bile Acid Cycling

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ABSTRACT: A viscous hydrocolloid (guar gum, GG; 2.5% of the diet) or a steroid sequestrant (cholestyramine; 0.5% of the diet) was included in semipurified diets containing 0.2% cholesterol to compare the cholesterol-lowering effects of each agent in rats. In the present model, GG significantly lowered plasma cholesterol (–25%), especially in the density < 1.040 kg/L fraction, whereas cholestyramine was less potent. Bile acid fecal excretion significantly increased only in rats fed cholestyramine, similar to the cecal bile acid pool; the biliary bile acid secretion was accelerated by GG, but not their fecal excretion, whereas GG effectively enhanced neutral sterol excretion. As a result, the total steroid balance (+13 μmol/d in the control) was shifted toward negative values in rats fed the GG or cholestyramine diets (–27 or –50 μmol/d, respectively). Both agents induced liver 3-hydroxy-3-methylglutaryl-CoA reductase, but cholestyramine was more potent than GG in this respect. The present data suggest that, at a relative low dose in the diet, GG may be more effective than cholestyramine in lowering plasma cholesterol by impairing cholesterol absorption and by accelerating the small intestine/liver cycling of bile acids, which is interestingly, accompanied by reduction of bile acid concentration in the large intestine.
Lipids 33, 765–771 (1998).

Several studies have indicated that the dietary intake of nonstarch polysaccharides can lower circulating cholesterol levels in humans as well as in experimental animals (1–3). Various compounds have been documented: cereal brans, gums, resistant starch, or compounds displaying sequestrant properties such as cholestyramine (a resin) or β-cyclodextrin (4–7). Guar gum (GG) has been shown to be particularly effective as a cholesterol-lowering agent: in the small intestine this neutral galactomannan has the capacity to form gels which may trap organic materials such as bile acids or sterols (8). GG, a hydrocolloid, is not markedly digested in the upper part of the digestive tract, but is extensively broken down in the large intestine by the host microflora, yielding short-chain fatty acids (SCFA) that are soon absorbed.

The cholesterol-lowering effect of fermentable carbohydrates may be explained by various mechanisms: (i) impairment of cholesterol absorption in the small intestine, (ii) binding of bile acids by hydrocolloids, with subsequent interruption of the enterohepatic bile acid cycling and increased fecal steroid excretion (9–11), (iii) impairment of the passive reabsorption of bile acids in the large intestine by insolubilizing these compounds (luminal pH acidification, entrapping to various insoluble structures, bacterial dehydroxylation into apolar metabolites), and (iv) production of specific metabolic effects, especially in the liver on lipid metabolism.

Previous investigations have established that the cholesterol-lowering effect of dietary fibers depends on their capacity to accelerate the fecal excretion of steroids and on their fermentability (12). The question was raised whether hydrocolloids such as GG could act, to some extent, as steroid sequestrants in the digestive tract. Both GG and cholestyramine produce greater fecal sterol excretion (13) and, in human subjects, increase bile acid synthesis (14). But in contrast to cholestyramine, GG is devoid of ion-exchange capacity and, in some experiments, hydrocolloids and cholestyramine showed contrasting effects on bile acid excretion (15).

The aim of the present work was to examine whether GG exerts effects different from those of cholestyramine on the enterohepatic bile acids cycling and on the control of plasma cholesterol and liver metabolism.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (IFFA-CREDO, l'Arbresle, France) weighing approximately 150 g were fed for 21 d semipurified diets distributed as a moistened powder. The control diet contained the following (in g/kg diet): casein (L. François, Paris, France), 150; corn oil, 100; cholesterol, 2; wheat starch, 678; mineral and vitamin mixes (Usine d'Alimentation Rationnelle, Villemoisson/Orge, France), 60 and 10, respectively (Table 1). The first experimental group contained 25 g of GG/kg diet at the expense of wheat starch, and

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Abbreviations: GG, guar gum; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; SCFA, short-chain fatty acid.

TABLE 1
Composition (g/kg) of the Experimental Diets

Diet group	Control	Guar gum	Cholestyramine
Wheat starch	678	653	673
Casein	150	150	150
Corn oil	100	100	100
Cholesterol	2	2	2
Guar gum	0	25	0
Cholestyramine	0	0	5
Mineral mixture	60	60	60
Vitamin mixture	10	10	10

the second contained 5 g cholestyramine/kg diet. GG flour (Meyproguar) of high MW ($\approx 2 \times 10^6$) was purchased from Meyhall (Kreuzlingen, Switzerland). Two animals were housed per cage and maintained in temperature-controlled rooms (22°C), with the dark period from 2000 to 0800 h. Rats were maintained and handled according to the recommendations of the Institutional Ethics Committee (Clermont-Ferrand University). The body weight of rats was recorded every 48 h during the experimental period; food intake and fecal excretion were recorded over three 2-d periods throughout the last 10 d.

Sampling procedures. Rats were killed at the end of the dark period, at which time cecal fermentations are still very active. They were anesthetized with sodium pentobarbital (40 mg/kg) and maintained at 37°C. An abdominal incision was made, and blood (1 mL) was drawn from both the portal vein and the abdominal aorta. The blood of each animal was placed in plastic tube containing heparin and centrifuged at $10,000 \times g$, for 5 min. After centrifugation, plasma was removed and kept at +4°C for lipid and lipoprotein analysis. After blood sampling, the cecal content was removed and weighed; two samples of it were transferred to microfuge tubes and immediately frozen at −20°C.

The small intestine was clamped at the pylorus and the ileal-cecal junction, removed, stripped of mesentery and fat, and weighed. The small intestine was halved to facilitate handling, and the content of each section was emptied into a preweighed tube by finger stripping and then frozen at −20°C. A portion of liver was freeze-clamped and stored at −80°C for the measurement of liver lipids. Two grams of liver were homogenized in 4 mL of an ice-cold buffer 1 (50 mmol/L Tris-hydrochloride, 250 mmol/L sucrose, 50 mmol/L EDTA, 2 mmol/L dithiothreitol, and 2 μmol/L leupeptin, pH 7.2) with a Potter-Elvejhem homogenizer (Braun, Melsungen, Germany) at moderate speed. The homogenate was first centrifuged at $10,000 \times g$ (15 min, 4°C); the resulting supernatant was then centrifuged at $100,000 \times g$ (60 min, 4°C). Pellets were resuspended in 2 mL of chilled buffer 1. The centrifugation procedure was repeated, and the resulting pellets were homogenized in 1 mL of buffer 2 (sucrose, 100 mmol/L; KCL, 50 mmol/L; K phosphate, 40 mmol/L; EDTA, 30 mmol/L; dithiothreitol, 1 mmol/L; pH, 7.2). The microsomal preparation was stored at −80°C until measurement of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity. The microsomal content of proteins was determined using the Pierce BCA reagent kit (Interchim, Montluçon, France).

Surgical procedure. In a separate series of anesthetized rats, a mid-line laparotomy was performed and the bile duct was exposed and ligated distally. The bile duct was then catheterized with a PE10 polyethylene catheter (Biotrol, Paris, France), and bile was allowed to drain for 30 min and then collected for two 30-min periods into preweighed vials cooled on ice. Bile volume was determined gravimetrically.

Analytical procedures. SCFA concentrations were measured by gas–liquid chromatography after ethanolic extraction of samples on supernatants ($8,000 \times g$, 5 min at 4°C) of cecal content. Bile acid analysis was conducted either directly on bile, or from cecal content, small intestine content or feces extracted by a two-step procedure. For this purpose, 1-vol sample was first dispersed in 10 vol of ethanolic KOH (0.5 M) using a Polytron disintegrator (Lucerne, Switzerland) and extracted at 70°C for 2 h, then 1 vol of this suspension (typically 2.5 mL) was redispersed in 4 vol of ethanolic KOH and reextracted at 70°C for 2 h. Bile acids were quantified using the reaction catalyzed by the 3-(α-hydroxysteroid dehydrogenase (E.C. 1.1.1.50; Sigma, St. Louis, MO). Sterol analysis was conducted by gas–liquid chromatography. Neutral steroids were extracted three times with 1 mL of hexane from a 100-μL aliquot of the alkaline ethanolic extract. After addition of 5-α-cholestane as an internal standard, the solvent was evaporated under a stream of N_2 and the residue dissolved in hexane. Portions (0.5 μL) of this extract were injected into a gas chromatograph (Delsi 330, Paris, France) equipped with a 12 m × 0.25 mm (i.d.) fused-silica capillary column (BP10; SGE, Villeneuve-St.-Georges, France) and a flame-ionization detector. Helium was used as a carrier gas, and the sterols were separated isothermally at 260°C. Sterols were calculated from the peak areas relative to the peak area of the internal standard. Triglycerides (Biotrol, Paris, France) and total cholesterol (BioMérieux, Charbonnières-les-Bains, France) were determined in plasma and bile (only cholesterol) by enzymatic procedures.

Plasma lipoproteins were separated on a density gradient by preparative ultracentrifugation (16), in a TST 41.14 swinging-bucket rotor (Kontron, Zürich, Switzerland) at $100,000 \times g$ for 36 h (15°C). Because of low level of plasma low density lipoprotein and the partial overlapping of high density lipoprotein (HDL)1 and HDL2 fractions in rats, only two fractions were considered: the fraction of density < 1.040 kg/L (chiefly triglyceride-rich lipoproteins, together with some low density lipoprotein) and the fraction of density > 1.040 kg/L (HDL). The cholesterol content of each fraction was determined by the method described above. Liver triglyceride and cholesterol were extracted and analyzed as described by Mazur *et al.* (17), and a control serum (Biotrol-33 Plus) serving as the control of analysis accuracy was treated similar to samples.

Enzyme activities. The activity of HMG-CoA reductase (E.C. 1.1.1.34) was determined on microsomal fractions as described by Wilce and Kroone (18). Labeled mevalonolac-

tone was separated from unreacted HMG-CoA by column chromatography using AG1-X8 resin (200–400 mesh, formate form; BioRad, Paris, France). Specific radioactivity of the enzyme was expressed in pmole of [3-^{14}C]HMG-CoA transformed in [^{14}C]mevalonolactone per min per mg of microsomal protein, after correcting for recovery of [^{3}H]mevalonolactone from the column.

Calculation and data analysis. The cecal pool was calculated as: cecal concentration (mmol/L) × cecal content volume (mL). Values are given as the means ± SEM and, where appropriate, significance of difference ($P < 0.05$) between mean values was determined by analysis of variance coupled with the student Newman-Keuls' Test.

RESULTS

Effects of diets on body weight, cecal development, cecal pH, and cecal fermentations. As indicated in Table 2, there were no significant differences in body weight between control rats and rats fed 2.5% GG or 0.5% cholestyramine.The cecal weight was significantly enhanced in rats fed the GG diet (+72%) with a similar increase of cecal wall weight. Only the GG diet caused an acidification of the cecal content (pH 6.60 compared to 7.00 for control rats). Rats fed the GG diet had cecal SCFA concentration of 135 mmol/L, compared to about 90 mmol/L for rats adapted to the control or the cholestyramine diet. Similarly, the total SCFA pool was significantly enhanced only in rats fed GG diet (+178% compared to the control); futhermore, there was a higher molar proportion of propionate in rats fed GG (38%, vs. 24 and 29% for the control and cholestyramine-fed rats, respectively).

Effects of diets on biliary secretion and fecal excretion of acidic and neutral sterols. As shown in Table 3, the biliary flow was higher (not significantly) in rats fed the GG diet than in the control rats, and the biliary bile acid secretion was significantly enhanced only in rats fed GG (+36% above the control values). Futhermore there was a significant increase in biliary cholesterol excretion (+60% above the control values) in rats fed GG diets and a slight increase with rats fed a cholestyramine diet. The bile acid content of small intestines in rats fed GG and cholestyramine diets was slightly higher than in the control rat (about +35%). The cecal pool of bile

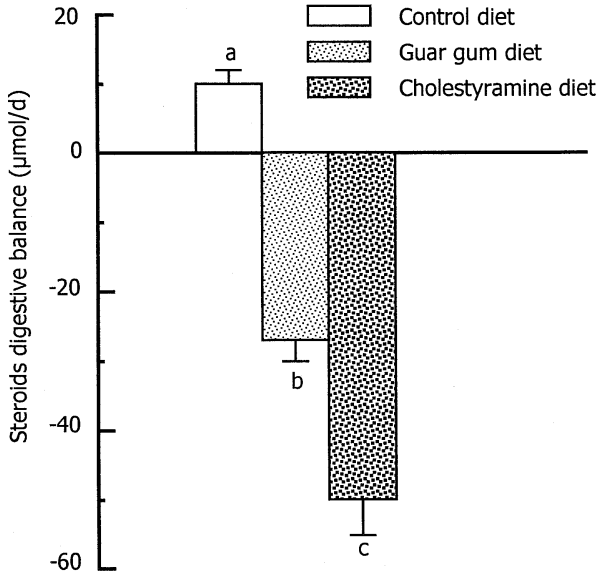

FIG. 1. The steroid balance is referred to as: cholesterol intake – total steroids excretion. Each value is the mean ± SEM, $n = 10$. Bars not sharing a common letter are significantly different ($P < 0.05$).

acids in rats adapted to the GG diet was similar to that found in control rats (14.8 µmol); but it was significantly increased in rats fed the cholestyramine diet (18 µmol). There was a substantial excretion of sterols (chiefly coprostanol) amounting to 61 µmol/d in the control diet, 123 µmol/d in the GG diet, and 106 µmol/d in the cholestyramine diet. Coprostanol excretion was significantly enhanced in rats fed the GG and cholestyramine diets (87 and 71 µmol/d, respectively, vs. 39 µmol/d for the control diet). Taking into account the daily cholesterol intake (116–131 µmol/d), it appears that the sterol net absorption was markedly lowered by GG and cholestyramine (8 and 11 µmol/d, respectively, vs. 55 µmol/d for the control); as a result, the apparent cholesterol digestibility was reduced (6 and 9% for GG and cholestyramine diets, respectively, compared to 47% for the control diet). Fecal bile acid excretion was enhanced only with the cholestyramine diet (+45% compared to the control diet). Furthermore, total steroid excretion was significantly enhanced with GG and cholestyramine diets (+53 and +62%, respectively). The total steroid balance (Fig. 1) was +13 µmol/d in the control diet;

TABLE 2
Effects of the Diets on the Final Body Weight and Cecal Digestive Parameters[a]

Diet group	Control	Guar gum	Cholestyramine
Final body weight (g)	323 ± 10	307 ± 6	322 ± 7
Cecal weight (g)	2.24 ± 0.16^{a}	3.77 ± 0.32^{b}	2.71 ± 0.18^{a}
Cecal wall weight (g)	0.63 ± 0.01^{a}	0.85 ± 0.03^{b}	0.69 ± 0.05^{a}
Cecal pH	7.00 ± 0.07^{a}	6.60 ± 0.03^{b}	7.11 ± 0.06^{a}
Cecal SCFA concentration (mM)	91 ± 7^{a}	135 ± 9^{b}	88 ± 6^{a}
Cecal SCFA pool (µmol)	114 ± 6^{a}	318 ± 10^{b}	118 ± 5^{a}
Molar ratio of cecal SCFA (acetate/propionate/butyrate)	(62:24:12)	(46:38:16)	(62:29:10)

[a]Each value is the mean ± SEM, $n = 10$. Values within a row that are not followed by a common superscript letter are significantly different ($P < 0.05$). SCFA, short-chain fatty acid.

TABLE 3
Effects of the Diets on Bile and Intestinal Bile Acid Concentration, and on the Fecal Excretion of Steroids[a]

Diet group	Control	Guar gum	Cholestyramine
Bile			
Bile flow (mL/h)	1.06 ± 0.08[a]	1.26 ± 0.32[a]	0.95 ± 0.12[a]
Bile acid flux (μmol/h)	39 ± 4[a]	53 ± 5[b]	33± 4[a]
Cholesterol flux (μmol/h)	0.45 ± 0.05[a]	0.72 ± 0.09[b]	0.68 ± 0.07[b]
Small intestine			
Bile acid pool (μmol)	16.5 ± 1.4[a]	22.4 ± 3.2[b]	25.7 ± 4.0[b]
Cecum			
Bile acid concentration (mM)	9.3 ± 0.8[a]	4.4 ± 0.6[b]	8.3 ± 0.6[a]
Bile acid pool (μmol)[b]	14.8 ± 1.4[a]	14.0 ± 1.7[a]	18.0 ± 1.2[b]
Fecal excretion			
Neutral sterols (μmol/d)	61 ± 6[a]	123 ± 10[b]	106 ± 4[b]
Cholesterol/coprostanol (%)	36:64	29:71	33:67
Bile acids (μmol/d)	42 ± 2[a]	35 ± 2[a]	61 ± 4[b]
Total steroids (μmol/d)	103 ± 8[a]	158 ± 12[b]	167 ± 13[b]
Cholesterol intake[c] – neutral sterol excretion (μmol/d)	55 ± 3[a]	8 ± 5[b]	11 ± 3[b]

[a]Each value is the mean ± SEM, $n = 10$. Values within a row that are not followed by a common superscript letter are significantly different ($P < 0.05$).
[b]The cecal bile acid pool (μmol) was calculated: [bile acid] × cecal content, namely the difference between total cecal weight and the cecal wall weight.
[c]Cholesterol intake was (in μmol/d): 116 ± 3 (control), 131 ± 5 (guar gum), and 117 ± 6 (cholestyramine).

whereas it was negative for GG and cholestyramine diets (–27 and –50 μmol/d, respectively).

Effects of diets on plasma and liver lipids concentration and HMG-CoA reductase activity. As shown in Table 4, the plasma cholesterol concentration was significantly decreased in rats fed the GG and cholestyramine diets compared to control rats (–25 and –13%, respectively); there was no significant triglyceride-lowering effect. In rats fed the control diet (0.2% of cholesterol), the major part of plasma cholesterol was found in the density < 1.040 kg/L (Fig. 2). GG effectively lowered cholesterol in this fraction (–51%), but cholestyramine was less effective (–30%). The cholesterol of density > 1.040 kg/L fraction was not significantly modified by any of the diet conditions. Furthermore GG and cholestyramine diets decreased cholesterol concentration in the liver. HMG-CoA reductase activity significantly increased with the GG and cholestyramine diets (+86 and +339% vs. the control, respectively). Liver triglycerides were not significantly affected.

DISCUSSION

Numerous studies have demonstrated that soluble fibers like GG exert a cholesterol-lowering effect in human or animal models (19,20) when there is a substantial dietary supply of cholesterol ranging from 0.2 to 1% (21,22). Similarly, in this present study by using 0.2% cholesterol diets, GG significantly lowered plasma cholesterol concentration (–25%), but cholestyramine was apparently less effective (–13%). The cholesterol-lowering effect of GG is probably connected to its ability to elevate the viscosity in the small intestine lumen (2,23), which results in a decreased intraluminal mixing or increased thickness of the unstirred water layer (2,13). Such changes would affect cholesterol absorption, hence neutral sterol excretion. Bile acid-binding capacity is questionable for GG since it is devoid of suitable polar groups ($–NH_3^+$, $–COOCa^+$) and of polysaccharidic cavity (or helical structure) to include steroids; nevertheless, some data *in vitro* sug-

TABLE 4
Effects of the Diets on Lipid Metabolism Parameters[a]

Diet group	Control	Guar gum	Cholestyramine
Plasma			
Cholesterol (mM)	1.94 ± 0.06[c]	1.45 ± 0.05[a]	1.69 ± 0.04[b]
Triglyceride (mM)	2.25 ± 0.36[b]	1.94 ± 0.21[b]	1.49 ± 0.19[a]
Liver			
Cholesterol (mg/g liver)	5.6 ± 0.5[b]	3.6 ± 0.5[a]	3.1 ± 0.4[a]
Triglyceride (mg/g liver)	10.0 ± 1.2	8.3 ± 0.5	8.9 ± 1.5
Microsomal HMG-CoA reductase (pmol/min·mg protein)	20 ± 2[a]	37 ± 5[b]	85 ± 7[c]

[a]Each value is the mean ± SEM, $n = 10$. Values within a row that are not followed by a common superscript letter are significantly different ($p < 0.05$). HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

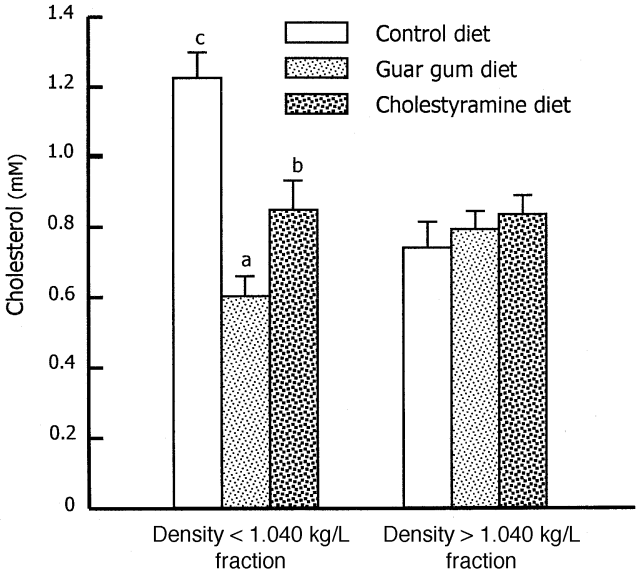

FIG. 2. Changes in cholesterol concentration of lipoprotein fractions, in control rats and rats adapted to guar gum or cholestyramines. Each value is a mean ± SEM of three pools of rat plasma representing 10 animals per pool. The fractions with a density lower than 1.040 kg/L correspond chiefly to triglyceride-rich lipoproteins, with a minor contribution of low density lipoprotein. The fractions with a density higher than 1.040 kg/L correspond essentially to high density lipoprotein. Bars not sharing a common letter are significantly different ($P < 0.05$).

gest that this capacity does exist for GG (14). On the other hand, bile-acid binding by cholestyramine is effective (24), but it binds a relatively small portion of trihydroxy bile acids which dissociate to varying extents from the sequestrants in the terminal ileum (25).

Notably in this study, fecal bile acid excretion did not significantly increase in rats fed the GG diet, although their biliary secretion (+36%) did increase, which is contrary to reports, particularly in hamster or guinea pig models, indicating that bile acid excretion is more responsive to dietary hydrocolloids than sterol excretion (15,21). The fact that fecal bile acid excretion was not elevated by GG probably reflects a lower availability of exogenous cholesterol, since GG apparently lowers cholesterol absorption.

GG diets also may increase both biliary secretion and intestinal bile acid pools (26–28). In the present experiments, the cecal bile acid pool was not enlarged in rats fed the GG diet, compared to fiber-free control rats, in spite of a higher rate of hepatic secretion. With higher levels of GG in the diet (5% or higher), this cecal pool was significantly increased, even if the fecal bile acid excretion was marginally increased (22). More efficient bile acid recovery in the small intestine is needed. Bile acid recovery and biosynthetic pathways are coordinately regulated and could be responsive to the size of the bile acid pool in enterohepatic circulation (29). Some data support the view that ileal bile acid transport could be increased with dietary fermentable carbohydrates (22,30). The mechanisms involved in this adaptive response could be either the hypertrophy of the ileal mucosa, along with an elongation of small intestine (31,32) affording higher capacity of reabsorption or upregulation of the ileal transporter, which is activated by cholic acid (33). The fact that an intestinal bile acid pool increase elicited by GG represents an effective stimulus is unproven. The large intestine also contains a noticeable bile acid pool (22) of which most is insolubilized due to several factors (acidic luminal pH, high Ca concentration, binding on microorganisms) and the remainder is reabsorbed by a passive (but relatively efficient) process. With its effectiveness depending on its capacity to impair bile acid reabsorption from the large bowel along with small intestine reabsorption (34), cholestyramine increases the bile acid cecal pool and the fecal excretion rate, greater than the control.

In control rats, dietary cholesterol drastically subdued HMG-CoA reductase basal activity, but GG and cholestyramine significantly activated this enzyme. Although the activation was greated with cholestyramine than with GG, cholestyramine was less effectively hypocholesterolemic in the present model. In rats adapted to 0.3% cholesterol diets, hepatic cholesterolgenesis could be reactivated in pectin- or psyllium-fed rats (35). Acceleration of the enterohepatic cycling of bile acids with GG may tend to counteract HMG-CoA reductase activation (36), which could explain more-effective cholesterol-lowering with GG than with cholestyramine even though the fecal excretion of bile acids was lower with GG than with cholestyramine. Another difference between GG and cholestyramine is that GG is broken down to SCFA by the large intestine microflora. In fact, with the relatively low GG level used in the present experiment, it seems unlikely that SCFA make a major contribution to liver metabolism, although some specific effects of propionate on lipid metabolism have been proposed (37). Also, soluble fibers such as those in GG tend to limit the postprandial increase of insulin (38,39), which is an important factor in the regulation of HMG-CoA reductase activity (40).

In conclusion, it appears that, in the present rat model fed low-cholesterol diets, a moderate percentage of GG (2.5%) may be more effective than 0.5% cholestyramine to lower plasma cholesterol. The fact that GG mainly affects the cholesterol digestive balance and lowers bile acid availability in the large intestine is enlightening by suggesting that an accelerated bile acid transfer from the ileum to the large intestine is not the unique mechanism whereby fibers stimulate bile acid secretion by the liver, in parallel to activation of cholesterol 7α-hydroxylase. Accordingly, Pandak *et al.* (41) postulated the existence of a factor likely to downregulate the liver cholesterol 7α-hydroxylase (possibly an intestinal peptide) that would be secreted or absorbed from the intestine in the presence of bile acids; whether fibers could interfere with such a process awaits further investigation. Also, this study suggests that bile acid reabsorption may be increased along with enlargement of the intestinal pool, even in the presence of viscous compound such as GG; it is still unproven if this reflects enhanced passive reabsorption along the small intestine or some upregulation of ileal transport. Bile acids are considered as potential initiators of colonocyte proliferation

(42,43), and some concerns have been raised about the risk of increasing bile acid transfer into the colon for the purpose of plasma cholesterol-lowering, especially with high-fat diets. In this respect, moderate concentrations of GG seem interesting because they combine effective cholesterol-lowering and limited transfer of bile acids into the colon. Nevertheless, even if this transfer were to be increased, fermentable hydrocolloids would also elicit processes in the large intestine (acidification, adsorption, *in situ* reabsorption) which tend to maintain luminal bile acids at a low level.

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[Received February 23, 1998, and in final revised form May 20, 1998; revision accepted June 8, 1998]

Surface Composition Regulates Clearance from Plasma and Triolein Lipolysis of Lipid Emulsions

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ABSTRACT: Sphingomyelin (SM) and cholesterol (Chol) are major surface lipid constituents of plasma lipoproteins. We investigated the effects of SM and Chol on the plasma clearance of lipid emulsions as a model for lipoprotein particles in rats. The presence of Chol facilitated the removal of emulsion particles from plasma, whereas SM delayed particle removal. Preinjection of lactoferrin, an inhibitor of the apolipoprotein E (apoE) receptor, revealed that the differences in clearance of emulsions were due to the differences in affinity for the apoE receptor. Measurement of apolipoprotein binding suggested that the balance of apoE and apoC (apoC-II and apoC-III) bound to emulsions caused the difference in plasma clearance of emulsion particles. That is to say, SM in the emulsion surface decreased binding of apoE, which led to a longer circulation of emulsion particles in plasma. Chol, on the other hand, decreased the ratio of apoC to apoE, which may have promoted emulsion uptake through the apoE receptor. We also examined *in vitro* lipolysis using immobilized lipoprotein lipase (LPL) in a heparin affinity column. Lipolysis rates were significantly reduced by the incorporation of SM into the emulsion surface, but not by the incorporation of Chol, indicating that SM in the lipoprotein surface is an important lipid component regulating LPL-mediated lipolysis. Our results suggest that the presence of SM and Chol in the lipoprotein surface plays an important role in the circulation behavior and LPL-mediated lipolysis of lipid emulsions through their effect on the selectivity of plasma protein binding.
Lipids 33, 773–779 (1998).

Lipid emulsions composed of triacylglycerol (TG) and phosphatidylcholine (PC) have been routinely used in parenteral nutrition for almost 30 yr. Recently, lipid emulsions were used as drug carriers in drug delivery systems (1). For example, the delivery of prostaglandin E_1 in injectable lipid emulsions has been proven, and is commercially available (2,3). However, it is likely that this type of emulsion is rapidly metabolized by lipoprotein lipase (LPL) and is readily taken up by the liver after intravenous injection. Because the release of drugs in blood is considered to depend on both the lipolysis of TG and the clearance of the particles from plasma, control of these metabolic processes is necessary in order to improve lipid emulsions as injectable drug carriers.

Emulsion droplets have a size and lipid composition similar to chylomicrons and have also been used as models for plasma lipoproteins. The metabolism of protein-free lipid emulsions in rats is comparable to that of chylomicrons (4,5). On entering the plasma, lipid emulsions rapidly acquire apolipoproteins, such as apoA-I, C-II and E, from circulating lipoproteins. It is thought that apoC-II is an activator of LPL, and apoE is necessary for recognition by lipoprotein receptors. On the other hand, an inhibitory effect on hepatic uptake through apoE-specific receptors by apoC-I, C-II, and C-III has been reported (6–11). The metabolism of lipid emulsions can be affected by the selective binding of these apolipoprotein to lipid particles.

Within a blood compartment, TG-rich lipoproteins are converted into remnants through the hydrolysis of TG by LPL (12). These remnants are subsequently taken up by the liver, although hepatic removal appears to be accomplished by several overlapping mechanisms (13). Lipolysis of TG in chylomicrons produces cholesterol (Chol)-enriched remnant particles (14). Presumably, the amount of Chol in the lipoprotein surface is an important factor influencing the metabolism of lipoproteins. In addition, differences in sphingomyelin (SM) content among plasma lipoproteins (15–19) may affect both lipolysis by LPL at endothelial sites and recognition by lipoprotein receptors. However, minimal information is available on the physiological role of SM in lipoprotein metabolism. We assume that the lipid composition of lipoproteins or lipid emulsions plays a crucial role in the metabolism of the particles. In other words, it must be possible to regulate both the clearance from plasma and triolein (TO) lipolysis of artificial lipid emulsions by modulating the lipid composition of an emulsion surface.

Experimental emulsions often contain liposome-like particles (20), which may affect the metabolism of emulsions. In this study, we prepared isolated emulsions without liposomes and evaluated the effects of Chol and SM in the emulsion surface on the lipolysis and clearance from plasma in rats. The selectivity of apolipoprotein binding was estimated, and its

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Abbreviations: apoC, summary term for apoC-II and apoC-III; apoC-II, apolipoprotein C-II; apoC-III, apolipoprotein C-III; apoE, apolipoprotein E; Chol, cholesterol; FFA, free fatty acids; LPL, lipoprotein lipase; PC, phosphatidylcholine; SM, sphingomyelin; TG, triacylglycerol; TO, triolein.

relevance to plasma clearance is discussed in this paper. These results are useful both for the deeper understanding of lipoprotein metabolism and for the development of improved lipid-emulsion drug carriers.

EXPERIMENTAL PROCEDURES

Materials. Egg yolk PC was kindly provided by Asahi Kasei Co. (Tokyo, Japan). The purity (over 99.5%) was determined by thin-layer chromatography. Egg yolk SM, Chol, and TO were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without purification. Fatty acid-free bovine serum albumin and sodium heparin were obtained from Sigma. Cholesteryl 1-pyrenedecanoate was purchased from Molecular Probes Inc. (Eugene, OR). Bovine lactoferrin was purchased from Wako Pure Chemicals (Kyoto, Japan). Heparin affinity columns (HiTrap® Heparin, 1 mL) were obtained by Pharmacia Biotech (Uppsala, Sweden).

Preparation of emulsions. We prepared lipid emulsions by the method described previously (20) using a high-pressure emulsifier (Nanomizer; Nanomizer Inc., Tokyo, Japan). Briefly, PC, Chol, SM and a fluorescent probe, cholesteryl 1-pyrenedecanoate, were mixed in chloroform. After evaporation of the solvent, the mixture was dried in a vacuum, resuspended in 30 mL of buffer [10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.01% (wt/vol) NaN_3] and successively emulsified [under a pressure of 1000 kg/cm^2 for 6 min, at 60°C for TO–PC, TO–PC/SM (2:1) emulsions; 15 min, at 30°C for TO–PC/Chol (3:2) emulsions]. After removing contaminating vesicles by ultracentrifugation, homogeneous emulsion particles were obtained. We confirmed the complete separation of emulsion particles from vesicles by ^{31}P nuclear magnetic resonance spectra in the presence of praseodymium as described previously (20). All types of emulsion particles were characterized by determining their particles size and lipid composition. The weight-averaged particle size of each emulsion was 110 ± 20 nm as determined from quasi-elastic light-scattering measurements (Photal LPA-3000/3100; Otsuka Electronic Co., Osaka, Japan). Phospholipid concentration was determined by the phosphorus assay according to the method of Bartlett (21). SM concentration was determined by thin-layer chromatography. The concentration of Chol was determined using enzymatic assay kits free cholesterol E-test Wako obtained from Wako Pure Chemicals. As a result, we confirmed that the surface lipid composition of isolated emulsions was the same as the surface lipid composition of starting lipid mixtures.

In vivo *experiments.* Emulsions were labeled with a fluorescent probe, cholesteryl 1-pyrenedecanoate, in phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.4). The probe to TO molar ratio was 1:500. Male Wistar rats (Shimizu Laboratory Supplies Co., Ltd.) weighing approximately 200 ± 20 g were fasted overnight. Rats were anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, Chicago, IL), and fluorescence-labeled emulsions were injected into the exposed femoral vein as a bolus of 25-μmol emulsion TO (*ca.* 22 mg TO) in 0.5 mL irrespective of emulsion composition and size. Blood samples of 250 μL were collected from the carotid vein at different time points and immediately centrifuged to separate the plasma. To remove the proteins in plasma, 600 μL of chloroform/methanol 1:1 (vol/vol) was added to 100 μL of each plasma sample. The fluorescence intensity of the solvent phase was measured by an Hitachi (Tokyo, Japan) F-4500 spectrofluorometer. Plasma emulsion concentration was calibrated by the fluorescence intensity of known concentration of emulsions. TO concentration in plasma was determined through enzymatic assay by the TG-G test Wako (Wako Pure Chemicals). The plasma clearances of emulsion particles and emulsion TO were simulated with a mono-exponential curve-fitting program. The initial concentration at time zero was estimated by extrapolating from the fitting curve. The concentration of emulsion particles and emulsion TO remaining in plasma were represented as a percentage of the initial blood concentration. The plasma clearances obtained by fluorescence probe and enzymatic assay were consistent with those measured using radiolabeled glycerol tri[1-^{14}C]oleate and 1, 2-^{3}H-cholesteryl hexadecylether (results not shown).

Apolipoprotein binding. Binding amounts of apoC-II, C-III, and E to lipid emulsions were determined by the ultracentrifugation method as described previously (22). Human plasma was centrifuged for 1.5 h at 27,000 rpm in a Beckman (Fullerton, CA) SW 28 rotor to remove the chylomicron-containing top fraction. Emulsions were incubated with chylomicron-free plasma for 20 min at 37°C. The final concentration of emulsion TO was 3.2 mM, which corresponded to the initial emulsion concentration in plasma immediately after the injection in *in vivo* experiments. The mixtures were ultracentrifuged for 20 min at 32,000 rpm in a Beckman SW 50.1 rotor. The equilibrium concentration of apolipoprotein in the bottom fraction was determined in single radical immunodiffusion assay plates, purchased from Dai-ichi Pure Chemicals (Tokyo, Japan). The amounts of apoC-II, C-III, and E binding were calculated from the difference between the apolipoprotein concentration before and after ultracentrifugation.

Lipolysis assay with heparin affinity column. Purified bovine milk LPL suspended in 3.8 M ammonium sulfate, 0.02 M Tris-HCl, pH 8.0, was purchased from Sigma Chemical Co. LPL solution was prepared according to the previously described methods (23). LPL was immobilized in a heparin affinity column. Emulsions (final concentration of TO was 1 mM) in 3 mL of Tris-HCl buffer [4% fatty acid-free bovine serum albumin, 180 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.01% (wt/vol) NaN_3, pH 8.5] containing 30 vol% of human plasma were circulated through the affinity column (1.65 μg, LPL) at 1.2 mL/min using a peristaltic pump. The temperature was kept constant at 37°C by a thermostat. Free fatty acids (FFA) released during circulation were measured at the indicated times by the enzymatic colorimetric reagent, NEFA C (Wako Pure Chemicals).

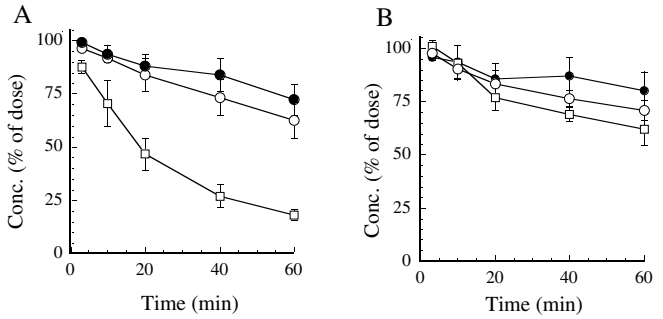

FIG. 1. Plasma clearance of lipid emulsions in rats without (A) or with (B) preinjection of lactoferrin. ○, triolein (TO)–phosphatidylcholine (PC) (control); ●, TO–PC/sphingomyelin (SM) (2:1 molar ratio); □, TO–PC/cholesterol (Chol) (3:2) emulsions. Concentration of emulsion particles is represented as percentage of dose. Initial concentration at time zero was estimated by extrapolating from a fitting curve. Data represent the mean ± SD of five or six rats.

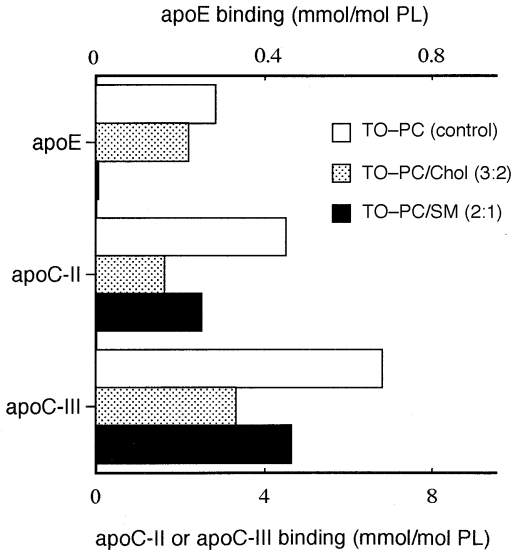

FIG. 2. Effects of SM and Chol on apolipoprotein (apo) binding to emulsions. Open bar, TO–PC (control); dotted bar, TO–PC/Chol (3:2 molar ratio); closed bar, TO–PC/SM (2:1) emulsions. Experiments on two separate preparations were performed in duplicate, each giving similar results. PL, phospholipids. See Figure 1 for other abbreviations.

RESULTS

Effect of Chol and SM on plasma clearance of emulsion particles. Figure 1A shows plasma clearance after injection of fluorescence-labeled emulsions. We prepared three types of labeled emulsions: TO/PC (control); TO–PC/Chol (molar ratio, 3:2); and TO–PC/SM (2:1). Approximately 60% of the injected control emulsions was detected in the plasma 1 h after injection. TO–PC/Chol emulsions disappeared more rapidly than the control emulsions, with only 20% of the injected emulsions remaining after 1 h. In contrast, the removal of emulsion particles was delayed when SM was incorporated into the surface. Two hours after emulsion injection, 25.1 ± 3.9 and 51.0 ± 5.9% of TO–PC and TO–PC/SM (2:1) emulsions remained in plasma, respectively. SM has a significant effect on emulsion clearance.

To elucidate the difference in plasma clearances caused by Chol or SM in the emulsion surface, we investigated the effects of lactoferrin (70 mg/kg rat) on emulsion removal from plasma. In previous studies, lactoferrin was shown to selectively block the apoE-mediated uptake of chylomicrons and very low density lipoproteins in rats (24–26). This lactoferrin effect has been attributed to an arginine/lysine-rich sequence at position 25-31 that resembles the binding site (amino acids 142–148) of apoE (27). Figure 1B shows plasma clearance of injected emulsions with preinjection of lactoferrin 1 min prior to emulsion injection. The presence of lactoferrin had little effect on the clearance of control emulsions, while the clearance of TO–PC/Chol emulsions was delayed effectively. Also, the presence of lactoferrin had almost no effect on the clearance of TO–PC/SM emulsions. Thus, all types of emulsions were removed from plasma at similar rates in the presence of lactoferrin.

Apolipoprotein binding to lipid emulsions. In artificial lipid emulsions as well as in TG-rich lipoproteins, the binding of exchangeable apolipoprotein to the surface of lipid particles plays a crucial role in their metabolism. To investigate the effect of the lipid composition of the emulsion surface on the binding behavior of apolipoprotein, we examined the binding of apoC-II, C-III, and E to the three emulsion types

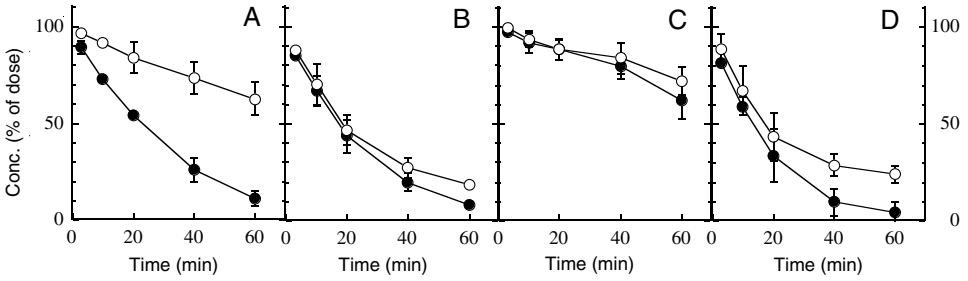

FIG. 3. Plasma clearance of emulsion particles and emulsion TO. ○, Emulsion particle concentration of injected dose as determined by fluorescence measurement. ●, TO concentration of injected dose determined by enzymatic assay. Initial concentrations at time zero of emulsion particles and emulsion TO were estimated by extrapolating from a fitting curve. (A) TO–PC (control); (B) TO–PC/Chol (3:2); (C) TO–PC/SM (2:1), (D): TO–PC (diameter is 200 nm) emulsions. Data represent the mean ± SD of five or six rats. See Figure 1 for abbreviations.

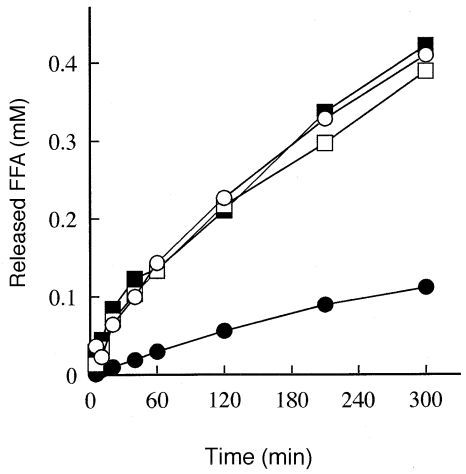

FIG. 4. Released free fatty acid (FFA) during lipolysis with lipoprotein lipase (LPL) in heparin affinity column. ○, TO–PC (control); ●, TO–PC/SM (2:1, molar ratio); □, TO–PC/Chol (3:2). ■, TO–PC (diameter is 200 nm) emulsions. The mixtures of plasma and emulsions in Tris buffer were circulated through the immobilized LPL attached with columns. Temperature was 37°C. TO concentration of lipid emulsions was 1 mM. Each value is a mean of duplicated determinations. See Figure 1 for other abbreviations.

in human serum. Figure 2 shows the effect of Chol and SM on the amount of apolipoprotein binding per surface phospholipid. Incorporation of SM into the emulsion surface drastically reduced the amount of apoE binding, while only a slight decrease was observed in the case of Chol incorporation. On the other hand, the amounts of apoC-II binding to TO–PC/Chol and TO–PC/SM emulsions were about one-third and one-half of the control values, respectively. We also found that the binding behavior of apoC-III was similar to that of apoC-II, with Chol reducing the binding of apoC more effectively than SM.

Plasma clearance of emulsion TO. In metabolic processes of lipid emulsions, the emulsion core, namely TO, is hydrolyzed by LPL on endothelial cells. We investigated the plasma clearance of core TO as well as that of emulsion particles (Fig. 3). Emulsion TO was removed from plasma by two processes: (i) the lipolysis of TO at the endothelial surface of capillaries, and (ii) the uptake of emulsion particles by the organs. Consequently, the difference between TO and emulsion particle concentration corresponds to the fraction of lipolysis *in vivo*. For control emulsions, TO in cores disappeared from plasma faster than the emulsion particles, indicating a significant degree of TO lipolysis in plasma. For TO–PC/Chol (3:2) and TO–PC/SM (2:1) emulsions, the small difference in clearance of emulsion particles and TO indicates minimal hydrolysis of emulsion TO by LPL. The rapid uptake of emulsion particles affects the lipolysis of emulsion TO. To determine the relationship between the clearance of emulsion particles and the amount of lipolysis *in vivo*, we examined the metabolism of large emulsion particles (diameter: 200 nm) composed of TO and PC in rats. As shown in Figure 3D, large emulsion particles were removed from plasma as rapidly as TO–PC/Chol (3:2) emulsions, and TO lipolysis was reduced to a level similar to TO–PC/Chol (3:2) emulsions.

Lipolysis in vitro. We measured FFA released during circulation of emulsions through immobilized LPL in a heparin affinity column. This lipolysis assay has the advantage of excluding the effects of particle-uptake by the organs as occurs *in vivo*, and allows the comparison of LPL activity with different emulsions. Figure 4 shows the time course of FFA released during lipolysis with LPL in a heparin affinity column. TO–PC/Chol (3:2) emulsions and large emulsion particles were hydrolyzed at rates similar to control emulsions, although both emulsions had low susceptibility to LPL *in vivo*. In contrast, as shown in Figure 4, lipolysis was negligible in TO–PC/SM (2:1) emulsions. This suggests that the key component in the lipoprotein surface regulating lipolysis was not Chol but SM. The presence of Chol is a determinant of early liver-uptake (Fig. 1), but has little influence on LPL activity (Fig. 4).

DISCUSSION

Phospholipid liposomes coexist with emulsion particles in commercially produced lipid emulsions and sonicated emulsions composed of TG and PC (28–32). We have shown the coexistence of liposomes and emulsion particles on the basis of a monolayer/bilayer equilibrium (33). To evaluate the emulsion kinetics in rats, it is necessary to separate excess liposomes completely, otherwise, coexisting liposomes may distort the clearance of emulsions through association with apolipoprotein or by other mechanisms. We prepared isolated lipid emulsions by ultracentrifugation and confirmed the absence of liposomes by the nuclear magnetic resonance method. As shown in Figure 1A, about 60% of the injected TO–PC (control) emulsions remained in rat plasma at 1 h after injection, which is compatible with results reported by Redgrave *et al.* (34) using isolated emulsions without liposomes.

We discovered that surface Chol promotes the removal of emulsions from plasma, while SM delays clearance of emulsions. In addition, the removal of TO–SM emulsions was slower than that of TO–PC/SM (2:1) emulsions (two hours after injection, 57.1 ± 9.4% of TO–SM emulsions remained in plasma.). Lipid emulsions in blood are mainly subject to uptake by the reticuloendothelial system and by apoE-recognizing receptors. Lactoferrin inhibits the apoE-mediated interaction of lipoproteins *in vivo* (24–26). Figure 1B suggests that the difference in emulsion removal (Fig. 1A) is due to the difference in the affinity with the apoE-mediated receptor. In fact, SM in the emulsion surface inhibited apoE binding to emulsions (Fig. 2) and delayed emulsion uptake by the liver. On the other hand, Chol in the emulsion surface decreased the ratio of apoC to apoE on the emulsions (Fig. 2) and promoted apoE-mediated uptake. The ratio of apoE to apoC correlated better with lipoprotein uptake than the absolute apoE content (13). Chol-containing emulsions are cleared from plasma rapidly because of a decrease in the inhibition effect of apoC on the apoE function. SM and Chol are lipid components regulating

the circulation time of emulsion particles in plasma through their effect on the selectivity of apolipoprotein binding.

Apolipoprotein are thought to interact surface PC layers with nestled helices. Apolipoprotein binding is likely to be reduced by the increase in the order of phospholipid layer. We proposed that either SM or Chol increases the order of emulsion surface and thereby reduces the total apolipoprotein binding to the lipid surface. The mechanism of the modulation of apolipoprotein binding in Chol- or SM-containing emulsions remains to be fully clarified. We have, however, obtained data indicating that the binding of apoC-II is regulated predominantly by the acyl chain order of surface lipids, whereas surface properties other than the acyl chain order may be important for the binding of apoE (Saito, H., and Arimoto, I., unpublished data). These differences in binding behavior may lead to differentials binding to emulsions depending upon the incorporation of Chol or SM.

Within a blood compartment, TG-rich lipoproteins are converted into remnants through the hydrolysis of core TG by LPL, and concomitantly become enriched in Chol at the lipoprotein surface (14). These remnants are subsequently taken up by the liver, although the precise mechanism of recognition is still unclear. Our results suggest that Chol enrichment resulting from TG hydrolysis reduces the binding of apoC to chylomicrons and leads to a rapid uptake through the apoE receptor and a termination of lipolysis by LPL. In addition, it may be possible that chylomicrons are metabolized and taken up by the liver more rapidly than very low density lipoproteins because the former contain little SM at the particle surface. Our results reveal physiologically significant evidence that the circulation behavior of lipoprotein particles is regulated by the lipid composition in the lipoprotein surface.

Emulsion TG is hydrolyzed by LPL located at the surface of the capillary endothelium before the lipid particles are taken up by organs. Lipolysis of lipid emulsions *in vivo* has been evaluated by the difference between the clearance of TO and emulsion particles (34,35) and by considering the competition between lipolysis and organ uptake. That is to say, if the uptake of the particles by organs is very rapid, LPL at endothelial sites will be unable to fully hydrolyze emulsion TG because the concentration of LPL substrate in plasma is decreasing too quickly.

As shown in Figure 3, the concentration of particles and TO of TO–PC/Chol (3:2) emulsions decreased concurrently in rat plasma. But it seems that Chol does not inhibit enzymatic activity; it promotes the removal of particles from plasma, resulting in a reduced amount of lipolysis. In fact, large emulsion particles which were removed rapidly from plasma appeared to show a low level of lipolysis (Fig. 3). To examine this in detail, LPL activity was determined in experimental conditions under which emulsion particles were circulating at all times without removal. As shown in Figure 4, we measured the lipolysis of different emulsions by using immobilized LPL in a heparin affinity column. This revealed that TO–PC/Chol (3:2) emulsions and large emulsions (diameter: 200 nm) were hydrolyzed at rates similar to the control emulsions. This supports the hypothesis that lipid particles which are rapidly removed from plasma are lipolyzed only to a limited extent *in vivo*. We also found that only SM-containing emulsions inhibited LPL susceptibility (Fig. 4). This is consistent with data in Figure 1 showing that SM decreased the lipolysis of TO despite the lengthy circulation of emulsion particles in the blood. From these results, it was concluded that SM is an important factor in regulating LPL activity in lipoprotein metabolism. On the contrary, Chol, which is a major component of plasma lipoproteins, was subject to enzymatic reaction in almost the same pattern as the control (Fig. 4). This is consistent with results showing that kinetic parameters of the LPL reaction were not affected by the addition of Chol (22).

ApoC-II is a well-known activator of LPL (36,37), and apoC-III exhibits an inhibitory effect upon LPL-mediated lipolysis (36). In these studies, however, apoC-III interfered with apoC-II in the activation of LPL only when the apoC-III concentration was extremely high compared with the physiological concentration (36). In addition, Wang *et al.* (38) have reported that apoC-II concentration is neither a determining factor in the *in vivo* hydrolysis of TG-rich lipoproteins nor a factor in the expression of hypertriglyceridemia. Also, very little apoC-II is required to maximally activate LPL (23,36, 39). The decrease in apoC binding by Chol and SM, therefore, is thought to have minimal influence on the LPL-mediated lipolysis. We recently showed that the inhibition of TO lipolysis by SM is attributed both to low affinity for, and reduced catalytic activity of, LPL on emulsion particles containing the phospholipid (23). Our results suggest that the content of Chol or SM in the plasma lipoprotein surface plays different roles in the regulation of clearance from plasma and the susceptibility to LPL in lipoprotein metabolism.

Emulsions of defined composition and metabolic behavior are of value not only to mimic plasma lipoproteins, but also to be vehicles for the delivery of lipophilic drugs to targeted organs. We found that emulsions containing SM were less susceptible to LPL and were very slow to be taken up by the liver after intravenous injection. Emulsions containing SM may, therefore, be appropriate carriers of lipophilic drugs which must circulate in the blood for a long time without hydrolysis of the core lipid. Chol-containing emulsions, on the other hand, would be useful for the rapid accumulation of lipophilic drugs in organs (mainly liver).

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture; by a fund from the Human Science Foundation of Japan; by a grant from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists; by a grant from the San-Ei Gen Foundation for Food Chemical Research; and by a grant from the Cosmetology Research Foundation.

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[Received February 13, 1998, and in final revised form May 12, 1998; revision accepted June 8, 1998]

Microsequencing of Bovine Cerebrospinal Fluid Apolipoproteins: Identification of Bovine Apolipoprotein E

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ABSTRACT: In studies of bovine plasma lipoproteins, apolipoprotein E (apoE) was not found associated with α-lipoproteins isolated over a broad range of densities. However, studies of cerebrospinal fluid (CSF) lipoproteins from other mammals have shown that apoE is a major apolipoprotein associated with high density lipoprotein, a fact that prompted us to determine if this were also the case in bovine CSF. CSF samples were obtained from animals with a surgically implanted catheter. Most analyzed samples were obtained from cows at various stages of the postpartum period; however, a few samples also were obtained at term or during pregnancy. Analyses of isolated ultracentrifugal fractions by polyacrylamide gel electrophoresis revealed the presence of two apo, with the expected molecular weights for apoE and apoA-I. By using both matrix-assisted laser desorption mass spectrometry and microsequencing techniques, we demonstrated that these apo are indeed apoE and apoA-I.

Lipids 33, 781–786 (1998).

The Shores (1) were the first to report on a novel apolipoprotein (apo) enriched in arginine amino acids and associated with very low density lipoproteins (hydrated densities less than 1.006 g/mL) in both humans and cholesterol-fed rabbits (2). They referred to this protein as the "arginine-rich protein." Subsequently, Swaney *et al.* (3), in their studies of lipoproteins, reported that a similar protein with a molecular weight of 35,000 was present on rat high density lipoprotein (HDL) (hydrated densities between 1.063 and 1.21 g/mL). This protein has since been designated apoE. In a variety of mammals, apoE is associated with α-lipoproteins, particularly those having hydrated densities within the low density lipoprotein (LDL) (hydrated densities between 1.020 and 1.063 g/mL) density range. This occurs when the ultracentrifugally isolated LDL is electrophoretically heterogeneous, i.e., the fractions contain both α- and β-lipoproteins (4–9). In most cases (8–11), the protein moiety of these α-lipoproteins contains apoA-I as the major apo, and apoE is one of the minor apo. Notable exceptions are rat α-migrating LDL which can be heavily enriched in apoE (12). By contrast, bovine α-LDL do not contain any discernible amounts of this apo (10).

With the onset of lactation, the concentration of cholesterol in bovine plasma can increase rapidly within 2 wk from 50 to over 300 mg/dL (13,14), due exclusively to changes in the levels of the main carrier of cholesterol, α-lipoproteins (10,15), with roughly 30% having hydrated densities between 1.020 and 1.063 g/mL (10). These α-lipoproteins, being elevated in concentrations throughout a wide range in density, have proven suitable for detailed characterization (10). When separated on a density gradient, compositional differences in the fractions were observed in both the lipid and protein moieties, but a protein corresponding in molecular weight to apoE was not detected in any of the fractions (10). Cordle *et al.* (16) also were unable to detect apoE in their studies of bovine plasma lipoproteins. However, an apo having a molecular weight between 36,000 and 40,000 was found associated with triacylglycerol-rich lipoproteins which are present in low concentrations in bovine plasma (17,18). To detect this protein the lipoproteins had to be isolated from large plasma volumes (17,18). Grummer *et al.* (18) in their studies proposed this protein to be apoE. Subsequent to these studies, two groups have published the sequence for bovine apoE based on analyses of hepatic cDNA (19,20).

In contrast to plasma where apoA-I is the major apo associated with HDL, Roheim *et al.* (21) in their studies of cerebrospinal fluid (CSF) HDL found that both apoE and apoA-I were the predominant apo. Pitas *et al.* (22) later reported apoE to be associated with lipoproteins within the HDL density range, primarily HDL_2 (hydrated densities between 1.090 and 1.121 g/mL) in canine CSF and HDL_3 (hydrated densities between 1.121 and 1.21 g/mL) in human CSF.

MATERIALS AND METHODS

Two sets of CSF samples were obtained from nine cows, which were part of the research herd at the experimental station of Texas A&M in Beeville, Texas. The initial set was obtained from six crossbred (*Bos taurus* × *B. indicus*; 1/2 Angus

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Abbreviations: apo, apolipoprotein; CSF, cerebrospinal fluid; HDL, high density lipoprotein; LDL, low density lipoprotein; MALD-MS, matrix assisted laser desorption mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

× 1/4 Brahman × 1/4 Hereford), first-calf heifers 28–30 mon of age. Heifers in various stages of the peripartum and postpartum periods were maintained in lots measuring 25 × 9 m and fed hay and concentrate to maintain late pregnant and lactating females in excellent body condition (23). The second set consisted of CSF obtained postpartum (between 11 and 53 d) from three pluriparous crossbred (Brahman × Hereford, F_1) cows, 8 to 10 yr of age, and maintained as described above. Twelve samples, six from each of two cows, were drawn on different days, and four samples were obtained from a third cow on four other days.

Sampling and preparation of CSF. Indwelling third ventricle cannulae were installed surgically with animals under halothane anesthesia as previously described (24). Samples were taken with a tuberculin syringe before, during, and after parturition as determined by the experimental protocol. Sampling intervals ranged from 1 min to 6 h. Aseptic technique was employed at all times. A solution (50 μL) containing EDTA (37.2 g/L) and the oxytocinase inhibitor, phenanthroline (1 g/L), was added to each collection vial. Vials were then turned upside down, allowing the fluid to run off and residual fluid to dry on the walls. Samples were immediately placed on ice, within 30 min frozen at −70°C until assayed, and then sent to the University of California at Los Angeles (Los Angeles, CA) where they were centrifuged in a tabletop centrifuge for 15 min. The recovered volumes varied from 0.5 to 2.0 mL. Two pools were formed from samples in the initial set to obtain baseline lipid data on whole CSF as well as ultracentrifugally isolated lipoproteins. Pools consisted of samples drawn either at parturition or between the 17th and 21st day postpartum. In the case of a sixth cow, from which three samples were obtained (in the third trimester, at parturition and at 5 d of postpartum), only ultracentrifugal isolation of CSF lipoproteins was done, as was the case for samples received in the second set.

Enzymatic analyses of CSF lipids. Concentrations of cholesterol and triacylglycerol in CSF samples were measured at 490 nm using a Vmax Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). Triacylglycerol analyses were done by mixing 600 μL of Sigma enzymatic reagent (Sigma Chemical Co., St. Louis, MO) with 100 μL of samples or diluted standards of 10- and 20-fold to concentrations of 20 and 10 mg/dL, respectively. Cholesterol analyses were done by mixing 600 μL of an enzymatic reagent, prepared as described by Puppione and Charugundla (25), with 40 μL of sample. Standards were diluted 16-fold to a concentration of 12.5 mg/dL. Measured volumes were dispensed into 1.0-mL tubes using a Cavro 190 IQ pipetter/dispenser (Cavro Scientific Instruments, Sunnyvale, CA). Following incubation at 37°C, 175 μL aliquots of the solution were dispensed in triplicate into 96 "Flat bottom" well plates (Costar, Cambridge, MA) that were then read in the plate reader. From the absorbance values of the standards, the concentrations were calculated on an IBM PS/2 computer using software from Molecular Devices. The dispensing of the incubated enzymatic solutions was done with a Biomek 2000 (Beckman Instruments, Fullerton, CA).

Isolation of CSF lipoproteins. The density of the CSF samples was raised to 1.21 g/mL by mixing a concentrated NaBr solution (density = 1.316 g/mL) in the proportions of 1 part sample to 2 parts NaBr solution. Total volume in each tube consisted of 6 mL of this mixture and 2 mL of a NaBr solution having a density of 1.21 g/mL. Lipoproteins were isolated using a 50 Ti Beckman rotor (Beckman Instruments). Ultracentrifugation was done at 40,000 rpm for 24 to 30 h and at 16°C. The top 0.5 to 1.0 mL was recovered from each tube. All salt solutions used for ultracentrifugation contained Na_2EDTA (0.04%), NaN_3 (0.05%), and Gentamycin (0.005%). The densities of salt solutions, prepared according to Lindgren (26), and of the infranates were measured at 26°C using a DMA 02D Mettler/Paar Densitometer (Graz, Austria) to an accuracy of ± 0.0002 g/mL.

Electrophoretic analyses. Centricon-10 concentrators (Amicon, Inc., Beverly, MA) were used both to concentrate the lipoproteins to approximately eightfold over concentrations in the CSF and to reduce the salt concentration with 0.196 molal NaCl as solvent. Apolipoproteins were separated on 12.5% polyacrylamide minigels with 1% sodium dodecyl sulfate in the buffer, prepared as described by Weber and Osborne (27) modifying the procedures of Laemmli (28). Human apo A-I, carbonic anhydrase, ovalbumin, and albumin were run as standards. Gels were stained with Coomassie Brilliant Blue R-250, destained with an aqueous methanolic (45% MeOH) containing 10% acetic acid, and dehydrated in a Model 583 gel dryer (BioRad, Richmond, CA) after remaining in water overnight. Then scans were taken with a Color One Scanner (Apple Computer, Inc., Cupertino, CA).

Microsequencing of CSF apo. Proteins were resolved on a 12.5% polyacrylamide gel (Bio-Rad Mini Protean) and stained with Amido Black 10B (29). After destaining, the bands were excised from the gel with a razor blade and prepared for trypsin digestion according to Jenö *et al.* (30). The gel slices were prepared for trypsin digestion by preincubation in three solutions for short periods: twice for 15 min in 40% aqueous *n*-propanol; twice for 15 min in 50% aqueous acetonitrile, 50 mM triethylammonium bicarbonate (pH 8.5); and for 1 min in 50 mM triethylammonium bicarbonate (pH 8.5). Then 50 μL of the same buffer containing 1 μg of trypsin was added and the digestion proceeded overnight at 37°C . After removing the supernatant, the gel slices were extracted twice with 0.1% aqueous trifluoroacetic acid at 55°C. The pooled supernatants and extracts were resolved by reversed-phase high-performance liquid chromatography (Hewlett-Packard 1090L, Palo Alto, CA) as described by Fischer *et al.* (29). The absorbance of column fractions (*ca.* 100 μL) was measured at 210 nm, and major fractions were analyzed by matrix-assisted laser desorption mass spectrometry (MALD–MS) (32) on a Bruker Reflex time-of-flight mass spectrometer (Karlsruhe, Germany) in the reflector mode as described in detail elsewhere (31). By using an automated PE Procise 494 Protein Sequencer (Applied Biosystems, Foster City, CA) (32), chemical sequence analyses of selected peptide fraction were carried out.

RESULTS

Lipid concentrations in bovine CSF. To analyze for CSF lipids, two separate pools were prepared using samples obtained at parturition and postpartum (days 17–21). Cholesterol levels, which were low for both pools, were twofold higher during the postpartum period (11 ± 0.04 μg/mL) than at parturition (5 ± 0.17 μg/mL). Triacylglycerols were not detected in these pools.

CSF apo distribution. Lipoproteins were isolated ultracentrifugally from bovine CSF at a density of 1.21 g/mL. Analyses of isolated fractions by SDS-polyacrylamide gel electrophoresis (PAGE) revealed two apo in all of the 15 samples which were obtained 2 wk or longer postpartum. One of the apo comigrated with human apoA-I (MW 28,000) (Fig. 1). The other apo with mobility intermediate to ovalbumin and carbonic anhydrase was calculated to have a molecular weight of 38,000 and was seen as a doublet, suggesting that this protein had undergone posttranslational glycosylation similar to what has been described for human apoE (33). The 38 K apo was not always observed when samples obtained around the peripartum period were analyzed. As can be seen from Figure 1, the amount of protein observed for the 11th day postpartum was lower than at the other times. But in other cases (data not shown) in which the 28 K apo was clearly seen, the 38 K apo was either absent or present in low amounts. In the only cow from which CSF was drawn during pregnancy and at term, no detectable amounts of the 38 K band were detected during pregnancy and a faint band was observed at term. The 38 K apo band also was not detected when lipoproteins from a pool of three CSF samples obtained at parturition from three cows were analyzed.

Sequencing of the major apo in bovine CSF. Two procedures were used separately to identify the two apo in bovine CSF. Initially, the apo were separated by PAGE, and the two bands noted above were excised from the gel. Following incubation with trypsin, as described in the Materials and Methods section, tryptic fragments were separated by high-performance liquid chromatography. Molecule masses of major fractions were determined by MALD-MS which revealed the presence of tryptic fragments predicted to be derived from bovine apoE and bovine apoA-1. To confirm this assignment, three of the apoE fragments were chemically sequenced and found to be in agreement with published data derived from cDNA analysis (19,20). Tryptic digestion of bovine apoA-I yielded seven fragments, and sequence data were obtained on two of these. These data are summarized in Tables 1 and 2.

DISCUSSION

Studies of various mammals have shown that the major site of synthesis of apoE is the liver (35–39), with additional synthesis occurring in extrahepatic tissue, including the brain (35–39). Brzozowska *et al.* (20) found this also to be true for the bovine. In addition to the brain, other extrahepatic sites of synthesis were the small intestine, lung, kidney, and mammary gland (20). Recently Ndikum-Moffor *et al.* (40) identified the corpus luteum as site of bovine synthesis of both apoE and apoA-I.

The extent to which apoE and apoA-I participate in lipid homeostasis in the CSF remains to be demonstrated. Concentration of CSF lipids is quite low, contrary to that in plasma. Pitas *et al.* (22) reported that the cholesterol value range was 2.1–6.6 μg/mL in human CSF and had mean values of 9–10 μg/mL in canine CSF. In our study of bovine CSF, the cholesterol concentrations were comparable to canine levels during the postpartum period and intermediate to human values at parturition.

Because apoE had not been detected in association with HDL in bovine plasma, we wanted to determine whether lipoproteins containing apoE could be isolated from bovine CSF. Analyses by PAGE revealed a 38,000 molecular weight

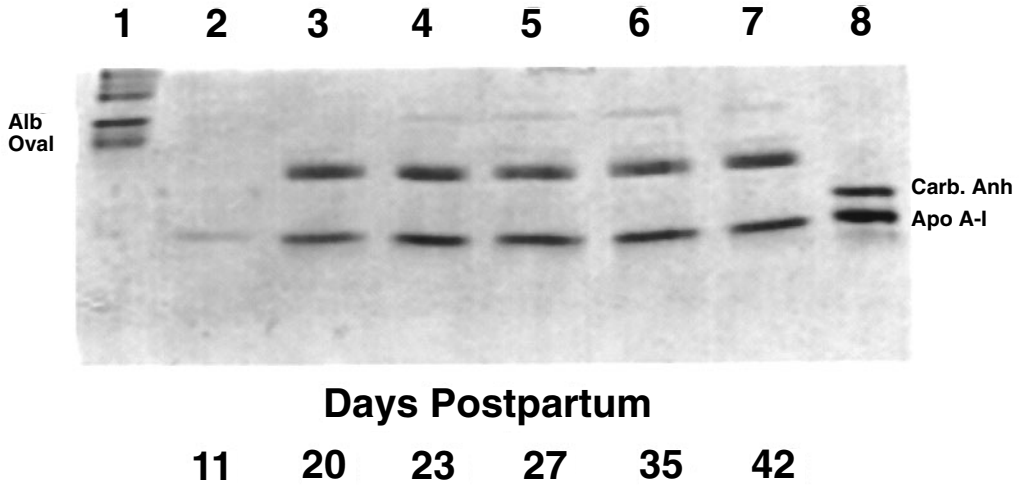

FIG. 1. Distribution of bovine cerebrospinal fluid (CSF) apolipoproteins in ultracentrifugally isolated fractions. Standards are in lanes 1 and 8. Distributions for fractions isolated from postpartum CSF are in lanes 2 through 7. Days postpartum are listed at the bottom of the various lanes; apo, apolipoprotein.

TABLE 1
Identification of Bovine CSF Apolipoproteins by Mass Spectrometry

HPLC number fraction	Observed mass (*m/z*)	Calculated mass (Da)[a]	Predicted sequence[b]
		Bovine apolipoprotein E	
20	1422.2	1421.70	LAVYQAGASEGAER
21	1033.8	1033.54	LQAEAFQAR
24	1218.0	1217.63	FGPLVEQGQSR
73	899.6	899.44	FWDYLR
78	1855.0	1853.90[c]	LRSWFEPLVEDMQR
		Bovine apolipoprotein A-I	
41	1305.6	1305.64	QQLAPYSDDLR
46	1017.8	1017.54	VAPLGEEFR
	1040.7	1040.61	AKPVLEDLR
	1267.0	1266.64	VQPYLDEFQK
	1431.0	1430.74	VAPLGEEFREGAR
48	1264.9	1266.64	VQPYLDEFQK
	1395.0	1394.70	VQPYLDEFQKK

[a]Calculated monisotopic $[M + H]^+$, based on sequence data obtained from cDNA for bovine apoE (19,20) or bovine apoA-I (34).
[b]Sequence of tryptic fragments based on data obtained from cDNA for bovine apoE (19,20) or bovine apoA-I (34).
[c]Fragment was oxidized; the mass of three oxygens was added. CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; apo, apolipoprotein.

protein. We then utilized MALD-MS and microsequencing techniques, basing our analyses on published sequences obtained from analysis of cDNA, to identify both apoE and apoA-I. Prior to our studies, there were other reports on the presence of apoE in bovine CSF. Using commercially available bovine CSF as a source of lipoproteins for studying the development of nerve cells, Bellosta *et al.* (41) reported that PAGE analysis revealed a protein band that the authors identified as being apoE, presumably based on its mobility. In a postmortem study of bovine CSF lipoproteins, Jones *et al.* (42) and Hochstrasser *et al.* (43), using two-dimensional gels, found apoE to be elevated in animals afflicted with bovine spongiform encephalopathy. In our study of live normal cows, we were able to observe apoE by PAGE after the lipoproteins had been isolated ultracentrifugally and concentrated approximately eightfold.

Our strategy for the characterization of the bovine CSF apo on ultracentrifugally isolated lipoproteins included the determination by MALD-MS of molecule masses of tryptic fragments, which were matched to the masses of hypothetical tryptic fragments that can be generated from bovine apoA-I and apoE. For selected fragments, we also determined the amino acid sequence by automated Edman degradation. Both sets of data were found to be in excellent agreement, allowing identification of these apo.

Microsequencing techniques also were used by Ndikum-Moffor *et al.* (40) to identify apoE and apoA-I secreted by explant cultures of bovine corpus luteal slices. In their study, they found that apoE mRNA was expressed only during days 2 and 3 of the estrous cycle, but apoA-I mRNA levels did not vary throughout the cycle. During pregnancy, these authors (40) found that apoA-I mRNA was expressed, with higher levels toward the later stages. ApoE mRNA, however, was not expressed during pregnancy. In our study, most of the PAGE analyses were done on samples obtained 2 wk or later postpartum. However, in the few samples obtained during the peripartum period, apoA-I was always observed, but not apoE. Because the brain is considered to be the synthesis site of the apoE associated with CSF lipoproteins, hormonal changes taking place following the delivery of the calf might influence the synthesis of apoE in the brain of the cow. Supraphysiological doses of estrogen were given to rodents to understand how hormonal changes might be affecting apoE and influencing its possible role in the development of Alzheimer's disease (44,45). The brains of rats and mice were found to respond to 17-β estradiol by increasing the synthesis of both apoE and the corresponding mRNA (44,45). It is yet undetermined if physiological hormonal changes in cows are affecting levels of CSF lipoproteins containing apoE.

TABLE 2
Identification of Bovine CSF Apolipoproteins by Microsequencing

HPLC fraction number	Predicted sequence[a]	Sequence determined[b]
	Bovine apolipoprotein E	
21	LQAEAFQAR	LQAEAFQAR
73	FWDYLR	XXDYL
78	LRSWFEPLVEDMQR	XXSXFEPLVEXXQ
	Bovine apolipoprotein A-I	
41	QQLAPYSDDLR	QQLAPYSDDLR
48	VQPYLDEFQK	VQPYLDEFQK

[a]Sequence of tryptic fragments based on data obtained from cDNA for bovine apoE (19,20) or bovine apoA-I (34).
[b]An X appears when no identification could be made. See Table 1 for abbreviations.

ACKNOWLEDGMENTS

Apolipoprotein identification by mass spectroscopy and microsequencing at The Salk Institute was made possible by Shared Equipment Grant 1 S10 RR11404-01A1 from the National Institutes of Health. W. Fischer was supported in part by the Foundation for Medical Research. G. Williams and O. Gazal were supported by USDA-NRICGP grant 94-37203-0924 and The Texas Agricultural Experiment Station. Authors also wish to thank Sarada Charugundla for doing the lipid analyses.

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[Received March 3, 1998, and in final revised form and accepted June 15, 1998]

Remodeling of Phospholipid Fatty Acids in Mitochondrial Membranes of Estivating Snails

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ABSTRACT: The effects of estivation on the phospholipid-specific fatty acid composition of mitochondrial membranes in the hepatopancreas of the terrestrial snail *Cepaea nemoralis* were investigated. The fatty acid composition of all phospholipids was significantly altered in snails estivating for 6 wk, indicating that substantial remodeling occurs. The most profound changes occurred in cardiolipin (CL). CL of estivating snails was 13-fold more saturated, contained 9-fold more monoenes, and had 45% fewer polyenes than in active snails. These differences were due, in part, to a reduction in linoleic acid (18:2n-6) content of CL from estivators. As in mammals, CL of active snails appears to preferentially incorporate 18:2n-6, which accounts for 60% of the acyl chains in this phospholipid. This proportion was reduced by 50% in estivators. Changes in the fatty acyl content of other phospholipids of estivating snails included increased monoenes in phosphatidylethanolamine (PE) and phosphatidylinositol, reduced ratios of n-3/n-6 polyenes in PE and phosphatidylcholine (PC), and an increased n-3/n-6 ratio in phosphatidylserine (PS). Arachidonic acid (20:4n-6) levels were reduced in PS but increased in CL and PC. Taken together, these alterations to fatty acid composition are consistent with decreased biological activity of membrane-related processes which occur in conjunction with the reduction of mitochondrial aerobic metabolism observed during estivation.

Lipids 33, 787–793 (1998).

Depression of basal metabolic rate is a common physiological strategy, allowing organisms to prolong the duration of their tolerance of suboptimal environmental conditions (1,2). Profound metabolic rate depression occurs during hibernation, torpor, and estivation. Suppression of mitochondrial respiration is a key component of strategies to reduce basal metabolic rate. Indeed, in hibernating ground squirrels, electron transfer is inhibited by 70 to 80% at the site of ubiquinol/cytochrome c_1, and adjustments of mitochondrial membrane composition are implicated in the mediation of this reduction (3). Changes to mitochondrial membrane composition have been studied in mammalian hibernators (4). However, hibernation and torpor are characterized by reductions in both metabolic rate and body temperature, and thus it is difficult to attribute changes of membrane phospholipid composition to either of these parameters. Several authors have attempted to overcome the confounding effects of temperature and metabolic rate reduction by comparing membrane compositional changes in active and quiescent overwintering animals (5,6), thus controlling for responses induced by lowered temperature. Nonetheless, interspecies differences still remain. Estivation, however, provides a superior model for studies of this nature. It typically occurs during environmental drought as a means to avoid desiccation (1). Metabolic rate reduction occurs in the absence of temperature change or significant cellular water loss. The extent of metabolic depression in estivators is at least as great as that observed during hibernation and torpor. Estivating terrestrial snails, for example, typically undergo a metabolic rate reduction of 85% (2).

As the functional mileu of the electron transport chain and many of the transporters and channels associated with oxidative phosphorylation, the mitochondrial membranes represent a potentially powerful site for the regulation of metabolism. Mitochondrial membranes are composed principally of proteins and phospholipids, and the function of many of the proteins associated with the membranes is responsive to, and in many cases dependent upon, surrounding phospholipids (7,8). Altering the composition of associated phospholipids can effect large changes in protein function (7). Modifications to the phospholipid environment include substitution of one phospholipid species for another, and/or modification of the acyl chains of particular phospholipids. Previously, we demonstrated that changes in the absolute and proportional amounts of individual phospholipids were altered in estivating snails. Here, we present results which indicate that a phospholipid-specific fatty acid remodeling occurs in mitochondrial membranes which is consistent with reduced rates of metabolic functions during estivation.

MATERIALS AND METHODS

Experimental animals. Several *Cepaea nemoralis* were collected in early summer, kept in terraria in the laboratory for *ca.* 6 wk, and fed a diet of "iceberg" lettuce. A group of these snails was removed to a dry terrarium, and food was withheld to induce estivation which lasted for 6 wk.

Mitochondrial isolation. Hepatopancreas from active or estivating snails was excised and immersed in 10 vol of mitochondrial isolation buffer (100 mM sucrose, 20 mM Hepes),

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Abbreviations: CL, cardiolipin; H_{II}, inverse hexagonal phase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

0.5% bovine serum albumin (BSA), pH 7.5), and homogenized by three passes with a Potter-Elvehjem homogenizer (Fisher Scientific Ltd., Whitby, Ontario, Canada). Homogenates were centrifuged at $150 \times g$ for 10 min and the pellet discarded. The remaining supernatant was centrifuged at $5000 \times g$ for 10 min, and the pellet "washed" by repeating this step twice. This procedure consistently gave a recovery of *ca.* 85% of mitochondria (9).

Purity of the mitochondrial preparation and recovery of mitochondria did not differ significantly ($P > 0.05$) between active and estivating snails based on the distribution of cytochrome c oxidase (mitochondrial marker), peroxidase (peroxisomal marker), and the proportional content of the phospholipid sphingomyelin (used to mark nonmitochondrial membranes, including nuclear membrane, endoplasmic reticulum, and lysosomal and plasma membranes). Recoveries of total cytochrome oxidase activities in the "mitochondrial fraction" of active and estivating snails were 82.4 ± 5.1 and 88.8 ± 4.0% ($n = 4$), respectively. Only 3.5 ± 1.0% of total peroxidase activity was recovered in the mitochondrial fraction of active snails, and 3.8 ± 0.8% ($n = 4$) in estivating snails. The proportional content of sphingomyelin in the mitochondrial fraction was 2.4 ± 1.5% ($n = 7$) in active snails and 4.2 ± 1.5% ($n = 8$) in estivators.

Total mitochondrial lipids were extracted by the method of Bligh and Dyer (10). Phospholipids were separated from neutral lipids and each other by thin-layer chromatography and methylated, as in Holub and Skeaff (11). Bands were visualized by using dichlorofluorescein. There were no band overlaps. An internal standard (17:0) was added to the phospholipid fatty acids prior to methylation. Individual fatty acids from each phospholipid fraction were separated and identified by gas chromatography using a reversed-phase DB-225 fused silica column (J&W Scientific, Folsom, CA) as described by Glémet and Ballantyne (12).

A small sample (1 g) of the diet (lettuce) was homogenized in 50 mM imidazole buffer using three 10-s bursts with a Polytron PT10 unit (Kinematica GmbH, Luzern, Switzerland). Lipid extraction of the homogenate was as above. Individual fatty acids were separated and quantified as above.

Chemicals. Chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Ltd. and were of the highest purity available. The fatty acid standard was obtained from Nu-Chek Prep Inc. (Elysian, MN) and was augmented by the addition of a menhaden extract.

Statistical analysis. Proportional phospholipid fatty acid compositional data were arcsine transformed where necessary to normalize the data and were compared by using Student's *t*-tests. *P*-values were adjusted (based on the number of tests made and degrees of freedom) to compensate for the use of multiple *t*-tests in comparing the fatty acid compositions of each phospholipid from active and estivating snails (13).

RESULTS

The snail diet (lettuce) was composed primarily of three fatty acids, 16:0, 18:2n-6 and 18:3n-3, which combine to account for 83% of total fatty acids (Table 1). 18:2n-6 was the most abundant fatty acid in lettuce, at almost 42% of total. Lettuce contained a large proportion (64%) of 18-carbon fatty acids, a lower proportion (29%) of 16-carbon fatty acids, and trace amounts of 14, 20, 22 and 24-carbon fatty acids.

Phospholipids from hepatopancreas mitochondria of active snails contained lower proportions (47%) of 18-carbon fatty acids than dietary lettuce, and greater proportions of their elongation products, especially 20-carbon fatty acids (37%). Some of the most common fatty acids in hepatopancreas mitochondrial phospholipids of active snails are 18:2n-6 (18%), 18:3n-3 (8%), and 20:4n-6 (18%) (Table 2).

The fatty acid composition of mitochondrial phospholipids was altered dramatically in estivating snails (Table 2). The proportion of 18:2n-6, 18:3n-3, and 18:4n-3 decreased by 60,

TABLE 1
Percentages of Individual Fatty Acids in Lettuce (average of two determinations)[a]

Fatty acid	Amount (mol %)
Nonessential	
14:0	0.82
14:1	0.12
16:0	25.04
16:1	3.65
18:0	1.81
18:1	1.92
20:0	0.40
20:1	0.41
22:0	0.88
22:1	0.93
24:0	1.27
24:1	0.73
n-3 Polyunsaturated	
18:3n-3	15.92
18:4n-3	2.69
20:3n-3	0.24
22:5n-3	0.32
22:6n-3	0.11
n-6 Polyunsaturated	
18:2n-6	41.93
20:2n-6	0.86
20:3n-6	n.d.
20:4n-6	0.23
22:2n-6	n.d.
22:4n-6	n.d.
22:5n-6	0.48
Total	100
Total saturates	30.21
Total monoenes	7.75
Total polyenes	62.76
n-3 Polyenes	19.26
n-6 Polyenes	43.50
n-3/n-6	0.44
Mononenes/polyenes	0.12
Unsaturation index	157.33
Chain length	17.00

[a]n.d., Not detectable.

TABLE 2
Percentages of Individual Fatty Acids in Cardiolipin of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.27 ± 0.14	5.26 ± 2.35
14:1	0.49 ± 0.43	2.60 ± 0.87
16:0	0.86 ± 0.23	9.07 ± 2.46
16:1	0.78 ± 0.42	12.18 ± 3.64
18:0	0.60 ± 0.31	5.96 ± 2.41
18:1	1.32 ± 0.47	5.59 ± 2.06
20:1	0.08 ± 0.04	2.70 ± 0.65[b]
23:0	0.11 ± 0.05	3.33 ± 1.47[b]
18:3n-3	20.58 ± 0.87	8.85 ± 2.04[b]
18:4n-3	8.57 ± 1.45	4.07 ± 1.64
20:3n-3	0.63 ± 0.08	n.d.
20:5n-3	0.18 ± 0.07	1.14 ± 0.89
22:5n-3	n.d.	0.19 ± 0.19
22:6n-3	n.d.	0.34 ± 0.20
n-6 Polyunsaturated		
18:2n-6	60.19 ± 1.67	29.68 ± 5.84[b]
20:2n-6	0.85 ± 0.08	1.31 ± 0.29
20:3n-6	0.71 ± 0.13	1.25 ± 0.17
20:4n-6	3.67 ± 0.27	5.11 ± 0.98[b]
22:2n-6	n.d.	0.11 ± 0.11
Total	100	100
Total saturates	1.85 ± 0.48	24.75 ± 6.29[b]
Total monoenes	2.67 ± 0.64	23.07 ± 4.69[b]
Total polyenes	95.48 ± 1.11	52.18 ± 9.40[b]
n-3 Polyenes	29.99 ± 1.43	14.58 ± 3.98
n-6 Polyenes	65.49 ± 1.85	37.60 ± 6.34[b]
n-3/n-6	0.46 ± 0.03	0.37 ± 0.09
Monoenes/polyenes	0.03 ± 0.01	0.81 ± 0.31
Unsaturation index[c]	240.76 ± 2.91	161.64 ± 22.52[b]
Chain length[d]	17.23 ± 0.04	17.16 ± 0.20

[a]Values are presented as means ± SE; n.d. = not detectable.
[b]Significantly different from active values, $\alpha = 0.0034$.
[c]Unsaturation index = $\sum m_i \cdot n_i$; where m_i is the mole percentage and n_i is the number of C–C double bonds in fatty acid "i".
[d]Mean chain length = $\sum f_i \cdot c_i$; where f_i is the mole fraction and c_i is the number of carbon atoms in fatty acid "i".

61, and 63%, respectively, in hepatopancreas mitochondria of estivating snails. 18-carbon fatty acids constituted only 30% of total fatty acids in estivators, compared with 47% in active snails (see above). These changes were offset by greater proportions of shorter chain fatty acids (14- and 16-carbons) and longer chain (22-carbons) fatty acids, which increased from 10 to 18% and from 5 to 9%, respectively. The phospholipid fatty acids of estivating snails contained significantly more monoenes and fewer polyenes. The reduced polyene content of mitochondrial membranes from estivators resulted primarily from a lower content of n-3 fatty acids, the proportional occurrence of which was reduced by 51%.

The greatest differences between the fatty acid composition of active and estivating snail mitochondria occur in the major constituent phospholipids of the mitochondrial membranes [phosphatidylcholine ((PC), phosphatidylethanolamine (PE) and cardiolipin (CL), which constitute 37.7, 35.2, and 11.4% of total phospholipids in mitochondria from the hepatopancreas of active snails (14)]. Perhaps the most profound alterations of fatty acid composition occurred in CL (Table 3). A 50% reduction in 18:2n-6 content was observed in CL which, in combination with a 57% decrease in 18:3n-3, accounted for a 55% decrease in the total polyene content of CL from estivating, compared to active, snails. While polyene levels decreased, saturated fatty acids increased 13-fold, and monoenes increased 9-fold. This resulted in a 33% reduction in CL unsaturation index.

Unsaturation index was not altered during estivation in any other phospholipid species. However, in PE, the proportion of monoenes increased 48%, due in part to an almost fourfold increase in 16:1 content (Table 4). Membranes of estivators contained 43% fewer n-3 polyenes, largely due to significant reductions in 18-carbon n-3 fatty acids. This resulted in a lowering, by 43%, of the n-3/n-6 ratio in estivating snail mitochondria.

TABLE 3
Percentages of Individual Fatty Acids in Phosphatidylethanolamine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	1.74 ± 0.76	2.37 ± 0.71
14:1	1.30 ± 0.36	2.00 ± 0.64
16:0	2.56 ± 0.20	4.70 ± 0.71
16:1	1.67 ± 0.34	6.25 ± 0.84[b]
18:0	15.28 ± 0.90	9.36 ± 0.58[b]
18:1	4.76 ± 0.24	3.84 ± 0.62
20:0	0.45 ± 0.11	0.57 ± 0.09
20:1	3.26 ± 0.15	4.20 ± 0.23
23:0	0.82 ± 0.09	1.37 ± 0.16[b]
n-3 Polyunsaturated		
18:3n-3	4.06 ± 0.22	1.24 ± 0.23[b]
18:4n-3	1.26 ± 0.19	0.43 ± 0.07[b]
20:3n-3	3.42 ± 0.63	1.10 ± 0.30
20:4n-3	0.08 ± 0.08	0.74 ± 0.27
20:5n-3	2.34 ± 0.28	2.20 ± 0.42
22:5n-3	0.31 ± 0.08	0.29 ± 0.13
22:6n-3	0.45 ± 0.02	0.85 ± 0.14
n-6 Polyunsaturated		
18:2n-6	10.41 ± 0.79	5.10 ± 0.40[b]
20:2n-6	7.91 ± 0.17	10.14 ± 0.58
20:3n-6	3.60 ± 1.21	5.68 ± 0.77
20:4n-6	27.59 ± 1.87	28.49 ± 1.71
22:4n-6	2.12 ± 0.07	3.09 ± 0.18[b]
22:5n-6	4.58 ± 0.29	5.99 ± 0.17
Total	100	100
Total saturates	20.85 ± 1.62	18.37 ± 1.45
Total monoenes	10.99 ± 0.21	16.29 ± 1.51[b]
Total polyenes	68.16 ± 1.49	65.34 ± 2.71
n-3 Polyenes	11.93 ± 0.87	6.84 ± 0.49[b]
n-6 Polyenes	56.22 ± 1.26	58.50 ± 2.33
n-3/n-6	0.21 ± 0.02	0.12 ± 0.01[b]
Monoenes/polyenes	0.16 ± 0.01	0.26 ± 0.04[b]
Unsaturation index[c]	243.85 ± 6.90	247.84 ± 8.22
Chain length[d]	18.91 ± 0.07	18.97 ± 0.11

[a–d]See Table 2 for footnotes.

TABLE 4
Percentages of Individual Fatty Acids in Phosphatidylcholine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.45 ± 0.14	1.18 ± 0.63
14:1	1.95 ± 1.25	0.98 ± 0.45
16:0	7.32 ± 0.38	6.64 ± 0.28
16:1	1.45 ± 0.56	2.57 ± 0.68
18:0	2.38 ± 0.12	4.06 ± 0.40[b]
18:1	13.93 ± 0.88	11.22 ± 1.14
20:0	0.32 ± 0.03	0.50 ± 0.10
20:1	3.37 ± 0.39	4.60 ± 0.40
23:0	0.21 ± 0.18	0.10 ± 0.05
n-3 Polyunsaturated		
18:3n-3	8.14 ± 0.44	2.37 ± 0.50[b]
18:4n-3	2.83 ± 0.47	1.00 ± 0.15[b]
20:3n-3	3.77 ± 0.44	1.70 ± 0.12[b]
20:4n-3	n.d.	0.12 ± 0.09
20:5n-3	1.35 ± 0.20	0.90 ± 0.15
22:6n-3	1.10 ± 0.16	2.35 ± 0.58
n-6 Polyunsaturated		
18:2n-6	19.69 ± 1.05	9.09 ± 0.62[b]
20:2n-6	12.62 ± 0.42	16.11 ± 0.64[b]
20:3n-6	2.53 ± 0.44	4.58 ± 0.67
20:4n-6	12.36 ± 0.59	21.01 ± 0.91[*b]
22:2n-6	n.d.	n.d.
22:4n-6	0.39 ± 0.03	0.60 ± 0.04
22:5n-6	3.65 ± 0.22	8.24 ± 0.66[b]
Total	100	100
Total saturates	10.77 ± 0.44	12.48 ± 1.13
Total monoenes	20.70 ± 1.30	19.38 ± 1.50
Total polyenes	68.53 ± 1.64	68.14 ± 2.06
n-3 Polyenes	17.28 ± 1.16	8.50 ± 0.67[b]
n-6 Polyenes	51.26 ± 1.59	59.64 ± 2.22
n-3/n-6	0.34 ± 0.03	0.15 ± 0.02[b]
Monoenes/polyenes	0.31 ± 0.03	0.29 ± 0.03
Unsaturation index[c]	222.82 ± 3.90	246.51 ± 6.74
Chain length[d]	18.33 ± 0.08	19.03 ± 0.12[b]

[a–d]See Table 2 for footnotes.

TABLE 5
Percentages of Individual Fatty Acids in Phosphatidylserine of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.45 ± 0.24	3.07 ± 1.54
14:1	0.93 ± 0.45	2.56 ± 0.90
16:0	7.26 ± 0.80	7.51 ± 2.31
16:1	2.14 ± 0.61	4.92 ± 1.94
18:0	25.40 ± 1.40	20.15 ± 4.56
18:1	5.96 ± 0.50	7.77 ± 2.08
20:0	1.34 ± 0.41	2.40 ± 0.45
20:1	11.07 ± 2.33	5.55 ± 1.79
23:0	0.14 ± 0.11	0.78 ± 0.36
n-3 Polyunsaturated		
18:3n-3	9.57 ± 0.47	25.35 ± 9.71
18:4n-3	2.06 ± 0.47	1.70 ± 0.68
20:3n-3	0.57 ± 0.33	n.d.
20:5n-3	0.26 ± 0.13	n.d.
22:6n-3	0.19 ± 0.10	2.35 ± 0.58
n-6 Polyunsaturated		
18:2n-6	2.95 ± 0.55	3.03 ± 0.91
20:2n-6	2.03 ± 0.35	1.49 ± 0.70
20:3n-6	2.17 ± 0.41	1.43 ± 0.47
20:4n-6	23.87 ± 1.32	11.34 ± 1.35[b]
22:4n-6	0.16 ± 0.06	n.d.
22:5n-6	1.49 ± 0.25	0.85 ± 0.30
Total	100	100
Total saturates	34.60 ± 0.89	33.91 ± 5.58
Total monoenes	20.10 ± 1.60	20.81 ± 4.62
Total polyenes	45.30 ± 1.34	45.29 ± 9.87
n-3 Polyenes	12.64 ± 0.85	27.13 ± 9.94
n-6 Polyenes	32.67 ± 1.64	18.16 ± 2.31[b]
n-3/n-6	0.40 ± 0.04	1.84 ± 0.70
Monoenes/polyenes	0.45 ± 0.04	0.75 ± 0.20
Unsaturation index[c]	181.18 ± 3.97	167.04 ± 23.87
Chain length[d]	18.24 ± 0.09	16.93 ± 0.42

[a–d]See Table 2 for footnotes.

No statistically significant changes to proportions of saturates, monoenes, or polyenes were observed in any other phospholipids. However, PC showed a similar decrease (51%) in n-3 fatty acid content in estivators (Table 5). This occurred primarily through significant reductions to 18:3n-3, 18:4n-3, and 20:3n-3 contents during estivation. The n-3/n-6 ratio of PC from estivating snails was thus decreased by 56%.

PC was the only phospholipid to show a change in average fatty acid chain length between experimental groups. PC from mitochondria of estivating snails contained longer fatty acyl chains on average than in active snails.

The n-3/n-6 polyene ratio appeared to be 4.5-fold greater in phosphatidylserine from estivating snails (Table 6), though a relatively large standard error made this statistically insignificant at $\alpha = 0.0034$. The altered value of the n-3/n-6 ratio was largely due to a statistically significant 50% decrease in 20:4n-6 and a concomitant, but not significant, 2.6-fold increase in 18:3n-3.

In phosphatidylinositol (PI), only 20:1 and 16:1 content were altered in estivators (Table 7). Proportions of both increased, which was reflected in a doubling of the proportion of monoenes in PI, though this was also not significant at $\alpha = 0.0034$.

Arachidonic acid (20:4n-6) was present in trace amounts in lettuce (Table 1), but occurred as a major constituent fatty acid of all phospholipids, except for CL, where it accounted for less than 4% of total fatty acids in active snails. 20:4n-6 was enriched 70% in PC during estivation, while PS 20:4n-6 content decreased by 52%.

DISCUSSION

The phospholipid composition of mitochondrial membranes from *C. nemoralis* hepatopancreas is dramatically altered during estivation. Many aspects of this remodeling indicate a reduced biological activity of the mitochondrion during estivation, when metabolic rate is reduced to about 15% of normal

TABLE 6
Percentages of Individual Fatty Acids in Phosphatidylinositol of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
Nonessential		
14:0	0.80 ± 0.45	4.68 ± 1.65
14:1	0.62 ± 0.32	1.39 ± 0.70
16:0	24.07 ± 0.94	19.05 ± 2.36
16:1	2.61 ± 0.58	7.89 ± 1.51[b]
18:0	26.45 ± 0.89	23.67 ± 1.18
18:1	4.95 ± 1.00	7.46 ± 1.05
20:0	1.16 ± 0.10	0.93 ± 0.20
20:1	2.60 ± 0.15	5.36 ± 0.73[b]
23:0	0.20 ± 0.10	0.19 ± 0.16
n-3 Polyunsaturated		
18:3n-3	4.21 ± 0.26	2.67 ± 0.41
18:4n-3	1.12 ± 0.16	0.94 ± 0.45
20:3n-3	1.01 ± 0.26	0.51 ± 0.27
20:4n-3	n.d.	n.d.
20:5n-3	0.67 ± 0.11	0.43 ± 0.17
22:6n-3	0.09 ± 0.06	0.30 ± 0.12
n-6 Polyunsaturated		
18:2n-6	2.32 ± 0.30	1.05 ± 0.35
20:2n-6	1.63 ± 0.24	1.57 ± 0.20
20:3n-6	2.52 ± 0.49	2.48 ± 0.57
20:4n-6	22.59 ± 0.77	18.03 ± 2.68
22:4n-6	0.04 ± 0.04	0.14 ± 0.6
22:5n-6	0.32 ± 0.13	1.27 ± 0.42
Total	100	100
Total saturates	52.68 ± 1.64	48.52 ± 2.89
Total monoenes	10.78 ± 1.41	22.10 ± 2.31
Total polyenes	36.54 ± 1.58	29.38 ± 4.05
n-3 Polyenes	7.10 ± 0.37	4.85 ± 0.88
n-6 Polyenes	29.44 ± 1.31	24.54 ± 3.42
n-3/n-6	0.24 ± 0.01	0.20 ± 0.04
Monoenes/polyenes	0.30 ± 0.04	0.98 ± 0.29
Unsaturation index[c]	142.42 ± 4.74	131.04 ± 14.64
Chain length[d]	17.86 ± 0.05	17.62 ± 0.25

[a–d]See Table 2 for footnotes.

TABLE 7
Percentages of Individual Fatty Acids in Phosphatidylinositol of Hepatopancreas Mitochondria from Active and Estivating *Cepaea nemoralis*[a–d]

Fatty acid	Active (n = 7)	Estivating (n = 8)
14:0	0.80 ± 0.45	4.68 ± 1.65
14:1	0.62 ± 0.32	1.39 ± 0.70
16:0	24.07 ± 0.94	19.05 ± 2.36
16:1	2.61 ± 0.58	7.89 ± 1.51[b]
18:0	26.45 ± 0.89	23.67 ± 1.18
18:1	4.95 ± 1.00	7.46 ± 1.05
18:2n-6	2.23 ± 0.30	1.05 ± 0.35
18:3n-3	4.21 ± 0.26	2.67 ± 0.41
18:4n-3	1.12 ± 0.16	0.94 ± 0.45
20:0	1.16 ± 0.10	0.93 ± 0.20
20:1	2.60 ± 0.15	5.36 ± 0.73[b]
20:2n-6	1.63 ± 0.24	1.57 ± 0.20
20:3n-6	2.52 ± 0.49	2.48 ± 0.57
20:4n-6	22.59 ± 0.77	18.03 ± 2.68
20:3n-3	1.01 ± 0.26	0.51 ± 0.27
20:4n-3	n.d.	n.d.
20:5n-3	0.67 ± 0.11	0.43 ± 0.17
22:0	n.d.	n.d.
22:1	n.d.	n.d.
22:2n-6	n.d.	n.d.
23:0	0.20 ± 0.10	0.19 ± 0.16
22:4n-6	0.04 ± 0.04	0.14 ± 0.6
22:5n-6	0.32 ± 0.13	1.27 ± 0.42
22:5n-3	n.d.	n.d.
22:6n-3	0.09 ± 0.06	0.30 ± 0.12
24:0	n.d.	n.d.
24:1	n.d.	n.d.
Total	100	100
Total saturates	52.68 ± 1.64	48.52 ± 2.89
Total monoenes	10.78 ± 1.41	22.10 ± 2.31
Total polyenes	36.54 ± 1.58	29.38 ± 4.05
n-3 Polyenes	7.10 ± 0.37	4.85 ± 0.88
n-6 Polyenes	29.44 ± 1.31	24.54 ± 3.42
n-3/n-6	0.24 ± 0.01	0.20 ± 0.04
Monoenes/polyenes	0.30 ± 0.04	0.98 ± 0.29
Unsaturation index[c]	142.42 ± 4.74	131.04 ± 14.64
Chain length[d]	17.86 ± 0.05	17.62 ± 0.25

[a–d]See Table 2 for footnotes.

(1,2). These changes are consistent with the inhibition of certain mitochondrial membrane-bound proteins, a stabilizing of the bilayer, and a reduced propensity toward hexagonal phase formation.

Perhaps the most dramatic changes to constituent fatty acids occurred in CL, a unique phospholipid which typically is found exclusively in the mitochondrial inner membrane (7). CL is found in close association with certain mitochondrial proteins, including the mono-, di-, and tricarboxylate carriers, and also carnitinepalmitoyl translocase, cytochrome c oxidase, ADP/ATP exchanger, and phosphate transporter (7). Many of these proteins do not function, or function submaximally, in its absence. In many cases, the ability of CL to stimulate protein function is dependent upon its fatty acyl composition (7,15,16). For example, several studies have demonstrated a specific requirement of the respiratory chain enzyme cytochrome c oxidase for CL with 18:2n-6 acyl chains. In rats fed diets deficient in 18:2n-6, which results in CL 18:2n-6 being replaced with other fatty acids (17), mitochondrial function (18), and cytochrome c oxidase activity (15) are both decreased by as much as 50%. This loss of activity has been shown to result directly from the presence of 18:2n-6-deficient CL, as activity can be fully recovered when delipidated cytochrome c oxidase is reconstituted with 18:2n-6/18:2n-6 CL (15). In estivating *Cepaea*, cytochrome c oxidase activity is reduced by 85% (14). This could be mediated in part by the significant reduction of CL 18:2n-6 content. As in mammals, *Cepaea* CL was particularly enriched in 18:2n-6, which accounted for 60% of all fatty acids in CL of active snails. The 18:2n-6 content of CL from estivating *Cepaea* decreased by 50%. A similar decrease in CL 18:2n-6 content in rats fed an 18:2n-6-deficient diet results in a 26% decrease in cytochrome c oxidase activity (15), suggesting that, during estivation, the reduced 18:2n-6 content of CL may play a role in

the observed reduction of cytochrome c oxidase activity. The activities of other CL-requiring mitochondrial membrane proteins which have specific interactions with 18:2n-6 acyl chains (7,15,16) may be similarly suppressed.

The altered fatty acyl composition of CL changes the molecular geometry of this phospholipid, and thus its functional properties in the membrane (19,20). CL of estivating snails was threefold more saturated, contained ninefold more monoenes, and had 45% fewer polyenes than that of active snails. Thus, the unsaturation index of CL from estivating snails was 33% reduced from control values. The more highly saturated CL found in estivating snails effects changes in the molecular geometry of CL which decrease its propensity to form nonlamellar structures, like the inverse hexagonal (H_{II}) phase (19,20). H_{II} phase-favoring phospholipids affect the structure and physical properties of membranes (8,21–23). These, in turn, can modulate the function of specific membrane proteins (24), an interaction illustrated by the requirement of a minimal proportion of hexagonal phase-preferring lipids for proper function of membrane-bound proteins, like rhodopsin in the visual system (25).

Other modifications to mitochondrial membrane composition during estivation also suggest greater bilayer stability. The mitochondrial membranes of estivating snails are characterized by an increased proportion of monoenes in PE. They also contain lower proportions of the H_{II} phase-preferring phospholipids, PE and CL (14). The other major constituent of mitochondrial membranes, PC, shows an increased average acyl chain length. Taken together, these changes suggest that membrane remodeling during estivation selects against H_{II} phase-preferring phospholipids, resulting in the adoption of a more stable lamellar phase in the phospholipid bilayer of estivators. H_{II} phase-preferring phospholipids are known to be important in a number of biological functions which are key to anabolic and catabolic processes, including trafficking of membrane fragments and proteins, fusion, and mitochondrial contact sites (8,24,26–29). The compositional changes which lower the tendency for formation of nonbilayer structures in mitochondrial membranes appear to be related to the depressed metabolism, and therefore reduced rates of these processes in estivating *Cepaea*.

Other aspects of the phospholipid compositions of mitochondria from estivatings snails are typical of those observed in association with reduced metabolic rates. The proportional content of monoenes in CL, PE, and PI was greater in estivating snails, due to the replacement of polyenes, like 18:2n-6 and 18:3n-3, with 16:1 and 18:1. Similarly, increased proportions of phospholipid monoenes have been shown to correlate strongly with reduced metabolic rates in other organisms (30,31). The specific mechanism underlying this correlation remains unknown, and the effects of phospholipid incorporation of monoenes are complicated. While modeling studies indicate that greater incorporation of monoenes in the *sn*-1 position can induce looser packing arrangements (32), dimonoenoic phospholipids are better able to form highly ordered phases than di-polyenoic phospholipids (22). Thus, the neighboring fatty acyl chain will determine how increased monounsaturation affects phospholipid properties. The impact of increased monoene incorporation in PE, PI, and CL on membrane bilayer stability is, therefore, uncertain without positional information. The reported association between monoenes and metabolic rate in various systems is, however, an interesting phenomenon that warrants further investigation.

The n-3/n-6 ratio of membrane phospholipid fatty acids was reduced in estivating snails, primarily due to decreased proportions of 18:3n-3 and 18:4n-3 in PE and PC. Similarly, lower proportional contents of n-3 polyenes are characteristic of the mitochondrial membranes of animals with lower metabolic rates (30,31). This has been demonstrated allometrically in mammals (31) and through comparisons of reptiles with mammals (30). In contrast, higher levels of n-3 polyunsaturated fatty acids are associated with superior recovery from postischemia reperfusion in rat heart (33), when oxygen levels may be expected to be abnormally high. Thus, the significance of phospholipid n-3 content may be related to the cellular oxygen concentrations which characterize different metabolic rates and physiological states. Lower relative levels of n-3 polyenes in estivating snails thus appear to be related to the metabolic rate reduction in these animals.

Changes in phospholipid fatty acid composition induced by estivation may not be attributable solely to the effects of elongation or desaturation. Mitochondria of estivating snails contain fewer phospholipids than those of active animals. These phospholipids do not appear to be lost from the cell (14). This suggests that phospholipids may be removed from the mitochondria and sequestered elsewhere in the cell (14). We have observed that a significant reduction in total mitochondrial phospholipid content occurs during estivation. Preferential removal of specific phospholipid or fatty acid species from the membrane thus provides a mechanism for altering phospholipid-specific fatty acid composition which is independent of elongating and desaturating processes.

In summary, the dramatic changes in phospholipid-specific fatty acid composition demonstrated here are consistent with profound reductions in the rates of membrane-associated processes during estivation. These results also suggest that estivating snails are a valuable model for studies of the relationship between metabolic rate and mitochondrial membrane composition. The results of this study should be extended to vertebrate estivators and to investigations of the functional properties of mitochondrial and cellular membranes during estivation.

ACKNOWLEDGMENTS

We wish to thank Amy Bourns and Jason McLeod for their assistance in the laboratory. This work was supported by a Natural Sciences and Engineering Research Council operating grant to JSB.

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[Received September 19, 1997, and in final revised form and accepted June 8, 1998]

Changes in Tissue Polyunsaturated Fatty Acids with Age, in Spontaneously Hypertensive Rats

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ABSTRACT: The relationship between the biosynthesis of long-chain fatty acids and their distribution in the key organs of hypertension is of considerable interest because of their role in the production of vasoactive eicosanoids and their effects on membrane properties. The present study analyzed the fatty acid compositions of the total lipids in the kidney, aorta, heart, and hepatocytes of 1-, 3-, and 6-mon-old spontaneously hypertensive rats (SHR) and their normotensive controls, Wistar Kyoto rats (WKY) by capillary gas chromatography . The major changes concerned the polyunsaturated fatty acids (PUFA). The percentage of arachidonic acid (AA) was significantly greater in the 1-mon-old SHR kidney than in the WKY kidney, but it was lower at 3 and 6 mon. The percentage of eicosapentaenoic acid was very low in the SHR kidney. The results for the aorta were similar, with marked decreases in 18:2n-6 and 18:3n-3 in SHR aged 1 and 6 mon. Despite a higher proportion of 18:2n-6 and AA at 6 mon, there was no major change in the SHR heart lipids. The fatty acid spectrum in the liver provides additional evidence for the previously reported inhibition of desaturase activities in SHR. Thus, this study shows that the PUFA composition is modified differently in different tissues in SHR, and this may be related to the pathogenesis of hypertension in these animals.

Lipids 33, 795–801 (1998).

Both humans and animals suffering from genetic hypertension have abnormal fatty acid metabolism (1–7). The synthesis of polyunsaturated fatty acids (PUFA) in the liver of spontaneously hypertensive rats (SHR) differs significantly from that of normotensive Wistar Kyoto (WKY) rats, depending on the age of the animals and the pathogenesis of hypertension (7–9). The activities of liver microsomal desaturases, which are key enzymes in PUFA synthesis, are greatly reduced, and this becomes more severe once the rats are hypertensive (after 3 mon of age) than during the prehypertensive period. These abnormalities may be linked to the altered membrane properties and functions that occur in hypertension (3,10,11), as membrane viscosity can be affected by small changes in fatty acid composition (12). Ollerenshaw *et al.* (13) showed that the erythrocyte membranes of patients with essential hypertension have a reduced linoleic acid (LA) content, while the arachidonic acid (AA) and oleic acid contents are above normal in subjects consuming similar diets.

Abnormalities of fatty acid metabolism may have implications for the local synthesis of eicosanoids, which depends greatly on the availability of C_{20} PUFA precursors (dihomo-γ-linolenic acid, DGLA, 20:3n-6; AA, 20:4n-6; eicosapentaenoic acid, EPA, 20:5n-3), arriving *via* the circulation. Their release from membrane phospholipids (PL) is the rate-limiting step in the synthesis of vasoactive eicosanoids involved in the regulation of blood pressure. These include thromboxane A_2, derived from AA, which is a powerful vasoconstrictor; prostaglandin E_2 and prostaglandin I_2, derived from the same precursor, which have the opposite effect; and prostaglandin E_1, derived from DGLA (14–18).

Consequently, the relationship between changes in liver PUFA synthesis and the PUFA status of organs in SHR involved in the pathology of hypertension has to be considered. The present work examines the distribution of PUFA in several tissues involved in hypertension, the heart (19), kidney (20–22) and thoracic aorta (23,24), as well as in the isolated hepatocytes of SHR and WKY rats. Measurements were made on rats aged 1, 3, and 6 mon to detect changes linked to the pathogenesis of hypertension (before 3 mon of age) and the blood pressure.

MATERIALS AND METHODS

Male SHR and WKY normotensive rats aged 3 or 11 wk were purchased from Iffa Credo (Domaine des Oncins, L'Arbresle, France), and fed *ad libitum* on a commercial standard diet (Souriffarat, UAR, Villemoisson Sur Orge, France; weight in g/kg pellets: protein 210; carbohydrate 535; fat 40; cellulose 45; moisture content 115; ash 55; minerals 27.8; vitamins: retinol 16,000 IU/kg, cholecalciferol 2,000 IU/kg, vitamin E 500 mg/kg; fatty acid composition of fat (% wt/wt of total): palmitic acid, 16:0: 10.1; palmitoleic acid, 16:1n-7: 0.9; stearic acid, 18:0: 2.7; oleic acid, 18:1n-9: 26.9; LA, 18:2n-6: 49.2; linolenic acid, 18:3n-3: 0.2; others: 10.0) and given tap water *ad libitum*. The schedule was 12 h dark/12 h light.

The systolic blood pressure of prewarmed, conscious rats was measured before killing, using a tail pulse transducer (M+M Electronic Inc., Basel-Müncheenstein, Switzerland).

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Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Blood pressure was determined for each animal by taking the average of five measurements.

Groups of four animals from each strain aged 1, 3, and 6 mon were anesthetized with sodium pentobarbital (Sanofi, Paris, France). Hepatocytes were prepared by the method of Seglen (25) and suspended in Krebs-Henseleit bicarbonate buffer containing 1% fatty acid-free bovine serum albumin. Hearts, kidneys, and thoracic aortas were quickly removed, weighed and frozen. They and the isolated hepatocytes were stored at −80°C until analysis. They were cut into thin slices, homogenized in 15 mL chloroform/methanol (2:1, vol/vol). Total lipids were extracted by the method of Folch *et al.* (26), saponified, and methylated by heating in 14% boron trifluoride in methanol at 80°C for 20 min (27). Fatty acid methyl esters were then extracted with 2 mL isooctane, separated by gas–liquid chromatography (Packard, model 417 gas–liquid chromatograph equipped with a flame–ionization detector and a 30-m capillary glass column coated with Carbowax 20M), and identified by their relative retention times (fatty acid standards: gas–liquid chromatography model mixture reference standards; Nu-Chek-Prep, Elysian, MN). Results (mole %) were expressed as arithmetic means with standard errors (mean ± SE) for each group.

Statistical significance of differences between means was assessed using the Student's *t* test. Values were considered significant when $P < 0.05$ and very significant when $P < 0.01$.

RESULTS

Systolic blood pressure. SHR blood pressures (mm Hg) were significantly higher (135 ± 4 at 1 mon, 221 ± 8 at 3 mon, and 232 ± 10 at 6 mon) than those of WKY at all three ages studied: 107 ± 6 (1 mon), 141 ± 7 (3 mon), and 146 ± 8 (6 mon). Blood pressure became abnormally high in SHR at the age of 2 mon (about 150 mm Hg). Hypertension was definitively established at the age of 3 mon (221 ± 8 mm Hg) and remained more or less constant thereafter.

Fatty acid composition of kidney total lipids. The fatty acid composition of the kidney total lipids is shown in Table 1. At each age, changes were seen between the SHR and WKY groups. At 1 mon of age, kidneys from the SHR had lower amounts of 18:2n-6 (16.5 vs. 18.3 mole %), 18:3n-3 (0.35 vs. 0.66 mole %), and less 20:5n-3 (0.50 vs. 0.77 mole %) compared to the WKY rats, but higher levels of 20:4n-6 (26.6 vs. 20.4 mole %). The 3 mon-old SHR had a higher concentration of LA (18.1 vs. 16.0 mole %) than the WKY rats, but there was no significant difference in their AA contents. The 3-mon-old SHR had significantly lower levels of 22:5n-3 (docosapentaenoic acid, DPA) than the WKY kidneys (0.43 vs. 0.53 mole %), but interestingly there were no differences in the levels of docosahexaenoic acid (DHA, 22:6n-3) in these tissues at this age. The 6 mon-old SHR had lower concentrations of AA (21.2 vs. 26.0 mole %), 22:5n-6 (0.15 vs. 0.48 mole %), EPA (0.15 vs. 0.75 mole %), DPA (0.36 vs. 0.82 mole %), and DHA (1.78 vs. 3.41 mole %) than WKY rats of the same age. SHR had significantly more 18:0 (8.8 vs. 7.2 mole %) than the WKY at the age of 1 mon, less 18:0 at the age of 3 mon, and significantly more 16:0 (23.2 vs. 20.0 mole %) and 18:1n-9 (15.8 vs. 12.6 mole %) than WKY at 6 mon.

The PUFA/saturated fatty acid (SFA) ratio decreased in SHR with age, while it increased in WKY. The 18:2n-6 concentration tended to increase with age in SHR, whereas 20:4n-6 decreased. The corresponding fatty acids of the n-3 family changed similarly. With respect to WKY rats, 20:4n-6

TABLE 1
Fatty Acid Composition of Kidney Total Lipids[a]

	1 mon		3 mon		6 mon	
Fatty acid	WKY	SHR	WKY	SHR	WKY	SHR
16:0	25.59 ± 1.08	24.30 ± 0.57	22.20 ± 0.25	22.25 ± 0.33	20.03 ± 0.34	23.17 ± 0.42[c]
16:1n-7	4.99 ± 0.57	3.44 ± 0.72	3.90 ± 0.32	4.39 ± 0.44	2.41 ± 0.48	3.20 ± 0.40
18:0	7.23 ± 0.40	8.83 ± 0.57[b]	11.22 ± 0.15	10.21 ± 0.46[b]	11.73 ± 1.16	12.60 ± 0.35
18:1n-9	14.91 ± 0.52	12.72 ± 0.90[b]	12.73 ± 1.04	11.81 ± 0.57	12.63 ± 1.53	15.76 ± 0.74[b]
18:2n-6	18.29 ± 1.08	16.50 ± 0.40	16.08 ± 0.10	18.11 ± 0.28[c]	19.04 ± 0.76	18.63 ± 0.46
18:3n-3	0.66 ± 0.12	0.35 ± 0.04[b]	0.43 ± 0.06	0.49 ± 0.07	0.50 ± 0.08	0.52 ± 0.04
20:1n-9	0.07 ± 0.03	0.07 ± 0.02	0.16 ± 0.02	0.08 ± 0.00[c]	0.15 ± 0.08	0.17 ± 0.01
20:2n-6	0.34 ± 0.06	0.35 ± 0.04	0.20 ± 0.07	0.27 ± 0.02	0.28 ± 0.04	0.29 ± 0.03
20:3n-6	0.74 ± 0.05	0.92 ± 0.11	0.83 ± 0.03	0.75 ± 0.05	0.66 ± 0.09	0.60 ± 0.04
20:4n-6	20.35 ± 0.96	26.57 ± 1.43[c]	26.54 ± 1.43	25.93 ± 0.73	25.97 ± 2.67	21.22 ± 0.94[b]
20:5n-3	0.77 ± 0.04	0.50 ± 0.05[c]	0.67 ± 0.09	0.48 ± 0.04[b]	0.75 ± 0.13	0.15 ± 0.02[c]
22:4n-6	0.40 ± 0.05	0.44 ± 0.09	0.51 ± 0.03	0.48 ± 0.06	0.83 ± 0.22	0.56 ± 0.03[b]
22:5n-6	0.33 ± 0.13	0.08 ± 0.06[b]	0.10 ± 0.02	0.10 ± 0.03	0.48 ± 0.20	0.15 ± 0.04[b]
22:5n-3	0.42 ± 0.16	0.43 ± 0.06	0.53 ± 0.03	0.43 ± 0.02[c]	0.82 ± 0.34	0.36 ± 0.04[b]
22:6n-3	3.12 ± 0.32	3.46 ± 0.29	2.94 ± 0.11	3.13 ± 0.24	3.41 ± 0.71	1.78 ± 0.18[b]
SFA	33.08	33.13	33.42	32.46	31.75	35.77
PUFA	45.40	49.59	48.82	47.04	52.74	44.23
PUFA/SFA	1.37	1.50	1.46	1.45	1.66	1.24

[a]Values (mol %) are given as mean values ± SEM for four rats.
[b,c]Significantly different from the value for the WKY control group at $P < 0.05$, $P < 0.01$, respectively. Abbreviations: WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat; SFA, total saturated fatty acids; PUFA, total polyunsaturated fatty acids.

was higher and 18:2n-6 lower in 3-mon-old than in younger rats, and the opposite occurred in the 6-mon-old rats.

Fatty acid composition of aorta total lipids. The fatty acid composition of aorta total lipids is shown in Table 2. The most important changes in the fatty acid composition in this tissue concerned LA, AA, and EPA. There was a highly significant decrease in LA (to 13.1) and in EPA and a slight increase in AA in 1-mon-old SHR as compared to WKY. By the time the rats had reached 3 mon 20:4n-6 was significantly elevated (3.6%) in SHR, while adrenic acid (22:4n-6) and DHA were only slightly higher in SHR than in WKY. Six-mon-old SHR had significantly lower LA (22.7%) and 18:3n-3 (1.1%) than WKY (30.0 and 1.5%) and slightly higher AA. The SHR had significantly more 16:1n-7 (5.8%) and 18:1n-9 (22.3%) at 1 mon than did WKY (4.1 and 19.9%), but they had significantly less 18:1n-9 (27.4%) at the age of 3 mon than did WKY (32.2%). The SHR had significantly more 16:0 and 16:1n-7 (24.8 and 9.9%) than WKY (21.3, 8.0%) at 6 mon.

The PUFA/SFA ratio increased greatly at the age of 6 mon in both strains. In relation to age, the fatty acid profiles for both strains were similar, in spite of a smaller increase in 18:1n-9 in SHR at 3 mon than in WKY, and a smaller increase in 18:2n-6 at 6 mon. The fatty acid profile of 6-mon-old WKY was very different from that of SHR and of the younger WKY rats.

Fatty acid composition of heart total lipids. The fatty acid composition of heart total lipids is shown in Table 3. Levels of 18:3n-3, 20:5n-3 and 22:6n-3 were similar in the SHR and WKY groups at all time periods studied. Levels of the n-6 fatty acids showed no major difference between these two groups at 1 and 3 mon of age except that SHR aged 3 mon had significantly lower 22:5n-6 and 22:5n-3 than WKY. By 6 mon of age, 20:4n-6 and the C_{22} fatty acids were significantly higher in the SHR, compared to the WKY group. In contrast, the levels of the 20:2n-6 and 20:3n-6 fatty acids were markedly reduced in this tissue from the SHR group.

The PUFA/SFA ratio increased in SHR with age. With respect to the effect of age, the amounts of 18:2n-6 were significantly lower and those of 22:6n-3 significantly higher in 6 mon-old WKY than in 1- and 3-mon-old rats. Stearic acid tended to increase with age, while 18:1n-9 remained unchanged. SHR had a similar profile, except that 20:4n-6 was higher at 6 mon than at the two other ages.

Fatty acid composition of hepatocyte total lipids. The fatty acid composition of hepatocyte total lipids is shown in Table 4. In this tissue, the major changes in the fatty acid levels between SHR and WKY groups were observed at the age of 3 mon, whereas there was no significant difference between 1 mon-old SHR and WKY for any of the fatty acids. At 3 mon of age, the SHR showed marked increases in the levels of 18:2n-6 (23.8 vs. 18.0 mole %), 18:3n-3 (0.8 vs. 0.4 mole %), and 22:6n-3 (7.5 vs. 6.5 mole %) compared to the WKY hepatocytes. In contrast, levels of 20:4n-6 (16.4 vs. 22.8 mole %) and its precursor fatty acid, 20:3n-6 (0.5 vs. 0.8 mole %), were lower in this group compared to the WKY animals. Hepatocytes from 3 mon-old SHR had significantly lower 16:0 (23.5 vs. 25.7 mole %) and 18:0 (8.8 vs. 13.3 mole %) than WKY, but more 18:1n-9 (13.5 vs. 7.3 mole %).

The PUFA/SFA ratio was higher in 3- and 6-mon old SHR than in WKY. Most of the differences in the fatty acid profiles of the two strains affected 18:2n-6 and 20:4n-6. LA was much higher and 20:4n-6 lower in 3-mon-old SHR than in WKY. The amount of 18:2n-6 increased regularly with age in WKY, while that of 20:4n-6 decreased. There was also a sharp decrease in 18:0 and a sharp increase in 18:1n-9 in 3-mon-old SHR.

Changes in the distribution of C_{20} PUFA and their C_{18} precursors in WKY and SHR organs. Considering changes in C_{20}

TABLE 2
Fatty Acid Composition of Aorta Total Lipids[a]

	1 mon		3 mon		6 mon	
Fatty acid	WKY	SHR	WKY	SHR	WKY	SHR
16:0	29.06 ± 1.36	30.54 ± 2.09	29.62 ± 0.78	29.80 ± 0.59	21.35 ± 0.57	24.80 ± 0.52[c]
16:1n-7	4.12 ± 0.31	5.84 ± 0.36[c]	8.20 ± 0.62	9.46 ± 0.53	8.01 ± 0.30	9.92 ± 0.58[b]
18:0	11.50 ± 0.49	10.42 ± 0.56	7.36 ± 0.61	6.24 ± 0.37	3.71 ± 0.67	3.71 ± 0.26
18:1n-9	19.92 ± 0.41	22.27 ± 0.77[b]	32.18 ± 0.76	27.44 ± 0.91[c]	25.58 ± 0.65	26.59 ± 1.97
18:2n-6	18.04 ± 1.11	13.13 ± 0.61[c]	15.84 ± 0.68	16.18 ± 0.48	30.03 ± 1.94	22.75 ± 0.89[c]
18:3n-3	0.79 ± 0.05	0.61 ± 0.11	0.83 ± 0.05	0.79 ± 0.05	1.46 ± 0.18	1.06 ± 0.07[b]
20:1n-9	0.43 ± 0.16	0.22 ± 0.09	0.35 ± 0.05	0.28 ± 0.02	0.31 ± 0.03	0.16 ± 0.03[b]
20:2n-6	0.41 ± 0.04	0.38 ± 0.06	0.16 ± 0.01	0.25 ± 0.02[c]	0.23 ± 0.01	0.19 ± 0.03
20:3n-6	0.63 ± 0.11	0.56 ± 0.09	0.14 ± 0.01	0.21 ± 0.04	0.21 ± 0.02	0.21 ± 0.05
20:4n-6	7.36 ± 0.98	8.06 ± 1.53	1.61 ± 0.06	3.65 ± 1.03[b]	4.10 ± 0.46	5.43 ± 1.17
20:5n-3	0.15 ± 0.05	0.03 ± 0.03[b]	0.09 ± 0.02	0.13 ± 0.06	0.16 ± 0.02	0.13 ± 0.03
22:4n-6	1.26 ± 0.27	1.30 ± 0.15	0.34 ± 0.02	0.61 ± 0.18	0.34 ± 0.05	0.47 ± 0.07
22:5n-6	1.49 ± 0.36	1.66 ± 0.80	0.10 ± 0.03	0.13 ± 0.03	0.19 ± 0.07	0.51 ± 0.18
22:5n-3	0.86 ± 0.04	1.01 ± 0.37	0.27 ± 0.02	0.43 ± 0.16	0.62 ± 0.50	0.90 ± 0.22
22:6n-3	2.20 ± 0.26	2.17 ± 0.59	0.69 ± 0.10	1.16 ± 0.34	1.94 ± 0.18	1.57 ± 0.40
SFA	40.56	40.96	36.98	36.04	25.06	28.51
PUFA	33.10	28.90	20.05	23.52	39.27	33.22
PUFA/SFA	0.82	0.70	0.54	0.65	1.57	1.16

[a]Values (mol %) are given as mean values ± SEM for four rats.
[b,c]Significantly different from the value for the WKY control group at $P < 0.05$, $P < 0.01$, respectively. For abbreviations see Table 1.

TABLE 3
Fatty Acid Composition of Heart Total Lipids[a]

	1 mon		3 mon		6 mon	
Fatty acid	WKY	SHR	WKY	SHR	WKY	SHR
16:0	15.31 ± 1.27	15.46 ± 0.64	12.54 ± 0.45	12.90 ± 0.89	14.49 ± 0.39	13.05 ± 0.64[b]
16:1n-7	1.72 ± 0.60	1.67 ± 0.31	1.22 ± 0.05	1.34 ± 0.09	1.10 ± 0.12	1.27 ± 0.24
18:0	11.72 ± 1.47	12.06 ± 1.40	13.77 ± 0.80	13.33 ± 0.58	14.68 ± 1.75	11.68 ± 1.70
18:1n-9	9.79 ± 1.14	10.56 ± 0.56	9.61 ± 0.41	9.86 ± 0.38	10.86 ± 1.35	10.01 ± 1.15
18:2n-6	28.18 ± 1.36	27.86 ± 1.52	30.47 ± 0.71	30.57 ± 0.94	24.02 ± 1.43	26.01 ± 1.51
18:3n-3	0.50 ± 0.10	0.45 ± 0.04	0.28 ± 0.02	0.29 ± 0.01	0.35 ± 0.04	0.42 ± 0.05
20:1n-9	0.21 ± 0.02	0.22 ± 0.04	0.16 ± 0.01	0.15 ± 0.04	0.20 ± 0.02	0.18 ± 0.04
20:2n-6	0.36 ± 0.04	0.33 ± 0.02	0.26 ± 0.03	0.23 ± 0.04	0.26 ± 0.02	0.19 ± 0.02[b]
20:3n-6	0.47 ± 0.04	0.47 ± 0.01	0.33 ± 0.02	0.33 ± 0.04	0.27 ± 0.01	0.24 ± 0.02[b]
20:4n-6	17.08 ± 1.10	16.71 ± 0.49	16.86 ± 0.58	17.12 ± 0.60	16.17 ± 0.87	20.29 ± 1.57[b]
20:5n-3	0.20 ± 0.05	0.18 ± 0.01	0.26 ± 0.03	0.18 ± 0.04	0.13 ± 0.02	0.12 ± 0.02
22:4n-6	1.08 ± 0.12	0.94 ± 0.12	0.86 ± 0.14	1.02 ± 0.23	0.78 ± 0.03	0.83 ± 0.07[b]
22:5n-6	0.81 ± 0.14	0.87 ± 0.12	0.65 ± 0.10	0.41 ± 0.07[b]	0.64 ± 0.27	1.28 ± 0.04[b]
22:5n-3	1.86 ± 0.17	1.67 ± 0.11	2.25 ± 0.11	1.94 ± 0.04[b]	2.50 ± 0.11	2.58 ± 0.13
22:6n-3	10.12 ± 0.79	9.95 ± 0.25	10.26 ± 0.26	10.04 ± 0.50	13.19 ± 0.58	11.63 ± 1.36
SFA	27.03	27.52	26.31	26.22	29.17	24.72
PUFA	60.65	59.41	62.48	62.12	58.30	63.58
PUFA/SFA	2.24	2.16	2.37	2.37	2.00	2.57

[a]Values (mol %) are given as mean values ± SEM for four rats.
[b,c]Significantly different from the value for the WKY control group at $P < 0.05$, $P < 0.01$, respectively. For abbreviations see Table 1.

and C_{18} PUFA profiles in WKY and SHR organs, LA in aorta and kidney of SHR (1 and 6 mon) was lower than in WKY. It was higher in the SHR heart (6 mon), hepatocyte (1 and 3 mon) and kidney (3 mon). The AA was usually higher in the SHR aorta and heart (at 6 mon) than in WKY. It was higher in the SHR kidney during the prehypertensive period (1 mon), but the amounts in the kidney and hepatocytes of adult SHR were similar to or less than those in WKY. Linolenic acid was also lower in SHR than in WKY during the prehypertensive period (1 mon), particularly in the SHR aorta and kidney, but it was markedly higher in the SHR hepatocytes (3 mon). EPA was quite often lower in SHR organs than in WKY, particularly in the SHR aorta (1 mon), kidney (1 and 6 mon), and hepatocytes (6 mon).

TABLE 4
Fatty Acid Composition of Hepatocyte Total Lipids[a]

	1 mon		3 mon		6 mon	
Fatty acid	WKY	SHR	WKY	SHR	WKY	SHR
16:0	19.61 ± 1.30	19.97 ± 0.60	25.72 ± 0.14	23.55 ± 0.71[b]	21.69 ± 2.70	20.22 ± 2.00
16:1n-7	0.81 ± 0.50	1.03 ± 0.10	2.70 ± 0.32	2.48 ± 0.32	1.61 ± 0.20	1.55 ± 0.30
18:0	15.30 ± 1.30	14.32 ± 1.10	13.30 ± 0.16	8.83 ± 0.81[c]	14.92 ± 1.20	13.96 ± 0.90
18:1n-9	10.20 ± 1.50	10.81 ± 1.50	7.28 ± 1.68	13.51 ± 1.32[c]	10.88 ± 0.70	9.55 ± 2.60
18:2n-6	13.95 ± 1.20	15.77 ± 1.10	18.04 ± 0.64	23.81 ± 1.20[c]	18.68 ± 1.30	18.27 ± 1.30
18:3n-3	0.32 ± 0.01	0.42 ± 0.00	0.38 ± 0.02	0.77 ± 0.06[c]	0.53 ± 0.10	0.44 ± 0.10
20:1n-9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:2n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3n-6	0.77 ± 0.10	0.85 ± 0.20	0.80 ± 0.06	0.50 ± 0.04[c]	0.67 ± 0.10	0.59 ± 0.10
20:4n-6	22.99 ± 1.10	20.44 ± 0.90	22.81 ± 1.14	16.44 ± 1.61[c]	21.82 ± 2.50	21.50 ± 1.80
20:5n-3	0.68 ± 0.20	0.58 ± 0.10	0.52 ± 0.09	0.46 ± 0.08	0.58 ± 0.20	0.40 ± 0.20
22:4n-6	0.71 ± 0.10	1.14 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	0.53 ± 0.20	2.19 ± 1.30[b]
22:5n-6	1.34 ± 0.20	1.23 ± 0.70	0.38 ± 0.06	0.45 ± 0.10	0.89 ± 0.20	1.01 ± 0.60
22:5n-3	2.23 ± 0.30	2.64 ± 0.50	1.46 ± 0.04	1.62 ± 0.08	1.15 ± 0.10	1.92 ± 0.20[b]
22:6n-3	11.10 ± 0.70	10.80 ± 1.20	6.51 ± 0.22	7.48 ± 0.48[b]	6.06 ± 0.70	8.40 ± 0.40[b]
SFA	34.91	34.29	39.02	32.38	36.61	34.18
PUFA	54.09	53.87	50.89	51.52	50.91	54.72
PUFA/SFA	1.55	1.57	1.30	1.59	1.39	1.60

[a]Values (mol %) are given as mean values ± SEM for four rats.
[b,c]Significantly different from the value for the WKY control group at $P < 0.05$, $P < 0.01$, respectively. For abbreviations see Table 1.

DISCUSSION

The proportions of fatty acids which are associated with membrane structure or which may be involved in the regulation of blood pressure (such as 18:2n-6, 20:3n-6, 20:4n-6 and 20:5n-3) were altered in SHR animals, compared to the levels of these fatty acids observed in tissues from the WKY group. These alterations do not reflect different nutritional status, as we did not observe differences in food consumption between WKY and SHR groups during the experiment. Similarly, no significant body weight difference appeared between the two groups of rats during the entire experiment, as shown in our previous studies (7,8).

The kidney cannot convert 18:2n-6 to 18:3n-6 in either normotensive or hypertensive rats (28), so that the precursors of series 1 and 2 prostaglandins (20:3n-6 and 20:4n-6) need to be provided *via* the circulation from the liver, the major site of fatty acid desaturation and elongation. The high content of 20:4n-6 in the kidneys of 1-mon-old SHR suggests that this fatty acid, or its Δ6 desaturated precursor, is highly incorporated. Alternatively, substantially less AA may be released by phospholipases A_2 and C in SHR. Okamoto *et al.* (22) showed that the AA content of the phospholipids (PL) increases with aging in SHR. Our findings on the fatty acid composition of total lipids in the kidney do not reflect such change, as the amount of AA was lower in SHR than in WKY aged 3 and 6 mon. This apparent difference may be explained by the results of Murthy *et al.* (29), who showed that PL acyltransferase activity is lower in the SHR kidney than in WKY, suggesting greater acylation of PUFA to the triglycerides rather than the PL fraction. However, our results agree with our previous results (7,8) showing that more arachidonate is synthesized by hepatocytes in 1-mon-old SHR than in WKY and that liver microsomal n-6 Δ6 and Δ5 activities decrease in SHR with age.

The kidney of SHR also contains less 20:5n-3 at all ages from 1 mon to 6 mon. At 1 mon, the higher proportion of AA in SHR kidney might contribute to an increase of the ratio n-6/n-3 PUFA. In spite of a conversion into vasodilatory eicosanoid (prostaglandin I_2), AA is a precursor for eicosanoids with potent vasoconstrictor effects, like thromboxane A_2 and isoprostanes, while DGLA and EPA are the precursors of the vasodilators prostaglandins E_1 and I_3 but not of any known vasoconstrictors. Consequently, the SHR kidney seems to contain a higher proportion of one type of eicosanoid precursor (AA) during the prehypertensive period (before 3 mon) and lower proportions of vasodilator precursors of other eicosanoids (DGLA and EPA) when there is hypertension (after 3 mon). DGLA could also inhibit the conversion of AA to vasoactive leukotrienes *via* its 15-hydroxy derivative (30). On the other hand, several experiments have shown that the capacity to synthesize vasodilator prostaglandins in SHR was impaired *in vivo* (31) and that SHR do not produce more prostaglandin I_2 than WKY rats (32). Consequently, PUFA could be involved in blood pressure regulation indirectly or even independently of the prostanoid system, *via* their influence on the biosynthesis of diradylglycerols and phospatidic acid, key intracellular messengers involved in the mediation of agonist-induced vascular smooth muscle cell contraction (33). Thus, an imbalance in the distribution of PUFA in the kidney of 1-mon-old SHR might contribute to the development and to the maintenance of hypertension in these rats. On the other hand, the lower level of DPA observed in SHR kidney at 3 mon of age is not followed by a decrease of its DHA metabolite, suggesting that the Δ4 desaturation system is not altered. Nevertheless, the significant fall in proportion of DPA observed in 6-mon-old SHR was associated with a subsequent fall of DHA.

Although the proportion of AA in the mesenteric vessels of SHR is lower than in controls (34), the fraction of AA in the SHR aorta is higher than in controls at 1 and 3 mon. These results are similar to those for the kidney and indicate that the SHR aorta has the potential to synthesize more cyclooxygenase products than the aorta of age-matched WKY or may undergo changes in the incorporation of PUFA in the *sn*-2 position of membrane phospholipids. This is in agreement with previous data showing an increased synthesis of AA metabolites by SHR aortic vascular segments *in vitro* taken from rats that had been hypertensive for 2–3 mon (35). The sharp decreases in the proportions of LA and α-linolenic acid in the SHR aorta at 1 and 6 mon of age are in agreement with the results of Dominiczak *et al.* (11) in cultured vascular smooth muscle cells from the stroke-prone SHR. The change may contribute to the reduced membrane fluidity of vascular smooth muscle in hypertensive rats (11).

Relatively little information is available on the effect of hypertension on PUFA metabolism in the heart. Lipid alterations, like PL deficiency and lower mass of associated fatty acids, have been reported for newborn SHR heart myocytes (36), suggesting that intrinsic factors, probably genetically determined, are active in the first few days of life in the absence of any neurohumoral regulation or hemodynamic stress. The present study shows that these alterations seem to disappear after weaning, as the heart proportions of 18:2n-6, 20:4n-6, 20:3n-6, and 20:5n-3 in SHR and WKY were similar at the ages of 1 and 3 mon. Nevertheless, the proportions of 18:2n-6 and 20:4n-6 were higher in 6-mon-old SHR than in WKY. It has been reported that cardiac performances were more impaired with age in hypertensive rats than in age-matched normotensive animals, and that young SHR exhibited normal or greater cardiac performances than WKY (37). The decreased cardiac performance with age might be related to changes in membrane lipid composition, which could have a number of effects on receptor function, transport proteins, electrolyte balance, eicosanoid production, enzyme activities, mitochondrial respiration, and/or other membrane-associated processes. The lower cytoplasmic fatty acid-binding proteins in the SHR heart (38) could contribute to an alteration in heart PL turnover, leading to the storage of n-6 PUFA.

We have previously reported that the Δ6 and Δ5 desaturase activities in the liver microsomes of 3-mon-old SHR are inhibited (9). The results from the present study support these obser-

vations, with higher liver 18:2n-6 and lower liver 20:4n-6 proportions in 1- and 3-mon-old SHR than in their homologous WKY. But the transformation of radiolabeled linoleate by isolated hepatocytes does not correlate with these results (7); it was consistently higher in hepatocytes from SHR than in those from WKY aged 1 mon, and similar in hepatocytes from rats aged 3 and 6 mon. These discrepancies suggest that the cellular metabolism of newly formed AA is altered. These changes were associated with elevated 18:3n-3 in 3-mon-old SHR, which may influence the balance between n-3 and n-6 essential fatty acids metabolism in the SHR liver, as the conversion of 18:2n-6 to 20:4n-6 is known to be inhibited by 18:3n-3 (39,40).

In conclusion, these results show that the PUFA composition of the organs and tissues involved in hypertension, like the kidney, heart and aorta, is influenced by local factors, which change with the age of the animal, and does not solely reflect their provision *via* the circulation. The different fatty acid proportions in the SHR organs and tissues studied may alter tissue function, the range of the prostaglandins being produced, and the synthesis of key intracellular messengers. Nevertheless, the changes observed in fatty acid composition of total lipids can result partially from changes in lipid class composition across the studied tissues, and in that way, such data can only be interpreted with caution. It is now necessary to investigate the fatty acid profiles of lipid classes—particularly of different types of PL—and how these changes can influence the PUFA cascade. In particular, the synthesis of the vasodilatory epoxyeicosatrienoic acids, recently demonstrated in smooth muscle (41), heart (42) and kidney (43), may be of significance in hypertension. Whether dietary PUFA might correct local imbalances and be useful for treating hypertension is worthy of further study.

ACKNOWLEDGMENTS

The authors thank Joseph Gresti for technical assistance and advice during the study. This work was partly supported by a grant from the French Ministry of Education and Research, and Bayer Pharma Ltd., France.

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[Received June 24, 1997, and in final revised form June 4, 1998; revision accepted June 23, 1998]

A New Conjugated Linoleic Acid Isomer, 7 *trans*, 9 *cis*-Octadecadienoic Acid, in Cow Milk, Cheese, Beef and Human Milk and Adipose Tissue

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ABSTRACT: The identity of a previously unrecognized conjugated linoleic acid (CLA) isomer, 7 *trans*, 9 *cis*-octadecadienoic acid (18:2) was confirmed in milk, cheese, beef, human milk, and human adipose tissue. The 7 *trans*, 9 *cis*-18:2 isomer was resolved chromatographically as the methyl ester by silver ion–high-performance liquid chromatography (Ag^+–HPLC); it eluted after the major 9 *cis*, 11 *trans*-18:2 isomer (rumenic acid) in the natural products analyzed. In the biological matrices investigated by Ag^+–HPLC, the 7 *trans*, 9 *cis*-18:2 peak was generally due to the most abundant minor CLA isomer, ranging in concentration from 3 to 16% of total CLA. By gas chromatography (GC) with long polar capillary columns, the methyl ester of 7 *trans*, 9 *cis*-18:2 was shown to elute near the leading edge of the major 9 *cis*, 11 *trans*-18:2 peak, while the 4,4-dimethyloxazoline (DMOX) derivative permitted partial resolution of these two CLA isomers. The DMOX derivative of this new CLA isomer was analyzed by gas chromatography–electron ionization mass spectrometry (GC–EIMS). The double bond positions were at Δ7 and Δ9 as indicated by the characteristic mass spectral fragment ions at *m/z* 168, 180, 194, and 206, and their allylic cleavages at *m/z* 154 and 234. The *cis*/*trans* double-bond configuration was established by GC–direct deposition–Fourier transform infrared as evidenced from the doublet at 988 and 949 cm^{-1} and absorptions at 3020 and 3002 cm^{-1}. The 7 *trans*, 9 *cis*-18:2 configuration was established by GC–EIMS for the DMOX derivative of the natural products examined, and by comparison to a similar product obtained from treatment of a mixture of methyl 8-hydroxy- and 11-hydroxyoctadec-9 *cis* enoates with BF_3 in methanol.

Lipids 33, 803–809 (1998).

The major conjugated linoleic acid (CLA) isomer present in cow milk (1–9), human milk (10,11), cheese (2,3,5,12–16), beef (17), and human adipose tissue (18,19) was reported to be 9 *cis*, 11 *trans*-octadecadienoic acid (18:2), or rumenic acid. CLA is a collective term referring to all conjugated geometric and positional isomers of 18:2. Parodi (1) determined the major 9 *cis*, 11 *trans*-18:2 isomer in milk by a combination of gas chromatography (GC) and argentation thin-layer chromatography, partial hydrazine reduction, and ozonolysis. In addition to the 9 *cis*, 11 *trans*-18:2, Ha *et al.* (2) reported seven more CLA isomers (10 *cis*, 12 *trans*-18:2; 10 *trans*, 12 *cis*-18:2; 11 *cis*, 13 *cis*-18:2; 9 *cis*, 11 *cis*-18:2; 10 *cis*, 12 *cis*-18:2; and 9 *trans*, 11 *trans*-18:2/10 *trans*, 12 *trans*-18:2) in cheese based on comparing equivalent chain length data with published results (20), and GC–chemical ionization mass spectrometry of the fatty acid methyl esters (FAME). Recently, Lavillonnière *et al.* (16) identified five additional minor CLA isomers in cheese (8 *cis*, 10 *trans*-18:2; 8 *cis*, 10 *cis*-18:2; 8 *trans*, 10 *trans*-18:2; 11 *trans*, 13 *trans*-18:2; and 11?,13?-18:2). They partially reduced the CLA mixture with hydrazine, isolated the resulting monounsaturated fatty acids by silver nitrate thin-layer chromatography and identified the fatty acids by gas chromatography–electron ionization mass spectrometry (GC–EIMS) as their 4,4-dimethyloxazoline (DMOX) derivatives. These authors also evaluated the total CLA mixture by GC–EIMS.

In the past decade, unequivocal identification of CLA isomers was very limited for the following reasons. Firstly, in almost all reports, the identifications of GC peaks were solely based on comparing observed GC retention times with those of ill-defined commercial CLA mixtures, without independent confirmation of provisional assignments by chemical or spectroscopic methods. Secondly, acid-catalyzed methylation procedures, which were reported to increase *trans*,*trans* CLA isomers (8), were not avoided. Thirdly, even with 100-m GC capillary columns, the resolution of all the CLA isomers has not been achieved (16,21). Therefore, other confirmatory techniques were necessary to identify CLA isomers. Diagnostic ions in the EI mass spectra observed for a mixture of the 4-phenyl-1,2,4-triazoline-3,5-dione adducts (22) were suc-

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Abbreviations: Ag^+–HPLC, silver ion–high-performance liquid chromatography; *cis/trans,* refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC–DD–FTIR, gas chromatography–direct deposition–Fourier transform infrared; GC–EIMS, gas chromatography–electron ionization mass spectrometry.

cessfully used to identify double-bond positions in CLA mixtures. GC–EIMS with selected ion monitoring was used to suggest the presence of different CLA isomers in an unresolved mixture of four 4-methyl-1,2,4-triazoline-3,5-dione adducts of CLA (23). Full mass scan data allowed the identification of individual isomers in conjugated fatty acid mixtures resolved by GC of 4-methyl-1,2,4-triazoline-3,5-dione derivatives (24). Reconstructed ion profiles of the isomer-specific ions for CLA DMOX were used to distinguish between the signals of different CLA isomers in the present study. This permitted the determination of double-bond positions for partially resolved CLA isomers. Recently, we modified a silver ion–high-performance liquid chromatography (Ag^+–HPLC) method by Adlof *et al.* (25). For the first time, using the modified method, we separated 12 geometric and positional CLA isomers found in a commercial mixture (15). This proved to be an essential complementary chromatographic technique for the identification of CLA isomers.

In the present communication, a newly identified CLA isomer found in milk, cheese, beef, human milk, and adipose tissue matrices was resolved as the FAME derivative by Ag^+–HPLC, and as the DMOX derivative by GC. This CLA isomer was identified as 7 *trans*, 9 *cis*-18:2 by GC–EIMS and GC-direct deposition-Fourier transform infrared (GC–DD–FTIR) spectroscopy. The *trans*,*cis* geometry was established by GC–EIMS for the DMOX derivative of the natural products examined, and by comparison with a known 7 *trans*, 9 *cis*-18:2 product.

MATERIALS AND METHODS

A mixture of CLA isomers was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Several pure CLA isomers were obtained as their free fatty acids from Matreya Inc. (Pleasant Gap, PA). Acetonitrile and hexane were ultraviolet grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol (95%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane (TMS-DAM) in hexane was obtained from TCI America (Portland, OR). Anhydrous $NaOCH_3$/methanol was purchased from Supelco, Inc. (Bellefonte, PA). Cow milk was available from a previous study (9). Cheese and beef products were purchased locally. The biological specimens were human subcutaneous adipose tissues. They were a gift from Dr. H.J. Boehles (Johann-Wolfgang-Goethe University Clinic, Frankfurt, Germany) and were obtained from a group of male and female children, aged 1–13 yr, with inguinal hernia. Human milk was a gift from Dr. G. Jahreis (Friedrich Schiller University, Jena, Germany).

Lipid extraction. Milk lipids were extracted using a modified Folch procedure as described previously (8). Cheese was extracted with diethyl ether/petroleum ether (1:1) after homogenization with ethyl alcohol in the presence of potassium oxalate. The total cheese lipids were then dried over anhydrous Na_2SO_4. Beef lipids were extracted with chloroform/methanol as described previously (26). Human adipose tissue was extracted as described previously (19).

Preparation of FAME. The total lipids from milk, cheese, beef, and human adipose tissue (20–70 mg) were methylated using anhydrous $NaOCH_3$/methanol (15). The FAME were analyzed directly by GC and Ag^+–HPLC. The free fatty acids were methylated with a 10% trimethylsilyldiazomethane as described previously (15,27).

Ag^+–HPLC. The HPLC equipment and conditions were the same as described previously (15). A ChromSpher 5 Lipids semipreparative (10 mm i.d. × 250 mm stainless steel; 5 µm particle size) and an analytical (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size) silver impregnated columns were used (Chrompack, Bridgewater, NJ). The mobile phase was 0.1% acetonitrile in hexane and was operated isocrati-

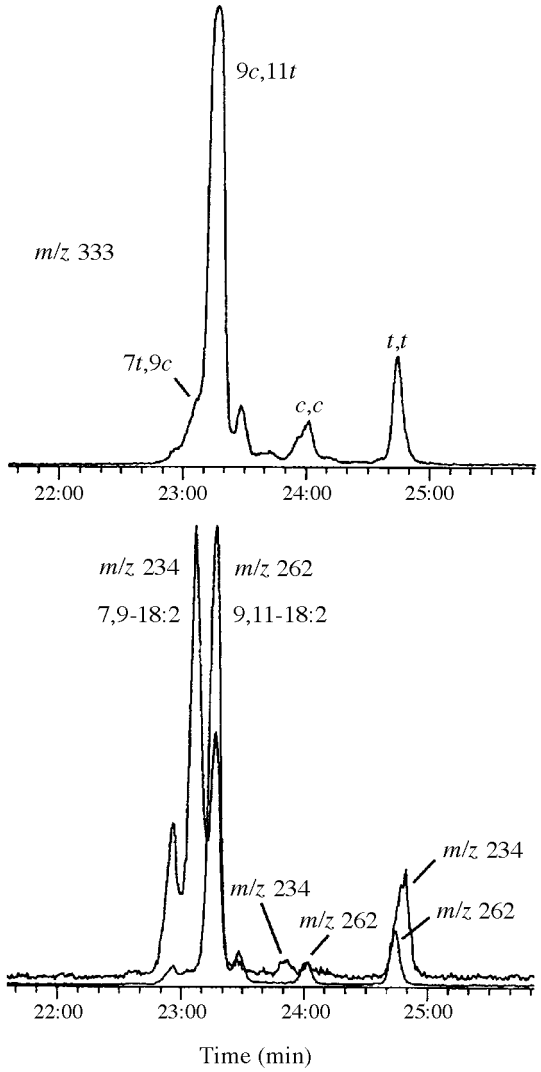

FIG. 1. Partial gas chromatography–electron ionization mass spectrometry (GC–EIMS) chromatogram of conjugated fatty acid 4,4-dimethyloxazoline (DMOX) derivatives from human adipose tissue showing the reconstructed ion profiles for *m/z* 333 (top); and *m/z* 234 and 262 (bottom). In the *cis*/*trans* region, the first two peaks (*m/z* 234) were due to 7 *cis*, 9 *trans*-18:2, 7 *trans*, 9 *cis*-18:2, respectively, and the third one (*m/z* 262) was due to 9 *cis*, 11 *trans*-18:2.

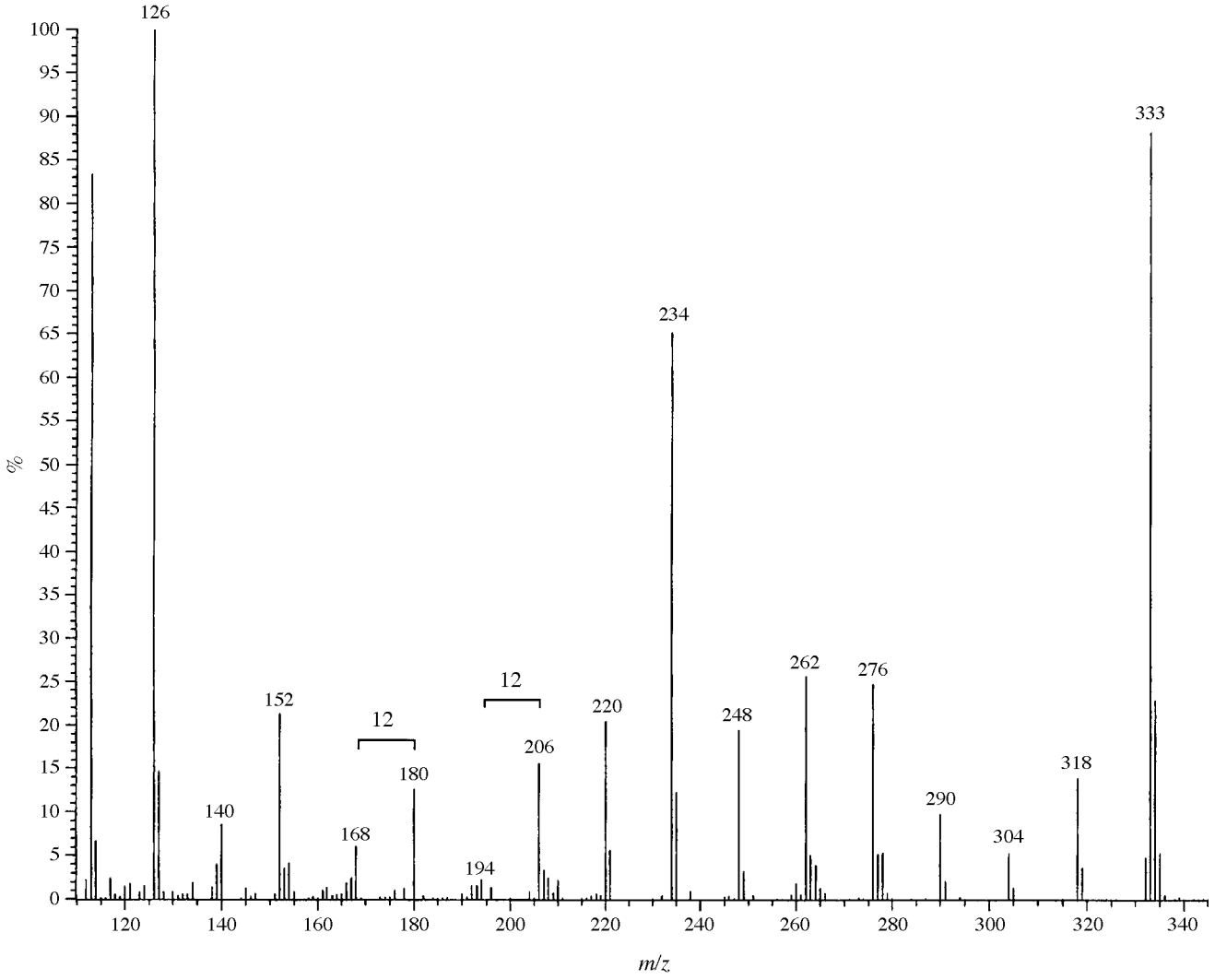

FIG. 2. GC–EIMS spectrum of the DMOX derivative of 7 *trans*, 9 *cis*-18:2 from cow milk. For abbreviations see Figure 1.

cally. The flow rates were 4.0 mL/min and 1.0 mL/min, for the semipreparative and analytical columns, respectively. Detection was at 233 nm.

DMOX derivatives. DMOX derivatives were prepared as described previously (15,28,29).

Instrumentation. The GC (8), GC–EIMS (15,28), and GC–DD–FTIR (30) instruments and conditions were given previously.

RESULTS AND DISCUSSION

The *m/z* 333 GC–EIMS ion profile for the DMOX derivatives of fatty acids from human adipose tissue (Fig. 1 top) showed the presence of a small peak emerging just ahead of that of the major 9 *cis*, 11 *trans*-18:2 isomer. A similar *m/z* 333 GC-EIMS ion profile was observed for the fatty acid DMOX derivatives of all other natural matrices investigated. The mass spectrum for this new peak is shown in Figure 2 for cow milk. The EI mass spectrum of the new compound exhibited an abundant molecular ion at *m/z* 333, characteristic of 18:2 DMOX derivatives. An abundant ion at *m/z* 126, followed by a homologous series of ions separated by gaps of 14 mass units, with intervals of 12 mass units found between *m/z* 168 (C6) and 180 (C7), and between *m/z* 194 (C8) and 206 (C9), indicated the presence of two double bonds at Δ7 and Δ9. In addition, the mass spectrum showed two favorable allylic cleavage ions at *m/z* 154 and 234, supporting a 7,9-18:2 structure. The abundant ion at *m/z* 152 is due to an anomaly (31), which remains unexplained. The reconstructed ion profile for the characteristic allylic ion *m/z* 234 showed two peaks due to 7,9-18:2 isomers that eluted before the one at *m/z* 262 for the 9 *cis*, 11 *trans*-18:2 isomer in Fig. 1 (bottom). The possible geometric configuration of the two 7,9-18:2 isomers will be discussed below. These results demonstrated that the first two eluting peaks (*m/z* 234) did not arise from the adjacent major 9 *cis*, 11 *trans*-18:2 isomer (*m/z* 262). Techniques, such as GC equivalent chain length (2), and partial hydrazine reduction followed by GC separation of the resultant 18:1 iso-

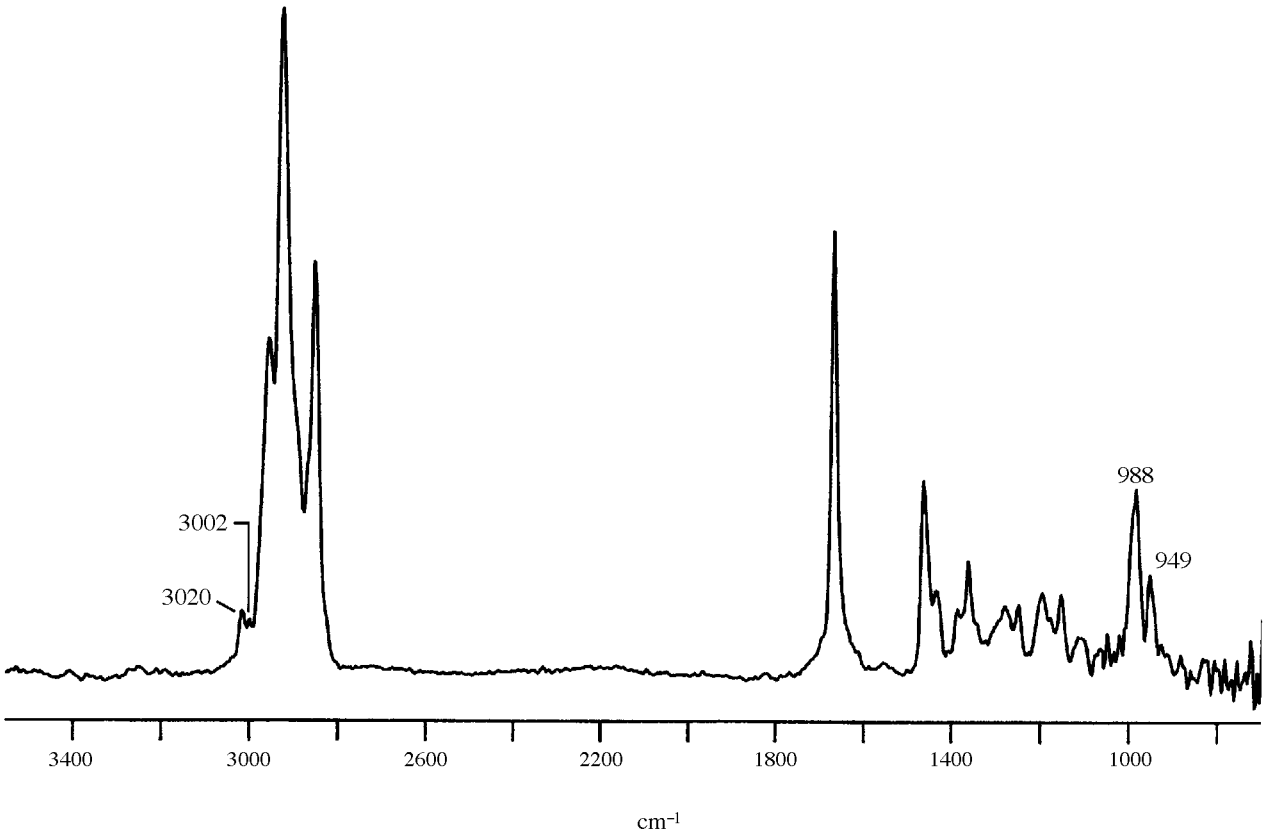

FIG. 3. Gas chromatography–direct deposition–Fourier transform infrared (GC–DD–FTIR) spectrum of 7 *trans,* 9 *cis*-18:2 DMOX derivative from cheese. For other abbreviation see Figure 1.

meric mixtures (16), did not identify the 7,9-18:2 isomer in cheese. A possible reason for the latter is that the 7 *trans*-18:1 FAME coeluted with the 6 and 8 *trans*-18:1 FAME, and the 7 *trans*-18:1 DMOX coeluted with the 9 *trans*-18:1 DMOX, even on long polar capillary columns (32).

The geometric configuration of the 7,9-18:2 DMOX derivatives was established by GC–DD–FTIR, as shown for cheese in Figure 3. The observed IR spectrum exhibited the characteristic doublet for conjugated *cis*/*trans* dienes at 988 and 949 cm^{-1} and absorptions at 3020 and 3002 cm^{-1} (19,33). The IR spectrum of 7,9-18:2 was similar to that of 9 *cis*, 11 *trans*-18:2. However, FTIR was not able to distinguish between a *cis*,*trans* or a *trans*,*cis* configuration.

Ag^+–HPLC was shown to separate the different *trans*,*trans*, *cis*/*trans*, and *cis*,*cis* CLA isomers of 8,10-18:2, 9,11-18:2, 10,12-18:2, and 11,13-18:2 (15), and is shown for comparison in Figure 4 (top). The 7,9-18:2 isomer in the natural matrices investigated was clearly separated by Ag^+–HPLC, eluting in the *cis*/*trans* region after the major 9 *cis*, 11 *trans*-18:2 peak, and was generally the most abundant of the minor CLA positional isomers. Figure 4 shows typical Ag^+–HPLC separations for cow milk, cheese, beef, and human adipose tissue and human milk. The relative elution position of this peak is consistent with a *cis*/*trans* configuration found by IR for this compound. To demonstrate that this peak was not due only to the 8 *trans*, 10 *cis*-18:2 isomer (Fig. 4, top), which eluted at about the same retention volume as that of the *cis*/*trans* 7,9-18:2 peak (Fig. 4, bottom five chromatograms), a representative cheese sample was fractionated as FAME by semipreparative Ag^+–HPLC. The fraction enriched in the *cis*/*trans* 7,9-18:2 isomer was further analyzed as the DMOX derivative by GC–EIMS. The results obtained were similar to those shown in Figures 1 and 2, thus confirming the double bond positions of this new isomer. The difference in retention time between the 8 *trans*, 10 *cis*-18:2 and *trans*/*cis* 7,9-18:2 was also demonstrated chromatographically by coinjecting increasing amounts of a commercial CLA mixture (Fig. 5A) added to a beef fat extract (Fig. 5B). With increased amounts of the commercial CLA mixture, the 8 *trans*, 10 *cis*-18:2 emerged as the more abundant and before the 7,9-18:2 CLA isomer in the coinjected mixture (Fig. 5, bottom). These data do not indicate the presence or absence of the 8 *trans*, 10 *cis*-18:2 isomer in the natural products investigated.

The absolute configuration of the 7,9-18:2 isomer was established by using two methods. The DMOX derivatives of geometric CLA isomers were separated by GC (19); the 9 *cis*, 11 *trans*-18:2 eluted before the 9 *trans*, 11 *cis* isomer (19). Based on this evidence, the first of the two *m/z* 234 peaks in Fig. 1 (bottom) was due to 7 *cis*, 9 *trans*-18:2, while the sec-

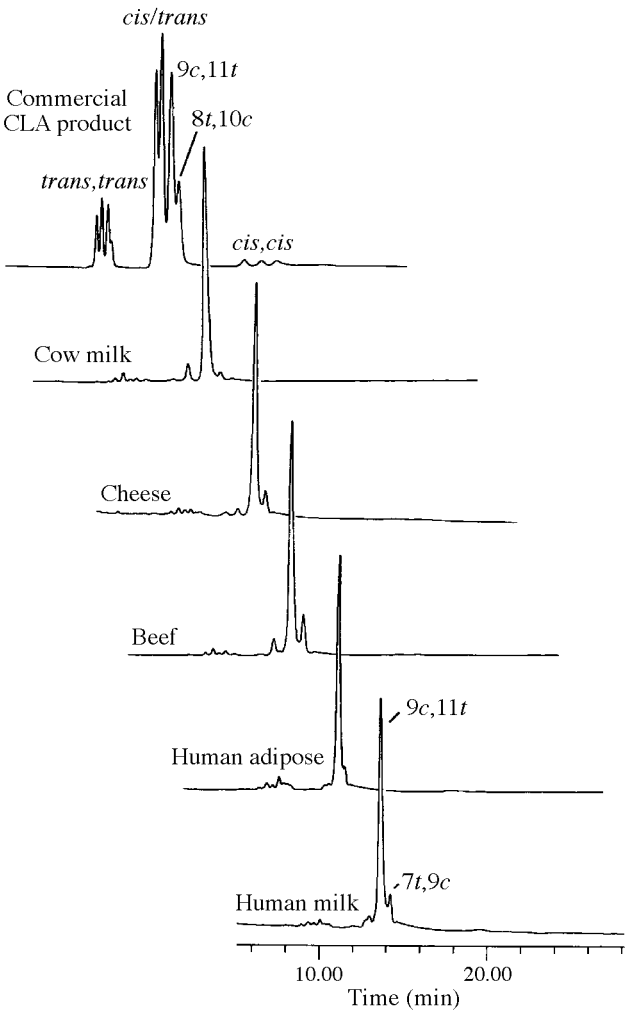

FIG. 4. Silver ion–high-performance liquid chromatography (Ag^+–HPLC) chromatograms of a commercial conjugated linoleic acid (CLA) mixture containing 12 CLA isomers and of cow milk, cheese, beef, human adipose tissue, and human milk. Concentration-dependent variations in resolution were observed between the 9,11-18:2 and the 7,9-18:2 *cis/trans* isomers, but the elution sequence was not altered.

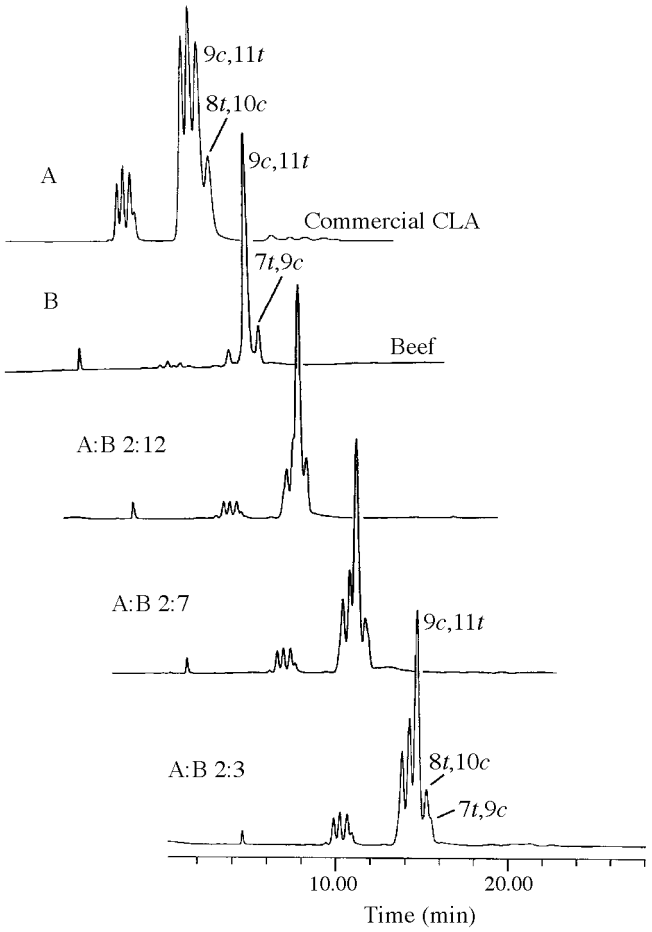

FIG. 5. Ag^+–HPLC chromatographic distinction between the 7 *trans*, 9 *cis*-18:2 isomer found in total beef fat and the 8 *trans*, 10 *cis*-18:2 isomer present in a commercial CLA mixture. Coinjection of these mixtures indicated that 7 *trans*, 9 *cis*-18:2 elutes after 8 *trans*, 10 *cis*-18:2. For abbreviations see Figure 4.

ond, more abundant, peak was due to 7 *trans*, 9 *cis*-18:2. Further independent confirmation was obtained from a mixture of methyl 8-hydroxy- and 11-hydroxyoctadec-9-*cis*-enoates treated with BF_3, as reported previously (28). The products of this reaction included two 7,9-18:2 isomers: 7 *trans*, 9 *cis*-18:2 and 7 *trans*, 9 *trans*-18:2 (28). Analysis of this mixture as DMOX derivatives by GC showed that the 7 *trans*, 9 *cis*-18:2 coeluted with the peak of the new CLA isomer (data not shown), thus establishing its identity. A similar result was also found for the 7 *trans*, 9 *trans*-18:2 (*vide infra*). Ag^+–HPLC did not separate the *cis/trans* geometric isomers.

In addition, Figure 1 is also consistent with the presence of trace levels of 7 *trans*, 9 *trans*-18:2, 7 *cis*, 9 *trans*-18:2, and 7 *cis*, 9 *cis*-18:2, based on the GC–EIMS reconstructed ion profile of the allylic ion *m/z* 234 for the DMOX derivatives of human adipose tissue. This recontructed ion profile permitted the identification of the 7 *trans*, 9 *trans*-18:2 isomer that was not resolved by GC. Furthermore, the last eluting *trans*, *trans* Ag^+–HPLC peak observed for the different specimens investigated had the same retention volume as that of the 7 *trans*, 9 *trans*-18:2 product of the BF_3 reaction with methyl 8-hydroxyoctadec-9-*cis*-enoate. This result was consistent with the presence of a trace of 7 *trans*, 9 *trans*-18:2 in these matrices. The identities of minor CLA isomers will need to be fully confirmed by direct measurements. The relative abundance of the 7 *trans*, 9 *cis*-18:2 isomer in a few select examples of natural products was determined by Ag^+–HPLC (Table 1).

In conclusion, a new CLA isomer was found in milk (cow and human), cheese, beef, and human adipose tissue, and it was generally prominent among the minor CLA isomers ranging in concentration from 3 to 16% of the total CLA isomers. This CLA isomer would therefore also be expected in all ruminants and dairy products, and in humans and animals that consume them. The isomer was shown to be 7 *trans*, 9 *cis*-18:2 based on GC–EIMS, GC–DD–FTIR and Ag^+–HPLC results. These results emphasize the need to use the most polar GC columns available, such as long CP-Sil 88 or

TABLE 1
Amount of 7,9-18:2 FAME Isomer, Relative to Total CLA FAME, in Natural Products as Determined by Ag^+–HPLC[a]

	CLA Isomers (% of total CLA)			
Natural product	9*c*,11*t*	7*t*,9*c*	Other *c*/*t*	Total *t*/*t*
Human milk[b]	79.7–84.0	5.9–9.9	4.3–7.3	3.8–6.4
Cow milk[b]	82.1–86.8	2.8–5.3	4.2–6.2	4.6–6.4
Sheep milk	86.0	6.0	2.7	5.3
Cheese[c]	80.0–83.5	4.6–11.1	1.6–5.8	5.8–9.2
Beef	74.8	15.8	4.8	4.2
Human adipose tissue[b]	83.2–87.4	2.9–5.1	1.8–2.0	6.6–10.5

[a]Abbreviations: FAME, fatty acid methyl ester; CLA, conjugated linoleic acid; Ag^+–HPLC, silver ion–high-performance liquid chromatography.
[b]Ranges obtained for five different samples.
[c]Ranges obtained for five types of cheese.

SP2560 capillary columns, to resolve as many CLA isomers as possible (21). Furthermore, we found it essential to confirm the identity of CLA isomers by GC–EIMS as their DMOX derivatives. Optimal GC conditions (21) allowed the resolution of 7 *trans*, 9 *cis*-18:2 and 9 *cis*, 11 *trans*-18:2 as DMOX derivatives. Erroneous conclusions may result if CLA assignments are solely based on chromatographic retention times. Ag^+–HPLC will become a necessary complementary method for the identification of the different geometric and positional CLA isomers including those present at trace levels (15). The 7 *trans*, 9 *cis*-18:2 isomer in biological matrices and dairy products may arise by desaturation of the 7 *trans*-18:1 as demonstrated by Pollard *et al.* (34).

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[Received May 7, 1998; and in final revised form and accepted July 6, 1998]

Analysis of Ubiquinone and Tocopherol Levels in Normal and Hyperlipidemic Human Plasma

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ABSTRACT: The type and amount of lipophilic antioxidants in plasma of hyperlipidemic patients are of great importance since they play a central role in preventing deleterious oxidation of blood lipids and proteins. Isolation and quantitation of lipophilic antioxidants from hyperlipidemic plasma samples meet great obstacles because of increased levels of various intermediary lipid products. This study was designed to develop a rapid and efficient extraction and separation procedure for simultaneous analysis of ubiquinone-9 and -10 as well as α-, δ-, and γ-tocopherol isomers. The levels of ubiquinone-10, α- and γ-tocopherol were analyzed in human plasma samples using high-performance liquid chromatography. Lipid extraction was performed by petroleum ether/methanol/water. After phase separation, ubiquinone was reduced to ubiquinol by sodium borohydride and the lipids were separated on a C18 column. A binary gradient with solvents containing lithium perchlorate was used, and an electrochemical detector was employed for quantitation. This procedure was also efficient for the analysis of antioxidant lipids in samples containing a large number of accumulated and interfering lipid intermediates. Thus, the procedure described here is useful for efficient and rapid quantitation of ubiquinones and tocopherols in human plasma samples, especially those originating from hyperlipidemic patients.
Lipids 33, 811–815 (1998).

Ubiquinone (UQ) is an obligatory constituent of the mitochondrial respiratory chain and, consequently, a well-studied lipid (1). During recent years, an additional function for UQ has been discovered: this lipid in its reduced form (ubiquinol) is the only endogenously synthesized lipid-soluble antioxidant in animal tissues (2). UQ is also found in blood, associated with low and high density lipoproteins (3,4). Since the antioxidant function is dependent on the reduced state of the lipid, an enzymatic mechanism is obviously present both in blood and in various cells for regeneration of the oxidized form (5,6). The blood antioxidant level is important in preventing cholesterol oxidation, preventing damage during reperfusion injury and also in ameliorating inflammation (7,8).

α-Tocopherol, the major lipid-soluble antioxidant present in blood, originates from the diet (9). In contrast to various tissues where ubiquinol is the dominating antioxidant, the concentration of α-tocopherol in the blood is 10 times higher than that of UQ (10). While α-tocopherol inhibits lipid peroxidation by quenching LOO· radicals, ubiquinol has been considered to influence both the initiation and propagation steps (2).

The use of high-performance liquid chromatography (HPLC) has considerably improved the separation and quantitation of UQ in various lipid extracts. Because UQ cannot be subjected to alkaline hydrolysis (11), an efficient extraction procedure was difficult to find since those that are used are time-consuming and cannot be applied to a large number of samples. Various chromatographic procedures have been employed to purify the samples and analyze the amount of UQ by using an ultraviolet (UV) detector to monitor the absorbing carbon-carbon double bonds of the isoprenoid sidechain or the characteristics of the benzoquinone ring (12–14). However, a large number of lipid metabolites, present in hyperlipidemic blood, absorb UV light at low wavelengths and prevent simultaneous quantitation of both UQ and tocopherols. Methods are additionally available which avoid most of these obstacles, such as coupled-column liquid chromatography (15,16), but these are time-consuming and necessitate special equipment.

In this study, we describe a rapid extraction procedure followed by a separation of the reduced UQ and tocopherols by reversed-phase HPLC using a binary gradient. In this system, by using an electrochemical detector, a broad range of lipid soluble antioxidants can be analyzed simultaneously. An additional advantage of the procedure is that the numerous lipid metabolites in the hyperlipidemic blood do not interfere with the quantitation of the antioxidant lipids.

MATERIALS AND METHODS

Chemicals. All solvents used in this study were obtained from Merck (Darmstadt, Germany) and were of HPLC grade. UQ and tocopherols used in the standard mixture or employed as internal standards were obtained from Sigma Chemical (St. Louis, MO). The other chemicals were pro analysis grade (Merck).

Plasma samples. The material examined consisted of 18 hyperlipidemic and 21 control samples. After overnight fasting, venous human blood samples were collected in he-

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Abbreviations: HPLC, high-performance liquid chromatography; UQ, ubiquinone; UV, ultraviolet.

parinized vacutainers. EDTA-containing tubes were equally efficient. The tubes were centrifuged immediately to prepare plasma, which was stored at −80°C prior to use in the various experiments. No antioxidants were added to the samples.

Lipid extraction. Samples of plasma (0.2 mL) were taken for lipid extraction; and 5-μL internal standards (2 nmol δ-tocopherol and 1 nmol UQ-9) dissolved in chloroform/methanol (2:1, vol/vol), 0.2 mL distilled water, 3.6 mL methanol, and 2.4 mL petroleum ether (boiling point 40–60°C) were added. The samples were vortexed vigorously for 30 s and centrifuged at 800 × *g* for 5 min to obtain phase separation. The upper solvent phase was transferred into another tube and dried under nitrogen.

HPLC analysis. The lipid residue was dissolved in 0.1 mL chloroform/methanol (2:1, vol/vol) and 2 μL 0.5 M sodium borohydride (dissolved in 0.2% NaOH) was added to a final concentration of 10 mM to reduce the UQ. This was required for determination of the total amount of UQ-10 with the electrochemical detector. The sample was vortexed for 5 s and incubated at room temperature for 10 min. Then 20 μL of this mixture was analyzed by HPLC (Shimadzu LC-6A with C-R4A Chromatopac system; Shimadzu, Tokyo, Japan). UQ and tocopherols were separated using a reversed-phase Microsorb C-18 column (3 μm, 4.6 mm × 10 cm; Rainin Instrument Co. Inc., Woburn, MA) (17). The column was protected by using a filter (Rheodyne 0.5 μm × 3 mm, Cotah, CA). A binary gradient was employed with a flow rate of 1.5 mL/min, from the initial 90% methanol/water (9:1, vol/vol) (A solvent) to 100% methanol/2-propanol/*n*-hexane (2:1:1, by vol) (B solvent). Both solvents contained 20 mM lithium perchlorate as an electrolyte and were degassed with helium. During the first 5 min, 90% A and 10% B solvents were employed. Between 5 and 15 min, a linear gradient was applied up to 50% B solvent; between 15 and 23 min, the B concentration was additionally increased to 53%; and between 23 and 26 min, a step gradient was used to reach 100% B concentration. During an additional 4 min, only B solvent was utilized. The levels of UQ and tocopherols were monitored with an electrochemical detector (Shimadzu L-ECD-6A) applying a potential of + 0.7 V vs. Ag/AgCl. The peak areas of compounds were compared to the peak areas of standard compounds with known concentrations. The peak areas were calculated using the computer programme of C-R4A Chromatopac (Shimadzu).

For comparison, UQ was also analyzed in all samples by injecting the lipid extract into the HPLC using a reversed-phase system with a C-18 Hypersil ODS column, (3 μm, 4.6 mm × 6 cm; Hewlett-Packard, Böblingen,Germany) (14). In this system, UQ was detected by UV absorption at 275 nm. The absorbance value of 0.04 AUFS corresponding to the recorder full scale was used for detection. Before injection, ferric chloride (final concentration 0.25 mM) was added to the samples in order to determine the total oxidized UQ-10 pool. A linear gradient was started with 100% methanol/water (9:1, vol/vol) and continued to 100% methanol/2-propanol/*n*-hexane (2:1:1, by vol) during a time course of 15 min.

The recovery studies demonstrated a range of 89–96%. In a separate series, aliquots of the same sample were quantitated at 8–12 different occasions. The recoveries were calculated by using internal standards. The difference between the samples did not exceed 8%.

RESULTS

The concentration of UQ can be determined by HPLC using its UV absorption at 210 nm based on the presence of double bonds. Organic compounds containing double bonds generally exhibit high absorption peaks in the UV region because the electrons in unsaturated bonds are easily excited. Because the carbon-carbon double bonds in the isoprenoid side-chain of UQ are numerous, this method has a high sensitivity. Tocopherols have rather high absorption peaks at this wavelength but other lipophilic compounds have similar retention times. A more specific but less sensitive approach is to use UV detection at 275 nm, which is the absorbance maximum for UQ in the oxidized form. Tocopherols have at this wavelength very low absorption and cannot be quantified. In normal human plasma, UQ-10 could be easily quantitated at this wavelength (Fig. 1A). In various types of hyperlipidemic conditions, however, a large number of additional absorbing peaks appeared on the chromatogram, eliminating the possibility of identification and quantitation of UQ (Fig. 1B).

By using appropriate conditions, as described in the Materials and Methods section, complete isolation and analysis of both tocopherols and UQ could be performed. In this procedure, the lipids were extracted by a mixture of petroleum ether and methanol. After phase separation, the phospholipids were removed, as they partitioned to the methanol phase. The extraction of neutral lipids was complete and rapid. As comparison, the samples were also extracted with prolonged treatment with chloroform/methanol (2:1, vol/vol) and also with ethanol. With all three procedures, the recoveries were identical. It is of utmost importance to use an appropriate concentration of sodium borohydride (Table 1). Low concentrations of the reducing agent were unsufficient to reduce UQ completely, whereas at high concentrations α-tocopherol was markedly degraded. The highest concentrations tested also caused breakdown of UQ. The optimal range was found to be 5–10 mM.

By designing an efficient gradient system and using an electrochemical detector, HPLC separation of a mixture containing standards of δ-, γ-, and α-tocopherols, as well as UQ-9 and UQ-10, resulted in a complete separation of all constituents (Fig 2A). When the same hyperlipidemic plasma as that shown in Fig. 1B was analyzed using an electrochemical detector, the two plasma tocopherols, the γ- and α-forms, were well separated (Fig. 2B). They were also distinguishable from the δ-form which was added as an internal standard. In the same gradient system, UQ-10 was eluted after 22 min, well separated from the internal standard, UQ-9. A very similar HPLC pattern was obtained when a large number of plasma samples originating from patients suffering from different types of hyperlipidemic conditions were investigated.

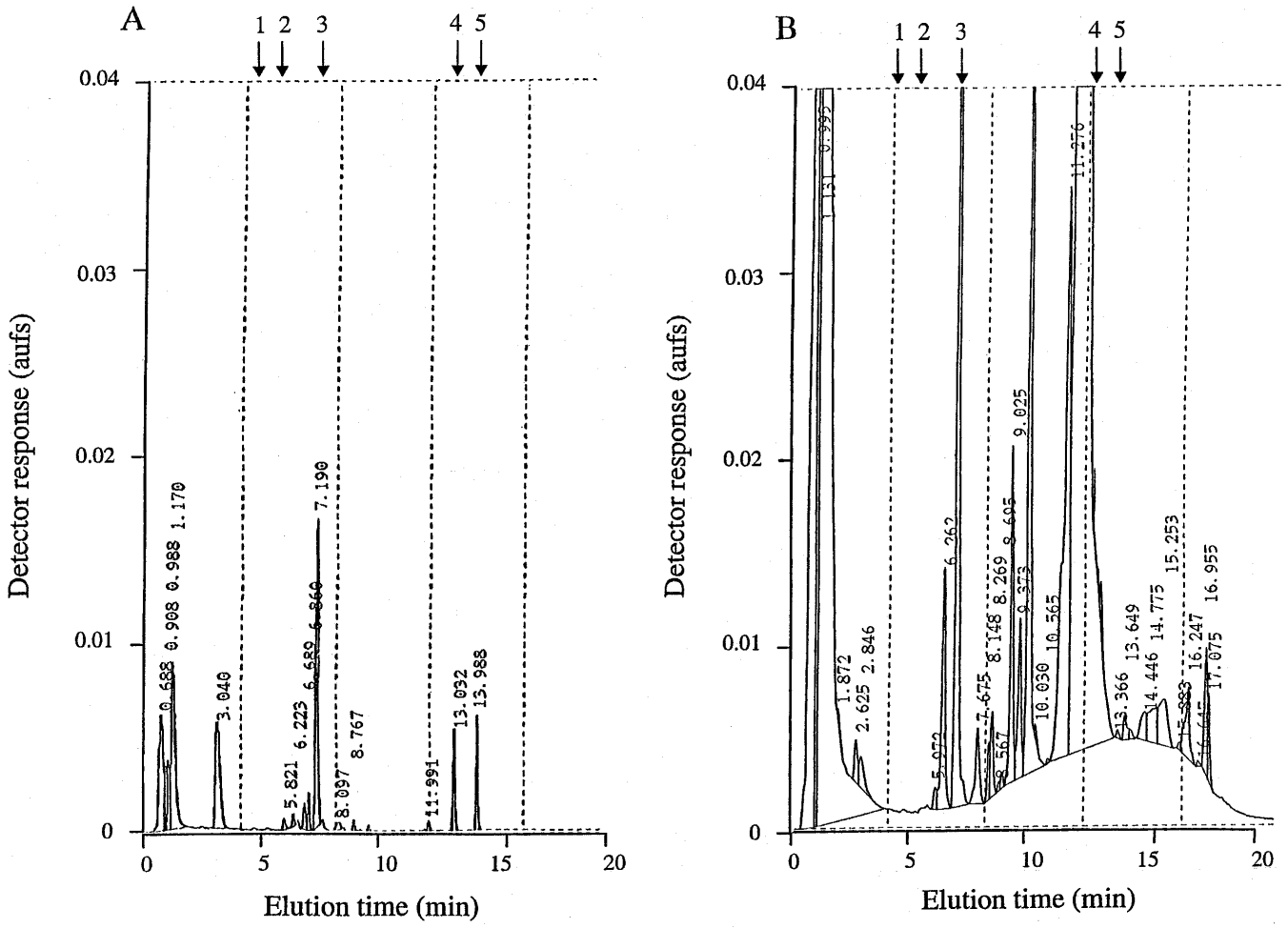

FIG. 1. Analysis of plasma ubiquinone (UQ) with high-performance liquid chromatography using ultraviolet absorption at 275 nm. (A) Normal human plasma. (B) Human plasma from a hyperlipidemic patient. Elution times: 1, δ-tocopherol; 2, γ-tocopherol; 3, α-tocopherol; 4, UQ-9; 5, UQ-10. The individual peaks were identified by comparing the retention times with those of the standards.

Also, normolipidemic plasma samples in this system exhibited a similar pattern as the hyperlipidemic ones but the lipid amounts were lower (Fig. 2C). The reliability of the method employed is demonstrated in Table 2. Analyses of plasma samples from 18 hyperlipidemic patients and 21 control subjects showed that the plasma levels of UQ-10 as well as α- and γ-tocopherols were significantly higher in hyperlipidemic patients compared to control subjects.

TABLE 1
Effect of Sodium Borohydride on the Reduction of α-Tocopherol and Ubiquinone

$NaBH_4$ Final concentration (mM)	Recovery (%)	
	α-Tocopherol	Ubiquinone
0	100	2
0.5	100	45
1.0	100	95
5.0	100	100
10	100	100
100	91	100
200	66	87
500	5	27

DISCUSSION

Recent developments concerning the antioxidant function of UQ have created a considerable interest in the quantitation of this lipid. Because of the low blood levels of UQ and the presence of other components with similar chromatographic properties, UQ isolation and quantitation is still a difficult task. The large number of lipid metabolites which accumulate in human blood in various hyperlipidemic conditions causes interference, not only when UV absorption is used to detect the double bonds but also when the absorbing characteristics of the benzoquinone ring are utilized. However, normal human blood, free from accumulating pathological lipid products, can be studied by using UV detection at 275 nm.

Petroleum ether efficiently extracted the lipids from various blood samples. In the presence of methanol, partition of the solvent phases was obtained providing a simple way of removing the phospholipids. In this way, the sample preparation required less than 10 min. We used sodium borohydride to reduce UQ to ubiquinol, which could be detected with high sensitivity using an electrochemical detector. When fresh samples are analyzed, the degree of reduction in the *in vivo*

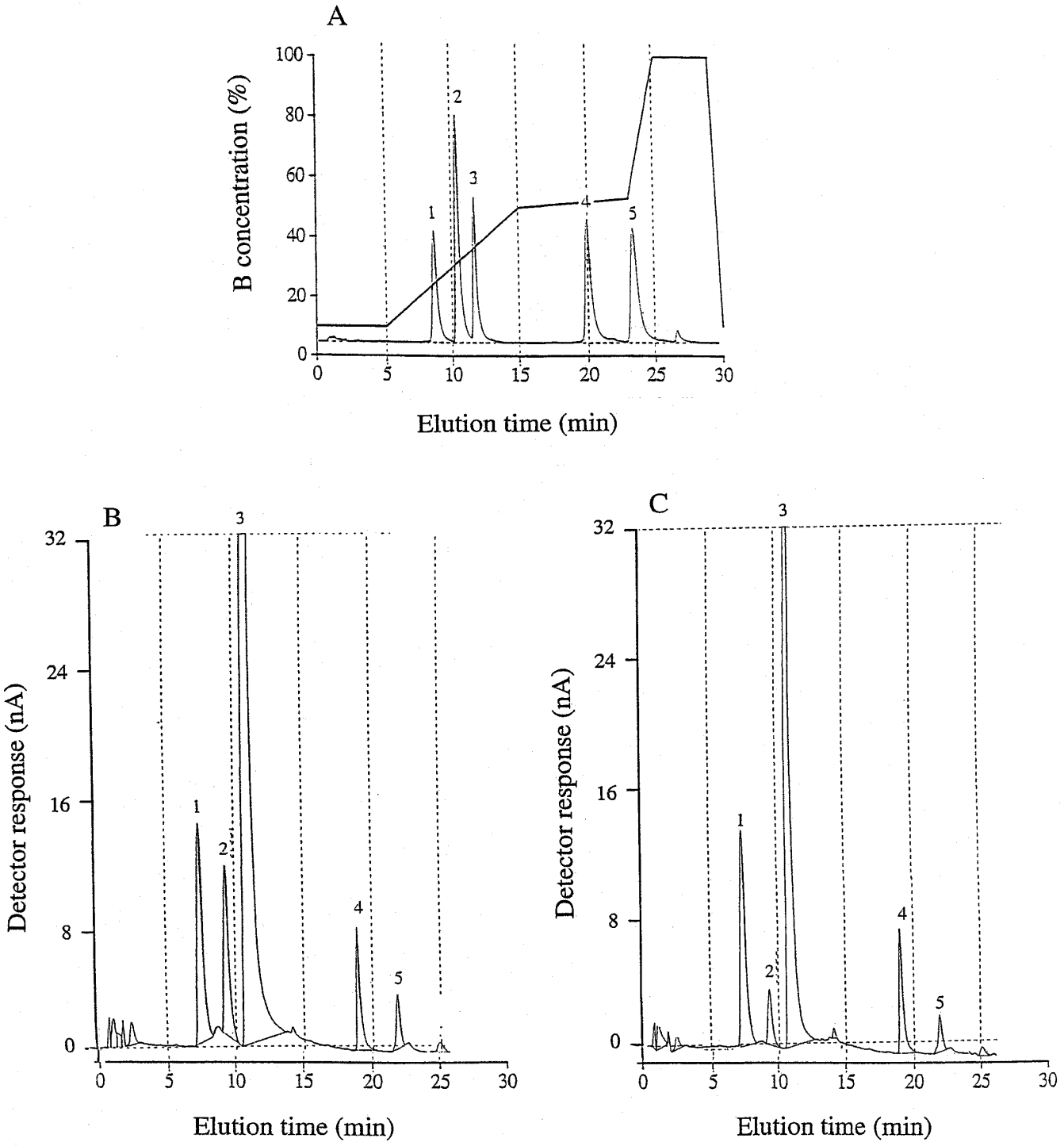

FIG. 2. Separation and quantitation of redox lipids from hyperlipidemic human plasma. The procedure used is described in the Materials and Methods section. The gradient pattern of B solvent [methanol/2-propanol/*n*-hexane (2:1:1, by vol)] is given as a straight line. (A) Separation of standards. (B) Chromatography of lipid extract from plasma sample of a hyperlipidemic patient (also shown in Fig. 1B). Chromatography of a normolipidemic plasma sample. 1, δ-tocopherol; 2, γ-tocopherol; 3, α-tocopherol; 4, UQ-9, 5, UQ-10. For abbreviation see Figure 1.

state can also be investigated. In earlier studies, we found that about 60% of blood UQ is in reduced form. Upon storage, the extent of reduction is decreased (18). The difference between the amounts of ubiquinol, analyzed with and without sodium borohydride treatment, can be used for the calculation of the amount of the oxidized form of UQ. Since only the reduced UQ is effective as an antioxidant, the redox state of this lipid is often of great physiological interest (19,20).

The two main types of redox lipids present in blood are

TABLE 2
Plasma Levels of Lipophilic Antioxidants in Hyperlipidemic Patients and Control Subjects[a]

	Patients	Control subjects
Ubiquinone-10	1.54 ± 0.59	0.75 ± 0.51[b]
α-Tocopherol	69.5 ± 17.3	38.4 ± 11.6[b]
γ-Tocopherol	10.9 ± 6.4	3.3 ± 1.1[b]

[a]The values are given as μmol/L. The results are means ± SD (n = 18 for hyperlipidemic and n = 21 for control plasma samples).
[b]$P < 0.001$ (Student's t-test).

UQ and tocopherols. Certain species possess only one dominant UQ homolog with a specific isoprenoid side-chain length, but in many cases an additional minor component, differing by one isoprene, coexists (21). In the case of tocopherols, the α-form is dominant, but the γ-form is often additionally present. When internal standards, comprising UQ with a different chain length and δ-tocopherol (which is practically absent in animal tissues), were included in the blood sample, the HPLC procedure employed separated all the five components well. The procedure described could be used in various studies to investigate the distribution and function of the two major classes of lipid-soluble antioxidants.

A range of chromatographic separations has previously been employed to remove contaminating lipid intermediates and to allow quantitation of UQ at the wavelength of 210 nm (21). The specificity of measurements, based on the analysis of the benzoquinone ring (275 nm), is considerably higher. This method is less sensitive than that using the absorption at 210 nm, but the relatively low background obtained represents an additional advantage. In this way, detection with an UV detector is a useful procedure in many cases. In blood samples with accumulated lipid intermediates, the utilization of an electrochemical detector is the only practical useful procedure.

ACKNOWLEDGMENTS

The work was supported by grants from the Swedish Heart-Lung Foundation and the Swedish Medical Research Council.

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[Received November 12, 1997, and in final revised form and accepted July 1, 1998]

COMMUNICATION

Evidence That Commercial Calf and Horse Sera Can Contain Substantial Amounts of *trans*-10,*cis*-12 Conjugated Linoleic Acid

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ABSTRACT: We analyzed fetal calf, newborn calf, horse, and adult cow sera for conjugated linoleic acid (CLA). All sera samples contained CLA, but the amounts varied. The predominant isomer was *cis*-9,*trans*-11 CLA but some samples appeared to contain substantial amounts of an isomer with the retention time of *trans*-10,*cis*-12 CLA.
Lipids 33, 817–819 (1998).

CLA is the acronym for a class of positional and geometric conjugated dienoic isomers of linoleic acid. The term was coined by Ha *et al.* (1) when they reported biological activity (i.e., anticarcinogenic activity) associated with CLA isolated from grilled ground beef or produced from linoleic acid by base-catalyzed isomerization. Since then, substantial interest has developed in the biochemical actions of CLA and its potential application to foods, feeds, and pharmaceuticals (2).

While numerous isomers have been described in various commercial CLA products (3,4), the major isomer in the diet is *c*-9,*t*-11 CLA (where *c* = *cis* and *t* = *trans*) (5) because dairy products are the major dietary source of CLA, and virtually all the CLA in dairy products is present as the *c*-9,*t*-11 isomer (6). Only small amounts of the *t*-10,*c*-12 CLA isomer are observed in dairy products (7). McGuire *et al.* (8) reported that human milk also contains mostly *c*-9,*t*-11 CLA with only small amounts of the *t*-10,*c*-12 CLA isomer. The *c*-9,*t*-11 CLA is also the major isomer in beef products, but beef consistently appears to contain the *t*-10,*c*-12 CLA isomer as well, in varying levels (5).

Given the prominence of the *c*-9,*t*-11 CLA isomer in the diet and especially in dairy products, this isomer may be the most biologically active form of CLA (5). However, it is important to note that this assumption has not been proved. We are utilizing cultured mammalian cells in our investigation of this issue (9). Since bovine and/or horse sera are commonly added to cell culture media, we undertook an investigation of the natural occurrence of CLA isomers in these products sold for cell culture use.

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Abbreviation: CLA, conjugated linoleic acid.

Five lots of fetal calf sera, three lots of newborn calf sera, and three lots of horse sera were purchased from commercial sources (Table 1, footnote *a*). Sera were also obtained from four cows maintained at the Dairy Forage Research Center, University of Wisconsin (UW)–Madison (Madison, WI). The sera were analyzed for CLA as described (5). Briefly, sera samples were extracted with chloroform/methanol (2:1) and methylated with 4% HCl in methanol at 60°C for 20 min. Heating to 60°C, as opposed to 80°C (7), substantially reduces isomerization artifact with this methylation procedure (5; Park, Y., and M. Pariza, unpublished observations). Additionally only trace amounts of the *t*-9,*t*-11 isomer were sometimes detected.

Methyl esters of fatty acids were analyzed with gas chromatography and identified by comparison with CLA standard prepared in this laboratory. The procedure utilizes a Hewlett-Packard 5890 series II (Palo Alto, CA) fitted with a flame-ionization detector and 3396A integrator. A Supelcowax-10 (Supelco, Bellefonte, PA) fused-silica capillary column (60 m × 0.32 mm i.d., 0.25 μm film thickness) was used and oven temperature was programmed from 50 to 185°C, increased 20°C per min, held for 80 min, increased 2°C per min to 225°C, and held for 60–100 min. This method has been validated with individually synthesized authentic CLA isomer standards (10).

The results are shown in Table 1. Total CLA in the commercial fetal and newborn calf sera ranged from 0.181–0.495% of fatty acids, which is in general agreement with the levels of total CLA usually reported for dairy and beef fat (5–8, unpublished observations). The mean total CLA content from the UW–Madison cows was 0.151%. Interestingly, the horse sera samples exhibited substantial variation. Sample 1 contained exceptionally high levels of total CLA whereas samples 2 and 3 were considerably lower (Table 1).

Both the *c*-9,*t*-11 CLA isomer and a peak with the retention time of the *t*-10,*c*-12 CLA isomer were detected in all of the commercial fetal and newborn calf sera, and in horse serum sample 1 (Table 1). In general the putative *t*-10,*c*-12 isomer content in these sera samples appeared to be higher than expected based on dairy fat analyses (5–8, and unpublished observations). By contrast the only isomer detected in the sera of the UW–Madison cows and in horse sera samples 2 and 3 was *c*-9,*t*-11 CLA.

TABLE 1
The *cis*-9,*trans*-11- and Putative *trans*-10,*cis*-12-CLA in Commercial Calf and Horse Sera[a]

	Fatty acid (%)		
	Total CLA	*c*-9,*t*-11-CLA	*t*-10,*c*-12-CLA
Fetal calf sera			
1	0.404	0.331 (82.0)[b]	0.073
2	0.375	0.295 (78.6)	0.080
3	0.495	0.363 (73.4)	0.132
4	0.341	0.256 (75.0)	0.085
5	0.181	0.159 (87.8)	0.022
Newborn calf sera			
1	0.379	0.301 (79.5)	0.078
2	0.224	0.193 (86.2)	0.031
3	0.246	0.212 (86.0)	0.035
Horse sera			
1	0.926	0.530 (57.3)	0.396
2	0.058	0.058 (100)	n.d.[c]
3	0.044	0.044 (100)	n.d.
Cow sera[d]	0.151	0.151 (100)	n.d.

[a]Fetal calf sera 1, 2, and 4, newborn calf sera 1 and 2, and horse sera 1 and 3 were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf sera 3 and 5, newborn calf serum 3, and horse serum 2 were from Biowhittaker (Walkersville, MD). CLA, conjugated linoleic acid.
[b]Numbers in parentheses are percentages of *cis*-9, *trans*-11-CLA.
[c]n.d., not detected.
[d]Mean from four cows (range 0.137–0.183%) maintained at the University of Wisconsin–Madison Dairy Forage Research Center.

The data of Table 1 beg the question of the source of the putative *t*-10,*c*-12 isomer in the commercial sera samples. It is well documented that linoleic acid is converted to *c*-9,*t*-11 CLA by several species of rumen bacteria (11–16) and by a strain of *Lactobacillus reuteri* isolated from rat colon (17). Verhulst *et al.* (18) reported that *Propionibacterium acnes* converts linoleic acid to *t*-10,*c*-12 CLA, but to what extent this finding pertains to the data of Table 1 is unclear.

Evidence exists for the generation of *c*-9,*t*-11 CLA by mammalian enzymes: a double bond can be introduced into vaccenic acid (*t*-11-octadecenoic acid) by the action of Δ-9 desaturase, to generate *c*-9,*t*-11 CLA (19,20). Also, *c*-9,*t*-11 CLA may be generated *in situ via* carbon-centered free radical oxidation (21). However, to our knowledge there is no known mechanism whereby the *t*-10,*c*-12 CLA isomer might be generated by mammalian enzymes. Moreover, the apparent finding of exceptionally high total CLA and putative *t*-10,*c*-12 CLA in horse serum sample 1 is intriguing in that the horse has a hind-gut fermentation area, where long-chain fatty acid absorption is expected to be minimal.

The commercial bovine sera used in developing the data of Table 1 were from fetal or newborn calves, whereas the UW–Madison cows were adult animals. The horse sera samples in Table 1 likely also came from adult animals (personal communication from the supplier, Sigma Chemical Co., St. Louis, MO). Hence, possibly undefined nutritional and/or physiological factors, as well as age, may be involved in the apparent accumulation of the *t*-10,*c*-12 CLA isomer in sera.

We are currently exploring these possibilities.

ACKNOWLEDGMENTS

The authors thank Drs. Larry Satter and Tilak Dhiman of the UW–Madison Dairy Forage Research Center for providing the adult cow sera samples. This research was supported in part by gift funds administered through the University of Wisconsin–Madison Food Research Institute. The authors are inventors of CLA-use patents assigned to the Wisconsin Alumni Research Foundation (WARF).

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[Received March 31, 1998, and in final revised form and accepted July 6, 1998]

Lectin May Contribute to the Atherogenicity of Peanut Oil

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ABSTRACT: Peanut oil is unexpectedly atherogenic for rats, rabbits, and primates. The lesions it produces are more fibrous than fatty. The mechanism underlying the atherogenicity of peanut oil has been elusive. Randomization of peanut oil reduces significantly its atherogenic properties, but native and randomized peanut oils have similar rates of lipolysis, and rats fed the two oils absorb and transport lipids in a similar fashion. Peanut oil differs from other oils in having a relatively high lectin content, and the randomization process markedly reduces the lectin content as well. The biologically active lectin of peanut oil has an affinity for glycoproteins found specifically on arterial smooth muscle cells. Peanut lectin has been shown to stimulate growth of smooth muscle and pulmonary arterial cells. Vigorous washing of peanut oil reduces its lectin content by 46%. Compared to rabbits fed cholesterol and peanut oil, rabbits fed cholesterol and washed peanut oil exhibited less severe atherosclerosis in the aortic arch (by 9%) and in the thoracic aorta (by 31%). The data suggest that peanut oils' endogenous lectin may contribute significantly to its atherogenic properties.

Lipids 33, 821–823 (1998).

Peanut oil has been shown to be unexpectedly atherogenic when fed to rats (1,2), rabbits (3), or monkeys (4,5) on an atherogenic diet. Peanut oil is more atherogenic than corn oil even when fed as part of a cholesterol-free atherogenic diet (6). Steiner and Dayton (7) found that a diet containing 50–75% ground peanuts was hypercholesterolemic and hyperlipoproteinemic for rabbits, resulting in some aortic sudanophilia. We found that when peanut oil was randomized (autointeresterified) it became significantly less atherogenic for rabbits (8) and vervet monkeys (5). The aortic lesions of the rabbits fed peanut oil contain a relatively small amount of lipid and are characterized by thick, fibromuscular plaques.

The unexpected atherogenicity of peanut oil and its reversal by randomization could not be ascribed to its triglyceride structure since the five principal triglycerides of native (mol%) and randomized (mol%) peanut oil (listed respectively, as follows) contained oleic and linoleic acids almost exclusively (9): OOO (16.3) and OOL (15.9); OLO (11.8) and OOO (13.1); OOL (9.1) and OLL (9.6); POO (8.0) and OLO (7.9); PLO (7.6) and POO (5.3); OLL (6.6) and LOL (4.8) where O = oleic acid; L = linoleic acid; P = palmitic acid. Our recent studies (10) suggest that the presence of palmitic acid at the *sn*-2 position of a triglyceride increases its atherogenic potential, but the principal fatty acids present at the *sn*-2 position of peanut oil are linoleic and oleic. Lipolysis rates of peanut oil, randomized peanut oil, and corn oil are similar (11). Lymphatic absorption of cholesterol is the same in rats fed native or randomized peanut oil (12,13). We began to investigate the possibility that peanut lectin might predispose this oil to become atherogenic. We found that peanut oil had 22% greater lectin-like activity than soybean oil, 129% more than corn oil, and 57% more than randomized peanut oil (14).

MATERIALS AND METHODS

Male, New Zealand White rabbits (8/group), initial weight 3–3.5 kg, were fed a diet containing 92% commercial ration, 6% oil, and 2% cholesterol. This diet was chosen so that results would be comparable to earlier studies (3,8). After 60 d the rabbits were bled under light anesthesia, then killed, and livers and aortas removed. Serum total cholesterol and triglycerides were analyzed using commercial kits (Sigma, St. Louis, MO). Aliquots of liver were homogenized in chloroform/methanol 2:1 (15), and the lipid extract was analyzed for free and total cholesterol (16) and triglycerides (17). Aortas were graded visually on a 0–4 scale (18).

Peanut oil (10 L) was mixed with 5 L of phosphate-buffered saline (pH 7.3) for 2 h then separated by centrifugation. The washed peanut oil (8 L) was mixed with 1.6 L high- purity water for 2 h then separated by centrifugation. Lectins present in the oils were determined as described previously (14) using the method of Lotan *et al.* (19) in which various concentrations of phosphate-buffered saline extracts of the oils are used to agglutinate human erythrocytes. Presence of lectins in the oils was verified by polyacrylamide gel electrophoresis (20). Lectin-like activity (μg/kg) of the oils used was: corn oil, 24.0; peanut oil, 55.0; and washed peanut oil, 29.5.

RESULTS AND DISCUSSION

Necropsy results are given in Table 1. There were no significant differences in any parameter. Effects of the fats on atherosclerosis are presented in Table 2. Severity of aortic arch atherosclerosis in rabbits fed peanut oil was 17% greater than that seen in corn oil and 10% greater than in rabbits fed

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TABLE 1
Necropsy Results. Rabbits Fed 2% Cholesterol and 6% Corn Oil (CO), Peanut Oil (PNO), or Washed Peanut Oil (WPNO) (for 60 d)[a]

	Group		
	CO	PNO	WPNO
Number	8/8	7/8	8/8
Weight gain (g)	694 ± 126	585 ± 101	691 ± 71
Liver weight (g)	128 ± 6	120 ± 7	125 ± 6
Liver as body weight %	4.36 ± 0.16	4.21 ± 0.19	4.28 ± 0.19
Serum lipids (mmol/L)			
Cholesterol	85.2 ± 8.6	99.9 ± 14.8	92.5 ± 9.0
Triglycerides	3.06 ± 0.68	2.03 ± 0.37	2.55 ± 0.61
Liver lipids (mmol/100 g)			
Cholesterol	1.88 ± 0.12	1.67 ± 0.11	1.89 ± 0.20
Ester %	66.0 ± 2.8	64 ± 3.3	65 ± 4.7
Triglycerides	0.07 ± 0.006	0.05 ± 0.003	0.06 ± 0.003

[a]Data ± SEM.

TABLE 2
Severity of Atherosclerosis in Rabbits Fed 2% Cholesterol and 6% CO, PNO, or WPNO (for 60 d) (graded on a 0–4 scale)

	Group		
	CO (24.0)[a]	PNO (55.0)[a]	WPNO (29.5)[a]
Aortic arch	3.25 ± 0.27	3.79 ± 0.15	3.44 ± 0.18
Thoracic aorta	2.00 ± 0.33	2.71 ± 0.26[b]	1.88 ± 0.26[b]

[a]Lectin-like activity, μg/kg.
[b]$P < 0.05$ using the Mann-Whitney test. All values ± SEM. See Table 1 for abbreviations.

washed peanut oil. Severity of atherosclerosis in the thoracic aorta of rabbits fed peanut oil was 36% greater than in those fed corn oil and 44% greater than in those fed washed peanut oil ($P < 0.05$, Mann-Whitney test). The oils used in this experiment were analyzed for lectin-like activity, which in peanut oil was 129% higher than in corn oil and 86% higher than in washed peanut oil. The presence of lectin-like activity provides a rational reason for the atherogenicity of peanut oil. That activity is reduced significantly during the randomization process as is the oil's atherogenicity.

Sanford and Harris-Hooker (21) showed that peanut lectin stimulated growth of smooth muscle and pulmonary arterial cells but not aortic endothelial cells. The structure of peanut lectin has recently been described by Banerjee *et al.* (22). Peanut lectin exhibits a specificity for D-galactose residues and preferentially for the β-D-Gal(1-3)D-Gal-NAC sequence which occurs on arterial smooth muscle cells. Peanut lectin, in the presence of hypercholesterolemia, could enhance proliferation of arterial smooth muscle cells. Testing the effects of peanut lectin added to atherogenic diets containing other oils would be of interest.

ACKNOWLEDGMENTS

We are indebted to Dr. Mark Bieber and Mr. Ted Ziegert of the BestFoods Technical Center for providing the intact and washed peanut oil. Supported, in part, by a Research Career Award (HL-00734) from the National Institutes of Health.

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[Received July 21, 1997, and in final revised form and accepted July 1, 1998]

Isolation and Structure of Glucosylceramides from the Starfish *Cosmasterias lurida*

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ABSTRACT: From the water-insoluble lipid fraction of the methylene chloride/methanol extract of the starfish *Cosmasterias lurida,* two new glucosylceramides together with a known glucosylceramide, ophidiacerebroside E, were isolated by chromatographic procedures and characterized by spectroscopic (^{1}H and ^{13}C nuclear magnetic resonance, mass spectrometry) methods. The new compounds were identified as (2*S,* 3*R,* 4*E,* 8*E,* 10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxyheptadecanoyl)amino]-9-methyl-4,8,10-octadecatriene (**3**) and (2*S,*3*R,*4*E,*8*E,*10*E*)-1-(β-D-glucopyranosyloxy)-3-hydroxy-2-[(*R*)-2-hydroxyoctadecanoyl)amino]-9-methyl-4,8,10-octadecatriene (**4**). *Lipids 33,* 825–827 (1998).

Among echinoderms, glycosphingolipids and sphingosine derivatives have been isolated from starfish (1,2), sea urchin (3), and sea cucumber (4). To date, the structures which have been elucidated contain a 2-hydroxy fatty acid chain, the doubly unsaturated sphingosine 4,8-sphingadiene as well as the saturated phytosphingosine 4-hydroxysphingosine, and one hexose unit (characteristic of glucosylceramides) or more than one hexose units (characteristic of ceramide oligosaccharides). Five cytotoxic glucosylceramides containing a novel sphingosine base 2-amino-1,3-dihydroxy-9-methyl-4,8,10-octadecatriene were isolated from the starfish *Ophidiaster ophidianus* (5).

As for the constituents of *Cosmasterias lurida* Philippi, a very common starfish collected in cold waters off the Patagonian coast of Argentina, we have described the isolation and the determination of structure of two novel steroidal monoglycoside sulfates (6) and four new sulfated asterosaponins (7). In this paper we report on the isolation and characterization of glucosylceramides from the starfish *C. lurida*.

MATERIALS AND METHODS

Fresh specimens of *C. lurida* were collected at San Antonio Oeste, Río Negro, Argentina, and identified by Dr. Alejandro Tablado from the Museo de Ciencias Naturales "Bernardino Rivadavia," Buenos Aires, Argentina, where a voucher specimen is preserved (No. 31231). The animals (14 specimens, 4 kg wet weight) were kept frozen until worked up. The frozen animals were homogenized in methylene chloride/methanol (1:2, vol/vol) (5 L) and centrifuged. After concentration *in vacuo*, the residue was partitioned between H_2O and ethyl acetate/*n*-buthanol (2:1, vol/vol), and the organic layer was concentrated and chromatographed on a silica gel column using methylene chloride/methanol/water (8:1.3:0.1, by vol) as the eluent followed by recrystallization from methanol to give the crude glucosylceramide mixture CL-1 (125 mg, 0.003%). Normal-phase thin-layer chromatography (TLC) was performed on silica gel F_{254} (Merck, Darmstadt, Germany) with methylene chloride/methanol/ethyl acetate/water (8.5:1.5:2:0.1, by vol). Reversed-phase TLC was performed on C_{18} silica gel 60 F_{254} with methanol. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in a Bruker AC-200 (Karlsruhe, Germany) spectrometer. Chemical shifts are listed in δ (ppm) relative to tetramethylsilane employed as internal standard. Coupling constants (*J*) are presented in Hz. ^{13}C NMR spectra are full decoupled. Gas chromatography–mass spectrometry (GC–MS) analyses were performed on a VG TRIO-2 (Manchester, United Kingdom) instrument. Fast atom bombardment mass spectra (FABMS) were registered in a VG-ZAB BEQ spectrometer. Preparative high-performance liquid chromatography (HPLC) was carried out in a Micromeritics chromatograph (Norcross, GA) with a solvent delivery system model 750, a Thermo Separation Products refractive index detector (Fremont, CA), and a Phenomenex Ultracarb ODS 20 column (250 × 10 mm, 5μ) (Torrance, CA) eluting with absolute methanol at a flow rate of 3.0 mL/min.

RESULTS AND DISCUSSION

A water-insoluble lipid fraction, which was obtained from the methylene chloride/methanol extract of the starfish *C. lurida,* was treated with organic solvents followed by normal-phase column chromatography to give the fraction CL-1, showing a single spot on normal-phase and three spots on reversed-phase TLC. CL-1 exhibited the characteristic signals of a sphingosine-type glucosylceramide possessing 2-hydroxy fatty acid and β-glucopyranose moieties in the ^{1}H and ^{13}C

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Abbreviations: FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LCB, long-chain base; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

TABLE 1
^{1}H and ^{13}C Nuclear Magnetic Resonance Chemical Shifts of CL-1 and Glucosylceramides 3, 4, and 6 in CD_3OD

Carbon	^{1}H (mult.)/(Hz)	^{13}C
1a	3.70 (*dd, J* = 10.2, 3.3)	69.9
1b	4.11 (*dd, J* = 10.2, 5.4)	
2	3.98 (*m*)	54.4
3	4.13 (*bt, J* = 7.3)	72.1[a]
4	5.52 (*dd, J* = 15.3, 7.3)	131.8
5	5.75 (*dt, J* = 15.3, 6.4)	132.7
6	2.15 (*m*)	33.0
7	2.08 (*m*)	35.5
8	5.34 (*t, J* = 7.0)	127.8
9	—	134.1
10	6.03 (*d, J* = 15.5)	135.2
11	5.55 (*dt, J* = 15.5, 6.4)	130.9
19	1.70 (*s*)	175.5
1′	—	72.3[a]
2′	3.98 (*m*)	12.8
1″	4.2 (*d, J* = 7.7)	105.4
2″	3.20 (*dd, J* = 8.8, 7.7)	74.9
3″	[b]	78.2[a]
4″	[b]	71.3
5″	[b]	78.3[a]
6″a	3.66 (*dd, J* = 11.3, 4.2)	62.5
6″b	3.86 (*bd, J* = 11.3)	

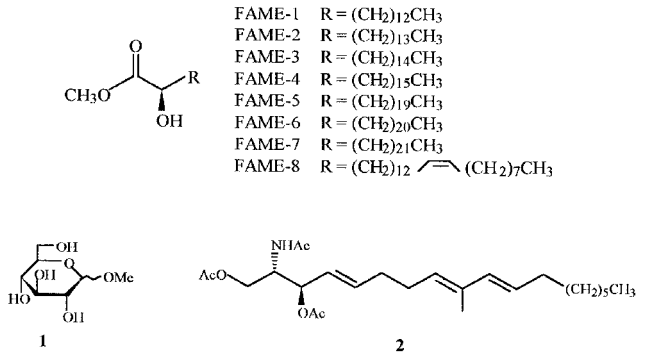
[a]Assignments may be reversed.
[b]Signals superimposed with the solvent.

NMR spectra (Table 1). Before separation of the mixture CL-1 into individual glucosylceramides, the fatty acid constituents and long-chain base moieties of these molecular species were investigated. CL-1 was methanolized with methanolic hydrochloric acid to yield a mixture of fatty acid methyl esters (FAME) and a mixture of long-chain base (LCB) together with methyl-D-glucopyranoside (8).

The FAME mixture exhibited ^{1}H and ^{13}C NMR signals due to normal 2-hydroxy FAME including a monounsaturated compound. Analysis of the FAME mixture by GC–MS showed the presence of eight compounds (FAME 1–8); they were characterized as methyl (2*R*)-hydroxy-pentadecanoate, -hexadecanoate, -heptadecanoate, -octadecanoate, -docosanoate, -tricosanoate, -tetracosanoate, and -tetracosenoate by comparing their spectral data with those reported (9). The position of the double bond in methyl-(2*R*)-hydroxy-tetracosenoate (FAME-8) was determined by GC–MS analysis of the dimethyl disulfide derivative (10) according to peaks at *m/z* 490 (M^+), 317, and 173. The double bond in FAME-8 was considered to have (*Z*) configuration as indicated by the signal observed at δ 27 ppm in the ^{13}C NMR spectrum of the FAME mixture due to the methylene carbons next to the olefinic carbons (11). Thus, the fatty acid composition of CL-1 was characterized as shown in Scheme 1, by the FAME, methyl-glucopyranoside (**1**), and acetylated LCB (**2**).

Mixture of FAME. ^{1}H NMR ($CDCl_3$): δ 0.87 (*t, J* = 6.3 Hz, 3H, terminal CH_3), 1.25 (*bs*, long chain -CH_2-), 3.77 (*s*, 3H, $COOCH_3$), 4.17 (*m*, 1H, H-2). ^{13}C NMR ($CDCl_3$): δ 14.2 [$(CH_2)_nCH_3$], 27.0 ($CH_2CH{=}CHCH_2$), 52.5 ($COOCH_3$), 70.6 [CH(OH)], 130.0 (CH=CH), 176.0 ($COOCH_3$).

The LCB mixture was acetylated to give the LCB acetate



SCHEME 1

mixture Ac-LCB and analyzed by GC–MS, showing the presence of (2*S*, 3*R*, 4*E*, 8*E*, 10*E*)-2-acetamido-1,3-diacetoxy-9-methyl-4,8,10-octadecatriene (**2**) as the major component (Scheme 1) as determined by comparing MS, ^{1}H NMR, and optical rotation data with those reported for ophidiacerebrosides containing the same base (5).

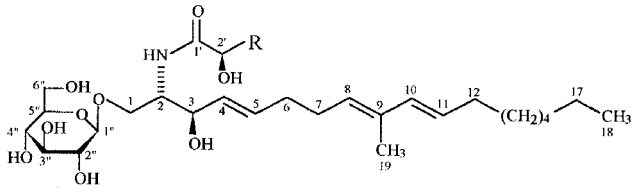
2-Acetamido-1,3-diacetoxy-9-methyl-4,8,10-octadecatriene (**2**). Gas–liquid chromatography retention time 22.0 min. ^{1}H NMR ($CDCl_3$): δ 0.87 (*t*, *J* = 6.9 Hz, 3H, terminal CH_3), 1.25 [*bs*, 8H, $(CH_2)_4CH_3$)], 1.69 (*s*, 3H, CH_3-19), 1.95 (*s*, 3H, CH_3CO), 2.12 (*s*, 6H, CH_3CO), 4.04 (*dd*, *J* = 12.3, 3.3 Hz, 1H, H-1a), 4.31 (*dd*, *J* = 12.3, 5.2 Hz, 1H, H-1b), 4.41 (*m*, 1H, H-2), 5.30 (*t*, *J* = 7.3 Hz, 1H, H-3), 5.35 (*m*, 2H, H-4, H-8), 5.57 (*dt*, *J* = 15.0, 6.4 Hz, 1H, H-11), 5.75 (*dt*, *J* = 15.3, 6.6 Hz, 1H, H-5), 5.99 (*d*, *J* = 15.0 Hz, 1H, H-10). MS *m/z* = 435 (M^+), 375 (M–60), 336, 256, 243, 144.

CL-1 (90 mg) was separated repeatedly by reversed-phase HPLC into four major fractions **3**–**6** each displaying a single peak on reversed-phase HPLC (Table 2). Fractions **3**, **4,** and **6** revealed single molecular ion peaks in their negative fast atom bombardment mass spectra, but fraction **5** exhibited three molecular ion peaks. Each fraction was methanolized and the FAME analyzed by GC–MS; a single FAME was detected from fractions **3** (FAME-3), **4** (FAME-4) and **6** (FAME-7), and three kinds of FAME were obtained for fraction **5** [FAME-5 (33%), FAME-6 (18%), and FAME-8 (39%)]. In taking the molecular masses of the four fractions and the fatty acid components into account, the LCB component of **3**, **4**, **5**, and **6** must be assigned as 2-amino-1,3-dihydroxy-9-methyl-4,8,10-octadecatriene. Accordingly, only

TABLE 2
Masses, High-Performance Liquid Chromatography RT and Negative FABMS of Glucosylceramides from *Cosmasterias lurida*

Fraction	Mass (mg)	RT (min)	Negative FABMS[a] (*m/z*)
3	1.8	15.5	738 (M-H), 576 (M-H-glucose)
4	12.6	23.4	752 (M-H), 590 (M-H-glucose)
5	16.0	51.2	808 (M_1-H),646 (M_1-H-glucose)
			822 (M_2-H), 660 (M_2-H-glucose)
			834 (M_3-H), 672 (M_3-H-glucose)
6	5.2	63.8	836 (M-H), 674 (M-H-glucose)

[a]RT, retention time; FABMS, fast atom bombardment mass spectra.



3 R = $(CH_2)_{14}CH_3$
4 R = $(CH_2)_{15}CH_3$
5 R = $(CH_2)_{19}CH_3$, $(CH_2)_{20}CH_3$, $(CH_2)_{12}$–CH=CH–$(CH_2)_7CH_3$
6 R = $(CH_2)_{21}CH_3$

SCHEME 2

three fractions are homogeneous, and they are assigned to compounds **3**, **4**, and **6** (Scheme 2). As glucosylceramides **3**, **4** and **6** differed only in the chain length of their α-hydroxy fatty acid constituents, they showed the same characteristic ^1H and ^{13}C NMR signals due to C-1–C-11, C-1′, C-2′, C-1″–C-6″ of a 1-*O*-β-glucopyranoside of a (2*S*,3*R*,4*E*,8*E*, 10*E*)-2-amino-1,3-dihydroxy-9-methyl-4,8,10-octadecatriene ceramide possessing a (2*R*)-hydroxy fatty acid (Table 1). The assignment of ^1H NMR signals was determined by ^1H–^1H correlation spectroscopy and ^1H NMR decoupling experiments. The conjugated diene structure was also supported by an ultraviolet band at 225 nm. Glucosylceramides **3** and **4** are new compounds, and compound **6** (ophidiacerebroside E) has been reported previously as a minor component in the starfish *O. ophidianus* (5). The natural occurrence of the ceramide present in glucosylceramide **4** was recently reported as the 1-sulfate derivative from the bryozoan *Watersipora cucullata* (12).

ACKNOWLEDGMENTS

We are most grateful to Dr. Alejandro Tablado (Museo de Ciencias Naturales "Bernardino Rivadavia," Buenos Aires) for the taxonomic identification of *C. lurida*. We also thank UMYMFOR and LANAIS-EMAR (CONICET-FCEN) for spectroscopic analysis and the International Foundation for Science and the Universidad de Buenos Aires for partial financial support.

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[Received January 27, 1998, and in final revised form May 15, 1998; revision accepted June 24, 1998]

The Signature 10-Hydroxy Stearic Acid Thought to Correlate with Infectivity in Oocysts of *Cryptosporidium* Species Is an Artifact

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ABSTRACT: Heating or freezing leads to loss in infectivity of oocysts of *Cryptosporidium parvum* toward neonatal BALB/c mice and is reflected in the profile of the polar lipid fatty acids. Upon loss of infectivity, the ratio of polar lipid to neutral lipid fatty acid decreased and the relative proportions of 18:1n-9 also decreased; proportions of 18:2n-6 and 20:5n-6 increased, whereas the proportions of 16:0 remained constant with freezing. During these investigations, a novel fatty acid, 10-OH 18:0, was discovered in the glycolipid fraction. The identification of a fatty acid unique to species of *Cryptosporidium* was thought to provide a specific biomarker for this organism. *Cryptosporidium* also demonstrated fluctuations in absolute quantities of 10-OH 18:0 with events that lead to loss of infectivity. This led to the presumed correlation of this biomarker with infectious *Cryptosporidium*. The 10-OH 18:0 was putatively localized at the *sn*-2 position of phosphatidylethanolamine. High-performance liquid chromatography/electrospray ionization mass spectrometry revealed that the 10-OH 18:0 existed principally in the free fatty acid form. Herein, we establish that the free fatty acid 10-OH 18:0 was, in actuality, an artifact of the procedures for sample preparation.

Lipids 33, 829–833 (1998).

A major problem in the potable water industry is the detection, identification, and determination of the presence of infectious protozoan parasites. *Cryptosporidium* is of special concern as it is widespread in surface water samples, has extreme resistance to disinfection by chlorine, completes its infectious cycle only in human or mammalian hosts, and is potentially fatal to immunocompromised individuals who become infected (1). Successful methods must be highly sensitive, as the presence of as few as 10–30 oocysts may initiate infections. The method must also be highly specific to *Cryptosporidium*, sufficiently rapid so as to be potentially useful to the water industry, and provide an indication of viability/infectivity. To date, existing methodologies for the detection of *C. parvum* oocysts in drinking water are not satisfactory (2). With the discovery that freezing *C. parvum* oocysts at –70°C, which renders them noninfectious to neonatal BALBC/c mice (3), induced shifts in the polar lipid/neutral lipid ratio, a shift in fatty acid composition, a decrease in cholesterol, and a loss in an unusual fatty acid 10-hydroxy stearic acid (10-OH 18:0) (4), a potentially quantitative biomarker was proposed. Identification of this fatty acid as unique to species of *Cryptosporidium* would provide a signature lipid biomarker (SLB) in complex environmental matrices such as water distribution system biofilms. For this reason, the focus of the assay was shifted to the highly unusual fatty acid, 10-OH 18:0. Previous investigators had shown that the traces of 10-OH 18:0 were localized at the *sn*-2 position of phosphatidylethanolamine (PE) (5). However, the combined efforts of parent/derived fragment ion mass spectrometry and high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) by R.S. Burkhalter and C.A. Smith revealed that the 10-OH 18:0 existed in the free fatty acid form (6). Free fatty acids are extremely unusual in nature and the potential of 10-OH 18:0 as a unique "signature" was explored. Unfortunately, subsequent experimental evidence revealed the true origin of 10-OH 18:0. The 10-OH 18:0 was found to be an extractable component of the disposable rubber pipette bulbs used during purification and was not found as a lipid component of *Cryptosporidium* oocysts.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. The standards (3-hydroxy eicosanoic acid, 12-hydroxy stearic acid, and 6-hydroxy stearic acid) were purchased from Matreya (Pleasant Gap, PA). Chromatography-grade solvents of chloroform, methanol, acetone, and water were also used (4,5).

Rubber bulbs. Amber rubber bulbs (2-mL, catalog number 14065B; Fisher Scientific Co., Pittsburgh, PA) were utilized with glass pipettes to transfer solutions.

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Abbreviations: CID, collision-induced dissociation; FAB, fast ion bombardment; GC, gas chromatography; HPLC/ESI/MS, high-performance liquid chromatography/electrospray ionization/mass spectrometry; PE, phosphatidylethanolamine; 10-OH 18:0, 10-hydroxy stearic acid.

Parasites. Oocysts of *C. parvum* used in this study were purified from calf feces as previously described (4,5).

Lipid extraction. Membrane filter retentates or lyophilized sediments were extracted at room temperature and atmospheric pressure with the one-phase chloroform/methanol/phosphate buffer (50 mM, pH 7.4), 1:2:0.8 by vol, solvent system as previously described (7–9). All solvents were of gas chromatography grade and obtained from Baxter Scientific Products (McGaw Park, IL). All glassware was washed in a 10% (vol/vol) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed five times with tap water and then five times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C. Glass filter loaded samples were extracted in separatory funnels containing the chloroform/methanol/phosphate buffer, 1:2:0.8 single phase extractant 4 h before adding chloroform and deionized water (final solvent ratios, chloroform/methanol/ phosphate buffer/water; 1:1:0.4:0.5 by vol) to form two phases. The organic phase was then collected and dried under a stream of N_2 at 37°C.

Purification of lipids. The total lipid extract was dissolved in chloroform (~ 0.5 mL) and separated using a silicic acid column (10-cm column length, 0.5-cm inner diameter, 100–200 mesh particle size). Each sample was loaded onto the column in a minimal volume of chloroform (100–200 μL) and then eluted in batches with 5 mL of chloroform, 5 mL acetone, and 5 mL of methanol so as to elute neutral, glyco-, and polar lipids, respectively (9). Prepacked silicic acid columns (Burdick and Jackson inert solid phase extraction system #7054G; Muskegon, MI) are also a commercial alternative. Bulk elution of the neutral lipids with chloroform was followed by recovery of the free fatty acids in acetone or glycolipid fraction. For each fraction, the solvent was removed under a stream of nitrogen. The lipids were stored at –20°C until analysis.

HPLC/ESI/MS. The acid 10-OH 18:0 migrates in reverse-phase high-performance liquid chromatography (C-18 Alltech Altima 5 μm particle size, 150 mm in length, 1 mm diameter column; Deerfield, IL) with a mobile phase consisting of methanol/ultrapure water/aqueous ammonia (100:6:1) at a flow rate of 50 μL/min with a retention time of 12–14 min typical of hydroxylated free fatty acids (3-OH 20:0). In contrast, PE elutes at 20–22 min under these conditions. Those components eluting with a retention volume typical of free fatty acids were nebulized into the ESI inlet of either the VG (Micromass Inc., Beverly, MA) Quattro II triple quadrupole mass spectrometer or the Micromass Platform II single quadrupole mass spectrometer. Analyses were performed in the negative mode of ionization so as to promote fragmentation resulting from cleavage of the two-acyl functionalities with charge retention on the free fatty acids formed through acyl cleavage. Initially, elevated cone voltages were utilized so as to promote fatty acyl cleavage from intact phospholipids. However, upon realizing that the biomarker of interest existed in the free fatty acid form, low orifice voltages were utilized so as to promote "soft" ionization. Tuning was performed such that maximum sensitivity was attained while achieving a minimum of unit resolution at a *m/z* value of 300 atomic mass units (a.m.u.) (resolution = 300). For the present study, resolution values of between 750 and 1,000 easily fulfilled this criterion. Narrow mass scans in the continuum mode of data acquisition were performed over a mass range spanning 320 to 270 a.m.u. at a scan rate of 0.4 s with a interscan delay of 0.1 s. Alternatively, selected ion monitoring of mass 299 could be utilized, which provided increased sensitivity. The continuum mode of data acquisition was utilized as this is the only means to obtain "true" signal averaging.

Mass spectral optimization studies found no significant gain in sensitivity upon the addition of organic bases. This is advantageous, as reverse-phase columns are generally not stable at pH values greater than 9. Addition of water to methanol resulted in a slight loss in sensitivity with model synthetic compounds. The initial mobile phase of methanol/water/aqueous ammonia was utilized so as to resolve free fatty acids from PE. As it will be explained later, the establishment that the novel biomarker was indeed present as a free fatty acid and that no PE species were present in the glycolipid fraction lends itself to a variation of the chromatographic conditions. Conversion to a mobile phase consisting of 100% methanol would be more efficient in that the analysis time would be reduced with a slight gain in sensitivity. Tuning in the negative mode of ionization may vary. But for the present study, the voltage applied across the capillary was –2.13 kV, the high voltage lens was set at 0.2 kV, and the initial cone voltage was set at –22 V so as to promote "soft" ionization. The nitrogen drying gas and nebulizing gas flow rates were set at 300 and 25 L/h. respectively. The temperature of the ESI source was set at 120°C. The photomultiplier detector was set to its maximum gain value.

Extracted ion chromatograms at a *m/z* value of 299 were monitored. Ion currents for 299 in the 11–14 min range were diagnostic of 10-OH 18:0 in the free fatty acid form. Ion currents in the range of interest were averaged with background subtraction resulting in a diagnostic mass spectrum.

RESULTS

Evidence that the 10-OH 18:0 is a free fatty acid. As previously stated, earlier investigators found the 10-OH 18:0 localized at the *sn*-2 position of PE (5). However, utilization of the derivative ion scanning of specific parent ion capabilities of the triple quadrupole mass spectrometer in the negative ionization mode did not support this. Examination of those parent ions that result in a fragment ion corresponding to 10-OH 18:0 revealed no parents which could correspond to PE or any possible phospholipid. Secondly, neutral loss mass scans for a neutral loss of 141 of the acetone fraction in the positive mode of ionization revealed that this fraction contained no detectable quantities of PE species. PE was found to be located in the methanol (polar lipid) fraction through neutral loss mass scans. The retention behavior of 10-OH 18:0 on the HPLC/ESI/MS system was described in the experimental section. The acid 10-OH 18:0 was found to coelute in the free fatty acid region (12–14 min) of the chro-

matogram with a synthetic standard of 3-OH 20:0. Elution of the free hydroxylated fatty acids as a class was clearly resolved from PE which eluted as a class at 20–22 minutes. Additional evidence as to the true nature of 10-OH 18:0 was supplied through examination of the ion current for a *m/z* value of 299 at both high and low orifice voltages. At high orifice voltages, the ion current for 10-OH 18:0 was found to be over two orders of magnitude less than at low orifice voltages (1.63×10^8 peak height units at −22 V vs. 8.53×10^5 peak height units for −200 V). With synthetic phospholipids, the −200 V cone voltage was found to maximize the dissociation of the acyl groups of phospholipids resulting in a maximal ion current for the fatty acyl constituents at maximal orifice voltage settings. Lower cone voltages (−22 V) resulted in maximal ion currents for the intact molecular species. This observation provided additional evidence that 10-OH 18:0 existed as an intact molecular species and was not acylated to any phospholipid. The combined evidence of parent ion MS, neutral loss mass scans, HPLC retention behavior, and effects of variation of the orifice voltage clearly established 10-OH 18:0 existed not as an acyl constituent of PE but as a free fatty acid.

Evidence that the free fatty acid is an eighteen-carbon fatty acid with a hydroxyl functionality localized at the 10 position. Theoretically, several fatty acids could produce a *m/z* value of 299 in the negative ion mode of operation. Obviously, any monohydroxylated 18-carbon fatty acid with the hydroxyl group at positions 2–17 would yield a deprotonated molecular ion at 299. Other possibilities include but are not limited to a 17-carbon monoenoic fatty acid with a sulfhydryl (-SH) substituent as well as the polyenoic fatty acid 20:6n-3. Unambiguous assignment of the free fatty acid as 10-OH 18:0 was accomplished through comparison of the parent/derivative ion mass spectrum of 10-OH 18:0 with synthetically available standards (6-OH 18:0 and 12-OH 18:0). The first quadrupole acted as the mass filter, allowing only ions with a *m/z* value of 299 to pass into the second mass analyzer. Collision-induced dissociation (CID) of the parent molecule was accomplished in the hexapole collision chamber between the first and third quadrupoles in the presence of 0.5 Torr of argon at a collision energy of 30 eV. Diagnostic fragments were monitored by scanning the third quadrupole mass analyzer. Hydroxylated fatty acids produced low-energy CID fragment ions which were indicative of hydroxy-substituted fatty acids as a class at a *m/z* value of 281 (loss of H_2O from the hydroxyl moiety) and at a *m/z* value of 253 (loss of formic acid). Nonhydroxylated free fatty acids (palmitic acid) do not undergo any significant fragmentation under similar conditions. The site of the hydroxy functionality was confirmed to be in the 10 position through the examination of two charge-remote fragment ions that overlapped at a *m/z* value of 141 for 10-OH 18:0. The two charge-remote fragmentation processes were confirmed through examination of the daughter ion spectra of two other mid-chain, branched hydroxylated fatty acids, 6-OH and 12-OH 18:0, under identical conditions. Analogous peaks expected and observed for 12-OH 18:0 were at *m/z* values of 113 and 169. For 6-OH 18:0, fragment ions at *m/z* values of 197 and 85 were observed. The nature of the two charge-remote fragmentation events has been discussed (10). However, to summarize, fragmentation of hydroxylated fatty acids under low-energy conditions leads to fragmentation events that are distinct from previously published high-energy CID processes (11). Also, those low-energy CID fragments which are produced are indicative of hydroxylated fatty acids as a class as well as of the site of hydroxysubstitution.

Sources of 10-OH 18:0. The identification of a fatty acid unique to species of *Cryptosporidium* by Schrum *et al.* (5) was critical to the application of this technique toward complex environmental matrices. However, conventional GC/MS methodologies lack the sensitivity necessary for use of this technique as a method capable of detecting an infectious dose of *Cryptosporidium.* It was hoped that through the application of the more sensitive technique of ESI MS, a more sensitive assay might result. Also, through the identification of those endogenous 10-OH 18:0 acylated phospholipids, a great deal of specificity to the assay should be gained. Identification of the 10-OH 18:0 as a free fatty acid as opposed to an acylated component of PE was initially thought to be a fortuitous event as hydroxylated free fatty acids are extremely rare in nature. The free fatty acid nature of the biomarker was subsequently exploited for expeditious recovery and analysis by HPLC/ESI/MS with the expected gains in method sensitivity and specificity.

It was only after many repetitions of this assay that the true origin of 10-OH 18:0 was discovered. Quantities of 10-OH 18:0 extracted from oocysts of *Cryptosporidium* fluctuated wildly. Occasionally, no 10-OH 18:0 was detected from extracted oocysts of *Cryptosporidium*. The random appearance of 10-OH 18:0 in one of our sample blanks was the first indication that this compound was generated through sample handling. The observation of free 10-OH 18:0 in a sample blank was initially thought to have arisen from some interfering contaminant. However, formation of the trimethylsilyl ether adduct of the methyl ester of the sample blank resulted in an analyte possessing chromatographic behavior and a mass spectrum identical to that analyte previously described by Schrum *et al.* (5) as 10-OH 18:0.

An additional benefit of adapting the identification of the target analyte to HPLC/ESI/MS was the decrease in sample handling. This should result in a much more rapid assay with less risk of sample contamination due to the decrease in sample handling. The extraction procedures are simple and straightforward. The method involves the liquid/liquid extraction of organic soluble lipids followed by the class fractionation of neutral, glyco-, and polar lipids over silicic acid columns. The simplicity of the assay eased the efforts to localize the source of contamination once 10-OH 18:0 was realized to be an artifact of sample isolation and preparation.

All organic solvents (methanol, chloroform, acetone) used in the assay were concentrated from an initial volume of 100 mL to a final volume of 100 μL and injected onto the HPLC/ESI/MS system. Only HPLC-grade solvents were used for the extraction and fractionation of samples. All organic solvents were found to contain no 10-OH 18:0. The 10 mL of

phosphate buffer used in the liquid/liquid extraction procedures was extracted by the method of Bligh and Dyer (8), as was 10 mL of Millipore (Milford, MA) water. They, too, were found to contain no 10-OH 18:0. This eliminated the source of contamination as arising from the solvents utilized for extraction and fractionation.

Those soaps and sterilization agents utilized to clean glassware were also examined; 1 mL of Fisherbrand solvent-free concentrator, 1 mL of Fisherbrand vesphenne II, and 1 mL of a 1:1 (vol/vol) mixture of the two were extracted by the method of Bligh and Dyer (8). No 10-OH 18:0 was identified from any of these three samples.

The disposable amber rubber pipette bulbs (Fisherbrand 14 065B) were then examined. A set of five rubber bulbs was extracted, and all were found to contain authentic free acid 10-OH 18:0. This was confirmed through both HPLC/ESI/MS analysis and GC/MS separation and identification of the trimethylsilyl ether adducts of the methyl ester of 10-OH 18:0.

So as to eliminate the possibility that 10-OH 18:0 was also a lipid component of *Cryptosporidium* species as well as the rubber bulb, two samples of 1×10^8 oocysts of *Cryptosporidium* previously found to contain 10-OH 18:0 were extracted. At no time were disposable rubber bulbs utilized. Both sets of experiments were found to contain no detectable quantities of 10-OH 18:0 through HPLC/ESI/MS and GC/MS analysis when disposable rubber bulbs were not used in the manipulations. This established that the target analyte was not an interfering contaminant but an artifact of the sample preparation procedures.

DISCUSSION

The putative occurrence of such an unusual component as 10-OH 18:0 in *Cryptosporidium* (4,5) led us to investigate the complex native form of this fatty acid in *C. parvum*. When the new studies by R.S. Burkhalter and C.A. Smith showed that the 10-OH 18:0 was a free fatty acid and not as an ester in PE (6), it was then realized that the fast atom bombardment (FAB) mass spectrum of PE presented by Schrum *et al.* (5) actually provided no evidence supporting the presence of 10-OH 18:0 as an acyl constituent in PE. This spectrum was obtained under positive ionization conditions, which will not produce fragment ions corresponding to fatty acyl constituents. Positive-ion FAB MS should provide protonated molecular ions and diglyceride ions after loss of the polar head group as a neutral species. FAB MS performed in the negative ion mode of operation would be expected to generate fatty acids with charge retention after acyl cleavage. In short, the fragment ion at 299 in the positive ion FAB mass spectrum of the major PE did not correspond to a fatty acyl constituent 10-OH 18:0. The enzymatic hydrolysis of PE recovered by thin-layer chromatography and subsequent derivatization with electron-withdrawing groups for negative ion detection by GC/MS (6) achieved the sensitivity to detect 10-OH 18:0, but the 10-OH 18:0 was apparently added during the preparation as a contaminant and was not in the PE.

When adaptation of HPLC/ESI/MS techniques revealed 10-OH 18:0 to be a free fatty acid, the putative signature became even more unique. Efforts were focused to characterize the target analyte of interest more completely and to establish lower limits of detection. Difficulty in producing a calibration curve for oocyst number vs. content of 10-OH 18:0 became a major problem. This was rationalized as being due to sample heterogeneity after microscopic examination of the preparations showed the oocysts clumped together and attached to fibers that were in these purified fecal preparations. The clumping increased with storage at 4°C. Difficulty in dispersing the oocysts reproducibly was thought to be the problem. Unfortunately, the random nature of the appearance of the 10-OH 18:0 prevented the appearance of the analyte in a sample blank until many repetitions were performed. Clearly when examining for traces of specific analytes such as found in 10–30 oocysts, comprehensive blank analyses which test for each step of the procedure must be rigorously performed. If repeated controls of lipids from a bacterium known not to contain 10-OH 18:0 had been utilized, the artifact might have been detected earlier.

Lipid biomarkers and Cryptosporidium *oocyst infectivity.* There is reproducible evidence of a correlation between loss of infectivity and oocyst metabolism in *C. parvum*. R. Fayer (personal communication; USDA, ARS, LPSI Parasite Immunology Laboratory, Beltsville, MD) has shown a direct correlation between the loss in amylopectin content of *C. parvum* oocysts and infectivity to BALB/c neonatal mice. There is a greater than threefold decrease in polar lipid fatty acids (PLFA) (from 3.8 to 0.75 pmoles/10^3oocysts) with a shift in patterns (4). With loss of infectivity by freezing there is a striking decrease in the proportion of 18:1n-9*c*, an increase in 18:2n-6 and 20:3n-6 with little change in the proportion of 16:0. During freezing of *C. parvum* oocysts, the phospholipids and cholesterol decrease, polyenoic PLFA accumulate, and polyenoic fatty acids in the polar lipids and neutral lipids increase. Lipid shifts have been shown to be excellent markers for the nutritional/physiological status in bacteria (12). Identification of lipid shifts with infectivity is a tool that will be useful in research studies of infectivity, but overlap in lipid composition with algae and other components of drinking water community requires that the oocytes be highly purified before analysis prior to use as a monitoring system for infectious *Cryptosporidium*. Patterns of PLFA of purified oocysts can used to differentiate between *C. parvum* and the nonhuman pathogens *C. baileyi, C. serpentis*, and *C. muris* (6).

ACKNOWLEDGMENTS

We thank HRA 699-517-B-94 “Risk Reduction in Drinking Water Distributor Systems by On-Line Monitoring of Pathogen Ecology for Quantitative Evaluation of Mitigation Procedures” from the National Water Research Institute (Fountain Valley, CA), for supporting initial phases of this work and Microbial Insights, Inc. for continuing support.

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[Received April 21, 1998, and in final revised form July 7, 1989; revision accepted July 9, 1998]

Rumenic Acid: A Proposed Common Name for the Major Conjugated Linoleic Acid Isomer Found in Natural Products

Sir:

At the last American Oil Chemists' Society meeting in Chicago, May 10–13, 1998, there was a formal discussion period after a day-long series of presentations on conjugated linoleic acid (CLA) attended by about 100 participants. One of the topics discussed was the possible naming of the major CLA isomer, *cis*-9, *trans*-11-octadecadienoic acid—found in milk, other dairy products, and meats of ruminant animals—as rumenic acid. The name rumenic acid has been proposed by Peter W. Parodi and is supported by a number of other scientists.

There was extensive debate on this topic. CLA is a mixture of many positional and geometrical isomers of conjugated octadecadienoic acids both in natural products and in commercial preparations. In natural products, the predominant isomer (≥80% of total CLA) is *cis*-9, *trans*-11, whereas in commercial preparations the number and proportion of the isomers can vary widely depending on the conditions of preparation.

The major arguments presented against naming any CLA isomer were: (i) the term CLA has been in common use for nearly two decades; (ii) *cis*-9, *trans*-11 appears also to be formed outside the rumen by desaturation of *trans*-11-18:1; (iii) the active isomer has yet to be identified; (iv) there appears to be evidence that *cis*-9, *trans*-11 isomer may not be the only active CLA isomer, therefore; how shall we name them?

On the other hand, the major arguments for naming *cis*-9, *trans*-11-octadecadienoic acid rumenic acid were: (i) this is the major naturally occurring conjugated fatty acid in milk, other dairy products, and meats from ruminants; (ii) a major natural component can be named regardless of whether any biological activity has been ascribed to it; (iii) the name would avoid the misconception that it is a CLA having a methylene-interrupted double bond system, and (iv) it is an easy name, associated with the major place of origin, and may thus be less confusing.

Additional names were suggested, such as bovinic acid. However, there was agreement that this name did not encompass the broad spectrum of natural products containing *cis*-9, *trans*-11-octadecadienoic acid, for example, from sheep and other ruminants.

There was no complete consensus, but half the participants said they would use the new name in their future publications. We therefore recommend naming *cis*-9, *trans*-11-octadecadienoic acid as rumenic acid.

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[Received June 23, 1998, and in final revised form and accepted July 13, 1998]

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Analysis of Novel Hydroperoxides and Other Metabolites of Oleic, Linoleic, and Linolenic Acids by Liquid Chromatography–Mass Spectrometry with Ion Trap MS^n

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ABSTRACT: Linoleate is oxygenated by manganese-lipoxygenase (Mn-LO) to 11*S*-hydroperoxylinoleic acid and 13*R*-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid, whereas linoleate diol synthase (LDS) converts linoleate sequentially to 8*R*-hydroperoxylinoleate, through an 8-dioxygenase by insertion of molecular oxygen, and to 7*S*,8*S*-dihydroxylinoleate, through a hydroperoxide isomerase by intramolecular oxygen transfer. We have used liquid chromatography–mass spectrometry (LC–MS) with an ion trap mass spectrometer to study the MS^n mass spectra of the main metabolites of oleic, linoleic, α-linolenic and γ-linolenic acids, which are formed by Mn-LO and by LDS. The enzymes were purified from the culture broth (Mn-LO) and mycelium (LDS) of the fungus *Gaeumannomyces graminis*. MS^3 analysis of hydroperoxides and MS^2 analysis of dihydroxy- and monohydroxy metabolites yielded many fragments with information on the position of oxygenated carbons. Mn-LO oxygenated C-11 and C-13 of 18:2n-6, 18:3n-3, and 18:3n-6 in a ratio of ~1:1–3 at high substrate concentrations. 8-Hydroxy-9(10)epoxystearate was identified as a novel metabolite of LDS and oleic acid by LC–MS and by gas chromatography–MS. We conclude that LC–MS with MS^n is a convenient tool for detection and identification of hydroperoxy fatty acids and other metabolites of these enzymes.

Lipids 33, 843–852 (1998).

Mass spectrometry (MS) has been extensively used for analysis of oxygenated metabolites of arachidonic and linoleic acids, which are formed by lipoxygenases, prostaglandin H (PGH) synthases, and cytochromes P450. These metabolites are prostaglandins and thromboxanes, leukotrienes, lipoxins, hydroxyeicosatetraenoic acids (HETE), allene oxide, jasmonic acid, regioisomeric epoxides, vicinal diols, and hydroxy fatty acids (1–3). Mass spectrometric analysis of eicosanoids and lipoxins has mainly been performed by gas chromatography (GC)–MS, which provides structural information and can be used to quantify metabolites in minute amounts (4,5).

GC–MS has some drawbacks. It requires derivatization, and it is not suitable for direct analysis of many labile metabolites, e.g., hydroperoxy fatty acids. In recent years, liquid chromatography (LC)–MS has become a routine method thanks to robust ionization techniques, e.g., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) (4,6). Many fatty acid metabolites have already been studied by LC–MS (4,7–13).

In this report we use LC–MS with MS^n to study the fatty acid metabolites of two unique enzymes: linoleate diol synthase (LDS) with ferriheme (15) and linoleic acid 13*R*-lipoxygenase (Mn-LO) with a manganese metal center (16). These enzymes were isolated from mycelium (LDS) and from the culture broth (Mn-LO) of the "take-all" fungus (*Gaeumannomyces graminis*), which is a devastating pathogen of wheat (14).

LDS converts linoleate sequentially to 8*R*-hydroperoxylinoleate (8-HPODE) through an 8-dioxygenase by insertion of molecular oxygen (17,18) and to 7*S*,8*S*-dihydroxylinoleate (7,8-DiHODE) through a hydroperoxide isomerase by intramolecular oxygen transfer (19,20). LDS will also oxygenate oleic and α-linolenic acids, but not γ-linolenic acid (18). The reaction mechanism of LDS has features in common with PGH synthases, e.g., formation of ferryl intermediates and a tyrosyl radical during catalysis (21).

Mn-LO metabolizes linoleic acid to 11*S*-HPODE and to 13*R*-HPODE (16,22). Its oxygenation mechanism thus differs from iron lipoxygenases (22). Mn-LO will also oxidize α- and γ-linolenic acids but not oleic acid (16). The oxygenation of unsaturated C_{18} fatty acids by Mn-LO and by LDS is summarized in Table 1. These oxylipins have been characterized mainly by GC–MS (16–18,22).

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Abbreviations: APCI, atmospheric pressure chemical ionization; 7,8-DiHODE, 7S,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 7,8-DiHOME, 7*S*,8*S*-dihydroxy-9*Z*-octadecenoic acid; 7,8-DiHOTrE, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; ESI, electrospray ionization; GC–MS, gas chromatography–mass spectrometry; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; 8-H(P)ODE, 8*R*-hydro(pero)xy-9*Z*,12*Z*-octadecadienoic acid; 11-H(P)ODE, 11*S*-hydro(pero)xy-9*Z*,12*Z*-octadecadienoic acid; 13-H(P)ODE, 13-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid; 8-H(P)OME, 8*R*-hydro(pero)xy-9*Z*-octadecenoic acid; 8-HPOTrE, 8*R*-hydroperoxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 11-H(P)OTrE(n-6), 11*S*-hydro(pero)xy-6*Z*,9*Z*,12*Z*-octadecatrienoic acid; 11-H(P)OTrE, 11*S*-hydro(pero)xy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 13-H(P)OTrE, 13-hydro(pero)xy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 13-H(P)-OTrE(n-6), 13-hydro(pero)xy-6*Z*,9*Z*,11*E*-octadecatrienoic acid; LC–MS, liquid chromatography–mass spectrometry; LDS, linoleate diol synthase; Mn-LO, manganese lipoxygenase; PGH, prostaglandin H; RP–HPLC, reversed phase–HPLC.

TABLE 1
Overview of the C_{18} Fatty Acid Metabolites Formed by Mn-LO and by LDS

Fatty acid	Mn-LO	LDS
18:0	Not a substrate	Not a substrate
18:1n-9	Not a substrate	8*R*-HPOME 7*S*,8*S*-DiHOME 8-Hydroxy-9(10)epoxy-stearic acid[a]
18:2n-6	11*S*-HPODE 13*R*-HPODE	8*R*-HPODE 7*S*,8*S*-DiHODE
18:3n-3	11*S*-HPOTrE[b] 13*R*-HPOTrE	8*R*-HPOTrE 7*S*,8*S*-DiHOTrE
18:3n-6	11*S*-HPOTrE(n-6) 13*R*-HPOTrE(n-6)	Not a substrate

[a]Identified in this study.
[b]Identified in this study and tentatively in Reference 16. Other metabolites were identified by gas chromatography–mass spectrometry and chemical methods as described (Refs. 16,17,22). Arachidonic acid is oxygenated neither by LDS nor by Mn-LO (Refs. 16,17). Abbreviations: Mn-LO, manganese lipoxygenase; LDS, linoleate diol synthase; 8*R*-HPOME, 8*R*-hydroperoxy-9*Z*-octadecenoic acid; 7*S*,8*S*-DiHOME, 7*S*,8*S*-dihydroxy-9*Z*-octadecenoic acid; 11*S*-HPODE, 11*S*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid; 8*R*-HPODE, 8*R*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid; 13*R*-HPODE, 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 7*S*,8*S*-DiHODE, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid; 11*S*-HPOTrE, 11*S*-hydroperoxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 8*R*-HPOTrE, 8*R*-hydroperoxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 13*R*-HPOTrE, 13-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 7*S*,8*S*-DiHOTrE, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 11*S*-HPOTrE(n-6), 11*S*-hydroperoxy-6*Z*,9*Z*,12*Z*-octadecatrienoic acid; 13*R*-HPOTrE(n-6), 13-hydroperoxy-6*Z*,9*Z*,11*E*-octadecatrienoic acid.

Wheelan *et al*. (8) systematically performed low-energy fast atom bombardment tandem MS^2 of monohydroxy substituted oleic, linoleic, linolenic, and arachidonic acids. ESI and tandem MS have also been used for MS^2 analysis of HETE and dihydroxyeicosatetraenoic acids, epoxyeicosatrienoic acids, and certain hydroperoxides of linoleic and arachidonic acids (7,9–12). Structurally informative and abundant product ions were formed by fragmentation processes, which were influenced by the position of the hydroxy and epoxy groups and by the double bonds.

The first aim of the present study was to evaluate LC–MS with MS^n as a tool for analysis of hydroperoxy-, hydroxy- and dihydroxy-substituted linoleic acids, which are formed by two novel enzymes, Mn-LO and LDS. The interpretation of the mass spectra of the linoleate hydroperoxides was not straightforward without comparison with structurally related compounds. We therefore studied some of the metabolites in deuterated methanol/water and performed a systematic study of different unsaturated C_{18} fatty acid metabolites, which are formed by Mn-LO and LDS. MS^3 spectra of hydroperoxy fatty acids and MS^2 spectra of mono- and dihydroxy fatty acids were found to provide sufficient information for identification.

MATERIALS AND METHODS

Materials. Mn-LO and LDS were purified to homogeneity as described (15,16). The acids 18:1n-9 (99%) and 18:3n-3 (99%) were from Merck (Darmstadt, Germany); 18:2n-6 (99%) and 18:3n-6 (99%) were from Sigma (St. Louis, MO) and Nu-Chek-Prep (Elysian, MN), respectively. The labeled compounds [1-^{14}C]18:2n-6 (55 Ci/mol) and [1-^{14}C]18:1 (55 Ci/mol) were from Amersham Corp. (Amersham, United Kingdom). The compounds 8*R*-HPODE, 8*R*-HODE, and 7*S*,8*S*-DiHODE were prepared and characterized as described (18). Compounds 11*S*-HPODE, 13*R*-HPODE, 13*R*-HPOTrE, 11*S*-HPOTrE(n-6), and 13*R*-HPOTrE(n-6) were prepared from biosynthesis with Mn-LO as described (16,22). Hydroperoxy fatty acids were reduced to alcohols with $NaBH_4$ (16). $C^2H_3O^2H$ (99.5%) and ^2H_2O (99.5%) were from Merck.

Incubations with Mn-LO or LDS. Fatty acids (0.25 mM) were incubated with purified Mn-LO or purified LDS as described (15,16). Radiolabeled fatty acids were used in some experiments. After extractive isolation (without acidification of Mn-LO metabolites) on octadecasilane silica (SepPak/C_{18}; 23), the metabolites were either separated by thin-layer chromatography (18) or directly analyzed by LC–MS with a reversed-phase–high-performance liquid chromatography (RP–HPLC) column.

LC–MS analysis. The pump for HPLC was from Thermo Separation Products (SpectraSYSTEM P2000; San Jose, CA) equipped with a degasser (Uniflow Degasys DG-1310, Tokyo, Japan). The column contained octadecasilane silica (5 μm, 250 × 2 mm or 150 × 2 mm; Kromasil 5 C_{18} 100A, Phenomenex, Torrance, CA) and a guard column (Opti-Gard, 1 mm C_{18}; Optimize Technologies, Oregon City, OR). The RP–HPLC columns were eluted with methanol/water/acetic acid, 80:20:0.01, at 0.2 mL/min. The effluent first passed an ultraviolet detector (Kratos Spectroflow 757, Ramsey, NJ) equipped with an integrator (Merck Hitatchi D-2500, Tokyo, Japan) and then into an ion trap mass spectrometer (LCQ; Finnigan MAT, San Jose, CA), where it was subject to APCI or ESI. The vaporizer temperature for APCI was 450°C. The targets for automatic gain control were usually set at 5×10^7 for full MS and at 1×10^7 for MS^n. For ESI the capillary temperature was usually 230°C. Negative ions were monitored and prostaglandin $F_{1\alpha}$ (Upjohn, Kalamazoo, MI) was used for tuning. Compounds in methanol/water, 1:1, or in deuterated methanol/water were infused into the mass spectrometer with a syringe pump at a rate of 50–100 ng per min (10–20 μL/min).

GC–MS analysis. Capillary GC–MS was performed with an ion trap mass spectrometer (ITS 40, Finnigan) as described (23). Methyl ester, trimethylsilyl ether, and chlorohydrin derivatives were prepared as described (23,24).

RESULTS

General observation and nomenclature. APCI–MS resulted in fragmentation of the hydroperoxides (M) with three strong signals, $[M - 1]^-$, $[M - 19]^-$ and $[M - 17]^-$, which corresponded to the carboxylate anion of the hydroperoxide ($[M - H]^-$), a fragment due to loss of water from the carboxylate anion of the

hydroperoxide ($[M - H - H_2O]^-$), and the carboxylate anion of the corresponding alcohol ($[M - H - O]^-$). The reduction of the hydroperoxide to the alcohol might be due to the high temperature (450°C) of the APCI source. In contrast, ESI–MS usually showed only two of these signals, $[M - 1]^-$ and $[M - 19]^-$. For convenience, the carboxylate anion $[M - 1]^-$ is often designated A^-. This carboxylate anion was subject to MS^2 (or MS/MS) analysis, i.e., A^- was first isolated in the ion trap and then subjected to collision-induced fragmentation, and the full mass spectrum of the daughter ions was recorded. This procedure was designated MS^2 A → full-scan. By analogy, MS^3or (MS/MS/MS) A → B → full-scan designates isolation and collision-induced fragmentation of *m/z* A^-, isolation of one of its daughter ions *m/z* B^-, collision-induced fragmentation of *m/z* B^- and recording of a mass spectrum of the daughter ions of B^-. For hydroperoxides, MS^2 A → full-scan yielded mainly *m/z* $[A - 18]^-$, whereas MS^3 A → [A – 18] → full-scan yielded a mass spectrum usually with many intense ions. Some ions were formed by loss of CO_2 and/or water, whereas other fragments appeared to be formed by cleavage in the vicinity of the oxygenated carbon.

APCI or ESI of mono- and dihydroxy fatty acid metabolites yielded essentially similar results. MS^2 A → full-scan provided sufficient fragmentation for structural identification. All compounds showed loss of water, loss of CO_2, or a combination of the two. The metabolites with an 8-hydroxy group formed a typical ion (*m/z* 157) by α-cleavage of the C-8-C-9-bond with transfer of a proton to the unsaturated side, whereas cleavage of the C-8-C-7 bond was not noticed. 7,8-Dihydroxy metabolites were fragmented both between C-9 and C-8 and between the hydroxyl carbons (C-7 and C-8) with transfer of a proton to the unsaturated side. The bisallylic hydroxy metabolites formed two informative ions containing the carboxy group, which differed by 28 amu. They were apparently due to α-cleavage with proton transfer to the adjacent double bond, and both could be further fragmented by loss of carbon dioxide. Finally, MS^2 A → full-scan of microgram amounts of metabolites sometimes resulted in a mass spectrum with an intense negative ion at *m/z* [A + 1]. This might be due to too high a setting of the automatic gain control target causing "space charge," since reanalysis of smaller amounts of material yielded a mass spectrum without the negative ion at *m/z* [A + 1].

LDS and oleic acid. RP–HPLC and LC analysis of metabolites of 18:1n-9 formed by LDS is shown in Figure 1. The two major metabolites eluted at 9.5 and 10.5 min, respectively. GC–MS analysis of the first eluting compound, marked I, showed it to be 7*S*,8*S*-dihydroxy-9*Z*-octadecenoic acid (7,8-DiHOME). The second eluting metabolite (II) was identified as 8-hydroxy-9(10)epoxystearate (see below). Small amounts of a mixture of 8*R*-hydroperoxy-9*Z*-octadecenoic acid (8-HPOME) and 8*R*-hydroxy-9*Z*-octadecenoic acid (8-HOME) eluted after about 18 min (data not shown) as judged from ESI–MS and MS^2 analysis with signals at *m/z* 313 (100%) and 295 (313 – 18; about 5%) due to 8-HPOME, and *m/z* 297 (30%) due to 8-HOME.

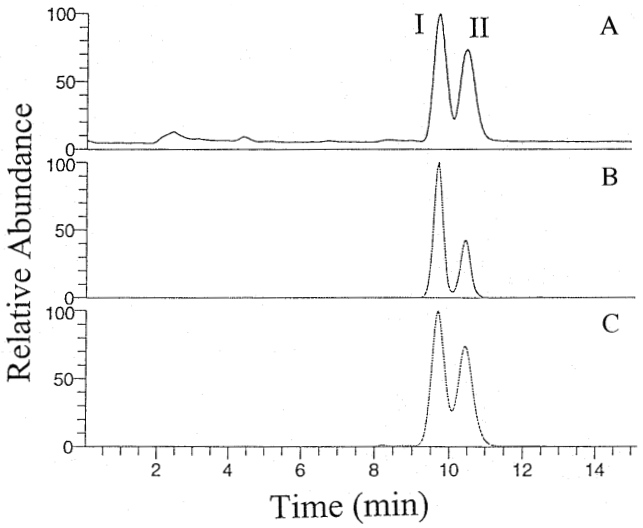


FIG. 1. Liquid chromatography–mass spectrometry (LC–MS) analysis of major metabolites formed by linoleate diol synthase (LDS) and oleic acid. Trace A shows total ion current (TIC), trace B, a mass chromatogram (*m/z* 313) of the carboxylate anion of 7*S*,8*S*-dihydroxy-9*Z*-octadecenoic acid (7,8-DiHOME), and trace C, TIC of MS^2 313 → full-scan. The peak with 7,8-DiHOME is marked I. The peak marked II was found to contain 8-hydroxy-9(10)epoxystearic acid. For explanation of MS^2 see text.

8R-HOME (M_w 298). MS^2 297 → full-scan yielded two strong signals, *m/z* 279 (297 – 18) and 157. The signal at *m/z* 157 is also present in the corresponding mass spectrum of 8-HODE, and it is likely due to $O{=}CH{-}(CH_2)_6{-}COO^-$, which might be formed by α-cleavage between C-8 and C-9 with transfer of a proton to the unsaturated fragment.

7S,8S-DiHOME (M_w 314). MS^2 313 → full-scan showed signals, *inter alia*, at *m/z* 295 (313 – 18), 277 (295 – 18), 251 (295 – 44, loss of CO_2), 233 (251 – 18), 197, 173 [possibly due to $CHO{-}CHOH{-}(CH_2)_5{-}COO^-$ formed by α-cleavage], 155 (173 – 18), 143 [possibly $OCH{-}(CH_2)_5{-}COO^-$], and 137 (155 – 18). This MS^2 spectrum is shown in Figure 2A. The structure of 7,8-DiHOME was confirmed by GC–MS (17).

8-Hydroxy-9(10)epoxystearic acid (M_w 314). MS of this metabolite, marked II (Fig. 1), showed a strong signal at *m/z* 313 (A^-), which suggested a molecular weight of 314. The carboxylate anion at *m/z* 313 shifted to 314 in deuterated methanol/water, which suggested that the metabolite contained one hydroxyl group and possibly an epoxy group. MS^2 313 → full-scan yielded *m/z* 295 (313 – 18), 277 (295 – 18), 187 [possibly $O{=}CH{-}CHOH{-}(CH_2)_6{-}COO^-$ formed by α-cleavage between C-9 and C-10], 171, 157 [possibly $O{=}CH{-}(CH_2)_6{-}COO^-$], and 141 as some of the most intense signals (Fig. 2B). In deuterated methanol/water, MS^2 314 → full-scan yielded signals at *m/z* 296 and 295, 278 and 277, and in the lower mass range *m/z* 188 [possibly $O{=}CH{-}CHO^2H{-}(CH_2)_6{-}COO^-$], 171, 157, 158, and 142. One deuterium atom was thus retained in the large carboxyl-group containing fragment (*m/z* 187) and to some extent in the small fragments (*m/z* 157 and 158).

GC–MS analysis of the trimethylsilyl ether methyl ester derivative yielded a mass spectrum at a C value of 20.5 with

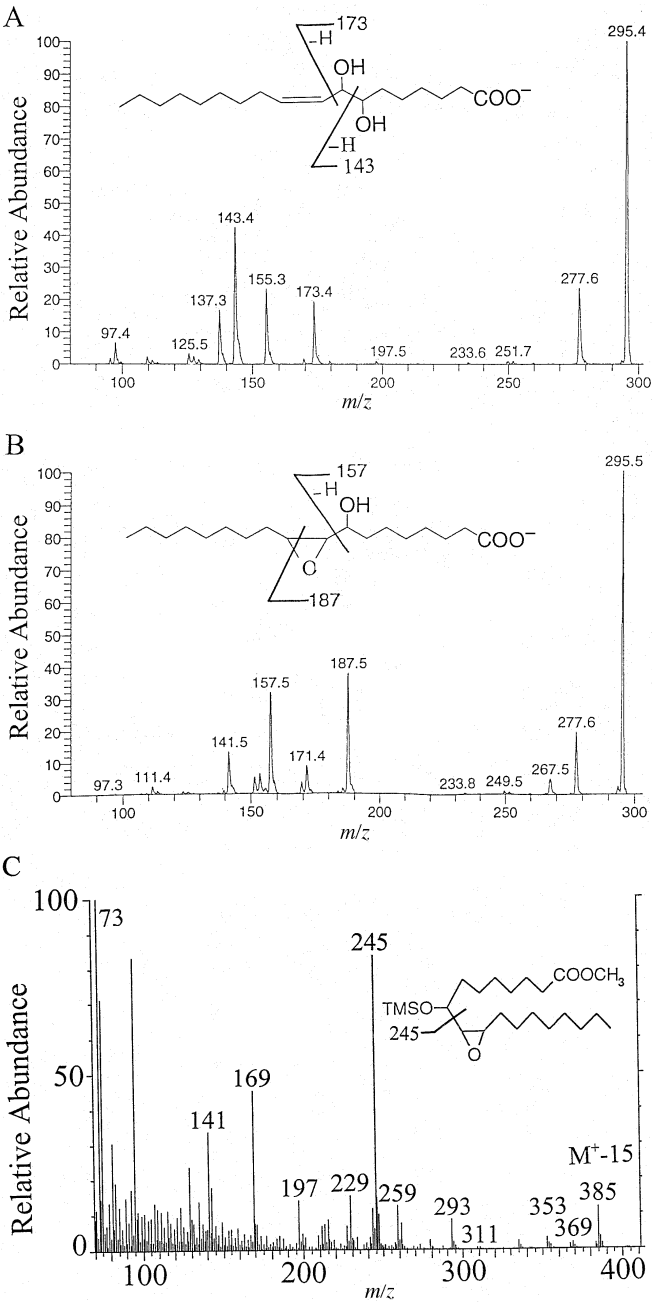


FIG. 2. LC–MS and gas chromatography–mass spectrometry (GC–MS) analysis of the two major LDS metabolites of oleic acid. (A) MS^2 analysis of the carboxylate anion (*m/z* 313) of 7,8-DiHOME (MS^2 313 → full-scan), which is marked I in Figure 1. (B) MS^2 analysis of metabolite II in Figure 1 (MS^2 313 → full-scan). The inset shows the proposed structure of metabolite II, 8-hydroxy-9(10)epoxystearate. (C) GC–MS analysis of the methyl ester trimethylsilyl ether (TMS) derivative of metabolite II with electron impact ionization. The inset shows this derivative of 8-hydroxy-9(10)epoxystearic acid. For other abbreviations see Figure 1.

signals, *inter alia*, at *m/z* 385 ($M^+ - 15$), 369 ($M^+ - 31$), 353, 311 [$M^+ - 89$, loss of $\cdot OSi(CH_3)_3$], 293 (311 – 18), 259, 245 {80%; possibly $[(CH_3)_3Si–O–CH_2–(CH_2)_6–COOCH_3]^+$}, 197, 169 (40%), and in the lower mass range at *m/z* 141, 129, 95, and 73 (base peak). The mass spectrum is shown in Figure 2C. Both the LC–MS and GC–MS analyses were thus consistent with an epoxy alcohol. For further confirmation, chlorohydrin adducts were prepared. The chlorohydrin and methyl ester trimethylsilyl ether derivative of this metabolite yielded two regioisomeric compounds (C value 22.4 and 22.6). Both spectra showed signals, *inter alia*, at *m/z* 245 $\{[(CH_3)_3Si–O–CH_2–(CH_2)_6–COOCH_3]^+\}$, and at 383 [$M^+ -$ 125, possibly loss of HCl and $\cdot OSi(CH_3)_3$], which were consistent with hydroxyl groups at C-8 and either C-9 or C-10 and chlorine at either C-10 or C-9. The LC–MS and GC–MS analysis of the metabolite and the formation of these chlorohydrins suggested that it was an epoxy alcohol, 8-hydroxy-9(10)epoxystearic acid.

LDS and linoleic acid. LDS metabolized linoleic acid to 7,8-DiHODE as the main metabolite and to only small amounts of 8-H(P)ODE as shown in Figure 3. An epoxy alcohol could not be detected. 8-HODE and 8-HPODE eluted

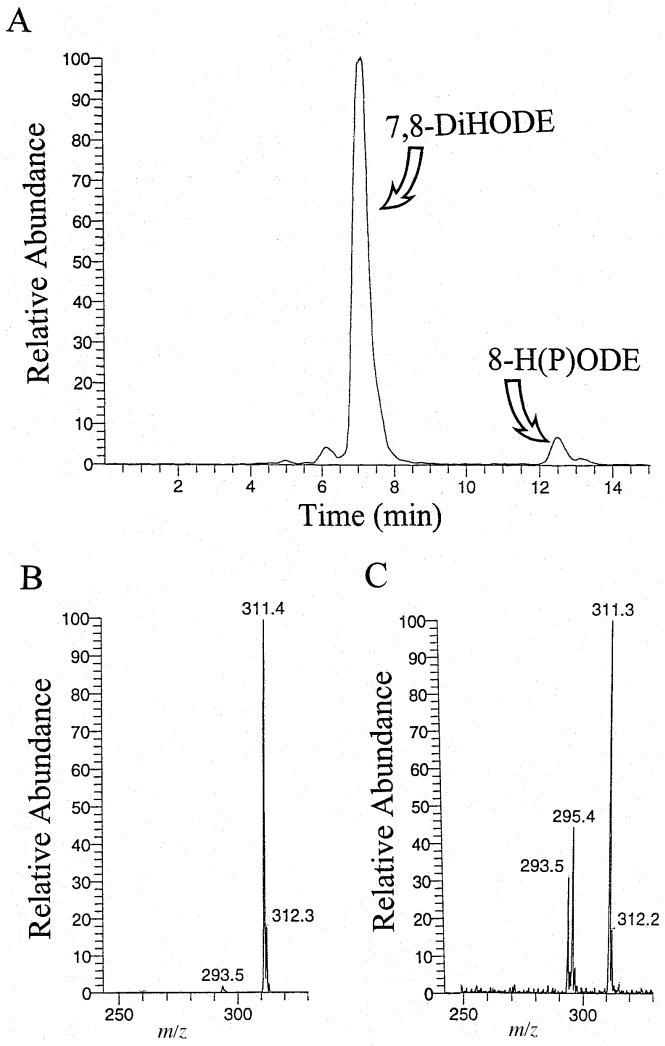


FIG. 3. LC–MS analysis of major metabolites formed by LDS and linoleic acid. (A) Chromatogram with TIC. The peaks containing 7*S*,8*S*-dihydroxy-9*Z*,12*Z*-octadeadienoic acid (7,8-DiHODE) and 8*R*-hydro(pero)xy-9*Z*,12*Z*-octadecadienoic acid [8-H(P)ODE] are marked in the figure. (B) Electrospray–MS (ESI–MS) analysis of the material in the peak marked 7,8-DiHODE. (C) ESI–MS analysis of the material in the peak marked 8-H(P)ODE. See Figure 1 for other abbreviations.

together on RP–HPLC. LC–MS analysis suggested that this peak contained 8-HPODE and 8-HODE in approximately a 3:1 ratio as judged from the intensities of *m/z* 311 and 293 (311 – 18), and of *m/z* 295, respectively (Fig. 3C). We confirmed that ESI–MS analysis of 8-HPODE did not generate a signal at *m/z* 295.

*8*R-*HPODE (M_w 312).* ESI–MS showed a strong signal at *m/z* 311 (A^-) and a weaker signal at *m/z* 293 (311 – 18). MS^2 311 → full-scan yielded *m/z* 293 (311 – 18) and 275 (293 – 18) as main signals. MS^3 311 → 293 → full-scan showed trivial signals due to losses of water, CO_2, and water plus CO_2. The MS^3 spectrum is shown in Figure 4A. Signals were also noted at *m/z* 223 [293 – 70, possibly loss of $CH_3(CH_2)_3CH$], 195 [293 – 98, possibly loss of $CH_3(CH_2)_4CH{=}CH_2$], 183 [293 – 110, possibly loss of $CH_3(CH_2)_4CH{=}CH_2{-}CH$], 171 [unidentified; isobaric with $CH_3{-}C(=O){-}(CH_2)_6{-}COO^-$], 163, 153 (171 – 18), 127 (171 – 44), and 111. Many of these fragments might conceivably be explained by collision-induced dehydration of 8-HPODE to 8-ketolinoleate (*m/z* 293) in the ion trap. MS^2 analysis of 8-ketolinoleate (MS^2 293 → full-scan) will be needed to confirm this. In deuterated methanol/water, ESI–MS showed signals at *m/z* 312 (311 + 1) and 293 (312 – 19, loss of ^2HHO), whereas MS^2 312 → full-scan yielded *m/z* 294 (312 – 18) and a weak signal at 275 (294 – 19), indicating different fragmentation mechanisms in MS and in MS^2.

*8*R-*HODE (M_w 296).* APCI–MS of 8-HODE showed *m/z* 295 (A^-) and weak signals at *m/z* 277 (295 – 18) and 157 [possibly $O{=}CH{-}(CH_2)_6{-}COO^-$], whereas ESI–MS mainly showed signals at *m/z* 295 and 157. MS^2 295 → full-scan yielded strong signals at *m/z* 277, 157, and weak signals at 251 (295 – 44), 182, and 139 (157 – 18). The mass spectrum is shown in Figure 4B.

In deuterated methanol/water, MS^2 296 → full-scan yielded *m/z* 157 and 277 as the main signals, and a weak signal at 252 (296 – 44). These results suggest that deuterium of the hydroxy group was not retained in the fragment *m/z* 157 [*cf.* 8-hydroxy-9(10)epoxystearate above].

7S,8S-DiHODE (M_w 312). The mass spectrum was dominated by *m/z* 311 (A^-), but a signal was also noted at *m/z* 293 (311 – 18). MS^2 311 → full-scan yielded signals at *m/z* 293 (311 – 18), 275 (293 – 18), 173 [possibly due to $OCH{-}CHOH{-}(CH_2)_5{-}COO^-$], 155 (possibly 173 – 18), 143 [possibly $OCH{-}(CH_2)_5{-}COO^-$], and 137 (155 – 18). This MS^2 spectrum is shown in Figure 4C. MS^3 311 → 293 full-scan showed signals, *inter alia*, at *m/z* 275 (293 – 18), 257 (275 – 18; possibly due to loss of water from the carboxyl group), 249 (293 – 44), 177, and 143.

In deuterated methanol/water, MS of 7,8-DiHODE showed signals as expected from a compound with two hydroxyl groups at *m/z* 313, 294 (313 – 19), and 275 (294 – 19). MS^2 313 → full-scan yielded *m/z* 294, 276 (294 – 18), 275 (294 – 19), 174 [possibly $OCH{-}CHO^2H{-}(CH_2)_5{-}COO^-$], and 156 (174 – 18). The intensities of *m/z* 276 and 275 appeared to vary with the collision energy. MS^3 313 → 294 → full-scan yielded *m/z* 276 (294 – 18), 258 (276 – 18), 250 (294 – 44), 177, and 143.

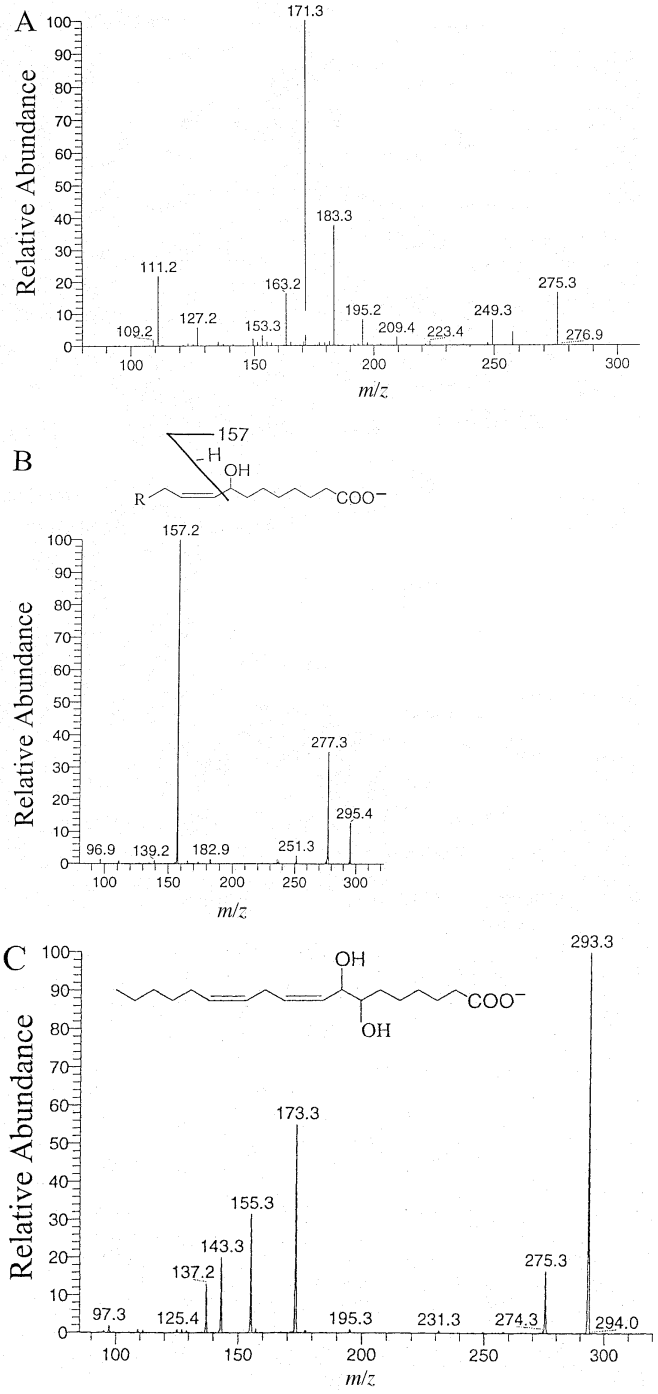


FIG. 4. MS^n of the carboxylate anions of metabolites of LDS and linoleic acid. (A) MS^3 analysis of the carboxylate anion (*m/z* 311) of 8-HPODE (MS^3 311 → 293 → full-scan). (B) MS^2 analysis of the carboxylate anion (*m/z* 295) of 8-HODE (MS^2 295 → full-scan). The inset shows the structural formula of 8-HODE where R designates $CH{=}CH{-}(CH_2)_4CH_3$. (C) MS^2 analysis of the carboxylate anion (*m/z* 311) of 7,8-DiHODE (MS^2 311 → full-scan). For abbreviations see Figures 1 and 3.

LDS and α-linolenic acid. LDS metabolized 18:3n-3 to one major metabolite, 7*S*,8*S*-dihydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid (7,8-DiHOTrE), so efficiently that its pre-

cursor [8*R*-hydroperoxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid (8-HPOTrE)] could not be detected.

7S,8S-DiHOTrE (M_w 310). MS^2 309 → full-scan showed signals at *m/z* 291 (309 – 18), 273 (291 – 18), 247 (291 – 44), 173 [possibly OCH–CHOH–$(CH_2)_5$–COO^-], 155 (possibly 173 – 18), 143 [possibly OCH–$(CH_2)_5$–COO^-], and 137 (possibly 155 – 18). The MS^2 spectra of 7,8-DiHOME, 7,8-DiHODE, and 7,8-DiHOTrE differed mainly in relative intensities of the main ions in the lower mass range.

Mn-LO. Like many other lipoxygenases, Mn-LO will oxygenate 18:2n-6, 18:3n-3, and 18:3n-6 but not 18:1n-9 (Table 1). Significant amounts of corresponding alcohols could not be detected. Mn-LO can convert the 11*S*-hydroperoxy fatty acids to 13*R*-hydroperoxy fatty acids (22). We will focus on the 11-hydroperoxy metabolites of Mn-LO, since 13*S*-hydroperoxy fatty acid metabolites are well-known products of soybean lipoxygenases and have been studied by MS^2 (8,9,12).

Mn-LO and linoleic acid. The two metabolites, 11*S*-HPODE and 13*R*-HPODE, which are formed from 18:2n-6 by Mn-LO, were separated by RP–HPLC as shown by the inset in Figure 5A. As judged from the total ion current, 11*S*-HPODE and 13*R*-HPODE were formed in a ratio of ~1:3.

11S-HPODE (M_w 312). APCI–MS showed signals at *m/z* 311 (A^-), 295 (possibly due to reduction of 11-HPODE to 11-HODE in the APCI source) and 293 (311 – 18), whereas the corresponding analysis with ESI–MS yielded 311 (base peak) and a strong signal at 293 (311 – 18; Fig. 5A). MS^2 311 → full-scan yielded *m/z* 293 (311 – 18) as the main ion and signals at 267 (311 – 44), 197, and 183 (unidentified) (Fig. 5B). MS^3 311 → 293 → full-scan yielded several fragments as shown in Figure 5C with signals, *inter alia*, at *m/z* 275 (293 – 18), 249 (293 – 44), 233 (293 – 44 – 18), 195 (293 – 98, possibly loss of C12–C18 and a hydrogen), 177 (possibly 195 – 18), 167, 153, 151 (possibly 195 – 44), 141, and 123 (possibly 167 – 44). A few fragments might be visualized as derived from the carboxylate side chain of an 11-keto-linoleic acid intermediate (*m/z* 293) and formed by α-cleavage, e.g., *m/z* 195 by cleavage between C-11 and C-12 with transfer of a proton to the unsaturated C-12. The ion at *m/z* 167 might conceivably be formed by cleavage between C-11 and C-10 with transfer of a proton to C-11.

11S-HODE (M_w 296). APCI–MS showed signals at *m/z* 295 (A^-) and 277 (295 – 18), whereas the latter ion was weak in ESI–MS. MS^2 295 → full-scan yielded many fragments (Fig. 5D). In addition to *m/z* 277 (295 – 18) and 251

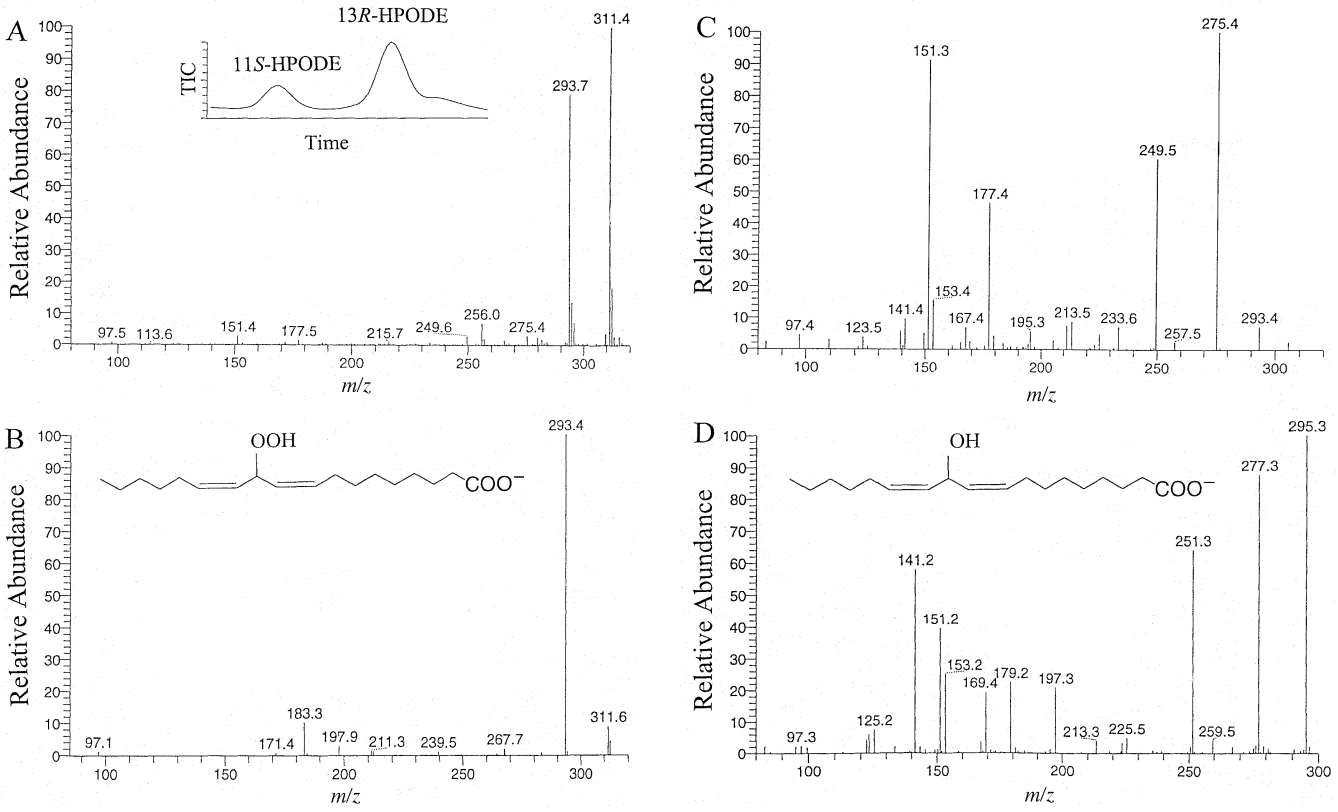


FIG. 5. LC–MS analysis of 11*S*-hydroperoxy-9*Z*,12*Z*-octadecadienoic acid (11*S*-HPODE) and formed by manganese lipoxygenase (Mn-LO). (A) MS of 11-HPODE. The inset shows separation of 11-HPODE and 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HPODE) by reversed-phase–high-performance liquid chromatography–mass spectrometry. (B) MS^2 analysis of the carboxylate anion (*m/z* 311) of 11-HPODE (MS^2 311 → full-scan). (C) MS^3 analysis of the carboxylate anion (*m/z* 311) of 11-HPODE (MS^3 311 → 293 → full-scan). (D) MS^2 analysis of the carboxylate anion (*m/z* 293) of 11*S*-hydroxy-9*Z*,12*Z*-octadecadienoic acid (11-HODE) (MS^2 293 → full-scan) with a low collision energy (24%). For other abbreviation see Figure 1.

(295 – 44), signals were noted at 197 [A^- – 98, possibly OCH–CH=CH–$(CH_2)_7COO^-$], 195 (weak, collision energy-dependent signal), 179 (possibly 197 – 18), 171, 153 (possibly 197 – 44), 151, and 141 [unidentified; this signal was also noted in the corresponding mass spectrum of 11-HOTrE(n-6)]. In deuterated methanol/water, ESI–MS of [$^2H_2$]11-HODE yielded *m/z* 296, 252 (296 – 44), and 233 (252 – 19). MS^2 296 → full-scan yielded *m/z* 278 (296 – 18) with a variable and less intense signal at 277 (296 – 19), 252 (296 – 44), 197, 198, 180, 170, 141, and 142.

*13*R-*HPODE (M_w 312).* APCI–MS yielded three strong signals [*m/z* 311 (A^-), 295 and 293], whereas ESI–MS mainly showed the signals at *m/z* 311 and 293. MS^2 311 → full-scan yielded *m/z* 293 (100%), but also intense signals at 267 (311 – 44), 195, and 171. MS^3 311 → 293 → full-scan yielded several strong signals, e.g., *m/z* 275 (293 – 18), 249 (293 – 44), 195, 179, 177, 141, and, in the lower mass range, 113. When 13-HPODE was dissolved in deuterated methanol/water, the MS signal with ESI was obtained at *m/z* 312, as expected, and MS^2 312 → full-scan yielded a signal at *m/z* 293 (loss of ^{2}HHO). ESI–MS^2 spectra of linoleic acid hydroperoxides formed by soybean lipoxygenase (presumably mainly 13*S*-HPODE) has been published (9), and our analysis of 13*R*-HPODE with MS^2 311 → full-scan and MS^3 311 → 293 → full-scan yielded many identical fragments also in the lower mass range.

*13*R-*HODE (M_w 296).* The mass spectrum of 13-HODE was similar to that reported with low-energy fast bombardment tandem MS (8). For a comparison, MS^2 295 (A^-) → full-scan yielded signals, *inter alia*, at *m/z* 277 (A^- – 18), 195 (A^- – 100, possibly due to loss of O=CH–C_5H_{11}), and 179. In deuterated methanol/water, MS^2 296 → full-scan yielded *m/z* 277, 196, 180, and 179. The signal at *m/z* 196 indicated a proton transfer from the hydroxy substituent as reported by Wheelan *et al.* (8).

Mn-LO and α-linolenic acid. The increase in light absorption at 235 nm during incubation of 0.25 mM 18:3n-3 with Mn-LO (0.5 μg) at room temperature is shown in Figure 6A. LC–MS analysis after 1 and 4 min incubation indicated that 11-HPOTrE and 13-HPOTrE were formed in a ratio of ~1:2. 11-Hydroperoxy metabolites will be converted to 13-hydroperoxy metabolites by Mn-LO, and it is therefore essential to have excess of substrate present in order to trap the 11-hydroperoxy intermediate (22).

*11*S-*HPOTrE (M_w 310).* MS^2 309 → full-scan yielded *m/z* 291 (309 – 18; 100%). MS^3 309 → 291 → full-scan yielded many fragments as shown in Figure 6B, e.g., *m/z* 273 (291 – 18), 247 (291 – 44), 211, 203, 195, 165, 177 (195 – 18), 165, 151, 149, and 139.

*11*S-*HOTrE (M_w 294).* MS^2 293 → full-scan yielded strong signals at *m/z* 275 (293 – 18), 249 (293 – 44), 213, 197 [base peak; likely OCH–CH=CH–$(CH_2)_7$–COO^-], 177, and 151 (169 – 18) as shown in Figure 6C. In addition, signals were noted at *m/z* 179 (possibly 197 – 18), 169, 167, and 153 (possibly 197 – 44). Many signals in the lower mass range were also present in the corresponding mass spectrum of 11*S*-HODE.

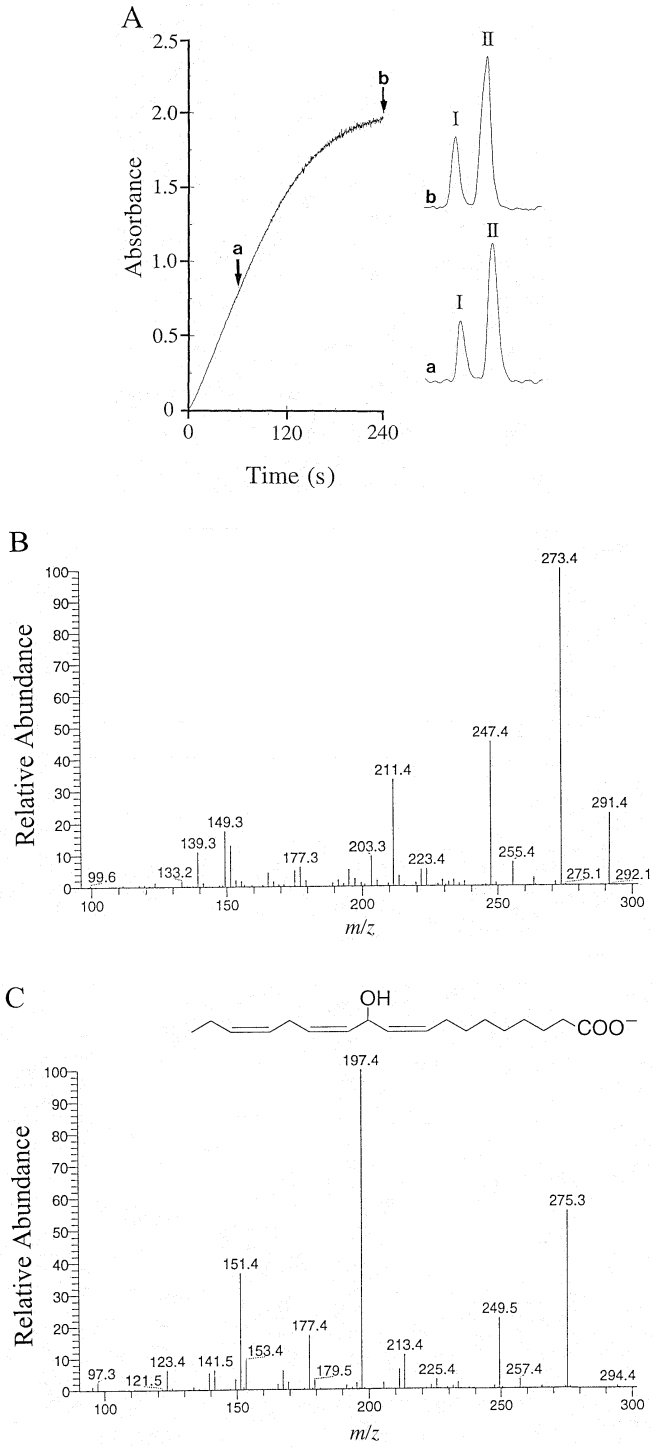


FIG. 6. Metabolism of α-linolenic acid by Mn-LO. (A) Increase in light absorption at 235 nm due to formation of 13-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-HPOTrE) during incubation of Mn-LO and 18:3n-3. The insets show the TIC from LC–MS analysis of 11*Z*-hydroperoxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid (11-HPOTrE) and 13-HPOTrE formed after 1 and 4 min of incubation (marked a and b). Peak I contains 11-HPOTrE and peak II 13-HPOTrE. (B) MS^3 analysis of the carboxylate anion (*m/z* 309) of 11-*S*-HPOTrE (MS^3 309 → 291 → full-scan). (C) MS^2 analysis of the carboxylate anion (*m/z* 293) of 11-hydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid (MS^2 293 → full-scan). For other abbreviations see Figures 1 and 5.

*13*R-*HPOTrE (M_w 310).* APCI–MS yielded *m/z* 309 (A^-), 293, and 291 (309 – 18). The signal at *m/z* 293 was not noted during ESI–MS. MS^2 309 → full-scan yielded mainly *m/z* 291 (309 – 18), and many weak signals, e.g., 273, 247, 209, and 197, which were in agreement with a MS^2 analysis of 13*S*-HOTrE (9). MS^3 309 → 291 → full-scan yielded strong signals at *m/z* 273 (291 – 18), 262, 247 (291 – 44), 221, and 177.

Mn-LO and γ-linolenic acid. The two Mn-LO metabolites of 18:3n-6 were separated by RP–HPLC and analyzed by LC–MS with ESI. The total ion current indicated that 11-HPOTrE(n-6) and 13-HPOTrE(n-6) were formed in ratio of ~1:1.

*11*S-*HPOTrE(n-6) (M_w 310).* MS^2 309 → full-scan yielded *m/z* 291 (309 – 18; 100%). MS^3 309 → 291 → full-scan yielded many fragments, e.g., *m/z* 273 (291 – 18), 247 (291 – 44), 229 (291 – 44 – 18), 225, 175, 170, 149, 139, and 125. A comparison with the corresponding mass spectrum of 11-HPODE shows that some nontrivial fragments of 11*S*-HPOTrE(n-6) were decreased by 2 amu, e.g., *m/z* 175 and *m/z* 149.

*11*S-*HOTrE(n-6) (M_w 294).* MS^2 293 → full-scan yielded *m/z* 275 (293 – 18), 249 (293 – 44), 231 (293 – 44 – 18), 195 (weak), 177 (possibly 295 – 18), 167, 151 (possibly 195 – 44), 141, and 123 (possibly 167 – 44). The presence of *m/z* 177 and 167 in the MS^2 spectrum of 11-HOTrE(n-6) and *m/z* 179 and 169 in the corresponding mass spectrum of 11-HODE suggests that these fragment pairs contain the carboxylate side chains and are formed by α-cleavage as discussed above.

*13*R-*HPOTrE(n-6) (M_w 310).* MS^2 309 → full-scan yielded *m/z* 291. MS^3 309 → 291 → full-scan yielded signals at *m/z* 273 (291 – 18), 247 (291 – 44), 193, 177, 165, and in the lower mass range 123 and 113.

*13*R-*HOTrE(n-6) (M_w 294).* MS^2 293 → full-scan showed a strong signal at *m/z* 275 (293 – 18) and a weaker signal at 193 [293 – 100, possibly α-cleavage with proton transfer to the unsaturated side chain (9)]. This MS^2 spectrum was similar to that reported for 13*S*-HOTrE(n-6) (9).

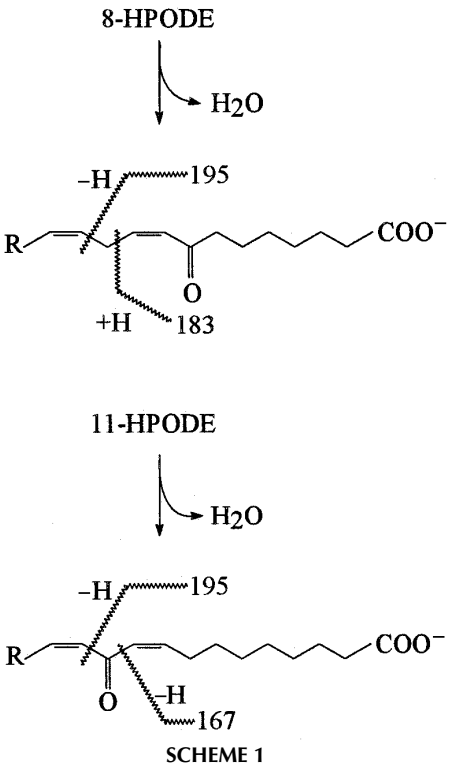
DISCUSSION

The two most well-characterized fatty acid dioxygenases are lipoxygenases of plants and animals and PGH synthases of mammals (1,2). The fungus *G. graminis* contains two unique dioxygenases, which seem to be related to lipoxygenases and to PGH synthases, respectively. Mn-LO oxidizes linoleic, α-linolenic, and γ-linolenic acids at C-11 and at C-13, whereas LDS oxidizes oleic, linoleic, and α-linolenic acids at C-8 to hydroperoxy metabolites, which then can be isomerized by LDS to 7,8-dihydroxy metabolites (Table 1). We report here the LC–MS analysis of these oxylipins and some new observations on the two enzymes.

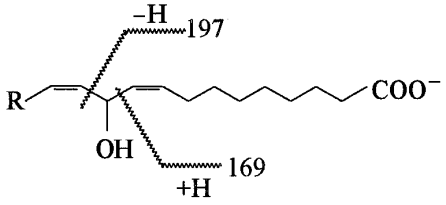
Mn-LO formed hydroperoxides with virtually no formation of the corresponding alcohols. LDS appeared to form only small amounts of 8-hydroxy fatty acids in comparison with 8-hydroperoxy and 7,8-dihydroxy fatty acids. LDS contains heme (16), which might reduce hydroperoxides to alcohols, and formation of significant amounts of 8-hydroxy fatty acids was therefore not unexpected.

LC–MS analysis of the fatty acid hydroperoxides formed by purified Mn-LO and by purified LDS allowed structural identification. MS yielded the carboxylate anion, and MS^2 of the carboxylate anion showed mainly loss of water. However, MS^3 analysis (MS^3 A → [A – 18] → full-scan) yielded an extensive fragmentation, which could be used to identify the 8-, 11-, and 13-hydroperoxy metabolites. For example, 8-HPODE showed characteristic ions with high intensity at *m/z* 183 and 171, 11-HPODE at *m/z* 177 (195 – 18) and 151 (195 – 44), and 13-HPODE at *m/z* 179 and 113. The mechanism of the collision-induced fragmentation of hydroperoxides during MS^3 analysis (MS^3 A → [A – 18] → full-scan) remains to be determined. The structure of the dehydrated hydroperoxide anion (*m/z* $[A - 18]^-$), which is isolated in the ion trap, is unknown, but it might be the anion of the corresponding keto compound. It will therefore be of interest to perform MS^2 analysis (MS^2 A → full-scan) of 8- and 11-ketolinoleate to determine similarities in fragmentation with the MS^3 fragmentation of 11-HPODE and 8-HPODE indicated below [Scheme 1; R designates $CH_3(CH_2)_4$].

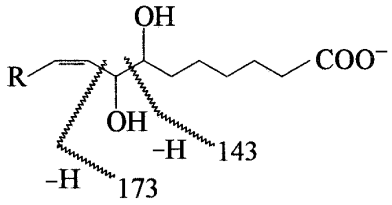
APCI and ESI yielded similar results with the hydroxy and dihydroxy fatty acids, but the hydroperoxides were partly reduced to alcohols during APCI. ESI is a milder ionization technique, and we used ESI for analysis of hydroperoxides. Mn-LO converted 18:2n-6, 18:3n-3, and 18:3n-6 to 11-hydroperoxy and 13-hydroperoxy metabolites. 18:3n-3 is metabolized twice as fast as 18:2n-6, and much faster than



SCHEME 1



SCHEME 2



SCHEME 4

18:3n-6 (16). When the fatty acid substrates are consumed, 11-hydroperoxy metabolites are converted to 13-hydroperoxy metabolites by Mn-LO (22). Under conditions with substrate excess, we found that Mn-LO converted 18:2n-6, 18:3n-3, and 18:3n-6 to 11-hydroperoxy and 13-hydroperoxy fatty acid metabolites in relative amounts (as judged from ion intensities), which ranged from almost 1:1 (18:3n-6) to 1:3 (18:2n-6) as judged from LC–MS analysis.

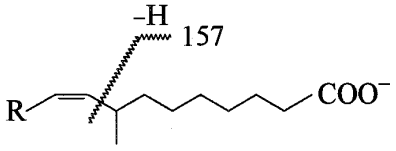
We also performed LC–MS analysis of the corresponding 8-, 11-, and 13-hydroxy fatty acids. MS^2 analysis (MS^2 A $\rightarrow$ full-scan) yielded characteristic mass spectra, and the structure of many nontrival fragments could be deduced. 13-HODE showed strong signals at *m/z* 195 and 179, whereas 11-HODE showed characteristic ions, *inter alia*, at *m/z* 179 and 169. These ions might be formed by cleavage at either side of C-11 (Scheme 2).

All the 8-hydroxy fatty acids yielded one fragment (*m/z* 157) due to α-cleavage between C-8 and C-9 and transfer of a proton to the unsaturated side chain (Scheme 3).

The 7,8-diols also formed fragments based on (-cleavage between C-8 and C-9 with proton transfer to C-9, e.g., *m/z* 173. Cleavage also occurred between the hydroxyl groups (*m/z* 143) as illustrated by Scheme 4. The analysis of [$^2H_3$]-derivatives supported this fragmentation.

A new finding was that oleic acid was oxidized by LDS to an epoxy alcohol along with 7,8-dihydroxyoleic acid as major metabolites. Recent work on the catalytic mechanism of LDS suggests many similarities with PGH synthases. LDS and PGH synthases will form two ferryl oxygen intermediates and a tyrosyl radical during catalysis as judged from stopped flow experiments and electron paramagnetic resonance spectrometry (2,21). One of these ferryl oxygen intermediates can apparently catalyze two reactions with oleic acid as a substrate, *viz*., hydroxylation at C-7 of 8-HOME to 7,8-dihydroxyoleic acid or epoxidation of the double bond of 8-HOME to 8-hydroxy-9(10)epoxystearic acid. We could not detect epoxidation of any other fatty acids by LDS.

In summary, LC–MS with MS^n was found to be convenient for studies on the metabolism of fatty acids by Mn-LO and LDS. This method may also be valuable for analysis of related metabolites in other biological systems. Biosynthesis of 8-HODE has also been reported by *Laetisaria arvalis*, *Aspergillus nidulans,* and *Leptomitus lacteus* (25–27) and by rat liver micromes (23). 11-HODE and bisallylic HETE are formed from linoleic and arachidonic acids by rat and human hepatic microsomes and by recombinant human cytochromes P450 (23,28–30). LC–ESI–MS with MS^2 and MS^3 options might be useful for analysis of hydroxy and hydroperoxy fatty acids and structurally related metabolites from many different sources.



SCHEME 3

ACKNOWLEDGMENT

Supported by the Swedish Medical Research Council (6523).

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[Received April 24, 1998, and in final revised form August 4, 1998; revision accepted August 12, 1998]

Characterization of Phospholipase D Activity in Bovine Photoreceptor Membranes

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ABSTRACT: Phospholipase D (E.C. 3.1.4.4.) was detected in isolated bovine rod outer segments (ROS) and its properties determined. The enzyme activity was assayed using either a sonicated microdispersion of 1,2-diacyl-*sn*-[2^{3}H]glycerol-3-phosphocholine (PC), or [^{14}C]ethanol. Using [^{3}H]PC and ethanol as a substrate, we were able to detect the hydrolytic properties as well as the transphosphatidylation reaction catalyzed by phospholipase D (PLD): formation of [^{3}H]phosphatidic acid and phosphatidylethanol [^{3}H]PtdEt; whereas with [^{14}C]ethanol or [^{3}H]glycerol in the absence of exogenous PC, only transphosphatidylation reactions were detected (formation of [^{14}C]PtdEt or [^{3}H]phosphatidylglycerol, respectively). The use of varying concentrations of [^{3}H]PC and 400 mM of ethanol gave an apparent K_m value for PC of 0.51 mM and a V_{max} value of 111 nmol × h^{-1} × (mg protein)$^{-1}$. The activity was linear up to 60 min of incubation and up to 0.2 mg of protein. The optimal ethanol concentration was determined to be 400 mM, with an apparent K_m of 202 mM and a V_{max} value for ethanol of 125 nmol × h^{-1} × (mg protein)$^{-1}$. A clear pH optimum was observed around 7. PLD activity was increased in the presence of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate or sodium deoxycholate and inhibited with Triton X-100. The enzyme activity was also activated in the presence of Ca^{2+} or Mg^{2+} (1 mM) although these ions were not required for measuring PLD activity. The high specific activity of PLD found in purified ROS compared to the activity found in other subcellular fractions of the bovine retina suggests that this enzymatic activity is native to ROS. The present report is the first evidence of PLD activity associated with photoreceptor ROS.
Lipids 33, 853–860 (1998).

The retinal rod photoreceptor is a highly specialized structure in visual transduction. The outer segment of this cell undergoes dynamical renewal and shedding of the disc membranes (1). While new discs are added at the base of the rod outer segments (ROS), old discs at the apical tip are phagocytized by the retinal pigment epithelium (2,3). The metabolism of lipids in ROS is quite distinct from that of protein, especially when considering their turnover rates. ROS phospholipids are renewed by membrane as well as by molecular replacement (3). This molecular replacement could involve the exchange of entire molecules mediated by exchange proteins from outside the ROS (4), and molecular retailoring such as fatty acid exchange and modification of base groups (5).

A special feature of ROS membranes is their particular lipid composition, very rich in long- and very long chain polyunsaturated fatty acids in their phospholipids (6,7). The metabolism of these ROS phospholipids and their role in the photoreceptor physiology have not yet been clearly defined.

Several enzymatic activities involved in lipid metabolism and molecular retailoring of phospholipids, such as phosphatidate phosphohydrolase (PAPase) (8), phospholipase A_2 (9–13), acyltransferase (14), phospholipase C (15,16), diacylglycerol (DG) kinase and phosphoinositide kinases (17,18) and phosphatidylethanolamine *N*-methyltransferase (19), have been described in ROS.

In view of the dynamic role of phospholipids in membrane function, we decided to study the properties of enzymatic modification to their polar portion in ROS. We speculate that the base exchange enzymes and phospholipase D (PLD) could be responsible for the biological transformation of protein microenvironments, probably regulating membrane function by modification of the polar head group of phospholipids (20).

PLD is the enzyme responsible for the hydrolysis of phosphatidylcholine (PC) which leads to the generation of choline and phosphatidic acid (PA). PA can be further hydrolyzed by phospholipase A_2 to form the proposed second messenger lysophosphatidic acid, or by PAPase to form DG. Both enzymes responsible for the hydrolysis of PLD products have been extensively characterized in ROS (8–13). In the last few years PLD has been implicated in novel signal transduction pathways, as described in eukaryotic cells, and, moreover, PLD is regulated by small G proteins (21,22), protein kinase(s) (23,24), and PLD activation has also been implicated in intracellular traffic of membranes (25,26).

This paper demonstrates the first evidence that PLD activity is present in isolated ROS membranes and determines kinetic parameters of the enzyme as well as other aspects of transphosphatidylation reactions in ROS.

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Abbreviations: CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; DG, diacylglycerol; NaDOC, sodium deoxycholate; PA, phosphatidic acid; PAPase, phosphatidate phosphohydrolase; PC, phosphatidylcholine; PG, phosphatidylglycerol; PLD, phospholipase D; PtdEt, phosphatidylethanol; ROS, rod outer segments; Triton X-100, *t*-octylphenoxypoly ethoxyethanol.

MATERIALS AND METHODS

[2-^{3}H]Glycerol (14.5 Ci/mmol) and [^{14}C]ethanol (4.5 Ci/mol) were supplied by New England Nuclear DuPont (Boston, MA). Phosphatidylethanol (PtdEt) standard was purchased from ICN Biomedicals (Costa Mesa, CA). PLD from cabbage and all other chemicals not specifically mentioned were obtained from Sigma Chemicals (St. Louis, MO).

Isolation of purified ROS. Fresh bovine eyes were obtained from a local abattoir (Trans Link, General Cerri, Argentina) and stored in crushed ice within 10 min of the animal's death. Bovine eyes were dark-adapted for 1–2 h. All subsequent procedures were conducted under dim red light and carried out at 2–4°C. The preparation containing crude ROS was then purified by discontinuous density gradient centrifugation as described by Kühn (27). This procedure gave ROS (band I) retained at the 0.84/1.00 M density interface, broken ROS contaminated with mitochondria and rod inner segments (band II), retained at the 1:00/1.14 M density interface, and a pellet composed of non-ROS membranes. The purity of ROS membrane preparation (band I) was controlled by the ratio of absorbance at 278 and 500 nm after solubilization in a 70 mM potassium phosphate buffer (pH 7) containing 1% of Emulphogene. Values of 2.3 ± 0.2 were typically obtained for this ratio. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28) was also used to check the purity of the membranes. Even in heavily overloaded gels, rhodopsin consituted 85% of photoreceptor membrane proteins. Thin-layer chromatography of photoreceptor membrane lipids in overloaded plates showed no cardiolipin, suggesting nondetectable contamination with mitochondria. On the other hand, an enrichment in long- and very long chain polyunsaturated fatty acid esterified to dipolyunsaturated molecular species in PC was observed as a characteristic in bovine ROS (6,7). Purity of populations of ROS in particular batches was determined by assaying marker enzyme activities in all fractions of the gradient, as previously described by Roque and Giusto (19). NADPH-cytochrome c reductase (microsomal marker) and cytochrome c oxidase activities were measured in band I, band II, and the pellet of the gradient. The mitochondrial marker enzyme was enriched in band II and pellet, whereas cytochrome c reductase was very low in band I and presented the highest activity in the pellet. These results led us to confirm that band I (purified ROS) was only slightly contaminated by either microsomes or mitochondria (less than 5%).

Preparation of other subcellular fractions. The crude mitochondrial and microsomal fractions were obtained from the remainder of the retinas, which were homogenized in a Potter-Elvehjem homogenizer with 0.32 M sucrose in 50 mM Tris-HCL (pH 7.4) with 1 mM EDTA to a final volume of 10%. Homogenization was performed by 10 up-and-down strokes by means of a motor-driven Teflon pestle. The homogenates were centrifuged at 1,000 × *g* for 15 min and resuspended to produce the crude nuclear pellet. The pooled supernatants were then spun at 11,500 × *g* for 20 min and washed twice to produce a crude mitochondrial pellet. The supernatants were centrifuged at 130,000 × *g* for 45 min to produce microsomes as a pellet and the cytosol as the supernatants.

Preparation of radioactive substrate. The [^{3}H]PC was obtained from bovine retinas. These were incubated for 2 h at 37°C with 10 μCi/retina of [2-^{3}H]glycerol (14.5 Ci/mmol) as previously described (29). Lipids were extracted from the tissue according to Folch *et al.* (30). [^{3}H]PC was isolated by one-dimensional thin-layer chromatography on silica gel H using chloroform/methanol/water (65:25:4, by vol) and eluted therefrom according to Arvidson (31). To assess the purity of [^{3}H]PC, we rechromatographed it by two-dimensional thin-layer chromatography according to the method of Rouser *et al.* (32). Radioactive [^{3}H]PC had a specific activity of 0.1–0.2 Ci/ mol.

Preparation of PtdEt standard. PtdEt standard was prepared from egg lecithin with cabbage PLD, according to a published procedure, with slight modifications (33). The reaction mixture contained 1.3 mM egg PC, 0.1 M sodium acetate buffer, (pH 5.6), 38 mM $CaCl_2$, 0.5 mM sodium dodecyl sulfate, 3 M ethanol, and 300 μg of cabbage PLD, in a total volume of 1 mL. After 2 h of incubation at 30°C, the reaction was stopped with chloroform/methanol (2:1, vol/vol), and the lipids were extracted and processed according to Folch *et al.* (30). Extract was evaporated to dryness under nitrogen, the residue dissolved with chloroform and then applied to a column of 1 g of heat-activated silicic acid. Lipids were eluted with 40 mL of chloroform/methanol (97:3 vol/vol), chloroform/methanol (47:3 vol/vol), chloroform/methanol (22:3 vol/vol), and chloroform/methanol (2:1 vol/vol). PtdEt was present in the following eluates: chloroform/methanol (47:3 vol/vol) and chloroform/methanol (22:3 vol/vol). Our standard preparation showed the same R_f as PtdEt standard from ICN Biomedicals.

Enzyme assay. ROS PLD activity was determined by measuring the formation of [^{3}H]PtdEt and [^{3}H]PA from [^{3}H]PC. [^{3}H]PC was dried under a stream of nitrogen and resuspended in assay buffer; this aqueous microdispersion was sonicated until the solution became clear. The standard incubation mixture contained: 1 mM [^{3}H]PC (0.15 Ci/mol), added as previously described, 40 mM HEPES (pH 6.8), 25 mM KF, 400 mM ethanol, and 200 μg of ROS membrane protein in a total volume of 250 μL. The assay mixture was sonicated, in a sonication bath, for 30 s prior to incubation. The assays were carried out for 60 min at 37°C. Reactions were terminated by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). The lipids were then extracted according to Folch *et al.* (30) and were washed using an upper phase containing 0.1 M sulfuric acid (34). Blanks were prepared identically, except that chloroform/methanol was added to the membranes before adding the substrate. Alternatively, blanks were prepared by using membranes boiled for 5 min and incubated as described above. Both blank preparations yielded similar values of activity (less than 2% of the activity observed in the experimental samples); hence, the first preparation was used on a regular basis.

Transphosphatidylation reactions in ROS were studied by the production of [^{14}C]PtdEt or [^{3}H]phosphatidylglycerol (PG)

using [^{14}C]ethanol or [^{3}H]glycerol as substrate, respectively. The transphosphatidylation assay contained 5 μCi of [^{14}C]ethanol (4.5 Ci/mol) or 5 μCi [^{3}H]glycerol (14.5 Ci/mol) in 40 mM HEPES (pH 6.8), 25 mM KF, 2 mM sodium oleate, and 200 μg of ROS membrane protein in a total volume of 250 μL.

Isolation of lipids. PA and PtdEt were separated by one-dimensional thin-layer chromatography on silica gel H and developed with chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, by vol), up to 70% of the plate (35). When [^{3}H]PC was used as substrate, the chromatogram was rechromatographed up to the top of the plate using hexane/diethyl ether (70:30, vol/vol) as the developing solvent, to separate the neutral lipids. PG was separated by one-dimensional thin-layer chromatography on silica gel H using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol). Lipids were visualized by exposure of chromatograms to iodine vapors, and were then scraped off the plate into vials for counting by liquid scintillation.

Other methods. Radioactivity corresponding to lipids was determined in a liquid scintillation spectrometer after the addition of 0.35 mL of water and 5 mL of 4% Omnifluor in toluene/Triton X-100 (*t*-octylphenoxypoly ethoxyethanol; 4:1, vol/vol).

Proteins were determined according to the method of Bradford (36), and lipid phosphorus by the method of Rouser *et al.* (32).

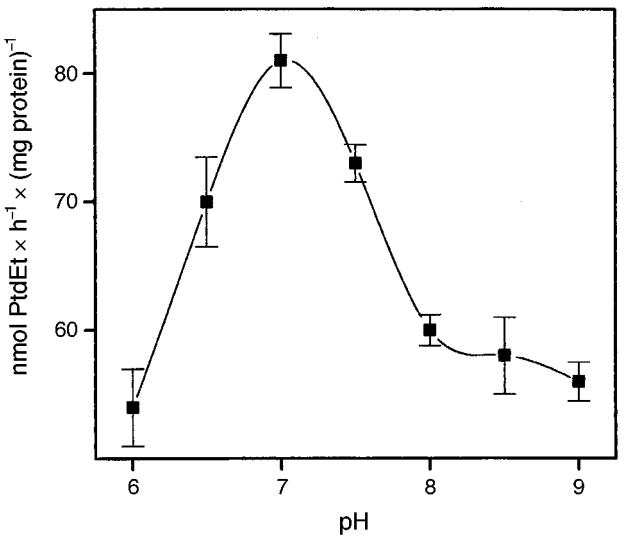

FIG. 1. Rod outer segment (ROS) phospholipase D (PLD) activity as a function of pH. Purified ROS membranes (200 μg) were incubated with 25 mM KF, 1 mM dithiothreitol (DTT), 1 mM [^{3}H]phosphatidylcholine (PC), 3 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS), 0.2 mM $CaCl_2$, and 400 mM ethanol in the following buffers: 40 mM Tris-maleate (pH 6–7.5); 40 mM Tris-HCl (pH 8–9). The activity is expressed as the mean ± SD of four or five independent samples. Other abbreviation: PtdEt, phosphatidylethanol.

RESULTS

Effect of time and protein concentration on PLD activity. PLD activity was detected in ROS preparations using a sonicated microdispersion of [^{3}H]PC obtained from bovine retinas as decribed above, either in the presence of 400 mM ethanol, or [^{14}C]ethanol or [^{3}H]glycerol without exogenous PC. The production of [^{14}C]PtdEt was protein concentration- and time-dependent, i.e., it was linear up to 60 min of incubation and up to 200 μg of protein in the incubation medium. [^{3}H]PG production was linear up to 200 μg of protein in the reaction mixture.

Effect of pH on PtdEt formation. To determine the optimal pH for PLD activity, ROS membranes were incubated at 37°C for 60 min with 1 mM [^{3}H]PC, 3 mM {3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate} (CHAPS), 1 mM dithiothreitol, 25 mM KF, 0.2 mM $CaCl_2$, and 400 mM ethanol, using different buffers in order to provide a range of pH between 6 and 9 (Fig. 1). Maximal activity was observed around pH 7. However, activity at lower and higher pH value was 50% of the activity observed at the optimal pH.

Effect of Mg^{2+} and Ca^{2+} on ROS PLD activity. To determine whether PLD activity was Mg^{2+}-dependent, ROS membranes were incubated with 1 mM [^{3}H]PC, 25 mM KF, 1 mM dithiothreitol, 3 mM CHAPS, and 400 mM ethanol as a function of Mg^{2+} concentrations (0–8 mM). The controls (0 mM Mg^{2+}) were incubated in the presence of 5 mM EDTA. PtdEt production was maximal at 1 mM of Mg^{2+} (Fig. 2). PA production was less than 15% of the PtdEt generated at every Mg^{2+} concentration assayed. PtdEt and PA production decreased drastically (40% of the production observed at 1 mM Mg^{2+}) at higher concentrations of this ion.

To determine the optimal concentration of Ca^{2+} for PLD activity, ROS membranes were incubated under the conditions decribed above in the presence of increasing concentrations of Ca^{2+} (0–8 mM) (Fig. 2). The controls (0 mM Ca^{2+}) were incubated in the presence of 5 mM EGTA. At 1 mM of Ca^{2+} the highest PA and PtdEt production was detected, and PLD activity was significantly inhibited (30% of the activity observed at 1 mM Ca^{2+}) as the concentration of this ion increased. PA production was less than 10% of PtdEt production at every Ca^{2+} concentration assayed.

Effect of PC concentration on ROS PLD activity. PtdEt formation increased as the PC concentration in the mixture increased. The production of PtdEt was linear up to 1 mM of PC concentration (Fig. 3A). PA generated by PLD activity also increased and was linear up to 1 mM of PC (Fig. 3B). The data obtained from Figures 3A and 3B (PtdEt and PA production) were plotted into a Lineweaver-Burk plot (Fig. 3C). The V_{max} was 111 nmol × h^{-1} × (mg protein)$^{-1}$, and the apparent value of K_m was 0.51 mM.

Effect of ethanol concentration on ROS PLD activity. The effect of varying ethanol concentrations on PtdEt, and PA formation was studied. The optimal ethanol concentration for PtdEt formation was 400 mM. At higher ethanol concentrations, product generation was inhibited. The Lineweaver-Burk plot, constructed with data from the sum of PtdEt and PA, shows an apparent K_m of 202 mM and a V_{max} of 125 nmol × h^{-1} × (mg protein)$^{-1}$.

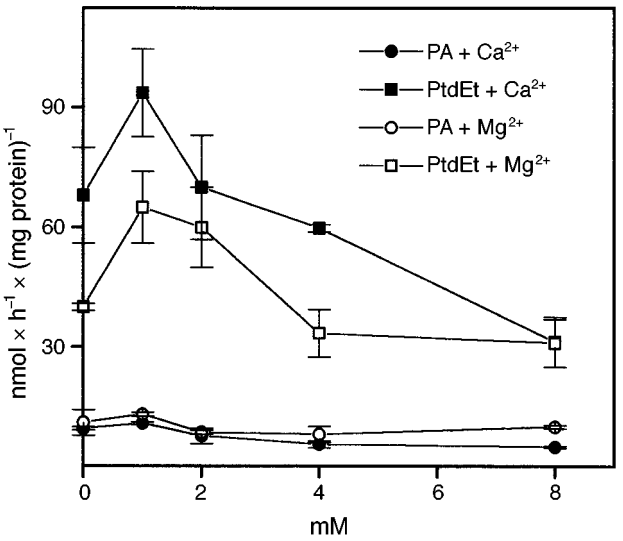

FIG. 2. ROS PLD activity as a function of Mg^{2+} and Ca^{2+} concentrations. Purified ROS membranes (200 µg) were incubated with 25 mM KF, 1 mM DTT, 1 mM [^{3}H]PC, 3 mM CHAPS, and 400 mM ethanol, in the presence of 40 mM HEPES (pH 6.8) and varying concentrations of $MgCl_2$ or $CaCl_2$. The activity is expressed as the mean ± SD of three independent samples. PA, phosphatidic acid; for other abbreviations see Figure 1.

PLD activity in the presence of oleic acid and detergents. The activators employed in this study include Triton X-100, CHAPS, sodium deoxycholate (NaDOC), polyoxyethylenesorbitanmonooleate (Tween 80), and sodium oleate (Fig. 4). Only CHAPS and NaDOC were found to significantly activate PLD from ROS membranes. Maximal stimulation (300% increase) occurred around the critical micelle concentration (CMC) of CHAPS, that is, 5 mM, although an important activation occurred below its CMC (50% increase). When NaDOC was added to the incubation mixture, the maximal stimulation occurred at its CMC (2.5 mM). Tween 80 only stimulated PLD activity at very low detergent concentrations (0.1 mM), whereas Triton X-100 slightly inhibited PLD activity (by 5% with respect to the control) at every concentration assayed.

Sodium oleate has been reported as a possible activator of PLD activity in rat brain (35). For PLD from ROS membranes, the oleic acid concentration required for maximal activation was 2 mM (27% increase). But the stimulation was less pronounced than in the presence of certain detergents (CHAPS and NaDOC) at similar concentrations. (Fig. 4).

When assays were carried out using [^{3}H]PC as substrate, CHAPS, 3 mM (0.2%, wt/vol), was the chosen activator because it producd a 50% increase and because this tensioactive agent is widely used in the study of visual protein systems (37). The maximal activation with CHAPS (300% increase with respect to control) was observed around its CMC.

PLD activity in ROS and retinal fractions. Table 1 shows the specific activities of PLD in fractions obtained by fractionation of crude ROS and retina. Crude ROS, obtained by shaking the retina in a 40% sucrose buffer solution, diluting and centrifuging, were layered onto a discontinuous sucrose

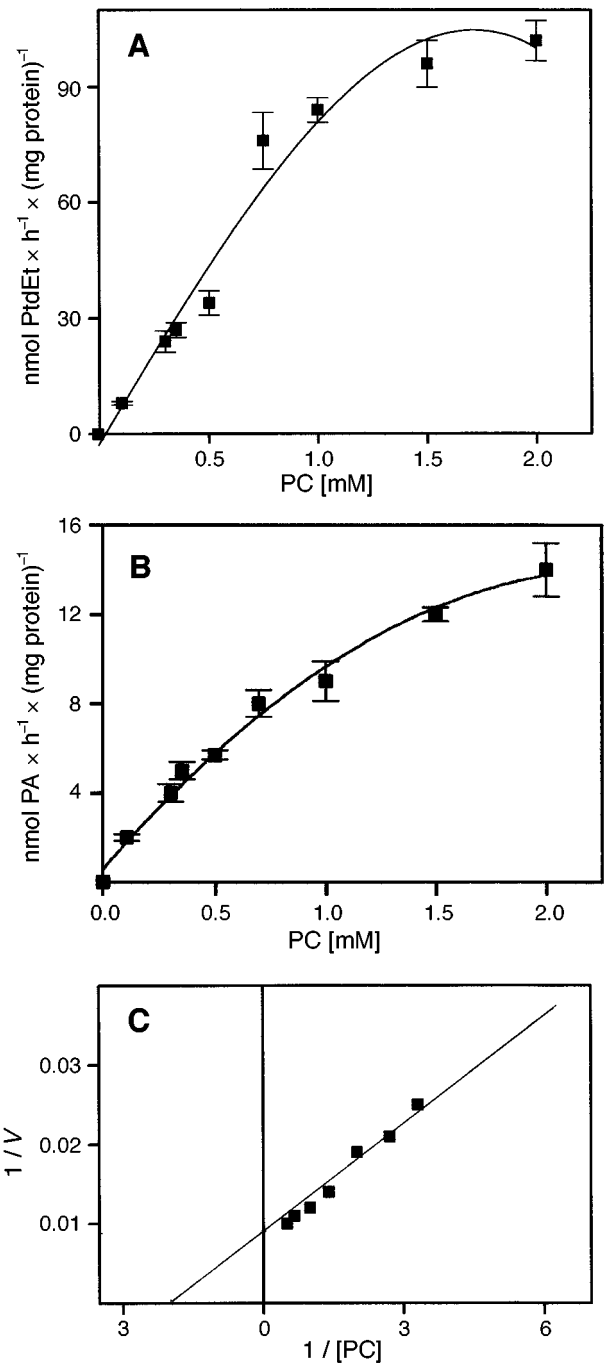

FIG. 3. ROS PLD activity as a function of PC concentration. Purified ROS membranes (200 µg) were incubated in 40 mM HEPES (pH 6.8), 25 mM KF, 1 mM DTT, 3 mM CHAPS, 0.2 mM $CaCl_2$, 400 mM ethanol, and varying concentrations of [^{3}H]PC. (A) PtdEt formation as a function of PC concentration. The activity is expressed as the mean ± SD of four or five independent samples. (B) Effect of PC concentration on PA production. The activity is expressed as the mean ± SD of four or five independent samples. (C) Double reciprocal plot of the data from plots A and B (the sum of PA plus PtdEt) were made according to Lineweaver and Burk to obtain apparent K_m and V_{max} values. For abbreviations see Figures 1 and 2.

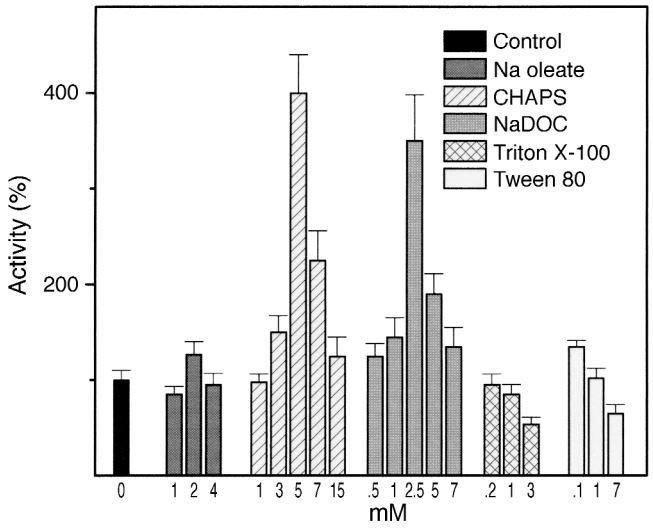

FIG. 4. Effect of detergents and sodium oleate on ROS PLD activity. The reaction mixture contained 200 µg of ROS protein, 40 mM HEPES (pH 6.8), 25 mM KF, 1 mM DTT, 0.2 mM $CaCl_2$, 1 mM [^{3}H]PC, and the indicated concentration of sodium oleate, sodium deoxycholate (NaDOC), CHAPS, *t*-octylphenoxypolyethoxyethanol (Triton X-100), or polyoxyethylenesorbitanmonooleate (Tween 80). The percentage of activity is presented as the mean ± SD of five or six independent samples.

TABLE 1
Phospholipase D Activity in Rod Outer Segment (ROS) Membranes and Retinal Subcellular Fractions[a]

Fraction	nmol PtdEt × h^{-1} × (mg protein)$^{-1}$	Protein (mg)
Band I (0.84/1.00 M interface)	40 ± 5.2	2.54
Band II	29 ± 0.31	0.88
Pellet	27 ± 0.98	3.90
Crude ROS	22 ± 1.9	9.98
Total homogenate	51 ± 6.8	210.00
P_1 (crude nuclei)	22 ± 1.09	92.36
P_2 (crude mitochondria)	35 ± 5.92	19.40
Microsomal	59 ± 6.02	14.23
Cytosolic	25 ± 1.47	65.64

[a]Band I (purified ROS), band II, and pellet of the gradient and retinal subcellular fractions were obtained as described in the Materials and Methods section. In every fraction assay, the reaction mixture contained 200 µg of protein, 40 mM HEPES (pH 6.8), 25 mM KF, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, 1 mM [^{3}H] phosphatidylcholine, 3 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 400 mM ethanol in a total volume of 250 µL. The enzymatic activity is expressed as the mean ± SD of four independent samples. Typical protein content of total homogenate from 10 retinas was 210 mg. PtdEt, phosphatidylethanol.

gradient. Band I, obtained at the 0.84/1.00 M interface, contained purified ROS and showed the highest specific activity of PLD with respect to band II (1.00/1.14 M interface) and pellet. In addition, marker enzyme activities were also assayed in all fractions, with the purpose of determining possible mitochondrial or microsomal contamination of ROS membranes (band I) of the gradient. Whereas the mitochondrial marker enzyme (cytochrome c oxidase) was enriched in band II and pellet [60 and 35 nmol × min^{-1} × (mg protein)$^{-1}$, respectively], the cytochrome c reductase activity (the microsomal marker enzyme) was very low in band I and band II [0.5 and 1 nmol × min^{-1} × (mg protein)$^{-1}$, respectively] and presented the highest activity in the pellet [3.6 nmol × min^{-1} × (mg protein)$^{-1}$]. These results demonstrated that band I (purified ROS) was only slightly contaminated with microsomes or mitochondria (lower than 5%) (19).

Table 1 also shows the specific activities of PLD in crude nuclear fraction (P_1), crude mitochondrial fraction (P_2), microsomal fraction and cytosolic fraction, obtained from the subcellular fraction of the remainder of the retina. The nuclear pellet contained not only a heterogeneous population of nuclei and tissue fragments but also an aggregation of mitochondria, derived from the photoreceptor inner segment, fragmental outer segments, and isolated photoreceptor terminals (pedicle synaptosomes). The crude mitochondrial fraction also contained fragmented photoreceptor outer segments and synaptic pedicles together with many free mitochondria, conventional nerve-ending particles, peroxisomes, lysosomes and occasional bipolar terminals (38). PLD activity was determined with exogenous [^{3}H]PC by measuring [^{3}H]PtdEt and [^{3}H]PA production. The microsomal fraction showed the highest PLD specific activity. The activity found in band I (purified ROS) was as high as 60% of the activity present in microsomes.

DISCUSSION

PLD activity has been described and characterized in a great number of mammalian tissues (39–41). It has also been proposed that such enzymatic activity could be implicated in multiple processes such as membrane intracellular trafficking (21,42), generation of second messengers such as PA, DG and lysophosphatidic acid (20), choline supply for acetylcholine synthesis in rat brain (43) and exocytic hormone release in pancreatic islet cells (44), and participation in events occurring during neutrophil activation (45) and in leukemic cell differentiation (46), among many other cellular events.

The present work demonstrates the presence of PLD activity in isolated ROS from bovine retinas. The activity of PLD observed in ROS [50 nmol × h^{-1} × (mg protein)$^{-1}$] is in agreement with that described for rat brain synaptosome (34). In our ROS membranes, prepared by discontinuous sucrose gradient centrifugation, the activity of PLD was as high as 66% of that found in microsomes. It has been shown that ROS membranes present minimal contamination, with cytochrome c reductase a microsomal marker enzyme (19). This indicates that PLD activity is located in ROS and does not constitute a contamination from other fractions. The majority of contaminants in ROS preparations arise from excessively vigorous homogenization procedures employed too early during the isolation process, with subsequent steps often not able to completely separate ROS from contaminants because of entrapped particles. The method used to obtain our ROS preparations involves gentle homogenization, and the buffer used has moderate ionic strength, thus allowing us to retain the soluble and peripheral protein of the ROS.

We have also established that the optimal pH for PLD activity is 7.0, and this value agrees with the findings of Kanoh *et al.* (40). The presence of ions such as Mg^{2+} and Ca^{2+} stimulates PLD activity. The optimal concentration was 1 mM for both ions. At 1 mM of Ca^{2+}, PtdEt production was increased by 44% with respect to the control (5 mM EGTA). In the presence of 1 mM Mg^{2+}, the activity was increased by 50% with respect to the control (5 mM EDTA). However, enzymatic activity was clearly detected in the absence of these ions [50–60 nmol $\times$ h^{-1} $\times$ (mg protein)$^{-1}$].

When the enzyme was assayed with increasing PC concentrations, the maximal activity was detected at 1 mM PC, the apparent value of K_m for PC being 0.51 mM and the V_{max} being 111 nmol $\times$ h^{-1} $\times$ (mg protein)$^{-1}$. The effect of increasing ethanol concentrations was examined, and the K_m for ethanol was 202 mM (for the sum of PtdEt and PA). V_{max} values, obtained with ethanol [125 nmol $\times$ h^{-1} $\times$ (mg protein)$^{-1}$], are in agreement with the V_{max} values obtained with PC. These values of K_m are similar to those of Kobayashi and Kanfer (35) for synaptosomal brain PLD.

ROS PLD was investigated in the presence of natural or synthetic tensioactive agents, of which the most effective in activating the enzyme were CHAPS and NaDOC. Oleic acid was a poor activator of ROS membrane PLD when [^{3}H]PC was used as a substrate. However, oleic acid is the most effective activator for synaptosomal PLD in rat brains (35). Kanoh *et al.* (40) have studied brain PLD activity from different species (bovine, rat, and rabbit), and they found that PLD activity in bovine brain is poorly detected when enzyme activity is assayed in the presence of oleic acid or Triton X-100. These findings are in accordance with our findings on PLD activation in bovine ROS. Triton X-100, a nonionic detergent, which does not usually break protein–protein interactions, did not affect ROS PLD activity. Stimulation of PLD activity in ROS is very important (300% increase) in the presence of CHAPS, a zwitterionic detergent, or in the presence of NaDOC (250% increase), a bile salt-like anionic detergent that is denaturing and effective at breaking protein–protein interactions. Whether the stimulatory effect of detergents, such as CHAPS and NaDOC, on the PLD activity is due to direct action on the enzyme or on the substrate cannot be distinguished from the present experiments. We also propose that mild perturbations of the membrane at the microenvironment of the enzyme do not interfere with its activity; on the contrary, this effect activates the enzyme.

To study transphosphatidylation reactions, characteristic of most PLD (32), ROS membranes were incubated in the presence of [^{14}C]ethanol or [^{3}H]glycerol in order to obtain [^{14}C]PtdEt or [^{3}H]PG, respectively. [^{14}C]PtdEt production was time- and protein concentration-dependent. These assays were carried out in the presence of oleic acid as activator because in the absence of exogenous PC, oleic acid was effective in allowing measuring of the enzymatic activity.

Base exchange enzymes and PLD have a common characteristic: their capacity to modify the polar portion of phospholipids. Fliesler and Anderson (48) demonstrated the existence of base exchange enzymes in retina, and they suggested their presence in ROS. The presence of both enzyme activities, PLD and base exchange, could play an important role in phospholipid metabolism in vertebrate photoreceptors, as well as in the lipid–protein interactions in ROS membranes.

Bazán *et al.* (49) have suggested that in photoreceptor membranes the pool of PA may arise from phospholipid degradation by a PLD. In addition, PA is the precursor of DG, and this neutral lipid may participate in the fusion of synaptic vesicles to presynaptic membranes during neurotransmission (50). Furthermore, PA breakdown by PAPase has been clearly demonstrated in ROS (6). It is interesting to speculate that PA, produced by PLD, in ROS may be involved in a signal transduction mechanism, and that it could then be hydrolyzed by PAPase to yield DG. DG has been established as an intracellular second messenger, and it has been demonstrated recently that DG is capable of activating a novel characterized ROS protein kinase C isozyme (51).

Fusion mechanisms between disc membranes and plasma membrane have been described in ROS (52), and the implication of several PLD isozymes regulated by small G proteins in the membrane fusion processes (21,42,45,47) is well known. On the other hand, the presence of small G proteins in ROS (53,54) provides a starting point for further studies aimed at understanding the regulation and physiological functions of PLD in photoreceptor membranes.

ACKNOWLEDGMENTS

This research was supported by the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET) and the Secretaría General de Ciencia y Tecnología, Universidad Nacional del Sur, Argentina. Norma M. Giusto is a research member of the CONICET and Gabriela A. Salvador is a research fellow of the CONICET. The authors are very grateful to Dr. Paula Castagnet for her critical reading of the manuscript.

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[Received May 15, 1998, and in final revised form August 10, 1998; revision accepted August 20, 1998]

Lipase Specificity Toward Some Acetylenic and Olefinic Alcohols in the Esterification of Pentanoic and Stearic Acids

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ABSTRACT: The esterification of five medium- and long-chain acetylenic alcohols (2-nonyn-1-ol, 10-undecyn-1-ol, 6-octadecyn-1-ol, 9-octadecyn-1-ol, and 13-docosyn-1-ol), seven olefinic alcohols (*cis*-3-nonen-1-ol, 10-undecen-1-ol, *cis*-6-octadecen-1-ol, *cis*-9-octadecen-1-ol, *trans*-9-octadecen-1-ol, *trans*-9, *trans*-11-octadecadien-1-ol, *cis*-9,*cis*-12-octadecadien-1-ol), and four short-chain unsaturated alcohols (allyl alcohol, 3-butyn-1-ol, 3-pentyn-1-ol, and *cis*-2-penten-1-ol) with pentanoic or stearic acid in the presence of various lipase preparations was studied. With the exception of 2-nonyn-1-ol, where Lipase AY-30 (*Candida rugosa*) was used as the biocatalyst, the esterification of C_{11}, C_{18}, and C_{22} acetylenic alcohols with pentanoic acid appeared to be generally unaffected by the presence of an acetylenic bond in the alcohol as relatively high yields of the corresponding esters (78–97%) were obtained. However, medium- and long-chain olefinic alcohols were discriminated by Lipase AY-30, Lipolase 100T (*Rhizomucor miehei*), and especially by porcine pancreatic lipase (PPL), when esterification was conducted with pentanoic acid. Esterification of medium- and long-chain acetylenic or olefinic alcohols with a long-chain fatty acid, stearic acid, was very efficient except when Lipase AY-30 and Lipolase 100T were used. Short-chain unsaturated alcohols were much more readily discriminated. 3-Pentyn-1-ol and 3-butyn-1-ol were difficult (<5% yield) to esterify with pentanoic or stearic acid in the presence of Lipase AY-30 and PPL, respectively. Very low yields (<26%) of esters were produced when 3-butyn-1-ol and 3-pentyn-1-ol were reacted with pentanoic or stearic acid, when catalyzed by lipase from *Candida cylindracea*. No reaction took place between 3-butyn-1-ol and stearic acid in the presence of Lipase AY-30. Esterification of short-chain acetylenic and olefinic alcohols was most efficiently achieved with Lipolase 100T (*Rhizomucor miehei*), Lipozyme IM20 (*Rh. miehei*), or Novozyme 435 (*Candida antarctica*) as the biocatalyst.

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Lipase as a biocatalyst in the esterification of fatty acids and in the hydrolysis of fatty esters has been extensively studied. Lipase specificity toward their substrates is important for metabolic studies, for synthesis of structured lipids, and for the isolation of useful fatty acids. Several review articles have been published detailing the wide variety of enzymes, substrates, and conditions used to accomplish such transformations (1–3), e.g., the lipase from *Brassica napus* L. discriminates against *cis*-4 and *cis*-6 unsaturated fatty acids and also against secondary and tertiary alcohols (4), while the lipase from *Candida parapsilosis* shows a high activity in the presence of esters with long-chain fatty acids and particularly unsaturated fatty acids with a *cis*-9 double bond (5).

The lipase reaction selectivity on the alcohol moiety of esters during hydrolysis and on the discrimination of different types of alcohols during esterification has also been reported. Most of these studies have focused on the chain length of saturated, methyl-branched, aromatic, and cyclic alcohols (6–12). Olefinic fatty alcohols are found in extracts of marine sponges, fish, and plants, e.g. , *cis*-3-nonen-1-ol and *cis*-3, *cis*-6-nonadienol are volatile components detected in watermelon (*Citrillus vulgaris*). These compounds are biosynthetic products derived from linoleic and linolenic acids, respectively (13,14). Long-chain fatty alcohols are synthesized in the brain from the corresponding long-chain fatty acid which is reduced by microsomal aldehyde reductase (15). The accumulation of C_{18} fatty alcohols in human breast cancer cells (MCF-7 cell line) suggests a deviation from the ordinary lipid metabolism in normal breast epithelial cells (16).

Because fatty alcohols are important in biological systems, this work investigated the effects that the unsaturation centers in fatty alcohols have on the alcohols' esterification rates to short- and long-chain fatty acids when various lipases or lipase preparations are used as biocatalysts. We recently reported the effects of the acetylenic bond in long- and medium-chain fatty acids in lipase-catalyzed esterification reactions with *n*-butanol: we showed that different lipases are able to discriminate acetylenic fatty acids according to the position of the triple bond in the alkyl chain of the fatty acid molecule (17).

MATERIALS AND METHODS

Immobilized lipases from *Rhizomucor miehei* (Lipolase 100T and Lipozyme IM 20) and *Candida antarctica* (Novozyme 435) were gifts from Novo Nordisk A/S (Hong Kong). Lipases from *Candida cylindracea* (CCL, type VII, 905 μ/mg) and porcine pancreatic lipase (PPL, type II, 179 μ/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Lipases from *Pseudomonas cepacia* (PS-D, type I) and *Candida*

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Abbreviations: NMR, nuclear magnetic resonance; PPL, porcine pancreatic lipase.

rugosa (Lipase AY-30) were gifts from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Allyl alcohol, 3-butyn-1-ol, *cis*-2-penten-1-ol, 3-pentyn-1-ol, *cis*-3-nonen-1-ol, 10-undecen-1-ol, 10-undecenoic acid, methyl 2-nonynoate, pentanoic acid, linoleic acid, oleic acid, and stearic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). *cis*-6-Octadecenoic and erucic acids were isolated from carrot and rapeseed oil, respectively. *trans*-9, *trans*-11-Octadecadienoic acid was prepared as described elsewhere (18).10-Undecynoic, 6-octadecynoic, 9-octadecynoic, and 13-docosynoic acids were synthesized by bromination–dehydrobromination reaction of the corresponding olefinic fatty acids as described elsewhere (19,20).

2-Nonyn-1-ol, 10-undecyn-1-ol, 6-octadecyn-1-ol, 9-octadecyn-1-ol, 13-docosyn-1-ol, *cis*-9-octadecen-1-ol, *cis*-9,*cis*-12-octadecadien-1-ol, and *trans*-9,*trans*-11-octadecadien-1-ol were obtained by lithium aluminum hydride reduction of the corresponding methyl esters (21). *cis*-6-Octadecen-1-ol was prepared by semihydrogenation of 6-octadecyn-1-ol over Lindlar catalyst (22). *trans*-9-Octadecen-1-ol was obtained by isomerization of *cis*-9-octadecen-1-ol with *p*-toluenesulfinic acid (23).

^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance DPX_{300} NMR spectrometer (Bruker, Fallanden, Switzerland) with the sample dissolved in deuteriochloroform. Gas–liquid chromatographic analyses were performed on a Hewlett-Packard (Hewlett-Packard Incorp., Palo Alto, CA) model 5890 instrument equipped with a fused silica capillary column coated with Nukol™ (Supelco Inc., Bellefonte, PA) (15 m, 0.53 mm diameter, 0.50 μm film thickness) as stationary phase at a column temperature of 200°C.

General method for the enzymatic esterification of acetylenic and olefinic alcohols as exemplified by the reaction of 9-octadecyn-1-ol with 1-pentanoic acid in the presence of CCL. A mixture of 9-octadecyn-1-ol (25 mg, 0.093 mmol), pentanoic acid (12.3 mg, 0.12 mmol), *n*-hexane (1.2 mL), and *CCL* (25 mg) was stirred at 40°C for 24 h in a glass culture tube. The reaction mixture was centrifuged to remove the lipase, and the *n*-hexane solution was loaded on a silica gel (6.0 g, 230–400 mesh; E. Merck, Darmstadt, Germany) in an *n*-hexane column (12 mm i.d.). The column was eluted with a mixture of *n*-hexane/diethyl ether (95:5 vol/vol, 50 mL). The eluate was evaporated under reduced pressure to give only 9-octadecynyl pentanoate (30 mg, 92%). The product was identified by gas–liquid chromatographic analysis and confirmed by NMR spectroscopic analysis. The results are graphically presented in Figures 1–7. Each bar represents the average percentage yield from at least two determinations. (Note: in the case of the esterification of short-chain alcohols with pentanoic acid, the eluate containing the esters after silica column chromatography was evaporated at atmospheric pressure.) In all other similar experiments, the amount of the respective fatty alcohol used was 25 mg, lipase 25 mg, and the amount of pentanoic or stearic acid was in 30% excess of the molar equivalent of the fatty alcohol.

Competitive enzymatic esterification of C_{18} olefinic alcohols with a mixture of pentanoic and stearic acids in the presence of PPL as exemplified by the reaction involving cis*-6-octadecen-1-ol.* A mixture of *cis*-6-octadecen-1-ol (40 mg, 0.15 mmol), pentanoic acid (15 mg, 0.15 mmol), stearic acid (40 mg, 0.14 mmol), *n*-hexane (1.6 mL), and PPL (30 mg) was stirred at 40°C for 24 h. The mixture of *cis*-6-octadecenyl pentanoate and stearate esters was isolated by column chromatography as described above. The ratio of each component was estimated from the relative intensities observed for the shifts of the C-2 carbon nuclei of the pentanoate and stearate esters in the ^{13}C NMR spectrum. The shift of the C-2 nucleus of *cis*-6-octadecenyl pentanoate appeared at δ_C 34.13, while the shift of the C-2 nucleus of *cis*-6-octadecenyl stearate appeared at δ_C 34.43.

The results of the competitive esterification experiment involving *cis*-6-octadecen-1-ol, *cis*-9-octadecen-1-ol, *trans*-9-octadecen-1-ol, *cis*-9,*cis*-12-octadecadien-1-ol, and *trans*-9,*trans*-11-octadecadien-1-ol are presented in Figure 5.

RESULTS AND DISCUSSION

A total of seven lipase preparations [Lipase AY-30 (*C. rugosa*), Lipolase 100T (*R. miehei*), PPL, CCL, PS-D (*P. cepacia*), Lipozyme IM 20 (*R. miehei*), Novozyme 435 (*C. antarctica*)] were used as biocatalysts in studying the esterification process.

In order to establish a general reaction time for the esterification for the proposed unsaturated alcohols with pentanoic or stearic acid, the esterification of *cis*-9-octadecen-1-ol with pentanoic or stearic acid was first studied over a period of 6, 12, 18, 20, 24, 30, 40, and 50 h using the various lipases. In each case the esterification process showed a gradual increase of ester formation. Generally, esterification of *cis*-9-octadecen-1-ol was more sluggish (about 23% after 12 h) with pentanoic acid than with stearic acid (about 60% after 12 h). However, after 20–24 h of reaction at 40°C, the esterification process reached a steady state. Little or no further esterification was observed when the reaction time was extended from 24–50 h, when a yield of about 95% of each ester was obtained. From this study it was concluded that a 24-h reaction period would be an ideal reaction time to study the esterification of the various acetylenic and olefinic alcohols with pentanoic or stearic acid.

The results of the esterification of acetylenic alcohols with pentanoic acid are given in Figure 1. 2-Nonyn-1-ol [9:1(2*a*)] [Note: The chain length of the alcohol is denoted by the first number, the number following the colon indicates the number of unsaturated center(s) present in the chain, the number within the brackets gives the position of the unsaturated center from the hydroxy function, and the letter (*a* = acetylene, *c* = *cis*, *t* = *trans,* and *e* = ethylenic) describes the type of unsaturated center(s) involved.] was significantly discriminated (43% yield), when Lipase AY-30 was used as the biocatalyst. This observation indicated that the effect of the acetylenic bond on the electronic nature of the hydroxy function seemed to affect the catalytic property of the enzyme. Lipase AY-30

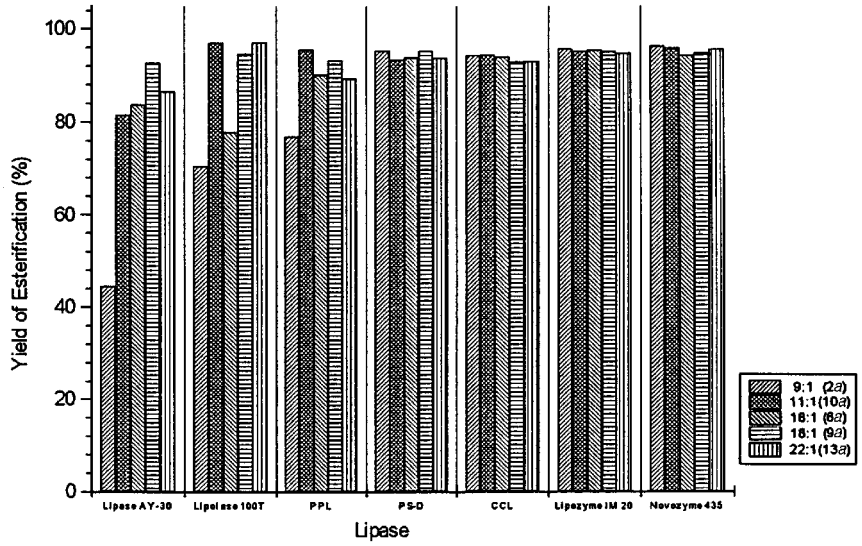

FIG. 1. Esterification yields of acetylenic fatty alcohols with pentanoic acid catalyzed by various lipases after 24 h. *a* = acetylene; PPL, porcine pancreatic lipase, and CCL, *Candida cylindracea* (Sigma Chemical Co., St. Louis, MO); PS-D, *Pseudomonas cepacia*, and Lipase AY-30, *Candida rugosa* (Amano Pharmaceutical Co. Ltd., Nagoya, Japan); Lipolase 100T, Lipozyme IM20, and Novozyme 435 (Novo Nordisk A/S, Hong Kong).

therefore could be considered as sensitive or discriminating against a propargyl system.

The esterification of the C_{11}, C_{18}, and C_{22} acetylenic alcohols with pentanoic acid in the presence of the other lipases appeared to be only slightly affected by the acetylenic bond in the alcohol as relatively high yields (78–97%) of esters were obtained. The presence of an acetylenic bond, irrespective of its position in the alkyl chain of the alcohol, did not seem to interfere significantly with the rate of esterification.

The esterification of seven olefinic alcohols [viz. 9:1(3*c*), 11:1(10*e*), 18:1(6*c*), 18:1(9*c*), 18:1(9*t*), 18:2(9*t*,11*t*), and 18:2(9*c*,12*c*)] with pentanoic acid was investigated next, and the results are shown in Figure 2. In the presence of Lipase AY-30, the esterification of 9:1(3*c*), 11:1(10*e*), and 18:1(9*t*) gave comparatively low yields (28, 40, and 48%, respectively) of the corresponding esters, while the remaining unsaturated C_{18} alcohols were esterified in good yields (60–75%). Lipolase 100T seemed to discriminate the monounsaturated C_{18} fatty alcohols,

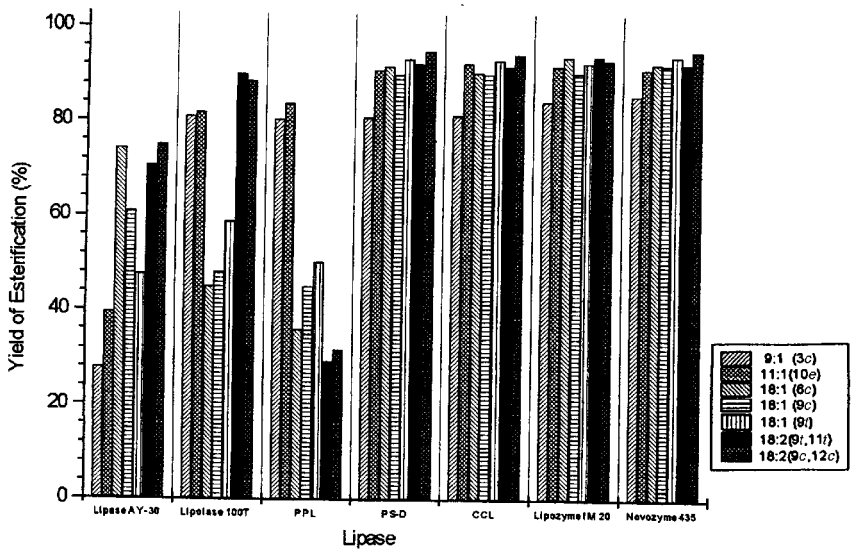

FIG. 2. Esterification yields of olefinic fatty alcohols with pentanoic acid catalyzed by various lipases after 24 h. *c = cis; t = trans; e* = ethylenic. See Figure 1 for other abbreviations and company sources and locations.

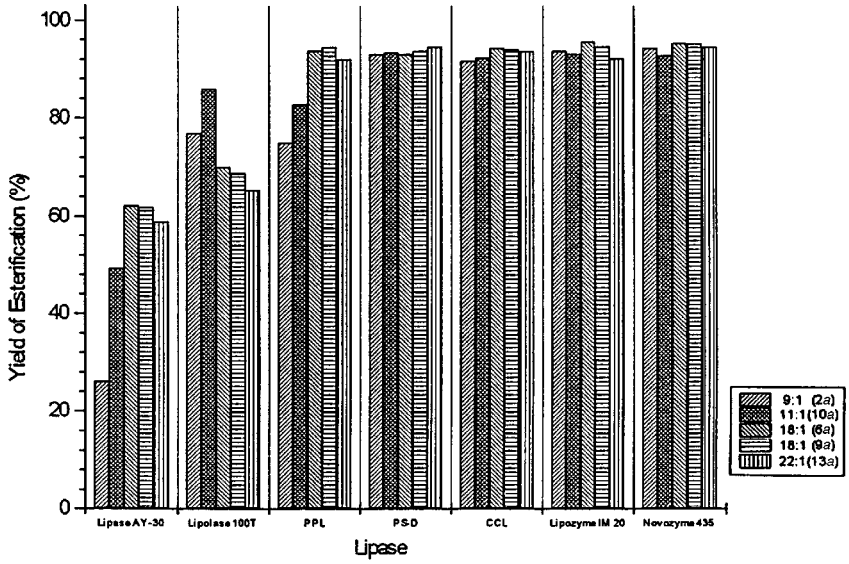

FIG. 3. Esterification yields of acetylenic fatty alcohols with stearic acid catalyzed by various lipases after 24 h. See Figure 1 for abbreviations and company sources.

but not the medium (C_9, C_{11}) and long-chain alcohols (C_{18}) containing two double bonds (methylene-interrupted or conjugated). Of interest was the result of the esterification conducted in the presence of PPL: the medium-chain olefinic alcohols [9:1(3*c*) and 11:1(10*e*)] were readily esterified, but the C_{18} and C_{22} unsaturated alcohols yielded only 29–50% of the corresponding pentanoate esters. Esterification of the olefinic alcohols in the presence of the other lipases furnished high yields of the pentanoate esters (average yield of 93%).

When the same sets of acetylenic and olefinic alcohols were subjected to esterification with stearic instead of pentanoic acid, the yields of the esters obtained were also generally very high (average 93%) for most lipases. Some significant discrimination was observed for Lipase AY-30 and Lipolase 100T: the yields of the stearate esters fluctuated between 26–86% (Fig. 3 and 4).

To further investigate the apparent differences in the ability of PPL to esterify olefinic C_{18} alcohols with short-vs. long-chain fatty acids, a competitive esterification reaction was carried out with each of the following alcohols [18:1(6*c*), 18:1(9*c*), 18:1(9*t*), 18:2(9*t*,11*t*), and 18:2(9*c*,12*c*)] using a mixture of pentanoic and stearic acids. The resulting mixture

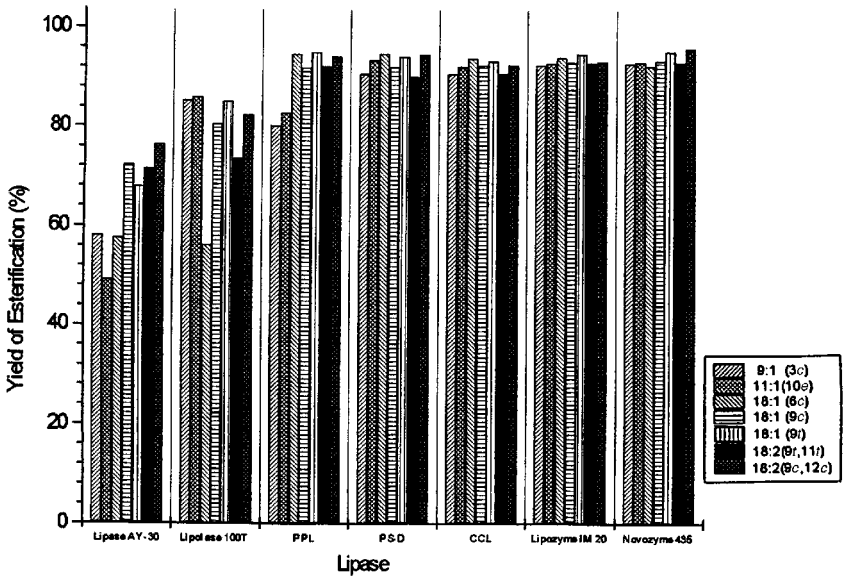

FIG. 4. Esterification yields of olefinic fatty alcohols with stearic acid catalyzed by various lipases after 24 h. See Figures 1 and 2 for company sources and abbreviations.

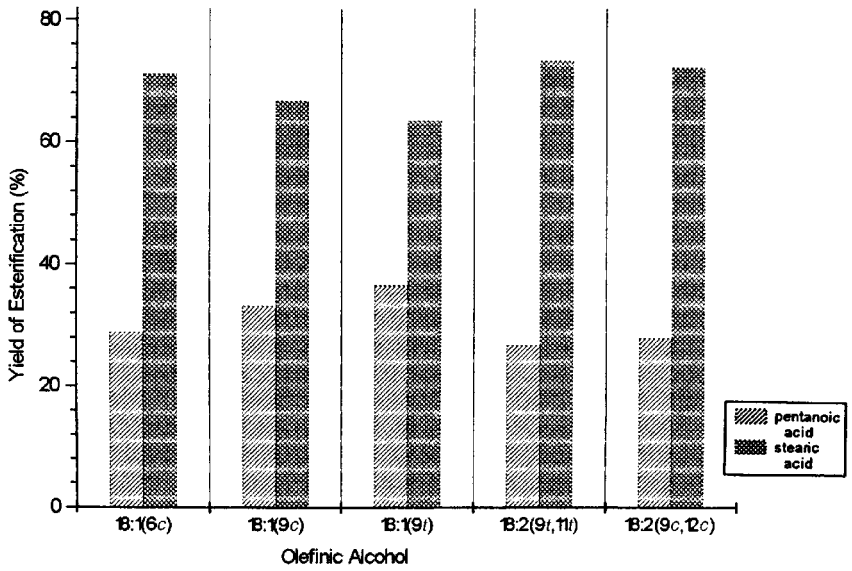

FIG. 5. Comparison of relative yields obtained from porcine pancreatic lipase-catalyzed esterification reaction between pentanoic and stearic acids with long-chain olefinic C_{18} fatty alcohols after 24 h. See Figure 2 for abbreviations.

of pentanoate and stearate esters was isolated by silica column chromatography. The ratio of the pentanoate to stearate esters was estimated from the intensities of the signals arising from the shifts of the C-2 carbon atoms of the ester mixture. The shift of the C-2 nucleus of the pentanoate ester appeared at δ_C 34.13, while the shift of the C-2 nucleus of the stearate ester appeared at δ_C 34.43. The amounts in percentage yield based on the individual fatty acid used are shown in Figure 5. In each case the amount of stearate ester was two- to threefold higher than that of the pentanoate ester. These results confirmed the initial observation that PPL favored the esterification of long-chain fatty acids (e.g., stearic acid) over short-chain fatty acids (e.g. pentanoic acid) with C_{18} olefinic alcohols.

To assess the effects of short-chain unsaturated alcohols on the rate of esterification with pentanoic and stearic acids,

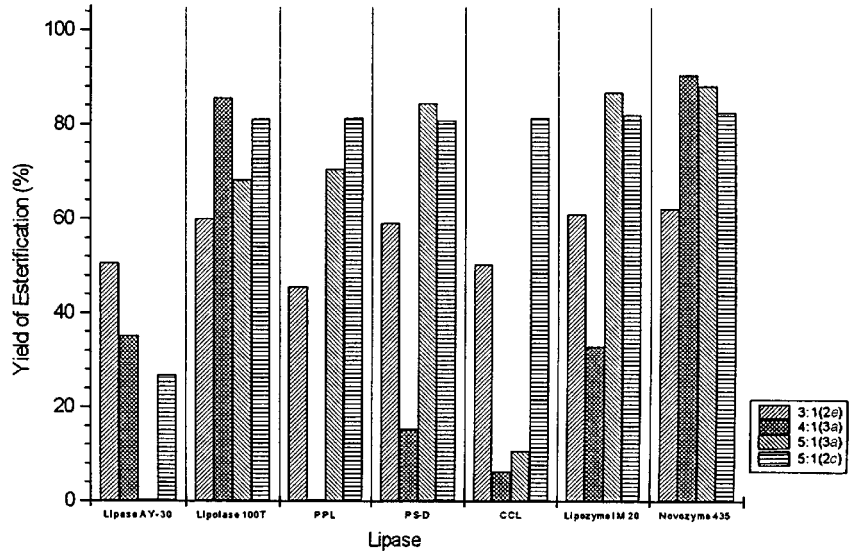

FIG. 6. Esterification yields of short-chain unsaturated alcohols with pentanoic acid catalyzed by various lipases after 24 h. See Figures 1 and 2 for abbreviations and Figure 1 for company sources.

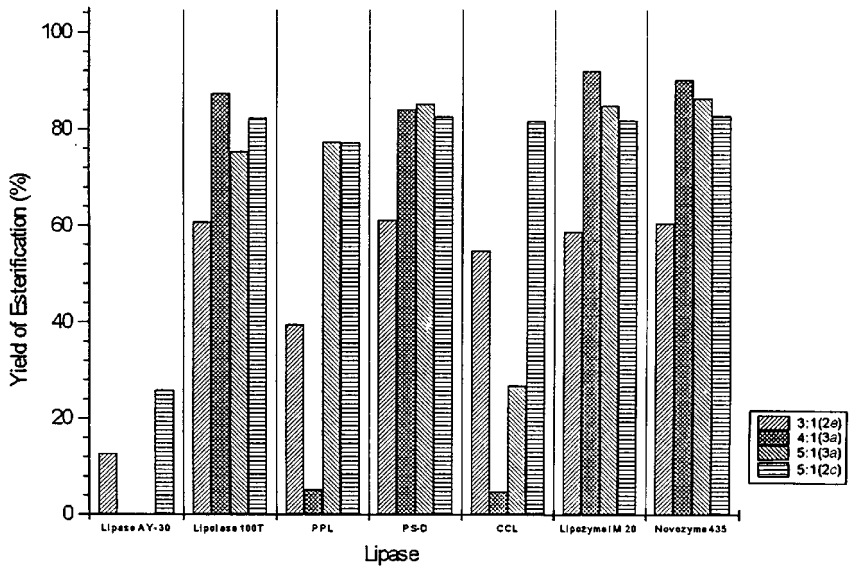

FIG. 7. Esterification yields of short-chain unsaturated alcohols with stearic acid catalyzed by lipases after 24 h. See Figures 1 and 2 for abbreviations and Figure 1 for company sources.

four unsaturated alcohols [3:1(2*e*), 4:1(3*a*), 5:1(3*a*), and 5:1(2*c*)] were selected. The results of the lipase-catalyzed esterification of such short-chain unsaturated alcohols with pentanoic or stearic acid are presented in Figures 6 and 7, respectively. The most striking feature in this study was seen in the case of Lipase AY-30, which was able to esterify 3:1(2*e*), 4:1(3*a*), and 5:1(2*c*) with pentanoic acid in yields varying from 27–51%. However, 5:1(3*a*) was not esterified with pentanoic acid in the presence of Lipase AY-30, and when pentanoic acid was replaced by stearic acid, only 3:1(2*e*) and 5:1(2*c*) were esterified in low yields (13 and 26%, respectively).

When PPL was used as the biocatalyst, 4:1(3*a*) was not esterified with pentanoic acid, and only 5% yield of the ester was obtained when pentanoic acid was replaced by stearic acid in this reaction. Low yields of ester production were found for 4:1(3*a*) and 5:1(3*a*) when CCL lipase was used. The other remaining lipases showed no difficulty in catalyzing the esterification of the short-chain unsaturated alcohols with either pentanoic or stearic acid.

From these results it is difficult to rationalize the precise mechanism involved in the enzymes during the esterification process. However, the data point to the fact that some of the lipases are relatively sensitive to the acetylenic or olefinic bonds, especially where this unsaturation is adjacent to the hydroxy function. Also, PPL appears to behave particularly sensitively toward short-chain fatty acid (pentanoic acid) during the esterification of such substrate with olefinic C_{18} fatty alcohols, probably due to the less well-bound nature of the short-chain fatty acid to the pancreatic lipase as compared to long-chain fatty acids, such as stearic acid.

This study concludes that medium- and long-chain acetylenic and olefinic alcohols are generally readily esterified in the presence of the seven lipase preparations studied, except in the case of PPL which shows discrimination against olefinic long-chain (C_{18}) alcohols. Short-chain (C_3–C_5) acetylenic or olefinic alcohols are best esterified with either pentanoic or stearic acid when catalyzed by Lipolase 100T, Lipozyme IM20, or Novozyme 435.

ACKNOWLEDGMENTS

This work has been supported by the Lipid Research Fund, the Research Grants Committee of the University of Hong Kong, and the Research Grant Council of Hong Kong.

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[Received December 1, 1997, and in final revised form June 24, 1998; revision accepted July 17, 1998]

The Triggering Signal Dictates the Effect of Docosahexaenoic Acid on Lymphocyte Function *in vitro*

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ABSTRACT: Docosahexaenoic acid (DHA) is an n-3 fatty acid beneficial to several human conditions including inflammation and autoimmune disease. To better understand the effect of DHA on immunity, we monitored the rise in cytosolic free calcium, interleukin 2 receptor (IL2R) expression, and proliferation of splenic lymphocytes triggered with three different stimuli in the presence or absence of DHA. We found that 10 μg DHA/mL suppressed concanavalin A-induced mitogenesis and the mixed lymphocyte reaction while concurrently enhancing proliferation stimulated with anti-Thy-1 antibodies. Proliferation, as measured by [^{3}H]thymidine incorporation after 2 to 5 d of culture, was affected by DHA, but earlier activation effects such as elevation of cytosolic free calcium and IL2R expression were not altered. These results imply that DHA incorporated into membrane phospholipids differentially affects the activity of distinct membrane-bound receptors and signaling molecules. This result suggests that DHA may be used to modulate immune responses selectively, e.g., to suppress undesired autoimmunity while maintaining protective immunity.
Lipids 33, 869–878 (1998).

There is considerable interest in long-chain n-3 polyunsaturated fatty acids (PUFA) as natural agents to control autoimmunity and destructive inflammatory responses. The two n-3 PUFA of greatest interest are docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) and eicosapentaenoic acid ($20{:}5^{\Delta 5,8,11,14,17}$), best recognized as components of oil from cold-water fish. DHA and eicosapentaenoic acid have similar although not identical activities, including suppression of arachidonic acid (AA, $20{:}4^{\Delta 5,8,11,14}$) metabolism and reduction of cancer growth (1,2). Their effects on immune cell function are puzzling and controversial, however. The predominant, but by no means exclusive, opinion is that they suppress lymphocyte proliferation and macrophage activation (3). The mechanisms of action of n-3 fatty acids are not well understood and may include effects on eicosanoid production, lipid peroxidation, and membrane structure. The influence of DHA on membrane structure is of particular interest to us because membranes are fundamental to all cells and provide an opportunity for DHA (in membranes) to participate in very diverse biological processes.

One likely reason for variable effects of n-3 PUFA on immune cells is the complexity of the immune system itself. The lymphocyte lineage is divided into T and B lymphocytes; T lymphocytes may be further distinguished (subdivided) by the expression of the membrane proteins CD4 and CD8. Lymphocytes may be immature or mature; they may be naïve or memory cells, depending on whether they have been exposed to antigen; and they may be driven by cytokines to distinct differentiation states, such as in the Th1/Th2 paradigm. There is substantial evidence that different cell types have distinct stimulation and costimulation requirements (4–6) and use nonidentical, though perhaps overlapping, signaling pathways (7–9). Thus, the outcome of membrane enrichment with n-3 fatty acids will depend on several variables, including the nature of the target cell, the presence of accessory cells, and the type, magnitude, and multiplicity of stimuli.

To use n-3 PUFA rationally and effectively to improve human health, we need a clearer understanding of their effects on immune function. In this report we focus on the effect of DHA on splenic T lymphocyte proliferation. T lymphocytes play key roles in immunity, orchestrating as well as implementing immune responses. We have chosen DHA for study because it is the longest, most unsaturated fatty acid found in abundance in nature and because of cells' propensity to retain this fatty acid in membrane phospholipids at the expense of n-6 and n-9 fatty acids (10). Membranes enriched in DHA display significant changes in membrane structure and function, including increased permeability and fusibility, formation of putative lipid microdomains, and alterations in membrane proteins (11–16). Because of the importance of the plasma membrane in lymphocyte activation, DHA-induced alterations in membrane structure may modulate lymphocyte function. We predict, furthermore, that DHA will have disparate effects on T cells activated through different membrane-associated receptors. Here we explore the importance of the triggering stimulus (lectin, alloantigen, and monoclonal anti-Thy-1 antibody) on modulation of lymphocyte activation and proliferation by DHA.

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; Con A, concanavalin A; DHA, docosahexaenoic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; IL2R, interleukin 2 receptor; PUFA, polyunsaturated fatty acid(s).

MATERIALS AND METHODS

Reagents. RPMI 1640 and Hanks' balanced salt solution were purchased from Gibco (Grand Island, NY), fetal bovine serum (FBS) and bovine calf serum were obtained from HyClone Labs (Logan, UT), concanavalin A (Con A) was purchased from Pharmacia (Piscataway, NJ), and all other cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-CD90 (anti-Thy-1.2; clone 30H12) and anti-CD25 [anti-IL2Rα; clone AMT13; phycoerythrin-labeled and fluorescein isothiocyanate (FITC)-labeled] were bought from Boehringer-Mannheim Biochemicals (Indianapolis, IN), anti-CD90 (anti-Thy-1; clone G7; unlabeled and FITC-labeled) was purchased from PharMingen (San Diego, CA), and anti-rat immunoglobulin G (IgG) (R-3756) was obtained from Sigma Chemical Co. Fluo-3 was obtained from Calbiochem (LaJolla, CA), fatty acids were purchased from Nu-Chek-Prep (Elysian, MN), [methyl-^{3}H]thymidine (2 Ci/mmole) was bought from Amersham Corp. (Arlington Heights, IL), and BetaMax ES scintillation fluid was purchased from ICN Biochemicals (Costa Mesa, CA).

Mice. Young adult BALB/c and C57BL/6 male mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and provided standard rodent chow (#5001; Purina, Richmond, IN) and water *ad libitum*. They were used in an approved protocol, housed in the AAALAC-accredited Animal Facility at the Indiana University—Purdue University at Indianapolis School of Science, and euthanized under the guidelines of the 1993 American Veterinary Medical Association Panel on Euthanasia.

Preparation of fatty acid-enriched media. To enrich medium with fatty acids from an ethanolic stock, commercial fatty acids in hexane were evaporated under a stream of nitrogen and then dissolved at levels of 25 to 100 mg/mL ethanol. The fatty acids were added gradually to RPMI 1640 medium supplemented with 100 units penicillin/mL, 100 µg streptomycin/mL, 25 mM HEPES buffer, and 2 mM glutamine ("supplemented RPMI") also containing 50 µM 2-mercaptoethanol and 5% FBS ("complete medium"), with mixing on a stir plate. The control medium received a comparable amount (0.05%) of ethanol vehicle only. The media were sterilized by filtration through 0.22-µm Millipore filters (Milford, MA) and used immediately. Ethanolic fatty acid stocks were covered with foil and stored at −20°C.

In some experiments, DHA-enriched medium was prepared by the method described by Spector and Hoak (17). Briefly, ≤5 mg of DHA in 5 mL hexane was added to Celite (0.1 mmol fatty acid per g Celite) and evaporated to dryness under nitrogen. The dried, DHA-coated Celite was mixed with supplemented RPMI containing 1% (wt/vol) bovine serum albumin (BSA) to produce the desired DHA concentration(s); in experiments shown here, the final DHA concentration was 50 µg/mL of BSA-containing RPMI. DHA was transferred from the Celite to the BSA in the medium by stirring the suspension, protected from light, at room temperature for 30 min. Celite was removed by centrifugation (20 min, $1360 \times g_{max}$, 4°C), and the medium was sterilized by filtration through a 0.22-µm Millipore filter. Medium was covered in foil and stored frozen. FBS and 2-mercaptoethanol were added to make complete medium just prior to use.

Cell cultures. Spleens were teased into single-cell suspensions, cell viability was measured by trypan blue exclusion, and the cells were resuspended at 5×10^6 cells/mL of complete medium (described above). As indicated in the Results section, complete medium was supplemented with DHA or AA, either complexed to BSA or in ethanol. For Con A stimulation, 5×10^5 BALB/c spleen cells/well were cultured in triplicate wells in 96-well flat-bottomed plates with various Con A concentrations in a final volume of 200 µL/well. For anti-Thy-1 stimulation, 5×10^5 BALB/c spleen cells were cultured in triplicate wells with 0 to 25 µg anti-Thy-1 (30H12 or G7) per mL of complete medium. With both Con A and anti-Thy-1 stimuli, the cultures were incubated for 48 h under standard culture conditions (in a 37°C, humidified, 5% CO_2 incubator), and [^{3}H]thymidine (0.5 µCi/well) was present during the last 4 h of incubation. Cultures were harvested onto glass-fiber filters with an automated cell harvester (Cambridge Technology, Watertown, MA), and radioactivity incorporated into DNA and present on the filters was measured by liquid scintillation counting in an LS1801 beta counter (Beckman Instruments, Fullerton, CA).

For primary mixed lymphocyte cultures, single-cell suspensions of C57BL/6 splenocytes were treated with 40 µg mitomycin C/mL for 30 min at 37°C, washed three times in Hanks' balanced salt solution, and resuspended at 5×10^6 cells/mL of complete medium plus DHA complexed to BSA or BSA alone. Five hundred thousand BALB/c responder splenocytes, prepared as described in the paragraph above, were mixed in triplicate wells with an equal number of mitomycin C-treated C57BL/6 stimulator splenocytes in a total volume of 200 µL/well. The plates were incubated for 5 d under standard culture conditions, with 0.5 µCi/well of [^{3}H]thymidine present during the last 24 h of incubation. The cultures were harvested onto filters, and radioactivity present on the filters was measured as described above. For secondary mixed lymphocyte cultures, ~1×10^8 naïve BALB/c splenocytes were cultured with mitomycin C-treated C57BL/6 splenocytes at a ratio of 10:1 in 60 mL of medium (either DHA-enriched or control medium) in two 75-cm^2 stationary flasks for 10 d, at which time the cells were harvested and washed by centrifugation ($500 \times g_{max}$, 10 min) in Hanks' balanced salt solution. The cells were restimulated for 3 d with mitomycin C-treated C57BL/6 splenocytes in 96-well plates as described above. [^{3}H]Thymidine (0.5 µCi/well) was added during the last 24 h of incubation, and radioactivity incorporated into DNA was measured as usual.

Intracellular free calcium. Fluo-3/AM was reconstituted in dimethylsulfoxide to a concentration of 1 µg/µL and stored covered with foil at −20°C. BALB/c spleen cells were cultured at a concentration of 5×10^6 cells/mL for 2 h in control or DHA-supplemented medium. Cells (1×10^6) from culture were resuspended in 1 mL of supplemented RPMI medium without serum, and 5 µL of fluo-3/AM was added to the cells, which were then incubated for 30 min in a humidified 37°C incubator with 5%

CO_2. In some tubes, G7 monoclonal anti-Thy-1 antibodies (4 µg) were added concurrently with the fluo-3/AM. After incubation, the tubes were placed in the sampling station of a Coulter Epics Elite ESP flow cytometer (Miami, FL) and immersed in a beaker containing 37°C water. By using forward and side scatter, a gate was established around the viable lymphocyte population; 525-nm fluorescence emission from the cells was measured for approximately 1 min to obtain baseline readings. Data collection was then paused, either 10 µg of Con A or 11.5 µg of anti-rat IgG (to crosslink cell surface-bound anti-Thy-1 antibodies) was added to the cells to stimulate an increase in intracellular free calcium, and data collection was resumed for an additional 9 min. Baselines were established so that 90% of the cells before stimulation were at or below the baseline. The percentage of cells responding was determined by measuring the percentage of cells above the baseline at 2 min after addition of the stimulus (i.e., at the maximal response).

Interleukin 2 receptor (IL2R) expression. Five million spleen cells contained in 1 mL were placed in each well of a 24-well plate and were incubated under standard culture conditions with 2 µg Con A/mL or 2 µg G7 anti-Thy-1 antibody per mL of control (ethanol alone) or ethanolic DHA-supplemented medium. After 24 h, the cells were harvested by centrifugation (340 × g_{max}, 10 min), resuspended in supplemented RPMI, and counted with a hemocytometer; viability was checked by trypan blue exclusion. One million cells were added to 1 mL of 0.1 M methyl α-D-mannopyranoside in supplemented RPMI containing 15% bovine calf serum and incubated at 4°C for 30 min. The cells were centrifuged at 340 × g_{max} for 5 min, the supernatant was discarded, and 1 mL of a solution containing 2 µg/mL of phycoerythrin- or FITC-labeled anti-CD25 (IL2R α chain) antibody in phosphate buffered saline was added to the pellet. The cells were incubated for 30 min at 4°C and then analyzed by flow cytometry. By using forward and side scatter, gates were set to exclude debris; the percentage of $IL2R^+$ cells was calculated with Immuno 4 software (Coulter Corp.), which compares the fluorescence of stimulated vs. unstimulated spleen cells.

Statistical methods. Student's *t*-test was used to compare two means, and analysis of variance was used for multisample analyses. A probability $P \leq 0.05$ was the criterion for statistical significance, i.e., accepting that measured values were not the same.

RESULTS

DHA suppresses Con A mitogenesis and the mixed lymphocyte reaction. Our objective was to explore the effect of DHA on early activation events and the subsequent proliferation of lymphocytes triggered with qualitatively and quantitatively different stimuli. To establish the DHA dose for subsequent experiments, we measured Con A-stimulated lymphocyte proliferation as a function of DHA concentration in culture medium (Fig. 1A). As anticipated from our previous work (18) and that of other investigators (19,20), DHA inhibited proliferation in a dose-dependent fashion. At 10 µg DHA/mL, lymphoproliferation was ~80% of control (ethanol vehicle only); the inhibition was reproducible and statistically significant but not so severe that proliferation was undetectable. We initially tested DHA delivered bound to BSA; because the

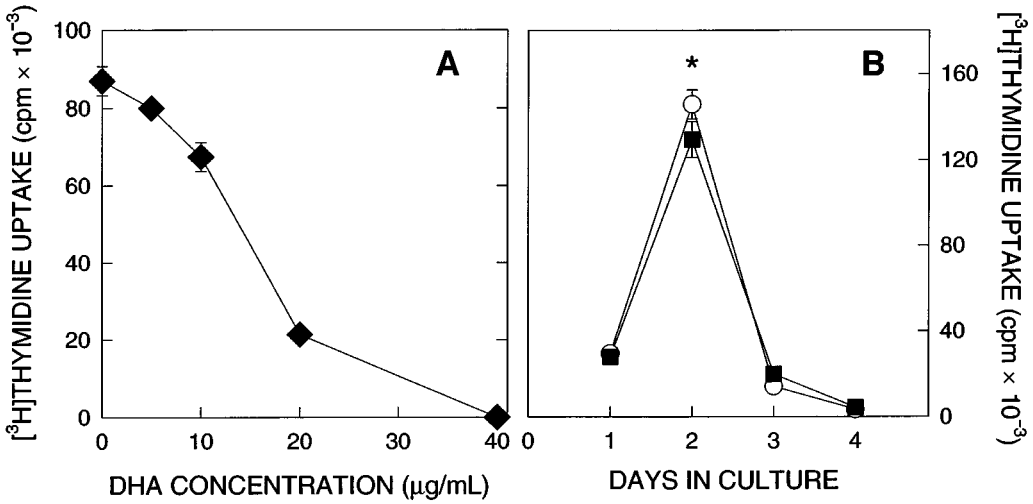

FIG. 1. Docosahexaenoic acid (DHA) decreases concanavalin A (Con A)-stimulated proliferation in a dose-dependent fashion without grossly altering the peak response. (A) Splenocytes (5×10^5 cells/well) were cultured for 2 d with 1.5 µg Con A/mL in medium supplemented with various DHA concentrations (as shown on the abscissa) or in the ethanol vehicle alone. [^{3}H]Thymidine (0.5 µCi/well) was present during the last 4 h of incubation. The data are expressed as means ± SD ($n = 3$); some error bars are smaller than the symbol. (B) Splenocytes (5×10^5 cells/well) were cultured with 2 µg Con A/mL in the presence (■) or absence (○) of 10 µg DHA/mL; there were no medium changes after the cultures were established. Cultures were harvested every day for 4 d, and [^{3}H]thymidine was present for the last 4 h of incubation. The data are expressed as means ± SD ($n = 3$). The asterisk denotes a significant difference between DHA-supplemented and control cultures ($P < 0.05$, analysis of variance).

two fatty acid addition methods produced comparable results, for technical convenience most experiments except mixed lymphocyte reactions were performed with 10 µg DHA/mL, delivered in ethanol. Figure 1B also demonstrates that DHA does not grossly alter the temporal kinetics of Con A-induced mitogenesis. The peak incorporation of [^{3}H]thymidine was on culture day 2, whether the culture contained control medium or medium supplemented with 10 µg DHA/mL. The decreased [^{3}H]thymidine incorporation on days 3 and 4 represents a combination of nutrient exhaustion (the medium is not replenished during the culture period) and normal down-regulation of the response, and thus all subsequent measurements were made on or before the second culture day.

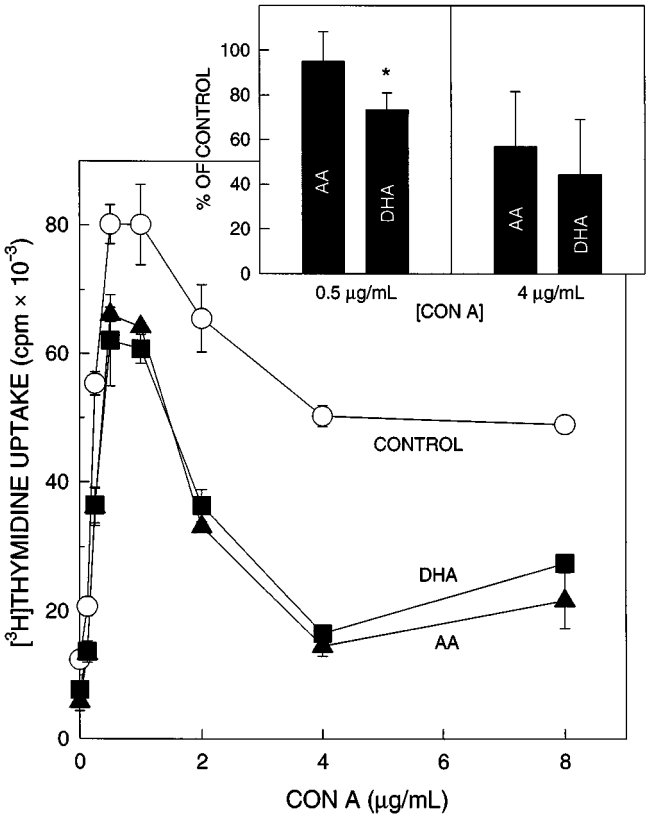

FIG. 2. DHA preferentially inhibits lymphoproliferation triggered by high Con A concentrations. Main figure: Splenocytes (5×10^5 cells/well) were cultured for 2 d in various Con A concentrations without fatty acid addition (○) or with 10 µg/mL of arachidonic acid (AA) (▲) or DHA (■) added in ethanol. [^{3}H]Thymidine (0.5 µCi/well) was added during the last 4 h of incubation, after which the cells were harvested and the incorporated radioactivity was measured by scintillation counting. Each symbol is the mean ± SD of triplicate wells; the graph is one representative experiment from three performed with control, AA- and DHA-supplemented media, and from >20 performed with DHA-supplemented and control media. Inset: The means ± SE of three independent experiments are shown for splenocytes cultured in the presence of 10 µg/mL of DHA or AA. The data are expressed as percentage of control (y-axis) for ease of presentation. Results for two Con A doses, 0.5 and 4.0 µg/mL, are shown to illustrate the greater fatty acid effect at higher Con A concentrations. The asterisk denotes greater inhibition of proliferation by DHA compared to AA ($P < 0.05$, analysis of variance). For abbreviations see Figure 1.

When we explored the inhibitory effect of DHA on cells stimulated with various Con A concentrations (Fig. 2), we observed that inhibition was more pronounced at higher (≥4 µg/mL) than lower (<1 µg /mL) Con A concentrations. In the representative experiment shown in the main portion of Figure 2, the level of proliferation stimulated by 0.5 µg Con A/mL in the presence of DHA was ~78% of control, whereas with 4 µg/mL, proliferation in DHA was ~33% of control. In a series of three experiments, DHA was slightly but significantly more inhibitory than AA (17 and 5% inhibition for DHA vs. AA, respectively; Fig. 2, inset), even though, at 10 µg/mL, the molar concentration of AA (33 µM) exceeded that of DHA (30.5 µM). To further explore this phenomenon, in the following experiments we test two other stimuli, allogeneic cells and monoclonal antibodies to Thy-1. Although the three stimuli may share some signaling pathways, the signals they induce are not identical (21,22).

Figure 3 summarizes results obtained when BALB/c splenocytes were cultured with allogeneic (C57BL/6) splenocytes, inactivated with mitomycin C, in the presence and absence of DHA. The primary response after 5 d of mixed lym-

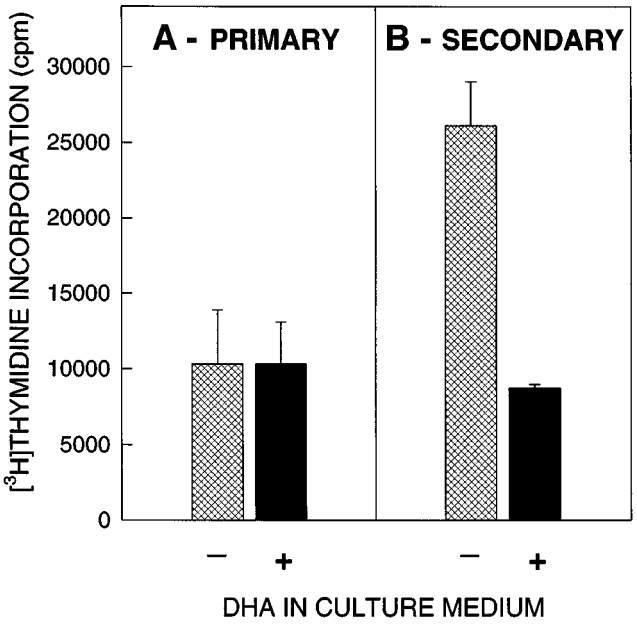

FIG. 3. Secondary mixed lymphocyte reactions are dramatically inhibited by DHA. (A) For the primary response, BALB/c splenocytes (5×10^5 cells/well) were cultured for 5 d with mitomycin C-inactivated C57BL/6 splenocytes (1:1) in microtiter wells with (solid bars) and without (hatched bars) bovine serum albumin-bound DHA at 50 µg/mL; [^{3}H]thymidine (0.5 µCi/well) was added for the last 24 h of incubation. (B) For the secondary response, BALB/c splenocytes were stimulated with mitomycin-inactivated C57BL/6 splenocytes at a ratio of 10:1 for 10 d in 75-cm^2 flasks with and without DHA supplementation, harvested, and restimulated for 3 d with mitomycin C-treated C57BL/6 stimulators as in the primary culture, with (solid bars) and without DHA (hatched). Primary and secondary cultures included unstimulated controls, the cpm of which have been subtracted as background from the cpm shown. The data are presented as means ± SE for 8 (primary response) and 5 (secondary response) experiments. For abbreviation see Figure 1.

phocyte culture was, on average, unaffected by DHA, whereas the secondary response, obtained by culturing splenocytes with stimulators in the presence or absence of DHA for 10 d and then restimulating under the same conditions, was drastically reduced by DHA. In fact, secondary responses in DHA-enriched medium were at the same levels as the primary response, that is, there was no evidence that antigen-specific clones had been expanded. Because of limited cell recovery we did not test secondary cultures established in the opposite medium, i.e., control cells restimulated in DHA-supplemented medium, and so on. DHA-induced suppression of the secondary mixed lymphocyte reaction was much more striking than that observed with Con A mitogenesis. Medium for mixed lymphocyte cultures was prepared with additional BSA (beyond that present in the FBS) to aid in fatty acid delivery, but we adjusted the amount of DHA appropriately; that is, 50 μg DHA/mL of BSA-supplemented medium produced the same inhibition of Con A-stimulated proliferation as 10 μg DHA/mL delivered in ethanol (data not shown). Although there was a small, not unexpected increase in background proliferation associated with the BSA supplement, the main differences between the mixed lymphocyte reaction and the Con A-stimulated mitogenesis were the nature of the stimulus and the duration of culture.

DHA enhances anti-Thy-1-induced proliferation. If culture duration, not the stimulus, were the determining factor in the effect of DHA, then DHA in a 2-d culture stimulated with something other than Con A should produce results similar to those seen for Con A (Fig. 2). We chose, as the alternative stimulus, antibody to Thy-1. Thy-1 is a glycosylphosphatidylinositol-linked glycoprotein implicated in T lymphocyte activation (23), through which signaling is accomplished *via* pathways overlapping with but not identical to those associated with Con A and T cell antigen receptors (21,22,24,25). Figure 4 demonstrates that, unlike Con A, anti-Thy-1-stimulated lymphocyte proliferation is augmented by the presence of DHA. With 3 to 6 μg anti-Thy-1/mL added at the beginning of culture, maximal proliferation was enhanced approximately twofold by DHA. This result was observed for two different anti-Thy-1 antibodies, G7 and 30H12, and in the absence of a crosslinking antibody. 30H12 is generally not stimulatory in the absence of crosslinking anti-IgG, however, DHA did augment the low level of proliferation triggered by 30H12 alone. We have reproduced this finding 10 times, both in BSA-supplemented and BSA-unsupplemented DHA-enriched media. This result also rules out extended culture periods as a critical component of the action of DHA, with the reservation that DHA-mediated suppression, but not enhancement, may require prolonged culture. Thus, we conclude that at least one aspect of the effect of DHA on proliferation is related to the stimulus.

DHA does not affect elevation of cytosolic free calcium or IL2R expression. Because the measure of proliferation, [^{3}H]thymidine incorporation 2 to 5 d after culture initiation, is late with respect to cells' first exposure to DHA, we were interested in monitoring earlier activation events. Our goal was to approximate where along the timeline from receptor occupation to thymidine incorporation (i.e., cell cycling) DHA exerts its influence. Two events were examined, calcium mobilization and IL2R expression, occuring within minutes and hours, respectively, of activation.

Table 1 demonstrates that the presence of DHA does not affect the percentage of splenocytes responding to either Con A or anti-Thy-1 by increasing intracellular free calcium. Approximately one-third to 40% of splenocytes respond to these

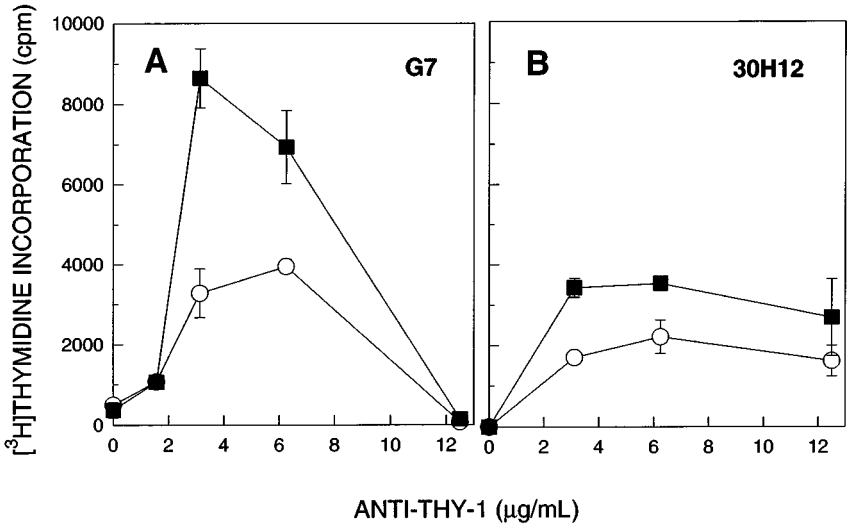

FIG. 4. In contrast to other stimuli, anti-Thy-1 evokes greater lymphoproliferation in the presence of DHA. Splenocytes (5×10^5 cells/well) were cultured in 10 μg DHA/mL (■) or ethanol vehicle alone (○), with various concentrations of monoclonal anti-Thy-1 antibodies G7 (A) or 30H12 (B). A secondary crosslinking anti-IgG was not used in either case. [^{3}H]Thymidine was added for the last 4 h of the 48-h culture period. The data shown are means ± SD for triplicate wells; one representative experiment is shown for each antibody.

TABLE 1
DHA Does Not Alter the Percentage of Lymphocytes That Elevate Calcium in Response to Mitogen[a]

	Calcium-mobilizing cells (%)	
Stimulus	Control	DHA^+
Con A	34.0 ± 3.7	33.4 ± 2.3
Anti-Thy-1	40.5 ± 3.6	39.6 ± 3.7

[a]Fresh splenocytes were incubated for 2 h in docosahexaenoic acid-supplemented (DHA^+) or control medium, washed, and loaded with the calcium-sensitive fluorescent probe fluo-3. The percentage of cells with fluorescence intensity above baseline after mitogen addition [calcium-mobilizing cells (%)] was measured by flow cytometry. For anti-Thy-1 stimulation, the fluo-3-loaded cells were pretreated with the G7 monoclonal anti-Thy-1 antibody, baseline fluorescence was measured, and crosslinking anti-rat IgG was then added to induce calcium mobilization. The percentage of cells responding with calcium mobilization was calculated at the peak of the response, 2 min after stimulus addition. The data are presented as means ± SE for three separate experiments; there were no significant differences between control and DHA^+ cells (Student's *t*-test). Con A, concanavalin A; IgG, immunoglobulin G.

T cell stimuli, consistent with the approximate proportion of T cells in mouse spleen. Table 2 shows that the peak calcium levels (average intensity of fluo-3 fluorescence 2 min after stimulation) were the same and elevated cytosolic free calcium was sustained equivalently (9 min after stimulation) in DHA-supplemented and control groups. The data are presented as ratios of stimulated/baseline to allow compilation of individual experiments. The mean numerical values for sustained fluorescence in anti-Thy-1-stimulated cells with and without DHA were 3.67 and 4.30, respectively, but statistical analysis suggested no significant difference ($P = 0.09$, Student's *t* test). Thus, there is no reason *a priori* to suspect that the effect of DHA on splenocyte proliferation is caused by altered calcium mobilization, an early activation event.

Expression of the IL2R α chain (CD25) is a lymphocyte activation marker detected several hours after cell triggering, but one that is dependent upon early activation events for relevant gene transcription. IL2R expression is not necessarily induced in parallel with other hallmarks of activation, such as elevated cytosolic calcium and IL2 synthesis (26). Thus, IL2R expression is an independent measure of the effects of DHA.

TABLE 2
DHA Does Not Affect the Calcium Levels Achieved in Response to Mitogen[a]

	Peak level		Sustained level	
Stimulus	Control	DHA^+	Control	DHA^+
Con A	6.87 ± 1.09	6.42 ± 0.12 ($P = 0.70$)	5.84 ± 0.46	5.12 ± 0.45 ($P = 0.37$)
Anti-Thy-1	4.57 ± 0.17	4.18 ± 0.43 ($P = 0.44$)	4.30 ± 0.23	3.67 ± 0.16 ($P = 0.09$)

[a]Cells described in Table 1 were analyzed for relative calcium levels (mean fluorescence channel) at baseline, the peak response (2 min after stimulus addition), and sustained response (after 9 min of stimulation). The data are presented as -fold fluo-3 fluorescence increase, i.e., the ratio of mean fluorescence channel after stimulation to mean fluorescence channel of the baseline. Statistical analyses revealed no significant differences between control and DHA+ groups (Student's *t*-test, $n = 3$ separate experiments). For abbreviation see Table 1.

In Table 3 we present the results of experiments in which splenocytes were cultured with Con A or anti-Thy-1 in the presence or absence of DHA. After 24 h, IL2R expression was measured by flow cytometry with fluorescent antibodies to CD25. The data indicate that DHA does not affect IL2R expression on splenocytes stimulated with either Con A or G7. The percentage of $IL2R^+$ cells and the mean channel fluorescence, an indication of surface receptor density, were equivalent in the presence and absence of DHA. Compared to Con A, anti-Thy-1 treatment led to fewer $IL2R^+$ splenocytes, with a lower receptor density per cell (Table 3), and less incorporation of [^{3}H]thymidine (Fig. 4), whereas the percentage of cells mobilizing calcium in response to anti-Thy-1 was greater than for Con A (Table 1), consistent with other reports dissociating intracellular free calcium responses from IL2R expression (26).

DISCUSSION

In this report we demonstrate that the activating stimulus dictates whether DHA has a suppressive or enhancing effect on lymphocyte proliferation. This result may help to reconcile apparently conflicting reports on the action of this fatty acid, and it implies that DHA may be used to modulate immune responses selectively. In addition, the data we show here are consistent with the effect of DHA being expressed late, i.e., during cell cycling, rather than during early activation events.

We entertained two interpretations for the differential effect of DHA on responses to low vs. high Con A doses and to mitogen or alloantigen vs. anti-Thy-1. First, there may be disparate populations responding; second, the stimulus (signaling pathway) may dictate the effect of DHA. These are, however, not mutually exclusive hypotheses; in fact, the disparate responses of various cell types imply differences in signaling. Evidence suggestive of multiple responding cell populations dates back decades ago when it was observed that purified T cell populations responded within a narrow, high Con A dose range, whereas T cells supplemented with adherent splenic or peritoneal exudate cells proliferated at lower Con A doses (27), indicating either multiple populations (the T cell population responding to low Con A doses required accessory cells) or one responding population requiring costimulation when the Con A dose was limiting. Even disease states yield clues for disparate populations responding to different Con A doses; lymphocytes from patients with light atopic dermatitis showed reduced Con A reactivity at low Con A concentrations (28). We have determined that DHA does not significantly alter the proportion of T ($Thy\text{-}1^+$) and B (surface immunoglobulin-positive) cells in unstimulated cultures (18), or the percentage of $CD4^+$ and $CD8^+$ T cells in Con A-stimulated cultures (preliminary data not shown). However, our results in Figure 3 strongly suggest that development of immunologic memory, as modeled by the secondary mixed lymphocyte reaction, is severely decreased by DHA. Naïve and memory T cells are a good example of different responding cell "types" with different signaling requirements. In contrast

TABLE 3
DHA Does Not Affect Interleukin 2 Receptor Expression on Splenocytes[a]

	Positive cells (%)		Mean fluorescence channel	
Stimulus	Control	DHA^+	Control	DHA^+
Con A	49.7 ± 7.7[b]	54.0 ± 6.7[b]	1.04 ± 0.08[c] 3.01[d]	1.08 ± 0.10[c] 2.3[d]
G7	29.1 ± 4.4[b]	28.5 ± 4.1[b]	0.76 (0.75–0.76)[e] 1.68[d]	0.80 (0.76–0.84)[e] 1.52[d]

[a]Splenocytes were stimulated with Con A or G7, or left unstimulated, in the presence (DHA^+) or absence (Control) of DHA. After 24 h, the cells were harvested and stained with FITC- or phycoerythrin-labeled anti-CD25 (to detect interleukin 2 receptor) for 30 min on ice. The viable cell population was gated, and fluorescence intensity was measured with a Coulter Elite ESP flow cytometer. Data from the background (stained, unstimulated cells) and experimental (stained, stimulated cells) were loaded into Coulter's Immuno 4 data analysis software, and the background histogram was automatically subtracted from experimental histograms to produce "Positive cells (%)."
[b]Mean ± SE (n = 3–4 separate experiments).
[c]Mean ± SE (n = 3 separate experiments, phycoerythrin-labeled anti-CD25).
[d]Single experiment, FITC-labeled anti-CD25.
[e]Mean (range), n = 2 separate experiments, phycoerythrin-labeled anti-CD25. There were no significant differences (Student's t-test) between DHA^+ and control groups having multiple samples. For other abbreviations see Table 1.

to memory cells, naïve T cells respond well to Con A, displaying elevated intracellular calcium and proliferation (29), are strictly dependent upon costimulation, especially through CD28/B7-2 interactions (5), and do not use IL12 in place of CD28/B7-2 ligation (30); naïve $CD4^+$ cells are not activated by IL15, and naïve $CD8^+$ T cells require exposure to their specific restricting class I major histocompatibility complex molecule to survive (4).

If memory cells have a lower threshold for activation, as suggested by this evidence, is DHA modulating the magnitude of the signal perceived by lymphocytes? That is, does DHA potentiate responses and thereby deliver superoptimal signals from mitogens and alloantigens, and bring weak signals (anti-Thy-1) into the optimal range? The data do not argue strongly for this interpretation. Neither the Con A dose-response curve (in the 0.25–1.0 μg/mL range) nor the anti-Thy-1 dose-response curve is left-shifted, as one would predict for potentiation. The sizable percentage of cells responding to anti-Thy-1 by elevating cytosolic free calcium or expressing CD25 suggests that anti-Thy-1 is not a weak signal in need of potentiation. Finally, the cells showing the most dramatic difference in DHA inhibition, naïve and memory T cells, have significant physiological differences beyond mere antigen dose preference.

The stimulus dose is not unimportant, however. Lymphocytes are stimulated to proliferate at low Con A concentrations but undergo apoptosis at high Con A concentrations (32). Not unexpectedly, intracellular activation events follow dose-dependent kinetics, e.g., substrates including ERK1, ERK2 and c-jun, are tyrosine-phosphorylated in a Con A dose-dependent fashion (32). Whereas binding of increasing amounts of Con A to the cell surface is a hyperbolic function, the resultant cellular responses are more complicated, i.e., sigmoidal, implying cooperativity (interaction) between receptors and other membrane proteins (33). Con A binds to and sequentially redistributes multiple sets of cell surface receptors, which are distinguished by the Con A concentration required for redistribution (first set, low dose; second set, high dose) (34). Con A receptors are present in small clusters, which bind succinyl-Con A (a dimeric rather than tetrameric form of the lectin) with high affinity, and co-existing large aggregates, which bind Con A with only low affinity (35). Ultimately, at very high Con A concentrations, competition between lectin molecules for the limiting number of surface receptors reduces Con A-induced receptor crosslinking and precludes a proliferative cell response.

What action of DHA may concurrently decrease mitogenesis and alloreactivity and increase responses through Thy-1? Viewing our results from the standpoint of the effect of DHA on membrane structure, we suggest that phospholipids containing DHA may act as annular lipids around membrane receptors, influencing receptor function; or may induce membrane microdomains into which membrane proteins, glycoproteins, and glycolipids may be selectively recruited or excluded. The potential scenario with DHA and Thy-1 is particularly interesting from a membrane structure standpoint. Thy-1 is a glycoprotein found on murine thymocytes and T cells, neurons of several species, fibroblasts, and rat mast cells (36), and is involved in T lymphocyte activation (23), inhibition of neurite outgrowth (37), and mast cell triggering (38). However, Thy-1 is lipid-linked, and therefore its signaling ability must be related to its ability to cluster with appropriate transmembrane proteins. Indeed, Thy-1 is found complexed with a 100 kDa transmembrane protein (39), as well as tyrosine kinases and a tyrosine phosphatase (40–42), in membrane domains poorly soluble in nonionic detergents. One may argue that the detergents used to isolate Thy-1 or the detergent-resistant domains, or the antibodies used to aggregate Thy-1 for cell activation, create an artificial Thy-1/domain association (43,44). However, the observation that most glycosylphosphatidylinositol-linked proteins (in contrast to transmembrane proteins) preferentially partition with certain lipids (cholesterol, sphin-

golipids, saturated fatty acids) during detergent extraction (45,46), implies that these proteins prefer a membrane environment different from other membrane proteins. Furthermore, functional lipid-linked Thy-1 and nonfunctional transmembrane Thy-1 (engineered with the transmembrane and cytoplasmic domains of NCAM-140) are found naturally clustered in distinct domains (47), strongly implying that membrane lipid domain formation may be a key element in regulating Thy-1 function. We have reported that DHA-containing phospholipids interact poorly with cholesterol, inducing the membrane to segregate into DHA-rich/cholesterol-poor and DHA-poor/cholesterol-rich domains (14). Thus, there is good reason to believe that membrane enrichment with DHA will induce lipid microdomain formation that promotes or stabilizes detergent-resistant (cholesterol-rich) domains. We speculate that its lipid anchor will cause Thy-1 to associate preferentially with select (cholesterol-rich) domains induced by DHA, thereby favoring Thy-1's interaction with signaling proteins and enhancing lymphoproliferation.

Much of the confusion surrounding n-3 fatty acids' effects on lymphocytes arises from technical differences in the experiments performed: free vs. esterified fatty acids, cell type, species, stimulus, and assay method. Free fatty acids obliterated the spike of intracellular free calcium (release from intracellular stores) in T cell lines (48,49); but once esterified into phospholipids, fatty acids did not affect intracellular stores but instead depressed the sustained calcium elevation, purportedly by activating the plasma membrane Ca^{2+}-ATPase and enhancing calcium extrusion (50,51). In our experiments, we expect that even the 2-h fatty acid exposure will produce significant esterification of DHA into phosphatidylethanolamine. In the myeloid leukemia T27A, for example, we found esterified DHA present after 2 h at almost one-third of the 24-h (plateau) level (52). Our results with normal mouse splenocytes agree in part with those of Chow *et al.* (50), in that the initial spike of cytosolic free calcium is unaffected by DHA enrichment; we did not, however, observe a depression of sustained calcium levels. These studies also differ with respect to stimuli and monitoring methods. Stimuli included anti-CD3 (49,50), Con A (48) (current study), and anti-Thy-1 (current study); fluorescence methods included flow cytometry (51) (current study), spectrofluorimetry (48–50), and the fluorophores Indo-1 (51), fura-2 (48–50), and fluo-3 (current study). The same argument that experimental differences may lead to different outcomes applies to the measurement of IL2R. Khalfoun *et al.* (19) found increased CD25 expression on phytohemagglutinin-stimulated human lymphocytes cultured *in vitro* for 72 h with DHA, whereas we did not detect such a difference in samples from a different lymphoid tissue of a different species, stimulated with different mitogens, and tested at a much earlier time. Our study is more similar to that of Calder and Newsholme (53), who stimulated rat lymphocytes with Con A for 48 h and found no difference in IL2R expression in the presence and absence of DHA.

Because DHA does not alter cytosolic free calcium elevation or IL2R expression but does affect [^{3}H]thymidine incorporation, it is tempting to speculate that DHA affects cell cycling. Progression through the cell cycle is coordinated with phosphatidylcholine synthesis to allow net phospholipid accumulation during S phase (54); interestingly, the incorporation of excess DHA into phosphatidylcholine instead of its usual phospholipid species, phosphatidylethanolamine, is associated with reduced cell proliferation (52). We have recently reported the apparent block or prolongation of S phase in DHA-treated Con A-activated lymphocytes (18), consistent with reports of n-3 fatty acid inhibition of S phase in cancer cells (55,56). This area is clearly ripe for more extensive study.

Overall, our data suggest that DHA may inhibit or enhance lymphocyte proliferation depending upon the stimulus used to elicit the response. We cannot at this point fully rule out that it is the magnitude of the signal rather than the particular membrane receptor(s) involved that is key to the differential effects of DHA, although, for reasons stated earlier, we favor the disparate membrane receptor theory. The possibility that enrichment of membrane phospholipids with DHA influences the protein and lipid composition of membrane domains associated with cell signaling is a very attractive one. A final consideration is that, although Con A and anti-Thy-1 both target T cells, the T cell subset responding to each stimulus may not be identical. We are currently exploring whether DHA influences the development or maintenance of Th1 and Th2 cells, two functionally disparate T cells thought to have different activation requirements. Armed with more complete information on the effects of DHA on T lymphocytes, we will be in a better position to utilize DHA to control immune responses.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute (RO1CA57212) and Phi Beta Psi Sorority.

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[Received April 21, 1998, and in final revised form June 25, 1998; revision accepted August 11, 1998]

Replacement of Partially Hydrogenated Soybean Oil by Palm Oil in Margarine Without Unfavorable Effects on Serum Lipoproteins

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ABSTRACT: We have compared the effects of three different margarines, one based on palm oil (PALM-margarine), one based on partially hydrogenated soybean oil (*TRANS*-margarine) and one with a high content of polyunsaturated fatty acids (PUFA-margarine), on serum lipids in 27 young women. The main purpose of the study was to test if replacement of *trans* fatty acids in margarine by palmitic acid results in unfavorable effects on serum lipids. The sum of saturated fatty acids (12:0, 14:0, 16:0) was 36.3% of total fatty acids in the PALM-diet, the same as the sum of saturated (12:0, 14:0, 16:0) (12.5%) and *trans* (23.1%) fatty acids in the *TRANS*-diet. This sum was 20.7% in the PUFA-diet. The content of oleic acid was 37.9, 35.2, and 38.6%, respectively, in the three diets, whereas linoleic acid amounted to 16, 13.5, and 27.3%, respectively. Total fat provided 30–31% and the test margarines 26% of total energy in all three diets. The subjects consumed each of the diets for 17 d in a Latin-square crossover design. There were no significant differences in total cholesterol, low density lipoprotein (LDL)-cholesterol and apolipoprotein B (apoB) between the *TRANS*- and the PALM-diets. High density lipoprotein (HDL)-cholesterol and apoA-I were significantly higher on the PALM-diet compared to the *TRANS*-diet whereas the ratio of LDL-cholesterol to HDL-cholesterol was lower, although not significantly ($P = 0.077$) on the PALM-diet. Total cholesterol, LDL-cholesterol, and apoB were significantly lower on the PUFA-diet compared to the two other diets. HDL-cholesterol was not different on the PALM- and the PUFA-diets but it was significantly lower on the *TRANS*-diet compared to the PUFA diet. Compared to the PUFA-diet the ratio of LDL- to HDL-cholesterol was higher on both the PALM- and the *TRANS*-diets whereas apoA-I was not different. Triglycerides and lipoprotein (a) were not significantly different among the three diets. We concluded that nutritionally, palmitic acid from palm oil may be a reasonable alternative to *trans* fatty acids from partially hydrogenated soybean oil in margarine if the aim is to avoid *trans* fatty acids. A palm oil-based margarine is, however, less favorable than one based on a more polyunsaturated vegetable oil.

Lipids 33, 879–887 (1998).

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Abbreviations: Apo, apolipoprotein; BMI, body mass index; E%, percentage of energy; HDL, high density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein (a); PALM-margarine, a hard zero-*trans* margarine containing 80% palm oil, 11% soybean oil, and 9% rapeseed oil; PHSO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids; PUFA-margarine, a commercial soft highly polyunsaturated margarine consisting of coconut, palm, refined sunflower, and refined rapeseed oils; *TRANS*-margarine, a hard margarine containing a high amount of *trans* fatty acids from PHSO, containing 56% PHSO, 34% refined rapeseed oil, and 10% refined soybean oil.

Partially hydrogenated vegetable oils constitute a major ingredient in margarine production. Such oils contain a number of geometrical (*cis-trans*) and positional isomers of mono- and diunsaturated fatty acids and saturated fatty acids. The content of *trans* unsaturated octadecenoic (C_{18}) fatty acids in margarines varies from 10.7 to 30.1% (1), and margarine is the most important single source of *trans* fatty acids in, for example, the Norwegian diet.

Trans fatty acids from partially hydrogenated vegetable oil increase serum total and low density lipoprotein (LDL) cholesterol (2–9), decrease high density lipoprotein (HDL) cholesterol (4,5), and increase lipoprotein (a) [Lp(a)] (6,9–11) in humans. Replacement of *trans* fatty acids by less atherogenic fatty acids in margarines is therefore desirable.

Palm oil, unlike soybean oil, can be used without hydrogenation to achieve a certain hardness of margarine products because of its semisolid texture at room temperature. Palm oil consists of 50% saturated and 50% unsaturated fatty acids and has a content of 43.8% palmitic acid and 10.6% linoleic acid (12). From the literature it appears that the effects of palmitic acid and palm oil on blood lipids are controversial. Hegsted *et al*. (13) reported that palmitic acid is cholesterol-increasing, but less so than myristic acid. Nestel *et al*. (6) found no difference in total and LDL cholesterol when an elaidic acid-rich diet was compared to one rich in palmitic acid. Ng *et al.* (14) found a palm oil diet not to be hypercholesterolemic in Malaysian volunteers. In a later study Ng *et al.* (15) found that dietary palmitic and oleic acids exert similar effects on serum cholesterol and lipoprotein profiles in normocholesterolemic men and women. Marzuki *et al.* (16) found no significant difference in effect on total, LDL, and HDL cholesterol when palm oil was compared to soybean oil. Choudhury

et al. (17) suggest that $C_{16:0}$ is not always a cholesterol-increasing fatty acid. They found no significant differences between palm olein and olive oil. On the other hand, Temme *et al.* (18) found a significant rise in total serum cholesterol with a diet rich in palmitic acid compared to one rich in oleic acid. One study in primates also indicated that palmitic acid increases total cholesterol (19).

As far as we know, no study has been published where the effects of palm oil on serum lipids have been directly compared to those of partially hydrogenated soybean oil (PHSO). In this study we compared the effects of three test margarines on serum lipids and Lp(a). One margarine with a high content of PHSO (*TRANS*-margarine), one of similar hardness but consisting of 80% palm oil (PALM-margarine), and one high in polyunsaturated fatty acids (PUFA-margarine). The aim of the study was to test whether palmitic acid can replace *trans* fatty acids in margarine without producing unfavorable effects on blood lipids and to see how a palm oil-based margarine compared nutritionally to one high in PUFA.

MATERIALS AND METHODS

Participants and their baseline characteristics. Thirty female home economics students were recruited in a strictly controlled dietary study. Criteria for inclusion were that the participants should be reliable and have a regular meal pattern. One person had been operated on for thyroid cancer (about three-quarters of the gland removed) and one had hypothyroidism. Both were well-regulated on thyroid hormone therapy. Two had a previous diagnosis of thrombosis and one of these had a lack of protein-S. Otherwise all participants were in good health with no history of diabetes, anemia, renal, hepatic or gastrointestinal disturbances, hypertension or intolerance, and all had normal dietary habits. None was taking any medication known to affect serum lipids except for oral contraceptives. We had no screening criteria with regard to smoking habits, age, physical activity, or body weight. All participants were requested to maintain their regular lifestyles and especially their usual extent of physical activities throughout the study. They were asked to abstain from alcohol consumption during the study period. In addition they were asked to report in a diary any deviation from their usual behavior.

Two persons withdrew during the study, and one participant had not fasted before blood sampling and this person also had problems with compliance and was excluded from the study. Thus, data from 27 subjects with a mean age of 27 yr (SD 5.8, yr range 19–42 yr) were evaluated. Seven of the women were taking oral contraceptives and 10 were smokers. Baseline levels of serum lipids and lipoproteins are presented in the Results section.

The average weight of the participants was 77.5 kg (SD 13 kg) ranging from 56.4 to 100 kg, and the body mass index (BMI) ranged from 20 to 36 kg/m^2 (mean 26.5 kg/m^2, SD 4.1 kg/m^2). Four persons had a BMI above 30. Eliminating the data of these four participants from the statistical analysis caused negligible changes in blood parameters except for total serum cholesterol (see below).

The protocol and the objective of the study were explained to the subjects in detail, and they gave their informed consent before entering the study. No payment was given except for free food during the study. The study protocol was approved by the Regional Committee for Ethics in Biomedical Research of Norway.

Habitual diet. The participants filled in a quantitative food frequency questionnaire (20) designed to cover the habitual diet, and each questionnaire was thoroughly checked. The calculated average amount of total fat in the habitual diet was found to be 31% of energy (SD 5.7%). The mean daily intake of saturated fatty acids, monounsaturated fatty acids, and PUFA was 29, 26, and 13.9 g, respectively, and the intake of linoleic and α-linolenic acids was 10.4 and 1.4 g, respectively. The calculated mean intake of cholesterol was 259 mg/d (SD 141 mg/d).

Study design. The study ran from January to March 1996, during periods of 17 d. Studies have demonstrated new stable levels of serum lipids and lipoproteins within 14 d on a controlled diet (21–23). Each person received the three diets in a sequence determined by assignment to one of six possible sequences as directed by a Latin-square design. The participants were fed simultaneously, and all three diets were fed in the three periods. The study was designed as an intraindividual crossover comparison of the effects on blood lipids of the three diets. In this way, variation due to residual effects of the previous diet or to drift of variables over time could be minimized. After the end of the test period, the participants crossed over to the next diet with a wash-out period of 1 wk. During this week the subjects returned to their normal eating habits.

Body weight without heavy clothes was monitored twice a week and read to the nearest 0.1 kg. Body height was measured without shoes and read to the nearest 0.1 cm. BMI was calculated as weight (kg)/height^2 (m)^2. Dietary compliance was checked daily by interview and diaries.

Test margarines. We used three different margarines in our study. We wanted to test a hard zero-*trans* margarine containing palm oil (PALM-margarine) with a hard margarine containing a high amount of *trans* fatty acids from PHSO (*TRANS*-margarine). The PALM-margarine was produced from 80% palm oil, 11% soybean oil and 9% rapeseed oil, and the *TRANS*-margarine from 56% PHSO, 34% refined rapeseed oil and 10% refined soybean oil. The third margarine was a commercial soft highly polyunsaturated margarine consisting of coconut oil, palm oil, refined sunflower oil and refined rapeseed oil (PUFA-margarine). Because of its high content of sunflower oil, this margarine contained more vitamin E than the other two (205 mg/kg vs. 81 and 100 mg/kg in the PALM- and the *TRANS*-margarines, respectively).

The fatty acid compositions of the three margarines are given in the Results section. The margarines were produced by the addition of water, vitamin A, vitamin D, NaCl, aroma (diacetyl, 0.002%), β-carotene as color, and emulsifier (soybean

lecithin, 0.3%). The PALM-margarine contained 16% water, the *TRANS*-margarine 15.7%, and the PUFA-margarine 18%.

Experimental test diets. The three diets were based on a 7-d menu and were calculated by using a computer-based, nutrient calculation program. They were designed to have the same nutrient composition except for the fatty acids and for the tocopherol content. The diets were calculated to contain 15.6 energy percent (E%) protein, 34 E% fat, and 50.4 E% carbohydrate. The fat from the background diet was calculated to supply a minimal amount of 6 E% while the test fat was planned to amount to 28 E%. The fat from the background diet came from meat, fish, dairy products, and cereals. The menu for the three experimental diets contained the same basic food items and differed only in the source of test margarine used for spreading, baking, and cooking. The test fats were also incorporated into the menus in several foods including bread, buns, porridge, and sauces.

Dinner was prepared and served under supervision in a dining room at the college from Monday through Friday. All meals including weekend meals were prepared at school. Supper and breakfast for the next day were sent home. Each Friday, weekend meals were packaged for home consumption. Perishable food was sent in frozen condition. During the controlled feeding periods, no foods other than those in the menu were allowed. If the participants lost weight or were hungry, they were allowed to eat buns with the same fat and energy composition as the rest of the diet. Coffee, tea, and mineral water with artificial sweeteners were allowed *ad libitum*. Initial daily caloric intake for each individual was calculated from estimates of energy requirements based on weight and height (24). Each diet was assigned a color code and an energy level. All foodstuffs were weighed for each individual subject. The subjects were supplied with free food to meet 100% of their mean daily energy requirements. The diet was calculated to contain 89.5 g fat per 10 MJ, of which 74 g derived from the margarines and 16 g from the background diet. The participants were informed before entering the study that they would be given three different types of fat in three different periods and that the type of fat would first be identified after the study.

Chemical analysis. Duplicate portions corresponding to a daily energy intake of 8.5 MJ were taken of the three diets. After homogenization and freeze-drying, the homogenates corresponding to seven consecutive days were pooled into one portion and kept frozen at –20°C until analyzed. The nitrogen content was determined by the Kjeldahl technique. The factor used for conversion of nitrogen content to protein was 6.25. The content of total fat was determined by chloroform–methanol extraction (25). The metabolizable energy content of the diets was determined as described by Andersson *et al.* (26).

For analysis of fatty acid composition, the lipid fractions of the duplicate portions were isolated by Soxhlet extraction of about 5 g of freeze-dried homogenates from each series. The solvent used was diethyl ether, analytical grade, and the extraction time was 4 h. Quantitatively, the lipid recovery was almost the same as extraction with the more polar solvent mixture chloroform–methanol (25). The fatty acids of the respective fat extracts were converted to methyl esters by the BF_3 method (27) and analyzed by gas chromatography as described in an earlier study (9).

The cholesterol content in the test fats and the homogenates was determined on the lipid extract as trimethylsilyl ether derivatives by gas chromatography (28). 5β,3α-Cholestanol was used as internal standard, and the separation between cholesterol, tocopherols, and plant sterols was acceptable on the nonpolar DB-5 capillary column used (30 m × 0.25 mm) (Alltech Associates, Inc., Deerfield, IL).

Blood sampling and analyses. Blood samples were taken after an overnight fast, before breakfast at the end of each period. A second fasting blood sample was taken from nine subjects that prolonged the experimental periods by one day. For these nine subjects the mean of the two values was used. During the start of the first period, baseline samples were taken. Serum was obtained by low-speed centrifugation within 1 h of venipuncture and stored at –70°C until analyzed.

Serum cholesterol and serum triglycerides were measured by enzymatic methods (29,30) using automated analyzer equipment (Hitachi 737; Hitachi Limited, Tokyo, Japan). LDL cholesterol was calculated using the equation of Friedewald *et al.* (31).

Serum HDL cholesterol was measured by an essentially similar enzymatic technique (29) after precipitation of the LDL fraction with dextran sulfate and magnesium chloride (Technicon Reagent T 0.1-2801-56, Tarrytown, NY). Serum apolipoprotein (apo) A-1 (Orion Diagnostika, Espoo, Finland) and serum apoB (Behringwerke Ag, Marburg, Germany) were both quantified immunoturbidimetrically using a seven-point standard curve and an automated enzyme analyzer (Cobas Fara; Hoffman-La Roche, Basel, Switzerland) essentially according to the manufacturer's instructions. Serum Lp(a) was quantified by a commercial kit [TintElize Lp(a); Biopool AB, Umeå, Sweden)] according to the manufacturer's instructions. The coefficients of variation were as follows: total cholesterol, 2%; HDL cholesterol, 5%; triglycerides, 3%; apoA-I, 6.3%; apoB, 5.5%, and Lp(a), 7.7%, at 100 mg/L, and 2.7% at 400 mg/L.

All lipid analyses were performed at the Clinical Chemistry Department and Clinical Research Unit, Ullevaal University Hospital, Oslo, Norway.

Statistical methods. Data were analyzed by repeated-measures analysis of variance for a crossover trial. When the analysis indicated a significant effect of diet ($P < 0.05$), the Bonferroni method was used for a pairwise comparison between the three diet groups. P values <0.05 were considered significant. All P-values are two-tailed. Plasma Lp(a) had a skewed distribution and was log transformed before pairwise comparisons were performed. Correlation coefficients (Pearson) between levels of serum lipids and apo are presented when suitable. Data analysis was performed using the statistical package SPSS 8.0 (SPSS Inc., Chicago, IL).

RESULTS

Dietary compliance. Two persons left the study after the first period because of illness. One participant had not fasted before blood sampling and also had problems with dietary compliance during the last period and was therefore excluded. Thus, of the 30 volunteers who entered the study, 27 completed according to schedule.

Compliance with the diets was judged by direct observation of consumption of weekday dinner, and by evaluation of food diaries. All 27 participants complied well, and only very small deviations from the diet were noted, e.g., one participant left out apples from the menu three times. The fasting body weights were significantly reduced ($P < 0.01$) during the first two periods (mean loss 1.9 and 0.47 kg, respectively). The weight loss was almost entirely confined to the four participants with BMI >30, who lost between 1.7 and 7 kg during the study period. In order to adhere to the "intention to treat" principle, the data from all 27 participants are given, but in addition the data were calculated after omission of the data from the four subjects with BMI >30.

Test diets. The three test margarines and the duplicate portions of the diets were analyzed, and the energy and protein contents were found to be identical in the three diets (Table 1). The energy contribution from fat was analyzed to be 31% in the PALM-diet, 30.1% in the *TRANS*-diet, and 30.4% in the PUFA-diet. This was slightly less than the calculated 34 E%. The PALM-diet contained 79.8 g fat per 10 MJ, the *TRANS*-diet 79.2 g fat, and the PUFA-diet 79.2 g fat per 10 MJ.

All three diets were very low in cholesterol; it was slightly lower in the *TRANS*-diet (67 mg/10 MJ) than in the PALM-diet (103 mg/10 MJ) and PUFA-diet (94 mg/10 MJ). The composition of fatty acids in margarines and duplicate portions of the diets is shown in Table 2. The PALM-diet contained 0.3% *trans* fatty acids, and the *TRANS*-diet 23.1% *trans* fatty acids, corresponding to 7% of energy.

The sum of the cholesterol-increasing saturated fatty acids (12:0, 14:0, and 16:0) in PALM-diet was the same (36%) as the sum of *trans* fatty acids and 12:0, 14:0, and 16:0 in the *TRANS*-diet. The PUFA-margarine had a content of 21% of 14:0–16:0 saturated fatty acids. The E% from 12:0, 14:0, 16:0 and *trans* fatty acids was in PALM-diet, *TRANS*-diet and PUFA-diet, 11, 11 and 6, respectively. The sums of *cis* 18:2 and 18:3 in the PALM-diet and the *TRANS*-diet were identical, 18%.

TABLE 1
Content of Energy and Nutrients of Duplicate Portions of the Test Diets[a]

	PALM-diet	*TRANS*-diet	PUFA-diet
Energy (MJ)	8.40	8.35	8.48
Protein (E%)	17.7	17.1	17.2
Fat (E%)	31.0	30.1	30.4
Carbohydrate (E%)	51.3	52.8	52.4
Cholesterol (mg)	86	56	80

[a]Portions corresponding to an estimated intake of 8.5 MJ/d were analyzed. Abbreviations: PALM-diet, diet using a hard zero-*trans* margarine containing palm oil; *TRANS*-diet, diet using a hard margarine containing a high amount of *trans* fatty acids from partially hydrogenated soybean oil; PUFA-diet, diet using a soft highly polyunsaturated margarine; E%, energy percentage.

Serum lipids and apo. Table 3 shows concentrations of total, LDL, and HDL cholesterol, apoB, apoA-1, and triglycerides at baseline and after the three different test diets. Table 4 shows the mean differences among the three diets and their statistical significance probabilities. Serum total and LDL cholesterol at baseline were higher than expected when compared to those on the experimental diets. This apparant discrepancy could be due to underestimation by the food frequency questionnaire of the fat intake, to the higher relative content of saturated fat and of cholesterol in the habitual diet, and to the fact that the experimental period started shortly after the Christmas holiday season.

There were no significant differences in total cholesterol, LDL cholesterol, and apoB between the *TRANS*- and the PALM-diets. HDL cholesterol and apoA-I were significantly higher on the PALM-diet compared to the *TRANS*-diet, while the ratio of LDL to HDL-cholesterol was lower on the PALM-diet, although this difference failed to reach statistical significance ($P = 0.077$).

Total cholesterol, LDL cholesterol, and apoB were significantly lower on the PUFA-diet compared to the other two diets. HDL cholesterol was not different on the PALM- and the PUFA-diets but was significantly lower on the *TRANS*-diet compared to the PUFA diet. The ratio of LDL to HDL cholesterol was higher on both the PALM- and the *TRANS*-diets compared to the PUFA-diet, although the difference between the PALM- and the PUFA-diets was significant only after exclusion of the four subjects with BMI >30 ($P = 0.063$ for $n = 27$ and 0.001 for $n = 23$). ApoA-I was not different on the PALM- and the *TRANS*-diets compared to the PUFA-diet.

Triglycerides and Lp(a) were not significantly different among the three diets. The Pearson coefficient of correlation between the baseline levels of apoB and LDL was 0.41 ($P = 0.04$) and between apoA and HDL was 0.73 ($P < 0.01$). The Pearson coefficient of correlation between the levels of apoB and LDL on the PALM-diet was 0.48 ($P = 0.01$) and between apoA-I and HDL was 0.86 ($P < 0.01$). The Pearson coefficient of correlation between the levels of apoB and LDL on the *TRANS*-diet was 0.28 ($P = 0.16$) and between apoA-I and HDL was 0.91 ($P < 0.01$). The Pearson coefficient of correlation between the levels of apoB and LDL on the PUFA-diet was 0.49 ($P = 0.01$) and between apoA-I and HDL was 0.87 ($P < 0.01$).

DISCUSSION

In this study we wanted to test whether palm oil could replace PHSO in margarine without causing unfavorable effects on serum lipids. *Trans* fatty acids in the *TRANS*-margarine were replaced by an equal amount of saturated fatty acids, mostly palmitic acid, in the PALM-margarine, and the sum of 12:0, 14:0, and 16:0 saturated fatty acids was equal to the sum of

TABLE 2
Fatty Acid Composition of the Test Margarines and the Corresponding Diets

	Fatty acid composition (% of total fatty acids)					
Fatty acid	PALM-marg	PALM-diet	*TRANS*-marg	*TRANS*-diet	PUFA-marg	PUFA-diet
8:0					0.2	
10:0					0.2	
12:0	0.2	0.5	0.2	0.5	3.2	2.6
14:0	0.9	1.6	0.2	1.0	1.4	1.8
16:0	37.2	33.9	8.7	11.0	12.5	15.8
16:1*c*	0.1	0.4	0.2	0.4	0.1	0.4
17:0		0.1	0.2	0.1	0.1	0.1
18:0	4.0	5.3	8.6	8.8	3.0	4.4
18:1*t*		0.1	27.0	22.6		0.2
18:1*c*	38.6	37.9	34.8	35.2	39.8	38.6
18:2*t/c*[a]		0.1	1.0	0.4	0.2	0.3
18:2*c*	15.6	16.0	13.6	13.5	30.8	27.3
18:3*c*	1.9	2.4	4.2	4.7	6.1	6.1
18:3*t/c*[b]	0.2		0.4		0.6	
20:0	0.4	0.4	0.4	0.3	0.4	0.2
20:1*t*		0.1		0.1		0.2
20:1*c*	0.4		0.6		0.7	
22:0	0.1	0.2	0.3	0.3	0.6	0.3
22:1*c*	0.1		0.1		0.2	
Sum *trans* fatty acids	0.2	0.3	28.4	23.1	0.8	0.5
Sum 12:0, 14:0, 16:0, and *trans* fatty acids	38.5	36.3	37.5	35.6	17.9	20.7
E% from 12:0, 14:0, 16:0		11.2		3.8		6.1
E% from *trans* fatty acids		0.1		7.0		0.2
E% from *cis* MUFA		11.8		10.6		11.7
E% from PUFA		5.7		5.5		10.2

[a]Include *trans,cis* and *cis,trans.*
[b]Include *trans,cis,cis* and *cis,cis,trans.* MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; for other abbreviations see Table 1.

the same fatty acids and *trans* fatty acids in the *TRANS*-margarine. The content of *cis* PUFA and *cis* monounsaturated fatty acids was approximately the same in the PALM- and the TRANS-diets. The results show that the PALM-diet increases HDL cholesterol compared to the *TRANS*-diet without having any significant effect on LDL cholesterol, leading to a slightly

TABLE 3
Serum Lipid and Lipoprotein Levels at Baseline and at the End of the Three Dietary Test Periods[a]

	Baseline	PALM-diet	*TRANS*-diet	PUFA-diet
Total cholesterol (mmol/L)	5.30 ± 0.93 (5.28 ± 0.99)	4.74 ± 0.66 (4.75 ± 0.71)	4.61 ± 0.70 (4.56 ± 0.72)	4.45 ± 0.64 (4.36 ± 0.64)
LDL cholesterol	3.26 ± 0.87 (3.22 ± 0.91)	2.90 ± 0.75 (2.89 ± 0.80)	2.88 ± 0.70 (2.80 ± 0.71)	2.61 ± 0.65 (2.50 ± 0.65)
HDL cholesterol	1.58 ± 0.40 (1.66 ± 0.36)	1.47 ± 0,32 (1.53 ± 0.29)	1.32 ± 0.29 (1.38 ± 0.27)	1.43 ± 0.28 (1.5 ± 0.24)
LDL/HDL cholesterol	2.27 ± 0.98 (2.07 ± 0.75)	2.09 ± 0.73 (2.02 ± 0.75)	2.26 ± 0.73 (2.13 ± 0.65)	1.88 ± 0.65 (1.74 ± 0.56)
Triglycerides (mmol/L)	1.03 ± 0.50 (0.90 ± 0.37)	0.90 ± 0.42 (0.81 ± 0.30)	0.92 ± 0.39 (0.86 ± 0.25)	0.89 ± 0.36 (0.78 ± 0.28)
ApoB (g/L)	1.24 ± 0.28 (1.2 ± 0.29)	1.11 ± 0.22 (1.11 ± 0.23)	1.11 ± 0.22 (1.10 ± 0.21)	1.04 ± 0.23 (0.81 ± 0.29)
ApoA-1 (g/L)	1.89 ± 0.33 (1.91 ± 0.32)	1.78 ± 0.27 (1.83 ± 0.25)	1.70 ± 0.26 (1.74 ± 0.26)	1.75 ± 0.25 (1.80 ± 0.24)
Lp(a) (mg/L)	313 ± 314 (309 ± 323)	309 ± 300 (308 ± 314)	340 ± 339 (342 ± 358)	326 ± 355 (331 ± 375)

[a]Values are given as mean ± SD. Two sets of data are presented, one that includes all subjects (n = 27) and one set (in parentheses) where four subjects with body mass indices >30 have been omitted (n = 23). LDL, low density lipoprotein; HDL, high density lipoprotein; apoB, apolipoprotein B; Lp(a), lipoprotein (a); for other abbreviations see Table 1.

TABLE 4
Differences in Serum Lipid and Lipoprotein Levels Between the Dietary Test Periods[a]

	Mean differences	*P* value[b]	95% Confidence interval
Total cholesterol (mmol/L)			
PALM-*TRANS*	0.12 (0.20)	0.449 (0.058)	–0.08, 0.32 (–0.01, 0.41)
PALM-PUFA	0.31 (0.40)	0.002 (0.00)	0.11, 0.51 (0.20, 0.59)
TRANS-PUFA	0.19 (0.20)	0.005 (0.017)	0.05, 0.34 (0.03, 0.36)
LDL cholesterol (mmol/L)			
PALM-*TRANS*	0.02 (0.10)	1.000 (0.647)	–0.17, 0.21 (–0.10, 0.30)
PALM-PUFA	0.29 (0.38)	0.006 (0.000)	0.07, 0.51 (0.17, 0.59)
TRANS-PUFA	0.27 (0.28)	0.000 (0.000)	0.14, 0.40 (0.13, 0.43)
HDL cholesterol (mmol/L)			
PALM-*TRANS*	0.15 (0.16)	0.003 (0.006)	0.05, 0.25 (0.04, 0.27)
PALM-PUFA	0.03 (0.03)	1.000 (1.000)	–0,05, 0.12 (–0.07, 0.14)
TRANS-PUFA	–0.11 (–0.12)	0.000 (0.000)	–0.17, –0.06 (–0.18, –0.06)
LDL/HDL cholesterol			
PALM-*TRANS*	–0.21 (–0.11)	0.077 (0.204)	–0.43, 0.02 (–0.31, 0.10)
PALM-PUFA	0.19 (0.28)	0.063 (0.001)	–0,01, 0.39 (0.09, 0.47)
TRANS-PUFA	0.39 (0.38)	0.000 (0.000)	0.26, 0.53 (0.24, 0.53)
Triglycerides (mmol/L)			
PALM-*TRANS*	–0.05 (–0.05)	0.155 (0.236)[c]	–0.13, 0.02 (–0.13, 0.03)
PALM-PUFA	0.03 (0.03)	0.448 (0.444)[c]	–0.05, 0.12 (–0.05, 0.12)
TRANS-PUFA	0.09 (0.08)	0.112 (0.154)	–0.01, 0.18 (–0.02, 0.18)
ApoB (g/L)			
PALM-*TRANS*	–0.003 (0.02)	1.000 (1.000)	–0.05, 0.04 (–0.03, 0.06)
PALM-PUFA	0.07 (0.08)	0.008 (0.001)	0.02, 0.12 (0.03, 0.14)
TRANS-PUFA	0.07 (0.07)	0.000 (0.001)	0.03, 0.11 (0.02, 0.11)
ApoA-I (g/L)			
PALM-*TRANS*	0.08 (0.09)	0.008 (0.01)	0.02, 0.15 (0.02, 0.16)
PALM-PUFA	0.03 (0.03)	1.000 (1.000)	–0.06, 0.13 (–0.07, 0.13)
TRANS-PUFA	–0.05 (–0.06)	0.251 (0.181)	–0.12, 0.02 (–0.14, 0.02)
Lp(a) (mg/L)[d]			
PALM-*TRANS*	–0.03 (0.04)	0.320 (0.346)[c]	–0.11, 0.05 (–0.13, 0.06)
PALM-PUFA	–0.02 (–0.02)	0.501 (0.559)[c]	–0.09, 0.05 (–0.08, 0.05)
TRANS-PUFA	0.01 (–0.02)	0.715 (0.613)[c]	–0.07, 0.10 (–0.07, 0.11)

[a]Two sets of data are presented, one that includes all subjects (n = 27) and one set (in parentheses) where four subjects with body mass indices >30 have been omitted (n = 23).
[b]Adjustment for multiple comparisons: Bonferroni.
[c]Not Bonferroni-adjusted.
[d]Log transformed data. For abbreviations see Tables 1 and 3.

more favorable ratio of LDL to HDL cholesterol. The results indicate that, from a nutritional point of view, palm oil may be used as a substitute for PHSO in margarine, which could contribute to a reduced content of *trans* fatty acids in food products. As expected, a margarine in which saturated fatty acids corresponding to 20% of total fatty acids were replaced by PUFA (the PUFA-margarine) had more favorable effects on blood lipids than the other two test margarines.

The 27 participants in this study were all young women. The diets were given in random order, and the women went into the study at different points in their menstrual cycles. Yu *et al.* (32) analyzed data from 18 studies, and their results indicate that the total and LDL cholesterol responses to dietary fatty acids for women and men are quite similar with the possible exception of HDL cholesterol. They found that unsaturated fatty acids may increase HDL cholesterol in men but not in women. In relation to our results this may indicate that the difference in HDL cholesterol between the PALM- and the PUFA-diets (Table 4) may have been slightly overestimated compared to what we might have seen if men had been studied. Otherwise, the results presented should be valid for both men and women.

We had no exclusion criteria concerning the BMI. Four individuals had a BMI above 30 but the others were within 85–120% of desirable BMI (≤25). It is difficult to maintain body weight in a metabolic study when BMI is over 30 (strong overweight). Judd *et al.* (8) included only subjects who were within 85–120% of BMI ≤25 in their study. Brussaard *et al.* (22) had six participants in their experiment who lost weight, between 3 and 7 kg. The data from these participants did not affect the conclusions from the experiment. The four participants in our study who were overweight with BMI >30 lost significant weight during the study period, in particular during the first period. Exclusion of these four persons from the calculations resulted in higher total cholesterol on the PALM-diet than on the *TRANS*-diet (mean difference 0.20 mmol/L, P = 0.058). Otherwise the results were not altered.

We did not find any significant difference between the PALM-diet and the *TRANS*-diet on the effect on LDL cholesterol. This result is in accordance with that reported by Nestel

et al. (6). In that study no significant difference in total or in LDL cholesterol was found with an elaidic acid-rich diet (18:1 *trans,* 7 E%) in 27 mildly hypercholesterolemic men compared to a palmitic acid diet. Our PALM-diet, like that of Nestel *et al.*, contained very little myristic acid, which is the most potent cholesterol-increasing fatty acid (14,32–36). Judd *et al*. (8) observed significantly higher total cholesterol and also higher, but not significantly, LDL cholesterol on a saturated fat diet (12:0, 14:0, and 16:0) compared to a high *trans* (6.6 E%) diet. Mensink and Katan (4) found a significant increase in total and LDL cholesterol on a saturated fat diet compared to *trans*-fatty acid diet. The saturated fat diets in both of these two studies contained higher amounts of lauric, myristic, and palmitic acids than the *trans* diets. Wood *et al.* (37) did not find a difference in total or LDL cholesterol between crude palm oil and a *trans* fatty acid-rich margarine. The difference in palmitic acid corresponded approximately to that of *trans* fatty acids in the two diets. The diets differed, however, both in oleic and linoleic acids, and their results (37) therefore cannot be directly compared to ours. Our results indicate that 18:1 *trans* fatty acids have an LDL cholesterol-increasing effect similar to that of palmitic acid.

Hayes and Khosla (38) suggested that the effect of 16:0 may vary depending on the amount of cholesterol in the diet. They suggested that when cholesterol intake was <300 mg/d in normocholesterolemic subjects the serum cholesterol response to dietary palmitic acid was negligible. At intakes of dietary cholesterol >400 mg/d (38), the serum cholesterol increased, probably because of down-regulation of the LDL receptor and probably maximizing very low density lipoprotein secretion. The results from our study indicate, however, that 16:0 is hypercholesterolemic even at cholesterol intake <300 mg/d.

As expected, total and LDL cholesterol were significantly lower on the PUFA-diet than on the PALM-diet. This is in accord with the results of several previous studies (39–41). Wardlaw and Snook (39) compared a diet rich in saturated fatty acids (butter) to corn and sunflower oils. When the intake of saturated fatty acids was reduced, a significant lowering in total and LDL cholesterol was observed. Cox *et al.* (40) studied the effect of butter, coconut oil, and safflower oil on serum lipids. Total and LDL cholesterol were significantly lower on the diet containing safflower oil and coconut oil compared to butter. Abbey *et al.* (41) exchanged saturated fatty acids with MUFA (raw almonds) and also with PUFA (walnuts). On both diets there was a significant reduction in total and LDL cholesterol.

Also compared to the *TRANS*-diet, the PUFA-diet reduced total and LDL cholesterol. This is in accord with the results of previous studies (4,5,42). Wood *et al.* (42) compared butter to hard and soft margarines. Soft margarine reduced total and LDL cholesterols significantly compared to hard margarine (*trans* fatty acids) and butter. In the studies of Mensink and Katan (4) and of Zock and Katan (5) *trans* fatty acids were found to increase total and LDL cholesterols significantly compared to oleic and linoleic acids, respectively.

Several reports indicate that 18:1 *trans* fatty acids decrease HDL cholesterol compared to saturated fat or to oleic acid (4,5,43,44). Also partially hydrogenated fish oil has a similar HDL cholesterol-decreasing effect (9). This HDL cholesterol-lowering effect was also observed in this study in that the lowest HDL cholesterol and apoA-I were observed on the *TRANS*-diet. The higher LDL to HDL cholesterol ratio observed on the *TRANS*-diet was mainly due to this effect on HDL cholesterol. Others have also noted the increasing effect of *trans* fatty acids on the LDL to HDL cholesterol ratio (4,43), which suggests that palm oil may be preferable to PHSO when considering the effects on the LDL/HDL ratio.

As a mechanism for the HDL-reducing effect of *trans* fatty acids, an increase in activity of cholesteryl ester transfer protein has been suggested (45,46). Increased activity of this enzyme may contribute to a rise in LDL cholesterol and a decrease in HDL cholesterol (46).

We found the highest HDL cholesterol on the PALM-diet although the difference between the PALM-diet and the PUFA-diet was not significant. Also others have found that HDL cholesterol may be significantly increased with intake of palmitic acids compared to oleic- or elaidic-rich diets (6,37,47). On the basis of available evidence (48–51), it may be assumed that the combined LDL cholesterol-increasing and HDL cholesterol-lowering effects of *trans* fatty acids contribute to increased risk for coronary heart disease, and it may be prudent to limit the use of partially hydrogenated vegetable oils in margarine production.

In this study we did not find any significant effect of *trans* fatty acids (7 E%) on Lp(a). A number of studies recently demonstrated that *trans* fatty acids increase Lp(a) when fed at a level of 7 E% or more (6,9–11). In an earlier study we also found increased levels of Lp(a) in young men after intake of a diet with PHSO (8.5 E% *trans* fatty acids) or a diet with partially hydrogenated fish oil (8 E% *trans* fatty acids) compared to a butter diet (9). In a smaller recent study the increasing effect on Lp(a) with partially hydrogenated fish oil did not reach significant level, presumably because of lack of power (52). As in the present study only women participated in that study, and it cannot be excluded that the effect of *trans* fatty acids on Lp(a) is more pronounced in men.

In conclusion, the results from our study indicate that, from a nutritional point of view, palm oil may be a reasonable alternative to PHSO in margarine production if the aim is to avoid *trans* fatty acids while maintaining the same degree of hardness. As expected, both margarines based on these oils were found to have less favorable effects on blood lipids than a more PUFA-rich soft margarine.

ACKNOWLEDGMENTS

We gratefully acknowledge the cooperation of the participants in the study. We thank our kitchen staff, especially Knud Detlefsen, Borgny Kirkholt and Siri Damman, for their assistance; Kari Solvoll for help with data handling of the food frequency questionnaires; Anita B. Mathisrud for taking blood samples; and Paula Yli-Jama for the vitamin E analyses. The study was part of the Nordic R&D Program for the Food Industry with financial support from The Nordic Industrial fund and the Norwegian food company Mills DA.

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[Received January 26, 1998, and in final revised form June 17, 1998; revision accepted July 17, 1998]

Different Degrees of Moderate Iron Deficiency Modulate Lipid Metabolism of Rats

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ABSTRACT: Severe iron deficiency affects lipid metabolism. To investigate whether moderate iron depletion also alters lipid variables—including lipid levels in serum and liver, hepatic lipogenesis, and fatty acid composition indicative of an impaired desaturation—we carried out experiments with rats fed 9, 13, and 18 mg iron/kg diet over a total of 5 wk. The study also included three pair-fed control groups and an *ad libitum* control group, fed with 50 mg iron/kg diet. The iron-depleted rats were classified as iron-deficient on the basis of reduced serum iron, hemoglobin concentration, and hematocrit. All moderately iron-deficient rats had significantly lower cholesterol concentrations in liver and serum lipoproteins than their pair-fed controls. Rats with the lowest dietary iron supply had higher concentrations of hepatic phosphatidylcholine (PC) and phosphatidylethanolamine (PE), lower activities of glucose-6-phosphate dehydrogenase, malic enzyme and fatty acid synthase, and higher triacylglycerol concentrations in serum lipoproteins than the corresponding pair-fed control rats. Moderate iron deficiency also depressed the serum phospholipid level. Moreover, several consistent significant differences in fatty acid composition of hepatic PC and PE occurred within moderate iron deficiency, which indicate impaired desaturation by Δ-9 and Δ-6 desaturases of saturated and essential fatty acids. We conclude that lipid variables, including cholesterol in liver and serum lipoproteins as well as fatty acid desaturation, reflect the gradations of iron status best and can be used as an indicator of the degree of moderate iron deficiency.

Lipids 33, 889–895 (1998).

Iron deficiency is a nutritional problem of worldwide importance, greatly contributing to human and animal morbidity. Experiments with animal models during the past decades have revealed a strong relationship between dietary iron and lipid metabolism. Some investigators have observed lipemia when anemia was produced by iron-deficient diets (1–3). Moreover, hepatic lipogenesis measured by means of ^{14}C-glucose incorporation was altered in livers of severely iron-deficient chicks and rats (2,3), and the fatty acid composition of tissues changed as a result of nutritional iron depletion (4–7). However, within these experiments, the results from one study often contradicted those from another. Many of these reports used either bleeding or diets with a maximum of 6 mg iron/kg to characterize metabolic defects associated with severe iron deficiency. However, moderate iron deficiency is more widespread than severe anemia. Only some single experiments point to the fact that moderate iron depletion also causes hyperlipemia (2), depresses hepatic lipogenesis (2), and increases essential fatty acids in rat tissues (4).

The objectives of the present study were to evaluate the effects of different degrees of moderate dietary iron depletion, by using diets with iron concentrations ranging from 9 to 18 mg iron/kg, on lipid concentrations of serum and lipoproteins, the liver lipid concentrations, the activities of hepatic lipogenic enzymes, and the fatty acid composition of individual liver phospholipids. Several investigations on iron-induced changes in lipogenesis utilized incorporation of labeled glucose into triacylglycerols (3,8), whereas enzymes involved in lipogenesis have been scarcely investigated in view of iron deficiency. One aspect of this study dealt with the effect of moderate iron deficiency on some enzymes involved in lipogenesis.

Many early iron deficiency studies did not take into consideration the fact that animals deficient in micronutrients often reduce voluntary food intake. Iron deficiency is therefore generally combined with an energy deficiency, which has been shown to affect lipid metabolism markedly. To eliminate differences in food intake we assigned iron-adequate pair-fed groups to each iron-deficient group.

MATERIALS AND METHODS

Animals and diets. In this experiment, weaned male specific pathogen-free Sprague-Dawley rats (WIGA GmbH, Sulzfeld, Germany) with an average body weight of 40 ± 2 g were fed diets moderately depleted of iron for 5 wk. For that purpose, the rats were divided into seven groups of 12 each. Three of these groups were made moderately iron-deficient by providing diets containing 9, 13, and 18 mg iron/kg. The iron supplementation of those diets was selected in such a way that disturbances in lipid metabolism were just detectable. The experiment included three pair-fed control groups and an *ad libitum* group, fed with 50 mg iron/kg diet that has been shown

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Abbreviations: HDL, high density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine (diacyl); VLDL, very low density lipoprotein.

to provide rats with adequate iron (9). The basal diet had 2.6 mg/kg iron and was adjusted to the experimental dietary iron concentrations by supplementation with iron as $FeSO_4 \cdot 7H_2O$. All the other components of the diet remained constant. The composition of the basal semisynthetic diet is given in Table 1. The diet was prepared from natural feedstuffs. All diets were fortified with recommended amounts of vitamins and minerals according to Reeves and coworkers (9). Rats were housed individually in a controlled environment, in Macrolon cages (Becker GmbH, Castrop-Ruxel, Germany) in a room maintained at 24°C with a humidity of 60%. All rats were kept under conditions of controlled lighting with a daily 12-h light/dark cycle and had free access to drinking water (double-distilled water, supplemented with 0.14 g/L sodium chloride to adjust osmolarity to that of tap water). Care and treatment of rats followed recommended guidelines (10). At the end of the experimental period of 5 wk, 12 h after the last feeding, the rats were killed by decapitation after light anesthesia with diethyl ether. Blood for hematological measurements was collected into EDTA-coated vials. Blood for determination of serum iron and lipoproteins was collected into untreated tubes. The liver and brain were promptly excised. Serum and tissue samples were stored at –80°C until analyzed.

Iron analyses. Iron concentration in serum was measured directly by aspirating a dilute solution (1:5) into the flame of the atomic absorption spectrometer. Iron concentration of each sample was calculated from standard curves using various dilutions of an iron standard solution (1000 ± 2 mg/L) from Merck (Darmstadt, Germany). All specimens were analyzed in duplicate. The coefficient of variation for duplicate analyses was typically below 2%.

Hematological measurements. Hematological variables including erythrocyte count, hematocrit, and hemoglobin concentration were determined with a Coulter Counter and a hemoglobinometer (Coulter Electronics GmbH, Krefeld, Germany).

TABLE 1
Composition of Basal Diet[a]

Ingredient	g/kg diet
Casein	200
Cornstarch	328
Sucrose	300
Fiber (cellulose)	30
Soybean oil	50
Coconut oil	30
Vitamin mixture[b]	20
Mineral mixture[c]	40
DL-Methionine	2

[a]Basal diet analyzed had 2.6 mg/kg iron.
[b]Vitamins/kg diet: 4000 IU *all-trans* retinol; 1000 IU cholecalciferol; 150 mg *all-rac*-α-tocopherol; 1.47 mg menadione sodium bisulfite; 5 mg thiamine hydrochloride; 7.20 mg riboflavin; 6 mg pyridoxine hydrochloride; 15 mg Ca pantothenate; 30 mg nicotinic acid; 1.38 g choline chloride; 0.2 mg folic acid; 0.2 mg D-biotin; 25 mg cyanocobalamin; sucrose to 20 g.
[c]Minerals/kg diet: $Na_2HPO_4 \cdot 2H_2O$, 3.15 g; KH_2PO_4, 3.82 g; KCl, 4.77 g; $MgCl_2 \cdot 6H_2O$, 4.24 g; $CaCO_3$, 12.49 g; $NiSO_4 \cdot 6H_2O$, 4.48 mg; $ZnSO_4 \cdot 7H_2O$, 131.9 mg; $CuSO_4 \cdot 7H_2O$, 23.6 mg; $MnSO_4 \cdot 5H_2O$, 30.8 mg; KI, 0.26 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.28 mg; $Na_2SeO_3 \cdot 5H_2O$, 0.50 mg; $CrCl_3 \cdot 6H_2O$, 9.61 mg; NH_4VO_3, 0.23 mg; $Na_2SiO_3 \cdot 5H_2O$, 37.76 mg; H_3BO_3, 2.86 mg; NaF, 2.21 mg; $(CH_3COO)_2Pb \cdot 3H_2O$, 0.73 mg; sucrose to 40 g.

Lipid extraction and analysis. Liver lipids were extracted with a hexane/isopropanol mixture (3:2, vol/vol, containing butylated hydroxytoluene as antioxidant) as described by Hara and Radin (11). Hepatic phospholipids were separated by high-performance liquid chromatography as described by Eder and coworkers (12) and collected with a fraction collector (model 201; Gilson, Villiers le Bel, France). Hepatic phospholipids separated by high-performance liquid chromatography were methylated with boron fluoride/methanol reagent (13). Fatty acid methyl esters were separated by gas chromatography using a Hewlett-Packard HP 5890 gas chromatographic system (Hewlett-Packard, Taufkirchen, Germany), fitted with an automatic on-column injector, a flame ionization detector, and a CP-Sil 88 capillary column (50 m × 0.25 mm internal diameter, film thickness 0.2 μm; Chrompack, Middleburg, The Netherlands). The oven temperature program used was as described by Eder and Kirchgessner (14). The detector temperature was 300°C. Fatty acid methyl esters were identified by comparing their retention times with those of individual purified standards and quantified with heptadecanoic acid methyl ester as internal standard (15). The amounts of individual hepatic phospholipids [phosphatidylcholine (PC) and phosphatidylethanolamine, diacyl (PE)] were calculated by the amount of their bound fatty acids. For measurement of liver total triacylglycerols and total cholesterol, liver lipids were extracted with hexane/isopropanol (3:2, vol/vol) and then dissolved in a chloroform/Triton X-100 mixture (1:1, vol/vol) as described by De Hoff *et al.* (16). After evaporating the chloroform under vacuum, total triacylglycerols and total cholesterol were determined by adding test reagents for fully enzymatic spectrophotometric assay of triacylglycerols (Merck, ref. 14354) and cholesterol (Boehringer, Mannheim, Germany, ref. 816302).

Activities of lipogenic enzymes. Approximately 4 g of liver was homogenized in 10 mL of 0.25 M sucrose solution (in 0.1 M phosphate buffer, pH 7.4) using a Potter-Elvehjem homogenizer. Homogenates were centrifuged (105,000 × *g* for 1 h at 4°C), and the supernatants were used for enzyme assays. All the enzymes were assayed by spectrophotometric methods. Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (17), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) (18), and malic enzyme (E.C. 1.1.1.40) (19) were determined by the rate of NADP reduction. In the assay of glucose-6-phosphate dehydrogenase, activity of 6-phosphogluconate dehydrogenase was inhibited by maleimide (17). Fatty acid synthase was determined by the rate of malonyl-CoA-dependent NADPH oxidation (20). Citrate cleavage enzyme (E.C. 4.1.3.8) was determined from NADH oxidation (21).

Serum lipids and lipoproteins. Blood was allowed to clot for at least 30 min before centrifugation. Serum samples were obtained by centrifugation at 4°C for 10 min at 1100 × *g*. Lipoproteins in serum as very low density lipoproteins (VLDL; density < 1.006 kg/L), low density lipoproteins

(LDL; density 1.006–1.063 kg/L) and high density lipoproteins (HDL; density 1.063–1.210 kg/L) were separated by the method of Kupke and Wörz-Zeugner (22) using density gradient ultracentrifugation with a Beckman air-driven ultracentrifuge and an A-100 fixed angle rotor at 100,000 × *g* for 2.5 h (Beckman Instruments, Palo Alto, CA). The adjustment of the density of serum with KBr solution to separate HDL was done according to a method of Terpstra *et al*. (23). According to adjustment of the density of serum, lipoproteins at the top of the tube were aspirated with a Beckman Airfuge Tube Fractionator (Beckman Instruments) and removed. The lipoprotein fractions at the bottom of the tubes were analyzed enzymatically for total cholesterol, triacylglycerols, and phospholipids by using an autoanalyzer (Hitachi 704, Boehringer, Mannheim, Germany) and Boehringer kit reagents.

Statistical analysis. The effect of iron concentration in the diet was evaluated by analysis of variance and compared for statistical significance ($P < 0.05$) by Fisher's multiple range test. All data in the present text are expressed as means ± SD.

RESULTS

Weight gain and iron status. Weight gain, food intake, and iron status of iron-deficient and iron-adequate rats are shown in Table 2. Rats receiving 9 mg iron/kg diet had a lower body weight gain than the corresponding pair-fed controls, and daily food intake was depressed compared to the *ad libitum*-fed control group. Body weight gain and food intake of the groups fed 13 or 18 mg iron/kg diet did not differ from their pair-fed controls and the *ad libitum*-fed group, respectively. All iron-dependent hematological parameters, including hemoglobin concentration, hematocrit, and iron concentration in serum, decreased markedly by week 5 in rats on the iron-deficient diets, demonstrating that the moderate iron deficiency was well developed 5 wk after the experiment started. Iron status did not differ between iron-adequate groups.

Liver lipids. The hepatic triacylglycerol concentration was unaffected by moderate dietary iron depletion (Table 3). The most striking difference in hepatic lipids was a lower cholesterol concentration in iron-deficient rats compared to their corresponding pair-fed controls. Concentrations of phospholipids (PC and PE) were elevated in rats fed 9 mg iron/kg diet compared to all the other groups.

Activities of hepatic lipogenic enzymes. Rats receiving 9 mg iron/kg diet had lower activities of glucose-6-phosphate dehydrogenase, malic enzyme, and fatty acid synthase than their corresponding pair-fed controls, whereas animals supplied with 13 and 18 mg iron/kg diet had enzyme activities near the control values (Table 3). The activities of 6-phosphogluconate dehydrogenase and citrate cleavage enzyme depended primarily on food intake and were reduced in livers of rats with the lowest iron supply and their pair-fed controls relative to all the other groups.

Serum lipids and lipoproteins. Iron deficiency led to considerable alterations in serum lipid and lipoprotein concentrations (Table 4). Rats receiving 9 mg iron/kg diet had a higher concentration of triacylglycerols in serum than their corresponding pair-fed controls. This elevation was primarily due to an increase in VLDL and LDL triacylglycerols, whereas triacylglycerol concentration in HDL remained unchanged. Triacylglycerols in serum and lipoproteins of the other iron-restricted groups and control groups did not differ. Cholesterol concentration in serum varied markedly with the dietary iron intake, and was reduced in all iron-inadequate rats compared to the corresponding pair-fed and *ad libitum* controls. The reduction of serum cholesterol level was caused by reduced VLDL, LDL and HDL cholesterol. Iron-inadequate rats without any reduction in food intake (groups fed 13 and 18 mg iron/kg diet) had a lower phospholipid concentration in serum and the lipoproteins than their pair-fed controls, whereas the phospholipid levels of rats receiving 9 mg iron/kg diet did not differ from their pair-fed controls.

Fatty acid composition of hepatic phospholipids. Iron deficiency influenced considerably the fatty acid composition of individual phospholipids, and the most striking differences were depressed proportions of total and individual monounsaturated fatty acids in PC and PE (Table 5). In agreement with total liver lipids, the proportions of total saturated fatty acids in hepatic PC and PE did not differ between the groups. Iron-deficient rats had higher proportions of 18:2n-6 in hepatic PC and PE than their pair-fed controls, and slightly decreased levels of 20:4n-6 (Table 6). Therefore, the ratio be-

TABLE 2
Body Weight Gain, Food Intake, and Iron Status of Moderate Iron-Deficient Rats and Their Pair-Fed Controls[a,b]

	Group/iron supply[c]						
Parameter	Fe – 9[d]	Fe + PF9[e]	Fe – 13[d]	Fe + PF13[e]	Fe – 18[d]	Fe + PF18[e]	Fe + *ad libitum*
Body weight gain (g)	226 ± 13^{g}	265 ± 7^{f}	286 ± 33^{e}	294 ± 16^{d,e}	306 ± 15^{d}	301 ± 22^{d,e}	292 ± 27^{d,e}
Food intake (g/d)	15.3 ± 1.4^{e}	15.3 ± 0.1^{e}	17.3 ± 2.1^{d}	17.3 ± 0.9^{d}	18.0 ± 1.0^{d}	17.6 ± 1.3^{d}	17.2 ± 1.4^{d}
Serum iron (μmol/L)	18.6 ± 4.8^{d}	52.7 ± 5.6^{f}	25.1 ± 7.9^{d}	54.8 ± 4.3^{f}	36.2 ± 7.0^{e}	59.6 ± 14.0^{f}	58.2 ± 13.3^{f}
Hemoglobin (g/L)	66.3 ± 8.4^{g}	152 ± 4.9^{d}	96.6 ± 12.5^{f}	150 ± 10.2^{d}	121 ± 21.4^{e}	146 ± 10.2^{d}	145 ± 21.0^{d}
Hematocrit (%)	22.6 ± 2.8^{g}	44.3 ± 1.2^{d}	31.7 ± 4.4^{f}	44.0 ± 3.1^{d}	37.6 ± 6.3^{e}	43.8 ± 3.2^{d}	43.5 ± 5.5^{d}

[a]Data are means ± SD, $n = 12$ for each group.
[b]Means were compared by Fisher's multiple range test; means not sharing a common roman superscript letter are significantly different at $P < 0.05$.
[c]Fe– means iron-deficient groups, Fe+ means iron-adequate groups, PF means control group pair-fed to the iron-deficient group.
[d]Fe – 9, Fe – 13, Fe – 18 mean iron-deficient groups fed 9, 13, or 18 mg iron/kg diet.
[e]Fe + PF9, Fe + PF13, Fe + PF18 mean iron-adequate groups (50 mg iron/kg diet) pair-fed to the iron-deficient groups.

TABLE 3
Lipid Concentrations and the Activities of Lipogenic Enzymes in Liver of Moderate Iron-Deficient Rats and Their Pair-Fed Controls[a,b]

	Group/iron supply[c]						
Parameter	Fe – 9	Fe + PF9	Fe – 13	Fe + PF13	Fe – 18	Fe + PF18	Fe + *ad libitum*
Hepatic lipids (μmol/g)							
Triacylglycerols	49.4 ± 17.1	45.3 ± 20.1	61.9 ± 32.5	54.4 ± 33.5	50.3 ± 22.9	61.0 ± 39.7	51.1 ± 23.3
Cholesterol	6.73 ± 0.62^{g}	9.25 ± 1.17^{e}	7.43 ± 1.22^{f,g}	10.0 ± 3.0^{e}	7.67 ± 1.14^{f,g}	9.79 ± 2.85^{e}	8.57 ± 1.30^{e,f}
PC	23.4 ± 4.7^{e}	19.4 ± 4.3^{f}	18.9 ± 3.9^{f}	18.0 ± 4.4^{f}	17.0 ± 2.3^{f}	18.6 ± 4.5^{f}	17.6 ± 3.6^{f}
PE (diacyl)	13.5 ± 3.14^{e}	11.3 ± 2.24^{f}	10.7 ± 2.20^{f}	9.78 ± 2.88^{f}	9.80 ± 1.50^{f}	9.97 ± 2.44^{f}	9.54 ± 2.49^{f}
Lipogenic enzymes[d]							
G6PDH	14.1 ± 4.9^{e}	26.5 ± 12.9^{f}	29.8 ± 11.3^{f}	33.3 ± 13.1^{f}	25.3 ± 9.1^{f}	31.8 ± 14.4^{f}	28.2 ± 14.6^{f}
6PGDH	65.0 ± 11.1^{e}	68.3 ± 18.0^{e}	86.7 ± 21.1^{f}	84.8 ± 28.5^{f}	80.3 ± 16.6^{e,f}	87.6 ± 18.8^{f}	79.3 ± 14.7^{f}
ME	4.93 ± 2.64^{e}	9.50 ± 3.79^{f}	9.75 ± 4.10^{f}	10.3 ± 4.2^{f}	8.17 ± 2.14^{f}	10.8 ± 3.7^{f}	8.51 ± 2.62^{f}
CCE	8.92 ± 3.25^{e}	12.2 ± 4.9^{e,f}	20.0 ± 5.7^{g}	18.5 ± 6.6^{g}	16.8 ± 6.6^{f,g}	20.3 ± 5.5^{g}	19.3 ± 7.6^{g}
FAS	1.44 ± 0.69^{e}	2.93 ± 0.93^{f}	4.28 ± 2.66^{f,g}	4.00 ± 2.01^{f,g}	3.40 ± 1.53^{f,g}	4.63 ± 1.79^{g}	3.86 ± 1.46^{f,g}

[a]Data are means ± SD, $n = 12$ for each group. See Table 2 for column heading explanations.
[b]Means were compared by Fisher's multiple range test; means not sharing a common superscript roman letter are significantly different at $P < 0.05$.
[c]Fe– means iron-deficient groups, Fe+ means iron-adequate groups, PF means control group pair-fed to the iron-deficient group.
[d]Activities of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and malic enzyme (ME) are expressed as nmoles NADP · reduced mg protein^{-1} · min^{-1} at 25°C; activity of citrate cleavage enzyme (CCE) is expressed as nmoles NADH oxidized · mg protein^{-1} · min^{-1} at 25°C; activities of fatty acid synthase (FAS) are expressed as nmoles NADPH oxidized · mg protein^{-1} · min^{-1} at 25°C.

tween 20:4n-6 and 18:2n-6 in PC and PE was markedly decreased by dietary iron deficiency. There were also some iron-induced alterations in n-3 polyunsaturated fatty acids, which showed an increase in most individual n-3 polyunsaturated fatty acids in PC and PE.

DISCUSSION

Since clinical iron deficiency is rarely as severe as can be achieved by feeding laboratory animals a diet maximally depleted of iron, we used diets only moderately depleted in iron in an attempt to mimic the human situation more closely. The alignment of food intake between the deficiency and the control group is necessary to isolate the symptoms of a trace element restriction and to make valid conclusions. In most iron deficiency studies both the iron-deficient and the iron-adequate diets were fed *ad libitum*. We have ascertained that even moderate iron-depleted rats receiving 9 mg iron/kg diet depressed voluntary food intake. The importance of such pair-fed groups consists in the fact that some iron effects would be otherwise over- or underestimated. This was apparent from the present findings showing that the activities of some lipogenic enzymes were reduced in iron-depleted rats compared to the iron-adequate rats fed the diet *ad libitum*, but not when the iron-adequate diet was fed in amounts equal to the depletion group. On the other hand, the triacylglycerol concentration in serum and VLDL of the rats with lowest iron supply was higher than that of their pair-fed controls and equal to that of the *ad libitum*-fed controls.

Excluding the discrepancies in food intake from the pre-

TABLE 4
Concentrations of Lipids (mmol/L) in Serum and Lipoproteins of Moderate Iron-Deficient Rats and Their Pair-Fed Controls[a,b]

	Group/iron supply[c]						
Serum/lipoprotein	Fe – 9	Fe + PF9	Fe – 13	Fe + PF13	Fe – 18	Fe + PF18	Fe + *ad libitum*
Triacylglycerols							
Serum	1.12 ± 0.32^{d}	0.76 ± 0.28^{e}	1.23 ± 0.38^{d}	1.30 ± 0.36^{d}	1.06 ± 0.17^{d}	1.26 ± 0.36^{d}	1.22 ± 0.32^{d}
VLDL	0.61 ± 0.27^{d}	0.36 ± 0.25^{e}	0.79 ± 0.35^{d}	0.79 ± 0.31^{d}	0.65 ± 0.23^{d}	0.82 ± 0.34^{d}	0.76 ± 0.32^{d}
LDL	0.31 ± 0.06^{d}	0.21 ± 0.07^{e}	0.24 ± 0.05^{e}	0.24 ± 0.07^{e}	0.22 ± 0.03^{e}	0.24 ± 0.06^{e}	0.25 ± 0.04^{e}
HDL	0.20 ± 0.06	0.21 ± 0.07	0.20 ± 0.04	0.22 ± 0.04	0.19 ± 0.04	0.20 ± 0.09	0.24 ± 0.05
Cholesterol							
Serum	1.61 ± 0.28^{g}	2.17 ± 13.9^{e,f}	2.05 ± 0.18^{f,g}	2.72 ± 0.81^{d}	2.02 ± 0.25^{f,g}	2.93 ± 0.71^{d}	2.58 ± 0.58^{d,e}
VLDL	0.29 ± 0.10^{f}	0.30 ± 0.09^{e,f}	0.33 ± 0.10^{e,f}	0.45 ± 0.19^{d}	0.25 ± 0.06^{f}	0.45 ± 0.20^{d}	0.42 ± 0.18^{d,e}
LDL	0.40 ± 0.09^{f}	0.50 ± 0.14^{e,f}	0.35 ± 0.08^{f}	0.60 ± 0.28^{d,e}	0.42 ± 0.09^{f}	0.66 ± 0.25^{d}	0.57 ± 0.19^{d,e}
HDL	1.04 ± 0.20^{f}	1.37 ± 0.28^{e}	1.37 ± 0.21^{e}	1.68 ± 0.47^{d}	1.35 ± 0.21^{e}	1.83 ± 0.35^{d}	1.59 ± 0.37^{d,e}
Phospholipids							
Serum	1.71 ± 0.18^{e}	1.83 ± 0.28^{e}	1.81 ± 0.17^{e}	2.28 ± 0.54^{d}	1.75 ± 0.15^{e}	2.37 ± 0.47^{d}	2.13 ± 0.45^{d}
VLDL	0.37 ± 0.11^{d,e}	0.30 ± 0.12^{e}	0.38 ± 0.10^{d,e}	0.46 ± 0.15^{d}	0.30 ± 0.07^{e}	0.46 ± 0.16^{d}	0.42 ± 0.20^{d}
LDL	0.22 ± 0.06^{f}	0.26 ± 0.08^{e,f}	0.20 ± 0.04^{f}	0.32 ± 0.16^{d,e}	0.23 ± 0.06^{f}	0.35 ± 0.12^{d}	0.31 ± 0.09^{d,e}
HDL	1.12 ± 0.12^{f}	1.26 ± 0.18^{e,f}	1.23 ± 0.18^{e,f}	1.50 ± 0.32^{d}	1.23 ± 0.10^{e,f}	1.56 ± 0.26^{d}	1.39 ± 0.28^{d,e}

[a]Data are means ± SD, $n = 12$ for each group. See Table 2 for column heading explanations.
[b]Means were compared by Fisher's multiple range test; means not sharing a common superscript roman letter are significantly different at $P < 0.05$.
[c]Fe– means iron-deficient groups, Fe+ means iron-adequate groups, PF means control group pair-fed to the iron-deficient group. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

TABLE 5
Proportions of Saturated and Monounsaturated Fatty Acids (mol/100 mol fatty acids) in Hepatic Phosphatidylcholine and Phosphatidylethanolamine (diacyl) of Moderately Iron-Deficient Rats and Their Pair-Fed Controls[a,b]

	Group/iron supply[c]						
Fatty acid	Fe – 9	Fe + PF9	Fe – 13	Fe + PF13	Fe – 18	Fe + PF18	Fe + *ad libitum*
Phosphatidylcholine							
14:0	0.69 ± 0.14^{d}	0.51 ± 0.16^{f}	0.62 ± 0.19^{d,e,f}	0.53 ± 0.14^{e,f}	0.63 ± 0.14^{d,e}	0.52 ± 0.11^{b,c}	0.51 ± 0.11^{f}
16:0	19.6 ± 1.3^{f}	25.0 ± 1.5^{d}	21.5 ± 2.1^{e}	24.7 ± 1.4^{d}	24.8 ± 0.7^{d}	24.5 ± 1.2^{a}	24.7 ± 1.7^{d}
18:0	25.9 ± 1.7^{d}	19.8 ± 2.3^{f}	23.9 ± 2.9^{e}	20.4 ± 1.6^{f}	20.1 ± 1.5^{f}	20.6 ± 1.7^{c}	20.9 ± 2.1^{f}
Total SFA	46.2 ± 0.8	45.3 ± 1.2	46.0 ± 1.5	45.6 ± 1.1	45.5 ± 1.3	45.6 ± 1.4	46.1 ± 1.9
16:1	0.87 ± 0.16^{f}	1.41 ± 0.26^{d}	1.16 ± 0.32^{e}	1.35 ± 0.24^{d}	1.28 ± 0.09^{d,e}	1.32 ± 0.19^{d,e}	1.34 ± 0.27^{d,e}
18:1	4.93 ± 0.49^{g}	6.49 ± 0.97^{d,e}	5.75 ± 0.71^{f}	6.54 ± 0.99^{d}	5.88 ± 0.37^{e,f}	6.51 ± 0.77^{d,e}	6.48 ± 0.95^{d,e}
Total MUFA	5.80 ± 0.59^{f}	7.89 ± 1.21^{d}	6.91 ± 0.98^{e}	7.89 ± 1.21^{d}	7.16 ± 0.34^{d,e}	7.83 ± 0.93^{d}	7.82 ± 1.17^{d}
Phosphatidylethanolamine (diacyl)							
14:0	0.12 ± 0.03	0.13 ± 0.04	0.14 ± 0.05	0.15 ± 0.02	0.14 ± 0.04	0.15 ± 0.04	0.15 ± 0.04
16:0	17.1 ± 2.0^{f}	17.1 ± 0.6^{e,f}	17.6 ± 2.1^{d,e,f}	18.8 ± 1.4^{d}	17.6 ± 1.1^{e,f}	18.3 ± 1.2^{d,e,f}	18.3 ± 1.5^{d,e}
18:0	30.8 ± 4.9^{d}	27.6 ± 1.1^{e}	27.6 ± 2.2^{e}	25.7 ± 1.6^{e}	27.4 ± 2.0^{e}	26.3 ± 1.5^{e}	26.6 ± 2.3^{e}
Total SFA	47.9 ± 6.0	44.8 ± 1.1	45.4 ± 1.6	44.7 ± 1.8	45.1 ± 2.0	44.7 ± 1.9	45.1 ± 2.4
16:1	0.31 ± 0.17^{f}	0.61 ± 0.18^{e}	0.66 ± 0.14^{d,e}	0.80 ± 0.13^{d}	0.68 ± 0.16^{d,e}	0.74 ± 0.25^{d,e}	0.78 ± 0.21^{d}
18:1	3.18 ± 0.38^{h}	4.85 ± 0.87^{d,e,f}	4.30 ± 0.99^{f,g}	5.31 ± 1.01^{d}	4.01 ± 0.69^{g}	4.62 ± 0.52^{e,f,g}	5.06 ± 0.86^{d,e}
Total MUFA	3.49 ± 0.50^{h}	5.46 ± 0.93^{d,e,f}	4.96 ± 1.07^{f,g}	6.11 ± 1.08^{d}	4.69 ± 0.78^{g}	5.36 ± 0.49^{e,f,g}	5.85 ± 0.90^{d,e}

[a]Data are means ± SD, $n = 12$ for each group. See Table 2 for column heading explanations.
[b]Means were compared by Fisher's multiple range test; means not sharing a common superscript roman letter are significantly different at $P < 0.05$.
[c]Fe– means iron-deficient groups, Fe+ means iron-adequate groups, PF means control group pair-fed to the iron-deficient group. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

sent findings, it is clear that dietary iron is associated with various aspects of lipid metabolism and that some alterations in lipid variables occur even in moderate iron deficiency. Parameters including hepatic cholesterol, phospholipids and cholesterol in serum and the lipoproteins, and fatty acid composition of hepatic total lipids and individual phospholipids were changed by only a slight iron deficiency. Only the condition of well-developed iron deficiency, with hemoglobin concentrations below 100 g/L, appears to be associated with a depressed capacity for lipid synthesis, as shown by the decreased activity of enzymes associated with lipogenesis and elevated triacylglycerol concentrations in serum and lipoproteins. Also, iron-deficiency anemia provoked by high-fat and high-copper diets, with hemoglobin concentrations about 85 g/L, has been shown to depress enzymes involved in lipogenesis (24).

However, it is difficult to compare the quality and extent of changes in lipid metabolism found in the present iron-deficiency study with those from other experiments because the changeability of lipid parameters also depends on the age, sex, and strain of the rats and the composition of the diets used in the experiment (1,2,25). Our results show hypertriglyceridemia in rats receiving the lowest level of dietary iron. The association of nutritional severe iron deficiency and lipemia has been already recognized by Amine and Hegsted (25) and Lewis and Iammarino (26), who found a rise in the plasma triacylglycerol content of chicks and weanling rats fed iron-deficient diets compared to the controls; the extent of their lipemia was a function of the degree of iron deficiency. However, in contrast to these findings, some authors found lowered triacylglycerols in serum of iron-deficient rats (27,28). A study by Lewis and Iammarino (26) demonstrating reduced activity of lipoprotein lipase suggests that lipemia in iron-deficient rats is due to impaired clearance of blood lipids. However, another report did not confirm a reduction of lipoprotein lipase activity due to iron depletion (3).

The depressed cholesterol level in liver and serum of the iron-depleted rats might be associated with the reduced activities of lipogenic enzymes involved in providing the reduced form of NADP for several pathways of synthesis. This can be supported by early results showing that the rate of lipid synthesis was depressed in iron-deficient rats (2). On the basis of the observed reduction in hepatic cholesterol level we also expected a lower triacylglycerol content in liver. However, hepatic triacylglycerol levels remained nearly unchanged by iron deficiency. The role of iron in mammalian metabolism includes a number of diverse functions which may also be responsible for the observed phenomena. Although there is no evidence for abnormal respiratory function of liver mitochondria in iron-deficient animals (3,29) and for changes in cytochrome oxidase activity (30), decreased carnitine levels observed in iron-deficient rats (31) may contribute to the observed findings. Besides, degeneration of parenchymal liver cells which has been reported in iron-deficient rats (25,32) could also be responsible for the alterations in liver lipid metabolism.

In the current study, few consistent significant differences in fatty acid composition, which indicate impaired desaturation of saturated and essential fatty acids by desaturases, occurred in moderate iron deficiency. This was apparent by the

TABLE 6
Proportions of Polyunsaturated Fatty Acids (mol/100 mol fatty acids) in Hepatic Phosphatidylcholine and Phosphatidylethanolamine (diacyl) of Moderately Iron-Deficient Rats and Their Pair-Fed Controls[a,b]

	Group/iron supply[c]						
Fatty acid	Fe – 9	Fe + PF9	Fe – 13	Fe + PF13	Fe – 18	Fe + PF18	Fe + *ad libitum*
Phosphatidylcholine							
18:2	12.8 ± 1.5^{d}	9.98 ± 1.44^{e}	9.49 ± 1.06^{e,f}	8.49 ± 1.79^{f,g}	9.67 ± 1.11^{e,f}	7.37 ± 1.11^{g}	8.04 ± 1.56^{g}
20:2	0.41 ± 0.16^{d}	0.29 ± 0.08^{e}	0.28 ± 0.09^{e}	0.28 ± 0.10^{e}	0.31 ± 0.08^{e}	0.31 ± 0.08^{e}	0.25 ± 0.06^{e}
20:3	0.52 ± 0.10^{d,e}	0.42 ± 0.05^{f}	0.56 ± 0.15^{d}	0.43 ± 0.16^{e,f}	0.47 ± 0.07^{d,e,f}	0.41 ± 0.11^{f}	0.44 ± 0.12^{e,f}
20:4	26.4 ± 1.1^{f}	28.8 ± 1.2^{e}	28.8 ± 1.3^{e}	29.6 ± 1.2^{d,e}	28.7 ± 1.4^{e}	30.2 ± 1.4	^{d}29.6 ± 1.9^{d,e}
22:4	0.19 ± 0.06	0.19 ± 0.05	0.16 ± 0.04	0.18 ± 0.04	0.19 ± 0.03	0.18 ± 0.04	0.16 ± 0.02
22:5	0.28 ± 0.06^{f}	0.47 ± 0.10^{d}	0.38 ± 0.06^{e}	0.44 ± 0.06^{d,e}	0.42 ± 0.06^{d,e}	0.42 ± 0.08^{d,e}	0.43 ± 0.08^{d,e}
Total n-6 PUFA	40.5 ± 1.0^{d}	40.1 ± 0.8^{d,e}	39.7 ± 1.3^{d,e,f}	39.4 ± 1.1^{e,f}	39.8 ± 0.7^{d,e,f}	38.9 ± 1.4^{f}	38.9 ± 1.4^{f}
20:4/18:2	2.08 ± 0.31^{f}	2.97 ± 0.63^{e}	3.08 ± 0.48^{e}	3.67 ± 1.00^{d}	3.02 ± 0.50^{e}	4.20 ± 0.71^{d}	3.85 ± 1.02^{d}
18:3	0.17 ± 0.05^{d}	0.08 ± 0.03^{g}	0.11 ± 0.03^{e}	0.09 ± 0.03^{e,f,g}	0.11 ± 0.03^{e,f}	0.10 ± 0.04^{e,f,g}	0.08 ± 0.02^{f,g}
20:5	0.15 ± 0.04^{d}	0.08 ± 0.03^{e}	0.14 ± 0.04^{d}	0.09 ± 0.02^{e}	0.07 ± 0.02^{e}	0.07 ± 0.02^{e}	0.09 ± 0.01^{e}
22:5	0.54 ± 0.13^{d}	0.33 ± 0.09^{g}	0.42 ± 0.05^{e,f}	0.38 ± 0.06^{e,f,g}	0.45 ± 0.10^{e}	0.37 ± 0.04^{f,g}	0.36 ± 0.05^{g}
22:6	6.71 ± 0.78	6.19 ± 0.93	6.77 ± 1.08	6.58 ± 0.78	6.98 ± 0.61	7.09 ± 0.62	6.65 ± 1.10
Total n-3 PUFA	7.56 ± 0.82	6.67 ± 0.88	7.44 ± 1.08	7.13 ± 0.74	7.61 ± 0.64	7.63 ± 0.59	7.18 ± 1.13
Phosphatidylethanolamine (diacyl)							
18:2	6.15 ± 1.56^{d}	4.78 ± 1.13^{e}	4.34 ± 0.58^{e,f}	4.44 ± 1.45^{e,f}	4.49 ± 0.89^{e,f}	3.58 ± 1.34^{f}	4.04 ± 0.96^{e,f}
20:2	0.39 ± 0.26^{d}	0.17 ± 0.06^{e}	0.22 ± 0.07^{e}	0.18 ± 0.10^{e}	0.26 ± 0.06^{e}	0.24 ± 0.10^{e}	0.20 ± 0.14^{e}
20:3	0.40 ± 0.24	0.28 ± 0.12	0.35 ± 0.12	0.28 ± 0.07	0.33 ± 0.13	0.25 ± 0.08	0.32 ± 0.13
20:4	23.6 ± 3.0^{f}	27.6 ± 1.0^{d}	25.2 ± 1.4^{e}	25.9 ± 2.2^{e}	25.2 ± 1.7^{e}	26.4 ± 0.9^{e}	25.8 ± 1.3^{e}
22:4	0.32 ± 0.07	0.28 ± 0.08	0.26 ± 0.04	0.28 ± 0.13	0.28 ± 0.06	0.33 ± 0.04	0.31 ± 0.07
22:5	0.57 ± 0.14^{e}	0.85 ± 0.20^{d}	0.79 ± 0.17^{d}	0.86 ± 0.30^{d}	0.84 ± 0.18^{d}	0.90 ± 0.26^{d}	0.93 ± 0.25^{d}
Total n-6 PUFA	31.4 ± 3.6	34.0 ± 1.6	31.2 ± 1.6	32.0 ± 3.0	31.4 ± 1.6	31.7 ± 2.1	31.6 ± 1.4
20:4/18:2	3.99 ± 0.74^{f}	6.10 ± 1.47^{e}	5.92 ± 0.85^{e}	6.51 ± 2.60^{e}	5.84 ± 1.27^{e}	8.06 ± 2.17^{d}	6.81 ± 2.03^{d,e}
18:3	0.14 ± 0.05^{d}	0.08 ± 0.02^{e}	0.09 ± 0.03^{e}	0.11 ± 0.04^{e}	0.10 ± 0.06^{e}	0.09 ± 0.02^{e}	0.09 ± 0.03^{e}
20:5	0.28 ± 0.11^{d}	0.12 ± 0.06^{f}	0.21 ± 0.05^{e}	0.16 ± 0.05^{f}	0.14 ± 0.05^{f}	0.11 ± 0.04^{f}	0.13 ± 0.04^{f}
22:5	1.11 ± 0.21^{d}	0.93 ± 0.30^{d,e,f,g}	1.00 ± 0.17^{d,e,f}	0.91 ± 0.18^{e,f,g}	1.07 ± 0.21^{d,e}	0.80 ± 0.28^{g}	0.86 ± 0.18^{f,g}
22:6	15.6 ± 3.1^{e,f}	14.6 ± 1.9^{f}	17.2 ± 1.8^{d,e}	16.1 ± 1.8^{d,e,f}	17.5 ± 2.3^{d}	17.3 ± 1.7^{d,e}	16.4 ± 1.8^{d,e}
Total n-3 PUFA	17.2 ± 3.3^{d,e}	15.8 ± 1.9^{e}	18.5 ± 1.8^{d}	17.3 ± 1.7^{d,e}	18.9 ± 2.3^{d}	18.2 ± 1.5^{d}	17.5 ± 1.9^{d}

[a]Data are means ± SD, $n = 12$ for each group. See Table 2 for column heading explanations.
[b]Means were compared by Fisher's multiple range test; means not sharing a common superscript letter are significantly different at $P < 0.05$.
[c]Fe– means iron-deficient groups, Fe+ means iron-adequate groups, PF means control group pair-fed to the iron-deficient group. PUFA, polyunsaturated fatty acids.

accumulation of linoleic and linolenic acid in hepatic individual phospholipids. Iron has been shown to be a structural component of Δ-6 desaturase (33), and the increased levels of linoleic and linolenic acids may be due to a decreased level of the chain elongation and desaturase activities necessary for the synthesis of arachidonic acid and eicosapentaenoic acids, which are essential substrates for the production of endoperoxides and thromboxanes. Iron is also present in the cytochrome and the terminal components of the stearyl CoA desaturase, which converts palmitic to palmitoleic acid and stearic to oleic acid (34). Individual hepatic phospholipids of iron-depleted rats from the present study had lower proportions of palmitoleic and oleic acids and higher proportions of stearic acid. Similar fatty acid alterations as a consequence of iron deficiency have been also found in liver total lipids (4,7,35), brain (36), and intestinal mucosa (4). Moreover, desaturase activity in livers of rats fed low-iron diets was significantly lowered (37). However, the study of Johnson *et al.* (6) did not confirm the requirement for iron in the Δ-9 pathway of fatty acid desaturation.

In conclusion, the current findings point to the fact that moderate nutritional iron depletion alters various aspects of lipid metabolism. Our study revealed that iron restriction first affects the lipid variables including cholesterol in liver and serum and fatty acid desaturation, and last the hepatic lipogenic enzymes and the triacylglycerol content in liver and serum. Therefore, the cholesterol concentration in serum and liver and the alteration in fatty acid composition of hepatic phospholipids best reflect the gradations of iron status and can be used as an indicator of the degree of iron deficiency.

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[Received April 8, 1998, and in final revised form and accepted August 4, 1998]

Effects of Long-Term Feeding of Marine Oils with Different Positional Distribution of Eicosapentaenoic and Docosahexaenoic Acids on Lipid Metabolism, Eicosanoid Production, and Platelet Aggregation in Hypercholesterolemic Rats

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ABSTRACT: Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were distributed mainly in the *sn*-1,3 positions of seal oil triglyceride and in the *sn*-2 position of squid oil triglyceride. Seal oil-rich or squid oil-rich fats having constant saturated/monounsaturated/polyunsaturated fatty acid (PUFA) and n-6/n-3 PUFA ratios were fed to exogenously hypercholesterolemic rats for 160 d. The control fat contained linoleic acid as the sole PUFA. Before starting the experimental diets, rats were orally treated with high doses of vitamin D for 4 d to accelerate atherogenesis. The percentage of arachidonic acid in phosphatidylcholine and phosphatidylethanolamine of liver, platelets, and aorta was lower in the marine oil groups than in the control group, seal oil being more effective than squid oil. Maximal platelet aggregation induced by collagen was significantly lower in both marine oil groups. Platelet thromboxane (TX) A_2 production induced by collagen or thrombin was markedly reduced by feeding seal or squid oils, the reduction being more pronounced in the seal oil than in the squid oil group. Aortic prostacyclin (PGI_2) production was the same among the three groups. The ratio of the productions of aortic PGI_2 and platelet TXA_2 was significantly higher in the seal oil than in the control group. Although there was no difference in intimal thickness among the three groups, the aortic cholesterol content was significantly lower in the marine oil groups than in the control group. These results showed that the main effects in rats of the different intramolecular distributions of EPA and DHA in dietary fats were on arachidonic acid content in tissue phospholipids and on platelet TXA_2 production.
Lipids 33, 897–904 (1998).

The Greenland Inuit who mainly live on seal have a low incidence of myocardial infarction (1). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are thought to be responsible for the physiological effects. These n-3 polyunsaturated fatty acids (PUFA) have lowering effects on triglyceride and cholesterol concentrations in plasma, on arachidonic acid content in various tissue phospholipids, and on thromboxane (TX) A_2 production in platelets (2,3). These functions are thought to contribute to the antiatherosclerotic effect of EPA and DHA (2,4). These PUFA are mainly distributed at the 1 and 3 positions in seal oil triglycerides. Several studies showed that differences in the intramolecular distribution of fatty acids in triglycerides may influence physiological functions of the fatty acids (5,6). However, there are few studies on physiological effects of the different intramolecular distributions of EPA and DHA in triglycerides (7,8).

In our previous study, dietary seal oil reduced plasma and liver triglyceride concentrations and arachidonic acid content in liver phospholipids more effectively than fish oil, in which EPA and DHA were located mainly at the *sn*-2 position (8). The ratio of the productions of aortic prostacyclin (PGI_2) and platelet TXA_2 stimulated by thrombin was significantly higher in rats fed seal oil than in those fed fish oil. These results suggest that seal oil may be more effective for suppressing thrombosis and hence atherosclerosis than fish oil.

In the present study, we investigated the effect of long-term feeding (160 d) of seal oil-rich and squid oil-rich oils to exogenously hypercholesterolemic (ExHC) rats, a strain of Sprague-Dawley rats which readily develops hypercholesterolemia on cholesterol feeding (9). EPA and DHA were located mainly at the *sn*-2 position in the squid oil. ExHC rats were orally treated with high doses of vitamin D to accelerate atherogenesis before starting a hypercholesterolemic diet. We previously observed that this treatment induces atherosclerotic lesions and cholesterol accumulation in rat aorta (9). For quantitative estimation of the effects of the structural differences in seal and squid oils, the dietary fats were designed to give constant ratios of saturated and monounsaturated fatty acids, PUFA, and n-6 and n-3 PUFA in these marine oil groups. The control fat contained linoleic acid as the sole PUFA.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ExHC, exogenously hypercholesterolemic; HDL, high density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGI_2, prostacyclin; PRP, platelet-rich plasma; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid-reactive substances; TX, thromboxane.

MATERIALS AND METHODS

Materials. Squid oil was obtained from the liver of *Todarodes pacificus*. Seal oil was from harp seal. These oils were purified to edible grade. Briefly, these oils dissolved in 5 vol of acetone were cooled to −80°C and the supernatants were concentrated and the obtained lipids were purified with silica gel chromatography. Fatty acid composition of squid and seal oils and the intramolecular distribution of fatty acids are shown in Table 1. EPA and DHA were mainly located at the *sn*-2 position of the triglyceride in squid oil; in contrast, 16:0 and 18:1 were at the *sn*-1,3 positions. In seal oil, EPA and DHA were mainly at the *sn*-1,3 positions and 16:1 and 18:1 were at the *sn*-2 position. Seal and squid oils contained cholesterol at 0.14 and 1.6% and α-tocopherol at 0.031 and 0.23%, respectively.

Safflower and coconut oils were obtained from Nissin Oil Mills Ltd., Tokyo, Japan, and Nacalai Tesque, Kyoto, Japan, respectively.

Animals and diets. ExHC rats originally donated by Takeda Chemical Industries (Osaka, Japan) were bred and maintained at Seac Yoshitomi (Fukuoka, Japan). Seven-week-old male ExHC rats weighing an average of 253 g were housed individually in an air-conditioned room (21–24°C, lights on 800–2000) and given free access to a nonpurified commercial diet (NMF, Oriental Yeast Co., Tokyo, Japan) for a week. The rats were given vitamin D_2 (200,000 IU/kg body weight) orally for four successive days as described previously (9). The vitamin D_2-treated rats were divided into three groups and maintained on a purified diet for 160 d. The experimental diets were prepared according to the recommendation of the American Institute of Nutrition (10) and contained (g/kg) cornstarch 355, casein 200, pregelatinized cornstarch 132, sucrose 100, fat 100, cellulose 50, mineral mixture (AIN-93G-MIX) 35, vitamin mixture (AIN-93-VX) 10, cholesterol 10, L-cystine 3, choline bitartrate 2.5, sodium cholate 2.5, and *tert*-butylhydroquinone 0.014. The mineral and vitamin mixtures were purchased from Nihon Nosan Kogyo (Tokyo, Japan). Dietary fats were designed to have a constant ratio of polyunsaturated/monounsaturated/saturated fatty acids ratio as shown in Table 2. The energy percentage of n-3 PUFA was 2.2 in the diets. The rats in the seal and squid oil groups were fed on average 190 mg/d of n-3 PUFA during the feeding period. Dietary fat in the control group was a mixture of coconut and safflower oils (76.1:23.9 w/w). Dietary fats in the seal and squid oil groups were mixtures of seal, coconut, and safflower oils (24.1:66.9:9.0 by wt) and squid, coconut, and safflower oils (26.1:65.0:8.9 by wt), respectively. Seal and squid oils contributed cholesterol to the diets at 3.4 and 42 mg/100 g diet, respectively. Because cholesterol was added at 1000 mg/100 g diet, the cholesterol contribution of seal and squid oils to lipid metabolism is minimal. The contribution of α-tocopherol from the seal and squid oils ws 0.75 and 6.1 mg/100 g diet, respectively. Rats were given only freshly prepared food each evening to prevent peroxidation of dietary fat. After the rats had been starved for 7 h (700–1400), they were killed under diethyl ether anesthesia by withdrawing blood from the abdominal aorta into a syringe containing 3.8% trisodium citrate.

Tissue preparation for determining atherosclerosis histologically. The entire aorta with its main branches was dissected from each animal and washed with physiological saline. Thoracic aorta between heart junction and right carotid artery was perfused with 20% neutral formalin buffer solution at pH 7.4, and preserved in the same solution before paraffin embedding in the routine manner to prepare 5 μm-thick serial sections. The sections were stained with orcein

TABLE 1
Fatty Acid Composition (mol%) and Triglyceride Structure of Squid and Seal Oils

	Squid oil			Seal oil		
Fatty acids	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
14:0	4.0	1.9	4.4	3.3	6.2	1.0
14:1	0.4	0.2	0.4	2.7	5.4	0.6
16:0	10.9	3.9	14.0	1.8	3.2	1.3
16:1	5.5	3.0	7.0	16.4	30.7	9.0
16:2	1.7	1.3	3.3	1.4	2.5	1.0
16:3	0.7	3.9	2.4	0.9	4.7	1.6
16:4	0.4	0.4	0.4	1.5	1.4	1.4
18:0	1.5	0.5	2.0	0.2	0.3	0.2
18:1n-9	11.2	5.8	14.7	13.4	20.1	11.3
18:1n-7	3.8	2.1	5.1	2.2	2.2	2.1
18:2n-6	1.5	1.6	1.8	2.4	5.8	1.0
18:3n-3	1.2	1.7	1.3	1.6	3.1	0.8
18:4n-3	2.5	2.2	2.6	6.2	3.8	7.3
20:1	6.2	4.3	7.5	3.7	1.2	4.9
20:4n-6	1.7	2.0	1.6	0.5	0.2	0.5
20:4n-3	1.0	1.4	0.7	1.0	0.5	1.3
20:5n-3	20.5	26.1	16.6	15.2	2.8	21.4
22:1n-9	3.0	2.1	3.5	0.7	0.1	1.0
22:5n-3	1.2	4.6	0.8	6.2	2.5	9.3
22:6n-3	21.2	31.2	9.9	19.0	3.3	23.1

TABLE 2
Fatty Acid Composition (wt%) of Dietary Fats[a]

	Group		
Fatty acids	Control	Squid oil	Seal oil
8:0	6.5	5.9	5.8
10:0	5.3	4.8	4.8
12:0	38.1	35.0	35.0
14:0	13.0	12.5	12.7
16:0	7.8	8.2	6.6
16:1	0.0	1.0	3.0
18:0	2.6	2.3	2.1
18:1n-9	7.6	7.3	8.2
18:2n-6	18.2	7.6	7.8
18:4n-3	—	0.5	1.1
20:1	—	1.3	0.8
20:5n-3	—	4.1	3.0
22:5n-3	—	0.3	1.4
22:6n-3	—	4.6	4.1
SFA	73.9	71.8	69.7
MUFA	7.8	10.3	12.3
n-6 PUFA	18.2	8.0	8.0
n-3 PUFA	—	9.9	10.0

[a]SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; —, less than 1 wt%.

and hematoxylin. Intimal thickness was measured by using a Nikon microscope and NIH Image as described (9).

Analytical methods. The triglyceride structure of squid and seal oils was analyzed by a Grignard degradation method as described previously (8). Briefly, triglyceride was reacted with 1 M ethylmagnesium bromide in diethyl ether for 25 s. After the addition of acetic acid to stop the reaction, the reaction mixture was extracted with diethyl ether. After the ether extract was concentrated by evaporating off, it was applied to a silica gel G thin-layer chromatography plate containing 5% boric acid, and the plate was developed with chloroform/acetone (96:4, vol/vol). 2-Monoglyceride and a mixture of 1- and 3-monoglyceride fractions were transmethylated with sulfuric acid/methanol (1:115, vol/vol). Fatty acid methyl esters were analyzed by gas–liquid chromatography on an Omegawax 320 capillary column (Supelco Japan, Tokyo, Japan; 30 m long, 0.25 μm film, 0.32 mm i.d., helium as carrier gas, split ratio of 1:80). Column, injector, and detector temperatures were 190, 250, and 250°C, respectively.

Plasma lipoproteins were prepared by ultracentrifugation as described previously (11). Plasma and lipoprotein lipids were determined by commercially available kits; Cholesterol C Test, Triglyceride G Test, and Phospholipid B Test (Wako Pure Chemicals, Osaka, Japan). Plasma high density lipoprotein (HDL)-cholesterol was determined with HDL-C Daiichi (Daiichi Pure Chemicals, Tokyo, Japan). Serum thiobarbituric acid-reactive substances (TBARS) were measured by LPO-Test (Wako Pure Chemicals). Tissues and thoracic aorta lipids were extracted by the method of Folch *et al.* (12). Cholesterol (13), triglycerides (14), and phospholipids (15) in the liver were analyzed as described previously. After saponification of aortic lipids, cholesterol was derivatized to the trimethylsilyl ether and then quantified by gas–liquid chromatography on a 3% OV-17 (GL Science, Tokyo, Japan) column (3). 5α-Cholestane (Nacalai Tesque) was used as an internal standard. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triglyceride in tissues were separated by thin-layer chromatography, and the fatty acid composition was analyzed by gas–liquid chromatography as described above.

Analysis of eicosanoids and platelet aggregation. Approximately 28 mg of abdominal aorta was incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) at 25°C for 30 min (8), and the concentration of 6-keto-prostaglandin $F_{1\alpha}$, a stable metabolite of PGI_2, was determined with a commercial enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Citrated plasma was centrifuged at $160 \times g$ for 10 min to obtain platelet-rich plasma (PRP), and platelet-poor plasma was obtained from the centrifugation of PRP at $1000 \times g$ for 15 min. PRP was stimulated by 10 μg/mL collagen, and platelet aggregation was measured with an automated platelet aggregation analyzer (Aggrecorder II; Kyoto Daiichi Kagaku, Kyoto, Japan) at 37°C. After 5 min, indomethacin (final concentration, 42 μM; Sigma-Aldrich Japan, Tokyo, Japan) was added to stop the production of TXA_2. Citrated blood was stimulated by thrombin (final concentration 1 U/200 μL citrated blood) for 5 min at 37°C, and the reaction was stopped by the addition of indomethacin. TXB_2, a stable metabolite of TXA_2, was measured with a commercial enzyme immunoassay kit (Cayman Chemical).

Statistical analysis. Data were analyzed by Duncan's new multiple-range test (16), and differences were considered significant at $P < 0.05$.

RESULTS

Growth, food intake, and liver weight. Body weights, food intake, and relative liver weights are shown in Table 3. There were no significant differences in body weight gain and food intake. Relative liver weight was significantly higher in the seal oil group than in the squid oil and control groups.

The concentrations of plasma and liver lipids and plasma TBARS. The concentrations of plasma and liver lipids are shown in Table 3. Total cholesterol, triglyceride, and phospholipid concentrations in plasma were significantly lower in the squid and seal oil groups than in the control group. The HDL-cholesterol concentration was significantly lower in the squid oil group than in the control group. The atherogenic index [total cholesterol − HDL-cholesterol)/HDL-cholesterol] in the seal oil group was significantly lower than indices in the other groups. The reduction of cholesterol and triglyceride concentrations in plasma was due to the decrease both in very low density lipoprotein and low density lipoprotein fractions (data not shown). The concentration of TBARS in plasma was significantly higher in the seal oil group than in the control group.

The liver triglyceride concentration was significantly lower in the squid and seal oil groups than in the control group. There were no significant differences in hepatic cholesterol and phospholipid concentrations among the three groups.

TABLE 3
Growth Parameters, Plasma Lipid Concentrations, Atherogenic Indices, and Thiobarbituric Acid-Reactive Substances

	Group		
	Control[a]	Squid oil[a]	Seal oil[a]
Growth parameters			
Initial body weight (g)	253 ± 6	250 ± 4	257 ± 3
Body weight gain (g)	329 ± 9	346 ± 10	354 ± 20
Food intake (g/d)	18.6 ± 0.3	18.9 ± 0.3	19.0 ± 0.4
Relative liver weight (g/100 g body weight)	5.26 ± 0.11[a]	5.22 ± 0.11[a]	5.74 ± 0.18[b]
Plasma lipid concentration			
Total cholesterol (mg/dL)	619 ± 68[a]	368 ± 28[b]	360 ± 46[b]
HDL-cholesterol (mg/dL)	49.8 ± 5.0[a]	33.7 ± 2.4[b]	44.8 ± 4.7[a,b]
Triglyceride (mg/dL)	206 ± 20[a]	112 ± 11[b]	132 ± 18[b]
Phospholipid (mg/dL)	402 ± 37[a]	203 ± 19[b]	228 ± 26[b]
Atherogenic index	11.5 ± 0.7[a]	10.5 ± 1.5[a]	7.21 ± 0.81[b]
Plasma TBARS (nmol/mL)	3.36 ± 0.37[a]	4.21 ± 0.38[a,b]	5.22 ± 0.44[b]
Liver lipid concentration (mg/g)			
Cholesterol	152 ± 7	156 ± 3	161 ± 4
Triglyceride	24.4 ± 1.4[a]	19.2 ± 0.9[b]	18.0 ± 0.4[b]
Phospholipid	22.3 ± 0.3	23.6 ± 0.4	23.8 ± 0.7

[a]Values represent mean ± SE, $n = 8$ rats. Values with different superscript roman letters are significantly different at $P < 0.05$. HDL, high density lipoprotein; atherogenic index: (total cholesterol – HDL-cholesterol)/HDL-cholesterol; TBARS, thiobarbituric acid-reactive substances.

Fatty acid composition in liver, platelets, and aorta. Fatty acid composition of PC and PE was measured in liver, platelets, and aorta (Tables 4–6). The percentage of arachidonic acid in the phospholipids of these tissues was significantly lower in the marine oil groups than in the control group. Seal oil was more effective for lowering the arachidonic acid content than squid oil. Percentages of linoleic acid in the aorta PC and platelet PC and PE were significantly higher in the seal oil group than in the squid oil group. As a result, the desaturation index [(20:4n-6 + 20:3n-6)/18:2n-6] was lower in the marine oil groups than in the control group, seal oil being more effective than squid oil. Percentages of n-3 PUFA in liver PC, aorta PE, and platelet PE were higher in the seal oil group than in the squid oil group. EPA and n-3 docosapentaenoic acid (22:5n-3) in aorta and platelet PE were higher in the seal oil than in the squid oil group.

TABLE 4
Fatty Acid Composition (wt%) of Phosphatidylcholine and Phosphatidylethanolamine in Liver

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acids	Control group	Fish oil group	Seal oil group	Control group	Fish oil group	Seal oil group
16:0	21.0 ± 0.4[a]	23.8 ± 0.5[b]	24.2 ± 0.5[b]	14.0 ± 0.9[a]	17.7 ± 1.1[b]	20.4 ± 1.4[b]
16:1	3.1 ± 0.2	3.0 ± 0.2	3.5 ± 0.2	1.7 ± 0.2	1.3 ± 0.1	1.4 ± 0.1
18:0	13.5 ± 0.2	13.1 ± 0.5	12.8 ± 0.3	16.7 ± 0.4[a]	16.3 ± 0.5[a]	14.3 ± 0.4[b]
18:1n-9	7.4 ± 0.3[a]	10.0 ± 0.3[b]	10.1 ± 0.3[b]	8.9 ± 0.8	7.4 ± 0.4	9.4 ± 0.7
18:1n-7	6.9 ± 0.1[a]	4.8 ± 0.1[b]	4.9 ± 0.2[b]	7.2 ± 0.3[a]	4.4 ± 0.1[b]	4.5 ± 0.1[b]
18:2n-6	20.1 ± 0.3[a]	18.1 ± 0.3[b]	19.5 ± 0.7[a,b]	12.7 ± 0.4[a]	8.3 ± 0.3[b]	9.9 ± 0.9[b]
Unknown	0.9 ± 0.3	0.9 ± 0.2	0.8 ± 0.1	1.4 ± 0.3	2.0 ± 0.4	1.3 ± 0.2
20:3n-6	4.2 ± 0.2[a]	1.4 ± 0.1[b]	1.7 ± 0.1[b]	2.8 ± 0.2[a]	0.6 ± 0.1[b]	0.7 ± 0.0[b]
20:4n-6	22.5 ± 0.3[a]	9.5 ± 0.3[b]	5.4 ± 0.2[c]	29.2 ± 0.7[a]	9.7 ± 0.5[b]	5.6 ± 0.4[c]
20:5n-3	0.0 ± 0.0[a]	7.0 ± 0.3[b]	7.7 ± 0.3[b]	0.0 ± 0.0[a]	8.4 ± 0.4[b]	8.8 ± 0.4[b]
22:4n-6	—	—	—	1.0 ± 0.2[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
22:5n-6	—	—	—	1.3 ± 0.3[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
22:5n-3	0.0 ± 0.0[a]	0.7 ± 0.1[b]	1.2 ± 0.1[c]	0.1 ± 0.1[a]	1.6 ± 0.4[b]	2.4 ± 0.1[c]
22:6n-3	0.4 ± 0.2[a]	7.7 ± 0.2[b]	8.2 ± 0.5[b]	3.1 ± 0.2[a]	22.6 ± 1.0[b]	21.2 ± 0.8[b]
n-6 PUFA	46.8 ± 0.3[a]	29.0 ± 0.5[b]	26.6 ± 0.8[c]	45.6 ± 1.3[a]	18.5 ± 0.7[b]	16.2 ± 1.3[b]
n-3 PUFA	0.4 ± 0.2[a]	15.5 ± 0.4[b]	17.1 ± 0.7[c]	3.2 ± 0.2[a]	32.5 ± 1.5[b]	32.5 ± 0.9[b]
Desat. index	1.33 ± 0.03[a]	0.60 ± 0.02[b]	0.37 ± 0.01[c]	2.56 ± 0.05[a]	1.25 ± 0.07[b]	0.66 ± 0.04[c]

[a]Mean ± SE, $n = 8$ rats. Values with different superscript roman letters are significantly different at $P < 0.05$. Desat. index, Desaturation index = [(20:3n-6 + 20:4n-6)/18:2n-6]. For other abbreviations see Table 2.

TABLE 5
Fatty Acid Composition (wt%) of Phosphatidylcholine and Phosphatidylethanolamine in Aorta

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acids	Control group	Fish oil group	Seal oil group	Control group	Fish oil group	Seal oil group
14:0	1.4 ± 0.1[a]	1.1 ± 0.1[b]	1.2 ± 0.1[b]	1.5 ± 0.1[a]	2.7 ± 0.4[b]	2.4 ± 0.5[a,b]
16:0	31.8 ± 0.4[a]	33.6 ± 0.6[b]	33.4 ± 0.5[b]	8.0 ± 0.3[a]	9.7 ± 0.2[b]	10.3 ± 0.2[b]
16:1	1.6 ± 0.1[a]	1.8 ± 0.1[a]	2.1 ± 0.1[b]	3.7 ± 0.2[a]	5.6 ± 0.5[b]	5.1 ± 0.4[b]
Unknown	—	—	—	1.4 ± 0.1	2.6 ± 0.6	1.7 ± 0.4
Unknown	—	—	—	8.7 ± 0.2[a]	7.7 ± 0.2[b]	7.3 ± 0.2[b]
16:4	—	—	—	2.0 ± 0.1[a]	1.2 ± 0.3[b]	1.6 ± 0.3[a,b]
18:0	15.1 ± 0.3	14.7 ± 0.2	14.6 ± 0.2	16.1 ± 0.2	15.7 ± 0.4	16.4 ± 0.4
18:1n-9	6.2 ± 0.2[a]	8.1 ± 0.3[b]	7.9 ± 0.3[b]	4.3 ± 0.2[a]	6.3 ± 0.6[b]	6.0 ± 0.4[b]
18:1n-7	6.5 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	1.4 ± 0.0[a,b]	1.1 ± 0.3[a]	1.7 ± 0.1[b]
18:2n-6	9.6 ± 0.4[a]	12.9 ± 0.2[b]	15.0 ± 0.2[c]	2.4 ± 0.3	3.2 ± 0.5	3.3 ± 0.3
20:3n-6	2.3 ± 0.1[a]	2.6 ± 0.1[b]	3.2 ± 0.1[c]	1.2 ± 0.1[a]	1.7 ± 0.1[b]	2.0 ± 0.1[c]
20:4n-6	23.7 ± 0.5[a]	11.1 ± 0.2[b]	8.5 ± 0.4[c]	36.4 ± 0.4[a]	21.1 ± 1.0[b]	18.1 ± 1.0[c]
20:5n-3	0.0 ± 0.0[a]	3.3 ± 0.1[b]	3.5 ± 0.2[b]	0.6 ± 0.3[a]	4.8 ± 0.2[b]	6.0 ± 0.4[c]
22:4n-6	0.8 ± 0.2[a]	0.2 ± 0.1[b]	0.1 ± 0.0[b]	8.0 ± 0.2[a]	1.4 ± 0.1[b]	1.0 ± 0.2[a,b]
22:5n-3	0.0 ± 0.0[a]	0.9 ± 0.1[b]	1.1 ± 0.2[b]	2.8 ± 0.3[a]	4.3 ± 0.3[b]	5.9 ± 0.4[c]
22:6n-3	0.4 ± 0.1[a]	2.7 ± 0.4[b]	2.5 ± 0.3[b]	1.8 ± 0.1[a]	11.0 ± 0.7[b]	11.3 ± 0.7[b]
n-6 PUFA	36.4 ± 0.6[a]	26.8 ± 0.2[b]	26.8 ± 0.4[b]	48.0 ± 0.5[a]	27.4 ± 0.7[b]	24.4 ± 0.8[c]
n-3 PUFA	0.4 ± 0.2[a]	6.8 ± 0.6[b]	7.0 ± 0.5[b]	5.1 ± 0.4[a]	20.1 ± 1.1[b]	23.2 ± 1.3[c]
Desat. index	2.73 ± 0.12[a]	1.06 ± 0.03[b]	0.78 ± 0.03[c]	17.4 ± 2.1[a]	8.20 ± 1.19[b]	6.43 ± 0.72[b]

[a]Mean ± SE, $n = 8$ rats. Values with different superscript roman letters are significantly different at $P < 0.05$. For abbreviations see Tables 2 and 4.

As shown in Table 7, percentages of DHA and EPA in liver triglycerides were significantly lower in the seal oil than in the squid oil group.

Eicosanoid production and platelet aggregation. As shown in Table 8, production of TXA_2 in PRP stimulated by collagen and in citrated blood stimulated by thrombin was much lower in the seal and squid oil groups than in the control group. Seal oil was more effective for lowering TXA_2 production than squid oil. The difference was particularly significant in the stimulation with collagen. Production of PGI_2 was not influenced by the dietary fats. As a result, the ratio PGI_2/TXA_2 was highest in the seal oil group and lowest in the control group. Maximal platelet aggregation by collagen was significantly lower in the squid and seal oil groups than in the control group.

TABLE 6
Fatty Acid Composition (wt%) of Phosphatidylcholine and Phosphatidylethanolamine in Platelets

	Phosphatidylcholine[a]			Phosphatidylethanolamine[a]		
Fatty acids	Control group	Fish oil group	Seal oil group	Control group	Fish oil group	Seal oil group
14:0	3.5 ± 0.2	2.9 ± 0.5	3.3 ± 0.5	0.9 ± 0.2[a]	1.6 ± 0.1[b]	1.5 ± 0.1[b]
16:0	52.2 ± 0.3[a,b]	52.3 ± 0.3[a]	51.1 ± 0.5[b]	4.5 ± 0.1[a]	5.2 ± 0.2[b]	5.2 ± 0.2[b]
16:1	1.9 ± 0.1[a]	2.5 ± 0.1[b]	2.9 ± 0.1[c]	6.3 ± 0.2	6.4 ± 0.2	6.5 ± 0.1
Unknown	—	—	—	4.5 ± 0.2[a]	5.5 ± 0.1[b]	5.4 ± 0.1[b]
Unknown	—	—	—	3.2 ± 0.1	3.0 ± 0.1	3.1 ± 0.1
16:4	—	—	—	3.0 ± 0.1[a]	2.2 ± 0.1[b]	2.5 ± 0.1[c]
18:0	6.0 ± 0.1	5.6 ± 0.2	5.7 ± 0.2	13.4 ± 0.2[a]	12.1 ± 0.2[b]	12.1 ± 0.2[b]
18:1n-9	4.9 ± 0.1[a]	7.0 ± 0.1[b]	7.2 ± 0.2[b]	3.5 ± 0.1[a]	4.2 ± 0.1[b]	4.5 ± 0.1[c]
18:1n-7	6.5 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	1.4 ± 0.0[a,b]	1.1 ± 0.3[a]	1.7 ± 0.1[b]
18:2n-6	13.3 ± 0.2[a]	14.1 ± 0.4[a]	15.3 ± 0.5[b]	4.4 ± 0.1[a]	6.4 ± 0.1[b]	8.0 ± 0.2[c]
20:3n-6	2.2 ± 0.1[a]	0.7 ± 0.0[b]	0.8 ± 0.0[b]	1.6 ± 0.1[a]	0.9 ± 0.0[b]	1.1 ± 0.0[c]
20:4n-6	9.7 ± 0.2[a]	3.2 ± 0.1[b]	1.9 ± 0.1[c]	38.2 ± 0.3[a]	19.3 ± 0.3[b]	12.6 ± 0.3[c]
20:5n-3	0.0 ± 0.0[a]	4.5 ± 0.2[b]	4.5 ± 0.2[b]	0.0 ± 0.0[a]	19.5 ± 0.3[b]	22.9 ± 0.4[c]
22:4n-6	—	—	—	11.5 ± 0.2[a]	1.2 ± 0.1[b]	0.7 ± 0.0[c]
22:5n-3	0.0 ± 0.0[a]	0.5 ± 0.1[b]	0.6 ± 0.0[b]	0.2 ± 0.1[a]	6.6 ± 0.1[b]	7.4 ± 0.1[c]
22:6n-3	0.0 ± 0.0[a]	1.1 ± 0.0[b]	1.1 ± 0.1[b]	0.0 ± 0.0[a]	3.4 ± 0.1[b]	3.2 ± 0.1[b]
n-6 PUFA	25.3 ± 0.4[a]	18.0 ± 0.4[b]	17.9 ± 0.5[b]	55.6 ± 0.4[a]	27.8 ± 0.4[b]	22.4 ± 0.5[c]
n-3 PUFA	0.0 ± 0.0[a]	6.1 ± 0.1[b]	6.2 ± 0.2[b]	0.2 ± 0.1[a]	29.6 ± 0.5[b]	33.5 ± 0.4[c]
Desat. index	0.89 ± 0.02[a]	0.28 ± 0.01[b]	0.17 ± 0.00[c]	9.18 ± 0.23[a]	3.14 ± 0.06[b]	1.71 ± 0.02[c]

[a]Mean ± SE, $n = 8$ rats. Values with different superscript roman letters are significantly different at $P < 0.05$. For abbreviations see Tables 2 and 4.

TABLE 7
Fatty Acid Composition (wt%) of Triglyceride in Liver[a]

Fatty acids	Group		
	Control	Squid oil	Seal oil
14:0	3.1 ± 0.2[a]	2.0 ± 0.1[b]	2.0 ± 0.2[b]
16:0	21.4 ± 0.3	21.4 ± 0.3	21.8 ± 0.5
16:1	8.1 ± 0.6[a]	6.5 ± 0.2[b]	7.2 ± 0.2[a,b]
18:0	1.4 ± 0.1[a]	1.9 ± 0.1[b]	2.3 ± 0.1[c]
18:1n-9	33.9 ± 0.9[a]	35.9 ± 1.3[a]	40.9 ± 0.6[b]
18:1n-7	8.3 ± 0.2[a]	5.0 ± 0.2[b]	5.7 ± 0.2[c]
18:2n-6	22.0 ± 0.7[a]	13.7 ± 0.4[b]	13.6 ± 0.5[b]
20:3n-6	1.0 ± 0.0[a]	0.2 ± 0.0[b]	0.1 ± 0.0[b]
20:4n-6	0.9 ± 0.1[a]	0.3 ± 0.1[b]	0.2 ± 0.0[c]
20:5n-3	0.0 ± 0.0[a]	1.5 ± 0.2[b]	0.7 ± 0.1[c]
22:5n-3	0.0 ± 0.0[a]	1.7 ± 0.2[b]	1.1 ± 0.2[c]
22:6n-3	0.0 ± 0.0[a]	9.9 ± 1.0[b]	4.4 ± 0.7[c]
n-6 PUFA	23.9 ± 0.8[a]	14.2 ± 0.4[b]	13.9 ± 0.5[b]
n-3 PUFA	0.0 ± 0.0[a]	13.2 ± 1.4[b]	6.2 ± 1.0[c]

[a]Mean ± SE, $n = 8$ rats. Values with different superscript roman letters are significantly different at $P < 0.05$. For abbreviation see Table 2.

Intimal thickness and cholesterol concentration in aorta. Differences in the thickness of aortic intima were not statistically significant among the three groups (data not shown). Cholesterol content in aorta was significantly lower in the squid and seal oil groups than in the control group (1.91 ± 0.07, 2.06 ± 0.11, and 2.51 ± 0.21 μg/mg aorta in the squid oil, seal oil, and control groups, respectively).

DISCUSSION

We previously reported that triglyceride structures in dietary seal and fish oils were reflected by the structures in chylomicron triglycerides secreted from intestinal cells to lymph (8). EPA and DHA at the *sn*-1,3 positions in seal oil triglyceride were retained at 94 and 92% in lymph triglycerides collected for 3 h after the administration, respectively, and EPA and DHA at the *sn*-2 position in fish oil triglyceride was retained at 62 and 74%, respectively. A similar observation was reported by Christensen and Høy (17). Christensen *et al*. (7) also observed that the removal rate from plasma of radiolabeled cholesterol contained in chylomicrons following seal oil feeding was faster than that after fish oil feeding. In addition, the recovery of radiolabeled palmitic acid from the liver was significantly lower in rats fed seal oil than in animals fed fish oil. They also reported that fatty acids bound to 2-monoglycerides following hydrolysis of chylomicron triglycerides may affect the clearance rate of chylomicrons and chylomicron remnants (18,19). Based on these results, the structural difference of triglycerides of seal and squid oils may affect lipid metabolism differently.

EPA and DHA inhibit Δ6 desaturation of linoleic acid and hence reduce arachidonic acid content in tissue phospholipids (2). In the present study, the effects of feeding seal and squid oils on the arachidonic acid content in several tissue phospholipids differed (Tables 4–6). Although total n-3 PUFA content in the dietary fats was the same in the seal and squid oil groups, feeding of seal oil more effectively reduced the arachidonic acid content in liver, platelets, and aorta phospholipids than squid oil. Desaturation indices in these phospholipids were significantly lower in the seal oil group than in the squid oil group. Therefore, the reduction of arachidonic acid in these phospholipids may be due to the inhibition of Δ6 desaturation. Generally, an increase of n-3 PUFA content in the liver induces the inhibition of Δ6 desaturation of linoleic acid and hence the reduction of arachidonic acid (2). However, although total n-3 PUFA content contained in liver PC was slightly higher in the seal oil group than in the squid oil group, it was not increased in liver PE by feeding with seal oil. In addition, n-3 PUFA content in liver triglycerides of the seal oil group was only half that of the squid oil group. These observations indicate that total n-3 PUFA content in the liver of rats fed seal oil was lower than of those fed squid oil. Therefore, the reduction of arachidonic acid content in the seal oil group may have occurred without an increase of n-3

TABLE 8
Production of Aortic Thromboxane A_2 and Prostacyclin, and Platelet Aggregation[a]

	Group		
	Control	Squid oil	Seal oil
Production of platelet thromboxane A_2 (ng/mL)			
Collagen	492 ± 30[a]	150 ± 14[b]	74.6 ± 4.4[c]
Thrombin	555 ± 38[a]	145 ± 15[b]	101 ± 8[b]
Production of aortic prostacyclin (pg/mg aorta)			
	1019 ± 241	847 ± 264	937 ± 193
Ratio of aortic prostacyclin and platelet thromboxane A_2			
Collagen	2.19 ± 0.54[a]	5.99 ± 1.86[a,b]	10.1 ± 2.67[b]
Thrombin	2.11 ± 0.73[a]	5.77 ± 1.28[a,b]	9.60 ± 2.55[b]
Maximal aggregation (%)			
Collagen	48.4 ± 3.2[a]	40.8 ± 2.3[b]	40.7 ± 0.9[b]

[a]Mean ± SE, $n = 6$–8 rats. Values with different superscript roman letters are significantly different at $P < 0.05$. Prostacyclin and thromboxane A_2 were measured as 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, respectively. Collagen: final concentration of collagen was 10 μg/mL. Thrombin: final concentration of thrombin was 1 U/200 μL citrated blood. Maximal aggregation: platelet was stimulated by 10 μg/mL collagen.

PUFA content in the liver. It is not evident how the structural differences of the triglycerides may influence Δ6 desaturation of linoleic acid.

An alternative possibility is that the differences in n-3 PUFA composition between dietary seal and squid oils may cause the observed influence on fatty acid composition in the phospholipids. Octadecatetraenoic (18:4n-3) and docosapentaenoic (22:5n-3) acids were higher in the seal oil than in the squid oil (Table 1). However, the effect of these n-3 PUFA on arachidonic acid content in phospholipids has never been reported. Mechanisms for different effects between seal and squid oils on linoleic acid metabolism deserve further investigation.

In our previous study in which seal oil-rich or fish oil-rich diets were fed to rats for 3 wk, the reduction of arachidonic acid content in the seal oil group was observed in liver PC and PE but not in platelet and aorta PC (8). In the present study, the percentage of arachidonic acid was even lower in platelet and aorta PC and PE in the seal oil group than in the squid oil group. The desaturation and elongation of linoleic acid to arachidonic acid are performed mainly in the liver (20) and hence a large portion of arachidonic acid in phospholipids of extrahepatic tissues is supplied from the liver. Christensen and Høy (21) did not observe any influence that was attributable to structural differences in seal and fish oils on the incorporation rate of n-3 PUFA into and the arachidonic acid content of heart and kidney phospholipids of rats fed for 17 d (21). These observations suggest that the influence of the reduction of arachidonic acid in the liver would be slowly transferred to the extrahepatic tissues. Therefore, long-term feeding may be necessary to identify effects of structural differences of seal and squid oils.

The content of n-3 PUFA, in particular EPA and 22:5n-3, in platelet and aorta PE was higher in the seal oil group than in the squid oil group. The increase in EPA occurred in spite of the lower EPA content in dietary seal oil compared with squid oil. Chylomicron triglyceride incorporated from intestinal lymph into the bloodstream is hydrolyzed at *sn*-1 and *sn*-3 positions to free fatty acid and 2-monoglyceride by peripheral lipopotein lipase. A portion of the free fatty acids is incorporated into the peripheral tissues, and the remaining part is taken up by the liver. A portion of the 2-monoglyceride is transformed to 1-monoglyceride and then hydrolyzed to free fatty acid. The remaining portion of the 2-monoglyceride is incorporated into the liver with the chylomicron remnant. It is thought that because of low activity, if any, of the 2-monoglyceride pathway, 2-monoglyceride incorporated into the liver is hydrolyzed to free fatty acid and glycerol and then the fatty acid is partitioned to various lipid fractions. The extent of the partition of free fatty acids and 2-monoglyceride between the peripheral tissues and the liver is not clearly understood. It seems likely that the partition may depend to some extent on the species of fatty acids, in particular, EPA and DHA. Ackman (22) suggested that EPA and DHA at the 1 and 3 positions in fish oil would be incorporated mainly into the peripheral tissues rather than the liver. Our results support this suggestion, because n-3 PUFA, in particular EPA, were preferentially incoporated into aorta and platelets rather than liver in rats fed on seal oil compared to rats fed on squid oil.

Platelet TXA_2 production was effectively suppressed by feeding with either marine oils. In particular, seal oil was more effective than squid oil in reducing TXA_2 production. Thrombin and collagen had similar influences on TXA_2 production, because the correlation coefficient between the production of TXA_2 stimulated by thrombin and collagen was high (r = +0.914). The reduction could be induced by the decrease in arachidonic acid of platelet phospholipids, because a highly positive correlation between platelet TXA_2 production and arachidonic acid content has been reported (2). In the present study, the correlation coefficient between the production of platelet TXA_2 stimulated by thrombin and the arachidonic acid content in platelet PC was high (r = +0.96). We previously reported that, although both EPA and DHA reduced arachidonic acid content in phospholipids of various tissues, DHA was more effective than EPA (4). Our results suggested that DHA, in comparison with EPA, more efficiently suppresses Δ6 desaturation of linoleic acid and competes with arachidonic acid for the *sn*-2 position of phospholipids. However, the relationship between the differences in triglyceride structure and the different effects of EPA and DHA on arachidonic acid content in phospholipids is not known.

In contrast to TXA_2 production, although arachidonic acid content in aorta phospholipids was suppressed by the feeding with marine oils, in particular with seal oil, PGI_2 production was not influenced by the reduction of arachidonic acid. Aortic PGI_2 production is not dependent on arachidonic acid content in aorta phospholipids (2). Saito *et al.* (23) reported that EPA enhanced the production of PGI_2 by activating cyclooxygenase in cultured rat vascular smooth muscle cells. This may explain why the production of PGI_2 was not reduced, although arachidonic acid content in aorta phospholipids was lower in rats fed seal or squid oils. In any event, it is reasonable to think that seal oil is more effective to prevent thrombosis than squid oil, because the PGI_2/TXA_2 ratio was higher in the seal oil group than in the squid oil group.

Atherosclerotic lesions developed in the aortas of ExHC rats treated orally for 180 d with high doses of vitamin D and a high cholesterol/sodium cholate/olive oil diet (9). However, atherosclerotic development was slight in this study compared with the previous observation. Therefore, the effect of squid and seal oils on atherogenesis was not apparent, although the aortic cholesterol content was lower in rats fed seal and squid oils. In our previous study, serum cholesterol concentration increased more than 1000 mg/dL (9), whereas about 600 mg/dL was maximal in the present study. This may be one of the reasons why intimal thickening was slight. The difference of dietary fats likely caused the different effect on the serum cholesterol concentration. Instead of the olive oil, rich in oleic acid, used in the previous study, dietary fat rich in lauric, myristic and palmitic acids was used as a basal fat in the present study. These saturated fatty acids are known to

be hypercholesterolemic in humans and experimental animals (24). It is not clear why saturated fatty acids in our animal model were not hypercholesterolemic compared with olive oil.

In conclusion, the different intramolecular distribution of EPA and DHA in dietary fats mainly affected arachidonic acid content in tissue phospholipids and TXA_2 production in platelets of rats fed for a long time, although the influence on atherosclerosis was not clearly observed. The mechanism for these different effects between seal and squid oils remains to be clarified.

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[Received February 23, 1998, and in final revised form August 4, 1998; accepted August 10, 1998]

Effects of Dietary Methionine and Cystine on Lipid Metabolism in Hepatoma-Bearing Rats with Hyperlipidemia

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ABSTRACT: Abnormal lipid metabolism and its restoration by dietary methionine (Met) and cystine (Cys) were studied in Donryu rats subcutaneously implanted with an ascites hepatoma cell line of AH109A. The hepatoma-bearing rats exhibited hyperlipidemia characterized by rises in serum triglyceride and cholesterol levels. Decreased lipoprotein lipase (LPL) activities in epididymal adipose tissue, cardiac muscle, and gastrocnemius as well as increased fatty acid mobilization from adipose tissue were considered to be responsible for the hepatoma-induced hypertriglyceridemia, while increased hepatic cholesterogenesis and decreased steroid excretion into feces were thought to be responsible for the hepatoma-induced hypercholesterolemia. Dietary-supplemented Met or Cys reduced the AH109A-induced hypertriglyceridemia with suppression of fatty acid synthesis in the host liver. Met restored the fall of LPL activities, while Cys did not. Dietary Met or Cys also reduced the hypercholesterolemia with restoration of decreased bile acid excretion into feces. These results suggest that dietary Met or Cys is hypolipidemic in the hepatoma-bearing rats with slight differences in their modes of action.

Lipids 33, 905–911 (1998).

Various cancers developed in, for example, breast and colon affect serum lipoprotein profiles in humans (1,2). Hepatoma also induces abnormal serum lipid metabolism in humans (3,4) and animals (5,6). Rats subcutaneously implanted with an ascites hepatoma cell line of AH109A show hyperlipidemia (7,8) with a notable decrease in high density lipoprotein (HDL) fraction and an enormous increase in the very low density lipoprotein plus low density lipoprotein (VLDL + LDL) fraction (6–8) during growth of the hepatoma. AH109A-bearing rats thus provide us with an endogenously hyperlipidemic animal model that is quite distinct from cholesterol (Ch)-loaded animals that have been widely used as an exogenously hypercholesterolemic model. Dietary-supplemented amino acids such as sulfur amino acids (9) and basic amino acids (10) can reduce the hepatoma-induced hyperlipidemia.

Lipoprotein lipase (LPL) affects not only the catabolism of triglyceride (TG)-rich lipoproteins but also the quantity and quality of HDL (11). LPL activity often decreases in the tumor-bearing state (12). In the present study, we first examined whether tissue LPL activity would be decreased by AH109A implantation. Second, we studied the actions of methionine (Met) and cystine (Cys) on the hyperlipidemia and their modes of action, with special attention to changes in LPL activity. Dietary Met and Cys were found to reduce the hepatoma-induced hyperlipidemia with slight differences in their modes of action.

EXPERIMENTAL PROCEDURES

Materials. Sodium heparin was purchased from Wako Pure Chemical Industries (Osaka, Japan). [Carboxyl-^{14}C]triolein (original specific radioactivity = 4.1 GBq/mmol) was purchased from NEN Research Products (Boston, MA). Insta-Gel was the product of Packard Instrument Co., Inc. (Meriden, CT). [1-^{14}C]Acetic acid (sodium salt, original specific radioactivity = 2.04 GBq/mmol) was purchased from Amersham International plc (Buckinghamshire, United Kingdom). Cholesterol C-test Wako and Total Bile Acid test Wako were purchased from Wako Pure Chemical Industries.

Animals and diets. Male Donryu rats were obtained from NRC Haruna (Gunma, Japan) at 4 wk of age. The animals were individually housed in stainless-steel cages with wire bottoms in an air-conditioned room with a temperature of 22°C, a relative humidity of 60%, and an 8:00 A.M. to 8:00 P.M. light cycle and kept on a stock pellet diet (CE-2; CLEA Japan, Tokyo, Japan), followed by a 20% casein (20C) diet (8). The composition of the 20C diet was as follows: 20% casein, 5% corn oil, 51.3% α-cornstarch, 17% sucrose, 3.5% mineral mixture (AIN 76 composition) (13), 1% vitamin mixture (AIN 76 composition) (13), 0.2% choline bitartrate, and 2% cellulose powder. After preliminary feeding, rats were divided into six or four groups of similar body weights (experiment 1: 152.9 ± 3.3, 154.5 ± 5.6, 154.9 ± 5.6, 154.7 ± 3.9, 153.3 ± 4.7, or 154.6 ± 3.6 g, and experiment 2: 153.9 ± 4.7, 153.9 ± 3.5, 154.1 ± 2.7, or 154.1 ± 3.1 g, mean ± standard error), and the animals received an implantation of 5×10^5

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Abbreviations: 20C, 20% casein diet; Ch, cholesterol; Cys, cystine; FA, fatty acid; H, hepatoma-bearing group; HDL, high density lipoprotein; HSL, hormone-sensitive lipase; HTGL, hepatic triglyceride lipase; LDL, low density lipoprotein; Met, methionine; N, normal group; LPL, lipoprotein lipase; NEFA, nonesterified fatty acid; PBS(–), Ca^{2+}, Mg^{2+}-free.phosphate-buffered saline; PL, phospholipid; T-Ch, total cholesterol; TG, triglyceride; TNF, tumor necrosis factor-α; VLDL, very low density lipoprotein.

AH109A cells (provided by SRL, Tokyo, Japan) suspended in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS(−)] (0.5 mL/rat) in the back to produce a solid hepatoma (hepatoma-bearing group) (6). One group of animals received an injection of PBS(−) alone (0.5 mL/rat) and was designated as the normal group. In experiment 1, all the rats were kept on the 20C diet, and the rats in hepatoma-bearing groups were sacrificed on the 1st, 2nd, 4th, 7th, and 14th d after AH109A implantation, while those of the normal group were sacrificed on the day of PBS(−) injection (d 0). In experiment 2, the hepatoma-bearing rats were fed the 20C diet (control) and the 20C diet supplemented with either 1.2% L-Met or L-Cys for 14 d. Likewise, rats of the normal group were fed the 20C diet for 14 d. Water and each diet were available at all times. Animals were deprived of their diet at 9:00 A.M. on the 14th d but allowed free access to water until killing, which was performed 4 h later by decapitation. Blood was collected and left to clot at room temperature to obtain serum. The liver, epididymal adipose tissue, cardiac muscle, gastrocnemius, and solid hepatoma were quickly removed, washed with cold 0.9% NaCl, and blotted on filter paper. Aliquots of the liver, epididymal adipose tissue, cardiac muscle, and gastrocnemius were frozen in liquid nitrogen and stored at −70°C until analyzed.

Lipid analyses. Total lipids were extracted according to the procedure of Folch *et al*. (14) from the liver and solid hepatoma. After portions of the chloroform phase had been dried under nitrogen, Ch (15), TG (16), and phospholipid (PL) (17) were determined as previously described (7). The serum TG and PL levels were also determined as described above. The serum nonesterified fatty acid (NEFA) level was measured by the method of Kushiro *et al.* (18). The serum lipoproteins were separated into HDL and VLDL + LDL fractions by the precipitation method (6). The total Ch of unfractionated serum (T-Ch) and HDL (HDL-Ch) were determined by an enzymatic method using a Cholesterol C-test Wako, and the difference between T-Ch and HDL-Ch was regarded as (VLDL + LDL)-Ch.

Measurement of tissue lipolytic activities. LPL was extracted from epididymal adipose tissue, cardiac muscle, and gastrocnemius by the method of Noguchi *et al.* (19); the tissues weighing 300 mg were homogenized in 0.2 M Tris-HCl buffer (pH = 8.5) containing 10 U/mL of sodium heparin. Hepatic TG lipase (HTGL) was also extracted from the liver in the same way. Lipase (LPL and HTGL) activities were then estimated as described previously (20,21). Briefly, 0.1 mL of the above-mentioned enzyme source was mixed with 0.1 mL of substrate solution (arabic gum-emulsified Tris-HCl buffer, 0.2 M and pH = 8.0, containing rat serum to supply adequate apolipoprotein C-II as an activator for LPL, and 0.1 M and pH = 8.5 for HTGL) containing 925 Bq (= 55,500 dpm)/μmol/assay of [carboxyl-^{14}C]triolein. The mixture was incubated at 37°C for 1 h, followed by the extraction and counting of hydrolyzed [^{14}C]oleic acid. Hormone-sensitive lipase (HSL) activity in adipose tissue was also determined. From epididymal adipose tissue, HSL was extracted with 2 vol of 0.2 M phosphate buffer (pH = 7.0) by homogenization (22). The enzyme suspension (0.1 mL) previously incubated with 1 M NaCl for 10 min was mixed with 0.1 mL of substrate solution (arabic gum-emulsified 0.2 M phosphate buffer, pH = 7.0) containing 925 Bq/μmol/assay of [carboxyl-^{14}C]triolein and the mixture was incubated at 30°C for 1 h. At the end of the incubation period, hydrolyzed [^{14}C]oleic acid was extracted and radioactivity was counted after adding Insta-Gel. One unit of lipase activity was defined as 1 μmol of fatty acid (FA) released per hour.

Hepatic lipid syntheses. Total FA and Ch syntheses were measured using liver slices (23,24). Liver slices weighing 100–120 mg were placed in 1 mL of Krebs-Ringer phosphate buffer (pH = 7.4) containing 37 KBq/μmol/assay of [1-^{14}C]acetic acid, sodium salt. Each tube was gassed with 100% O_2, stoppered tightly with a screwcap, and incubated at 37°C for 2 h. At the end of the incubation period, 1 mL of 15% ethanolic KOH was added and the mixture was saponified at 75°C for 2 h. Nonsaponifiable lipids were extracted three times with petroleum ether, and digitonin-precipitable sterols were formed, isolated, washed, dissolved in methanol, and counted after addition of a toluene-based scintillator. The residual aqueous layer was acidified to pH = 2 with concentrated HCl, and total FA were extracted three times with petroleum ether. Pooled extracts were then dried under nitrogen, dissolved in chloroform, and washed twice with water. The chloroform layer was quantitatively transferred to a scintillation vial, dried, and counted as mentioned above.

Fecal steroid excretion. Feces were collected for 2 d before killing (days 12–14). Neutral sterols and bile acids were extracted from dried and ground feces after saponification according to the procedure of Yamanaka *et al.* (25). Fecal 3β-hydroxy neutral sterols and 3α-hydroxy bile acids were determined by enzymatic methods (26), using commercial kits as described previously (27).

Statistical analysis. Results were expressed as mean ± standard error. Statistical analysis was carried out by analysis of variance followed by Student's *t*-test, or Duncan's multiple-range test (28).

RESULTS

Experiment 1. Food intake and body weight gain per day after AH109A implantation were almost the same among the five hepatoma-bearing groups (food intakes for groups of the 1st, 2nd, 4th, 7th, and 14th d after AH109A implantation were 19.1 ± 0.9, 18.0 ± 0.8, 18.2 ± 0.8, 18.3 ± 1.1, and 19.3 ± 0.3 g/d, respectively, and body weight gains for those groups were 6.9 ± 0.7, 5.6 ± 0.7, 5.9 ± 0.4, 6.0 ± 0.6, and 6.0 ± 0.2 g/d, respectively). The solid hepatoma started to be palpable on the 5th d after implantation, and the absolute and relative weights on the 14th d were 5.8 ± 1.5 g/rat and 2.5 ± 0.7% of body weight, respectively.

Figure 1 shows the time course of the serum TG and Ch levels after AH109A implantation. Judging from the data obtained on 0, 1, 2, 4, 7, and 14 d after the implantation of AH109A, the serum TG level significantly increased the day

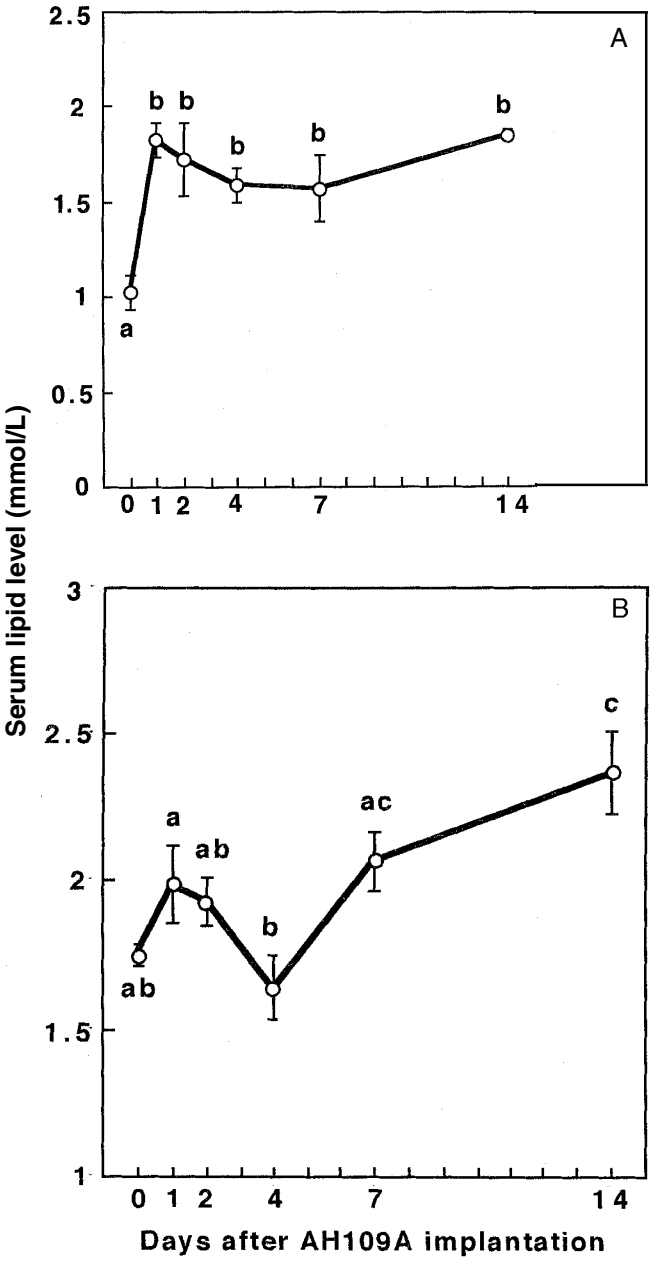

FIG. 1. Changes in the serum A: triglyceride (TG) and B: total cholesterol (T-Ch) levels with days after AH109A (SRL, Tokyo, Japan) implantation. Each value represents the mean of six rats. Vertical bars indicate standard errors. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.

following implantation and remained at a high level until the 14th d. The serum T-Ch level fluctuated during the first 7 d but rose significantly on the 14th d.

Figure 2 illustrates changes in the tissue TG lipase (LPL and HTGL) activities with days after AH109A implantation. LPL activity in epididymal adipose tissue decreased significantly the day after AH109A implantation and continued to decrease until the 14th d (10% of the control group). Cardiac muscle and gastrocnemius LPL activities also decreased the day after AH109A implantation and remained at low values until killing. HTGL activity in the liver showed no significant changes during the first week but dropped significantly on the 14th d.

Experiment 2. Table 1 shows food intake, body weight gain, liver and solid hepatoma weights, and lipid contents in the liver and hepatoma 14 d after AH109A implantation. Food intake, body weight gain, and liver weight were reduced by the presence of the hepatoma. Among the hepatoma-bearing groups, no significant changes in these parameters and hepatoma weight were noted. Liver TG content was decreased in the presence of the hepatoma, but the PL and Ch contents were not affected. The liver Ch content was increased by feeding Cys, but liver TG and PL contents and hepatoma lipid contents were not affected by feeding either Met or Cys.

Figure 3 represents changes in the serum lipid levels. As compared with the normal group, the serum TG, T-Ch, (VLDL + LDL)-Ch, PL, and NEFA levels were significantly higher in the hepatoma-bearing group (N vs. H). Conversely, the serum HDL-Ch level was lower in the hepatoma-bearing group than in the normal group (N vs. H). Dietary Met and Cys decreased the serum TG, PL, T-Ch, and (VLDL + LDL)-Ch levels. The HDL-Ch level was significantly increased by dietary Met, but dietary Cys had little effect on the level.

Figure 4 shows LPL activities in various tissues. In epididymal adipose tissue, cardiac muscle and gastrocnemius, these activities were all lower in the hepatoma-bearing group than in the normal group (N vs. H). In hepatoma-bearing groups, dietary Met restored the reduced LPL activity, but Cys did not.

As shown in Table 2, the HTGL activity was decreased in the presence of the hepatoma, while HSL activity was increased by hepatoma implantation. Dietary sulfur amino acids had no effect on these activities.

The lipid (FA and Ch) syntheses by liver slices are also shown in Table 2. Hepatic Ch synthesis was significantly stimulated by AH109A implantation, but FA synthesis was not (N vs. H). Dietary sulfur amino acids had no effect on Ch synthesis, while they suppressed FA synthesis.

As shown in Table 2, dry weight of feces and excretion of both neutral sterols and bile acids were significantly reduced by the presence of the hepatoma (N vs. H). Both dietary Met and Cys enhanced bile acid excretion, while these amino acids had no influence on neutral sterol excretion.

DISCUSSION

The serum TG level quickly increased the day following hepatoma implantation and was almost constant until the 14th d (Fig. 1). In contrast, LPL activity in various tissues rapidly decreased the day following the implantation. In the case of epididymal adipose tissue, LPL activity continued to decrease up to the 14th d (Fig. 2). These findings are not inconsistent with the results described by Damen *et al.* (12) who found a reduction of LPL activity in postheparin plasma in mice bearing a GRSL ascites tumor (a murine leukemia). LPL activity is concerned with serum TG clearance; a reduction of LPL activity is related to an increase in the serum TG level. Thus,

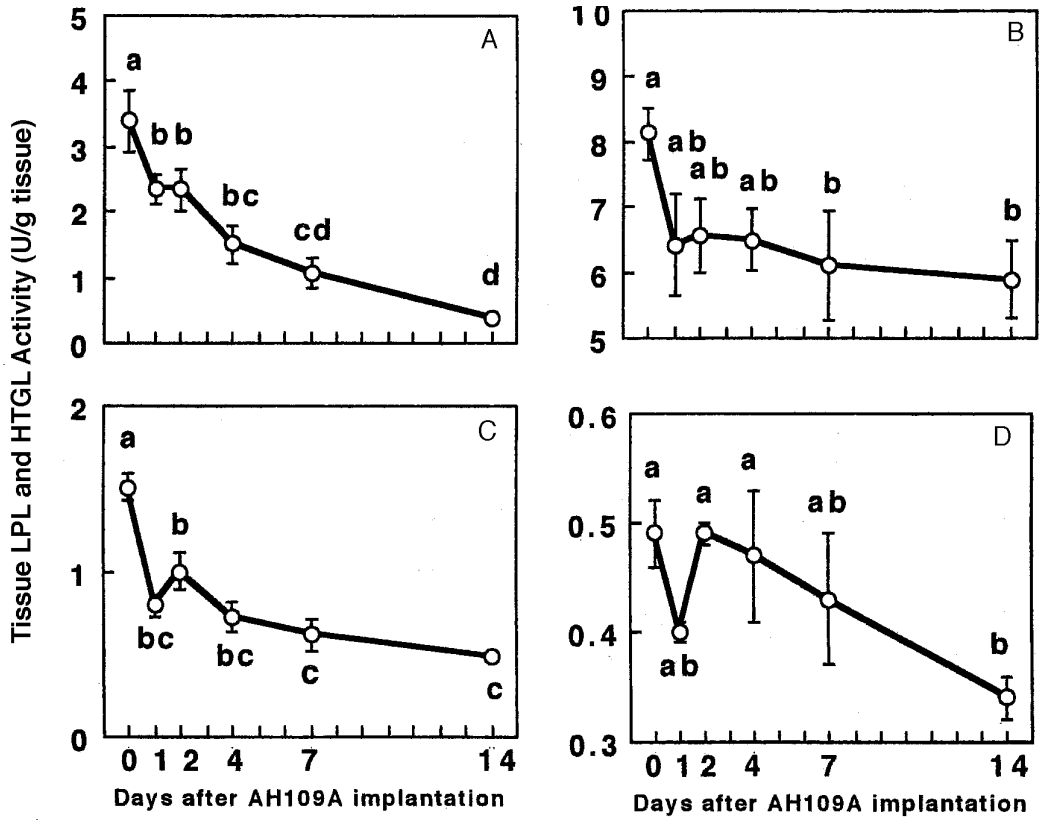

FIG. 2. Changes in tissue lipoprotein lipase (LPL) [A: epididymal adipose tissue (EAT), B: cardiac muscle, and C: gastrocnemius] and hepatic triglyceride lipase (HTGL) (D: liver) activities with time after AH109A implantation. Each value represents the mean of six rats. Vertical bars indicate standard errors. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test. See Figure 1 for company source.

the reduction of tissue LPL activity is considered to be one cause for the hypertriglyceridemia in AH109A-bearing rats. HSL in adipose tissue (Table 2) and the serum NEFA level (Fig. 3) increased significantly in hepatoma-bearing rats when compared with hepatoma-free (normal) rats. This increased FA mobilization from adipose tissue is thought to be another cause for the hepatoma-induced hypertriglyceridemia, which was reduced by ingestion of either Met- or Cys-fortified diet

TABLE 1
Initial Body Weight, Food Intake, Body Weight Gain, Weights and Lipid Contents of Liver and Hepatoma in Hepatoma-Bearing Rats[a]

	Normal (N)	Hepatoma-bearing (H)		
Measurement	20C	20C	20C + 1.2 Met	20C + 1.2 Cys
Initial body weight (g)	153.9 ± 4.7	153.9 ± 3.5	154.1 ± 2.7	154.1 ± 3.1
Food intake (g/14 d)	267.6 ± 7.0[b]	209.6 ± 7.7	180.9 ± 8.3	192.8 ± 11.5
Body weight gain (g/14 d)	112.8 ± 3.2[b]	84.9 ± 4.9	69.7 ± 6.8	75.8 ± 8.6
Liver weight (g)	12.4 ± 0.6[b]	9.8 ± 0.5	9.1 ± 0.3	9.0 ± 0.6
Hepatoma weight (g)	—	41.3 ± 2.6	37.8 ± 4.4	39.3 ± 2.8
Liver lipid content (μmol/g liver)				
Triglyceride	14.4 ± 2.1[b]	7.2 ± 1.5	5.7 ± 3.2	6.2 ± 2.2
Phospholipid	32.0 ± 0.8	31.5 ± 0.9	34.5 ± 1.0	34.0 ± 0.9
Cholesterol	5.2 ± 0.3	4.7 ± 0.0[c]	4.7 ± 0.3[c]	5.2 ± 0.3[d]
Hepatoma lipid content (μmol/g hepatoma)				
Triglyceride	—	1.7 ± 0.2	1.4 ± 0.2	2.1 ± 0.2
Phospholipid	—	7.4 ± 1.7	8.8 ± 1.4	7.7 ± 1.0
Cholesterol	—	5.7 ± 0.5	5.2 ± 0.3	5.4 ± 0.3

[a]Each value represents the mean ± standard error of six rats. Cys, cystine; Met, methionine; 20C, 20% casein diet.
[b]Significantly different from the hepatoma-bearing-20C diet group at $P < 0.05$ by Student's *t*-test. Values not sharing a common superscript letter (c,d) are significantly different at $P < 0.05$ by Duncan's multiple-range test.

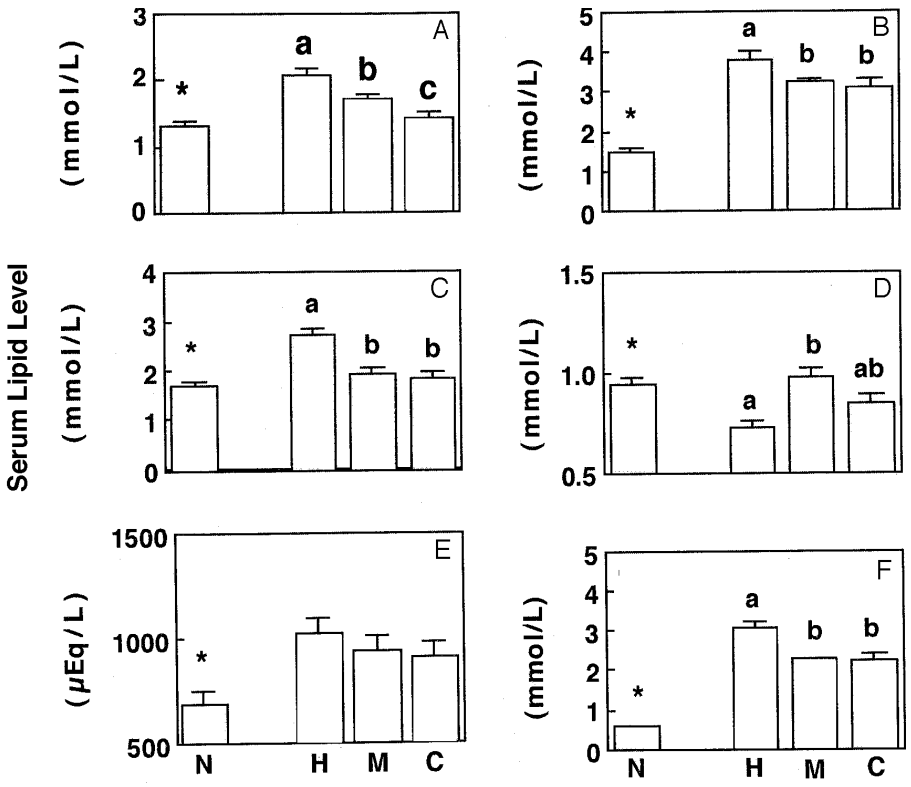

FIG. 3. Effects of dietary additions of either methionine (Met) or cystine (Cys) to a 20% casein diet (20C) on serum lipid levels in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical lines at the top of the bars indicate standard errors. *Significantly different from hepatoma-bearing-20C diet (H) group at $P < 0.05$ by Student's *t*-test. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test. A: TG, triglyceride; B: T-Ch, total cholesterol; C: PL, phospholipid; D: HDL-Ch, high density lipoprotein-cholesterol; E: NEFA, nonesterified fatty acid; F: (VLDL + LDL)-Ch, (very low density lipoprotein + low density lipoprotein)-cholesterol. N, normal group fed 20C; H, hepatoma-bearing group fed 20C; M, hepatoma-bearing group fed 20C + 1.2Met; C, hepatoma-bearing group fed 20C + 1.2Cys.

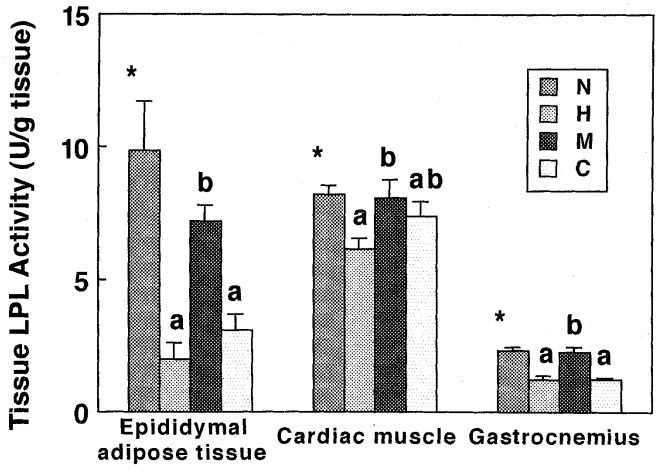

FIG. 4. Effects of dietary additions of either Met or Cys to a 20C on tissue LPL activities in hepatoma-bearing rats. Each value represents the mean of six rats. Vertical lines at the top of the bars indicate standard errors. *Significantly different from hepatoma-bearing-20C diet (H) group at $P < 0.05$ by Student's *t*-test. Values not sharing a common letter are significantly different at $P < 0.05$ by Duncan's multiple-range test. See Figure 3 for abbreviations.

(Fig. 3). Both sulfur amino acids suppressed hepatic FA synthesis in the hepatoma-bearing state (Table 2), but they failed to suppress the increased NEFA level in the serum. Met restored decreased LPL activity in the hepatoma-bearing state to the level of that in the normal state, while Cys did not (Fig. 4). The hypotriglyceridemic effect of Met may therefore be due to a suppression of hepatic FA synthesis and a restoration of decreased LPL activity in various tissues, whereas that of Cys may be due to a suppression of hepatic FA synthesis. Since the hypotriglyceridemic effect of Cys was stronger than that of Met (Fig. 3), a certain powerful mechanism(s), such as an increased FA oxidation in the liver, must operate in the case of Cys.

The hepatoma-induced hypercholesterolemia was characterized by a striking increase in the (VLDL + LDL)-Ch level and a slight but significant decrease in the HDL-Ch level (Fig. 3). An increase in hepatic cholesterolgenesis and a decrease in bile acid excretion into feces (Table 2) are considered to be responsible for the hepatoma-induced hypercholesterolemia. Both Met and Cys succeeded in improving the hypercholesterolemia by reducing the (VLDL + LDL)-Ch level

TABLE 2
Activities of Hepatic Triglyceride Lipase and Hormone-Sensitive Lipase, Hepatic Lipid Syntheses, and Fecal Steroid Excretion in Hepatoma-Bearing Rats[a]

	Normal (N)	Hepatoma-bearing (H)		
Measurement	20C	20C	20C + 1.2Met	20C + 1.2Cys
Hepatic triglyceride lipase activity (U/g tissue)				
Liver	2.4 ± 0.3[b]	1.2 ± 0.1	1.7± 0.2	1.4 ± 0.2
Hormone-sensitive lipase activity (U/g tissue)				
Epididymal adipose tissue	1.2 ± 0.1[b]	1.5 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Hepatic lipid syntheses (dpm × 10^{-4}/2 h/g liver)				
Fatty acids	9.2 ± 1.7	10.2 ± 2.1[c]	4.6 ± 0.5[d]	4.3 ± 1.0[d]
Cholesterol	3.2 ± 0.3[b]	14.5 ± 3.7	16.7 ± 3.5	12.7 ± 2.4
Fecal dry weight (g/2 d)	1.7 ± 0.2[b]	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
Steroid excretion (μmol/2 d)				
Neutral sterols	41.1 ± 4.7[b]	14.7 ± 2.1	11.3 ± 1.0	12.7 ± 1.4
Bile acids	26.5 ± 3.0[b]	9.9 ± 1.4[c]	16.4 ± 1.7[d]	17.3 ± 1.3[d]

[a]Each value represents the mean ± standard error of six rats. See Table 1 for other abbreviations.
[b]Significantly different from the hepatoma-bearing-20C diet group at $P < 0.05$ by Student's *t*-test. Values not sharing a common superscript letter (c,d) are significantly different at $P < 0.05$ by Duncan's multiple-range test.

(Fig. 3). The hypocholesterolemic actions of these two sulfur amino acids could be attributed to a restoration of decreased bile acid excretion into feces, like a Cys derivative, *S*-methyl-L-cysteine sulfoxide (29). In addition to these common actions of the two amino acids, Met restored the decreased HDL-Ch level in the hepatoma-bearing state to the normal level (Fig. 3). Since LPL deficiency induced by injecting antibody against LPL is associated with low level of HDL-Ch and occurrence of hypertriglyceridemia (11), the restoration effect of Met on HDL-Ch appears to be related to that on LPL activity in tissues. The restorative effect of Met on LPL activity also seems to contribute toward improvement of the hepatoma-induced hypercholesterolemia, since the hypercholesterolemia itself may be influenced by LPL activity.

LPL is known to be regulated by various factors. Insulin enhances LPL activity and mRNA level (30,31), while tumor necrosis factor-α (TNF) suppresses both (32,33). TNF productivity in resident macrophages from AH109A-bearing rats is found to be much higher than that from normal rats (34). Thus, the cytokine seems to be a factor that affects LPL activity and hence serum lipoprotein profiles, as suggested previously (8). To know how Met restores the decreased LPL activity in the hepatoma-bearing state is also important: suppression of an LPL-inactivating factor(s) like TNF, induction of LPL-activating factor(s) like insulin, or their combination. Further intensive studies are needed to clarify these aspects.

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[Received June 1, 1998, and in final revised form and accepted August 18, 1998]

Dietary Proteins Modulate the Effects of Fish Oil on Triglyceridemia in the Rat

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ABSTRACT: Sprague-Dawley rats were fed purified diets varying in both protein (20%) and lipid (11%) content for 28 d to verify the independent and interactive effects of dietary proteins and lipids on serum and hepatic lipids, and on tissue lipoprotein lipase (LPL) activity in both fasted and postprandial states. These diets consisted of either casein–menhaden oil, casein–coconut oil, soy protein–menhaden oil (SPMO), soy protein–coconut oil, cod protein–menhaden oil, or cod protein–coconut oil. A randomized 3 × 2 factorial design was used. A significant protein–lipid interaction was seen on serum triglyceride levels: menhaden oil, compared with coconut oil, induced a decrease in serum triglyceride levels when combined with soy protein but not when combined with cod protein and casein. The lower serum triglyceride concentrations observed in the SPMO-fed rats could be the result of decreased hepatic triglycerides when soy protein was compared with casein and when menhaden oil was compared with coconut oil. Total LPL activity in the heart was higher in menhaden oil-fed rats than in coconut oil-fed rats in the postprandial state. The higher LPL activity in the heart could, however, explain only 10% of the reduction of serum triglycerides, contributing slightly to the lowering effects of SPMO diet on serum triglycerides. Therefore, the present results indicate that dietary proteins can modulate the effects of fish oil on triglyceridemia in the rat, and that could be mainly related to specific alterations in hepatic lipid concentrations.
Lipids 33, 913–921 (1998).

Fish consumption has been inversely associated with the risk of coronary heart disease (1,2). These correlations with cardiovascular events appear to be mediated by the hypotriglyceridemic and antithrombogenic effects of n-3 polyunsaturated fatty acids present in fish oil (3,4). Plasma triglycerides are reduced by a decrease in hepatic triglyceride synthesis (5), a very low density lipoprotein (VLDL) synthesis (6), and an increase in VLDL and chylomicron degradation by lipoprotein lipase (LPL) (4,7,8).

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Abbreviations: CACO, casein-coconut oil; CAMO, casein–menhaden oil; CPCO, cod protein–coconut oil; CPMO, cod protein–menhaden oil; HDL, high density lipoproteins; LDL, low density lipoproteins; LPL, lipoprotein lipase; P/S ratio, polyunsaturated to saturated fatty acid ratio; SPCO, soy protein–coconut oil; SPMO, soy protein–menhaden oil; VLDL, very low density lipoproteins; VLM, *vastus lateralis* muscle.

Dietary proteins also have been shown to influence plasma lipid concentrations. Soy protein compared with casein and other animal proteins reduces plasma cholesterol in animal models (9,10) and in humans (11). Soy protein may also modulate the effects of dietary lipids on cholesterol metabolism. Indeed, when combined with corn oil, a source of n-6 polyunsaturated fatty acids, soy protein enhances the hypocholesterolemic effect of corn oil compared with butter, a source of saturated fatty acids (12). The effects of soy protein on triglyceride levels are less documented. However, since soy protein has been shown to reduce hepatic triglyceride (13) in addition to hepatic cholesterol concentrations, as well as hepatic triglyceride and cholesterol secretion into the blood (14), it may possibly accentuate the hypotriglyceridemic effect of fish oil.

The effects of dietary fish protein (which is a major nutrient in fish) on serum lipids have been compared to those of soy protein and casein. In rats, cod protein may decrease serum cholesterol and triglycerides when compared with casein (15). In rabbits, cod protein was shown to induce lower VLDL triglycerides (16) and higher high density lipoprotein (HDL) cholesterol (16,17) concentrations than casein and soy protein. These latter beneficial effects associated with decreased risk of coronary heart disease were related to increased activity of plasma postheparin LPL activity (18). A previous study in rabbits carried out in our laboratory also demonstrated that, when combined with cod protein, corn oil does not reduce total and HDL cholesterol, as it does when combined with either casein or soy protein (16), indicating that fish protein can interact with dietary lipids to modulate lipidemia. Because previous reports demonstrated that both fish oil (5) and fish protein (16) can reduce serum triglycerides, the determination of the combination of both nutrients possibly accentuating the hypotriglyceridemic response was important. Thus an attempt was made in rabbits to determine if such a combination of soy and cod proteins with fish oil may modulate serum and hepatic lipid concentrations (19). However, when fish oil was combined with either casein, fish protein or soy protein, an increasing effect of fish oil on triglyceridemia occurred, suggesting that the rabbit model we used was not the best animal model for studying the reducing effects of fish oil on triglyceridemia observed in humans (19). The present study was thus undertaken in the rat, which had a

reducing triglyceridemic response to fish oil comparable to that of humans (7,20).

Our working hypothesis was that dietary proteins can modulate the effects of fish oil on lipid metabolism. The aim of this study was to determine the distinct and interactive effects of three dietary proteins, casein, cod protein and soy protein, and of two dietary lipids, menhaden oil and coconut oil, on serum lipid levels. A 9% coconut oil plus 1% corn oil diet was used in the present study for comparison with the literature (5) and with one of our previous experiments (15). To gain insight into the mechanisms by which the diet may influence serum lipids, hepatic lipids and LPL activity in white adipose tissue, skeletal and heart muscles were assessed. Because insulin is a major modulator of lipogenesis and LPL activity, serum insulin levels were also determined.

MATERIALS AND METHODS

Experimental animals. Sprague-Dawley rats (120) (St-Constant, Québec, Canada) initially weighing *ca.* 200 g were housed individually in stainless-steel wire-bottom mesh cages. The temperature (20 ± 2°C) and humidity (45–55%) of the animal room were constant and the rats were kept under a daily inverted light–dark cycle (light: 2000 to 0800). During an adaptation period of 2 d in their new environment, the rats were fed a nonpurified commercial diet (rat chow No. 5075; Charles River Canada, St-Constant). They were then divided into six groups of 20 rats of the same average weights. Purified diets and water were provided once daily on an *ad libitum* basis for a period of 28 d. Food intake was measured daily and body weight was monitored three times a week. At the end of the experimental period, 10 rats of each group were killed by decapitation in the fasted state, and the remaining 10 rats were killed in the fed state. Two rats, one in the fasted state and one in the fed state, were eliminated before statistical analysis because of abnormally low growth rate. This protocol was approved by the animal ethics committee of Université Laval (Québec, Canada).

Purified diets. Powdered purified diets, varying in both protein (20%) and lipid (11%) sources, consisted of either casein–menhaden oil (CAMO), casein–coconut oil (CACO), soy protein–menhaden oil (SPMO), soy protein–coconut oil (SPCO), cod protein–menhaden oil (CPMO), or cod protein–coconut oil (CPCO). The composition of each purified diet is detailed in Table 1. Coconut oil and menhaden oil were supplied by ICN Biomedicals Canada Ltd. (Mississauga, Ontario, Canada), and corn oil was purchased from Mazola (Best Foods Canada, Montréal, Canada). According to manufacturer information, menhaden oil contained 28.2% saturated fatty acids, 23.0% monounsaturated fatty acids, 1.8% linoleic acid, 2.3% arachidonic acid, 16.0% eicosapentaenoic acid, 10.8% docosahexaenoic acid, and 5.23 mg cholesterol/g menhaden oil. Therefore, menhaden oil provided only 0.5 g cholesterol/kg in menhaden oil diets, and we considered this amount as negligible. Coconut oil contained 92.0% saturated fatty acids, mainly from lauric acid (48.5%), 6.5% monounsaturated fatty acids, and 1.5% polyunsaturated fatty acids. α-Tocopherol, butylated hydroxyanisole and butylated hydroxytoluene (ICN Biomedicals Canada Ltd.) were added to the diets as proposed by Fritsche and Johnston (21) to minimize the oxidation of n-3 and n-6 polyunsaturated fatty acids in menhaden oil, and n-6 polyunsaturated fatty acids in corn oil and coconut oil. Cholesterol was supplied by Sigma (Sigma Chemical Co., St. Louis, MO). Highly purified casein (85.8% protein), soybean protein isolate (87.6% protein), cornstarch, cellulose (Alphacel), and rat mineral mixture were purchased from ICN Biomedicals Canada Ltd., and vitamin mix was supplied by Teklad Test Diets (Madison, WI). The cod protein was prepared in our laboratory by freeze-drying cod fillets and diethylether delipidation for 24 h in an industrial Soxhlet-type apparatus (Canadawide Scientific, Montréal, Québec, Canada), rendering a cod protein isolate containing 93.7% protein. The protein content (N × 6.25) of casein, soy protein, and cod protein was assayed by the Kjeldahl method using a Kjeldahl-Foss autoanalyzer (Model 16210; Foss Co., Hillerod, Denmark), and the level of protein in the diets was adjusted at the expense of cornstarch to obtain an isonitrogenous content. The residual lipid content of casein (0.07%), soy protein (0.44%), and cod protein (0.19%) was determined with a Goldfish Lipid Extractor (Model 35001; Labconco Corporation, Kansas City, MO). The energy content of the diets was measured in an automatic adiabatic calorimeter (Model 1241; Parr Instruments, Moline, IL) and was similar in the CAMO (19.6 kJ/g), CACO (19.5 kJ/g), SPMO (19.8 kJ/g), SPCO (19.4 kJ/g), CPMO (19.1 kJ/g), and CPCO (19.1 kJ/g) diets.

At the end of the 28-d experimental period, all rats were food-deprived for 12 h and weighed. Six groups of 10 rats, which were first conditioned to meal eating, were fed for 30 min a meal similar to the experimental diet. These rats were killed by decapitation 2.5 h after the meal. The remaining rats were killed after the 12-h fast.

TABLE 1
Composition (g/kg) of the Purified Diets[a]

	CACO	CAMO	SPCO	SPMO	CPMO	CPCO
Ingredients						
Casein	233	233	—	—	—	—
Soy protein	—	—	228	228	—	—
Cod protein	—	—	—	—	213	213
Cornstarch	548	548	553	553	568	568
Cellulose	50	50	50	50	50	50
Coconut oil	100	—	100	—	100	—
Menhaden oil	—	100	—	100	—	100
Corn oil	10	10	10	10	10	10
Cholesterol	10	10	10	10	10	10
Minerals	35	35	35	35	35	35
Vitamins	10	10	10	10	10	10
Choline bitartrate	2	2	2	2	2	2
α-Tocopherol	1.2	1.2	1.2	1.2	1.2	1.2
BHA and BHT	0.4	0.4	0.4	0.4	0.4	0.4

[a]CACO = casein–coconut oil; CAMO = casein–menhaden oil; SPCO = soy protein–coconut oil; SPMO = soy protein–menhaden oil; CPCO = cod protein–coconut oil; CPMO = cod protein–menhaden oil. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

Serum, lipoprotein, and hepatic lipid analyses. Blood samples of all rats were collected in a 10-mL tube and centrifuged (2,000 × *g*, 4°C, 15 min) to isolate serum. Total cholesterol and triglycerides were determined by enzymatic methods using CHOD-PAP and triglyceride without free glycerol enzymatic kits provided by Boehringer Mannheim (Laval, Québec, Canada) respectively. HDL cholesterol was measured enzymatically with the CHOD-PAP kit after precipitation of VLDL and low density lipoproteins (LDL) with phosphotungstic acid and magnesium ions as described by Burstein *et al*. (22), using an HDL precipitant solution supplied by Boehringer Mannheim. Total (VLDL + LDL) cholesterol was determined by subtracting HDL cholesterol from total cholesterol.

The livers of fasted rats were removed, weighed, frozen in liquid nitrogen, and stored at −80°C. Hepatic lipids were then extracted by chloroform/methanol (2:1, vol/vol) according to Folch *et al*. (23), and determined enzymatically as described above.

Tissue LPL activity. Epididymal adipose tissue, *vastus lateralis* muscle (VLM), and heart of fasted and fed rats were removed and weighed. Approximately 50 mg from each of these tissues was homogenized in 1 mL of solution using all-glass Duall grinders (Kontes Glass, Vineland, NJ). The homogenization solution for adipose tissue consisted of 0.25 M sucrose, 12 mM deoxycholate, 10 mM tris(hydroxymethyl-aminomethane (Tris)-HCl, and 1 mM EDTA at pH 7.4. The homogenization solution for muscle tissues consisted of 1 M ethylene glycol, 50 mM Tris-HCl, 3 mM deoxycholate, 10 UI/mL heparin, and 5% (vol/vol) aprotinin (Trasylol; Miles Pharmaceuticals, Rexdale, Ontario, Canada) at pH 7.4. The homogenates of epididymal adipose tissue were centrifuged at 12,000 × *g*, 4°C for 20 min. The fraction between the upper fat layer and the bottom sediment was removed and diluted with 4 vol of a dilution solution similar to the homogenization solution but without deoxycholate. Homogenates of VLM and heart, as well as diluted samples of epididymal adipose tissue, were then quickly frozen and stored at −80°C until LPL measurements.

LPL activity was measured in these extracts using a method described by Deshaies *et al*. (24). Samples of 100 µL of tissue homogenates were incubated for 1 h at 28°C with 100 µL of a substrate mixture containing either 0.1 or 2 M NaCl. The substrate consisted of 0.2 M tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer, pH 8.6, containing 10 MBq/L [^{14}C]carboxytriolein, and 2.52 mM cold triolein emulsified in 5% gum arabic, 2% fatty acid-free bovine serum albumin, and 10% human serum as a source of apolipoprotein C-II. After incubation, free oleate released by LPL was separated from intact triolein. Sample radioactivity was then determined. LPL activity was calculated by subtracting lipolytic activity measured in a final NaCl concentration of 1 M (activity not due to LPL) from total lipolytic activity measured in a final NaCl concentration of 0.05 M.

Serum glucose and insulin. Serum insulin was measured by radioimmunoassay as described by Desbuquois and Aurbach (25). Serum glucose was measured with an autoanalyzer (YSI 2700 Select; Yellow Springs, OH).

Statistical analyses. The results are expressed as mean ± standard error of the mean and the significance level is $P \leq 0.05$. Data were subjected to an analysis of variance using the general linear model procedure of the Statistical Analysis System (SAS Institute, Cary, NC), according to a 3 × 2 factorial arrangement used to determine the main protein and lipid effects, as well as interactions among dietary proteins and lipids. When statistically significant protein and lipid effects as well as protein–lipid interactions were detected, Duncan's New-Multiple-Range test was performed to identify differences among diet groups. Data for serum triglycerides and (VLDL + LDL) cholesterol of fasted rats were logarithmically transformed to achieve normality of residues. However, data presented in tables and figures are not transformed.

RESULTS

Food consumption and weight gain. Table 2 shows the mean food intake and body weight gain of rats fed the various diets. At the end of the experimental period, food consumption and weight gain were similar for the six dietary groups of rats sacrificed either in the fasted or in the fed state. Food intake at the last meal was also the same for the six dietary groups.

Serum and hepatic lipids. Mean values of serum total, lipoprotein, and hepatic cholesterol of fasted rats are presented in Table 3. The overall analysis of variance and multiple comparisons are shown in the bottom half of the table. A significant protein effect was seen on total serum cholesterol concentrations, which were lower in rats fed soy protein and cod protein than in those fed casein. The lipid source induced significant effects on total and HDL cholesterol in the fasted state. Coconut oil diets provoked higher levels of total and HDL cholesterol than menhaden oil diets. Casein and cod protein induced higher hepatic cholesterol concentrations

TABLE 2
Food Intake and Weight Gain of Rats Fed the Purified Diets[a]

Dietary group	*n*	Food intake (g/d)	Food intake last meal (g)	Weight gain (g/28 d)
Fasted				
CACO	10	21.3 ± 0.8	—	128 ± 10
CAMO	10	21.4 ± 0.4	—	148 ± 11
SPCO	9	20.6 ± 0.6	—	106 ± 8
SPMO	10	21.1 ± 0.6	—	126 ± 8
CPCO	10	22.6 ± 0.5	—	136 ± 9
CPMO	10	21.8 ± 0.5	—	144 ± 11
Fed				
CACO	10	20.5 ± 0.5	4.7 ± 0.2	120 ± 7
CAMO	10	20.1 ± 0.5	4.1 ± 0.3	125 ± 5
SPCO	10	20.9 ± 0.4	4.0 ± 0.2	110 ± 6
SPMO	9	20.3 ± 1.0	3.9 ± 0.3	107 ± 9
CPCO	10	20.5 ± 0.5	4.0 ± 0.3	102 ± 11
CPMO	10	21.7 ± 0.3	4.6 ± 0.3	132 ± 7

[a]Values are means ± SEM. See Table 1 for abbreviations.

TABLE 3
Total Serum, Lipoprotein, and Hepatic Cholesterol Levels of Rats Fed the Purified Diets in the Fasted State[a]

Dietary group	Serum			Hepatic cholesterol (μmol/g)
	Total cholesterol (mmol/L)	(VLDL + LDL)-C (mmol/L)	HDL-C (mmol/L)	
CACO	2.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	99 ± 8
CAMO	1.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	99 ± 6
SPCO	1.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	56 ± 5
SPMO	1.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	69 ± 4
CPCO	1.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	75 ± 6
CPMO	1.6 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	85 ± 8
Sources of variations	ANOVA (*P* values)[b]			
Protein (P)	0.04	0.08	0.33	0.001
Lipid (L)	0.01	0.12	0.02	0.13
P × L	0.28	0.16	0.98	0.91
Comparisons[c]				
P	CA > SP CA > CP CP = SP	CA = SP CA = CP CP = SP	CA = SP CA = CP CP = SP	CA > SP CA > CP CP > SP
L	CO > MO	CO = MO	CO > MO	CO = MO

[a]Values are means ± SEM, n = 9–10 rats per dietary group. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL-C, high density lipoprotein-cholesterol; ANOVA, analysis of variance. See Table 1 for other abbreviations.
[b]$P < 0.05$ indicates significant protein or lipid effects or their interactions.
[c]Explanation of the symbols: =, no difference among the groups at $P < 0.05$; >, significantly higher than the group with which it is compared at $P < 0.05$; <, significantly lower than the group with which it is compared at $P < 0.05$.

than soy protein. However, hepatic cholesterol was higher in rats fed casein than in those fed cod protein. Neither an independent lipid effect nor a protein–lipid interaction was observed on hepatic cholesterol concentrations.

Table 4 shows the effects of the purified diets on serum and lipoprotein cholesterol of rats in the fed state. Significant protein effects were observed on serum cholesterol. Total, (VLDL + LDL) and HDL cholesterol were higher in the casein-fed rats than in the soy protein-fed rats. Total and HDL cholesterol were also lower after soy protein consumption when compared with cod protein consumption. In contrast to the lipid effect observed in the fasted state, the lipid source induced no significant effect on serum total and lipoprotein cholesterol levels in the postprandial state.

TABLE 4
Total Serum and Lipoprotein Cholesterol Levels of Rats Fed the Purified Diets in the Fed State[a]

Dietary group	Total cholesterol (mmol/L)	(VLDL ± LDL)-C (mmol/L)	HDL-C (mmol/L)
CACO	2.4 ± 0.1	1.4 ± 0.1	1.0 ± 0.1
CAMO	2.4 ± 0.2	1.3 ± 0.2	1.1 ± 0.1
SPCO	2.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
SPMO	1.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.0
CPCO	2.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
CPMO	2.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
Sources of variations	ANOVA (*P* values)[b]		
Protein (P)	0.01	0.05	0.02
Lipid (L)	0.26	0.30	0.29
P × L	0.50	0.56	0.10
Comparisons[c]			
P	CA > SP CA = CP CP > SP	CA > SP CA = CP CP = SP	CA > SP CA = CP CP > SP
L	CO = MO	CO = MO	CO = MO

[a–c]See Table 3 for footnotes.

Serum total triglycerides of rats in the fasted and fed states are presented in Figures 1 and 2, and hepatic triglycerides of rats in the fasted state are shown in Figure 3. As shown in Figures 1 and 2, a significant protein–lipid interaction was observed in the fasted and the fed states: there was a hypotriglyceridemic effect of menhaden oil compared to coconut oil when combined with soy protein but not when combined with either casein or cod protein. The lowest values of serum triglycerides in both the fasted and the fed states were thus observed when rats were fed SPMO. In the fasted state, serum triglyceride concentrations of SPMO-fed rats were lower than those of CACO- and SPCO-fed rats. In the fed state the effects between dietary proteins were more pronounced: when combined with coconut oil, cod protein induced lower serum triglycerides than soy protein, casein being intermediate, and when combined with menhaden oil, soy protein induced lower serum triglycerides than casein and cod protein. Proteins and lipids in the diets had independent effects on hepatic triglyceride concentrations (Fig. 3). Casein and cod protein caused similar triglyceride levels in the liver,

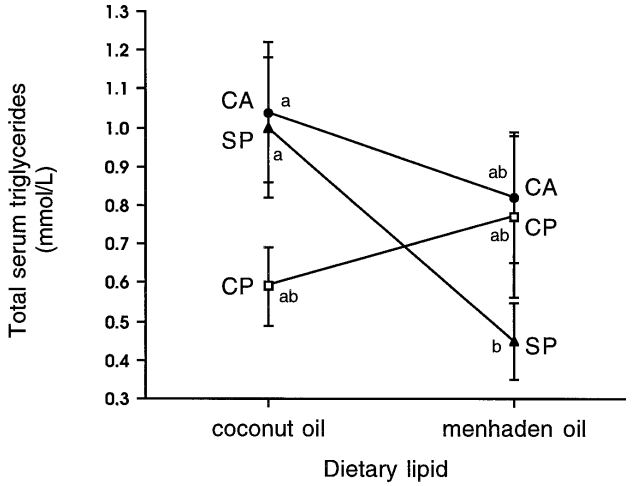

FIG. 1. Interaction between proteins and lipids in the regulation of total serum triglycerides (n = 9–10 rats per dietary group; P = 0.046) of rats fed the purified diets in the fasted state. CA = casein; SP = soy protein; CP = cod protein. Groups bearing different letters were significantly different at $P < 0.05$.

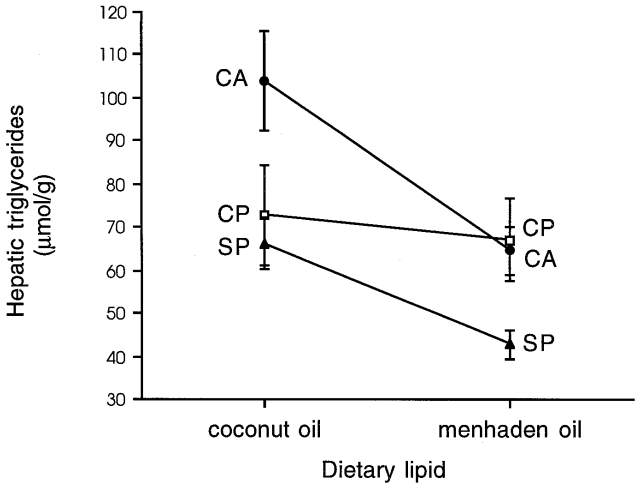

FIG. 3. Main protein and lipid effects on hepatic triglycerides of rats fed the purified diets in the fasted state (n = 9–10 rats per dietary group). P value for protein effect = 0.009, CA > SP, CA = CP, CP = SP; P value for lipid effect = 0.005, CO > MO; P value for protein–lipid interaction = 0.2. See Figure 1 for abbreviations.

but casein induced higher triglyceride concentrations than soy protein. The lipid effect was attributed to a diminution of hepatic triglycerides after menhaden oil compared to coconut oil feeding. Menhaden oil, compared with coconut oil, induced a greater diminution of hepatic triglyceride content when combined with casein (38%) and soy protein (34%) than when combined with cod protein (13%).

LPL activity. Table 5 shows LPL activity in epididymal adipose tissue, VLM, and heart of fasted and fed rats. In fasted rats, LPL activity in epididymal fat and VLM was modulated by significant protein effects. Total LPL activity in epididymal fat, expressed as µU/tissue, was higher following cod protein consumption than after casein or soy protein feeding. In the VLM, casein feeding induced higher LPL activities than either soy protein or cod protein feeding. No lipid or protein effect and no protein–lipid interaction was observed in the heart in fasted rats. However, in fed rats, the only significant effect was a lipid effect in the heart: LPL activity was higher when coconut oil was replaced by menhaden oil.

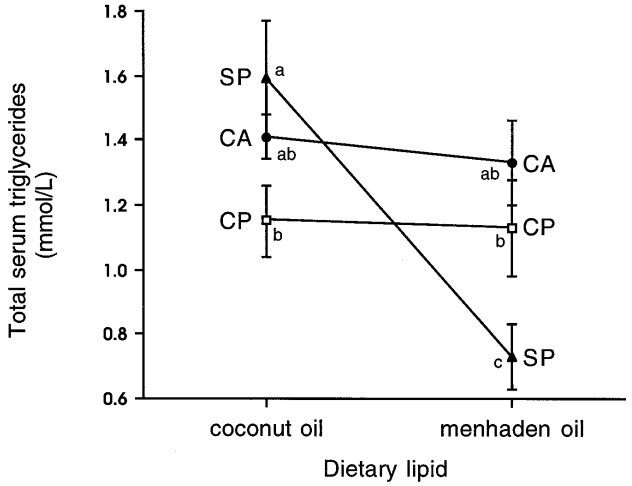

FIG. 2. Interaction between proteins and lipids in the regulation of total serum triglycerides (n = 9–10 rats per dietary group; P = 0.003) of rats fed the purified diets in the fed state. Groups bearing different letters were significantly different at $P < 0.05$. See Figure 1 for abbreviations.

Serum insulin and glucose. Serum insulin and glucose levels of fasted and fed rats are presented in Table 6. No significant effect of diet was observed on serum insulin or glucose levels. Pearson's correlation coefficients between insulin levels and total LPL activity in tissues were also calculated. LPL activity in epididymal adipose tissue was positively correlated with serum insulin levels in the fed state (n = 59, r = 0.38, P = 0.006), but no such correlation was seen in the fasted state (n = 59, r = 0.08, P = 0.58). Variations of insulinemia could explain about 14% (r^2 = 0.14) of variations of LPL activity in this tissue. No significant correlations were seen between LPL activity in VLM of fed rats (n = 59, r = 0.23, P = 0.09) and heart of fasted rats (n = 59, r = –0.23, P = 0.08) and serum insulin levels. Variations of insulinemia could explain only 4% (r^2 = 0.04) of variations of LPL activity in these tissues. There were no correlations between LPL activity in VLM of fasted rats (n = 59, r = –0.07, P = 0.63) and heart of fed rats (n = 59, r = 0.05, P = 0.71) and serum insulin levels.

DISCUSSION

The present study shows that dietary proteins and lipids affected serum cholesterol independently but induced interactive effects on serum triglycerides, resulting in a diminution of serum triglycerides when menhaden oil was combined with soy protein but not with either casein or cod protein. These results emphasize the importance of nutrient interactions in determining plasma lipid profiles and potential risks for cardiovascular disease. It appears from this study that dietary proteins modulate the effects of menhaden oil on serum

TABLE 5
Tissue LPL Activity of Rats Fed the Purified Diets[a]

Dietary group	Fasted			Fed		
	Epididymal adipose tissue (μU/tissue)[b]	*Vastus lateralis* muscle (μU/tissue)	Heart (μU/tissue)	Epididymal adipose tissue (μU/tissue)	*Vastus lateralis* muscle (μU/tissue)	Heart (μU/tissue)
CACO	12 ± 2	4.5 ± 0.7	23 ± 3	11 ± 2	4.3 ± 0.7	18 ± 1
CAMO	11 ± 2	5.1 ± 1.1	26 ± 2	11 ± 4	4.9 ± 0.9	25 ± 2
SPCO	11 ± 3	2.3 ± 0.3	26 ± 3	11 ± 2	5.4 ± 1.6	17 ± 1
SPMO	9 ± 1	3.6 ± 0.3	29 ± 3	9 ± 3	4.4 ± 0.9	22 ± 2
CPCO	15 ± 1	3.2 ± 0.6	28 ± 3	8 ± 2	3.4 ± 0.8	17 ± 1
CPMO	15 ± 2	2.8 ± 0.8	27 ± 3	10 ± 2	3.2 ± 0.6	24 ± 3
Sources of variations	ANOVA (*P* values)[c]					
Protein (P)	0.02	0.01	0.30	0.76	0.29	0.55
Lipid (L)	0.69	0.29	0.31	0.85	0.80	0.0002
P × L	0.77	0.53	0.61	0.80	0.58	0.75
Comparisons						
P	CA = SP	CA > SP	CA = SP	CA = SP	CA = SP	CA = SP
	CA < CP	CA > CP	CA = CP	CA = CP	CA = CP	CA = CP
	CP > SP	CP = SP	CP = SP	CP = SP	CP = SP	CP = SP
L	CO = MO	CO = MO	CO = MO	CO = MO	CO = MO	CO < MO

[a]Values are means ± SEM, n = 9–10 rats per dietary group in the fasted state and 9–10 rats per dietary group in the fed state. See Table 3 for abbreviations and symbol explanations.
[b]1 μU = 1 μmol nonesterified fatty acids released per hour of incubation.
[c]$P < 0.05$ indicates significant protein or lipid effects or their interactions.

triglycerides and that could be related to specific alterations in hepatic cholesterol and triglyceride concentrations. This concept is in good agreement with the results reported by Ikeda *et al*. (26), showing that the effects of dietary lipids on lipid metabolism may be modulated by the origin of dietary proteins in rats. Indeed, in their study, plasma and hepatic triglycerides were lower in hypercholesterolemic rats fed perilla oil (high in α-linolenic acid) than in those fed safflower oil (high in linoleic acid), and these effects were more pronounced with casein than with soy protein.

In the present study, although the fatty acid composition of coconut oil and menhaden oil differs greatly, no significant difference occurred in weight gain between the six dietary groups, either in the fasted or in the fed state. The linoleic acid requirement for growing rats is 1–2% of total calories (27), and the coconut oil and menhaden oil diets provided 1.60 and 1.67% of total calories from linoleic acid, respectively, therefore preventing a deficiency in linoleic acid in our dietary groups. However, the coconut oil diets contained only 0.1 g of α-linolenic acid/kg diet, which is lower than the minimal requirement for adult rats (1.3 g/kg diet) (28), while the menhaden oil diets provided 2 g of α-linolenic acid/kg diet. Nevertheless, a deficiency in α-linolenic acid is characterized in the rat by abnormal electroretinogram and impaired vision, but not by growth retardation, skin lesions, or fatty liver as is observed in linoleic acid deficiency (29). Consequently, the lower α-linolenic acid content of coconut oil diets in comparison with menhaden oil diets did not influence weight gain of rats, and the effects of diets on lipid variables in the present study can be attributed to the origin of dietary proteins and lipids.

The lowering effect of soy protein on serum and hepatic cholesterol we observed when compared with casein is in good accordance with the earlier findings of Hurley *et al.* (15) and Saeki and Kiriyama (30). Choi *et al*. (31) also found lower hepatic cholesterol concentrations after feeding soy protein in comparison with casein, when sardine oil was the

TABLE 6
Serum Insulin and Glucose Levels of Rats Fed the Purified Diets[a]

Dietary group	Fasted		Fed	
	Insulin (pmol/L)	Glucose (mmol/L)	Insulin (pmol/L)	Glucose (mmol/L)
CACO	319 ± 42	7.6 ± 0.2	677 ± 80	8.4 ± 0.1
CAMO	309 ± 27	7.6 ± 0.2	511 ± 104	8.5 ± 0.2
SPCO	265 ± 27	7.4 ± 0.1	509 ± 77	8.5 ± 0.2
SPMO	289 ± 62	7.3 ± 0.2	478 ± 101	8.5 ± 0.2
CPCO	261 ± 18	7.7 ± 0.3	379 ± 74	8.4 ± 0.2
CPMO	388 ± 65	7.9 ± 0.2	540 ± 69	8.4 ± 0.1
Sources of variation	ANOVA (*P* values)[b]			
Protein (P)	0.47	0.17	0.18	0.73
Lipid (L)	0.17	0.86	0.72	0.83
P × L	0.28	0.84	0.11	0.98
Comparisons[c]				
P	CA = SP	CA = SP	CA = SP	CA = SP
	CA = CP	CA = CP	CA = CP	CA = CP
	CP = SP	CP = SP	CP = SP	CP = SP
L	CO = MO	CO = MO	CO = MO	CO = MO

[a–c]Values are means ± SEM, n = 9–10 rats per dietary group in the fasted state and 9–10 rats per dietary group in the fed state. See Table 3 for other footnotes.

lipid source. The mechanism for the hypocholesterolemic effect of soy protein may involve a reduction in intestinal cholesterol and bile acids (32) absorption, which leads to an increased excretion of neutral steroids and bile acids in feces (32,33), a reduction in hepatic cholesterol concentrations (32), and a rise in the activity of apolipoprotein B/E receptors (32,33). Lower plasma insulin concentrations and insulin/glucagon ratio following soy protein feeding instead of casein feeding (32,33) may be implicated in these effects. However, the exact role of the soy protein constituents in the regulation of lipid metabolism is not clearly defined. Both the essential amino acids and nonessential amino acid components of soy protein may account for its effects on serum and hepatic lipids. One of the postulated mechanisms is that the lower lysine/arginine ratio of soy protein compared with that of casein may influence lipid metabolism by modulating serum concentrations of hormones, such as insulin and glucagon (34), and in turn the activity of specific enzymes involved in the lipolysis or the cholesterol and triglyceride synthesis. Indeed, Sugano *et al*. (35) showed that, in rats, serum glucagon levels increased proportionately with increasing amounts of arginine added to casein.

In the present study, cod protein induced a hypocholesterolemic effect similar to that of soy protein compared with casein in the fasted state. These results are in good agreement with those previously observed in rats (15,36). However, in this study hepatic cholesterol in rats fed cod protein was significantly different, being intermediate to hepatic cholesterol in rats fed casein and soy protein. Other studies in rats showed significantly higher hepatic cholesterol concentrations after cod protein feeding compared with soy protein feeding, but cod protein and casein did not induce statistically different effects in these studies (15,36). As with soy protein, it has been postulated that the lower lysine/arginine ratio of cod protein when compared with casein may have played a role in the hypocholesterolemic effect of cod protein (37). On the other hand, cod protein is rich in essential amino acids, and contains more lysine and methionine than casein and soy protein (38). These differences in amino acid content may be involved in the divergent effects of cod protein in comparison to soy protein and casein on hepatic cholesterol concentrations and other lipid parameters.

The hypotriglyceridemic impact of fish oil consumption is well documented. The n-3 long-chain polyunsaturated fatty acids present in fish oil decreased plasma triglyceride concentrations in a number of clinical (3,4) and rat (5,6,20) studies. Specifically the effects of n-3 polyunsaturated fatty acids appear to be attributed to a reduction of hepatic triglyceride synthesis (5,7) with impairment of VLDL assembly and secretion (6). Also the high polyunsaturated to saturated fatty acid (P/S) ratio of fish oil can contribute to the reduction of plasma triglycerides. Indeed, a diet rich in n-6 polyunsaturated fatty acids with a high P/S ratio (P/S ratio = 1.4) provoked a hypotriglyceridemic effect as compared to a diet rich in saturated fatty acids (P/S ratio = 0.07) (39). However, with the same P/S ratio (P/S ratio = 1.39), a diet rich in n-3 polyunsaturated fatty acids induced significantly lower triglyceride concentrations than a diet rich in n-6 polyunsaturated fatty acids (P/S ratio = 1.4) (39), showing that n-3 polyunsaturated fatty acids lower triglycerides by themselves, and not only by increasing the P/S ratio.

The present results demonstrate for the first time that the effects of fish oil on serum triglycerides can be modulated by dietary proteins. The lowering effect of fish oil on serum triglycerides is observed only when menhaden oil is combined with soy protein and is accompanied by a 34% diminution of hepatic triglyceride concentrations in fasted rats, strongly suggesting that the reducing effect of SPMO on serum triglycerides could be the result of a diminution of hepatic triglyceride synthesis and secretion into the blood. In the presence of coconut oil, soy protein already induced lower hepatic cholesterol and triglyceride concentrations than casein, confirming previous data obtained in rats by Terpstra *et al*. (40) and Hurley *et al*. (15) on hepatic cholesterol and by Iritani *et al*. (13) on hepatic triglycerides. The combination of soy protein plus menhaden oil accentuated the reducing effect of menhaden oil on hepatic triglycerides, suggesting that soy protein has an additive effect with fish oil on hepatic triglycerides, resulting in a greater diminution of serum triglycerides. Notably, the observation that LPL activity in the heart was inversely correlated with serum triglycerides in both fasted ($n = 59$, $r = -0.31$, $P = 0.02$) and fed ($n = 59$, $r = 0.34$, $P = 0.01$) rats suggests a relationship between triglyceride hydrolysis rate in the heart and serum triglyceride levels. However, the increase of LPL activity in the heart when rats were fed menhaden oil could explain only 10% of the diminutions of serum triglyceride levels ($r^2 = 0.096$ and 0.11 for fasted and fed rats, respectively), thereby contributing slightly to the lowering effects of SPMO diet on serum triglycerides. Baltzell *et al*. (7) also observed an increase in heart LPL activity in rats after a diet containing fish oil, compared with a diet containing corn oil, but there was no correlation with plasma triglyceride levels. In this study, no correlations were observed between LPL activity in epididymal fat or VLM and serum triglycerides.

The hypotriglyceridemic effect of fish oil was not reproduced in the fasted and fed states when combined with casein and cod protein. Menhaden oil combined with casein reduced only slightly and not significantly serum triglycerides by 21% in the fasted state and 6% in the fed state, despite a 38% reduction of hepatic triglycerides. These effects could result from a decrease in triglyceride synthesis in the liver without a concomitant reduction in the rate of triglyceride secretion from the liver to the blood. According to Lewis (41), both cholesterol ester and triglyceride hepatic content appear to be important in the regulation of VLDL secretion. Avramoglu *et al*. (42) showed that VLDL secretion correlates better with total mass of cholesterol ester within HepG2 cells than with the mass of intracellular triglycerides, and not only with newly synthetized cholesterol esters but also with stored cholesterol esters. Consequently, it is possible that the unchanged hepatic cholesterol content following menhaden oil compared

to coconut oil in combination with casein could have prevented a reduction of hepatic VLDL triglyceride secretion into the blood and maintained unchanged serum triglyceride concentrations.

The combination of menhaden oil plus cod protein provoked no reducing effect on serum triglycerides in fasted rats. The lack of a hypotriglyceridemic effect of menhaden oil with cod protein could be partly related to the low reducing effect of menhaden oil on hepatic triglycerides (13%) when combined with cod protein. According to Lewis (41), the supply of fatty acids to the liver is dependent not only on dietary fatty acids and *de novo* lipogenesis but also on endogenous free fatty acids released from fat stores in peripheral tissues and by lipolysis of circulating triglyceride-rich lipoproteins. In the present study, higher fasting LPL activity in epididymal fat tissue was shown in rats fed cod protein than in rats fed casein and soy protein. Similarly, higher plasma postheparin fasting LPL activity was observed in rabbits fed cod protein compared with those fed soy protein (18). These facts support the concept that cod protein enhances the supply of fatty acids to the liver from an increased intravascular lipolysis, attenuating the reducing effect of menhaden oil on hepatic VLDL triglyceride assembly and secretion into the blood. Further studies are now necessary to verify the rates of synthesis and secretion of triglycerides from the liver when rats are fed casein, cod protein, and soy protein in the presence of menhaden oil.

In contrast to the interactions outlined above was the lack of a serum insulin and glucose response to the diets. Our inability to detect insulin differences between diets in this study may be partly attributed to the high variability of insulin values in rats fed menhaden oil. Also, in rats, insulinemia reaches a peak in the postprandial state at 30 min to 1 h after the meal, and insulin determination was done in this study at only one time point, 2.5 h after the meal, not allowing under these conditions a complete investigation of insulin response. Thus, fasting and postprandial insulinemia in the present study do not seem to be responsible for the interaction of dietary proteins and lipids on triglyceridemia. A modulation of the hypotriglyceridemic effect of fish oil by dietary proteins through a regulation of LPL activity by plasma insulin levels was not observed in the present study.

In conclusion, the above results show that there are interactions between dietary proteins and lipids in the regulation of serum triglyceride levels in the rat. Indeed, soy protein enhanced the hypotriglyceridemic effect of fish oil, whereas casein and to a greater extent cod protein dampened it. The present results suggest that dietary proteins can modulate the effects of fish oil on serum triglyceride concentrations through hepatic cholesterol and triglyceride concentrations. The role of insulin levels in this modulation is still unclear. Further studies to determine the rates of synthesis and secretion of triglycerides, and the overall response of insulinemia and triglyceridemia by repeated measures in the postprandial state, would be of help to clarify the mechanisms by which dietary proteins modulate the hypotriglyceridemic effect of fish oil.

ACKNOWLEDGMENTS

The authors wish to acknowledge the invaluable professional assistance of Charles Lavigne and technical help of Rachel Gaudreault and Josée Lalonde. This research was supported by NSERC.

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[Received February 5, 1998, and in final revised form July 6, 1998; revision accepted July 27, 1998]

Carnitine Palmitoyltransferase I, Carnitine Palmitoyltransferase II, and Acyl-CoA Oxidase Activities in Atlantic Salmon (*Salmo salar*)

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ABSTRACT: Salmon farmers are currently using high-energy feeds containing up to 35% fat; the fish's capability of fully utilizing these high-energy feeds has received little attention. Carnitine is an essential component in the process of mitochondrial fatty acid oxidation and, with the cooperation of two carnitine palmitoyltransferases (CPT-I and CPT-II) and a carnitine acylcarnitine transporter across the inner mitochondrial membrane, acts as a carrier for acyl groups into the mitochondrial matrix where β-oxidation occurs. However, no reports are available differentiating between CPT-I and CPT-II activities in fish. In order to investigate the potential for fatty acid catabolism, the activities of key enzymes involved in fatty acid oxidation were determined in different tissues from farmed Atlantic salmon (*Salmo salar*), i.e., acyl-CoA oxidase (ACO) and CPT-I and CPT-II. Malonyl-CoA was a potent inhibitor of CPT-I activity not only in red muscle but also in liver, white muscle, and heart. By expressing the enzyme activities per wet tissue, the CPT-I activity of white muscle equaled that of the red muscle, both being >> liver. CPT-II dominated in red muscle whereas the liver and white muscle activities were comparable. ACO activity was high in the liver regardless of how the data were calculated. Based on the CPT-II activity and total palmitoyl-L-carnitine oxidation in white muscle, the white muscle might have a profound role in the overall fatty acid oxidation capacity in fish.
Lipids 33, 923–930 (1998).

Fatty acid β-oxidation occurs in two distinct organelles, i.e., peroxisomes and mitochondria. Mitochondria and peroxisomes are vital for sustaining life, but these organelles have evolved with distinct metabolic features. Fatty acid β-oxidation in peroxisomes, like mitochondria, generates acetyl-CoA through successive steps of dehydrogenation, hydration, and thiolytic cleavage. In contrast to mitochondrial β-oxidation, the first dehydrogenation step involves the reduction of O_2 to H_2O_2 by acyl-CoA oxidase, whereas in mitochondria the second dehydrogenation step reduces NAD^+ to NADH (1). The peroxisomal β-oxidation sequence, which is not coupled to a phosphorylating system, is not inhibited by cyanide (1). Mitochondrial β-oxidation of fatty acids is thought to be essentially complete, whereas this seems not to be the case in peroxisomes (2). Mitochondria are more abundant than peroxisomes in most animal cells and under normal conditions oxidize more than 90% of long-chain fatty acids (3).

The processes of lipid catabolism are less well known in fish (4,5), but teleosts, like higher vertebrates, store a considerable proportion of their triacylglycerol (TG) in a discrete abdominal tissue (6) and in adipocytes which are distributed throughout the muscle myosepta (7–9). In addition, a lipase which degrades long-chain TG occurs in red and white muscles of fish (10). During starvation, ketone bodies, but not nonesterified fatty acids, are an important fuel for muscle in elasmobranchs, whereas nonesterified fatty acids, but not ketone bodies, are an important fuel in teleosts (11,12). Both mitochondria and peroxisomes show a broad chain-length specificity of hepatic β-oxidation of fatty acids in fish (13–15). In an Antarctic fish (*Notothenia gibberifrons*) and another teleost (*Myoxocephalus octodecimspinosus*), substrate selectivities were broader for peroxisomal β-oxidation than for mitochondrial β-oxidation and the peroxisomal β-oxidation system could account for up to 30 and 50% of total hepatic β-oxidation, respectively (16,17). In addition to the liver, red muscle (18,19) and heart (20) also possess a high capacity for the oxidation of fatty acids, whereas the kidney and particularly the white muscle (21) have a lower ability to oxidize fatty acids. Atlantic salmon fed L-carnitine revealed a higher capacity to β-oxidize [1-^{14}C]palmitic acid in both liver cubes and isolated hepatocytes (22). The muscle metabolic organization of salmonids undergoes seasonal variation (23), and during cold conditions (24–26) and spawning (27) an enhanced capacity for lipid oxidation occurs.

In general, the amount of fat in the fish feed is continuously rising, and today a normal commercial salmonid feed contains around 30–35% fat. However, no reports exist as to fish handling these high-fat diets and the effect it will have on fat deposition and/or fatty acid catabolism in different tissues. Another important aspect is that in most cases the capacity to β-oxidize fatty acids in different tissues is expressed per gram wet weight

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Abbreviations: ACO, acyl-CoA oxidase (E.C. 1.3.3.6); BSA, bovine serum albumin; CPT-I, carnitine palmitoyltransferase I (E.C. 2.3.1.21); CPT-II, carnitine palmitoyltransferase II (E.C. 0.0.0.0); TG, triacylglycerol.

and/or per milligram protein (specific activity). The importance of considering tissue size was emphasized elsewhere (28–31); therefore, we have also presented the data per wet tissue. The aim of the present study was to study key enzymes involved in peroxisomal and mitochondrial β-oxidation in different tissues and to differentiate between carnitine palmitoyltransferase-I (CPT-I) and CPT-II activities in Atlantic salmon.

MATERIALS AND METHODS

Chemicals. [*N*-Me-^{14}C]L-carnitine was purchased from New England Nuclear (Boston, MA). 2′,7′-Dichlorofluorescein diacetate was obtained from Eastman Kodak Company (Rochester, NY). Palmitoyl-CoA, horseradish peroxidase, L-carnitine, and other cofactors were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and obtained from common commercial sources.

Fish. Five sexually immature adult Atlantic salmon (*Salmo salar*) weighing 4183 ± 687 g (69.6 ± 4.3 cm) were collected from Institute of Marine Research, Matre Aquaculture Research Center, Norway). The fish had been kept in net pens in the sea and fed a standard diet containing 29% lipid (Elite Granulat, T. Skretting A/S, Bergen, Norway). The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals (Bergen, Norway).

Preparation of tissue homogenates. When fully anesthetized (methomidate), the fish were killed by a blow to the head; and the liver and heart were removed, weighed, and homogenized with an Ultra-Turrax T25 to 20% (wt/vol) in ice-cold sucrose solution containing 0.25 M sucrose in 10 mM HEPES buffer and 1 mM EDTA, pH 7.4. Red muscle was sampled from one side of the fish and dissected free from skin, subdermal lipid, and underlying white muscle. White muscle was sampled on the same side directly below the dorsal fin toward the vertebral column. Red and white muscles were weighed and homogenized as described above. The resulting total homogenates were then centrifuged (1880 × *g* for 10 min at 2°C). The resulting postnuclear fraction (E-fraction) was collected, and some portions were used immediately to determine CPT-I activity. Other aliquots were stored at −80°C until analyzed.

Lipid analysis. Tissue lipids were determined in the E-fractions by enzymatic colorimetric methods. TG and cholesterol were determined according to Technicon Method no. SA4-0324L90 (32) and Technicon Method no. SA4-0305L90 (33) (Technicon Instruments, Tarrytown, NY), respectively. Phospholipids were measured by the method of bioMérieux (Marcy-l'Etoile, France) (34).

Enzyme activities. Fatty acid oxidation was determined in the E-fractions as acid-soluble products using [1-^{14}C]palmitoyl-L-carnitine as substrate (35). All samples were preincubated for 2 min at the different temperatures used before adding the substrate. After incubation for 10 min, oxidation was stopped by addition of 150 µL 1.5 M KOH; 25 µL fatty acid-free bovine serum albumin (BSA) (100 mg/mL) was then added to the suspension in order to bind nonoxidized substrate. Next, 500 µL of 4 M $HClO_4$ was added to precipitate nonoxidized substrates bound to BSA. The total solution was then centrifuged at 1880 × *g* for 10 min at 2°C. Aliquots of 500 µL were assayed for radioactivity.

The rate-limiting enzyme in peroxisomal β-oxidation, acyl-CoA oxidase (ACO) (E.C. 1.3.3.6), was determined according to Small *et al*. (36). Oxidation of 2′,7′-dichlorofluorescein diacetate was monitored by following the increase in A_{502} for 7 min after the samples had been preincubated for 3 min at the different temperatures used. CPT-I (E.C. 2.3.1.21) and CPT-II (E.C. 0.0.0.0) activities were measured essentially as described by Bremer (37). CPT-I is deeply anchored on the outer mitochondrial membrane and loses activity upon exposure to detergents but is stabilized by BSA (38,39). Briefly, the assay medium for CPT-I contained 70 mM KCl, 5 mM KCN, 100 µM palmitoyl-CoA, 10 µL BSA (10 mg/mL), 600 µg tissue protein, and 20 mM HEPES, pH 7.5. The reaction was started with 200 µM [*N*-Me-^{14}C]L-carnitine (1000 dpm/nmol). When included, malonyl-CoA (5 µM) was added to the assay mixture 15 min prior to the start of the reaction. CPT-II, which is localized on the inner surface of the inner mitochondrial membrane, is readily released by a variety of detergents (38,39). Assay conditions for determining CPT-II activity were identical except that BSA was omitted, 0.01% Triton X-100 was included, and the amounts of tissue protein were 400 µg (white muscle), 40 µg (red muscle), and 100 µg (heart and liver). The reaction was stopped with 1 mL 1 N HCL, and water-saturated 1-butanol was added to extract the product, i.e., palmitoyl-[^{14}C]carnitine. Aliquots were assayed for radioactivity on an LKB Wallac 1219 Rackbeta liquid scintillation counter. All enzyme assays were run in duplicate and performed under conditions where product formation was linear with respect to both the time of incubation and the amount of protein. Protein was determined using the Bio-Rad protein kit (Bio-Rad, Richmond, CA).

RESULTS

The fatty acid oxidation capacity and enzyme activities increased in all tissues with increasing assay temperatures but were practically not detectable at 50°C. The reason for this is probably that the organelle membranes had disintegrated, and the enzymes to some extent were denatured.

The mass of the different tissues is given in Table 1. Lipids and protein content of the E-fractions are shown in Table 2.

TABLE 1
Tissue Mass and Percentage of Body Mass[a]

Tissue	Mass (g)	Body mass (%)
Heart	5.1 ± 1.2	0.1
Liver	40.3 ± 8.4	1.0
Red muscle[b]	209 ± 34	5.0
White muscle[b]	2510 ± 412	60.0

[a]Tissue values are means ± SD, $n = 5$.
[b]Red and white muscle mass in Atlantic salmon were derived from the assumption that they occupy 5 and 60% of the total body mass, respectively (Ref. 64).

TABLE 2
Lipids and Protein Content in E-fractions from Various Organs[a]

Organ	Protein (mg/g tissue)	Triacylglycerol (μmol/g tissue)	Cholesterol (μmol/g tissue)	Phospholipids (μmol/g tissue)
Heart	28.7 ± 3.1	3.6 ± 0.8	2.1 ± 0.3	3.7 ± 0.5
Liver	94.4 ± 8.5	5.6 ± 1.4	4.9 ± 0.8	15.4 ± 2.2
Red muscle	20.3 ± 1.5	16.8 ± 5.9	1.4 ± 0.2	3.7 ± 0.4
White muscle	36.1 ± 4.9	16.0 ± 4.8	1.8 ± 0.2	2.1 ± 0.5

[a]All values are means ± SD, $n = 5$.

The amount of TG was high in muscle, whereas the liver contained high levels of cholesterol and especially phospholipids. In addition, the protein content of liver was *ca.* threefold higher than the other tissues.

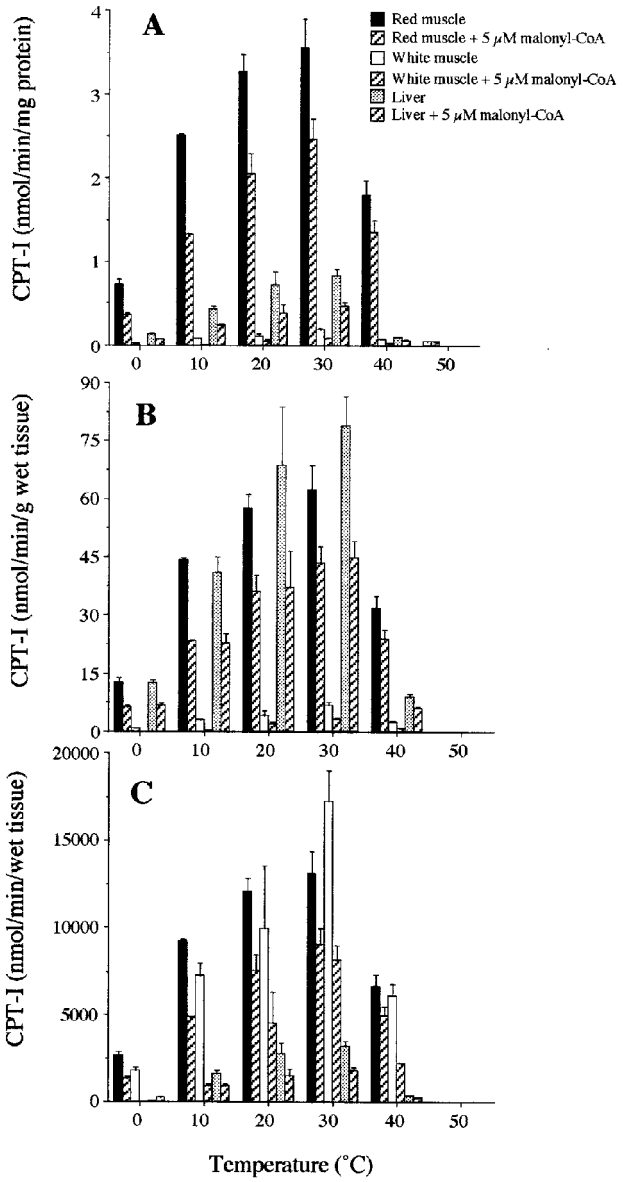

FIG. 1. Activity of carnitine palmitoyltransferase I (CPT-I) in different tissues of Atlantic salmon with varying assay temperatures. The results are expressed per milligram protein (A), per gram wet tissue (B), and per wet tissue (C). The values represent means ± SD ($n = 5$).

Figure 1 shows CPT-I activities in different tissues with varying assay temperatures. In panels A–C the CPT-I activity is expressed as nmol/min/mg protein (specific activity), nmol/min/g wet tissue, and nmol/min/wet tissue, respectively. Increased CPT-I activities with increasing temperature with a maximum at 30°C were observed for all tissues, and CPT-I was malonyl-CoA-sensitive. Above 30°C the enzyme activity declined and at 50°C no enzyme activity could be detected. The order of CPT-I activities in different tissues was red muscle >> liver > white muscle. If the results are calculated as nmol/min/g wet tissue, the CPT-I activity in liver equaled that of red muscle, whereas both were much greater than white muscle (panel B). However, by calculating the enzyme activity as nmol/min/wet tissue, a completely different picture emerged (panel C). The total enzyme activity in white muscle equaled that in red muscle and both were much greater than in liver.

Figures 2 to 4 are presented in the same manner as Figure 1. Figure 2 shows that CPT-II activity dominated in red muscle when expressed as specific activity, and the CPT-II activities in liver and heart were much greater than that of white muscle at all temperatures (panel A). As for CPT-I, the CPT-II activity in liver equaled that of red muscle when calculated as nmol/min/g wet tissue (panel B). The total CPT-II activity dominated in red muscle, whereas no differences were observed in liver and white muscle (panel C). The order of activity of the key enzyme in peroxisomal β-oxidation, i.e., acyl-CoA oxidase (ACO), was liver >> red and white muscle and heart (Fig. 3).

Figure 4 shows the fatty acid oxidation measured as acid-soluble products in E-fractions with palmitoyl-L-carnitine as a substrate. The total oxidation of palmitoyl-L-carnitine was highest in red muscle when calculated as nmol/min/mg protein (panel A) or as nmol/min/wet tissue (panel C). If the data are calculated as nmol/min/g wet tissue, the activities in liver equaled that of red muscle (panel B).

DISCUSSION

The heart, liver, and especially the red muscle, but not white muscle, are generally accepted as the most important tissues involved in fatty acid oxidation in fish (4,5,20).

In the present study we analyzed the rate of key enzymes involved in mitochondrial and peroxisomal β-oxidation of fatty acids in different tissues from Atlantic salmon. Enzyme activities are normally expressed per gram wet mass or per

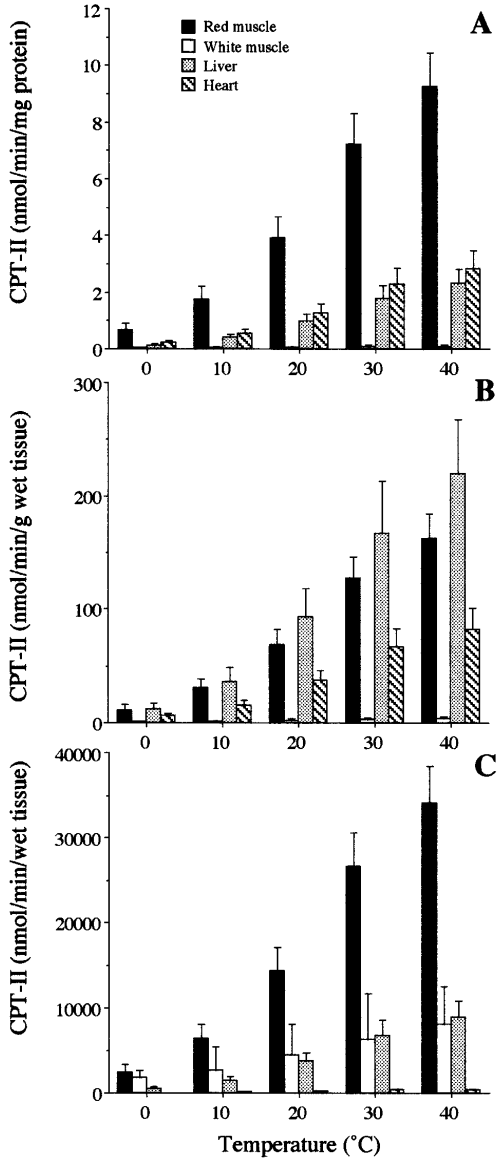

FIG. 2. Activity of carnitine palmitoyltransferase II (CPT-II) in different tissues of Atlantic salmon with varying assay temperatures. The results are expressed per milligram protein (A), per gram wet tissue (B), and per wet tissue (C). The values represent means ± SD (n = 5).

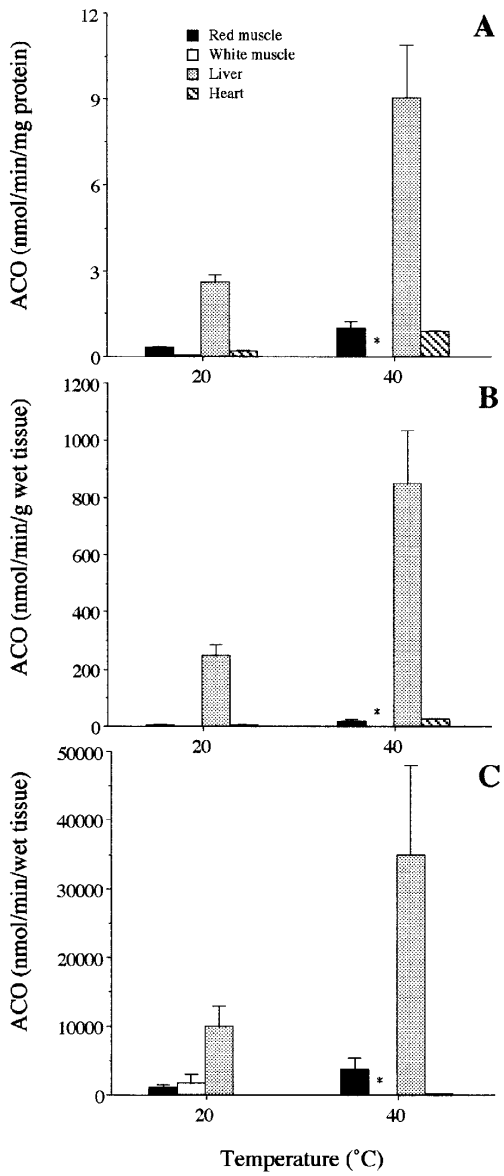

FIG. 3. Activity of acyl-CoA oxidase (ACO) in different tissues of Atlantic salmon with two assay temperatures. The results are expressed per milligram protein (A), per gram wet tissue (B), and per wet tissue (C). The values represent means ± SD (n = 5). *Denotes not determined. The reason for this was that the assay mixture at 40°C became cloudy and led to an unaccounted for unspecific activity.

milligram protein (specific activity), but we have included the rates per wet tissue, i.e., per organ of fish based on the information given in Table 1.

CPT has a wide distribution in the animal kingdom, and the concentration of the enzyme seems to be correlated to the metabolic activity of the animal (40). CPT-I and its sensitivity toward malonyl-CoA, an intermediate in fatty acid synthesis, are believed to regulate hepatic fatty acid oxidation and ketogenesis (41,42). Rodnick and Sidell (43) reported that CPT-I in red muscle of striped bass (*Morone saxatilis*) was malonyl-CoA-sensitive, a fact never before shown in Atlantic salmon. No data differentiating CPT-I and CPT-II activities from any fish have been published. Figure 1 shows that CPT-I was sensitive toward malonyl-CoA in all tissues analyzed, indicating that the fatty acid synthesis and fatty acid oxidation are regulated in the same manner as in mammalian tissues.

The liver contains about three times more protein as the other tissues (Table 2). This is reflected when the enzyme activities of hepatic CPT-I and CPT-II are expressed per gram wet tissue as the hepatic activities equaled that of red muscle (Figs. 1 and 2). In contrast, when hepatic CPT-I and CPT-II activities are expressed per milligram protein or per wet tissue, they do not reach the level of red muscle (Fig. 1).

Another interesting finding was observed when enzyme activities were expressed per wet tissue as the CPT-I activity

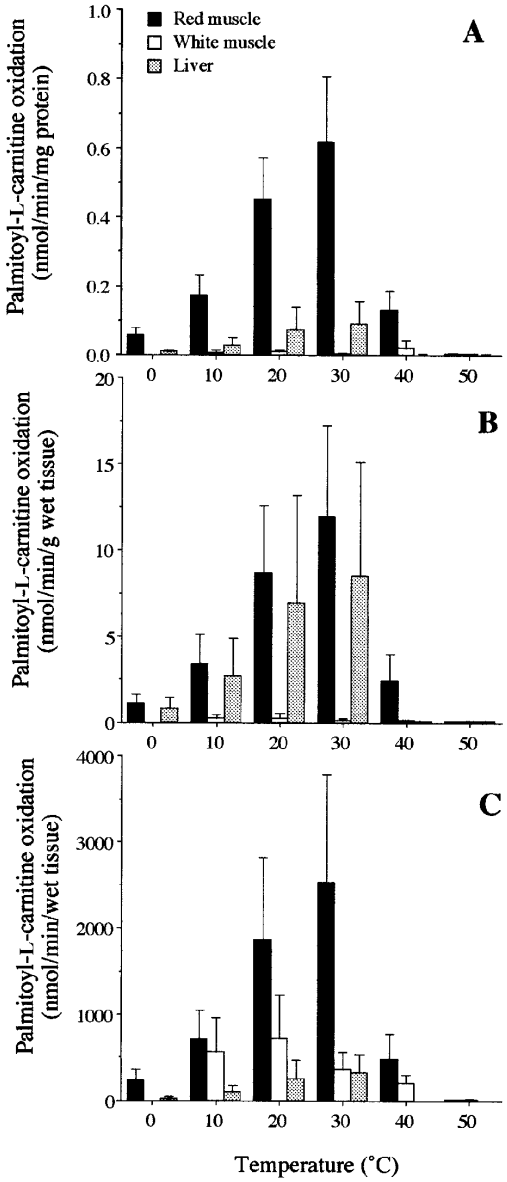

FIG. 4. Oxidation of palmitoyl-L-carnitine in different tissues of Atlantic salmon with varying assay temperatures. The results are expressed per milligram protein (A), per gram wet tissue (B), and per wet tissue (C). The values represent means ± SD (n = 5).

of white muscle was of the same magnitude as for red muscle (Fig. 1), whereas the CPT-II activity was much lower and comparable to the liver (Fig. 2). Importantly, CPT-I and CPT-II activities are found not only in mitochondria but also in other organelles, e.g., peroxisomes and microsomes (44,45). However, the role of CPT-II in other organelles remains uncertain (46,47), whereas mitochondrial CPT-II is involved in the process of β-oxidation, i.e., generates acyl-CoA from acylcarnitines in the mitochondrial matrix (Fig. 5).

By comparing Figure 2 with the oxidation of palmitoyl-L-carnitine (Fig. 4), which must be converted to acyl-CoA by CPT-II before it can undergo β-oxidation (Fig. 5), this study indicates that CPT-II activity in Atlantic salmon reflects the capacity of mitochondrial fatty acid oxidation and involvement of L-carnitine in mitochondrial long-chain fatty acid oxidation. In addition, adding L-carnitine to isolated mitochondria stimulated the oxidation of fatty acids in rainbow trout (*Salmo gairdneri*) (20), and supplementation of L-carnitine to Atlantic salmon led to an increased fatty acid oxidation capacity (22). Furthermore, accelerated growth and reduced body fat were reported for hatchery-reared sea bass (*Dicentrarchus labrax*) (48,49) and African catfish (*Clarias gariepinus*) (50) fed carnitine.

The peroxisomal ACO activity dominated in liver regardless of how the results are expressed. Thus this organ has a high capacity for peroxisomal β-oxidation, whereas red and white muscles seem to possess a limited ACO activity (Fig. 3).

The rate of key enzymes involved in the fatty acid oxidation system and the interpretation of their possible role in the metabolic pathways depend on how the data are expressed (51). After comparing the rate of palmitoyl-L-carnitine oxidation in red muscle from Atlantic salmon with hepatic fatty acid oxidation of palmitoyl-L-carnitine from other species, evidently this tissue possesses a high capacity to oxidize fat (Table 3).

In fish muscle, lipids exist in two forms: neutral lipids (TG) in lipid droplets distributed in cell cytoplasm as local energy stores and polar lipids (phospholipids) serving as major components of the cell membrane. Atlantic salmon stores most of its reserve of lipids (TG) in the muscle instead of the liver (54,55). Histological studies revealed that approximately 40% of white muscle TG was stored in myosepta, whereas the remaining 60% was localized to the perimysium (56–58), which is the connective tissue surrounding a bundle of muscle fibers. Involvement of white muscle in the higher range of sustained cruising speeds of active species was suggested by several authors (59–61). Moreover, the white muscle of pelagic species is functionally and structurally adapted for sustained aerobic activity with relatively abundant mitochondria being preferentially situated close to the source of gas and metabolite exchange (62,63). This is interesting as more than 60% of the total body mass of Atlantic salmon is composed of white muscle (64); thus, the overall metabolic capacities of this tissue might have been underestimated up to now.

Currently, salmon feed usually contains 30–35% fat, but with improving technology the amount of fat in salmon feed will most likely increase to 40–45%. Are salmon capable of metabolizing this added dietary fat? Moreover, how will different fat (oil) sources affect fat deposition vs. catabolism, i.e., storage or energy utilization? Results from a relatively limited number of studies performed on mitochondrial β-oxidation in fish suggest that a substrate preference exists (for review see Ref. 5); thus, the importance of selecting the right fat (oil) source with an optimal fatty acid composition for energy utilization becomes evident.

In conclusion, based on CPT-II activity and palmitoyl-L-carnitine oxidation, Atlantic salmon seem to possess a high capacity to utilize fat as an energy source. Mitochondrial β-

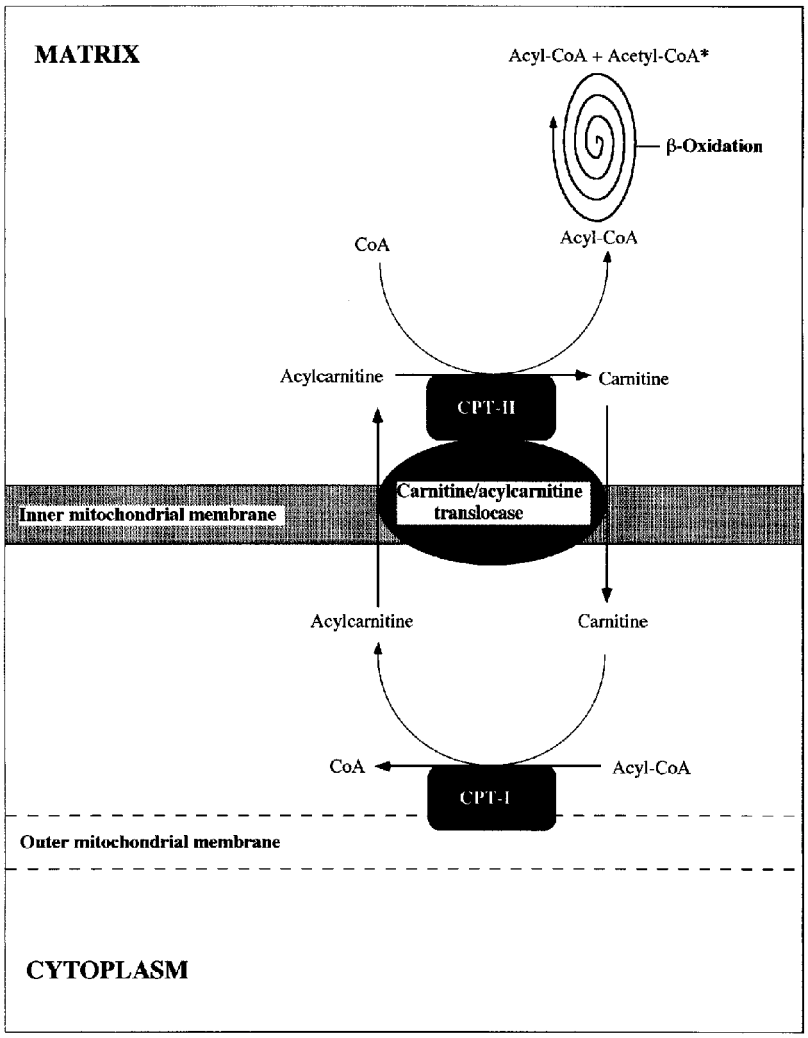

FIG. 5. Schematic presentation of the carnitine-dependent transfer of activated fatty acids (acyl-CoA esters) into mitochondria. CPT-I in the outer mitochondrial membrane converts acyl-CoA to acylcarnitine. This is a necessary step as acyl-CoA esters cannot penetrate the inner mitochondrial membrane. The outer membrane also contains the long-chain acyl-CoA synthetase (not shown). Carnitine/acylcarnitine translocase in the inner mitochondrial membrane exchanges acylcarnitine with carnitine. CPT-II on the inner surface of the inner mitochondrial membrane is responsible for the conversion of acylcarnitine to acyl-CoA. Acyl-CoA enters the β-oxidation sequence and leads to formation of acid-soluble products. *Denotes acid-soluble product. See Figures 1 and 2 for abbreviations.

TABLE 3
The Rate of Palmitoyl-L-Carnitine Oxidation Measured as Acid-Soluble Products in Different Species[a]

Species	Tissue	Palmitoyl-L-carnitine oxidation (nmol/min/mg protein)	Reference
Atlantic salmon	Red muscle	0.45[b]	
Hamster	Liver	0.41	35
Rabbit	Liver	1.47	52
Rat	Liver	0.22	53

[a]Values are expressed as means.
[b]Determined at 20°C, whereas the other values were measured at 30°C.

oxidation dominates in red muscle, but white muscle makes a significant contribution to the overall fatty acid oxidation capacity in fish.

ACKNOWLEDGMENTS

The authors are obliged to the technical staff at Matre Aquaculture Research Station and to Kari Helland, Kari-Elin Langeland, Svein Krüger, and Kari Williams for excellent technical assistance. This study was supported by The Research Council of Norway (grant no. 1160085/122).

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[Received March 23, 1998; and in final revised form July 16, 1998; revision accepted July 20, 1998]

Phospholipid Composition of the Granular Amebocyte from the Horseshoe Crab, *Limulus polyphemus*

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ABSTRACT: The phospholipid composition was determined for the amebocyte of the primitive arthropod *Limulus polyphemus*. The total fatty acid composition of the cells' lipids was analyzed by gas chromatography/mass spectrometry (GC/MS) of fatty acid methyl esters (FAME). The FAME analysis revealed high levels of 20-carbon polyunsaturated fatty acids (PUFA), especially arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acids. Almost 20% of the total lipid profile was comprised of dimethyl acetals of 16- to 20-carbon chain lengths, indicative of plasmalogens in the phospholipid pool. Phospholipids, analyzed by high-pressure liquid chromatography, included phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SPH), and cardiolipin (CL). PE and PC levels predominated at 42.2 and 36.3%, respectively. Smaller amounts of PS (9.0%) and PI (6.2%) were present, as well as low levels of SPH (4.6%), CL (1.6%), and trace amounts of lysophosphatidylcholine. The major phospholipid species, PE, PC, PS and PI, were collected and their molecular species were examined by electrospray–ionization mass spectrometry. The molecular species within the phospholipid classes reflected the high levels of PUFA seen in the total lipid profile. PI was mainly composed of 18:0*a*/20:4. Over half of the PS consisted of 18:0*a*/18:1 and 18:0*a*/20:4. The major PE species were 20:1*p*/20:5, 20:1*p*/20:4, 18:0*p*/20:5, and 18:0*p*/20:4. PC had the largest distribution of molecular species, and its most abundant species were 16:0*e*/20:5, 16:0*e*/20:4, and 16:0*p*/20:4. The presence of 16:0*e*/20:4 is the first documentation of a specific precursor to platelet-activating factor in an invertebrate hemocyte. Note: at the *sn*-1 position: [*a* = 1=*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-*O*-alk-1′-enyl (plasmalogen)].
Lipids 33, 931–940 (1998).

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Abbreviations: AA, arachidonic acid; CL, cardiolipin; DMA, dimethyl acetal(s); EPA, eicosapentaenoic acid; ESI/MS, electrospray–ionization/mass spectrometry; FAME, fatty acid methyl ester(s); GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography; IP_3, inositol-1,4,5-triphosphate; LPC, lysophosphatidylcholine; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid(s); SPH, sphingomyelin; phospholipid molecular species nomenclature, from Reference 1, *n:jk/s:t,* where *n* = number of carbons at the *sn*-1 position, *j* = number of double bonds in the *sn*-1 chain, *k* = the type of linkage at the *sn*-1 position [*a* = 1-*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-*O*-alk-1′-enyl (plasmalogen)], *s* = number of carbons at the *sn*-2 position, and *t* = the number of double bonds in the *sn*-2 chain.

The horseshoe crab, *Limulus polyphemus*, serves as a unique model for studying the lipid chemistry of invertebrate blood cells. The majority of lipid analyses in arthropods have involved studies on whole animals, muscle tissue, or specific organs owing to constraints on organism size and the amounts of blood available (2,3). The presence of as many as six circulating blood cells in arthropods further complicates the isolation and analysis of an individual cell type (4). In horseshoe crabs, large quantities of blood are readily available and a single circulating blood-cell type, the granular amebocyte (5), is present. Moreover, *Limulus* is considered to be a living fossil with blood cells regarded as "primordial immunocytes" because they possess functions comparable to those found in vertebrate leukocytes, such as platelets, macrophages, and B- and T-lymphocytes (4). These vertebrate leukocytes are especially active during an immunological challenge brought on by injury or infection. The activation of these blood cells during an immune response is associated with the production of fatty acid metabolites, termed eicosanoids, which are primarily generated from unsaturated 20-carbon polyunsaturated fatty acids (PUFA) cleaved from the cell's phospholipid membrane, notably di-homo-γ-linoleic acid (20:3), arachidonic acid (AA) (20:4), and eicosapentaenoic acid (EPA) (20:5). The presence of these PUFA in specific phospholipid pools in the cell membrane also has been implicated in accelerating membrane fusion (6). The *Limulus* amebocyte produces eicosanoids (7), and its rapid degranulation in the presence of pathogens is well established (8,9). Therefore, a better understanding of the phospholipid composition, as well as the molecular species within each phospholipid class, will provide a framework for interpreting some of the functions of this primitive undifferentiated cell. In these studies, we will report the phospholipid composition of the *Limulus* granular amebocyte.

MATERIALS AND METHODS

Chemicals and materials. The chloroform, methanol, water, hexane, isopropanol, and acetone used to extract, fractionate, and separate lipids were Fisher Scientific high-pressure liquid chromatography (HPLC)-grade (Pittsburgh, PA). The benzene was Fisher A.C.S.-certified grade and the ethanol was 200-proof dehydrated alcohol, U.S.P. punctilious (Quantum

Chemical Co., Anaheim, CA). Phospholipid, cardiolipin (CL), and sphingomyelin (SPH) standards were obtained from Sigma (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL).

Horseshoe crabs. The horseshoe crabs were purchased from either Marine Biological Laboratories (Woods Hole, MA) or Gulf Specimens (Panacea, FL) and maintained in tanks with fresh, flowing seawater and fed fresh local mussels, *Mytilus galloprovincialis,* collected by university divers from local piers. Blood samples were obtained within 1 wk of the animals' arrival. Animals with a prosoma, the larger anterior portion of the horseshoe crab which is followed by the smaller posterior opisthosoma, of 15–20-cm width were used.

Cell isolation. Prior to blood collection, the animals were cooled at 4°C for at 1 h. The animals were bled *via* cardiac puncture at the prosoma–opisthosomic joint using a sterile 16.5-gauge needle. Aliquots of blood (15 mL) were collected into sterile, silanized 50-mL Pyrex centrifuge tubes containing 10 mL of ice-cold saline (3.0% NaCl, buffered to pH 4.6 with citrate and EDTA) (5). The cells were briefly spun in a clinical centrifuge at room temperature (300 × *g*). The plasma/buffer supernatant was decanted, and the lightly pelleted cells were washed by resuspending in 10 mL of sterile 3% NaCl. Duplicate blood samples were obtained from each animal: one sample was used for fatty acid methyl ester (FAME) analysis and the other was extracted for phospholipid analysis. FAME were prepared directly from freshly isolated amebocytes to expedite analysis of the total lipid.

FAME analysis. The amebocyte's total cellular fatty acids were saponified and derivatized directly by a four-step process (10–12). The blood cells were isolated and washed, as detailed above, and then transferred into a 10-mL Pyrex test tube with a Teflon-lined lid and pelleted in a clinical centrifuge at room temperature (300 × *g*). The supernatant was decanted; the cells were lysed, and the lipids were saponified by adding 2 mL of 3.75 M NaOH/MeOH (50:50, vol/vol) and by heating at 100°C for 30 min. Samples were cooled, and the free fatty acids were methylated with the addition of 3 mL of 6 N HCl/MeOH (65:55, vol/vol) and with heating at 85°C for 10 min. The samples were cooled and 1 mL of hexane/CH_2Cl_2 (50:50, vol/vol) was added. The tubes were inverted end-over-end for 3–5 min. The aqueous layer was removed and 3 mL of 0.3 M NaOH was added. The tubes were inverted end-over-end for 10 min. The upper organic layer was transferred to an amber vial. The samples were placed under nitrogen for either immediate analysis by gas chromatography/mass spectrometry (GC/MS) or stored at −35°C until analyzed.

GC/MS analysis. FAME samples were analyzed on a Hewlett-Packard 5790 gas chromatograph (Palo Alto, CA) coupled to a VG 70SE double-focusing mass spectrometer using a DB-5MS column, 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA). A 1-μL aliquot of the FAME sample was injected. The DB-5 column was held at 50°C for 1 min, ramped to 125°C at 18°/min, and then ramped to 255°C at 3°/min. The sample chromatograms were compared to an authentic standard of mixed FAME (PUFA-2) (Matreya Inc., Pleasant Gap, PA) run under the same conditions as the samples. The mass spectra of the dimethyl acetal (DMA) peaks were compared with known spectra National Institute of Standards and Technology (NIST) library.

Lipid extraction and fractionation. The cells for the phospholipid analyses, isolated as described above, were transferred to a 15-mL, silanized Pyrex test tube with a Teflon-lined cap. The cells were pelleted, the supernatant was decanted and then the cells were resuspended in 1 mL of sterile 3% NaCl. The cells were extracted using a modification of Kates (13). A 3.75-mL aliquot of methanol/chloroform (2:1, vol/vol) was added to the cell solution. The samples were vortexed and placed on a rotary shaker for 2 h. The solution was centrifuged in a clinical centrifuge for 5 min, the supernatant was transferred to a new tube, and the pellet was reextracted in 4.75 mL of methanol/chloroform/water (2:1:0.8, by vol). The sample was centrifuged, as before, and the supernatant was pooled with the first extract. A 2.5-mL aliquot each of chloroform and water was added to the pooled extract. The samples were vortexed, centrifuged, and the lower aqueous layer was removed. An equal volume of benzene was added and samples were taken to dryness under N_2. The samples were immediately resuspended in chloroform/MeOH (4:1, vol/vol) and stored under N_2 at −35°C until fractionated.

The samples were dried and resuspended in chloroform before being fractionated on small silica columns in Pasteur pipets using Baker-analyzed 60–200 mesh silica gel (Phillipsburg, NJ). The columns were equilibrated in chloroform. The sample was applied to the column followed by 4 column volumes (8 mL) of chloroform to elute the neutral lipids, 16 column volumes (32 mL) of acetone to elute the glycolipids, and finally 4 column volumes (8 mL) of MeOH to elute the phospholipids and SPH. The fractions were dried down under N_2 and stored at −35°C until analyzed.

HPLC analysis of phospholipid classes. The phospholipid extracts were analyzed using the method developed by Patton *et al*. (14). The lipid classes were separated using a Hitachi 6200A HPLC (San Jose, CA) equipped with two Rainin Silica Microsorb-MV columns (Woburn, MA) in tandem (4.6 i.d. × 150 mm, 5 μm, 100 Å). The columns were preceded by an Upchurch precolumn packed with 30–40 μm PerisorbA (Oak Harbor, WA). The phospholipid peak elution was monitored at 205 nm. The separation was isocratic starting with a flow rate of 0.5 mL/min for the first 60 min and then increased to 0.8 mL/min for the remainder of the run (*ca*. 180 min). The solvent phase was water/1 M phosphate buffer (pH 7.4)/isopropanol/-hexane/EtOH/acetic acid (55:1.2:485:376:100:0.6, by vol). The peaks for each lipid class were collected. CL samples were pooled while phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and SPH peaks were collected individually for electrospray–ionization/mass spectrometry (ESI/MS) analysis to determine their molecular species. Phosphate does not easily volatilize and therefore interferes with ESI/MS analysis. To avoid problems, samples were briefly taken to dryness under N_2 and reextracted to remove phosphate (13,15).

ESI/MS analysis of molecular species within each phospholipid class. The fatty acid composition of the major phospholipid classes (PE, PC, PS, and PI) was analyzed using a modification of Kim *et al*. (16). The phospholipid classes were separated on a Phenomenex C18 minicolumn (2.0 i.d. × 150 mm, 5 µm, 80Å) preceded by a 30-mm guard column of the same material using a Michrom BioResources UMA HPLC System (Auburn, CA) at a flow rate of 400 µL/m. The samples were separated using a starting solvent phase of 0.5% ammonium hydroxide in water/MeOH/hexane (12:88:0) for 3 min followed by a linear gradient to water/MeOH/hexane (0:18:12) over a 30-min period. The column was maintained at 20–25°C.

The solvent flow was split postcolumn (1:3, vol/vol), and 100 µL of the HPLC effluent was directed into the inlet of an ESI probe of a Fisons VG Platform II single quadrupole mass spectrometer (Micromass Ltd., Altrincham, United Kingdom). The ESI/MS was tuned daily by direct infusion of dipalmitoyl PC (positive-ion mode) or PI (negative-ion mode) (2 µM). The skimmer cone voltage was set during tuning for moderate fragmentation (−60 to −70 V in negative-ion and 40–50 V in positive-ion mode). The mass collection range was set between 100 and 1000 amu for all samples. Sample ions were generated using N_2 nebulization-assisted electrospray with a source temperature maintained at 80°C. All spectra were collected in total scan mode and subjected to background subtraction.

PI and PS samples were analyzed in negative-ion mode. PE and PC peaks were analyzed in both positive- and negative-ion modes. The negative-ion analyses of PE and PC were used to determine the fatty acids associated with the major molecular ions generated in the positive-ion mode. A phospholipid molecular ion can represent one, sometimes two, but rarely all three of the phospholipid subclasses (diacyl, alkylacyl, and alkenacyl). The molecular species of the major peaks were identified through examination of their mass spectra. The remaining molecular species listed were calculated based on the molecular ion and any available fragmentation data from the negative-ion spectra (1). The molecular ion usually represents the entire phospholipid, either $[M + H]^+$ or $[M - H]^-$, depending on the analysis mode, the exception being PC in negative-ion mode that displays a pseudomolecular ion of [M − 15], corresponding to a loss of a methyl group from the choline headgroup (17). Since the phospholipids were separated by class prior to ESI/MS, the headgroup is known and any free fatty acids present in the mass spectra are generated from ester linkages at the *sn*-1 or *sn*-2 position. The FAME/DMA profile in the GC/MS analyses was also used to predict the likely diacyl and alkenylacyl species present in each sample. In the more complicated phospholipid classes, PE and PC, published tables of the three subclasses (diacyl, alkylacyl, and alkenylacyl) were used to help determine the molecular species of the minor peaks (18). In these tables, the molecular ion is calculated based on *sn*-1 and *sn*-2 linkages, as well as the total number of carbons and double bonds present in these positions. Since the double bond in the vinyl linkage of alkylacyl lipids is considered part of the linkage, it is not counted as a double bond in these tables (15,18).

Analysis of smaller phospholipid peaks. The smaller peaks present in the HPLC phospholipid separation (CL and SPH) were screened to identify major species present. The samples were separated by HPLC in a manner similar to the major phospholipid classes. The mass range was set between 100 and 1000 amu, except for CL samples where the range was extended to 2000 amu. These samples were screened in both positive- and negative-ion modes.

RESULTS

GC/MS analysis of FAME. The chromatogram of the FAME revealed that almost half (46%) of the amebocyte fatty acid content consisted of two 20-carbon PUFA, AA, and EPA (Table 1). The next most abundant species were the saturated and monounsaturated 18-carbon fatty acids at 12.9 and 11.6%, respectively. DMA made up nearly 20% of the total lipid. DMA are formed by the acid methanolysis of phospholipids containing vinyl groups in ether linkages. Therefore, the DMA here indicate that a considerable portion of the phospholipids contains ether lipids with alkenyl groups at the *sn*-1 position of the phospholipids. The DMA profile in the amebocyte indicates that eicosene (20:1) was the most com-

TABLE 1
Fatty Acid Composition of the *Limulus* Amebocyte[a]

Fatty acid	Percentage area
14:0	0.1 ± 0.1
15:0	trace
16:0	2.2 ± 0.2
16:1n-7	0.2 ± 0.1
16:0 DMA	1.4 ± 0.2
i/ai 17:1 or 17:1	0.2 ± 0.0
i/ai 17:0	0.2 ± 0.0
17:0	0.8 ± 0.1
17:0 DMA	0.4 ± 0.1
18:0	12.9 ± 0.4
18:1n-9	8.4 ± 0.5
18:1n-7	3.2 ± 0.2
18:2n-6	1.1 ± 0.2
18:0 DMA	5.9 ± 0.5
18:1 DMA	0.7 ± 0.1
19:1, *i/ai* 19:0[b]	3.9 ± 0.4
19:0	0.2 ± 0.1
20:0	trace
20:1n-9	0.8 ± 0.3
20:4n-6	25.4 ± 1.0
20:5n-3	20.5 ± 0.8
20:0 DMA	0.5 ± 0.1
20:1 DMA	9.5 ± 1.3
22:0	trace
22:4n-6	0.7 ± 0.1
22:5n-3	0.7 ± 0.1

[a]Values are the mean ± SEM, $n = 5$. Samples were derived from five separate individuals. Trace levels of fatty acid were considered to be less than 0.1%.
[b]This peak was a mixture of three molecular ions: 294, 310, and 312 amu.
DMA = dimethyl acetal; *i/ai* indicates iso- and anteiso-fatty acids.

TABLE 2
Phospholipid Composition of the *Limulus* Amebocytes[a]

Phospholipid class	Percentage area
Phosphatidylethanolamine	42.2 ± 1.0
Phosphatidylinositol	6.2 ± 0.5
Phosphatidylserine	9.0 ± 0.7
Cardiolipin	1.6 ± 0.5
Phosphatidylcholine	36.3 ± 0.7
Sphingomyelin[b]	4.6 ± 0.5

[a]The percentage area is the mean ± SEM of amebocyte preparations from seven animals.
[b]One of the samples analyzed by electrospray–ionization/mass spectrometry (ESI/MS) contained lysophosphatidylcholine.

mon alkyl chain in ether linkage (9.5%), with lesser amounts of octadecane (18:0) and hexadecane (16:0), 5.9 and 1.4%, respectively.

HPLC analyses of the phospholipid classes. A clean separation existed between the major phospholipid classes. Analysis of the phospholipid fraction revealed a greater proportion of PE (42.2%) than PC (36.2%) (Table 2), followed by lower levels of PS and PI at 9.0 and 6.2%, respectively. SPH (4.6%) and CL (1.6%) were also present in the amebocyte membranes. Lysophosphatidylcholine (LPC) was detected at trace levels in one of the SPH samples.

ESI/MS analyses of the molecular species within the major phospholipid classes. The levels of EPA and AA in the FAME analyses were reflected by the predominance of phospholipid species containing these fatty acids. To distinguish between alkyl and alkenyl linkages at the *sn*-1 position using ESI/MS without prior separation of these subclasses (13) is not possible, as previously stated, but the phospholipid molecular species can still be calculated based on the molecular ion, the headgroup, and any fatty acids that have fragmented from the phospholipid molecule, as seen in the negative-ion spectra. The diacyl and plasmalogen species agreed well with the species of FAME and DMA present in the total lipid (Table 1).

Examination of the negative-ion mass spectra for PI revealed only the molecular ion $[M - H]^-$ (Fig. 1A). To examine the fatty acid profile in the PI class, the sample also was analyzed at higher voltages (–80 to –100 V, data not shown). These spectra indicated 18-carbon *m/z* 281 (18:1) and 283

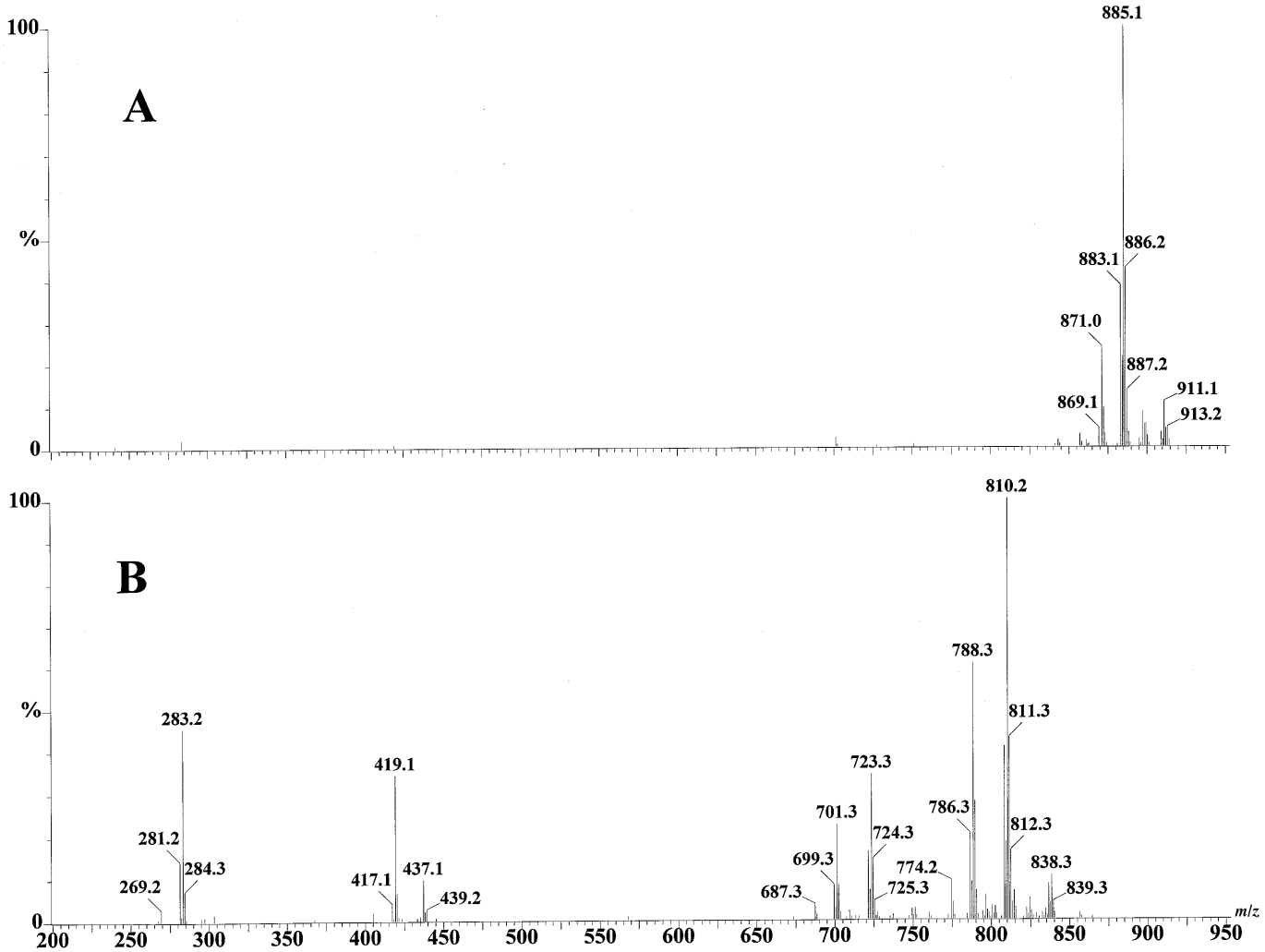

FIG. 1. Electrospray–ionization/mass spectrometry negative-ion mass spectra of the molecular ions and fragments of (A) phosphatidylinositols and (B) phosphatidylserines in the *Limulus* amebocyte.

(18:0), 20-carbon *m/z* 301 (20:5), 303 (20:4), 305 (20:3), 307 (20:2) and 309 (20:1), as well as *m/z* 313 (22:6), 315 (22:5), and 317 (22:4) were present. Greater than half of the PI (52.3%) consisted of a single species, 18:0*a*/20:4 (Table 3), where *a* = 1-*O*-acyl. Almost another quarter of the PI (21.4%) was composed of a mixture of the two species 18:0*a*/20:5 and 18:0*e*/20:4, where *e* = 1-*O*-alkylether. The PS negative-ion mass spectra displayed numerous fragments (Fig. 1B). The molecular ion $[M - H]^-$ was present, as well as ions corresponding to the loss of serine $[M - 88]^-$, and the loss of both serine and fatty acids (*m/z* 417–439) and free fatty acids (*m/z* 281, 283). There were two predominant species of PS, 18:0*a*/20:4 (*m/z* 810) and 18:0*a*/18:1 (*m/z* 788), at 33.8 and 22.7%, respectively (Table 3). The fatty acid composition of PS and PI species indicates that almost 90% of these lipids are diacyl species.

Analysis of the positive-ion mass spectra of the PE revealed the molecular ion $[M + H]^+$ and the loss of ethanolamine from the major species $[M - 142]^+$ (Fig. 2A). The negative-ion mass spectra (Fig. 3A) gave a molecular ion at $[M - H]^-$, as well as free fatty acids at *m/z* 281, 301, and 303. In contrast to the prevalence of diacyl species in the PI and PS fractions of the amebocyte, the PE molecular species consisted of plasmalogen (62%), diacyl (27%), and alkylacyl (11%) phospholipids (Table 4). The chains in the *sn*-1 position of ether lipids are in ether or vinyl ether linkage and are not readily cleaved from the phospholipid backbone during ESI/MS analysis. The general lack of saturated and monounsaturated 16- to 20-carbon free fatty acids in the negative-ion spectra further supports the predominance of ether lipids in this class. The major species were 20:1*p*/20:5 (27.3%) and 20:1*p*/20:4 (13.6%), where *p* = 1-*O*-alk-1′-enyl (plasmalogen). A significant amount of 18:0*p*/20:5 (13.7%) and 18:0*p*/20:4 (4.5%) also existed.

The positive-ion mass spectra for the PC yielded only molecular ions $[M + H]^+$ and the choline headgroup (*m/z* 184) (Fig. 2B). The negative-ion spectra for the PC were quite complicated. The pseudomolecular ion occurred at $[M - 15]$, which is indicative of demethylation of the choline headgroup (17) (Fig. 3B). The other peaks present included the losses of the quaternary nitrogen from the molecular ion (*m/z* 660–720), the loss of quaternary nitrogen and fatty acids (*m/z*

TABLE 3
Molecular Species Composition of the Amebocyte Phosphatidylserines (PS) and Phosphatidylinositols (PI) in Negative-Ion ESI/MS[a,b]

	Molecular species	*m/z*	Composition (%)
PS	18:1*a*/18:1	786.3	4.7 ± 0.6
	18:0*a*/18:1	788.3	23.0 ± 1.4
	18:0*a*/18:0	790.3	2.7 ± 0.2
	18:0*a*/20:5	808.3	3.3 ± 1.2
	18:0*a*/20:4	810.3	34.1 ± 1.0
	18:0*a*/20:3	812.3	5.9 ± 0.1
	18:0*a*/20:2	814.3	4.7 ± 0.7
	18:0*a*/22:5	836.3	4.4 ± 0.3
	18:0*a*/22:4	838.3	6.9 ± 0.8
	18:0*e*/18:1,18:0*p*/18:0	774.3	2.8 ± 0.1
	18:0*e*/20:4	796.3	1.0 ± 0.2
	18:0*e*/20:3	798.3	0.7 ± 0.2
	18:0*p*/20:1	800.3	1.3 ± 0.2
	18:0*e*/20:1	802.3	1.5 ± 0.5
	18:0*p*/22:4	822.3	0.8 ± 0.1
	18:0*e*/22:4	824.3	2.2 ± 0.3
PI	16:0*a*/20:4	857.1	1.1 ± 0.4
	18:0*a*/18:2	861.1	0.6 ± 0.1
	18:0*a*/20:5	883.1	14.7 ± 1.3
	18:0*a*/20:4	885.1	52.3 ± 1.8
	18:0*a*/20:3	887.1	7.3 ± 0.4
	18:0*a*/22:6, 18:1*a*/22:5	909.1	1.2 ± 0.1
	18:0*a*/22:5	911.1	4.5 ± 0.3
	18:0*a*/22:4	913.1	2.0 ± 0.1
	16:0*e*/20:4	843.1	0.8 ± 0.1
	18:1*p*/20:4, 18:0*e*/20:5	869.1	1.4 ± 0.2
	18:0*e*/20:4	871.1	6.7 ± 0.6
	18:0*e*/20:3	873.1	1.0 ± 0.1
	18:0*p*/22:5	895.1	0.7 ± 0.0
	18:0*e*/22:5	897.1	3.3 ± 0.3
	18:0*e*/22:4	899.1	2.4 ± 0.3

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid, where *a* = 1-*O*-acyl, *e* = 1-*O*-alkylether, and *p* = 1-alk-1′-enyl (plasmalogen).
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of four separate individuals, mean ± SEM. See Table 2 for abbeviations.

TABLE 4
Molecular Species Composition of the Amebocyte Phosphatidylethanolamines in Positive-Ion ESI/MS[a,b]

Molecular species	*m/z*	Composition (%)
16:0*a*/20:4	736.3	1.6 ± 0.4
16:0*a*/20:5	738.3	0.5 ± 0.1
18:1*a*/18:1	744.3	0.5 ± 0.2
18:0*a*/18:1	746.3	0.6 ± 0.1
18:0*a*/18:0	748.3	3.4 ± 0.5
18:1*a*/20:5	764.3	2.1 ± 0.2
18:0*a*/20:5	766.3	3.6 ± 0.6
18:0*a*/20:4	768.3	4.3 ± 0.6
18:0*a*/20:3	770.3	1.1 ± 0.1
18:1*a*/22:5	792.3	0.7 ± 0.1
18:1*a*/22:4, 18:0*a*/22:5	794.3	1.0 ± 0.1
18:0*a*/22:4	796.3	0.8 ± 0.1
20:0*a*/20:3	798.3	2.6 ± 0.4
20:1*a*/20:1	800.3	1.3 ± 0.2
20:0*a*/20:1	804.3	2.6 ± 0.2
16:0*p*/20:5	722.3	2.0 ± 0.5
16:0*p*/20:4	724.3	0.7 ± 0.1
18:0*p*/20:5	750.3	13.7 ± 0.9
18:0*p*/20:4	752.3	4.5 ± 0.3
18:0*e*/20:4	754.3	1.9 ± 0.2
18:0*e*/20:3	756.3	4.0 ± 0.4
18:0*e*/20:0	762.3	1.1 ± 0.2
20:1*p*/20:5	776.3	27.3 ± 2.8
20:1*p*/20:4	778.3	13.6 ± 0.9
20:0*e*/20:5	780.3	2.9 ± 0.2
20:0*e*/20:4	782.3	0.8 ± 0.5
20:1*e*/20:0	788.3	0.6 ± 0.1

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid. See Tables 2 and 3 for abbreviations.
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of five separate individuals, mean ± SEM.

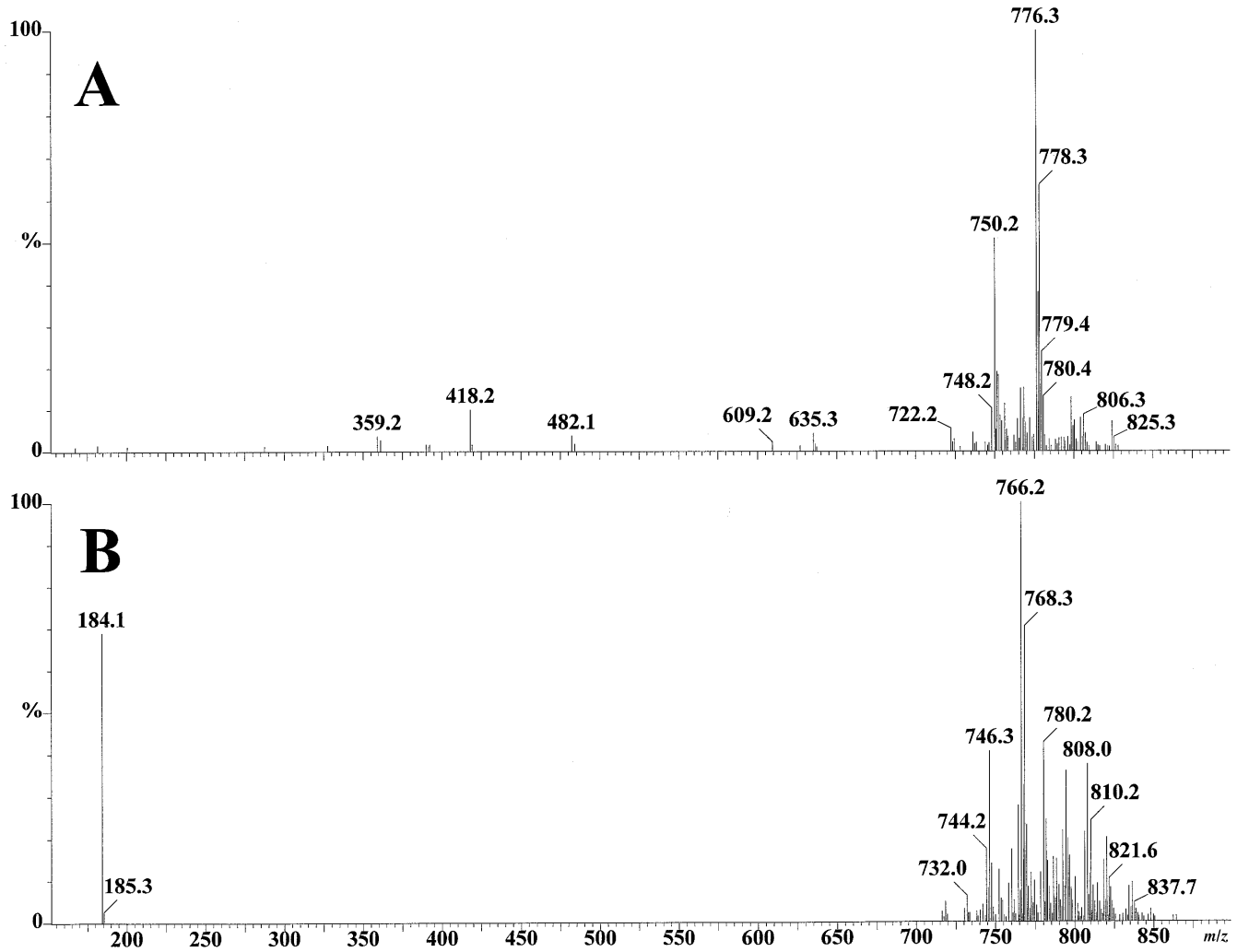

FIG. 2. Electrospray–ionization/mass spectrometry positive-ion mass spectra of the molecular ions and fragments of (A) phosphatidylethanolamines and (B) phosphatidylcholines in the *Limulus* amebocyte.

370–420), as well as the presence of large levels of free fatty acids (*m/z* 255–317). Whereas plasmalogen levels dominated in PE, the levels of the three subclasses in PC were almost evenly distributed. The composition of the PC species consisted of diacyl (39%), alkylacyl (35%), and plasmalogen (26%) phospholipids (Table 5). In comparison to the PE fraction, the presence of detectable 16:0 (*m/z* 255) and large 18:1 (*m/z* 281) in the negative-ion spectra mirrors the higher levels of diacyl phospholipid in the PC fraction. A broad distribution of molecular species was present in PC, but the three major species were a mixture of 16:0*e*/20:5 and 16:0*p*/20:4 (14.4%) and 16:0*e*/20:4 (8.9%).

Screening of CL, SPH, and LPC species. Small peaks in the phospholipid samples (<5%) were screened to identify major species present. The CL fraction was tested in both positive- and negative-ion modes (data not shown). In negative-ion mode, the mass spectra revealed mainly free fatty acids (20:5 > 20:4 ≫ 18:1 > 18:0 = 18:2 > 22:4 > 22:5). The positive-ion spectra showed a cluster of molecular ions in the range of *m/z* 1520–1660. The masses are most likely dipotassiated CL species resulting from the use of potassium phosphate in the HPLC solvent and potassium chloride in the wash buffer used to remove phosphate. The major masses present were *m/z* 1531, 1553, and 1579, indicative of CL with fatty acid mixtures of 18:1, 18:2, 20:4 and 20:5, which correlates with the fatty acid profile seen in the negative-ion mass spectra.

In the positive-ion mode scan of the SPH samples, an extracted ion profile of the choline headgroup, *m/z* 184, demonstrated the presence of six peaks (data not shown). The mass spectra of the five major peaks revealed apparent masses of *m/z* 675, 689, 703, 717 and 731, while the sixth minor peak had an apparent mass of *m/z* 661. Each of these apparent masses had an additional sodiated mass at $[M + 23]^+$. The incremental difference of 14 mass units between each major peak indicates a group of SPH with saturated carbon chains ranging from 13 (*m/z* 661) to 18 (*m/z* 731) carbons. In one of the four SPH samples tested, two minor LPC peaks were detected at *m/z* 496 (16:0 LPC) and *m/z* 524 (18:0 LPC). These LPC peaks displayed the characteristic cleavage of the

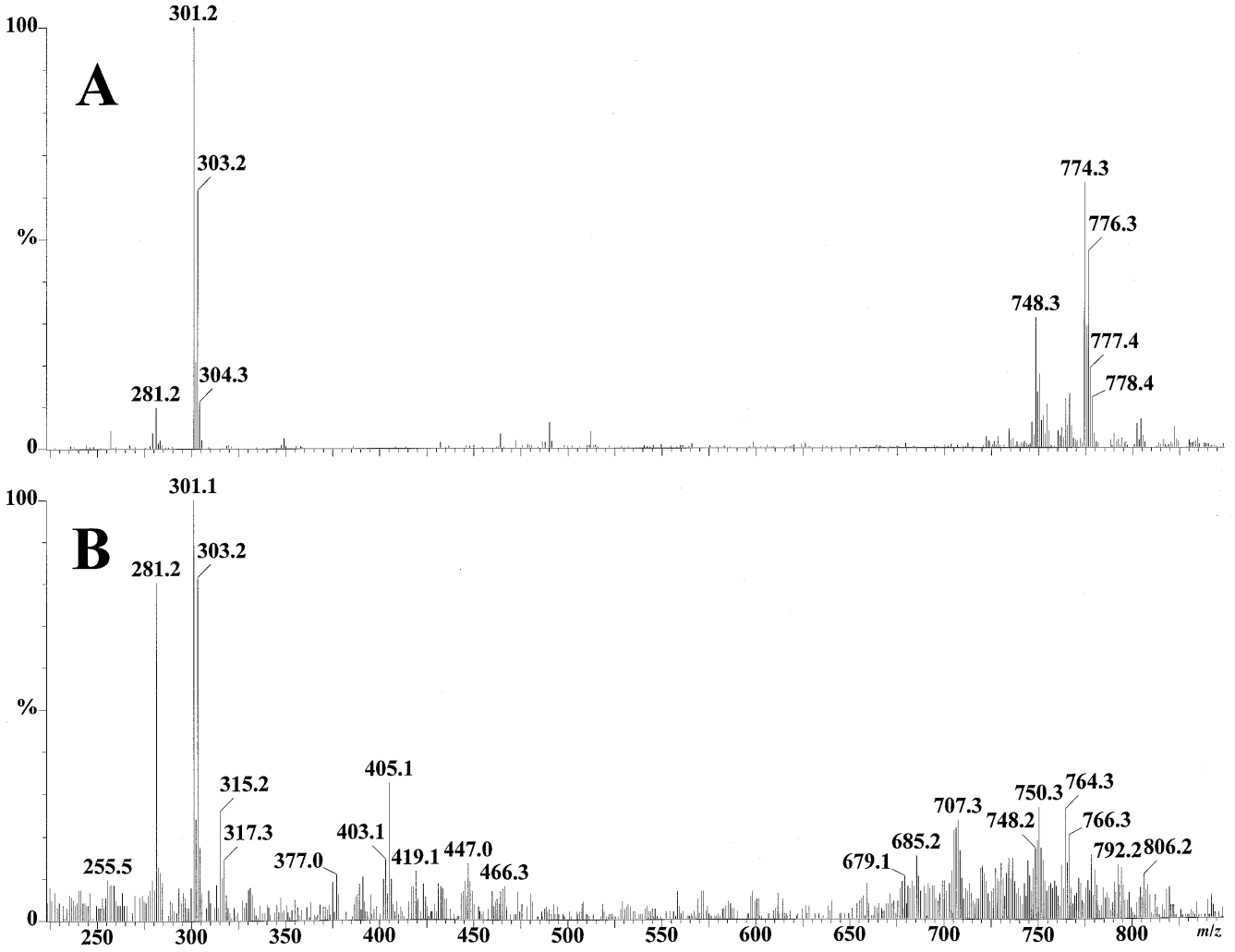

FIG. 3. Electrospray–ionization/mass spectrometry negative-ion mass spectra of the molecular ions and fragments of (A) phosphatidylethanolamines and (B) phosphatidylcholines in the *Limulus* amebocyte.

choline headgroup at *m/z* 184, the molecular ion $[M + H]^+$, the loss of water $[M - 18]^+$ peak, the addition of sodium $[M + 23]^+$, and the addition of potassium $[M + 38]^+$.

DISCUSSION

Analysis of FAME. At present, very few reports are available on the lipid composition of arthropod blood cells to compare with that of the *Limulus* amebocyte. One report is available for the mixed crab blood cells of *Carcinus meanas* of 20% EPA and 10% AA levels (19). *Limulus* amebocytes demonstrate significant levels of ether lipids. Phospholipids with alkenylether bonds (plasmalogens), and to a lesser extent those with alkylether bonds, are also common components in vertebrate blood cells and are most often found in the PC and PE classes (20,21). Though the levels of ether lipids are modest in mammals, in lower animals they can reach appreciable levels, especially the alkylacyl phospholipids. Horseshoe crabs are arthropods, though more closely related to arachnids than crustaceans. In Sugiara *et al.* (2), the tissues of two arachnids, a spider and a water scorpion, were analyzed, and they contained between 6.7 and 17.9% alkylacyl PC and between 13.7 and 22.7% alkylacyl PE, respectively. A marine prawn contained 7% alkylacyl PC and 7% alkylacyl PE. Alkylacylphospholipids can represent up to 20% of the lipid in marine organisms, and in extreme cases as much as 50–80% (2,3,22). The most commonly reported groups in the *sn*-1 position of ether lipids are 16:0, 16:1, 18:0 and 18:1 (23); however, the DMA profile in the amebocyte indicates that eicosene (20:1) is the most abundant group in ether linkage (9.5%), with lesser amounts of 18:0 (5.9%) and 16:0 (1.4%). The role of eicosene ether lipids is not known, but they have been detected in small amounts at the *sn*-l position in the ether lipids of human erythrocytes, adrenal glands, and placentas (23). We also compared the amebocyte to vertebrate white blood cells with similar functions, i.e., platelets and macrophages.

Analysis of the phospholipid class composition. In comparison to mammalian leukocytes, the PE levels of the amebocyte (42.2%) were about 60% greater than those seen in

TABLE 5
Molecular Species Composition of the Amebocyte Phosphatidylcholines in Positive-Ion ESI/MS[a,b]

Molecular species	*m/z*	Composition (%)
16:0*a*/16:1	732.3	1.2 ± 0.2
14:0*a*/20:5	752.3	2.8 ± 0.5
14:0*a*/20:4	754.3	1.3 ± 0.2
16:0*a*/18:2	758.3	1.1 ± 0.0
16:0*a*/18:1	760.3	2.0 ± 0.1
16:0*a*/18:0	762.3	0.8 ± 0.1
16:1*a*/20:5	778.3	1.5 ± 0.1
16:0*a*/20:5	780.3	4.8 ± 0.2
16:0*a*/20:4	782.3	2.8 ± 0.4
18:1*a*/18:2	784.3	0.5 ± 0.1
18:1*a*/18:1	786.3	2.1 ± 0.1
18:0*a*/18:1	788.3	2.0 ± 0.2
18:0*a*/18:0	790.3	0.9 ± 0.2
18:1*a*/20:5	806.3	2.5 ± 0.1
18:1*a*/20:4, 18:0*a*/20:5	808.3	4.5 ± 0.2
18:0*a*/20:4	810.3	3.3 ± 0.3
20:1*a*/18:2	812.3	1.2 ± 0.1
20:1*a*/18:1	814.3	1.2 ± 0.1
18:0*a*/22:6	834.3	1.0 ± 0.1
18:0*a*/22:5	836.3	1.0 ± 0.1
18:0*a*/22:4	838.3	0.6 ± 0.0
16:0*e*/16:0	718.3	0.7 ± 0.1
14:0*e*/20:5	738.3	1.1 ± 0.2
16:0*e*/18:2	744.3	2.6 ± 0.1
16:0*e*/18:1	746.3	6.1 ± 0.5
16:0*e*/18:0	748.3	1.4 ± 0.3
16:0*p*/20:5	764.3	4.2 ± 0.4
16:0*e*/20:5, 16:0*p*/20:4	766.3	14.4 ± 0.5
16:0*e*/20:4	768.3	8.9 ± 1.0
18:1*p*/18:1	770.3	1.0 ± 0.4
18:0*e*/18:2, 18:1e/18:1	772.3	1.6 ± 0.2
18:0*e*/18:1	774.3	1.5 ± 0.1
18:0*p*/20:5	792.3	2.7 ± 0.4
18:0*p*/20:4	794.3	4.7 ± 0.1
18:0*e*/20:4	796.3	2.3 ± 0.1
18:1*p*/20:1	798.3	0.7 ± 0.1
18:0*p*/20:1	800.3	1.3 ± 0.1
20:1*p*/20:5	818.3	1.5 ± 0.3
20:1*p*/20:4	820.3	2.4 ± 0.2
18:0*e*/22:5	822.3	1.1 ± 0.1
18:0*e*/22:4	822.3	0.6 ± 0.0

[a]Phospholipid nomenclature from Reference 1. The letter designates the linkage at the *sn*-1 position in the phospholipid. See Tables 2 and 3 for abbreviations.
[b]The percentage composition was based on the average relative intensities of each molecular ion from the analyses of five separate individuals, mean ± SEM.

platelets and macrophages (23–29%), whereas PC levels (36.3%) were at the lower end of the range seen in these leukocytes (35–46%) (24–26). The higher PE levels in the amebocyte may reflect differences in immune response between invertebrate and vertebrate lymphocytes. Even though the amebocyte can undergo diapedesis, like the macrophage or neutrophil, to reach the site of injury or counter microbial invasion, it is not phagocytic, and it encapsulates foreign bodies by degranulating and releasing clotting factors and antimicrobial substances (8). When compared to the platelet, the amebocyte can plug the site of injury, but unlike the platelet it degranulates initially allowing clot material to fill the site of injury, followed by continued migration of cells to the site of injury, infiltrating and replacing the clot material. These cells then degranulate, elongate, and serve as basis for the formation of new tissue or even carapace (27). The amebocyte PI (6.2%) and PS (9.0%) levels were roughly comparable to the amounts of PI and PS previously reported in platelets and macrophages, 6–8 and 5–11%, respectively. The amount of SPH present in the amebocyte, ~5%, was apparently low in relation to the range of 9–20% SPH detected in mammalian leukocytes (25,26). The amebocyte's CL levels (1.6%) were in a range similar to that reported in human and guinea pig macrophages (1–2.5%) (20,21).

Analysis of the phospholipid molecular species. The molecular species composition of the amebocyte phospholipids reported here reflects the key role this hemocyte plays in the immune defenses of this organism. Within the phospholipid classes, a number of overall similarities existed between the molecular species distribution of PS and PI in the amebocyte with vertebrate platelets and macrophages. In the human platelet, the predominant diacyl molecular species of PS are 18:0*a*/20:4 (41%) and 18:0*a*/18:1 (37%) (24), but in the mouse macrophage, PS has three major species 16:0*a*/18:1 (41.1%), 18:0*a*/18:1 (19.8%), and 18:0*a*/20:4 (11.1%) (21). The PS profile of the amebocyte was more similar to the platelet than the macrophage with its predominant PS species of 18:0*a*/20:4 (34%) and 18:0*a*/18:1 (23%). The major PI species of the amebocyte was 18:0*a*/20:4 (52.3%), and the level of this molecular species was comparable to the mouse macrophage 18:1*a*/20:4 levels (59.1%), but was lower than the human platelet that is almost three-quarters 18:0*a*/20:4 (71%). The level of 18:0*a*/20:4 PI is an important indicator of signal transduction processes as this PI species can act as a potential source for the biosynthesis of PI-4,5-bisphosphate. After cellular activation, PI-4,5-bisphosphate is hydrolyzed, and the diacylglycerol portion remains in the plasma membrane inner leaflet while inositol 1,4,5-triphosphate (IP_3) is released into the cytosol and stimulates the release of Ca^{2+} from the endoplasmic reticulum, which in turn activates numerous cellular processes (28). The recent detection of a putative IP_3 receptor (29) in the *Limulus* amebocyte reinforces the likelihood that there must be a potential source for IP_3 in the plasma membrane.

The major species of PE seen in the amebocyte differed markedly from those reported in vertebrate cells. Whereas diacyl species dominated in the PE lipid pools of mammalian leukocytes, ether lipids made up almost three-quarters of the amebocyte PE species. The major PE species reported in mammalian leukocytes were 16:0*a*/20:4, 18:0*a*/20:4, 16:0*a*/18:1, and 16:0*a*/18:2 (21,24,26). The predominant *Limulus* PE species, 20:1*p*/20:5 and 20:1*p*/20:4, were unusual with the eicosene (20:1) group in the *sn*-1 position. One earlier report, in the guinea pig macrophage, discussed small amounts of eicosenoic acid, mixed with 18:3, in ester linkage at the *sn*-1 position, but none in ether linkage (26).

Elevated levels of AA and EPA in the *sn*-2 position in plas-

malogen PE were reported in prior analyses of both *Limulus* nerve tissue and photoreceptor membranes (30,31). These levels of AA and EPA are quite significant to cellular functions. Glaser and Gross (6) demonstrated that the speed of membrane fusion is directly related to the amount of plasmalogen PE molecular species with PUFA in the *sn*-2 position, particularly AA. The rate of membrane fusion, using plasmalogen PE vesicles, is doubled when 20:4 is in the *sn*-2 position rather than 18:1. The predominance of PE plasmalogens species containing AA and EPA is likely to play a significant role in the rapid rate of degranulation of the *Limulus* amebocyte during an immune response to pathogens or external injury. With an open coelom, *Limulus* must be able to react quickly to injury or microbial invasion to preclude systemic contamination, and degranulation promotes the release of clotting and antimicrobial proteins (polyphemusins and antilipopolysaccharide factor) from the cell's granules (8). These levels of polyunsaturation may also affect the membrane fluidity of the amebocyte, as *Limulus* inhabits a broad range of habitats, from the continental shelf (~200 m) to shallow bays (≤5 m) where temperature and salinity ranges are quite extreme (2–3°C/32 ppt to +25°C/13 ppt) (32).

The PC species observed in the amebocyte, as with the PS and PI pools, were similar to those seen in mammalian leukocytes. The diacyl PC species that predominated in mammalian leukocytes were 16:0*a*/18:1, 16:0*a*/18:2, 16:0*a*/16:0, and 16:0*e*/20:4 (21,24,26). In *Limulus*, the pattern was similar, except the major species were alkylether and plasmalogens, 16:0*e*/20:5, 16:0*p*/20:4, 16:0*e*/20:4, and 16:0*e*/18:1. As reported for the PE species, high levels of AA and EPA were also detected in the PC fractions of *Limulus* nerve tissue (30%) and photoreceptor membranes (42%) (30,31).

In the present study of the amebocytes, the detection of 16:0*e*/20:4 as major species in the PC phospholipid pool is significant, as it is considered to be a precursor to platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-PC) (33). PAF-like lipids were detected in as many as 30 species of invertebrates (2,3), but this is the first documentation of the presence of a specific precursor to PAF in an invertebrate hemocyte. PAF is involved in both signaling and adhesive events between proinflammatory cells and vascular tissues (34). The presence of this potential precursor to PAF is a further indication that signal transduction pathways seen in modern vertebrate cells may also occur in this primitive invertebrate blood cell.

ACKNOWLEDGMENTS

The research presented in this paper is funded, in part, by a grant from the National Sea Grant College Program, National Oceanographic and Atmospheric Administration, U.S. Department of Commerce, under grant number NA-36RG0537, project number R/MP-71 through the California Sea Grant College System, and in part by the California State Resources Agency. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies. Jennifer MacPherson's support is funded through the Sea Grant Traineeship Program.

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[Received January 6, 1998, and in final revised form May 27, 1998; revision accepted August 10, 1998]

An Efficient Ultrasound-Assisted Zinc Reduction of Fatty Esters Containing Conjugated Enynol and Conjugated Enynone Systems

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ABSTRACT: Reduction of methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) with zinc in either aqueous *n*-propanol or water under concomitant ultrasound irradiation furnished a mixture of methyl 8-hydroxy-9*Z*,11*E*-octadecadienoate (**3a**) and methyl 8-hydroxy-9*Z*,11*Z*-octadecadienoate (**3b**) (96% yield). Reduction of methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) under similar conditions gave methyl 8-oxo-10-*Z*-octadecenoate exclusively (**4**, 70%). The latter compound was epoxidized and converted to a C_{18} furanoid fatty ester (**6**, methyl 8,11-epoxy-8,10-octadecadienoate) in 70% yield.
Lipids 33, 941–945 (1998).

Lindlar catalyst is widely used for the semihydrogenation of acetylenic compounds (1,2), but the reduction of conjugated diacetylenes or enynes with this catalyst has produced complex mixtures of dienes and monoenes (2,3). Morris *et al.* (4) reported the use of zinc powder in aqueous organic solutions for the reduction of conjugated enyne fatty esters. However, these reactions require long reaction times (16–43 h) to complete. To improve such reduction reactions, Petrier and Luche (5,6) used Zn-$NiCl_2$ as the catalyst for conjugated systems under concomitant ultrasonic irradiation. This observation prompted us to study the reduction of methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) and methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) with zinc under ultrasound conditions.

MATERIALS AND METHODS

Thin-layer chromatography (TLC) was performed on microscope glass plates coated with silica (*ca.* 0.1 mm thick), and a mixture of *n*-hexane/diethyl ether, 1:1 vol/vol, was used as the developer. Components on the microplates were viewed by exposing them to iodine vapor. Column chromatographic separations were accomplished using silica gel (Merck Art. 7730, type 60, 70–230 mesh; Darmstadt, Germany) as the adsorbent with mixtures of *n*-hexane/diethyl ether as the eluent. Infrared (IR) spectra were measured on a Bio-Rad FTS-7 Fourier-IR spectrometer (Richmond, CA) on neat samples placed between NaCl plates. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier-transform NMR spectrometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform ($CDCl_3$) (0.2–0.3 mM) with tetramethylsilane as the internal reference standard. Chemical shifts are given in δ-value in ppm downfield from tetramethylsilane. Mass spectra were recorded on a Finnigan Mat 95 mass spectrometer (70 eV; Finnigan Mat Corp., San Jose, CA). Ultrasonication was carried out using a 20 kHz (52 W/cm^2) ultrasound horn (Undatim Ultrasonic S.A., Louvain-la-neuve, Belgium).

Methyl santalbate (methyl 11-*E*-octadecen-9-ynoate) was obtained from methyl ricinoleate as reported (7), and methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) and methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) were obtained by oxidation (using selenium dioxide/*tert*-butyl hydroperoxide) of methyl santalbate as described elsewhere (8). Compound **2** could also be obtained from compound **1** by the two-phase chromic acid oxidation procedure (9) in 73% yield.

*Reduction of methyl 8-hydroxy- and 8-oxo-C_{18}-enynoate as exemplified by the reaction of methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) with zinc under ultrasound.* A mixture of compound **1** (300 mg, 0.98 mmol), zinc powder (30 g), and *n*-propanol/water (40 mL, 1:1, vol/vol) was placed in an air-jacketed glass cell (about 150 mL in volume) and sonicated for 15 min. The internal temperature of the reaction reached about 85°C at the end of the sonication period. The reaction mixture was filtered under suction and the zinc residue was washed with diethyl ether (200 mL). Water (100 mL) was added to the filtrate and the ethereal layer was isolated. The aqueous solution was reextracted with diethyl ether (2 × 50 mL) and the organic extracts were combined. The ethereal extract was washed with water (30 mL) and dried over anhydrous Na_2SO_4. The filtrate was evaporated and the residue was separated by silica column chromatography using a mixture of *n*-hexane/diethyl ether (3:2, vol/vol) to give a mixture of methyl 8-hydroxy-9*Z*,11*E/Z*-octadecadienoate isomers (**3a** and **3b**, 290 mg, 96%) as an oil. Retention factor (R_f) = 0.3 (*n*-hexane/diethyl ether, 1:1, vol/vol, as developer); IR

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Abbreviations: ^{13}C NMR, carbon-13 nuclear magnetic resonance spectroscopy; EI, electron impact; ^1H NMR, proton nuclear magnetic resonance spectroscopy; IR, infrared; R_f, retention factor; TLC, thin-layer chromatography.

(cm^{-1}): 3436, 1740, 1640, 1629, and 1530; ^1H NMR ($CDCl_3$ δ_H): 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.5 (*m*, 18H, C*H*$_2$), 1.6 (*s*, broad, O–*H*), 2.09, and 2.16 (*q, J* = 6.5 Hz, 2H, 13-*H*), 2.29 (*t, J* = 7.5 Hz, 2H, 2-*H*), 3.6 (*s*, 3H, COOC*H*$_3$), 4.57 (*m*, 1H, C*H*–OH), 5.26 (*t, J* = 9.9 Hz, 1H), 5.39 (*t, J* = 9.5 Hz, 1H), 5.52 (*m*, 1H), 5.76 (*m*, 1H), 6.02 (*t, J* = 11 Hz, 1H), 6.24 (*m*, 1H), and 6.29 (*m*, 2H) [olefinic protons]; ^{13}C NMR ($CDCl_3$, δ_C) : 14.10 (C-18), 22.62 (C-17), 24.87 (C-3), 25.16 (C-6), 27.45 (C-13 of **3b**), 28.91, 28.94, 29.07, 29.17, 29.48, 31.71(C-16), 32.86 (C-13 of **3a**), 34.06 (C-2), 37.32/37.38 (C-7), 51.47 (COO*C*H$_3$), 67.75/67.89 (C-8), 122.94, 125.02 (2C), 130.54, 131.39, 133.35, 134.64, 137.46, and 174.30 (C-1); mass spectral analysis [electron impact (EI), 70 eV], *m/z* (intensity): 310 (M^+, 12.6), 172 (7.7), 171 (77), 167 (59), 166 (13), 165 (36), 139 (29), 111 (42), and 83 (100, base peak). When water was substituted for aqueous *n*-propanol, compounds **3a** and **3b** were obtained in 70% yield after 25 min of sonication. The geometric isomers (**3a** and **3b**) could not be separated by silver-ion TLC into individual isomers.

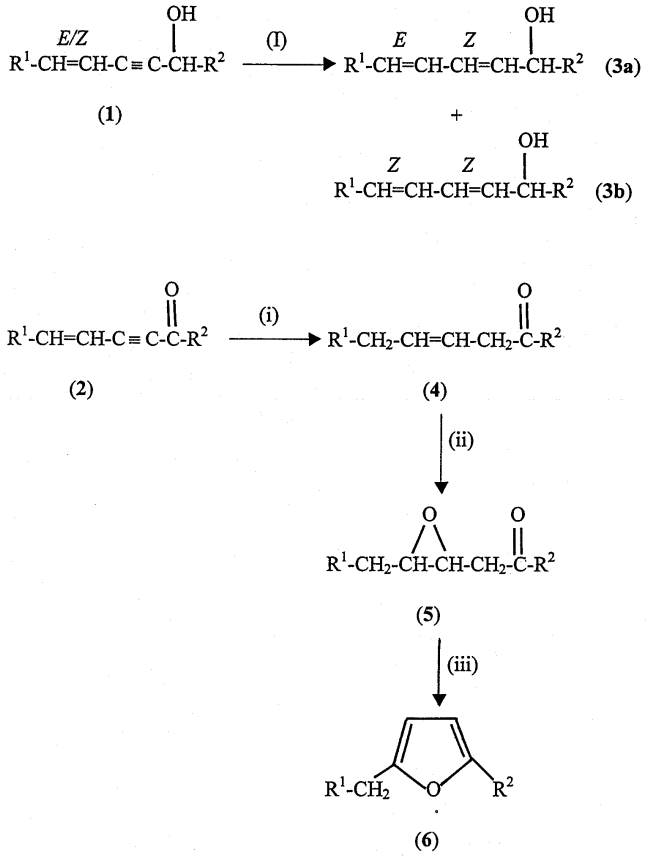
Reaction of methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) with zinc under ultrasound furnished methyl 8-oxo-10-*Z*-octadecenoate (**4**, 213 mg, 70%) as an oil. R_f = 0.5 (*n*-hexane/diethyl ether, 1:1, vol/vol, as developer); IR (cm^{-1}): 1739 (C=O, ester stretching), 1717 (C=O, oxo stretching), and 1636; ^1H NMR ($CDCl_3$, δ_H): 0.89 (*t*, 3H, C*H*$_3$), 1.3–1.6 (*m*, 18H, C*H*$_2$), 2.02 (quartet, *J* = 6.7Hz, 2H, 12-*H*), 2.29 (*t, J* = 7.5 Hz, 2H, 2-*H*), 2.43 (*t, J* = 7.4 Hz, 2H, 7-*H*), 3.14 (*d, J* = 6.2 Hz, 2H, 9-*H*), 3.6 (*s*, 3H, COOC*H*$_3$), and 5.59 (*m*, 2H, 10-*H*, 11-*H*); ^{13}C NMR ($CDCl_3$, δ_C): 14.10 (C-18), 22.66 (C-17), 23.55 (C-6), 24.79 (C-3), 27.52 (C-12), 28.83, 28.90, 29.19, 29.25, 29.36, 31.84 (C-16), 33.98 (C-2), 41.71 (C-9), 42.16 (C-7), 51.45 (COO*C*H$_3$), 120.88 (C-10), 133.73 (C-11), 174.16, and 209.05 (C-8); mass spectral analysis (EI, 70 eV), *m/z* (intensity): 310 (M^+, 0.5), 171 (100, base peak), 139 (17), 111 (23), 83 (23) and 69 (14). When the reaction was carried out in water with zinc, the temperature of the reaction rose to 98°C after 15 min of sonication. The yield of compound **4** by this procedure was 60%.

Transformation of compound **4** *to C_{18} furanoid fatty ester (***6***).* A mixture of methyl 8-oxo-10-*Z*-octadecenoate (**4**, 56 mg, 0.18 mmol), *m*-chloroperoxybenzoic acid (57 mg, 0.33 mmol), and dichloromethane (20 mL) was stirred at room temperature for 12 h. An aqueous solution of Na_2SO_3 (10%, 5 mL) was added to the reaction mixture. The organic layer was isolated and successively washed with aqueous $NaHCO_3$ (10%, 5 mL), water (10 mL), and dried over anhydrous Na_2SO_4. The filtrate was evaporated under reduced pressure to give crude methyl 8-oxo-10,11-epoxystearate (**5**, 46 mg) as an oil: R_f = 0.8 (*n*-hexane/diethyl ether, 7:3, vol/vol, as developer), IR (cm^{-1}) 1740, 1710, 1060; ^1H NMR ($CDCl_3$, δ_H): 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.8 (*m*, 20H, C*H*$_2$), 2.30 (*t, J* = 7.0 Hz, 2H, 2-*H*), 2.48 (*t, J* = 7.0 Hz, 2H, 7-*H*), 2.62 (*dd, J* = 6.0 Hz, 2H, 9-*H*), 2.8–3.0 (*m*, 1H, 11-*H*), 3.2–3.4 (*m*, 1H, 10-*H*), and 3.66 (*s*, COOC*H*$_3$); ^{13}C NMR ($CDCl_3$, δ_C): 14.0 (C-18), 22.5 (C-17), 23.6 (C-6), 24.9 (C-16), 26.4 (C-12), 28.0, 28.8, 29.0, 29.1, 29.3, 31.6 (C-16), 34.0 (C-2), 41.7 (C-9), 43.3 (C-7), 51.3 (COO*C*H$_3$), 52.3 (C-10), 56.4 (C-11), 174.1(C-1), and 208.1 (C-8).

A mixture of the crude compound **5** (46 mg), sodium azide (28 mg, 0.43 mmol), ammonium chloride (23 mg), ethanol (5 mL), and water (1 mL) was refluxed for 30 min. Water (5 mL) was added and the cooled reaction mixture was extracted with diethyl ether (2 × 25 mL). The ethereal extract was washed with water (20 mL) and dried over Na_2SO_4. The filtrate was evaporated and the residue was separated by preparative TLC (*n*-hexane/diethyl ether, 4:2, vol/vol, as developer) to give methyl 8,11-epoxy-8,10-octadecadienoate (**6**, 30 mg, 70%) as an oil. R_f = 0.9 (*n*-hexane/diethyl ether, 9:1, vol/vol, as developer); IR (cm^{-1}): 3100, 1741, 1639, 1566, and 1119; ^1H NMR ($CDCl_3$, δ_H): 0.87 (*t*, 3H, C*H*$_3$), 1.2–1.4 (*m*, 12H, C*H*$_2$), 1.6 (*m*, 6H, C*H*$_2$), 2.30 (*t, J* = 7.5 Hz, 2H, 2-*H*), 2.55 (*t, J* = 7.5 Hz, 4H, 7-*H*, 12-H), 3.66 (*s*, 3H, COOC*H*$_3$), and 5.8 (*s*, 2H, furan ring, 9-*H*, 10-*H*); ^{13}C NMR ($CDCl_3$, δ_C): 14.10 (C-18), 22.66 (C-17), 24.86 (C-3), 27.97, 28.08, 28.15, 28.79, 28.88, 29.07, 29.20, 30.32, 31.80 (C-16), 34.06 (C-2), 51.46 (COO*C*H$_3$), 104.80 (C-10), 104.91 (C-9), 154.33 (C-8), 154.71 (C-11), and 174.27 (C-1); mass spectral analysis (EI, 70 eV), *m/z* (intensity): 308 (M^+, 45), 277 (10), 237 (5), 223 (19), 193 (10), 180 (14), 179 (100, base peak), and 149 (18).

RESULTS AND DISCUSSION

The reactions and products from the zinc reduction of methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) and methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) are shown in Scheme 1. The substrates (**1** and **2**) consisted of mixtures of geometric isomers, because these mixtures were not able to be separated into individual isomers by silica column chromatography prior to reduction. Reduction of methyl 8-hydroxy-11-*E/Z*-octadecen-9-ynoate (**1**) with zinc in aqueous propanol (1:1, vol/vol) and under concomitant ultrasonic irradiation furnished a 96% yield of a mixture of methyl 8-hydroxy-9*Z*,11*E*-octadecadienoate (**3a**) and methyl 8-hydroxy-9*Z*,11*Z*-octadecadienoate (**3b**) as a viscous oil in about 1:1 ratio (by comparing the intensities of the signals in the ^{13}C NMR spectrum and integration ratios of the olefinic protons in ^1H NMR spectrum). These isomers could not be separated into individual isomers by silver-ion TLC. The ^1H NMR spectrum of the mixture of isomers showed a total of seven signals in the olefinic region with a multiplet at δ_H 6.29 where an intensity equivalent to the shifts of two protons (total olefinic protons equal to eight) existed. The ^{13}C NMR spectrum also revealed seven signals with the shifts of two carbon nuclei overlapping at δ_C 125.02. The overlapping signals in the ^1H and ^{13}C NMR spectra are connected as evident from the ^1H-^{13}C correlation spectroscopy correlation spectrum (Fig. 1). In view of the fact that the NMR spectra showed eight protons and eight carbons for the olefinic system, the conclusion could be that the reduction of compound **1** produced only two geometric isomers. These isomers were methyl 8-hydroxy-9*Z*,11*E*-octadecadienoate (**3a**) and methyl 8-hydroxy-9*Z*,11*Z*-octadecadienoate (**3b**). The presence of an *E*-double bond in



$R^1 = CH_3(CH_2)_5$; $R^2 = (CH_2)_6COOCH_3$

Reagents and conditions: (i) Zn powder, 50% aqueous *n*-propanol or water, ultrasound 15 min; (ii) *m*-chloroperoxybenzoic acid, CH_2Cl_2, 12 h, 20°C; (iii) NaN_3, NH_4Cl, EtOH, H_2O, reflux, 30 min.

SCHEME 1

position C-11/C-12 of compound **3a** [partial formula: –CH_2–CH=CH–CH=CH–CH(OH)–] was confirmed by the quartet at δ_H 2.09 (13-*H*), which was shown to be correlated to the carbon signal at δ_C 32.86 (*E*-double bond, C-13). Likewise the presence of a *Z*-double bond in position C-11/C-12 of compound **3b** could be confirmed by the quartet at δ_H 2.16, which was correlated to the carbon signal at δ_C 27.45. Similar chemical shift values for methylene-interrupted olefinic fatty esters with *E/Z* configurations have been reported (10). The carbon atom bearing the hydroxyl group gave two signals in the ^{13}C NMR spectrum (δ_C 67.75 and 67.89, C-8), due to the nonstereospecific hydroxylation of C-8 as mentioned elsewhere (8).

The attempt to assign the shifts of the various protons and carbon atoms of the olefinic system of the mixture of compounds **3a** and **3b** unambiguously was difficult. By referring to the chemical shifts of the protons and carbon nuclei of the olefinic system of C_{18} conjugated dienes as described elsewhere (11), we tentatively assigned the signals for olefinic protons and carbon atoms as follows: the signal at δ_H 6.29 (2H, correlated to two overlapping carbons at δ_C 125.02) and the signal at δ_H 6.24 (*m*, 1H, correlated to δ_C 122.94) are due to shifts from 10-*H*/C-10 of compound **3b** and 11-*H*/C-11 of compounds **3a** and **3b** in view of the low-carbon shift values and the high deshielded protons; the signal at δ_H 6.02 (*t, J* = 11Hz, 1H, correlated to δ_C 130.54) is likely due to signals from 12-*H*/C-12 (*E*-double bond) of compound **3a** in view of the high *J* value; the hydroxy at C-8 is expected to induce a strong deshielding effect on the adjacent carbon nuclei, which inferred that the signals at δ_H 5.76 (connected to δ_C 137.46) are most likely the results of the shifts of 9-*H*/C-9 of compound **3b**, and the signals at δ_H 5.52/ δ_C 134.64 to 9-*H*/9-C of compound **3a**; the signal at δ_H 5.39/ δ_C 133.35 is probably due to 12-*H*/C-12 of compound **3b** and the remaining signal at δ_H 5.26/δ_C 131.39 can therefore be assigned to the shifts of 10-*H*/C-10 of compound **3a**. The mass spectral analysis of compounds **3a/3b** showed a molecular ion of $M^+ = 310$, which agrees with the structure of a hydroxy-C_{18} diene methyl ester. The fragment ions at *m/z* = 167 (relative intensity 59%) [due to the cleavage of C-7/C-8] provided evidence of the position of the hydroxy group at C-8. The peak at *m/z* = 165 (36%) is due to the fragment $CH_3(CH_2)_5CH{=}CH{-}CH{=}CH{-}C{\equiv}O^+$, and the peak at *m/z* = 171 (77%) is due to the fragment $^+O{\equiv}C(CH_2)_6COOCH_3$, which are daughter ions supporting the position of the hydroxy group at the C-8 position of the alkyl chain.

Reduction of substrate **1** was also successfully carried out in pure water as the "solvent" medium, despite the fact that the substrate was not soluble in water. The reduction reaction carried out in water gave compound **3a/3b** in 70% yield. This result indicated that reactions of hydrophobic lipid molecules could be successfully accomplished in water under ultrasound (12,13). When the reaction was repeated under similar condition but under nonultrasonic condition, the substrate was recovered.

Reduction of methyl 8-oxo-11-*E/Z*-octadecen-9-ynoate (**2**) furnished an unprecedented methylene-interrupted oxo-ene derivative (**4**, methyl 8-oxo-10*Z*-octadecenoate) as a viscous oil. The presence of a methylene group between the olefinic bond and the oxo function in this compound was evident from the doublet (*J* = 6.2 Hz) at δ_H 3.14 in the ^1H NMR spectrum and from the signal at δ_C 41.71 (C-9) in the ^{13}C NMR spectrum. The spectral data indicated that the olefinic bond was of the *Z*-configuration, as the shift of the carbon atom of the methylene group adjacent to the olefin appeared at δ_C 27.52 (C-12) and also from the shifts of the olefinic carbon atoms at δ_C 120.88 (C-10) and 133.73 (C-11). The IR spectrum showed no evidence of the presence of any *trans*-olefinic bond as no absorption was found in the region of 960 cm^{-1}. The molecular ion $M^+ = 310$ agrees with the structure of compound **4**. The peaks at *m/z* = 171 (100%, base peak) and *m/z* = 139 (17%) are fragments resulting from the cleavage of C-8/C-9. These ion fragments confirmed the position of the oxo group at C-8 of the alkyl chain of the fatty ester.

In order to prove that compound **4** was indeed a methylene-interrupted oxo-ene derivative, the unsaturated center was epoxidized. And if this epoxy system were interrupted by a methylene group from the oxo function, cyclodehydra-

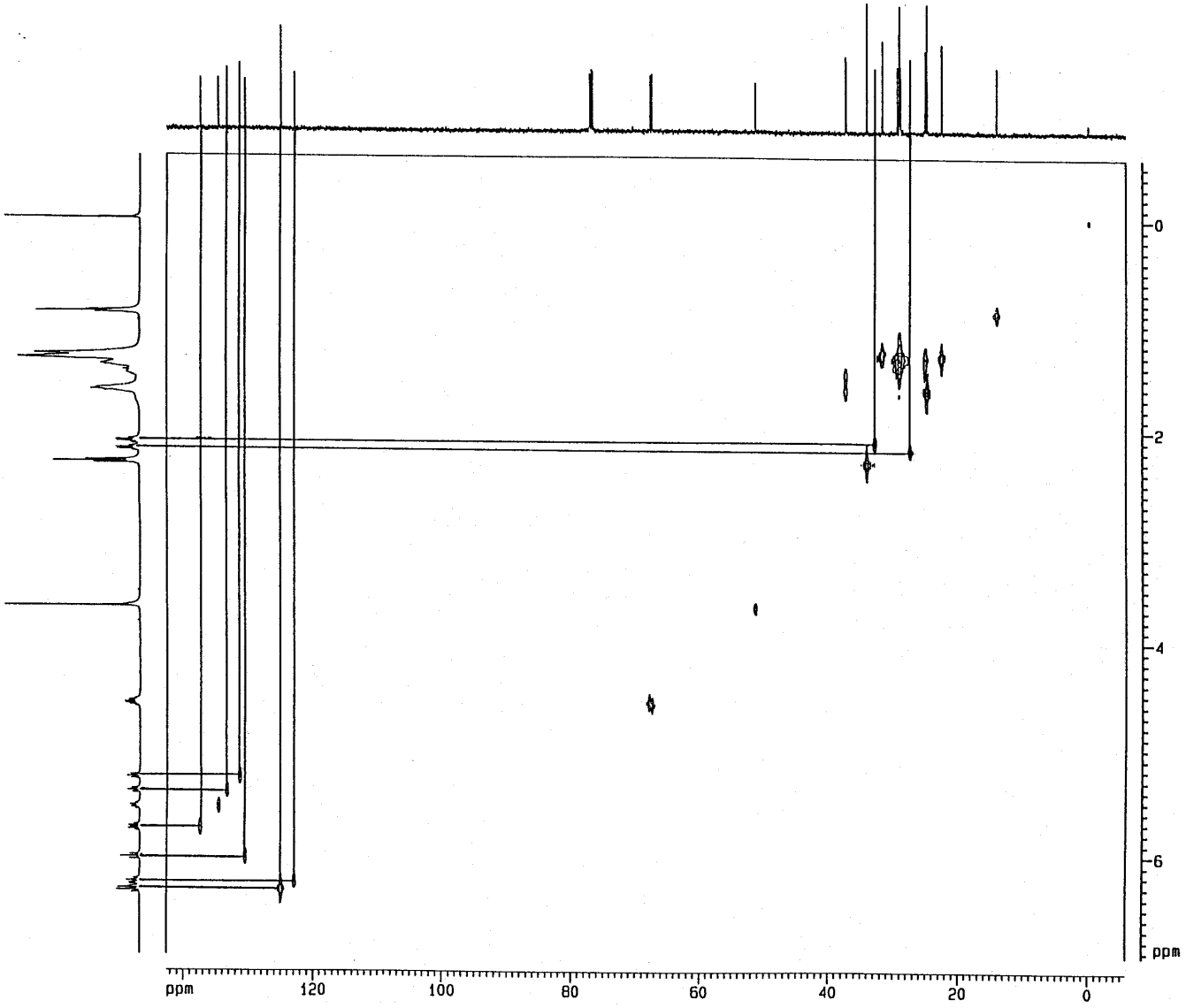


FIG. 1. Proton and carbon-13 nuclear magnetic resonance correlation spectrum of a mixture of compounds **3a** and **3b**.

tion of the epoxy-oxo system would result in the formation of a furanoid fatty ester product (14). Epoxidation of compound **4** furnished the corresponding epoxy derivative **5** as a viscous oil, which, after treatment with sodium azide in ammonium chloride, gave a 2,5-disubstituted C_{18} furanoid fatty ester (**6**, methyl 8,11-epoxy-8,10-octadecadienoate) in 70% yield as a viscous oil. The presence of the furan ring was confirmed by NMR spectroscopic analysis: δ_H 5.8 (furan ring protons) and δ_C 104.80, 104.91, 154.33, and 154.71 for the four furan ring carbon atoms. The position of the furan ring (straddling the C-8 and C-11 carbon atoms of the alkyl chain) was determined from the mass spectral fragmentation pattern, which gave a base peak at *m/z* 179 (100%) and a corresponding fragment ion *m/z* = 223. These ion fragments are characteristic of furanoid fatty esters and arise from the cleavage of the carbon-carbon β to the furan system (9,14). From these results zinc reduction of methyl 8-oxo-11-*E*/*Z*-octadecen-9-ynoate (**2**) was confirmed as furnishing methyl 8-oxo-10-*Z*-octadecenoate (**4**). Unlike the corresponding hydroxy-enynoate (compound **1**), reduction of the oxo-enynoate (compound **2**) appeared to have taken a novel and unexpected route to furnishing a single methylene-interrupted oxo-ene derivative (compound **4**).

These results indicate that zinc reduction of a conjugated oxo-enyne fatty ester furnishes a single methylene-interrupted *Z*-olefinic keto derivative, while a conjugated allylic hydroxy-enyne fatty ester gives a mixture of the corresponding hydroxy-diene isomers.

ACKNOWLEDGMENTS

We thank the Lipid Research Fund, the Research Grants Council of Hong Kong, and the Committee on Research and Conference Grants of the University of Hong Kong for financial assistance.

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[Received July 16, 1997, and in final revised form and revision accepted July 23, 1998]

Models of Antioxidant Protection Against Biological Oxidative Damage

Sir:

Among the many published works describing antioxidant protection against oxidation, there are a few that present enough data to construct a model or simulation model that brings out the relationships between the components that are measured. The research of Eder and Kirchgessner (1) and Allard *et al.* (2) are suitable for simple modeling. The applications and advantages of simple models and simulation models of biological oxidations have been described (3,4). For the research of Eder and Kirchgessner (1), two simple simulation models showing the relationships in the reactions are:

$$\text{TBARS} = \frac{\text{PUFA} \times \text{PEROX}}{\text{TOCOPHEROL} + \text{ANTIOXIDANT}} \quad [1]$$

$$\text{TBARS} = \frac{\text{PUFA} \times \text{PEROX}}{\text{LOG TOCOPHEROL}} \quad [2]$$

These models follow the basic relationship: Products of Peroxidation = Oxidizable Lipids/Antioxidants. TBARS are the thiobarbituric acid-reacting substances given in Table 2 (1). PUFA are the amounts of polyunsaturated fatty acids with three to six double bonds as given in Table 3 (1). PEROX is the peroxidizability of these PUFA. TOCOPHEROL is the amount of total tocopherol given in Table 2 (1). ANTIOXIDANT is the amount of antioxidants in addition to total tocopherol. The researchers present two bases for measuring tocopherol and TBARS, namely, dry matter and total lipid. When values of TBARS that were calculated from the models are compared to the experimental TBARS, there was good agreement. Application of regression analysis of the calculated values of TBARS vs. the experimental measurements of TBARS gives, for the first model using dry matter basis, $r = 0.79$, $P < 0.1$ and, for total lipid basis, $r = 0.89$, $P < 0.02$. Regression analysis for the second model for dry matter basis gives $r = 0.81$, $P < 0.05$ and for total lipid $r = 0.91$, $P < 0.02$.

For the research of Allard *et al.* (2), a simple simulation model giving the interrelationships between measured components is:

$$(\text{ETHANE} + \text{PENTANE}) + \text{TBARS} + \text{PEROXIDE} = \frac{(\text{PUFA} \times \text{PEROX}) + \text{CAROTENE}}{\text{LOG TOCOPHEROL} + \text{ASCORBATE} + \text{Se} \bullet \text{GSHPx}} \quad [3]$$

This model also follows the basic relationship: Products of Peroxidation = Oxidizable Lipids/Antioxidants. The terms TBARS, (PUFA × PEROX), and TOCOPHEROL are as described for the first model presented here and now using the data of Allard *et al.* (2). ETHANE + PENTANE use the combined data for breath ethane and pentane from Table 3 (2). PEROXIDE uses the data on the measurement of lipid peroxides of Table 3. An average of the four measurements (ETHANE + PENTANE) + TBARS + PEROXIDE can be defined as a lipid peroxidation index as indicated in Table 3. ASCORBATE is the amount of ascorbic acid reported in Table 1. Se · GSHPx is the activity of the enzyme selenium-glutathione peroxidase in Table 3 (2). Allard *et al.* (2) discuss some of the relationships in their data. This model uses 17 individual pieces of experimental data in an overall relationship. The lipid peroxidation indices are calculated *via* the model and compared to the indices calculated from the four pieces of experimental data of Table 3. The results are viewed in the format of the study of Allard *et al.* For the groups at week 0; menhaden oil + vitamin E, menhaden oil, olive oil + vitamin E, and olive oil, the percentages of the amounts calculated by the model compared to the amounts of the experimental lipid peroxidation indices are: 105, 110, 100, and 100%, respectively. These results show very good agreement. After the subjects had been fed the supplements for 6 wk, there were significant changes in the n-3 fatty acids and vitamin E. The same model was able to give a good fit to these main experimental data. For the groups at week 6: menhaden oil + vitamin E, menhaden oil, olive oil + vitamin E, and olive oil, the percentages of the amounts calculated by the model compared to the amounts of the experimental lipid peroxidation indices are: 91, 115, 83, and 97%, respectively. These examples of the application of simulation modeling show that modeling can be a valuable addition to many studies.

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[Received July 20, 1998; accepted August 20, 1998]

ARTICLE

Divergent Incorporation of Dietary *trans* Fatty Acids in Different Serum Lipid Fractions

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ABSTRACT: *Trans* fatty acids may be involved in atherosclerotic vascular diseases. We investigated the incorporation of dietary *trans* fatty acids and oleic acid into the serum triglycerides (TG), cholesterol esters (CE), and phospholipids (PL). Fourteen healthy female volunteers, aged 23.2 ± 3.1 yr (mean ± SD), body mass index 20.8 ± 2.1 kg/m^2 participated in this study. All subjects consumed both a *trans* fatty acid-enriched diet (TRANS diet) and an oleic acid-enriched diet (OLEIC diet) for 4 wk according to a randomized crossover design. Both experimental diet periods were preceded by consumption of a baseline diet for 2 wk which supplied 37% of total energy (E%) as fat: 18 E% from saturated fatty acids (SFA), 12 E% from monounsaturated fatty acids, and 6 E% from polyunsaturated fatty acids. Five E% of the SFA in the baseline diet was replaced by *trans* fatty acids (18:1*t* and 18:2*c,t* + 18:2*t,t,* where *c* is *cis* and *t* is *trans*) in the TRANS diet and by oleic acid (18:1n-9) in the OLEIC diet. After the TRANS diet, the proportions of 18:1*t* and 18:2*t* increased ($P < 0.001$) in all serum lipid fractions analyzed. The increase of 18:1*t* in TG and PL (1.80 ± 0.28 vs. 5.26 ± 1.40; 1.07 ± 0.34 vs. 3.39 ± 0.76 mol% of total fatty acids, respectively) was markedly higher than that in CE (0.44 ± 0.07 vs. 0.92 ± 0.26), whereas that of 18:2*t* was nearly the same in all three fractions. The proportions of palmitic, stearic, arachidonic, and eicosapentaenoic acids in TG, CE, and PL and that of oleic acid in TG and CE were decreased when compared with the baseline value. In contrast, the proportion of palmitoleic acid in TG and PL and that of linoleic acid in PL increased on the TRANS diet. After consumption of the OLEIC diet, the proportion of oleic acid increased in all three lipid fractions analyzed, and the percentage increase was nearly the same in all fractions. In contrast, the proportions of 18:1*t* in TG and PL and 18:2*t* in TG and CE decreased when compared with the baseline value. In conclusion, a moderate increase in dietary *trans* fatty acids resulted in a marked incorporation into serum lipids and decreased the conversion of linoleic acid to its more unsaturated long-chain metabolites. Analysis of 18:1*t* from serum TG and PL seems to reflect reliably the dietary intake of this fatty acid.

Lipids 33, 955–962 (1998).

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Abbreviations: CE, cholesterol ester; E%, total energy percentage; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; OLEIC diet, oleic acid-enriched diet; PL, phospholipid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglyceride; TRANS diet, *trans* fatty acid-enriched diet.

Trans fatty acids are unsaturated fatty acids that are found in the tissues and milk of ruminant animals (1). They are produced by the process of biohydrogenation of dietary polyunsaturated fatty acids (PUFA) in the rumen of ruminant animals (2). Larger amounts of *trans* fatty acids are found in certain types of margarines and shortenings. They are formed when vegetable oils, rich in PUFA, are partially hydrogenated to produce fats with better firmness and plasticity (3). Only small amounts of *trans* fatty acids occur in nature (4); e.g., milk contains 3% (range 2.7–3.4%) *trans* fatty acids of total fat. The predominant *trans* fatty acid in foodstuffs is elaidic acid (18:1Δ9*t*), and 18:1Δ10*t* and 18:1Δ11*t* are predominant isomers in partially hydrogenated oils. With a predominance at position 10, the monounsaturated fatty acids (MUFA) contain *trans* double bonds at positions 6–12. *Cis* double bonds are almost entirely at position 9 (2). Recent studies indicate that *trans* MUFA or hydrogenated fats, when substituted for oleic acid, increase low density lipoprotein and decrease high density lipoprotein cholesterol levels (5,6). In a prospective epidemiological study, a positive relationship was found between 18:1*t* intake and the risk for coronary heart disease (7); and strong positive associations between 25-yr death rates from coronary heart disease and average intake of the 18:1*t* were also reported (8). Methodological difficulties involved in the analysis of *trans* fatty acid isomers in food items have resulted in an inadequate and unreliable database to estimate dietary *trans* fatty acid intake. Analysis of the fatty acid composition of serum lipids is one approach to estimate the dietary intake of *trans* isomers. *Trans* fatty acid isomers were analyzed from serum triglycerides (TG), cholesterol esters (CE) and phospholipids (PL), total plasma, platelets, erythrocytes, and adipose tissue (6,9–13). The incorporation of *trans* fatty acids into all lipid fractions during a moderate dietary *trans* fatty acid intake was determined in animals (14), but not in humans. This study was undertaken to determine the impact of moderate *trans* fatty acid intake on the fatty acid profile of the TG, CE, and PL fractions of serum. Fatty acid profiles were assessed after the consumption of a diet in which part of the saturated fatty acids (SFA) was replaced with 18:1*t* (18:1*t*, *trans*-octadecenoate, elaidic acid) and 18:2*t* (18:2*c,t*, mono-*trans*-octadecadienoate + 18:2*t,t*, *trans*-*trans*-octadecadienoate). These data were compared to the fatty acid pattern of subjects after they consumed a diet in which

TABLE 1
Baseline Characteristics of the Study Subjects[a]

	n = 14
Age (yr)	23.2 ± 3.1
Height (cm)	165.7 ± 6.6
Weight (kg)	57.1 ± 5.6
BMI (kg/m^2)	20.8 ± 2.1
Fasting plasma glucose (mmol/L)	5.0 ± 0.3
Total cholesterol (mmol/L)	4.5 ± 0.5
Total triglycerides (mmol/L)	0.9 ± 0.3

[a]Values are means ± SD. BMI, body mass index.

part of SFA was replaced with 18:1 *cis* (18:1*c*, *cis*-octadecenoate, oleic acid).

SUBJECTS AND METHODS

Subjects. Fourteen healthy female volunteers aged 23.2 ± 3.1 yr (mean ± SD) participated in this study (Table 1). All had normal body weight with the mean body mass index of 20.8 ± 2.1 kg/m^2. Their average fasting plasma glucose was 5.0 ± 0.3 mmol/L, serum total cholesterol 4.5 ± 0.5 mmol/L, and TG 0.9 ± 0.3 mmol/L. Not one subject was taking medication known to affect lipid metabolism, and all had normal liver, kidney, and thyroid functions. Four of the study subjects used low-estrogen oral contraceptives during the study. The subjects were advised to maintain a constant level of physical activity during the study period. Subjects gave their informed consent for the study, and the study protocol was approved by the Ethics Committee of the University of Kuopio (Kuopio, Finland).

Study design. All subjects consumed both a *trans* fatty acid-enriched diet (TRANS diet) and an oleic acid-enriched diet (OLEIC diet) for 4 wk according to a randomized crossover study design. Eight of the subjects were on the TRANS diet during the first dietary period and six were on the OLEIC diet. Both experimental diet periods were preceded by the consumption of a baseline diet for 2 wk. Height, routine hematological measurements, and levels of serum creatinine, thyroxine, and liver enzymes were analyzed at the start of the study. Body weight was measured at 2-wk intervals. Samples for the determination of serum lipids and the fatty acid composition of serum lipid fractions were taken after a 12-h fast in the beginning and at the end of each diet period.

Experimental diets. Baseline diet and both experimental diets supplied 37% of total energy intake (E%) as fat, 48–50 E% as carbohydrates, and 14–16 E% as protein. The calculated fatty acid composition of the baseline diet was 16 E% from SFA, 14 E% from MUFA, and 6 E% from PUFA. During both experimental diet periods, 5 E% of SFA was replaced by either *trans* fatty acids (18:1*t* and 18:2*c,t* + 18:2*t,t*) in the TRANS diet or oleic acid (18:1n-9) in the OLEIC diet. The cholesterol content was *ca.* 300 mg/1000 kcal, and dietary fiber content *ca.* 11 g/1000 kcal in the TRANS and OLEIC diets (Table 2).

During the baseline diet, butter (17–33 g/d), olive oil (14–24 g/d), and sunflower oil (5–9 g/d) were used as main sources of fat. The absolute amounts included in the diet depended on the E% intake. The source of *trans* fatty acids in the TRANS diet was a *trans* fatty acid-enriched margarine (39–72 g/d, mean intake of 18:1*t* 8.25 ± 1.82 g/d and that of 18:2*c,t* + 18:2*t,t* 2.14 ± 0.47 g/d) specially prepared for this

TABLE 2
Composition of the Diets on the Basis of Food Records[a] During the Study

	Run-in period	TRANS diet	Washout period	OLEIC diet
Energy (kcal/d)	1838 ± 351	1818 ± 348	1873 ± 343	1828 ± 341
Carbohydrates (% of energy)	47.6 ± 3.0	46.6 ± 2.8	46.5 ± 2.6	45.6 ± 2.4
Protein (% of energy)	14.1 ± 0.6	13.9 ± 1.1	13.6 ± 0.8	14.6 ± 1.1
Fat (% of energy)	36.7 ± 3.0	36.6 ± 2.2	37.7 ± 2.0	37.9 ± 2.0
SFA (% of energy)	15.2 ± 1.1	11.0 ± 1.1	15.4 ± 1.1	10.9 ± 0.8
Palmitic acid (% of energy)	7.2 ± 0.6	5.5 ± 0.5	7.5 ± 0.4	6.0 ± 0.4
Stearic acid (% of energy)	2.3 ± 0.2	2.2 ± 0.2	2.5 ± 0.2	2.4 ± 0.2
MUFA (% of energy)[b]	13.4 ± 1.3	12.6 ± 0.9	13.9 ± 0.9	18.6 ± 0.8
Trans fatty acids (% of energy)[c,d]	—	5.1 ± 0.5[e]	—	—
PUFA (% of energy)	5.4 ± 0.6	5.2 ± 0.5	5.7 ± 0.8	5.8 ± 0.6
Linoleic acid (% of energy)	4.6 ± 0.4	4.4 ± 0.4	5.0 ± 0.7	4.8 ± 0.4
Cholesterol (mg/1000 kcal)	104 ± 18	105 ± 24	106 ± 16	107 ± 23
Fiber (g/1000 kcal)	14.1 ± 2.1	13.0 ± 2.4	12.8 ± 2.8	12.2 ± 2.8

[a]Data are based on a 4-d food record during run-in and washout periods and on a 7-d food record during the TRANS and the OLEIC diets.
[b]The intake of 18:1n-9 + n-7 during the TRANS diet was 13.1% of energy and during the OLEIC diet 17.0% of energy. Values are calculated from the fatty acid analysis of double portions, mean of seven samples.
[c]Includes 18:1*t*, 18:2*c,t*, 18:2*t,t*.
[d]The intake of *trans* fatty acids during the TRANS diet was 5.5% of energy and during the OLEIC diet 1.4% of energy. Values are calculated from the fatty acid analysis of double portions, mean of seven samples. Double portions were not collected during the run-in and washout periods.
[e]Derived from *trans* fatty acid-enriched margarine. —Not available. Values are mean ± SD. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

study. Fatty acid composition of the margarine was as follows: SFA, 16.0% of total fatty acids (palmitic acid 9.6%, stearic acid 5.0%), MUFA, 63.8% (oleic acid 41.4%, 18:1*t* 22.3%), and PUFA, 20.0% (linoleic acid 14.2%, 18:2*c,t* 2.4%, 18:2*t,t* 3%) (Raisio Group, Finland). In addition, a small amount of sunflower oil (0–1 g/d) was also included in the diet. The major source of oleic acid in the OLEIC diet was olive oil (29–51 g/d) (Raisio Group). In addition, sunflower oil (2–5 g/d) was also included in that diet. Except for the *trans* fatty acid-enriched margarine, and olive and sunflower oils, the diets were composed of common Finnish foodstuffs. The subjects received margarines, milk products, oils, and spreads free of charge.

The experimental diets were planned for six levels of energy intake: 1400, 1600, 1800, 2000, 2200, and 2400 kcal (5.9, 6.7, 7.5, 8.4, 9.2, and 10.1 MJ, respectively). The estimation of the energy requirement of the subjects was based on 3-d food records kept before the study. The subjects received both oral and detailed written instructions about the diets, specifying the amounts of individual foodstuffs by the main food groups, and they were asked, whenever possible, to weigh the food items on a digital scale before consumption. To assess dietary compliance, subjects kept 7-d food records (5 weekdays and 2 weekend days) during both experimental diet periods. During the run-in and washout diet periods, 4-d food records (3 weekdays and 1 weekend day) were kept. Nutrient intake was calculated by using the Micro-Nutrica dietary analysis program based on the database of the Finnish Social Insurance Institute. Food composition tables were based on values obtained from Finnish food analyses and values taken from international food composition tables (15). The data regarding *trans* fatty acids and oleic acid were not available in the food composition data. Therefore, two estimates are used in the results: one for *trans* fatty acid intake calculated from the intake of the *trans* fatty acid-enriched margarine, and the other for both *trans* fatty acid and oleic acid intakes calculated combining data from fatty acid analysis of double portions and food records.

Laboratory methods. Enzymatic colorimetric methods were used for the determination of cholesterol and TG from the whole serum (Monotest® Cholesterol and Triglyceride GPO-PAP; Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with an automated instrument (Kone Specific Clinical Analyzer; Kone Ltd., Espoo, Finland). The coefficient of variation between the measurements for serum total cholesterol using two different standards was 0.9% and for total TG was 0.9–1.0%. Plasma glucose was analyzed by glucose oxidase method (Glucose Auto & Stat, Model GA-110; Daiichi, Kyoto, Japan).

Analysis of fatty acid composition of serum lipids. After centrifugation of the samples at 3000 rpm at 5°C for 15 min, the plasma was transferred to plastic tubes and stored under nitrogen at −78°C until the time of analysis. The procedure applied to fatty acid analyses was described elsewhere (16). In short, in the fatty acid analysis the serum samples were extracted with chloroform/methanol (2:1, vol/vol), and the lipid fractions were separated by solid-phase extraction with aminopropyl columns. The fatty acids of serum TG, CE, and PL were transmethylated with 14% boron trifluoride in methanol and analyzed by gas chromatograph (HP 5890 Series II; Hewlett-Packard Company, Waldbronn, Germany) equipped with a fused-silica capillary column (100 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA) for analysis of *trans* fatty acids and with an HP-FFAP capillary column (25 m, 0.20 mm i.d., 0.33 μm film thickness; Hewlett-Packard, Palo Alto, CA) for analysis of other fatty acids. The molar percentage proportions of fatty acids in serum lipid fractions were calculated.

Statistical analysis. The data were analyzed with SPSS-PC (SPSS, Chicago, IL). Before further analysis, normal distribution of the variables was checked with the Kolmogorov-Smirnov test. An analysis of variance for repeated measurements was performed to assess whether there was any carryover effect. A nonparametric Wilcoxon test for paired samples was used to compare the results within diet periods and at the 4-wk time point between the TRANS and the OLEIC diets. All data are expressed as the mean ± SD.

RESULTS

Baseline characteristics of the subjects are presented in Table 1. No significant changes in body mass index occurred during the experimental diet periods (20.8 ± 1.9 vs. 20.6 ± 1.7 kg/m^2, beginning vs. end of the TRANS diet; 20.7 ± 2.0 vs. 20.6 ± 1.8 kg/m^2, beginning vs. end of the OLEIC diet). Serum total cholesterol tended to decrease in the TRANS diet (4.5 ± 0.5 vs. 4.2 ± 0.6 mmol/L, beginning vs. end, $P = 0.059$), whereas serum total TG did not change (0.9 ± 0.3 vs. 0.9 ± 0.3 mmol/L, beginning vs. end). In the OLEIC diet, both total cholesterol (4.8 ± 0.6 vs. 4.1 ± 0.6 mmol/L, $P < 0.001$, beginning vs. end) and total TG (0.9 ± 0.3 vs. 0.8 ± 0.3 mmol/L, $P = 0.020$, beginning vs. end) decreased significantly. The actual compositions of the two experimental diets and the baseline diet during run-in and washout periods are shown in Table 2. The goals of the experimental diets were well achieved. The mean energy intake remained stable during the study. The intake of protein and carbohydrates was similar during the diet periods. The OLEIC diet provided 1.3 E% more fat (nonsignificant) than the TRANS diet. The intake of PUFA was a little lower (nonsignificant) as well as that of palmitic acid ($P < 0.001$) in the TRANS diet compared to that in the OLEIC diet. The cholesterol and fiber contents of the diets were similar.

Fatty acid composition of serum lipids. The fatty acid compositions of serum TG, CE, and PL are presented in Tables 3, 4, and 5, respectively. No significant differences existed in the fatty acid composition of serum lipid fractions at baseline between the diet groups.

After consumption of the TRANS diet, both 18:1*t* and 18:2*t* were incorporated into all serum lipid fractions analyzed. The percentage increase of 18:1*t* in TG and PL was markedly higher than that in CE (197, 236, and 117%, respectively) (Fig. 1), whereas that of 18:2*t* was nearly the same in all three fractions (117, 114, and 94%, respectively).

TABLE 3
Fatty Acid Composition (mol% of total) of Serum Triglycerides During the *trans* Fatty Acid-Enriched Diet (TRANS diet) and Oleic Acid-Enriched Diet (OLEIC diet)[a]

Fatty acid	Number of weeks	TRANS diet	OLEIC diet	P[d]
14:0	0	2.58 ± 1.00	3.20 ± 1.11	
	4	2.45 ± 0.84	2.03 ± 0.72[b]	
16:0	0	26.42 ± 2.51	26.36 ± 2.00	
	4	24.24 ± 2.99[b]	24.66 ± 2.39[b]	
16:1n-7	0	5.45 ± 0.97	5.66 ± 1.13	
	4	6.16 ± 1.09[a]	5.24 ± 0.88	0.0303
18:0	0	3.10 ± 0.99	2.99 ± 0.30	
	4	2.57 ± 0.40[a]	2.62 ± 0.38[b]	
18:1n-9	0	36.99 ± 2.09	36.71 ± 2.12	
	4	35.05 ± 2.56[a]	41.13 ± 2.86[b]	0.0010
18:1n-7	0	2.25 ± 0.32	2.34 ± 0.41	
	4	2.36 ± 0.38	2.43 ± 0.35	
18:1t	0	1.80 ± 0.28	2.11 ± 0.56	
	4	5.26 ± 1.40[c]	1.59 ± 0.38[b]	0.0010
18:2n-6	0	15.70 ± 1.90	15.18 ± 1.90	
	4	16.11 ± 2.12	15.71 ± 1.86	
18:2t	0	1.00 ± 0.16	1.16 ± 0.16	
	4	2.12 ± 0.43[c]	0.96 ± 0.17[b]	0.0010
18:3n-6	0	0.30 ± 0.13	0.28 ± 0.13	
	4	0.27 ± 0.14[a]	0.30 ± 0.14	
18:3n-3	0	1.21 ± 0.28	1.09 ± 0.32	
	4	1.05 ± 0.31	1.06 ± 0.31	
20:3n-6	0	0.18 ± 0.06	0.17 ± 0.07	
	4	0.15 ± 0.06[a]	0.20 ± 0.06	0.0157
20:4n-6	0	0.89 ± 0.49	0.70 ± 0.22	
	4	0.62 ± 0.16[b]	0.68 ± 0.21	
20:5n-3	0	0.36 ± 0.24	0.27 ± 0.14	
	4	0.20 ± 0.10[a]	0.19 ± 0.08	
22:5n-3	0	0.30 ± 0.11	0.22 ± 0.10	
	4	0.23 ± 0.10	0.25 ± 0.10	
22:6n-3	0	1.49 ± 1.16	1.56 ± 1.15	
	4	1.16 ± 0.82	0.96 ± 0.48[a]	
Σn-3[1]	0	2.14 ± 1.42	2.05 ± 1.21	
	4	1.59 ± 0.88	1.40 ± 0.49[b]	
Σn-6[2]	0	1.37 ± 0.54	1.15 ± 0.34	
	4	1.04 ± 0.26[b]	1.17 ± 0.35	

[a]Values are means ± SD. Superscript letters a, b, c indicate a significant difference within diet periods, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively, and d indicates a significant difference between TRANS and OLEIC diets after the 4-wk time point; [1]without 18:3n-3; [2]without 18:2n-6.

TABLE 4
Fatty Acid Composition of Serum Cholesterol Esters (mol% of total) During *trans* Fatty Acid-Enriched Diet (TRANS diet) and Oleic Acid-Enriched Diet (OLEIC diet)[a]

Fatty acid	Number of weeks	TRANS diet	OLEIC diet	P[d]
14:0	0	0.94 ± 0.15	1.03 ± 0.18	
	4	0.85 ± 0.23	0.76 ± 0.18[b]	0.0413
16:0	0	11.97 ± 0.59	11.78 ± 0.63	
	4	11.43 ± 0.82[a]	11.34 ± 0.48[b]	
16:1n-7	0	3.48 ± 0.58	3.47 ± 0.59	
	4	3.36 ± 0.60	3.41 ± 0.75	
18:0	0	0.87 ± 0.20	0.92 ± 0.22	
	4	0.76 ± 0.18[a]	0.78 ± 0.15[a]	
18:1n-9	0	18.50 ± 1.57	18.10 ± 1.42	
	4	17.54 ± 1.68[a]	21.48 ± 2.28[b]	0.0015
18:1n-7	0	1.19 ± 0.14	1.15 ± 0.11	
	4	1.17 ± 0.11	1.16 ± 0.16	
18:1t	0	0.44 ± 0.07	0.44 ± 0.07	
	4	0.92 ± 0.26[c]	0.43 ± 0.06	0.0010
18:2n-6	0	54.00 ± 2.04	54.97 ± 2.26	
	4	55.63 ± 2.69	52.89 ± 3.13[b]	0.0035
18:2t	0	0.95 ± 0.12	1.02 ± 0.12	
	4	1.82 ± 0.31[c]	0.90 ± 0.08[b]	0.0010
18:3n-6	0	0.38 ± 0.18	0.42 ± 0.20	
	4	0.36 ± 0.16	0.55 ± 0.38[a]	0.0052
18:3n-3	0	0.60 ± 0.12	0.57 ± 0.15	
	4	0.52 ± 0.16[b]	0.54 ± 0.15	
20:3n-6	0	0.42 ± 0.15	0.44 ± 0.16	
	4	0.34 ± 0.12[c]	0.46 ± 0.20	0.0010
20:4n-6	0	4.34 ± 1.12	4.17 ± 1.02	
	4	4.05 ± 1.00[a]	4.13 ± 1.03	
20:5n-3	0	1.26 ± 0.99	0.88 ± 0.46	
	4	0.64 ± 0.40[a]	0.65 ± 0.34[a]	
22:6n-3	0	0.67 ± 0.28	0.64 ± 0.23	
	4	0.61 ± 0.23	0.54 ± 0.17[b]	0.0186
Σn-3[1]	0	1.93 ± 1.16	1.52 ± 0.63	
	4	1.25 ± 0.62[a]	1.19 ± 0.47[a]	
Σn-6[2]	0	5.14 ± 1.28	5.04 ± 1.25	
	4	4.74 ± 1.17[a]	5.14 ± 1.34	0.0186

[a]See Table 3 footnote.

The quantitative distribution of 18:1*t* and 18:2*t* to different serum lipid fractions during TRANS diet is presented in Table 6. The amount of 18:1*t* increased mostly in TG and PL while the greatest increase of 18:2*t* was seen in CE. The ratio of 18:1*t* to 18:2*t* in TRANS diet was about 3.9. After 4 wk on the diet, this ratio was 4.18 ± 1.07 in PL, 2.48 ± 0.30 in TG, and only 0.52 ± 0.19 in CE.

After 4 wk on the TRANS diet, the proportions of palmitic, stearic, and oleic acids in serum TG were decreased when compared with the baseline value (Table 3). The proportions of γ-linolenic, dihomo-γ-linolenic and arachidonic acids from n-6 PUFA and the proportion of eicosapentaenoic acid (EPA) from n-3 PUFA series decreased in TG. In contrast, the proportion of palmitoleic acid in TG increased on the TRANS diet when compared with the baseline value.

Similar changes were also found in the serum CE and PL (Tables 4 and 5) after consumption of the TRANS diet. The proportions of palmitic, stearic, arachidonic, and α-linolenic acids and of EPA decreased in CE and PL. Furthermore, the proportion of dihomo-γ-linolenic acid in CE decreased, and the proportions of palmitoleic and linoleic acids in PL increased on the TRANS diet when compared with the baseline value.

After consumption of the OLEIC diet, the proportion of oleic acid increased in all three lipid fractions analyzed, and the percentage increase was nearly the same in all fractions. In contrast, the proportions of 18:1*t* in TG and PL and 18:2*t* in TG and CE decreased on the OLEIC diet when compared with the baseline value (Fig. 1, Tables 3–5).

The 4-wk TRANS diet elevated most the quantitative amounts of 18:1*t* in PL (10.8 ± 3.3 vs. 32.6 ± 6.1) and TG (10.7 ± 3.4 vs. 31.2 ± 13.4), and the amount of 18:2*t* in CE (8.6 ± 1.2 vs. 16.1 ± 3.7) and TG (5.9 ± 1.8 vs. 12.7 ± 5.3). The OLEIC diet induced the most marked increase of oleic acid in CE (168.6 ± 22.8 vs. 178.7 ± 24.6) and PL (103.1 ±

TABLE 5
Fatty Acid Composition of Serum Phospholipids (mol% of total) During *trans* Fatty Acid-Enriched Diet (TRANS diet) and Oleic Acid-Enriched Diet (OLEIC diet)[a]

Fatty acid	Number of weeks	TRANS diet	OLEIC diet	P[d]
14:0	0	0.73 ± 0.27	0.76 ± 0.15	
	4	0.63 ± 0.14	0.57 ± 0.08[b]	
16:0	0	31.99 ± 2.21	32.35 ± 1.80	
	4	31.09 ± 2.37[a]	31.94 ± 1.80	
16:1n-7	0	0.98 ± 0.48	0.93 ± 0.23	
	4	1.12 ± 0.23[a]	0.88 ± 0.15	0.0052
18:0	0	11.28 ± 1.47	11.36 ± 1.34	
	4	10.07 ± 1.30[b]	11.30 ± 1.33	0.0023
18:1n-9	0	10.13 ± 2.04	9.81 ± 0.90	
	4	9.60 ± 0.98	11.61 ± 1.21[c]	0.0010
18:1n-7	0	1.46 ± 0.16	1.43 ± 0.12	
	4	1.45 ± 0.15	1.51 ± 0.21	
18:1t	0	1.07 ± 0.34	1.05 ± 0.22	
	4	3.39 ± 0.76[c]	0.88 ± 0.26[a]	0.0010
18:2n-6	0	23.43 ± 2.32	24.36 ± 1.95	
	4	24.87 ± 1.93[a]	23.70 ± 2.37	
18:2t	0	0.42 ± 0.19	0.39 ± 0.06	
	4	0.84 ± 1.56[b]	0.37 ± 0.10	0.0010
18:3n-6	0	0.16 ± 0.12	0.12 ± 0.04	
	4	0.12 ± 0.04	0.14 ± 0.03	
18:3n-3	0	0.28 ± 0.19	0.23 ± 0.07	
	4	0.20 ± 0.05[a]	0.21 ± 0.06	
20:3n-6	0	2.56 ± 0.83	2.82 ± 1.01	
	4	2.35 ± 0.73	2.91 ± 1.07	0.0063
20:4n-6	0	6.03 ± 1.38	5.75 ± 1.22	
	4	5.73 ± 1.05[a]	5.84 ± 1.14	
20:5n-3	0	1.36 ± 1.11	1.01 ± 0.51	
	4	0.70 ± 0.39[a]	0.72 ± 0.35[a]	
22:4n-6	0	0.12 ± 0.03	0.12 ± 0.03	
	4	0.14 ± 0.04	0.11 ± 0.04	0.0035
22:5n-3	0	0.60 ± 0.21	0.56 ± 0.20	
	4	0.54 ± 0.20	0.49 ± 0.18[b]	0.0043
22:6n-3	0	4.22 ± 1.24	3.88 ± 0.87	
	4	4.07 ± 1.14	3.69 ± 0.78	
24:0	0	0.67 ± 0.13	0.69 ± 0.11	
	4	0.67 ± 0.10	0.70 ± 0.13	
24:1n-9	0	1.58 ± 0.31	1.54 ± 0.31	
	4	1.29 ± 0.21[b]	1.54 ± 0.31	0.0052
Σn-3[1]	0	6.18 ± 2.11	5.44 ± 1.41	
	4	5.31 ± 1.63	4.91 ± 1.11	
Σn-6[2]	0	8.88 ± 1.91	8.82 ± 1.96	
	4	8.34 ± 1.58[a]	9.00 ± 1.93	0.0258

[a]See Table 3 footnote.

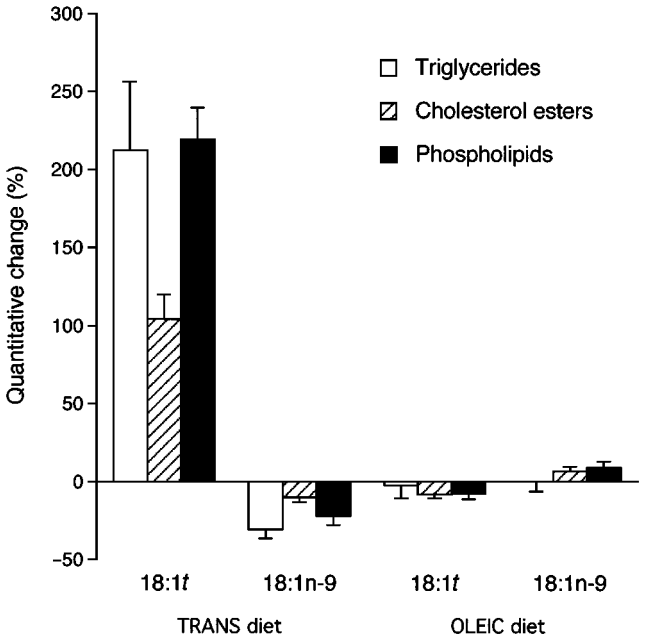

FIG. 1. Percentage changes of quantitative amounts from baseline after consumption of the TRANS and OLEIC diets for 18:1*t* and 18:1n-9, where *t* is *trans.*

16.9 vs. 111.7 ± 20.7). Notably, the average quantitative change of oleic acid in TG was negative (221.2 ± 68.2 vs. 220.2 ± 79.0).

After 4 wk on the OLEIC diet, the proportions of all SFA (myristic, palmitic, and stearic) decreased in TG and CE but only that of myristic acid in PL. In addition, the OLEIC diet led to the decreased proportions of docosahexaenoic acid in TG, linoleic acid and EPA in CE, and EPA and docosapentaenoic acid in PL. In CE an increased proportion of γ-linolenic acid was observed (Tables 3–5).

The proportions of both 18:1*t* and 18:2*t* in all three lipid fractions were higher after the TRANS diet when compared with those after the OLEIC diet. By contrast, the proportion of oleic acid was higher in all three fractions after the OLEIC diet when compared with that after the TRANS diet.

In general, the level of n-3 fatty acids in each of the lipid fractions assessed decreased after the subjects changed from the baseline to TRANS or OLEIC diets. In some cases these differences reached statistical significance.

The value of the sum of n-6 fatty acids (excluding linoleic acid) decreased in all lipid fractions on the TRANS diet compared to the baseline level, while this value remained unchanged on the OLEIC diet. The sums of n-6 fatty acids in CE and PL were lower at the end of the TRANS diet as compared with those at the end of the OLEIC diet. The proportion of dihomo-γ-linolenic acid in all lipid fractions was lower at the end of the TRANS diet when compared with the OLEIC diet.

The proportion of myristic acid in CE was higher and that of stearic acid in PL lower after consumption of the TRANS diet when compared with those at the end of the OLEIC diet. Of MUFA, the proportion of palmitoleic acid in TG and PL was higher after the TRANS diet than after the OLEIC diet. The proportion of linoleic acid in CE was higher, but that of α-linolenic acid lower after the TRANS diet.

DISCUSSION

In the present study the main dietary *trans* fatty acid, 18:1*t,* was preferentially incorporated in serum TG and PL, but to a lesser degree in CE, whereas dietary 18:2*t* induced a similar increase in all serum lipid fractions among young healthy women after 4 wk of moderate dietary intake of *trans* fatty

acids. The increase in the proportion of 18:1*t* was *ca.* threefold in serum TG and PL and twofold in CE. Also quantitative increases of 18:1*t* were highest in serum TG and PL. In contrast, the increase in the proportion of 18:2t was twofold, and quantitative increases were nearly the same in all lipid fractions reflecting identical incorporation of 18:2t in serum TG, CE, and PL.

The intake of *trans* fatty acids during the TRANS diet was *ca.* five times higher (5 E%) than the amount of *trans* fatty acids in the habitual Finnish diet (about 1 E%, that means 1.7 g/person/day and 1.9% of the total fat consumption), the main source (77%) being edible fats (17). Habitual diet includes *ca.* 80% of the dietary *trans* fatty acids as 18:1 isomer and 20% as 18:2 isomers (17). In the present study the *trans* fatty acid-enriched margarine consisted of *ca.* 80% *trans* fatty acid 18:1 isomer and *ca.* 20% *trans* fatty acid 18:2 isomers from all *trans* fatty acids, the proportion identical to the Finnish diet.

In some previous studies the incorporation of dietary *trans* fatty acids was analyzed in total plasma or serum lipids (9,18,19) or in serum TG (10,11,20), CE (12) or PL (11), in platelet PL (10,20), and erythrocytes (21), and in serum TG, CE, and PL in animal study (14). The *trans* fatty acid composition data of serum or plasma TG, CE, and PL of mothers, their infants and children, and estimated dietary *trans* fatty acid intake have been reported (22). In the present study the change from about 1 to 5 E% achieved an increase in the proportion of serum TG and PL and also in CE. The relative levels of incorporation of 18:1*t* into TG, PL, and CE are consistent with the isotope tracer data reported earlier (6). In rabbits, 18:1*t* from diet was incorporated into the fatty acids of serum TG, CE and PL, although the levels of incorporation were considerably under the level of *trans* fatty acid in the diet (14). Mensink and Hornstra (10) reported decreased proportion of 18:1*t* in serum TG from 3.5 ± 0.1 to 2.8 ± 0.1% when habitual intake of total *trans* fatty acids decreased from 4.7 ± 0.3 to 2.1 ± 0.2 E%.

Serum TG tends to reflect the fatty acid composition of the most recent meals, and the variation in the *trans* fatty acids for TG is larger than for other lipid fractions. Nestel and coworkers (18) reported a sevenfold increase in plasma elaidic acid concentration with the *trans* fatty acid diet (7 E%) during 11 wk in 27 mildly hypercholesterolemic men. However, total plasma lipid data are not considered a valid biomarker estimating dietary *trans* fatty acid intake over the long term. In contrast, serum PL reflects the habitual dietary intake over a period of several weeks, and according to the present results it would be one biomarker of dietary *trans* fatty acid intake in the long term. The 18:1*t* to 18:2*t* ratio was also closest to that in diet in PL.

Trans fatty acids behave like SFA, and they are preferentially incorporated into the 1-position in PL replacing SFA. In the present study, after consumption of 5 E% TRANS diet, the proportions of palmitic, stearic, and SFA decreased not only in PL but in TG and CE, as well, which indirectly supports the view of replacements. Oleic acid, but not 18:1*t*, competes with linoleic acid for incorporation into the 2-position of PL. Thus, on the TRANS diet a greater incorporation of linoleic acid into PL would be expected. This was, in fact, observed in our study subjects as in an earlier animal study (14), where after *trans* fatty acid feeding increased the amount of linoleic acid in CE and PL, but was found unchanged in TG when compared to 18:1 *cis* diet.

Our results suggest that *trans* fatty acids may be involved in the metabolism of n-6 fatty acids as a result of a reduced conversion of linoleic acid to its long-chain polyunsaturated metabolites (γ-linolenic, dihomo-γ-linolenic, and arachidonic acids) observed in the subjects fed the TRANS diet. These findings are compatible with inhibition of arachidonic acid formation by *trans* fatty acids as a result of inhibition of desaturase activity (23–27). Another explanation for the reduced proportion of n-6 metabolites is the selective incorporation of linoleic acid into PL. These changes could potentially result in the preservation of an unsaturated *cis* double bond-containing fatty acid in the 2-position of PL favoring lecithin cholesterol acyltransferase activity. In the present study the proportion of arachidonic acid decreased in all lipid fractions. In contrast, Wood and coworkers (19) noticed higher level of arachidonic acid in total serum lipids after *trans* diet (5.5 E% from *trans* fatty acids). This issue still needs to be resolved.

The proportion of n-3 fatty acids tended to decrease after subjects were switched from the baseline to either the TRANS or OLEIC diet. These findings suggest a shift in the fatty acid profile of the diet, and the dramatic alteration of the *trans* or MUFA content of the diet at the expense of n-3 fatty acids will be reflected in the fatty acid profile at all lipid fractions.

The proportion of oleic acid increased significantly in all lipid fractions of our study subjects reflecting good adherence to the OLEIC diet. Fatty acid compositions of serum TG and PL are considered good biomarkers of oleic acid intake because oleic acid predominates in TG (13). Our results support this finding although the percentile increase of oleic acid in all

TABLE 6
Total Serum Cholesterol and Triglycerides (mmol/L) and Quantitative Amounts (μg/mL) of 18:1*t* and 18:2*t* in the Serum Triglycerides, Cholesterol Esters and Phospholipids, and Changes from Baseline After Consumption of the TRANS Diet[a]

	Total cholesterol	Total triglycerides	Triglycerides		Cholesterol esters		Phospholipids	
			18:1*t*	18:2*t*	18:1*t*	18:2*t*	18:1*t*	18:2*t*
0 wk	4.5 ± 0.5	0.9 ± 0.3	10.69 ± 3.39	5.86 ± 1.80	4.00 ± 0.68	8.59 ± 1.24	10.78 ± 3.28	4.25 ± 1.78
4 wk	4.2 ± 0.6	0.9 ± 0.3	31.16 ± 13.44	12.72 ± 5.34	7.89 ± 1.32	16.07 ± 3.72	32.64 ± 6.07	8.14 ± 2.01
Change	0.2 ± 0.4	–0.0 ± 0.2	20.48 ± 13.36[c]	6.86 ± 5.34[c]	3.89 ± 1.70[c]	7.49 ± 3.78[c]	21.86 ± 6.84[c]	3.89 ± 2.54[c]

[a]See Table 3 footnote.

lipid fractions was relatively small, a finding consistent with previous studies (28). In the present study, the 5 E% increase in oleic acid intake induced a 12–18% increase of oleic acid in lipid fractions. Also Valsta *et al.* (29) reported about 7% increase in the proportion of oleic acid in serum TG, although the dietary intake of oleic acid (from olive oil) increased by 47%. Increased proportions of oleic acid in TG (30) and CE (31) were seen after oleic acid-enriched diets while Sarkkinen and coworkers (32) did not find any increase in the proportion of oleic acid in serum CE, erythrocyte, or platelet membranes when the increase of monoene intake was rather low.

The most plausible explanation for the decrease of 18:1*t* and 18:2*t* in serum lipid fractions after consumption of the OLEIC diet in the present study is the decreased intake since the amount of butter was smaller in the OLEIC diet than the baseline diet. Also the medium-fat milk products of the baseline diet were replaced by low-fat milk products in the OLEIC diet.

In conclusion, a moderate increase in dietary *trans* fatty acids results in a marked incorporation into serum TG and PL, and to a lesser degree in CE. In addition, our results suggest that 5 E% *trans* fatty acids in diet decrease the conversion of linoleic acid to its more unsaturated long-chain metabolites. Analysis of 18:1*t* from serum TG and PL seems to reflect reliably the dietary intake of this fatty acid.

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[Received May 19, 1998; and in final revised form and accepted August 26, 1998]

Identification of Conjugated Linoleic Acid Isomers in Cheese by Gas Chromatography, Silver Ion High Performance Liquid Chromatography and Mass Spectral Reconstructed Ion Profiles. Comparison of Chromatographic Elution Sequences

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ABSTRACT: Commercial cheese products were analyzed for their composition and content of conjugated linoleic acid (CLA) isomers. The total lipids were extracted from cheese using petroleum ether/diethyl ether and methylated using $NaOCH_3$. The fatty acid methyl esters (FAME) were separated by gas chromatography (GC), using a 100-m polar capillary column, into nine minor peaks besides that of the major rumenic acid, 9*c*,11*t*-octadecadienoic acid (18:2), and were attributed to 19 CLA isomers. By using silver ion–high performance liquid chromatography (Ag^+–HPLC), CLA isomers were resolved into seven *trans,trans* (5–9%), three *cis/trans* (10–13%), and five *cis,cis* (<1%) peaks, totaling 15, in addition to that of the 9*c*,11*t*-18:2 (78–84%). The FAME of total cheese lipids were fractionated by semipreparative Ag^+–HPLC and converted to their 4,4-dimethyloxazoline derivatives after hydrolysis to free fatty acids. The geometrical configuration of the CLA isomers was confirmed by GC–direct deposition–Fourier transform infrared, and their double bond positions were established by GC-electron ionization mass spectrometry. Reconstructed mass spectral ion profiles of the $m + 2$ allylic ion and the $m + 3$ ion (where m is the position of the second double bond in the parent conjugated fatty acid) were used to identify the minor CLA isomers in cheese. Cheese contained 7*t*,9*c*-18:2 and the previously unreported 11*t*,13*c*-18:2 and 12*c*,14*t*-18:2, and their *trans,trans* and *cis,cis* geometric isomers. Minor amounts of 8,10-, and 10,12-18:2 were also found. The predicted elution orders of the different CLA isomers on long polar capillary GC and Ag^+–HPLC columns are also presented.

Lipids 33, 963–971 (1998).

Conjugated linoleic acid (CLA) has been reported to lead to reduced carcinogenesis (1–5) and atherosclerosis (6,7), increased bone mass (8) and muscle mass (9–11), and to have antidiabetic properties (12) in laboratory animals. The term CLA refers to a mixture of positional and geometric conjugated octadecadienoic acid (18:2) isomers. The active isomer has been assumed to be 9*c*,11*t*-18:2 (also called rumenic acid) because it is the major CLA isomer present in milk (13–20), cheese (15,21–27), and meat (14,15,28) from ruminant animals. It is formed as an intermediate in the biohydrogenation of linoleic acid in the rumen (29–31), and possibly by Δ9 desaturation of vaccenic acid (11*t*-18:1) (32).

Besides the major 9*c*,11*t*-18:2 established by Parodi (13), there are a number of minor CLA isomers in cheese and milk. To date, their reported identities and levels remain questionable, because of inadequate chromatographic separations and confirmatory methods used to establish the double bond positions and configurations. Ha *et al.* (21) were the first to report six additional CLA peaks in cheese separated on a 60-m Supelcowax-10 gas chromatographic capillary column. Based on comparisons of gas chromatographic equivalent chain lengths with published data (33), they reported the presence of 10*c*,12*t*-18:2, 10*t*,12*c*-18:2, 11*c*,13*c*-18:2, 9*c*,11*c*-18:2, 10*c*,12*c*-18:2, 9*t*,11*t*-18:2, and 10*t*,12*t*-18:2 as fatty acid methyl esters (FAME) after BF_3 methylation. They determined the molecular weight of the CLA isomers by gas chromatography (GC)–chemical ionization mass spectrometry (MS), but were unable to identify CLA positional isomers by GC–MS using 4-phenyl-1,2,4-triazoline-3,5-dione derivatives. Nevertheless, their BF_3 methylation procedure, GC separation conditions, and equivalent chain length comparisons were subsequently used by many investigators (17,23,24,34), with variation in the methylation catalyst, i.e., HCl (15,22), tetramethylguanidine (25), or $NaOCH_3$ (35). Acid (BF_3 or HCl)-catalyzed methylations (8,15,17,21–24,34) were shown to lead to isomerization of *cis/trans* to *trans,trans* CLA isomers and the formation of methoxy artifacts (19), as well as CLA artifacts from allylic hydroxy fatty acids (36). Acid fat extraction procedures (22,25) may also lead to CLA isomer-

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Abbreviations: *c*; *cis*; *cis/trans*, refers to the same positional isomers that have either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC–DD–FTIR, gas chromatography–direct deposition–Fourier transform infrared; GC–EIMS, gas chromatography–electron ionization mass spectrometry; HPLC, high performance liquid chromatograph(y); *t, trans*.

ization. On the other hand, base-catalyzed methylation did not isomerize CLA isomers nor produce methoxy artifacts (19). The GC resolution of CLA isomers was improved by using 100-m polar capillary columns under optimal conditions, which resulted in the separation of 10 GC peaks attributed to CLA isomers (19,37).

Recently, Lavillonnière *et al.* (26) reported the identity of five additional minor CLA isomers in cheese (8*c*,10*t*-18:2, 8*c*,10*c*-18:2, 8*t*,10*t*-18:2, 11*t*,13*t*-18:2, and 11?,13?-18:2). They methylated cheese lipids with $NaOCH_3$, then partially reduced the isolated CLA mixture with hydrazine, separated the resultant monounsaturated (18:1) FAME by silver nitrate thin-layer chromatography, and analyzed them by GC. The positional 18:1 isomers were identified as 4,4-dimethyloxazoline (DMOX) derivatives by GC–electron ionization mass spectrometry (GC–EIMS). These authors also evaluated the total CLA DMOX mixture by GC–EIMS.

We have just reported the separation of 12 CLA isomers as their FAME from a commercial CLA mixture by using silver ion high-performance liquid chromatography (Ag^+–HPLC) based on their double-bond geometry (*trans,trans*, *cis/trans,* and *cis,cis*) and position (8,10-, 9,11-, 10,12-, and 11,13-18:2) (27). The Ag^+–HPLC method, together with reconstructed GC–EIMS ion profiles of isomer-specific ions for CLA DMOX derivatives, permitted us to identify the previously unrecognized and abundant minor isomer, 7*t*,9*c*-18:2, in milk, cheese, beef, and human milk and adipose tissue (38).

In the present communication, we report the accurate identification and composition of CLA isomers in cheese, including for the first time the previously unreported 11*t*,13*c*- and 12*c*,14*t*-18:2 isomers. Cheese lipids were fractionated by semipreparative Ag^+–HPLC, and the minor CLA isomers were identified by GC–EIMS and GC–direct deposition-Fourier transform infrared (GC–DD–FTIR) spectrometry.

MATERIALS AND METHODS

A mixture of CLA FAME was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Pure CLA isomers (9*c*,11*t*-18:2, 10*t*,12*c*-18:2, 9*c*,11*c*-18:2, and 9*t*,11*t*-18:2) were obtained as free fatty acids from Matreya Inc. (Pleasant Gap, PA). Acetonitrile and hexane were ultraviolet grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol (95%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from TCI America (Portland, OR). The cheeses were purchased locally.

Lipid extraction. Cheese was extracted using a published procedure (39). Briefly, 20 g cheese, 2 g potassium oxalate, and 100 mL ethyl alcohol were placed in a blender jar and homogenized for 3 min. The jar contents were poured into a 250-mL centrifuge tube, to which 50 mL diethyl ether and 50 mL petroleum ether were added. The contents were mixed well for 0.5 min after each addition of solvent. The mixture was centrifuged at 1700 rpm for 7 min at room temperature. The lower phase was reextracted two more times with 50 mL petroleum ether and diethyl ether (1:1, vol/vol). The combined organic phases were transferred into a 1000-mL separatory funnel containing 500 mL distilled water and 30 mL saturated sodium chloride. The combined organic extract was washed two more times with 100 mL distilled water. Any emulsion formed was broken up by the addition of saturated sodium chloride (2–5 mL), and allowed to stand for 30 min. The combined organic layer was poured through a glass column (2.5 cm × 50 cm) containing a bed of anhydrous sodium sulfate (15 cm). The organic solvent was removed using a rotary evaporator at 37°C and the total lipids were determined gravimetrically.

Preparation of FAME. Free fatty acids of CLA were dissolved in 1 mL of 20% methanol/benzene, placed in a reaction tube fitted with a Teflon-lined screw cap, and reacted with trimethylsilyldiazomethane for 30 min at room temperature (40). Unreacted trimethylsilyldiazomethane was destroyed by adding acetic acid with gentle swirling until the yellow color disappeared. After addition of H_2O (5 mL), 1 mL isooctane was added to extract the FAME which were subsequently dried over Na_2SO_4.

Portions of total lipids from cheese (20–70 mg) were placed into 15-mL reaction tubes fitted with a Teflon-lined screw cap. One milliliter of benzene was added to dissolve the lipids, followed by 4 mL anhydrous $NaOCH_3$. The tubes were flushed with nitrogen, then heated at 50°C for 10 min with occasional shaking. After completion of methylation, 0.2 mL of water was added, and the FAME were extracted with hexane, dried over Na_2SO_4, and analyzed directly by GC and HPLC.

HPLC. The HPLC (Waters 510 solvent delivery system; Waters Associates, Milford, MA) was equipped with an autosampler and 2-mL injection loop for the semipreparatory column and a 200-µL injection loop for the analytical column (Waters 717), an ultraviolet detector (Waters 486 tunable absorbance), a fraction collector (Waters LR 76413), and an operating system (Waters Millennium^{TM} version 2.15.01). Both ChromSpher 5 Lipids semipreparative (10 mm i.d. × 250 mm stainless steel; 5 µm particle size) and an analytical (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size) silver-impregnated column were used (Chrompack, Bridgewater, NJ). CLA isomers were measured at 233 nm. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically. The solvent flows were 4.0 and 1.0 mL/min, for the semipreparative and analytical columns, respectively. The flow was commenced for 0.5 h prior to sample injection. For solution containing about 20 mg/mL, typical injection volumes were 150–200 µL for the semipreparative column and 5–15 µL for the analytical column. Whenever necessary, the column was restored by flushing with 1% acetonitrile in hexane for 4 h followed by 0.5 h with 0.1% acetonitrile in hexane.

DMOX derivatives. In a reaction tube, 10–20 mg of methyl heptadecanoate (17:0) and *ca.* 1–2 mg of CLA FAME were added, and the mixture was hydrolyzed with 1 N KOH/95% ethanol at 50°C for 30 min. The free fatty acids were extracted with petroleum ether after the reaction was neutral-

ized with HCl. The free fatty acids were added to a screw-cap reaction tube (1 mL) and a threefold excess of 2-amino-2-methyl-1-propanol (w/w) was added. The tube was purged with argon, capped, and heated at 170°C for 0.5 h in an oven. The reaction mixture was transferred into a 250-mL separatory funnel containing 40 mL of petroleum ether and 50 mL of water and shaken vigorously. Saturated NaCl was added to break the emulsion. The aqueous layer was removed, and the petroleum ether was rewashed with water, dried over anhydrous Na_2SO_4 and concentrated to the desired volume under a stream of argon (36).

GC. The cheese FAME were analyzed by GC (model 5890; Hewlett-Packard, Palo Alto, CA) using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.2 µm film thickness; Chrompack Inc.). The column was held at 70°C for 4 min after injection, temperature-programmed at 13°C/min to 175°C, held there for 27 min, then temperature-programmed at 4°C/min to 215°C, and held there for 31 min. Hydrogen was the carrier gas, at a split ratio of 20:1.

GC–EIMS. The GC–EIMS was performed using a GC (Hewlett-Packard 5890, series II) coupled to a mass spectrometer (Autospec Q mass spectrometer) and a data system (OPUS 4000; Micromass, Manchester, United Kingdom). The GC–EIMS system utilized version 2.1 BX software. This system was used with a 50-m CP-Sil 88 capillary column described previously (41). The GC–EIMS conditions were: splitless injection with helium sweep restored 1 min after injection; injector and transfer lines' temperature 220°C; oven temperature was 75°C for 1 min after injection, then temperature-programmed 20°C/min to 185°C, held there for 15 min, then temperature-programmed 4°C/min to 220°C, and held there for 45 min.

GC–DD–FTIR. A Bio-Rad (Cambridge, MA) Tracer™ GC–FTIR 60A spectrometer system was used. This system was used with a 50 m CP-Sil 88 capillary column (42).

RESULTS AND DISCUSSION

Fat extraction from cheese. The total fat was extracted from cheese (39). In this method, potassium oxalate served as a grinding agent and formed calcium oxalate, which prevented calcium ions from causing emulsions. The total fat content of 11 commercial cheese products ranged from 14 to 37% (Table 1). For the extraction of fats and oils, acid conditions were avoided because they may lead to isomerization of the *cis/trans* to *trans,trans* CLA isomers. In a previous study (22) preliminary acid digestion may have contributed to the low content of 9*c*,11*t*-18:2, 48–68% (22), instead of 78–84% reported for selected cheese products (Table 1).

Methylation. The total lipids from cheese were methylated using $NaOCH_3$ to avoid any isomerization of conjugated dienes and formation of methoxy and CLA artifacts. Acid-catalyzed methylation using either BF_3, HCl or H_2SO_4 would lead to conversion of *cis/trans* to *trans,trans* CLA isomers. Reducing the temperature during methylation reduced the extent of this conversion (17,23,43), but the methylation, particularly of phospholipids, may not be complete (19). Therefore, to avoid isomerization and incomplete methylation of CLA, $NaOCH_3$ is recommended. If test samples contained significant amounts of free fatty acids, alkali hydrolysis followed by subsequent methylation using tetramethylsilyldiazomethane (40) was carried out and checked by thin-layer chromatography (19), and these steps are recommended. However, neither of these two methods will methylate sphingomyelin, since the *N*-acyl bond is resistant to alkali. In the present cheese study, we ignored the small contribution of CLA from sphingomyelin because its level in cheese was less than 0.1%, and the occurrence of CLA in sphingomyelin is generally the lowest compared to all the other phospholipids (37).

GC. The CLA region of the FAME GC trace for cheese (Fig. 1) was a complex mixture of 10 peaks attributed to many

TABLE 1
Lipid Content and Composition of Conjugated Linoleic Acid (CLA) Isomers in Commercial Cheeses[a]

Types of cheese	Lipid content[b] (%)	Total CLA[c] (%)	CLA isomers (%)[d] 9*c*,11*t*	7*t*,9*c* 8*t*,10*c*	11*t*,13*c*[e]	12*c*,14*t*	Total *t*,*t*
American processed cheese	16.5	0.46	79.35	10.17	1.71	0.67	8.11
Cheddar, sharp	29.5	0.54	82.58	8.31	2.14	0.61	6.36
Cheddar, extra sharp	23.4	0.52	77.72	12.13	0.90	0.66	8.6
Colby	29.7	0.40	82.01	9.89	0.98	0.63	6.48
Cream cheese	36.7	0.77	83.53	4.61	4.23	1.57	6.06
Feta	24.7	0.49	80.76	11.05	1.51	0.90	5.78
Monterey Jack	26.9	0.47	80.01	10.97	1.32	0.93	6.77
Mozzarella	16.5	0.47	78.07	11.11	0.88	0.77	9.16
Parmesan	25.1	0.38	80.83	10.01	1.09	0.63	7.44
Prepared cheese product	15.3	0.56	81.74	8.63	1.80	0.99	6.84
Processed, Cheddar	13.5	0.50	84.15	8.57	1.26	0.61	5.41

[a]As determined by silver ion–high-performance liquid chromatography (Ag^+–HPLC).
[b]Determined gravimetrically after total lipid extraction; see the Materials and Methods section.
[c]Total CLA fatty acid methyl ester (FAME) content (% of total FAME) in extracted lipids determined by gas chromatography (GC).
[d]CLA isomeric composition (% of total CLA FAME) determined by Ag^+–HPLC.
[e]See text regarding discussion about double-bond configuration.

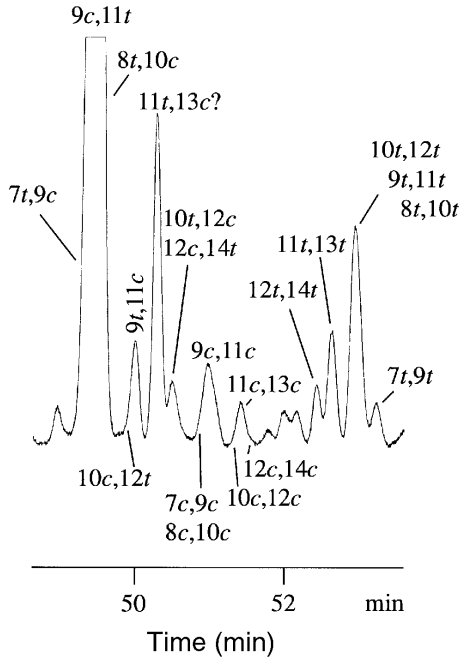

FIG. 1. The conjugated linoleic acid (CLA) region of a gas chromatography (GC) trace for cheese.

TABLE 2
Expected Gas Chromatographic Elution Order of Positional and Geometric CLA Fatty Acid Methyl Ester, or Dimethyloxazoline, Isomers on a 100 m CP-Sil 88 Capillary Column[a]

cis/trans-18:2[b]	*cis,cis*-18:2[c]	*trans,trans*-18:2[d]
7*c*,9*t*	(7*c*,9*c*)[e]	12*t*,14*t*
(6*t*,8*c*)	8*c*,10*c*	11*t*,13*t*
(8*c*,10*t*)	9*c*,11*c*	10*t*,12*t*
7*t*,9*c*	10*c*,12*c*	9*t*,11*t*
9*c*,11*t*	11*c*,13*c*	8*t*,10*t*
8*t*,10*c*	12*c*,14*c*	7*t*,9*t*
10*c*,12*t*		
9*t*,11*c*		
11*c*,13*t*		
10*t*,12*c*		
12*c*,14*t*		

[a]The elution order was: all the *cis/trans,* followed by all the *cis,cis,* followed by all the *trans,trans* CLA positional isomers. Many CLA isomers overlapped within each geometric group.
[b]The observed elution time of *cis/trans* CLA isomers increased as the Δ value of the *cis* double bond increased in the molecule. For a pair of *cis/trans* isomers in which the *cis* double bond has the same Δ value, the isomer with the lower Δ *trans* value eluted first. Therefore, it followed that for the same positional isomer, the *cis,trans* eluted before the *trans,cis* geometric isomer.
[c]The observed elution time of *cis,cis* CLA isomers increased with increased Δ values.
[d]The observed elution time of *trans,trans* CLA isomers increased with decreased Δ values.
[e]CLA isomers shown in parentheses were predicted. For abbreviations see Table 1.

more unresolved CLA isomers, even with a highly polar 100-m capillary column. The CLA isomers in the gas chromatogram were labeled based on an elution order that was verified recently (19,37,38) as well as in this study; see the predicted elution order in Table 2. Besides the major 9*c*,11*t*-18:2, there were minor peaks in the *cis/trans* region corresponding to 9*t*,11*c*- plus 10*c*,12*t*-18:2; 11*t*,13*c*-18:2; and a mixture of 10*t*,12*c*- plus 12*c*,14*t*-18:2. The 7*t*,9*c*,10*c*,12*c*- and 8*t*,10*c*-18:2 isomers eluted on the leading and the tailing edges of 9*c*,11*t*-18:2, respectively. In the *cis,cis* region (Fig. 1) there was one major peak consisting of a mixture of 7*c*,9*c*-, 8*c*,10*c*-, and 9*c*,11*c*-18:2, followed by a smaller peak due to 10*c*,12*c*-, 11*c*,13*c*-, plus 12*c*,14*c*-18:2. The *trans,trans* region showed four responses attributed to the FAME of 12*t*,14*t*-18:2; 11*t*,13*t*-18:2; a mixture of 10*t*,12*t*-, 9*t*,11*t* plus 8*t*,10*t*-18:2; and 7*t*,9*t*-18:2 (Fig. 1). To separate and confirm the identity of CLA isomers, complementary techniques, such as Ag^+–HPLC, GC–EIMS and GC–DD–FTIR were used.

Ag^+–HPLC. The separation of cheese FAME by Ag^+–HPLC is shown in Figure 2. The elution order observed by Ag^+-HPLC for a commercial mixture of 12 CLA isomers (27), plus those found in natural products (38, and the present study), are listed in Table 3. The main CLA isomer in cheese was 9*c*,11*t*-18:2. To determine the structure of the many minor CLA isomers, FAME were prepared from 1 g of cheese lipids and were fractionated using a semipreparative Ag^+–HPLC column. Five fractions were collected and those containing minor CLA isomers, Fractions 1, 2, 4 and 5, were shown after expanding the *y*-scale in Figure 2. The fractions were subsequently derivatized to DMOX and analyzed by GC–EIMS and GC–DD–FTIR.

GC–EIMS reconstructed ion profiles for DMOX derivatives of conjugated dienes. The fatty acid DMOX mass spectra consisted of a series of even-mass ions separated by 14 mass units due to successive losses of methylene units. A gap of 12 mass units between ions containing n–1 and n carbon atoms indicated the presence of a double bond between carbons n and n + 1 of the parent fatty acid (44). For monounsaturated fatty acids, more abundant peaks occurred due to allylic cleavages on either side of the gap of 12 mass units for ions containing $n - 2$ and $n + 2$ carbons (44). In addition, the $n + 3$ carbons fragment ion occurred in equal abundance to the one containing $n + 2$ carbons; the mechanism of formation of this ion remains unknown (45). Therefore, for conjugated dienes, prominent ions occurred at $n - 2$, $m + 2$ and $m + 3$, where n is the position of the first double bond and m is the position of the second double bond along the parent conjugated fatty acid chain. These three prominent ions were clearly recognizable in previous published spectra of CLA DMOX (27,46,47), and in the series of mass spectra of 12,14-18:2 to 8,10-18:2 isomers found in cheese (Fig. 3).

The characteristic abundant ions with $m + 2$ and $m + 3$ carbons in the GC–EIMS DMOX spectra of CLA isomers were used in this study to identify trace amounts of isomers, because the gap of 12 mass units was often too weak to observe. For example, all the minor CLA isomers in Figure 3, except for the major 9*c*,11*t*-18:2 isomer, were missing one or more of the expected ions of the conjugated system, but the allylic $m + 2$ and the $m + 3$ ions were clearly visible. The prominent ion profiles for the $m + 2$ and $m + 3$ pair of ions due to a spe-

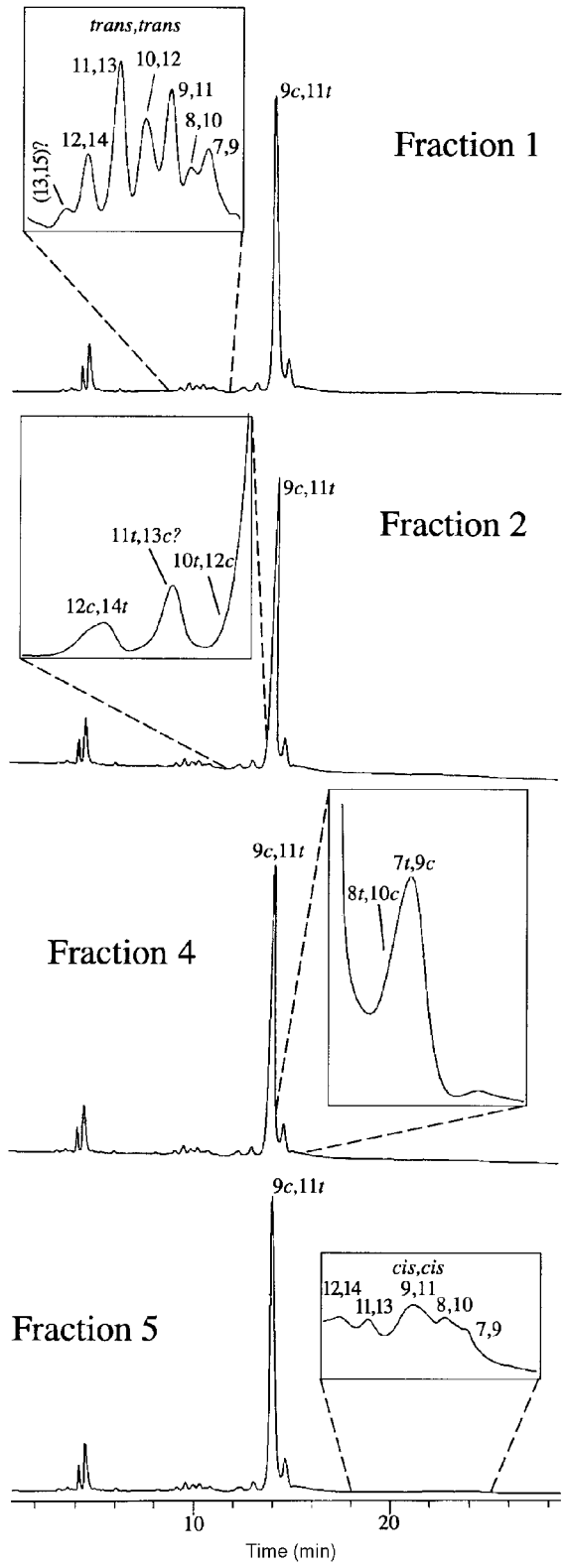

FIG. 2. Silver ion–high performance liquid chromatography (Ag^+–HPLC) profile for a commercial cheese product. Expanded traces are shown for Fractions 1 (A), 2 (B), 4 (C), and 5 (D). These fractions were collected by Ag^+–HPLC for further analysis.

TABLE 3
Elution Order of Positional and Geometric CLA FAME Isomers by (Ag^+–HPLC)[a]

trans,trans-18:2[b]	*cis/trans*-18:2[b,c]	*cis,cis*-18:2[b]
(13*t*,15*t*)d	(13,15 *c/t*)	
12*t*,14*t*	12,14 *c/t*	12*c*,14*c*
11*t*,13*t*	11,13 *c/t*	11*c*,13*c*
10*t*,12*t*	10,12 *c/t*	10*c*,12*c*
9*t*,11*t*	9,11 *c/t*	9*c*,11*c*
8*t*,10*t*	8,10 *c/t*	8c,10*c*
7*t*,9*t*	7,9 *c/t*	7*c*,9*c*

[a]The elution order was: all the *trans,trans,* followed by all the *cis/trans,* followed by all the *cis,cis* CLA positional isomers.
[b]The observed elution time of each group of geometric CLA isomers increased as the Δ values decreased.
[c]For the same positional isomer, the *cis,trans* and the *trans,cis* geometric isomers were not clearly separated under our experimental conditions.
[d]CLA isomers shown in parentheses were not confirmed. For abbreviations see Table 1.

cific CLA isomer were normalized and displayed. If these two ion profiles superimposed, then this GC peak was due to this specific CLA isomer. For nearly coeluting (or coeluting) CLA isomers, the reconstructed ion chromatograms showed a small (or lack of) difference in retention times. This reconstructed ion profile GC-EIMS method allowed the identification of most minor CLA isomers in cheese.

Identification of Ag^+–HPLC fractions. Fraction 1 consisted of a mixture of seven *trans,trans* CLA peaks (Fig. 2), as evidenced by the elution order of geometric CLA isomers previously found by Ag^+–HPLC (27) and verified by GC–DD–FTIR with characteristic =C-H stretching (3016 cm^{-1}) and deformation (990 cm^{-1}) vibrations for *trans,trans* CLA isomers. The GC–EIMS reconstructed ion profile for the molecular ion *m/z* 333 of the DMOX derivative of all CLA isomers is shown in Figure 4A. The total *trans,trans* CLA DMOX mixture exhibited three GC–EIMS peaks using a 50-m CP-Sil 88 capillary column. In using the reconstructed ion profile technique described above, the identify of the first small peak (Fig. 4C) was established as 12*t*,14*t*-18:2 (Fig. 3). The second CLA peak (Fig. 4B) was due to 11*t*,13*t*-18:2 (Fig. 3), and the third one (Fig. 4D) to 9*t*,11*t*-18:2 (Fig. 3). The CLA isomers 8*t*,10*t*- and 10*t*,12*t*-18:2 coeluted with 9*t*,11*t*-18:2 (reconstructed ion profile not shown), while the 7*t*,9*t*-18:2 eluted on the tail of the third peak [see reference (38), Fig. 1 bottom]. The first small peak in the Ag^+–HPLC chromatogram (Fig. 2, Fraction 1) would appear to be 13*t*,15*t*-18:2 based on the Ag^+–HPLC elution order, although this was not confirmed by GC–EIMS.

In Figure 3, GC–EI mass spectra were obtained from different cheese fractions, except the one for 10,12-18:2, because this *trans,trans* isomer coeluted by GC–EIMS with 9*t*,11*t*-18:2 (Fig. 4D), and the corresponding *cis/trans* and *cis,cis* isomers were very weak. However, its identity was confirmed from the reconstructed ion profiles for *m/z* 276 (*m* + 2) and 290 (*m* + 3) in each case. A mass spectrum of 10*t*,12*c*-18:2, shown for comparison in Figure 3, was selected from a previous study on the distribution of CLA isomers in pig lipid classes (37).

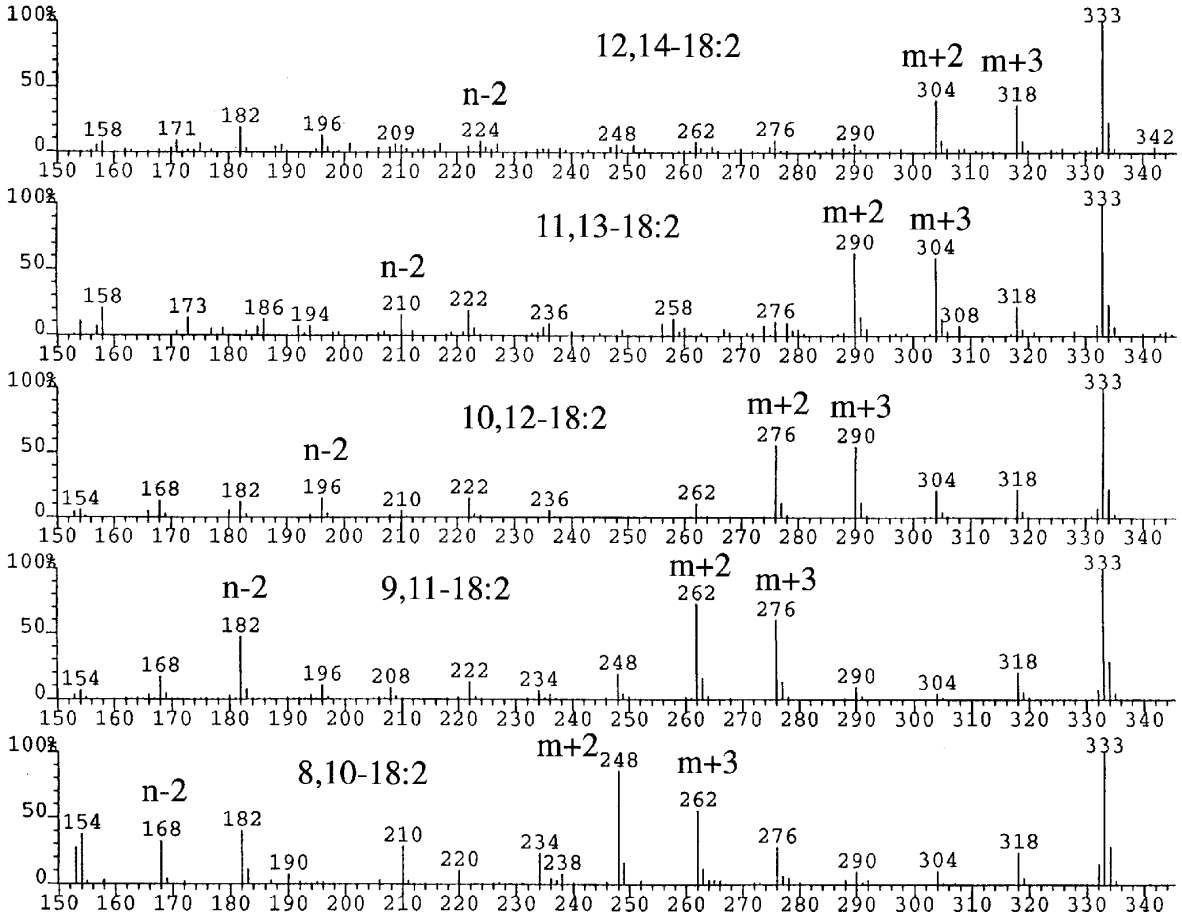

FIG. 3. GC–electron ionization mass spectra of the 4,4-dimethyloxazoline (DMOX) derivatives of 12*t*,14*t*-18:2 (Fraction 1), 11*t*,13*t*-18:2 (Fraction 1), 10*t*,12*c*-18:2 [reference (37), see text], 9c,11*t*-18:2 (Fraction 2), and 8*c*,10*c*-18:2 (Fraction 5). The allylic ions $n-2$ and $m+2$, and the $m+3$ ion are labeled for each of the positional CLA DMOX isomers; the molecular ion was *m/z* 333. For other abbreviations see Figure 1.

Fraction 2 (Fig. 2) showed two minor *cis/trans* CLA peaks eluting before the major 9*c*,11*t*-18:2, whose *cis/trans* geometric configuration was consistent with Ag^+–HPLC and GC–DD–FTIR data. In using the same reconstructed ion profile techniques described for the *trans,trans* CLA isomers, the peaks were shown to be due to 12,14- and 11,13-18:2. The EI mass spectra of the DMOX derivative of the 12,14- and 11,13-18:2 isomer were similar to the corresponding *trans,trans* CLA isomers shown in Figure 3. The geometric configuration of 12,14-18:2 was likely 12*c*,14*t*-18:2 based on the GC elution sequence and because it was possibly derived from biohydrogenation and isomerization of α-linolenic acid in the rumen. The geometric configuration of the 11,13-18:2 isomer in cheese was probably 11*t*,13*c*-18:2 based on the following considerations. On a 100-m CP Sil 88 column (Fig. 1), the 11,13-18:2 isomer in cheese eluted after the 11*c*,13*t*-18:2 isomer previously found in a Nu-Chek-Prep commercial mixture (27,37). In using two Ag^+–HPLC columns in series, the 11,13-18:2 isomer in cheese eluted before the 11*c*,13*t*-18:2 present in the Nu-Chek-Prep commercial mixture when these two lipid mixtures were coinjected (Sehat, N., unpublished data). Therefore, the relative elution sequences of the two *c/t* 11,13-18:2 isomers observed by both Ag^+–HPLC and GC are consistent with the presence of 11*t*,13*c*-18:2 in cheese. On the other hand, the fact that 11*t*,13*c*-18:2 eluted *before* 10*t*,12*c*-18:2 by GC cannot be reconciled with the expected GC elution sequence (Table 2), namely, 10*t*,12*c*-18:2 is expected to elute first, followed by 12*c*,14*t*-18:2 and then by 11*t*,13*c*-18:2. Therefore, the identification of the *c/t* 11,13-18:2 peak in cheese is only tentatively attributed to the 11*t*,13*c*-18:2 isomer. The double-bond configuration of these two *c/t* 11,13-18:2 geometric isomers (after isolation) still needs to be definitively confirmed by such methods as hydrazine reduction and GC separation of the resulting 18:1 fatty acids (26, and references cited therein). Trace amounts of 10*t*,12*c*-18:2 were generally not resolved chromatographically from the major 9*c*,11*t*-18:2 by Ag^+–HPLC because of the large differences in concentration between these two isomers.

Fraction 3 contained the major 9*c*,11*t*-18:2 (Fig. 2), which was not further investigated. Fraction 4 (Fig. 2) contained one *cis/trans* CLA peak. The main isomer under this minor peak

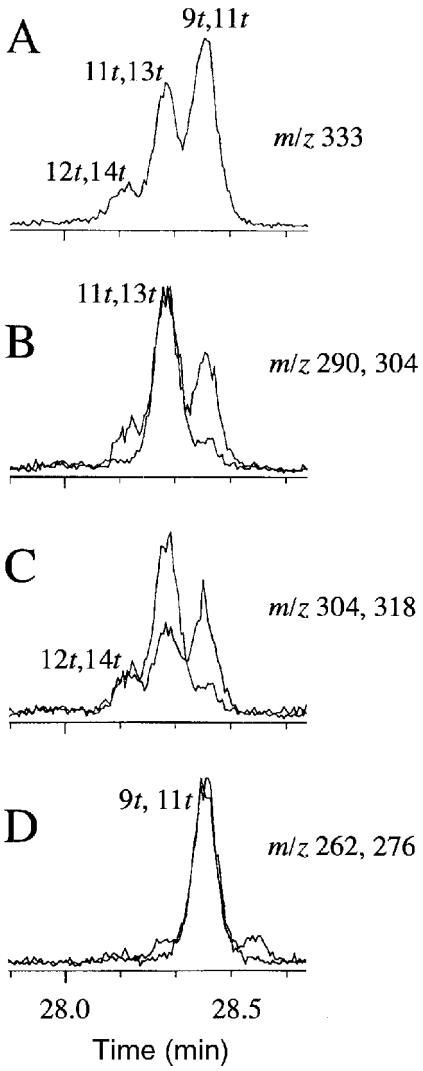

FIG. 4. Reconstructed ion chromatograms for DMOX derivatives of *trans,trans* CLA isomers in Fraction 1 isolated from cheese using Ag^+–HPLC showing the profiles for (A) the molecular ion *m/z* 333; (B) the allylic ion (*m* + 2) and the *m* + 3 ion for 11,13-18:2, (C) 12,14-18:2, and (D) 9,11-18:2. In each case these two ions were normalized for the CLA isomer indicated. For abbreviations see Figures 1–3.

was identified as 7*t*,9*c*-18:2 by GC–EIMS, GC–DD–FTIR and by comparison with partial synthesis, as reported recently (38). In addition to the 7*t*,9*c*-18:2, small amounts of 8*t*,10*c*-18:2 were evident under this peak by GC–EIMS.

Fraction 5 (Fig. 2) contained a mixture of five *cis,cis* CLA peaks as evidenced by their elution on Ag^+–HPLC (Table 3). The more predominant CLA isomers were identified by GC–EIMS as 9*c*,11*c*-18:2 and 8*c*,10*c*-18:2, with minor amounts of 12*c*,14*c*-, 11*c*,13*c*-, and 7*c*,9*c*-18:2. The EI mass spectrum of the DMOX derivative of the 8*c*,10*c*-18:2 isomer is shown in Figure 3. The peak expected for 10*c*,12*c*-18:2 was not observed, probably because it was too weak.

Composition of CLA isomers in cheese. The total CLA content of cheese fats was determined by GC and ranged from 0.4 to 0.8% (Table 1). Based on the separation of CLA isomers by Ag^+–HPLC, the compositions of the various cheese products are reported in Table 1. The concentration of the major 9*c*,11*t*-18:2 ranged from 78 to 84% (as percentage of total CLA) in the cheese products investigated. Among the minor CLA isomers, 7*t*,9*c*-18:2 was the most predominant at about 5 to 12% (containing undetermined small amounts of 8*t*,10*c*-18:2), followed by 11*t*,13*c*-18:2 at about 2%. The presence of 7*t*,9*c*-18:2 in the rumen is expected from Δ9 desaturation of 7*t*-18:1, and to a smaller extent from biohydrogenation and isomerization of γ-linolenic acid, while 12*c*,14*t*-18:2 is from biohydrogenation and isomerization of α-linolenic acid. The remainder was a mixture of total *trans,trans* (5 to 9%) and *cis,cis* (<1%) CLA isomers produced in the complex rumenal fluids.

Alternative methods used to determine CLA isomers in cheese by partial reduction with hydrazine, followed by GC separation of the resultant mixtures of octadecenoic acids (18:1) (26), did not lead to the identification of the 12,14- and 7,9-18:2 isomers. A possible reason for the latter is that 14*t*-18:1 is only partially resolved on polar capillary GC columns from 13*t*-18:1 as DMOX, and even less resolved as FAME (48). Similarly, the 7*t*-18:1 FAME coelutes with 6*t*- and 8*t*-18:1 FAME, while 7*t*-18:1 DMOX coelutes with 9*t*-18:1 DMOX (48).

The 11*t*,13*c*-18:2 isomer was found as a minor CLA component in cheese lipids. On the other hand, the 11*c*,13*t*-18:2 isomer was present at about 22% of total CLA in a commercial CLA mixture (27). The latter isomer, when included in the diet of pigs at the level found in commercial CLA mixtures, preferentially accumulated in heart lipids, particularly in heart and liver diphosphatidylglycerol (37). It would be of interest to investigate if 11*t*,13*c*-18:2 also accumulated in heart lipids and diphosphatidylglycerol, of pigs fed a cheese product containing 11*t*,13*c*-18:2 at about 1% of total CLA.

The availability of Ag^+–HPLC, GC–EIMS, GC–DD–FTIR, and very long polar capillary GC columns has provided the necessary tools to investigate the minor isomers of CLA in cheese products. Combinations of these methods plus reversed-phase HPLC will be required to identify the metabolites of CLA. In addition to 9*c*,11*t*-18:2 and the isomers reported previously by Ha *et al.* (21) and Lavillonniere *et al.* (26), the previously unrecognized isomers 11*t*,13*c*-18:2, 12*c*,14*t*-18:2, the prominent 7*t*,9*c*-18:2 (38), and their *trans,trans*, and *cis,cis* geometric isomers were found in this investigation. On the basis of our observed results in this and previous studies (19,37,38), the elution orders of CLA isomers on Ag^+–HPLC and long polar GC columns were determined (Tables 2 and 3).

ACKNOWLEDGMENT

Contribution number S009 from Southern Crop Protection, Food Research Center, Guelph, Ontario, Canada.

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[Received June 4, 1998, and in final revised form and accepted September 24, 1998]

Influence of Long-Chain Polyunsaturated Fatty Acids on Infant Cognitive Function[1]

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ABSTRACT: Long-chain polyunsaturated fatty acids (LCPUFA) are important for normal visual and cortical development. In a previous study of the effects of LCPUFA on cognitive function of term infants at the age of 3 mon, we indicated that infants with evidence of reduced growth parameters at birth and impaired attention control as manifested by a late peak fixation during infant habituation assessment may benefit from LCPUFA supplementation. The aim of this prospective study was to determine whether LCPUFA supplementation and late peak fixation are related to means-end problem-solving ability in these same infants at the age of 9 mon. Term infants (58) were randomized to one of two formulas containing either LCPUFA or no LCPUFA and completed 4 mon of feeding with their formula. Cognitive function was assessed at 3 mon of age by measures of infant habituation. Infants (20 LCPUFA and 20 no-LCPUFA) completed the problem-solving assessment at 9 mon. The no-LCPUFA group had lower scores on both measures of intention and number of solutions, but neither of these differences was significant. Analysis of covariance for the effects of group and peak fixation, covaried with gestation and birth weight, showed that the number of solutions was significantly reduced in the late peak-fixation infants receiving no LCPUFA ($P < 0.02$). Intention scores tended to be reduced in this group ($P < 0.06$). The late peak-fixation infants who received LCPUFA had solution and intention scores similar to early peak-fixation infants receiving LCPUFA or no LCPUFA. These findings suggest that in term infants who have reduced growth parameters at birth and who show evidence of impaired attention control, information processing and problem-solving ability in infancy may be enhanced by LCPUFA supplementation.
Lipids 33, 973–980 (1998).

Observational studies indicate that children who were breast fed are advantaged in cognitive and intellectual development compared to children who were fed formula milk (1–3). The mechanisms that underlie this advantage remain uncertain. Various constituents of human milk including hormones, growth-promoting factors, and nutrients were postulated as factors that may positively influence neural development. However, studies of children who were breast-fed or bottle-fed involve nonrandom assignment to these groups, and factors such as genetic and social-demographic variables, parenting skills, or quality of parent–child interaction may also contribute to differences in cognitive abilities. Attempts to statistically control for the potentially confounding effects of these factors may be inadequate (4–6).

Currently, one hypothesis involves the role of LCPUFA, especially arachidonic acid (AA) and docosahexaenoic acid (DHA), which are considered to have an important role in cellular physiology and are preferentially accreted by the infant brain during the last intrauterine trimester and the first months of life (7,8). Photoreceptor outer segment membranes in the retina contain uniquely high concentrations of DHA, their presence being important for normal photochemical activity of the visual pigment rhodopsin (9). DHA may also influence excitable membrane function by contributing to the modulation of membrane fluidity, particularly at the synapses (10). LCPUFA are present in human milk, but until recently were not available in formulas as it was assumed that they would be synthesized by the infants from the essential C18 fatty acids, n-6 linoleic acid and n-3 α-linolenic acid through elongase and desaturase enzyme systems. Young infants can synthesize AA and DHA (11,12), but low concentrations of AA and DHA in plasma and red cell membranes and low concentrations of DHA in cerebral cortex in formula-fed term and preterm infants compared to infants receiving human milk suggest that these enzyme systems may be inadequate to meet the infants' needs during the first months of life (13–15). The possibility that LCPUFA concentrations remain below optimum owing to metabolic insufficiency suggests that dietary supply of preformed LCPUFA may be important, particularly in infants who are fed formula.

Whether this relative deficiency of LCPUFA in formula-fed term infants, at a critical time of brain growth and development, is functionally relevant remains uncertain. Enhanced visual acuity scores were reported in term infants fed a DHA-supplemented formula compared to infants fed a standard formula, and acuity scores correlated with erythrocyte DHA (16–18). Four-month-old term infants who had received a formula supplemented with DHA and AA performed better in the Brunet-Lézine psychomotor development test compared to infants who were fed an identical formula containing no

[1]Based on a presentation at the AOCS Meeting on PUFA in Infant Nutrition: Consensus and Controversies, Barcelona, November 7–9, 1996.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; ANCOVA, analysis of covariance; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids. MAC, midarm circumference; OFC, occipito-frontal circumference.

LCPUFA supplement (19), although this advantage was not evident at 24 mon (20). However, standard tests of infant psychomotor development such as the Brunet-Lézine test and the Bayley Scales of Infant Development (21), when applied to normal populations in early infancy, provide scores that depend more on perceptual and motor abilities than on cognitive abilities (22). Standard tests of infant development correlate poorly both with scores obtained from the same tests applied in later infancy and with measures of childhood intelligence (23).

New assessments of infant cognitive function that correlate more closely with childhood intelligence were evaluated. Infant habituation, which is described as the decrease in attention to a stimulus over repeated presentations, is now one of the basic tools for studying memory and cognition in infancy (24,25). Studies showed a significant negative relation ship between infant habituation scores and childhood IQ, with infants who habituated with shorter "looking times" achieving higher IQ scores (21,25,26). Although the reported correlations are generally modest and some studies failed to demonstrate a significant relationship between habituation scores and IQ scores (27), measures of infant habituation reflect individual differences in cognitive function that demonstrate some stability throughout childhood (22,25,26,28,29).

Measures of "looking time" during habituation may reflect speed of information processing (25,29,30), and infants who show characteristics of faster processing (i.e., shorter looking times) have faster reaction times (31). Negative correlations between speed of decision-taking and IQ scores in children and adults implicate speed of processing as a component in the development of intelligence (32). Alternatively, "looking time" during habituation may be related to inhibitory processes involved in the control of attention, which was previously identified by differences in the pattern of habituation organization (28,33). Better attention control is characterized by a pattern of the peak fixation (longest fixation) occurring early in the sequence of fixations. Infants who have an early peak fixation may have a shorter looking time because they immediately attend maximally to the stimulus, inhibit attention to other less-salient stimuli, and are quicker to inhibit attention to the stimulus (disengage) when information processing is completed. In contrast, poorer attention control is shown by a pattern of the peak fixation occurring later in the sequence. Infants who have a late peak fixation may have a longer "looking time" because they are slower to pay maximal attention to the stimulus, are more easily distracted by other stimuli due to poorer inhibitory control, and take longer to disengage from the stimulus when processing is completed (28). Pattern of habituation organization was reported to remain stable for several months (34).

We reported on the effects of LCPUFA supplementation on measures of habituation in term infants at age 3 mon who demonstrated either an early or late peak fixation where an early peak fixation was defined as a peak fixation that occurred on the first fixation, and a late peak fixation was defined as a peak fixation that occurred on any fixation other than the first (35). Infants who demonstrated late peak fixations had significantly longer total "looking times" if they received no LCPUFA supplementation in comparison to infants who did receive LCPUFA supplementation. Infants with late peak fixations also had reduced growth parameters at birth compared to infants with early peak fixations. The total "looking times" of infants with early peak fixations were not influenced by LCPUFA supplementation. These data suggested that term infants who have reduced growth parameters at birth and who may have poorer inhibitory control of attention demonstrate less-efficient information processing if they are not supplemented with LCPUFA.

In a prospectively designed study, we studied all infants again at 9 mon of age to determine if LCPUFA supplementation and position of peak fixation at 3 mon were related to problem-solving ability. Infant means-end problem solving is described as the deliberate and planful execution of a sequence of steps to achieve a goal (36). This ability develops rapidly after 6 mon of age when infants begin to solve simple problems such as searching under a cover for a toy (37) or pulling a support to retrieve a toy resting on it (38,39). At 9 mon, infants begin to solve more complex problems which require completion of two intermediate steps to achieve a goal (40). Infant scores on a two-step problem measured at 9 mon correlate positively with childhood vocabulary and IQ scores measured at 3 yr (22,40).

The aims of this prospectively designed study were twofold: (i) to determine whether LCPUFA supplementation influences two-step problem-solving ability at 9 mon; and (ii) to determine whether problem-solving ability at 9 mon is related to early and late peak fixation at 3 mon.

EXPERIMENTAL PROCEDURES

Subjects. The study was conducted on term infants (birth weight 2500–4000 g; gestation 37–42 wk) who were randomized to one of two formulas containing either LCPUFA or no LCPUFA, who completed 4 mon of feeding with their formula, and who demonstrated either an early or a late peak fixation on the habituation assessment undertaken at 3 mon of age. The details of the habituation assessment have been described elsewhere (35). The problem-solving assessment described here was undertaken when infants were 9 mon of age.

Infant formulas. The infant formulas were commercially available (LCPUFA: Aptamil/Milupan (Milupa Ltd., Trowbridge, United Kingdom); no LCPUFA: Aptamil) and were identical apart from the fatty acid composition (Table 1). LCPUFA-supplemented formula was enriched with a fat blend (Milupan) derived from milk fat, vegetable oils, and egg lipids. Infant formulas were supplied as ready-to-feed by Milupa Ltd. Random assignment of infants to their formula was accomplished with a time-balanced randomization table.

Problem-solving assessment. The principles of this procedure have been described and evaluated (37–40). The infant sat on the parent's lap at the end of a table, and problems were presented on a tray (60 × 80 cm). The goal object was a small

TABLE 1
Formula Fatty Acid Composition (g/100 g total fatty acids)[a]

Fatty acid	LCPUFA formula	No-LCPUFA formula
12:0	4.9–5.6	4.8
14:0	5.6–5.9	5.3
16:0	26.1–26.8	25.0
18:1n-9	30.2–32.2	36.0
18:2n-6	11.5–12.8	11.4
18:3n-3	0.60–0.65	0.70
20:4n-6 (AA)	0.30–0.40	<0.10
22:6n-3 (DHA)	0.15–0.25	—

[a]Energy: 670 kcal/L from formula; 324 kcal/L from fat. LCPUFA, long-chain polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid.

toy which was placed on a 22 × 42 cm brown cloth and hidden under a cover, a blue cloth (6 × 6 cm). To familiarize infants with the materials and the procedure, we presented the cloths and toy on the tray individually, allowing two presentations of each object and 20 s for play.

Next, infants received pretests on the component steps of the two-step problem: first they pulled the cloth to retrieve the toy on the far end, and second they searched for the toy after they had seen it hidden under the cover. Infants were allowed 30 s to retrieve the toy and played with the toy for 20 s before receiving the next trial. Four trials were administered on each pretest. Finally, infants received four test trials on the two-step problem. The cloth was positioned 5 cm from the front edge of the tray. When the infant was looking, the toy was set down on the far end of the cloth, and the cover was placed over it. The tray was immediately pushed forward, and infants were allowed 30 s to retrieve the toy. All trials were recorded on videotape. Behavior was scored from the videotapes for evidence of intention. Intentional behavior was defined as behavior aimed at achieving the goal of retrieving the toy, rather than simply playing with the cloth or cover (37,38,40). Information on the problem-solving assessment may be obtained from the first author.

An "intention score" was defined as the total of the scores for each behavior, averaged across the four trials. For the two steps of the problem and three behaviors appropriate to the step, the scores were 0 (no intention), 1 (ambiguous and possible intention), and 2 (clear intention) (see Table 2). The total intention score for the entire problem could range from 0 to 12. Intentional solutions were trials on which each relevant behavior was scored as intentional (1 or 2). All videotapes were scored by one independent, trained observer. As a check on reliability, a second independent observer scored a random sample of 40% of assessments on the two-step task. The interobserver correlation for intention score was $r = .97$, and the proportion of agreements on the identification of intentional solutions was 94%. Because of the high level of reliability, the results were based entirely on the scores of the first observer.

Demographic, nutritional, and anthropometric data. We also obtained information on social class, housing, family size, maternal education, and maternal age. Social class was coded as six categories using the Office of Population Censuses and Surveys Standard Occupational Classification (41) (social class I; social class II; social class III nonmanual; social class III manual; social class IV; social class V and unemployed) and was based on occupation of the income-providing parent, or on the father's occupation if both parents were earning. Housing was coded as rented or owned. Family size was recorded as the number of surviving children in the family unit. Maternal education was recorded as the age when the mother left full-time education.

Mothers and infants were seen by a research nurse before discharge and at 1, 2, and 3 mon during home visits. The volume of formula milk consumed by the infant during the 3 d prior to each home visit was calculated by measuring all residual volumes of milk from the feeds over these periods. Information on the following infant anthropometric measures at birth and at 3 mon was obtained by the research nurse: weight; crown–heel length; occipito-frontal head circumference (OFC); subscapular skinfold; triceps skinfold; and midarm circumference (MAC). The ratio of MAC to OFC at birth, which was shown to detect intrauterine growth retardation in infants born with a birth weight in the "normal range,"

TABLE 2
Criteria for Scoring Behavior for Evidence of Intention on Each Step of the Two-Step Problem

	No intention (0)	Possible intention (1)	Clear intention (2)
Cloth step			
Cloth behavior	Play; cover not within reach	Hesitant pulling	Pull cloth
Fixation	Fixate away from cover	Fixate briefly away from cover	Fixate cover continuously
Cover retrieval	Ignore cover	Attempt to grasp	Pick up cover
Cover step			
Cover behavior	Play; cover not removed	Hesitant removal	Remove cover
Fixation	Fixate away from toy	Fixate briefly away from toy	Fixate toy continuously
Toy retrieval	Ignore toy	Attempt to grasp toy	Pick up toy

was calculated as MAC (cm)/OFC (cm) (42–44). Anthropometric measures were not obtained at 9 mon.

Mothers and researchers remained blind to the diet groups throughout the entire study period (i.e., until both the habituation assessment at 3 mon and problem-solving assessment at 9 mon were completed). The study was approved by the Tayside Committee on Medical Research Ethics (Dundee, United Kingdom).

Statistical methods. A sample size of 22 in each group was calculated to detect a difference of one intentional solution on problem-solving with a power of 90% at $P = 0.05$ level. A sample size estimate for the relationship of problem-solving at 9 mon to peak fixation at 3 mon could not be obtained because this relationship had never been studied. Comparisons between diet groups on demographic and nutritional variables were made with the Student's *t*-test for interval measures and chi-square test for categorical measures. Comparisons on anthropometric measures were made with analysis of covariance (ANCOVA) covaried with gestation. The effects of diet and peak fixation on measures of problem-solving were determined with ANCOVA covaried with gestation and birth weight. Interactions between the factors of diet and peak fixation were examined with analyses of simple effects covaried with gestation and birth weight. Additionally, the effect of diet on problem-solving scores at each level of peak fixation was examined with regression analysis. Independent, potentially confounding, variables included in these analyses were gestation, birth weight, gender, and social class. All analyses were run with SPSS Version 6.1.2 for Windows (45). A P value < 0.05 was considered as evidence of a significant difference.

RESULTS

The total enrollment for the habituation and problem-solving assessments was 58 infants (LCPUFA = 27; no-LCPUFA = 31); 18 infants were excluded from the study; 10 failed to complete the habituation assessment (mother failed to attend: LCPUFA = 1; no-LCPUFA = 3; infant became distressed: LCPUFA = 2; no-LCPUFA = 4); 8 failed to complete the problem-solving assessment (mother failed to attend: LCPUFA = 3; no-LCPUFA = 4; data lost through experimenter error: LCPUFA = 1); 40 infants completed both assessments (LCPUFA = 20; no-LCPUFA = 20). The characteristics of the LCPUFA and no-LCPUFA groups are shown in Table 3. The LCPUFA group had a significantly longer ges-

TABLE 3
Demographic, Anthropometric, Formula Intake Characteristics, and 9-Month Problem-Solving Scores of Infants Randomly Assigned to the LCPUFA and No-LCPUFA Formulas

	LCPUFA ($n = 20$)	No-LCPUFA ($n = 20$)	P
%/*n* in social classes I and II	0 (0)	5 (1)	1.00
Maternal age (yr)	26.2 ± 4.2[a]	27.7 ± 4.6	0.29
Maternal education (yr)	16.2 ± 0.6	17.1 ± 2.2	0.10
Family size (*n*)	1.6 ± 0.8	1.9 ± 1.2	0.44
Housing: rent/own	12/8	9/11	0.34
Male/female	7/13	8/12	0.74
Gestation (wk)	40.6 ± 1.1	39.7 ± 0.9	0.009
Age at assessment (d)	274.2 ± 2.7	275.2 ± 5.0	0.41
Anthropometry birth			
Crown–heel length (cm)	49.8 ± 1.9	50.0 ± 1.7	0.74
OFC (cm)	34.4 ± 1.1	34.3 ± 1.4	0.70
Subscapular skinfold (mm)	4.3 ± 0.9	4.5 ± 0.8	0.70
Triceps skinfold (mm)	4.2 ± 1.21	5.0 ± 0.7	0.03
MAC (cm)	10.5 ± 1.3	11.0 ± 1.0	0.07
Weight (g)	3261 ± 383	3360 ± 373	0.21
MAC/OFC ratio	0.31 ± 0.04	0.32 ± 0.03	0.11
3 mon			
Crown–heel length (cm)	60.3 ± 2.1	60.8 ± 1.8	0.54
OFC (cm)	40.4 ± 1.3	41.8 ± 5.1	0.33
Subscapular skinfold (mm)	6.8 ± 1.3	7.2 ± 1.3	0.86
Triceps skinfold (mm)	5.9 ± 2.1	7.1 ± 2.4	0.26
MAC (cm)	12.9 ± 1.0	13.1 ± 1.0	0.92
Weight (g)	5959 ± 650	6044 ± 683	0.82
MAC/OFC ratio	0.32 ± 0.02	0.32 ± 0.03	0.42
Formula intake (mL)			
1 mon	866 ± 267	808 ± 192	0.46
2 mon	857 ± 186	881 ± 223	0.73
3 mon	831 ± 243	822 ± 259	0.92
Problem-solving			
Intention score	7.7 ± 3.0	6.0 ± 3.7	0.06
Solutions (*n*)	1.7 ± 1.5	1.3 ± 1.3	0.15

[a]Mean ± SD; MAC/OFC, midarm circumference/occipito-frontal circumference. See Table 1 for other abbreviation.

tation than the no-LCPUFA group (6 d), but no significant differences existed on any other demographic and social variables. ANCOVA covaried with gestation showed that the no-LCPUFA group had a significantly greater triceps skinfold at birth ($P < 0.05$), but no other significant anthropometric or formula intake differences were present between the groups. The LCPUFA group had marginally higher scores on both measures of intention and number of intentional solutions (Table 3), but ANCOVA covaried with gestation showed that these differences were not significant.

Analysis of the relationship of 9-mon problem-solving scores of the LCPUFA and no-LCPUFA groups to early and late peak fixation on the 3-mon habituation assessment is shown in Table 4. ANCOVA on number of intentional solutions for the effects of diet and peak fixation, covaried with gestation and birth weight, showed a significant diet × peak fixation interaction ($P < 0.03$). Simple-effects analyses showed that the number of intentional solutions did not differ significantly between the early peak-fixation infants receiving or not receiving LCPUFA ($P = 0.18$). In contrast, the number of intentional solutions was significantly reduced in the late peak-fixation infants receiving no LCPUFA ($P < 0.02$).

This interaction was further examined by separate regression analyses on the number of intentional solutions in the early and late peak-fixation infants. Independent and potentially confounding variables included gestation, birth weight, gender, and social class. In the early peak-fixation infants, none of these variables was significantly related to number of intentional solutions. In the late peak-fixation infants, only diet ($P < 0.05$) and birth weight ($P < 0.05$) were significantly related to the number of intentional solutions. None of the other factors significantly increased the predictive power of the regression equation.

ANCOVA on intention scores for the effects of diet and peak fixation showed no significant main effects, and the diet × peak interaction was not significant ($P = 0.28$). Analysis of simple effects showed that, although intention scores did not differ between the early peak-fixation infants receiving either LCPUFA or no LCPUFA ($P = 0.92$), intention scores tended to be reduced in the late peak-fixation infants receiving no LCPUFA ($P = 0.051$).

DISCUSSION

The infants recruited to this study were randomly assigned to a formula either supplemented or not supplemented with LCPUFA. Infants were assessed at 3 mon of age by infant habituation measures, and analysis of the pattern of habituation identified infants who demonstrated either an early or a late peak fixation. Previously we found that at the age of 3 mon, infants who had a late peak fixation and who received no LCPUFA supplementation had increased total "looking times" in comparison to other groups. We have now demonstrated that at the age of 9 mon, infants with a late peak fixation at age 3 mon and who received no-LCPUFA supplementation had reduced two-step problem-solving ability in comparison to other groups. These findings are important because longer "looking times" on infant habituation and lower scores on infant two-step problem-solving were shown to be related to lower childhood IQ scores (22,29). Although the reported correlations between infant habituation and problem-solving scores and childhood IQ tend to be low and therefore account for a relatively small proportion of the variance in IQ scores (22), our findings suggest that provision of preformed dietary LCPUFA may have implications for the development of childhood intelligence.

Table 4 shows there was variation between the problem-solving scores of the diet and peak-fixation groups that may be related to the small numbers of subjects. However, problem-solving scores in both the LCPUFA and no-LCPUFA early peak-fixation groups and in the LCPUFA late peak-fixation group were within the normal range for 9-mon-old infants (40). The exception was the no-LCPUFA late peak-fixation group in which problem-solving scores were low. We had reported the association between late peak fixation and reduced growth parameters at birth (35). This finding suggests that factors affecting intrauterine growth may also affect the development of neural systems involved in the regulation of habituation and problem-solving. Concentration of DHA in brain tissue increases steadily during fetal development (8), and a positive correlation exists between fetal growth measurements such as weight and head circumference and circulating concentrations of LCPUFA (46). Possibly among these infants with reduced growth parameters and late peak fixations, some were LCPUFA-deficient at birth. If infant cognitive function is influenced by LCPUFA, then this may depend on factors that affect fetal LCPUFA status (e.g., maternal LCPUFA status during pregnancy), as well as the postnatal LCPUFA status of the infant. LCPUFA supplement had no effect on habituation or problem-solving in infants who tended

TABLE 4
Effect of LCPUFA Supplementation on 9-Mon Problem-Solving Scores in Term Infants with Either an Early or a Late Peak Fixation at 3 Mon

	Early peak fixation at 3 mon		Late peak fixation at 3 mon		ANCOVA		
Problem-solving scores at 9 mon	LCPUFA ($n = 9$)	No-LCPUFA ($n = 10$)	LCPUFA ($n = 11$)	No-LCPUFA ($n = 10$)	Diet P	Peak P	Diet X Peak P
Intention score[a]	6.9 ± 2.1	7.2 ± 3.6	8.3 ± 3.5	4.8 ± 3.6[b]	0.16	0.13	0.28
Intentional solutions (n)	1.1 ± 1.2	1.9 ± 1.4	2.2 ± 1.5	0.6 ± 0.7[c]	0.47	0.19	0.03

[a]Mean ± SD. ANCOVA, analysis of covariance. See Table 1 for other abbreviation.
[b]$P = 0.051$.
[c]$P < 0.05$.

to have normal growth parameters and who had an early peak fixation, which may be explained by some of these infants already being sufficiently endowed with LCPUFA and therefore not benefiting from their LCPUFA supplementation.

The no-LCPUFA formula provided only 0.34% of energy from α-linolenic acid (18:3n-3; ALA) and may have been deficient, but what level is optimal is unclear. Jensen *et al.* (47) showed that the percentage of DHA in erythrocyte phospholipids at age 120 d was significantly higher in infants fed a formula with ALA content constituting 3.24% of the total fatty acids, in comparison to infants fed formulas containing either 0.4, 0.95, or 1.7% ALA among which erythrocyte DHA content did not differ significantly. However, infants receiving formula with the highest level of ALA demonstrated poorer growth at age 120 d (48). The fact that the ALA composition of both LCPUFA and no-LCPUFA formulas in our study was similar suggests that the observed effects on infant cognitive behavior were due to LCPUFA supplementation rather than ALA deficiency.

Examination of problem-solving scores at 9 mon suggests that the benefits of early LCPUFA supplementation to term infants persisted beyond the period they received their supplemented formula. Similar benefits of early LCPUFA supplementation to preterm infants were reported in infants who received LCPUFA-supplemented formula until 2 mon and who showed improved scores on measures of visual attention at 12 mon (49). The absence of differences between the randomized diet groups in formula intake or anthropometric measures at 3 mon suggests that other nutritional factors were unlikely to have contributed to problem-solving ability. We do not have precise data on the timing and nature of solid feeding during the first months of life in these infants, but weaning foods had low-LCPUFA content and were unlikely to have influenced LCPUFA status (50).

How can we explain these effects of LCPUFA on infant cognitive behavior? Developmental psychologists have offered alternative interpretations for differences in looking times during infant habituation that consider processes involved in speed of information processing (25,29,30), inhibitory control of attention (28,33), and arousal or sensitization (51), although it is uncertain whether there are stable individual differences in sensitization that can account for differences in infants' "looking times." One possible explanation for our findings is that accumulation of LCPUFA in cell membranes in the central nervous system results in an increase in the speed at which infants are able to process information. Recently, two other randomized trials reported that DHA-supplemented preterm infants demonstrated shorter "looking times" during the novelty preference phase of the Fagan Test of Infant Intelligence compared to unsupplemented controls (49,52). Although no studies have investigated the relationship between speed of processing and infant problem-solving, infants who are faster at processing information may be more efficient at solving the two-step problem. These infants may more quickly identify the correct method for solving the problem, and more quickly achieve a solution before being distracted or forgetting the final goal.

Alternatively, LCPUFA may influence the development of neural structures involved in the inhibitory control of attention and disengagement or release of attention from a stimulus. Young infants show considerable difficulty at disengaging from one stimulus to orient to another, but disengagement improves substantially after 3 mon (53,54). Development of several cortical structures may be involved in this change, including parietal cortex, prefrontal cortex, and frontal eye fields (55). Improved maturation of these structures in infants who received LCPUFA-supplemented formula may have enhanced their capacity to release attention after sufficient information had been processed, which in turn would reduce their total "looking time" during habituation. Similarly, improved ability at releasing attention may assist infants to switch quickly from manipulating the cloth to manipulating the cover and solve the two-step problem. Evidence exists that development of prefrontal cortex is involved in the ability to solve means-end problems such as searching for a hidden object and reaching around a barrier (56,57), and early LCPUFA supplementation may contribute to prefrontal maturation. Although it is not possible to distinguish between the speed of processing or release of attention hypotheses in our present study, assessments of infant reaction times (31) and disengagement (53) in future research should help to identify the specific effects of LCPUFA on infant cognitive function.

Although this study was conducted on a relatively small number of infants and it remains to be shown whether similar effects can be obtained with a larger sample, the results suggest that term infants who have reduced growth parameters at birth and show evidence of impaired attention control as manifested by a late peak fixation during infant habituation assessment may selectively benefit from LCPUFA supplementation. We speculate that LCPUFA supplementation may enhance more-efficient information processing in these susceptible infants, and the data suggest that the effects persist beyond the period of supplementation. Further larger randomized studies are required to examine the relationship of maternal and infant LCPUFA status to infant cognitive function, to identify the characteristics of those infants who may benefit from LCPUFA supplementation, and to determine the relationship of LCPUFA supplementation to intellectual abilities in later childhood.

ACKNOWLEDGMENTS

The investigators wish to acknowledge the support they received from Milupa Ltd., UK, and to thank Alan Slater and Liz Brown for provision of computer software and advice on the habituation assessment, and Lyn Galbraith, Diana Ciupka, and Andrew Mirrlees for assistance with scoring the habituation and problem-solving assessments.

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[Received November 11, 1997, and in final revised form September 22, 1998; revision accepted October 2, 1998]

Tomato Lycopene and Low Density Lipoprotein Oxidation: A Human Dietary Intervention Study

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ABSTRACT: Increase in low density lipoprotein (LDL) oxidation is hypothesized to be causally associated with increasing risk of atherosclerosis and coronary heart disease. In recent epidemiological studies, tissue and serum levels of lycopene, a carotenoid available from tomatoes, have been found to be inversely related to risk of coronary heart disease. A study was undertaken to investigate the effect of dietary supplementation of lycopene on LDL oxidation in 19 healthy human subjects. Dietary lycopene was provided using tomato juice, spaghetti sauce, and tomato oleoresin for a period of 1 wk each. Blood samples were collected at the end of each treatment. Serum lycopene was extracted and measured by high-performance liquid chromatography using an absorbance detector. Serum LDL was isolated by precipitation with buffered heparin, and thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD) were measured to estimate LDL oxidation. Both methods, to measure LDL oxidation LDL-TBARS and LDL-CD, were in good agreement with each other. Dietary supplementation of lycopene significantly increased serum lycopene levels by at least twofold. Although there was no change in serum cholesterol levels (total, LDL, or high-density lipoprotein), serum lipid peroxidation and LDL oxidation were significantly decreased. These results may have relevance for decreasing the risk for coronary heart disease.

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Coronary heart disease is one of the leading causes of death in North America. Epidemiological, clinical, and biochemical studies indicate convincingly that increased serum low density lipoprotein (LDL) cholesterol concentration is associated with increased risk of atherosclerosis (1,2). Oxidative damage to LDL caused by reactive oxygen species may play an important role in the etiology of coronary heart disease (3–5). Antioxidants are believed to slow the progression of atherosclerosis because of their ability to inhibit LDL oxidation (6–9).

In recent epidemiological studies, lycopene levels in serum and adipose tissue were inversely associated with the risk of coronary heart disease (10–13). Lycopene is an antioxidant carotenoid without provitamin-A activity. Tomatoes and tomato products are the main sources of lycopene in the human diet. *In vitro* studies have shown it to be one of the most potent antioxidants (13–15) while epidemiological, animal, and tissue culture studies have indicated its anticarcinogenic potential (for review see Ref. 13). In humans lycopene levels are related to dietary habits and lifestyle (16). The aim of this study was to investigate the effect of dietary supplementation of lycopene on LDL oxidation in healthy human subjects.

MATERIALS AND METHODS

Subjects. Nineteen healthy subjects (10 male and 9 female), age group 25 to 40 yr (average age 29 yr), nonsmokers, not pregnant, and not taking any medication or vitamin supplements, were chosen for this study. Average weight and body mass index of the subjects were 67.6 ± 11.6 kg and 24.0 ± 2.8 kg/m^2, respectively. The subjects maintained their body weight and had no adverse symptoms during the entire study period.

Study design. A randomized, cross-over study design was used. All subjects completed all four treatments including a placebo (0 mg lycopene); 126 g spaghetti sauce (39.2 mg lycopene), provided by Hunt-Wesson Inc., Fullerton, CA; 540 mL tomato juice (50.4 mg lycopene), provided by H.J. Heinz Co. of Toronto, Ontario, Canada; and 1.243 g of 6% lycopene oleoresin from tomatoes (75.0 mg lycopene), provided by LycoRed Natural Product Industries Ltd., Beer-Sheva, Israel, in the form of capsules. Each treatment was for 1 wk with a 1-wk washout phase. Dietary lycopene in the form of tomato products or capsules was consumed once a day with a standardized breakfast consisting of pasta, margarine, grated Parmesan cheese, banana, and BECEL® margarine (Lipton, Toronto, Ontario, Canada) during the treatment period. Test products were consumed as a part of breakfast to standardize the delivery of lycopene. Subjects consumed their regular breakfast during the washout periods. They were advised to avoid consuming tomatoes and tomato products and any other sources of lycopene throughout the study period (treatment and washout phases) and were provided with a list of food products containing tomatoes as a guide. They also main-

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Abbreviations: BHT, butylated hydroxytoluene; CD, conjugated dienes; HDL, high-density lipoprotein; LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances.

tained their food records and a symptoms diary during the study period, which were used to check compliance. The study protocol was approved by the Human Ethics Committee of the University of Toronto. Blood samples were collected from overnight fasting subjects at the end of each treatment phase. Serum was separated from fresh blood and stored at −70°C for analysis. All the samples from one subject were analyzed at the same time to avoid the sample-handling variability.

Lycopene analysis. Serum lycopene was extracted using hexane/methylene chloride (5:1) containing 0.015% butylated hydroxytoluene (BHT) and analyzed by high-performance liquid chromatography using Vydac 201HS54 reverse-phase analytical column (The Separations Group, Hesperia, CA) and a mixture of acetonitrile/methanol/methylene chloride/water (7:7:2:0.16, by vol) as mobile phase (16,17). Lycopene was measured as single peak containing *all trans* and *cis* isomers using an absorbance detector set at 470 nm. An external standard of lycopene (Sigma Chemical Co., St. Louis, MO) was used as reference standard. The coefficient of variance was 4.0%.

Serum cholesterol analysis. Serum was analyzed for total cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol after magnesium chloride precipitation using a Technicon CH1000 (Technicon Inc, Tarrytown, NY) with chemical methods of the Lipid Research Clinic (LRC) Project (18). LDL cholesterol was derived using a modification of the Freidewald formula. The precision and accuracy for total cholesterol, triglyceride, and HDL cholesterol measurements were certified by the Centers for Disease Control—National Heart, Lung and Blood Institute (CDC—NHLBI) Lipid Standardization Program. Internal and external quality control procedures were followed (18).

LDL isolation. For LDL oxidation analysis, serum LDL were isolated by precipitation with buffered heparin (19). The LDL precipitate was separated by centrifugation at $1000 \times g$ and resuspended in saline for further analysis. LDL cholesterol contents were estimated enzymatically (20) using Cholesterol Assay Kit (Sigma Chemical Co.). The coefficient of variance was 1.7%.

Malondialdehyde analysis. Malondialdehyde, as a measure of lipid peroxidation, was estimated using the thiobarbituric acid (TBA) reaction. Results are expressed as TBA-reactive substances (TBARS). Freshly thawed serum or freshly prepared LDL samples were incubated with TBA and orthophosphoric acid, in the presence of BHT, for 45 min at 95°C, cooled to room temperature, and extracted with *n*-butanol. Absorbance of the butanol phase was measured at 535 nm (16,21,22). Results were calculated using the extinction coefficient 1.56×10^5 M^{-1}. Serum-TBARS are reported as μmol/L of serum and LDL-TBARS as mmol/mol LDL. The coefficients of variance for serum-TBARS and LDL-TBARS were 2.8 and 5.3%, respectively.

Conjugated diene (CD) analysis. CD analysis was also done to measure lipid peroxidation. Lipids from LDL were extracted by chloroform/methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm using the molar extinction coefficient 2.95×10^4 M^{-1} (23). LDL-CD are reported as mmol/mol LDL. The coefficient of variance was 2.5%.

Statistical analysis. All statistical calculations were performed by using Excel 5.0 (Microsoft Corp., Redmond, WA). *P* values were assessed by analysis of variance, Dunnett's test, two samples paired Student's *t*-test, and Student-Newman-Keuls multiple range test (SAS Institute, Cary, NC). *P* values of <0.05 were considered statistically significant. Results are expressed as mean ± SEM.

RESULTS

Figure 1 shows the effect of dietary lycopene on serum lycopene. Serum lycopene levels increased significantly with daily intakes of 126 g of spaghetti sauce (39.2 mg lycopene), 540 mL of tomato juice (50.4 mg lycopene), or 1.24-g capsule of tomato oleoresin (75 mg lycopene) as a part of breakfast for a period of 1 wk each over the placebo when no lycopene was consumed. The average increase in serum lycopene levels for any treatment over placebo was at least twofold. However, there were no differences in the serum lycopene levels between the different treatments.

Dietary intake of tomato products had no significant effects on either cholesterol (total, LDL, or HDL) or triglycerides. All dietary lycopene treatments significantly lowered serum LDL oxidation over the placebo (Fig. 2). Both LDL-TBARS and LDL-CD decreased significantly with each tomato product. The average decrease over placebo was 25% for LDL-TBARS and 13% LDL-CD for the tomato products treatment. There were no significant differences between

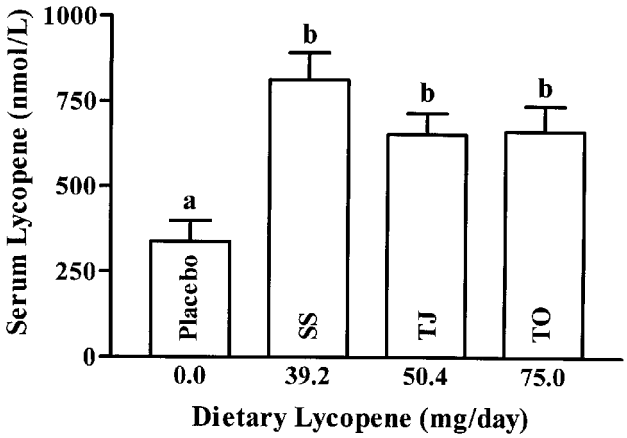

FIG. 1. Effect of dietary lycopene supplementation on serum lycopene concentration. Dietary lycopene was provided to healthy human subjects in the form of spaghetti sauce (SS), tomato juice (TJ), or tomato oleoresin capsules (TO) with a standardized breakfast for a period of 1 wk in a random order. Fasting blood samples were collected at the end of each treatment. Serum lycopene was extracted by hexane/methylene chloride and analyzed by high-performance liquid chromatography (16,17). Results are mean ± SEM. Bars with different letters are statistically significant ($P < 0.05$).

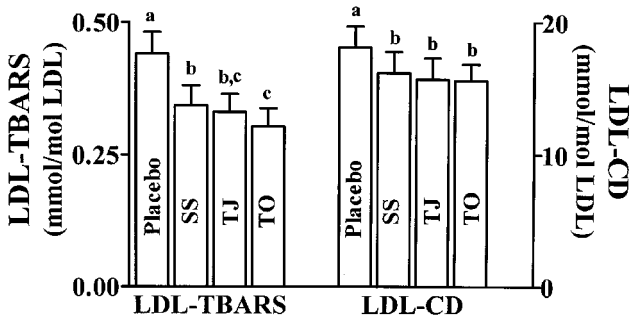

FIG. 2. Effect of dietary lycopene supplementation on serum low density lipoprotein (LDL) oxidation [LDL-thiobarbituric acid-reactive substances (TBARS) and LDL-conjugated dienes (CD)]. Dietary lycopene was provided to healthy human subjects in the form of SS, TJ, or TO with a standardized breakfast for a period of 1 wk in a random order. Fasting blood samples were collected at the end of each treatment. Serum LDL was separated by complexation with heparin. LDL oxidation was measured by spectrophotometric thiobarbituric acid-malondialdehyde and CD assay. Results are mean ± SEM. Bars with different letters are statistically significant ($P < 0.05$). For other abbreviations see Figure 1.

LDL-TBARS and LDL-CD between different sources of lycopene. Both methods of detecting LDL oxidation, LDL-TBARS and LDL-CD, yielded similar results and were highly correlated ($r = 0.89$, $P < 0.05$) with each other. Also LDL-TBARS were significantly correlated ($r = 0.90$, $P < 0.05$) with the serum-TBARS.

DISCUSSION

Our study indicates that human subjects absorb lycopene from traditional sources of processed tomato products including spaghetti sauce and tomato juice as well as from tomato oleoresin. Consuming the tomato products for 1 wk resulted in significantly higher levels of serum lycopene when compared to the placebo. Although no baseline (washout phase) measurements were made, the carryover effect is not important because plasma half-life of lycopene is about 2–3 d (24), and we allowed 1 wk washout during which no tomato products were consumed. Lycopene levels dropped significantly when the subjects consumed lycopene-free diet (16). Moreover, a randomized, cross-over study design provides for a balance in which the order of treatments was assigned to the subjects (25). The consumption of spaghetti sauce, containing much lower amounts of lycopene, resulted in an increase in serum lycopene almost identical to that with tomato oleoresin, which contained a higher lycopene amount, indicating a differential absorption rate for lycopene from different tomato products. These differences can probably be attributed to the differences in food processing. Lycopene from heat-processed tomatoes is more bioavailable than from fresh tomatoes (26).

Intake of tomato products protected the serum LDL cholesterol from oxidative damage and increased the serum lycopene levels. For oxidation analysis, the LDL were separated by precipitation following complexation with heparin at an isoelectric point. The technique was particularly chosen because of its simplicity and rapidity, which avoided artifactual oxidation during processing (27). Moreover, a highly significant correlation between precipitation method and ultracentrifugation or quantitative electrophoresis techniques for plasma LDL analysis had already been established (19,28).

TBARS and CD analyses were used to estimate serum LDL oxidation. Since TBARS assay as a measure of lipid oxidation lacks sensitivity and specificity, we modified the method in this study by using isolated LDL together with BHT during heating and butanol extraction of the pigment. These procedures contributed to increasing specificity and sensitivity of TBARS assay (22). Additional measurement of LDL oxidation was carried out by measuring total serum-TBARS and LDL-CD. The results obtained from these different analyses were in good agreement with each other. Highly significant correlations between LDL-TBARS and serum-TBARS and between LDL-TBARS and LDL-CD, as observed in this study, would further support the validity of using the TBARS test to measure oxidative stress. Our results indicate that both TBARS and CD are good indicators of LDL oxidation. Others reported similar results (29).

Several studies suggest that lipid peroxidation products are cytotoxic and genotoxic and play an important role in the etiology of several degenerative diseases (5–8,30–32). Oxidized LDL is involved in the formation of foam cells that eventually lead to the formation of arterial plaques (5,33), making oxidized LDL a risk factor for coronary heart disease. That the intake of lycopene lowered not only serum lipid peroxidation but also LDL oxidation may have an important physiological relevance in decreasing the risk for coronary heart disease.

In this study, the different tomato products were consumed in one or two servings per day for 1 wk. These consumption levels are easily achievable and are consistent with the current dietary guideline for healthful eating (34,35). Daily consumption of tomato products providing at least 40 mg lycopene was enough to alter LDL oxidation; however, intakes lower than this may also be effective. Future long-term studies should be undertaken in healthy human subjects as well as subjects at risk for coronary heart disease to obtain further information about the role of dietary lycopene in the prevention of the disease.

ACKNOWLEDGMENTS

We thank H.J. Heinz Co. of Canada and Hunt-Wesson Inc., USA for the supply of food products and support of the present work. Technical assistance from Charu Jain, Elena Speciani, and Zeeshan Waseem is deeply appreciated. We also thank Drs. Philip Conelly and David Jenkins for the serum lipid analysis and Dr. Letty Rao for critically reading the manuscript.

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[Received June 25, 1998, and in final revised form and accepted September 30, 1998]

Low pH Enhances the Transfer of Carotene from Carrot Juice to Olive Oil

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ABSTRACT: A current model for carotenoid transport and absorption in the gut proposes an initial solubilization in the oil phase of dietary emulsions followed by incorporation of the carotenoids in mixed bile salt micelles. To assess the relevance of the first stage of this model to what is observed *in vivo* we have examined the transfer of carotene from carrot juice to olive oil. Increased acidity enhanced the transfer from both whole juice and carotene crystals isolated from carrot chromoplasts. The transfer was significantly slower from whole juice. By using exogenous β-carotene and measuring its transfer to oil in the presence and absence of carrot juice we have demonstrated that the inhibition of the transfer in juice arises, at least in part, from soluble juice factors. The inhibition is relieved by a fall in pH, which leads to lowering of the electric potential at the oil/aqueous phase interface and aggregation of carrot tissue including crystalline carotene. Under conditions of low pH, oil droplets adhere to the tissue aggregates, allowing carotene to pass into the oil. Our results provide an explanation for why carotene absorption *in vivo* is depressed by conditions of low gastric acidity.

Lipids 33, 985–992 (1998).

There is evidence that carotenoids in plant foods are important not only as precursors of vitamin A but also for enhancement of cell-mediated immune responses (1,2) and for their antioxidant (3) and anticarcinogenic (4) potential. Therefore, there is interest in their bioavailability from plant foods and the mechanisms by which they are solubilized and transported in the gastrointestinal tract. *In vivo* studies have shown that the absorption of carotenoids from vegetables can be very low. For example, it may be as low as 1–2% from raw carrot (5), but cooking and small particle size usually lead to enhanced absorption (reviewed in Ref. 6).

The carotenoids fall into two classes: the xanthophylls, which have one or two polar groups, and the carotenes, which are completely apolar. The carotenes in plant foods such as carrots and tomatoes are present largely in crystalline form in membrane-bounded organelles, the chromoplasts (7). Given the stability of the crystalline state and the hydrophobicity of the carotenes, there is debate as to how they cross the aqueous phase in the gut for absorption into the body. It has been suggested (8) that they are solubilized in the oil phase of dietary emulsions and then transferred to mixed micelles before absorption into the enterocytes. If this hypothesis is correct, then the bioavailability of carotenes in the diet will be influenced by factors that control the transfer of the carotenes from the plant tissue into the oil phase. The aim of the work reported here was to examine one of these factors, the pH of the aqueous phase, using carrot juice as a source of carotenes.

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MATERIALS AND METHODS

Commercially available washed carrots were used and stored at 1–2°C at 98% humidity. Unless otherwise stated, the work reported in this paper was done on carrots which had been stored under these conditions for less than 2 mon. Nylon mesh with a nominal 63 μm hole size was from John Staniar & Co. (Manchester, United Kingdom). β-Carotene (95%) and olive oil (low acidity) were obtained from Sigma-Aldrich (Poole, United Kingdom). Spurr resin for embedding samples for electron microscopy was from Agar Scientific Ltd. (Stansted, United Kingdom). All other reagents were of Analar grade.

Carrots were peeled and the outer cortex juiced with a Moulinex juicer (Type CF1A). The juice was then filtered through one layer of nylon mesh (pore size 63 μm). For experiments using plastid-free juice, the juice was diluted to the required concentration with a solution containing 70 mM NaCl and 1 mM sodium azide and then centrifuged at 27,600 $\times g_{max}$ for 20 min at 4°C. The clear, straw-colored supernatant was used. Protein concentration in the plastid-free juice was measured by Bradford's microprotein assay (9).

A fraction enriched in chromoplast carotene was prepared from the carrot juice by differential centrifugation. A 40% dispersion of juice in 70 mM NaCl plus 1 mM sodium azide was centrifuged at 1300 $\times g_{max}$ for 20 min. The supernatant was recentrifuged at 7200 $\times g_{max}$. A centrifugation at 38,000 $\times g_{max}$ of the resulting supernatant yielded a pellet, the top of which was enriched in carotene crystals. These were washed in 20 vol of a solution that was 70 mM NaCl, 1 mM azide, 55 mM KCl and recentrifuged.

Carotene was measured by a modification of the method of Hart and Scott (10). Since α- and β-carotene are the major carotenoids in raw carrot, constituting 98.1 ± 0.4% (mean and standard deviation) of the total (the residue being 1.9 ± 0.4%

lutein) and α-carotene contributes only 25.0 ± 1.9% to this total (10), no attempt was made to distinguish between the carotenes. An aliquot of juice was vortexed with 1 mL *n*-hexane and 4 mL of tetrahydrofuran (THF)/methanol (MeOH) (1:1, vol/vol) containing 0.1% butylated hydroxytoluene. NaCl (25%) and water were then added to bring the aqueous NaCl concentration and volume to 10% and 1 mL, respectively. After mixing, the carotene extracted into the hexane phase was calculated from the absorbance at 450 nm using a Perkin-Elmer lambda 9 spectrophotometer. Calibration with known amounts of β-carotene gave the effective extinction coefficient, $\varepsilon^{1\ cm} = 0.152$ AU/μg. (Concentration = $A^{450}/(0.152 \times 537 \times V)$ μmoles/mL where V = juice volume in mL).

Transfer of carotene from juice to olive oil was measured in 50-mL, stoppered Quickfit flasks using 30 mL aqueous phase and 6 mL olive oil. The area of the undisturbed oil/water interface was 16 cm^2. The aqueous phase contained the juice at the required concentration, 1 mM sodium azide, and 70 mM NaCl to make up the volume to 30 mL. The mixture was adjusted to the required pH by the addition of HCl or NaOH solutions. It was pre-equilibrated for 20 min at 37°C in a Gallenkamp Plus II incubator with the shaking platform in orbital mode (setting 5.5). The oil was then added and the flasks returned to the shaking incubator. Samples of the oil phase (30–60 μL) were taken at intervals, centrifuged briefly at 10,000 × *g* to remove any entrained aqueous phase and carrot tissue. Carotene was measured by diluting aliquots of the oil (10 to 50 μL) into 1 mL *n*-hexane and measuring the absorbance at 450 nm using a mM extinction coefficient of 137.4 (10) .

In experiments where transfer of exogenous β-carotene was measured, crystalline β-carotene was dispersed in 20 mL oxygen-free water to a concentration of 1.6 to 2.3 mM by sonication using an MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, Sussex, United Kingdom) and a 9.5-mm probe at maximum power with 16 cycles of 15 s power on, 30 s power off. This gave a β-carotene dispersion, which by light microscopy was shown to have particles of size ranging from ≤0.4 to about 4 μm. The dispersion could be conveniently diluted into the experimental systems.

Carrot juice that had been incubated with oil was examined by light (including polarized light) microscopy. To assess quantitatively the adherence of oil drops to carrot tissue we used a hemacytometer (a calibrated counting chamber of known volume).

For transmission electron microscopy, samples were fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 3 h then centrifuged and resuspended in buffer three times. The samples were then centrifuged and the sediment mixed with just molten agarose which was quickly cooled in ice. The samples were then chopped into small cubes and postfixed in 1% aqueous osmium tetroxide for 4 h, dehydrated through an ethanol series, transferred to acetone, and then infiltrated and embedded in Spurr resin. Sections approximately 80 nm thick were cut with a diamond knife and collected on copper grids. After sequential staining with uranyl acetate (saturated solution in 50% aqueous ethanol) and lead citrate (11), the sections were examined and photographed in a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan).

The ζ potentials of olive oil droplets dispersed in plastid-free juice were measured with a Rank Brothers Mark II particle microelectrophoresis apparatus (Rank Brothers, Bottisham, Cambridge, United Kingdom) at 22–25°C. Olive oil (200 μL) was dispersed in 8 mL of the aqueous phase (adjusted to the required pH) by vortexing and the resulting emulsion transferred to a rectangular electrophoresis cell for mobility measurements. The ζ potentials were calculated from the Smoluchowski equation (12) with appropriate corrections for temperature variation effects on the dielectric constant and viscosity.

RESULTS

Particle size. Preliminary experiments indicated that, although the amount and rate of carotene transport from carrot tissue into oil was quite variable, one important controlling factor was particle size. Figure 1 shows a typical experiment illustrating the particle size effect. Here, carrot juice was filtered through a 63-μm mesh. The residue on the mesh was washed with a solution of 70 mM sodium chloride + 1 mM sodium azide until the washings were clear and colorless. The washings were combined with the filtrate, and transport of carotene from this mixture to oil was compared with that from the residue dispersed in 70 mM NaCl so that both contained the same concentration of carotene. It is clear that larger particles give less transport, particularly at longer times. To stan-

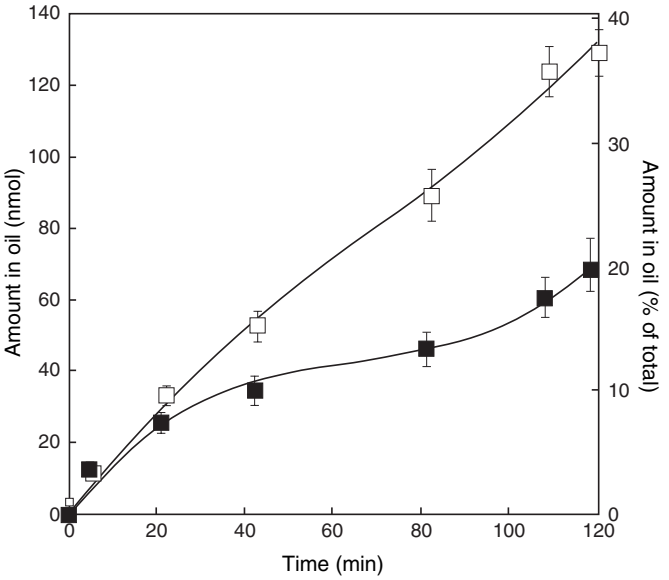

FIG. 1. The effect of particle size on carotene transfer from carrot juice to olive oil. The pH of the aqueous phase was 2.1, and the initial concentration of carotene 11.6 μM. (□) Juice filtered through 63-μm nylon mesh; (■) unfiltered residue from the filtration. The points represent the means of two experiments and the error bars the range of values.

dardize the particle size, subsequent work described in this paper used juice filtered through the 63-μm mesh.

Nature of the filtered juice. Electron microscopy showed that the filtered juice consisted largely of cell contents. There was extensive vesiculation. Some intact chromoplasts were present as well as carotene crystals both free and membrane-bounded. Following the terminology adopted by Ben-Shaul and Naftali (13) who described the ultrastructure of lycopene bodies in tomato chromoplasts, in this paper the following definitions will be used: "carotene crystal," a free crystal from a carrot chromoplast; "carotene body," the native carotene structure in carrot chromoplasts that is membrane-bounded; "chromoplast," the organelle containing the carotene bodies.

Concentration dependence of transport. Transport of carotene depended on the amount of filtered juice in the aqueous phase. Figure 2 shows the results when transport was measured at four different dilutions of the carrot juice. There is an approximately linear relationship between transport and the concentration of carotene (see the insert to Fig. 2).

pH dependence of transport. Low pH enhanced carotene transfer into oil. Figure 3 summarizes the results where transport into the oil was measured at two pH values (pH 2–2.4 and pH 6.2–6.6) after 20 min, and in most cases after 1 h and 2 h. In all these experiments the initial carotene concentration in the aqueous phase was 95 μM. It can be seen from Figure 3 that although low pH invariably leads to enhanced carotene transfer the magnitudes of the effect and the absolute values of carotene transfer are variable.

Reversibility of pH effect on transport. Since low pH could lead to breakdown of some component(s) affecting transport, we explored the reversibility of low pH treatment. Filtered juice mixtures were pre-incubated for 1 h at pH 2 and 6. One of the low pH mixtures was then adjusted to pH 6 and transport measured in this system and compared with transport measured in mixtures that were kept at the same pH as in the pre-incubations. Table 1 shows two representative experiments: the first one on carrots that had been stored for less than 2 mon, and the second one done on carrots that had been stored for more than 4 mon, when our macroscopic and microscopic observations (see below) indicated some changes in the properties of the carrot juice. It is clear from the data for carrots stored for the shorter time that low pH treatment does not affect transport subsequently measured at high pH. However, with the older carrots the effect of lowering the pH in the pre-incubation was irreversible. We have also observed the intermediate situation where there is partial reversal of the effect of low pH pretreatment.

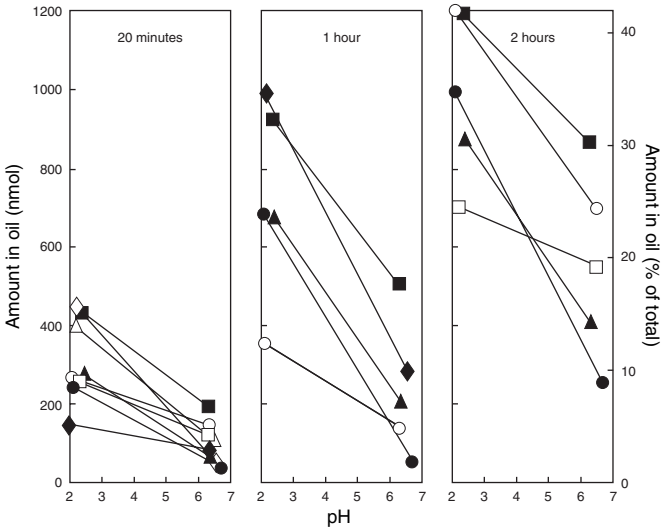

FIG. 3. Summary of carotene transfer from carrot juice to olive oil at low pH (2.03 to 2.37) and high pH (6.27 to 6.70). Each line joins the amount of transfer measured at 20 min, 1 and 2 h at the two pH values from the same batch of juice and does not represent the amount of transfer at any intermediate pH (see Fig. 6). Each experiment is given a different symbol. The initial concentration of carotene in the aqueous phase was 94 μM.

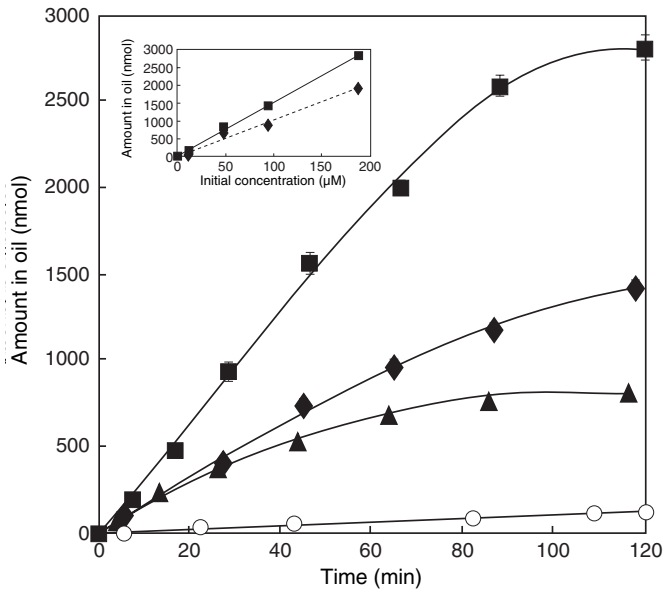

FIG. 2. The effect of concentration of carrot juice on carotene transfer to olive oil, pH 2.1. The initial concentrations of carotene in the aqueous phase were ■ 188, ◆ 94, ▲ 47, and ○ 11.6 μM. The error bars represent the range of amounts transferred measured for two replicates of the experiment. Transfer at the two lower concentrations was measured only once. The insert shows the data plotted as a function of the initial concentration of carotene in the aqueous phase, ■ at 2 h and ◆ at 1 h.

TABLE 1
The Reversibility of the pH Effect on Carotene Transport from Carrot Juice

	Carrots stored less than 2 mon		Carrots stored more than 4 mon	
	% Transferred at 20 min[a]	% Transferred at 60 min[a]	% Transferred at 20 min[b]	% Transferred at 60 min[b]
pH 2.1 throughout	9.2 (0.2)	17.2 (0.9)	10.7 (0.1)	21.8 (1.2)
pH 2.1–pH 6.2	4.9 (0.1)	9.5 (0.2)	11.2 (0.2)	21.7 (0.1)
pH 6.2 throughout	5.6 (0.5)	10.5 (0.7)	5.7 (0.2)	16.9 (0.7)

[a]Mean (range of values) for two replicates where the initial concentration of carotene in the aqueous phase was 95 μM.
[b]Mean (standard deviation) for three replicates where the initial concentration of carotene in the aqueous phase was 63.9 μM.

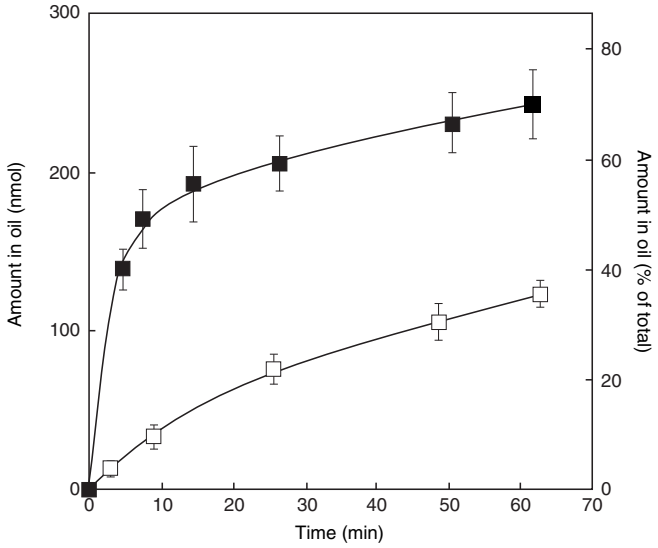

FIG. 4. Carotene transfer from isolated chromoplast measured at pH 2.1 ± 0.1 (■) and pH 6.5 (□). The initial carotene concentration in the aqueous phase was 11.6 μM. The error bars represent the SD in three experiments.

Partitioning of carotene from a fraction enriched in carotene crystals and carotene bodies. Figure 4 shows the rate of uptake of carotene from chromoplast carotene isolated from carrot juice. It is apparent that the partitioning is faster than that observed from carrot juice (compare Fig. 4 with Fig. 1). However, the results resemble those from carrot juice in that the partitioning is pH-dependent, being greater at low pH.

Effect of pH and carrot juice on exogenous β-carotene transport. The studies of transport using isolated chromoplast carotene from carrot showed that the process is inherently pH-sensitive. However, further work showed that the situation with carrot juice is rather more complicated and that there are factors in the carrot tissue that inhibit carotene transport, the inhibition being relieved by lowering the pH. This was shown by the following experiments: we dispersed pure β-carotene in an aqueous phase without juice and measured its rate of transport to oil at pH 2 and 6 (see Fig. 5a). The rate of transport of the β-carotene is independent of pH and significantly faster than endogenous carotene transfer from carrot juice. Then we added exogenous β-carotene at the same concentration to carrot juice and measured the transport of the added β-carotene plus the endogenous carotene and compared this transport to that measured with carrot juice alone. The results of the experiment are shown in Figure 5b. By fitting third-order polynomials to the data and subtracting the curves for endogenous carotene transport from those for exogenous plus endogenous carotene transport we could calculate the effect of the juice on the exogenous β-carotene transport. The calculated data are shown in Figure 5a. It is clear that the exogenous β-carotene transport is slowed by the presence of carrot juice and is enhanced by low pH.

Correlation of carotene transport with ζ potential of the oil. Transfer of carotene from the carrot juice to the oil phase involves penetration through the oil–water interface, which when unmodified is negatively charged (14). Therefore, we considered it likely that the effect of pH on carotene transport could be due to alterations in the electric potential at the

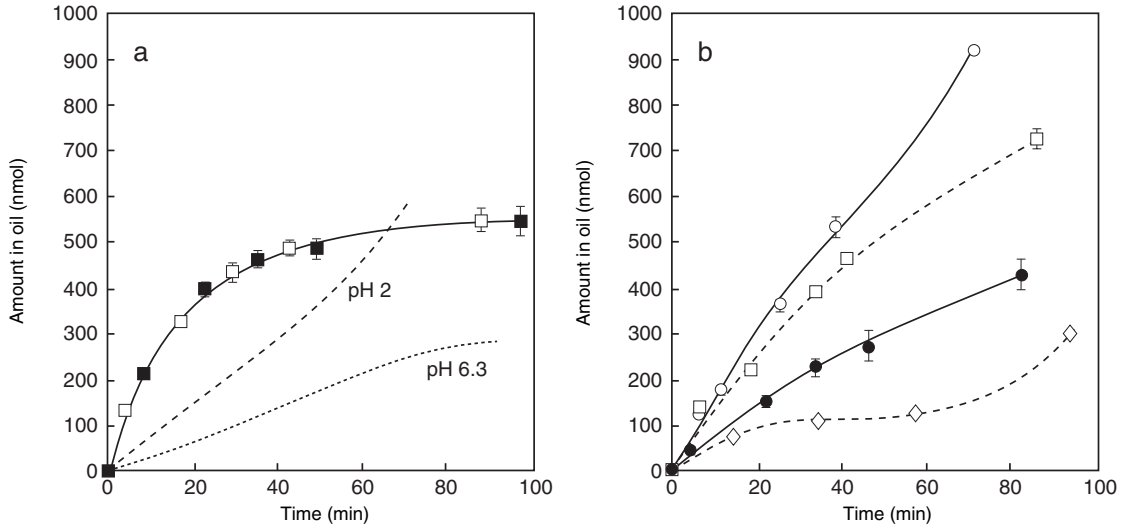

FIG. 5. Inhibition of exogenous β-carotene transfer to olive oil by carrot juice. (a) β-Carotene transfer to oil in the presence and absence of carrot juice. Transfer was measured at pH 2 (□) and pH 6.3 (■) from a solution of 70 mM NaCl, 55 mM KCl, and 1 mM sodium azide containing initially 25.3 μM β-carotene. The dotted lines show the transfer to oil of exogenous β-carotene added to carrot juice at pH 2 and pH 6.3 calculated from the data in b (see text for details). (b) Transfer of exogenous β-carotene and carotene from carrot juice to olive oil at pH 2 and 6.3: solid lines show data when exogenous β-carotene was added to carrot juice and the total amount of β-carotene and endogenous carotene from carrot juice was measured from an aqueous phase containing initially 25.3 μM added β-carotene + 47.5 μM carotene in juice (○) at pH 2, (●) at pH 6.3. The dotted lines show the amount of carotene transfer without added β-carotene (□) at pH 2, (◇)at pH 6.3.

oil/carrot juice mixture interface. A close approximation to the potential at an oil/aqueous phase interface is given by the ζ potential (15). This is the potential at the plane of shear as oil droplets move through the aqueous phase and is related to the electrophoretic mobility (12).

In three batches of carrot juice which were giving similar proportional enhancement of carotene transfer to oil with lowered pH, we measured the amount of transfer after 20 min incubation as a function of pH and the ζ potential of olive oil droplets dispersed in plastid-free juice. Figure 6 shows the results. Here the carotene concentration in the oil at each pH is expressed as a percentage of the concentration measured at pH 2. This normalization procedure obviates the difficulty of variability in the absolute amount of carotene transport between experiments.

Interpretation of the ζ potential against pH curve is complicated since potentially the contribution of changing adsorption at the interface as well as protonation of adsorbed ionizable group at the interface has to be considered. What is significant in the context of carotene transfer to oil is that, although the shapes of the curves of carotene transport as a function of pH, and ζ potential as a function of pH, have similar shape, they cannot be superimposed on the pH axis. Therefore, although it is possible that some soluble component in the carrot juice is important in determining the pH dependence of surface charge at the interface and hence ζ potential, the latter cannot be the only important factor in determining transfer of carotene into the oil phase.

Studies precluding solubilized carotene being the partitioned form of carotene. Plastid-free juice prepared at pH >6 was straw-colored. As the pH was lowered, the yellow color was progressively lost. To quantify this effect we incubated undiluted filtered juice for 20 min over a pH range from 2.42 to 6.20 and then prepared plastid-free juice samples and measured their absorbances at 450 nm. Table 2 shows the results together with the soluble protein concentrations. Both the absorbance and protein concentration fell as the pH was lowered. This loss of soluble protein and carotenoid was largely irreversible: raising the pH of juice incubated at pH 2.42 to 6.32 did not restore the original protein concentration or absorbance value in the plastid-free juice (see Table 2). Since carotene transport is highest at low pH when soluble carotenoid is at its lowest, it seems unlikely that this is the form in which it reaches the oil.

Macroscopic and microscopic observations. Light microscopy showed that the aqueous phases during and after incubations with oil contained oil droplets, some of which had orange crystals located at their surface. It was not possible to tell by light microscopy whether these crystals were actually carotene bodies. Lowering the pH precipitated cellular material containing the crystals (Fig. 7a). This precipitate was invariably present when carotene partitioning was high: for example, when at high pH after low pH treatment the low pH enhancement of transport was not reversed. The precipitate sedimented quite rapidly under $1 \times g$ and could be collected from the bottom of the flask after all the other material had been aspirated away. Entrained in the precipitate were oil droplets (arrows in Fig. 7a) associated with aggregates of cell contents with large amounts of adhering orange crystals (arrowheads). Material in juice at pH >6 sedimented overnight. This material was more diffuse with few aggregates and included many freely floating carotene crystals/bodies (arrowheads in Fig. 7b), which were absent in low pH sediments where carotene crystals were invariably associated with aggregated cellular material. Another distinct difference between low pH sediments and the aqueous phases from which they were prepared was the larger number of oil drops seen at low pH. Using a hemacytometer we found the ratio of the number of oil droplets per milliliter of juice incubated with

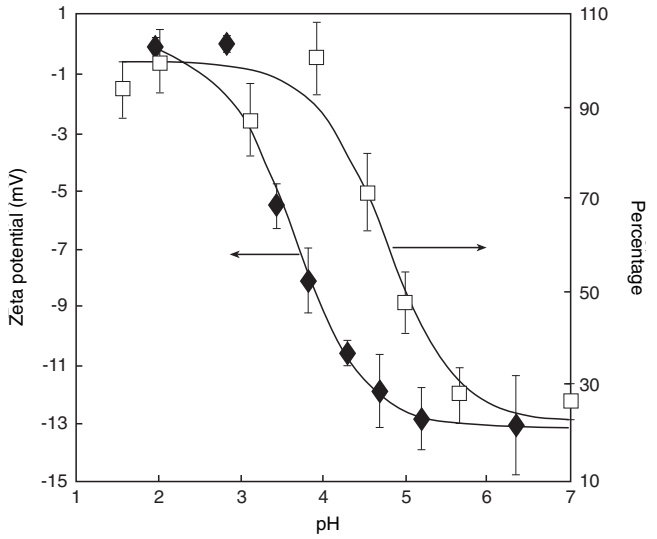

FIG. 6. The ζ potential (left-hand axis) of olive oil droplets dispersed in plastid-free carrot juice as a function of pH (◆). The solid line represents the equation $\zeta = -13.27 \times 10^{pH}/(10^{3.7} + 10^{pH})$ and is the best fit to the data. Also shown (right-hand axis) is the relative amount of carotene transport from carrot juice to olive oil as a function of pH (□). The amount transferred is expressed as a percentage of that measured at pH 2. The solid line is $\% = 100 - (78.06 \times 10^{pH})/(10^{4.8} + 10^{pH})$ and is the best fit to the data. The initial concentration of carotene in the aqueous phase was 94 μM.

TABLE 2
The Protein Concentration and Absorbance of Plastid-Free Juice as a Function of pH[a]

pH	Protein concentration (mg/mL)	$A_{450}{}^{1\ cm}$
6.20	0.73	1.01
5.36	0.40	0.252
4.45	0.094	0.029
3.30	0.044	0.005
2.42	0.034	0.007
2.42 → 6.32[b]	0.097	0.005

[a]Whole juice containing 1 mM sodium azide was incubated for 30 min at 37°C at the indicated pH values. Plastid-free samples were then prepared for protein and absorbance measurements.
[b]Juice incubated at pH 2.42 was adjusted to pH 6.32 and incubated for a further 15 min.

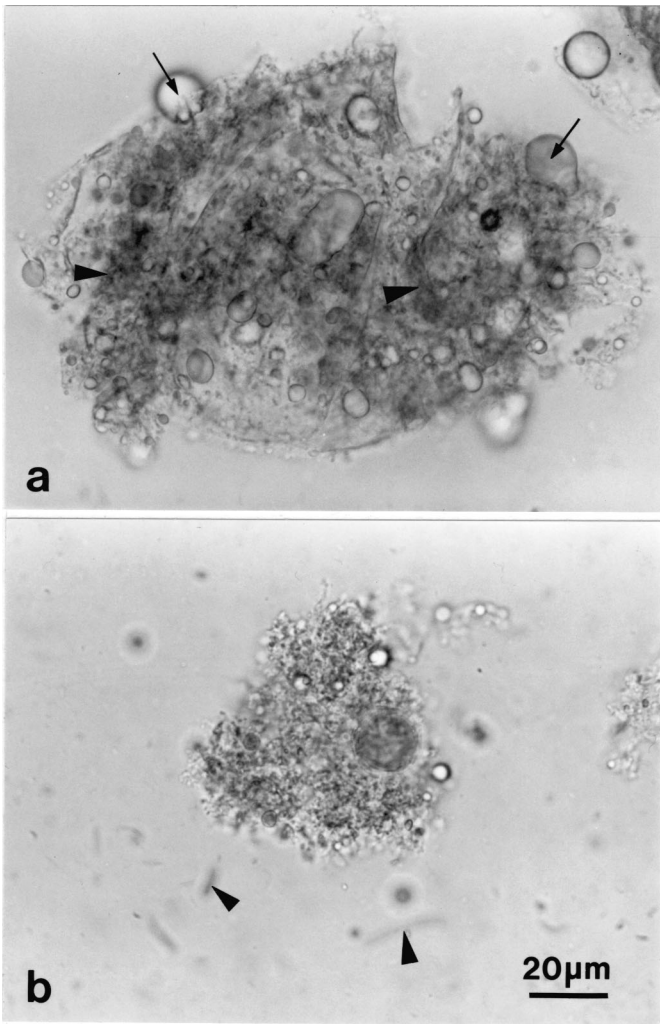

FIG. 7. Light microscopy of carrot juice incubated with olive oil. (a) Typical aggregate of carrot cell material seen at low pH (pH 2) showing entrained oil droplets (arrows) and carotene crystals/bodies (arrowheads). Note the absence of free crystals in the background surrounding the aggregate. (b) A rare aggregate seen at pH 6 surrounded by many free carotene crystals/bodies (arrowheads).

oil at pH 2.16 compared to the number at pH 6.24 was 3.0 ± 0.6. However, when we only counted droplets that were attached to carrot material this ratio rose to 10.0 ± 1.3.

The location of exogenous β-carotene in carrot juice at low and high pH was also examined. At low pH (2.17) the added β-carotene crystals were found entirely on aggregated cell contents. In contrast, at pH 6.32 the β-carotene crystals were uniformly distributed. On the rare aggregates that were present, the density of crystals was no higher than that found floating free. Thus, the location of added β-carotene crystals at different pH values mimics that of the endogenous carotene.

Electron microscopy confirmed and amplified these findings. Sediment from untreated filtered juice (Fig. 8a) appeared very similar to the sediment from filtered juice incubated with oil at pH 6.3 (Fig. 8b). Distinctive features in both were the presence of vesicles less than 1 μm in diameter as well as plastoglobuli and electron-opaque carotene crystals

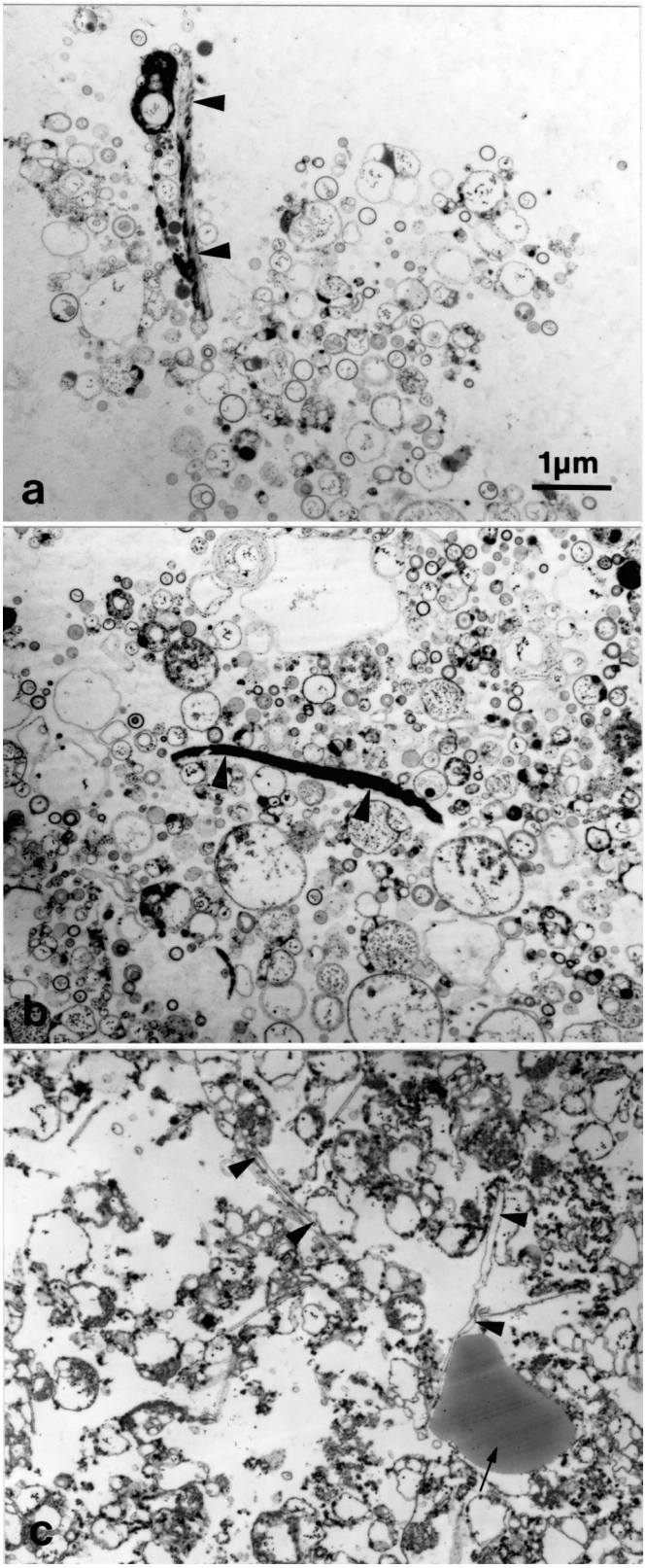

FIG. 8. Transmission electron micrographs of filtered carrot juice. (a) Untreated, (b) incubated with oil at pH 6.3, (c) incubated with oil at pH 2.2. Arrowheads in a and b indicate carotene bodies, and in c, carotene body membranes from which the carotene has been eluted. A distorted oil droplet is arrowed in c.

and carotene bodies (indicated by arrowheads in the figures), which had been released from the chromoplasts. There were no olive oil droplets visible. In contrast, filtered juice incubated with oil at pH 2.2 (Fig. 8c) showed the presence of adhering oil droplets (arrow in figure) and densely staining precipitated material (probably largely protein) concentrated around membranes. There were no electron-opaque carotene crystals, but there were needle-shaped membranous vesicles (indicated by arrowheads) which we have interpreted as carotene body membranes from which the carotene had been extracted by either the incubation with oil or the ethanol dehydration. Oil drops were frequently distorted and lying adjacent to the carotene body membranes.

DISCUSSION

The absorption of lipid-soluble vitamins from the gut is enhanced by acidic conditions (16–18) and by the presence of fat in the diet (19,20). Further, others have shown (21), using omeprazole to suppress gastric acid secretion in human subjects, that after a liquid meal containing fat and β-carotene there is an increased serum concentration of *trans* and *cis* β-carotene at low gastric pH compared to high gastric pH. That there is reduced uptake of β-carotene from consumption of carrot material compared to that when β-carotene is given in purified supplements has been shown in both humans (22) and ferrets (23).

The results presented in this paper suggest that these observations can be explained if an important step for absorption is partition of the carotene into an oil phase. In using either juice or a fraction of juice enriched in carotene, this partition is pH-dependent, being higher at low pH. In using purified β-carotene alone, partition is independent of pH. Further, the rate at which carotene partitions is much higher using β-carotene alone than carotene in carrot juice. However, if carrot juice is added to exogenous β-carotene, the β-carotene partitioning becomes pH-dependent and the rate of partitioning falls at both low and high pH. Our observations by microscopy indicate that the extent of precipitation of aggregated cell contents and the adherence of either carotene crystals/bodies or β-carotene crystals to these aggregates correlate with the amount and rate of transport into the oil.

There is a possibility that carotene in carrot juice could reach the oil phase as a water-soluble carotene–protein complex. Such a complex has been isolated from an enriched chromoplast fraction from carrot tissue (24). However, the low concentration of solubilized carotene and loss of protein in the aqueous phase (especially at low pH) during our experiments argues against solubilized carotene as the partitioned species. In this context it is significant that carotenoids are known to be unstable under acid conditions (25). Furthermore, our spectroscopic and high-performance liquid chromatographic studies (Rich, G.T., Fillery-Travis, A., and Scott, K.J., unpublished data) indicate that the noncrystalline, soluble components in carrot juice contain only trace amounts of carotene.

Our light microscope observations are consistent with the carotene in carrot juice passing directly into the oil phase from either carotene crystals or carotene bodies. We have noticed isolated carotene crystals/bodies adhering to oil–water surfaces. The concentration of crystals and oil droplets on precipitated cell components at low pH therefore provides a mechanism for the low-pH enhancement of carotene transport into the oil. It is a physical process whereby the crystals and oil droplets are brought close together so the carotene can pass through the oil–water interface and dissolve in the oil. The low ζ potential of the oil droplets at low pH aids this process. At pH values approaching neutrality, when the ζ potential is much larger, there will be significant repulsion between the oil droplets and the negatively charged cellular material.

We have shown the importance of particle size in determining carotene transfer into oil. This parallels other studies (5) showing that finely chopped vegetables give higher absorption of β-carotene compared to whole or sliced vegetables. However, organelle structures sequestering the carotene crystals cannot be the only factors in carrot juice inhibiting carotene transfer, because purified β-carotene partitioning is also inhibited by carrot juice. A possible candidate for this inhibitory factor is fiber. For example, various types of dietary fiber reduce the bioavailability of β-carotene in chicks (26). A similar effect has been shown with humans where pectin added to a meal containing purified β-carotene reduced the plasma β-carotene response (27). In general, the effect of high-fiber diets is to increase the fecal excretion of lipids (28) and carotene (29). This has been attributed to high-viscosity conditions limiting the absorption. Raw carrots contain about 2.5% dietary fiber of which 32 to 48% is soluble and includes pectin (30,31). It is possible that this is acting at the oil surface in our experiments inhibiting transfer of carotene into the oil. This idea is consistent with the rapidity with which carotene isolated from carrot juice partitions into the oil compared to the rate when juice is used. A similar effect has been observed by Zhou, Gugger, and Erdmann (32) who showed that ferrets fed carrot chromoplasts had higher serum and tissue carotene concentrations than ferrets fed carrot juice. Both the chromoplast isolation process and our preparation of chromoplast carotene involve washing which would remove soluble fiber. It is also possible that acid conditions cause some degradation of pectin which would contribute to the enhancement of carotene transport at low pH.

Our observations that the pH dependence of partitioning of carotene from carrot juice into oil mimics what is observed *in vivo* for carotene absorption from the gut contents suggest that an important step in chromoplast carotene absorption is solubilization in the gastric oil phase. This first stage is followed by solubilization in mixed bile salt micelles, whose concentration in the duodenum is stimulated by fat in the diet. A number of factors influence the solubility of carotene in mixed micelles (reviewed in Ref. 33). Important in the context of the present work is the observation that below pH 5 the solubility of carotene in mixed micelles decreases with a decrease in pH (34). We have suggested that fiber in carrot

juice inhibits carotene partitioning into oil. Similarly, this putative factor could also inhibit solubilization of carotene in bile salt micelles.

ACKNOWLEDGMENTS

This work was supported by a ROPA award to Annette Fillery-Travis and in part by the European Union, DG XII: FAIR project CT97-3100. We thank K. John Scott for high-performance liquid chromatography analysis and Sue Southon and Richard Faulks for helpful discussions.

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[Received June 22, 1998, and in final revised form and accepted September 21, 1998]

Effects of Feeding *Lunaria* Oil Rich in Nervonic and Erucic Acids on the Fatty Acid Compositions of Sphingomyelins from Erythrocytes, Liver, and Brain of the Quaking Mouse Mutant

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ABSTRACT: Feeding an oil from *Lunaria biennis* rich in 22:1n-9 and 24:1n-9 to homozygous quaking (qk.qk) mice caused a large increase in the percentage of 24:1n-9 and corresponding decreases in the percentage of 24:0 and 22:0 in sphingomyelins from liver, erythrocytes, and milk. Brain sphingomyelin from 2-wk-old qk.qk pups born to qk.qk mothers maintained on the *Lunaria* oil had essentially normal percentage of 24:1n-9 and 18:0, in contrast to pups born to mothers maintained on a control oil rich in 18:1n-9 whose brain sphingomyelin had a markedly reduced percentage of 24:1n-9 and an increased percentage of 18:0. After 2 wk and up to and beyond weaning, the qk.qk pups from *Lunaria*-fed mothers weaned on to the *Lunaria* diet had a markedly decreased percentage of 24:1n-9 in their brain sphingomyelin, accompanied by an increased percentage of 18:0, as compared to heterozygous quaking mice. However, the percentage of 24:1n-9 in brain sphingomyelin in qk.qk pups weaned on to the *Lunaria* diet continued throughout this period (2–8 wk postbirth) to be significantly higher than in qk.qk pups weaned on to the control diet. We conclude that dietary 24:1n-9 influences the fatty acid composition of brain sphingomyelin in qk.qk mice, but only *via* the mother in pre- or early postnatal animals. We further consider that the dietary effects may be elicited mainly in the sphingomyelin of nonmyelinated brain cells, and that the nervonic acid in myelin sphingomyelin may be formed mainly by chain elongation in oligodendrocytes from shorter chain fatty acid precursors.

Lipids 33, 993–1000 (1998).

The "quaking" mouse is a recessive autosomal mutant characterized by deficient myelination of the central nervous system (1). Lipids of the central nervous system of quaking mice have markedly reduced levels of cerebrosides and sulfatides, reduced levels of the major phosphoglycerides, and unaltered levels of sphingomyelin (1–3). Simultaneously, the brain cerebrosides, sulfatides, and sphingomyelin of quaking mice have markedly decreased levels of gadoleic acid (20:1n-9), behenic acid (22:0), lignoceric acid (24:0), and nervonic acid (24:1n-9) (2,4). Brain microsomes from quaking mice have a markedly reduced ability to convert behenyl CoA to lignocerate (5–7), due to a markedly reduced condensing enzyme activity (with both 20:0 and 22:0 CoA as substrates) (8).

Recently the quaking gene was cloned and shown to be a multifunctional gene involved in a specific set of developing tissues to link signal transduction with some aspect of RNA metabolism (9,10). Thus, the abnormalities of brain lipid metabolism in quaking mice, specifically the markedly reduced capacity to form lignoceric and nervonic acid from shorter chain precursors, are but one of a variety of outcomes of the quaking mutation. Nonetheless, the quaking mouse mutation remains a useful model system for investigating aspects of brain lipid metabolism, including influences of dietary-administered fatty acids on the fatty acid compositions of brain sphingolipids. The present study was undertaken to determine the extent to which long-chain monoenoic fatty acids, specifically 22:1n-9 and 24:1n-9, administered in the diet can access the brain of quaking mice and thereby modify the fatty acid composition of brain sphingomyelin.

MATERIALS AND METHODS

Animals and diets. Heterozygous quaking mice (qk.+), i.e., phenotypically normal animals, were purchased from the MRC Radiobiology Unit, Didcot, Oxfordshire, United Kingdom, and maintained on a standard laboratory chow supplied by Special Diet Services, Witham, Essex, United Kingdom. Homozygous quaking mice (qk.qk), i.e., animals expressing the quaking symptom, were bred from the heterozygous stock and weaned directly on to either a control diet or a test diet. These diets were purchased from ICN Biomedicals Ltd., Thame, Oxfordshire, United Kingdom, and consisted of the standard ICN diet AIN-93G (for growth, pregnancy, and lactation of rodents), which contains 7% w/w soy oil, supplemented with a further 3% w/w of either a high-oleic acid sunflower oil to produce the control diet, or an oil extracted from

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Abbreviations: GC, gas chromatography; HPTLC, high-performance thin-layer chromatography; qk.+, heterozygous quaking mice; qk.qk, homozygous quaking mice.

TABLE 1
Fatty Acid Compositions of the Test and Control Diets[a]

Fatty acid	Control diet	Test diet
16:0	10.8	8.0
16:1n-7	0.6	0.4
18:0	3.5	2.9
18:1n-9	38.8	22.3
18:2n-6	39.3	38.4
18:3n-3	5.4	5.4
20:0	0.8	0.7
20:1n-9	0.8	1.0
22:1n-9	0.0	13.8
24:0	0.0	0.0
24:1n-9	0.0	6.8

[a]Data are expressed as wt% of total fatty acids. The control diet contained 7% soy oil + 3% of a high-oleic acid sunflower oil. The test diet contained 7% soy oil + 3% of an oil from *Lunaria biennis.*

Lunaria biennis to produce the test diet. Both the high-oleic acid sunflower oil and the *Lunaria* oil were supplied by Croda Chemicals, Hull, Humberside, United Kingdom. The test diet differed from the control diet by having a reduced percentage of 18:1n-9 and correspondingly increased percentages of 22:1n-9 and 24:1n-9 in its lipid component (Table 1). Quaking mice pups were weaned, 19 d after birth, randomly on to the two diets, such that the sex ratios of the two groups were the same, and the mice were maintained on the two diets until they reached sexual maturity. A second generation of qk.qk mice was then bred from the first generation of qk.qk females and qk.+ males (as noted by other authors, qk.qk males did not breed successfully with qk.qk females), and the second-generation qk.qk pups were continued on the same diets as their qk.qk mothers. The results presented here for liver and blood are from first-generation mice and for brain and milk from second-generation mice. Groups of animals were sacrificed at times specified in individual Figures and Tables, and samples of brain, liver and heparinized blood were taken for analyses. Erythrocytes were sedimented from blood by centrifugation and washed twice in 0.9% wt/vol NaCl. Samples of liver, brain, and erythrocytes were either processed for analysis immediately, or frozen at −70°C and processed for analysis within weeks, with the same result. Milk was collected from lactating qk.qk mothers by manual expression after anesthetizing them and promoting milk flow by injecting oxytocin.

Lipid extraction and analyses. Total lipids were extracted from samples of brain, liver, erythrocytes, and milk by the method of Folch *et al.* (11). Lipid extracts were stored at –20°C under nitrogen between procedures. Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) of total lipids on silicic acid, using methyl acetate/propan-2-ol/chloroform/methanol/0.25% w/w aq. KCl (25:19:25:10:9, by vol) as solvent and copper acetate–phosphoric acid as a charring stain, and scanning densitometry performed on a Shimadzu CS-9000 instrument (Tokyo, Japan) (12). Sphingomyelin was routinely isolated from all tissues following mild alkaline transesterification of total lipid, so as to convert *O*-acyl glycerides into fatty acid methyl esters and water-soluble products, while leaving *N*-acyl sphingolipids intact (13). Sphingolipids and fatty acid methyl esters were extracted from the alkaline transesterification reaction medium after adding chloroform and methanol (14), and sphingomyelin isolated by HPTLC in methyl acetate/propan-2-ol/chloroform/methanol/0.25% w/w aq. KCl (25:19:25:10:9, by vol) as solvent. Triacylglycerols and phosphoglycerides (comprising phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) were isolated from milk total lipid in the same solvent. Individual lipid classes were detected on thin-layer plates by staining with 0.1% 2′,7′-dichlorofluorescein in 97% methanol containing 0.01% (wt/vol) butylated hydroxytoluene, eluted from the plates with chloroform/methanol (2:1, vol/vol) and dried under nitrogen prior to preparing fatty acid methyl esters for gas chromatographic (GC) analyses. Fatty acid methyl esters were prepared from sphingomyelin by reacting with 1% sulfuric acid in methanol for 24 h at 60°C, and from phosphoglycerides and triacylglycerols by reacting with 1% sulfuric acid in methanol for 16 h at 50°C. Fatty acid methyl esters were purified by HPTLC using hexane/diethyl ether/acetic acid (80:20:2, by vol) as solvent and analyzed by capillary GC using a Canberra-Packard 436 instrument (Berkshire, United Kingdom) fitted with a Sil 5CB column (50 m × 0.32 mm; Chrompack, London, United Kingdom) with hydrogen as carrier gas and a thermal gradient from 50 to 260°C (14). Individual fatty acid methyl esters were identified by comparison with authentic standards and by GC–mass spectrometry using a Fisons MD 800 instrument fitted with a DB-5MS column (15 m × 0.25 mm; J&W Scientific, Folsom, CA) with helium as carrier gas.

Statistical analyses. Data are expressed as means ± 1 SD with the total number of individual determinations (each on a separate animal) in parentheses. Significance of difference between dietary treatments was determined by Student's *t*-test.

RESULTS

The diet containing the test oil, which was rich in both erucic acid (22:1n-9) and nervonic acid (24:1n-9), readily altered the fatty acid compositions of sphingomyelin from erythrocytes and liver, as compared to the control diet, which was rich in oleic acid (18:1n-9) (Table 2). In the present study, mice were routinely weaned on to the experimental diets 19 d after birth. Effects of the two diets on liver and erythrocyte sphingomyelin were readily apparent 1 or 2 wk after weaning. The data in Table 2 show the maximal changes that occurred by 8 wk post-birth, i.e., *ca.* 5 wk post-weaning. Erythrocyte sphingomyelin had the same fatty acid composition in nonsymptomatic, qk.+ animals and symptomatic, qk.qk animals fed the control diet, with 22:0, 24:0, and 24:1n-9 being the major fatty acids, accounting for *ca.* 15, 30, and 20%, respectively, of the total fatty acids. The same situation held for livers of qk.+ and qk.qk mice fed the control diet except that 22:0, 24:0, and 24:1n-9 accounted for *ca.* 30, 25, and 15%, respectively, of the total fatty acids. Feeding the *Lunaria* oil rich in 22:1n-9 and 24:1n-9

TABLE 2
Effects of the Control and Test Diets on the Fatty Acid Compositions of Sphingomyelin from Erythrocytes and Liver in Heterozygous (qk.+) and Homozygous (qk.qk) Mice[a]

	Control diet		Test diet
Fatty acid	qk.+ Mice	qk.qk Mice	qk.qk Mice
Erythrocytes			
20:0	1.9 ± 0.1	1.7 ± 0.0[a]	0.5 ± 0.4[b]
22:0	15.4 ± 1.0	15.2 ± 0.5[a]	5.5 ± 0.4[b]
22:1n-9	0.2 ± 0.2	0.1 ± 0.1[a]	0.4 ± 0.1[b]
23:0	2.0 ± 0.1	1.9 ± 0.1[a]	1.0 ± 0.0[b]
24:0	30.7 ± 1.2	28.9 ± 0.3[a]	11.8 ± 1.9[b]
24:1n-9	18.2 ± 0.4	21.7 ± 0.5[a]	54.7 ± 4.8[b]
24:2n-6	8.2 ± 0.4	6.6 ± 0.5[a]	2.2 ± 0.4[b]
Liver			
20:0	2.0 ± 0.2	2.4 ± 0.1[a]	1.4 ± 0.1[b]
22:0	30.7 ± 2.9	30.9 ± 8.1[a]	13.5 ± 1.2[b]
22:1n-9	0.0 ± 0.0	0.2 ± 0.2[a]	0.7 ± 0.1[b]
23:0	5.3 ± 0.6	5.8 ± 0.5[a]	2.1 ± 0.4[b]
24:0	26.0 ± 2.8	24.7 ± 3.6[a]	6.5 ± 1.5[b]
24:1n-9	16.2 ± 1.4	14.4 ± 3.2[a]	56.0 ± 2.3[b]

[a]Data are expressed as wt% of total fatty acids and are means ± 1 SD of determinations on three separate animals weaned on to the respective diets and sacrificed 8 wk postbirth. Significant differences ($P < 0.05$) between qk.qk mice fed the control and test diets are indicated by different superscript letters (a,b) in a given row.

markedly affected the fatty acid compositions of sphingomyelin in both erythrocytes and liver of qk.qk mice. In both tissues there was a marked elevation of the percentage of 24:1n-9 and corresponding decreases in the percentage of 22:0 and especially of 24:0. These data establish that the *Lunaria* oil was readily digested and assimilated by the qk.qk mice and exerted a major influence on the fatty acid composition of sphingomyelin in both liver and erythrocytes.

The above experiment was performed on first-generation qk.qk mice, i.e., qk.qk mice born to qk.qk mothers maintained on conventional laboratory chow and then weaned on to the test and control diets and maintained on these diets for the rest of their lives. Analyses of brain sphingomyelin in these mice revealed minor changes only (data not shown). Therefore, maintained on the respective control and test diets, second-generation mice were bred from qk.+ fathers and from qk.qk mothers that had been maintained on the two diets since weaning. The remainder of the experiments were performed on these second-generation mice. Although difficulties were experienced in breeding the mice, no difficulties were experienced in maintaining the weaned mice on either the test or control diets and no mortalities occurred.

Table 3 shows the evolution of sphingolipids in the brains of quaking mice over the 2-mon period following birth; the first sampling time was at 2 wk (5 d before weaning), the earliest time we could reliably identify pups born from first-generation qk.qk females and qk.+ males as being quaking mutants. Normal qk.+ mice fed the control diet had a relatively constant percentage of sphingomyelin, *ca.* 3%, throughout the 8-wk study period. Qk.qk mice fed the control diet had the same percentage sphingomyelin as the qk.+ mice at 2 and 4 wk and only slightly increased percentage of sphingomyelin at 6 and 8 wk. Apart from a slight difference at 2 wk, qk.qk mice fed the test diet had the same percentage sphingomyelin as those fed the control diet. In contrast to the evolution of sphingomyelin, the evolution of both cerebrosides and sulfatides in the 2-mon period postbirth was markedly depressed in qk.qk mice compared to qk.+ mice when both were fed the control diet, and feeding the test diet to the qk.qk mice did not change their depressed levels of brain cerebrosides or sulfatides (Table 3). These findings held for both the hydroxy and nonhydroxy components of both cerebrosides and sulfatides (data not shown).

Figures 1–6 show the evolution of the principal fatty acids in sphingomyelin from the brain lipids of the second-genera-

TABLE 3
Levels of Sphingolipids in Brains of Quaking Mice Fed the Test and Control Diets[a]

	Weeks postbirth			
	2	4	6	8
Sphingomyelin				
qk.+ Fed control diet	2.8 ± 0.4[a]	3.1 ± 0.2[a]	3.1 ± 0.2[a]	3.1 ± 0.2[a]
qk.qk Fed control diet	2.9 ± 0.6[a]	3.4 ± 0.4[a]	3.7 ± 0.5[b]	3.7 ± 0.4[b]
qk.qk Fed test diet	2.1 ± 0.1[b]	3.5 ± 0.3[a]	3.5 ± 0.2[b]	3.6 ± 0.0[b]
Cerebrosides				
qk.+ Fed control diet	5.9 ± 1.2[a]	12.3 ± 0.8[a]	15.0 ± 1.8[a]	16.6 ± 0.8[a]
qk.qk Fed control diet	1.9 ± 0.8[b]	4.8 ± 0.9[b]	5.2 ± 0.9[b]	5.7 ± 1.2[b]
qk.qk Fed test diet	1.0 ± 1.0[b]	4.9 ± 1.0[b]	4.9 ± 1.0[b]	5.8 ± 0.8[b]
Sulfatides				
qk.+ Fed control diet	1.1 ± 0.7[a]	3.1 ± 0.3[a]	4.2 ± 0.3[a]	4.6 ± 0.4[a]
qk.qk Fed control diet	0.5 ± 0.2[b]	0.9 ± 0.9[b]	1.2 ± 0.9[b]	1.9 ± 0.4[b]
qk.qk Fed test diet	0.5 ± 0.5[b]	1.5 ± 0.7[b]	1.5 ± 0.7[b]	2.1 ± 0.5[b]

[a]Data are wt% of individual sphingolipids in total brain lipid and are means ± 1 SD of six determinations on separate second-generation animals. Significant differences ($P < 0.05$) within each of the three lipid classes are indicated by different superscript letters (a,b) in a given column.

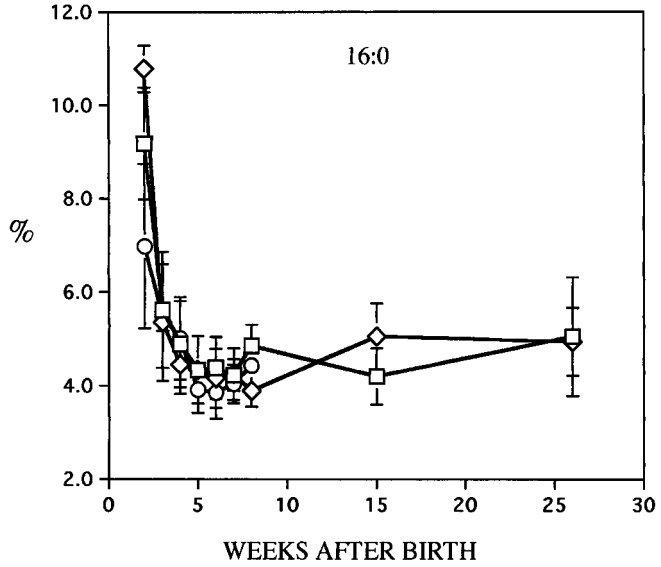

FIG. 1. Changes in the wt% fatty acid composition of brain sphingomyelin of normal (qk.+) and second-generation quaking (qk.qk) mice fed the control and test diets. Data are the means of six determinations on separate animals with the error bars representing 1 SD. □, qk.qk. control diet; ◇, qk.qk. test diet; ○, qk.+ control diet.

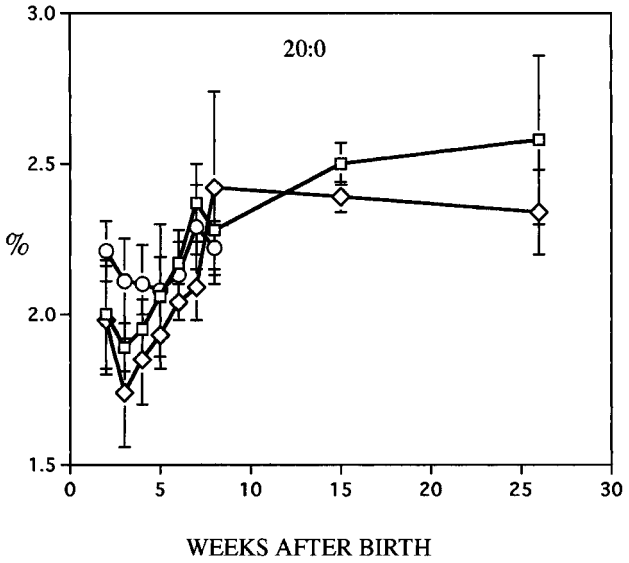

FIG. 3. See Figure 1 for caption details.

tion qk.qk mice postbirth. At the earliest sampling period, 2 wk postbirth, the percentage of 16:0 was elevated in qk.qk mice fed the control diet as compared to qk.+ mice fed the control diet (Fig. 1). In qk.qk mice fed the test diet, the level of 16:0 was further elevated compared to qk.qk mice fed the control diet. As sampling progressed through weaning, the levels of 16:0 fell in all cases to reach the same value by *ca.* 5 wk, after which no further change occurred (Fig. 1). Of the total fatty acids in all three groups of mice at 2 wk postbirth, 18:0 accounted for more than 70% with the level of 18:0 in qk.qk mice fed the control diet being higher than in both qk.+ mice fed the control diet and qk.qk fed the test diet (Fig. 2). The level then increased slightly in qk.+ mice fed the control diet, and substantially more so in qk.qk mice fed both the control and test diets which reached the same final level of 18:0 (Fig. 2). The initially low levels of 20:0 in qk.+ fed the control diet were even lower in qk.qk fed the same diet and qk.qk mice fed the test diet (Fig. 3). As the animals progressed through weaning, the level of 20:0 increased in all three groups of animals, more so in the qk.qk mice than the qk.+ mice and especially in qk.qk mice fed the control diet. However, differences between the test and control diets in the qk.qk mice were not significantly different. A much clearer

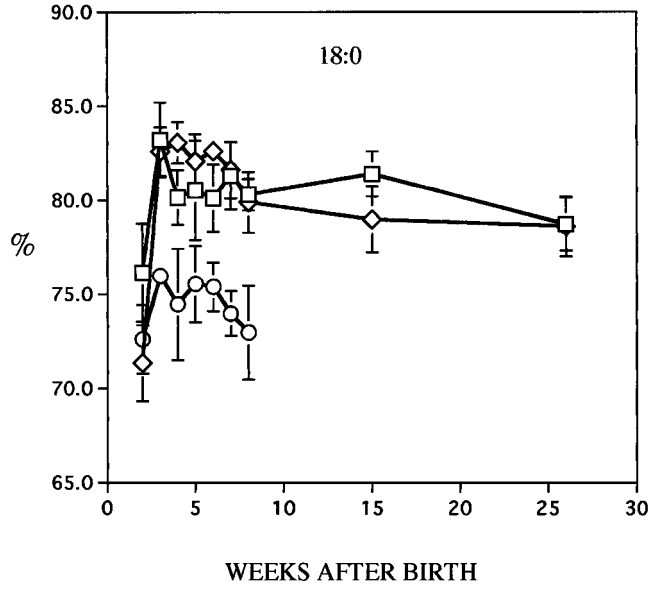

FIG. 2. See Figure 1 for caption details.

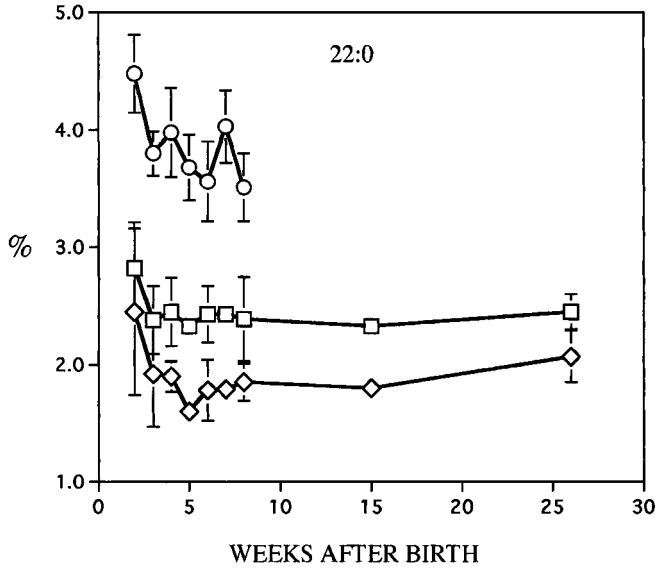

FIG. 4. See Figure 1 for caption details.

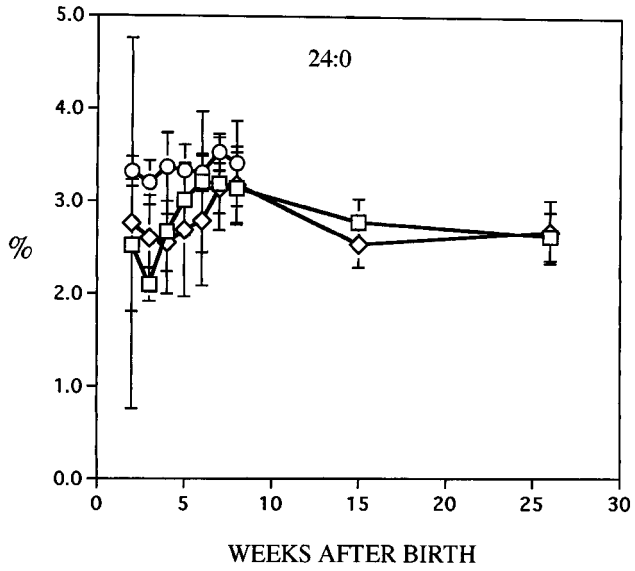

FIG. 5. See Figure 1 for caption details.

picture emerged for 22:0 (Fig. 4) where qk.+ mice fed the control diet had a consistently higher level than qk.qk mice fed the control diet, and these in turn had a consistently higher level of 22:0 than qk.qk mice fed the test diet. Clearly, levels of 22:0 in brain sphingomyelin are depressed in qk.qk mice and further depressed by administering the *Lunaria* oil diet. Levels of 24:0 were also initially depressed in qk.qk mice compared to qk.+ mice (Fig. 5) and, although the levels progressively increased in the qk.qk mice, differences between the test and control diets were not significantly different. Finally, Figure 6 shows that the initial level of 24:1n-9 was the same in qk.qk mice fed the test diet as in qk.+ mice fed the control diet, and substantially higher than in qk.qk mice fed the control diet. Further, while the level of 24:1n-9 rose progressively in qk.+ mice fed the control diet, it fell rapidly in qk.qk mice fed the test diet to reach a level that was consistently significantly ($P < 0.05$) greater than in qk.qk mice fed the control diet (Fig. 6).

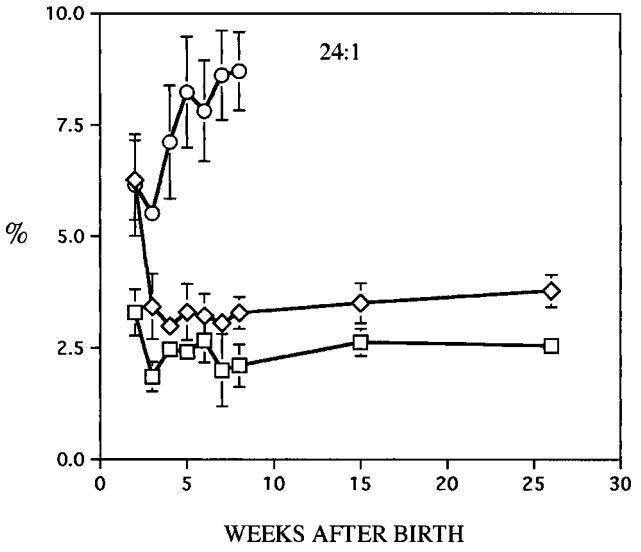

FIG. 6. See Figure 1 for caption details.

Last, because the test diet had clearly influenced brain sphingomyelin fatty acids in qk.qk mice sometime prior to weaning, we investigated the influence of the diet on lactating mothers' milk. Table 4 shows that the test diet markedly increased the level of 24:1n-9 and simultaneously depressed the levels of 22:0 and especially 24:0 in milk sphingomyelin, as compared to the control diet. Lipid class analyses established that 100 g of milk total lipid from qk.qk mice fed the test diet contained 0.6 g of sphingomyelin, 3.1 g of phosphoglycerides, and 87 g of triacylglycerols. The percentages of 24:1n-9 in the total fatty acids of these lipids were 43.9% in sphingomyelin, 0.7% in phosphoglycerides, and 1.6% in triacylglycerols. We conclude that, although sphingomyelin has by far the highest level of 24:1n-9 in the individual milk lipids of qk.qk. mice fed the test diet, the majority of the total 24:1n-9 present in their milk resides in the triacylglycerols.

DISCUSSION

The results of the present study are entirely consistent with previous studies of the quaking mouse mutant. Thus, levels of cerebrosides and sulfatides but not sphingomyelin are markedly reduced in the brain lipid of quaking (qk.qk) as compared to normal (qk.+) mice (1–3; Table 3). Moreover, qk.qk mice have reduced levels of 24:1n-9 in brain sphingomyelin due to a markedly reduced ability to convert C_{20} to C_{22} and C_{24} fatty acids (5–8). This is clearly seen here in the evolution of the fatty acid composition of sphingomyelin in qk.qk mice where, in the 2–8 wk period postbirth, there is a marked elevation in 18:0, and marked depressions of 22:0, 24:0, and especially 24:1n-9 (Figs. 1–6). The quaking gene is now known to be involved in fundamental developmental processes including nerve myelination (9,10), and it would be very surprising if normalization of the nervonic acid content of brain sphingolipids, as was attempted in the present

TABLE 4
Fatty Acid Composition of Milk Sphingomyelin from Quaking Mice Fed the Test and Control Diets[a]

Fatty acid	qk.qk Control diet	qk.qk Test diet
16:0	26.0 ± 1.6	27.1 ± 2.6
18:0	3.4 ± 0.9	2.5 ± 0.7
18:1n-9	5.6 ± 1.8	6.8 ± 3.9
18:2n-6	4.5 ± 1.2	5.4 ± 3.6
20:0	0.7 ± 0.7	0.5 ± 0.5
22:0	8.0 ± 0.5[a]	0.5 ± 0.4[b]
24:0	22.1 ± 2.8[a]	1.2 ± 0.4[b]
24:1n-9	14.1 ± 4.0[a]	43.9 ± 9.2[b]

[a]Data are wt% of total fatty acids in sphingomyelin and are means ± 1 SD for separate milk samples from three individual animals. Significant differences ($P < 0.05$) between control and test diets are indicated by different superscript letters (a,b) in a given row.

study, by itself ameliorated the condition. Nonetheless, the findings here are relevant to understanding the interplay between dietary fatty acids and *de novo* fatty acid biosynthesis in determining the final composition of brain sphingomyelin.

Our results establish that *Lunaria* oil is readily digested and assimilated as evidenced by its marked effects on the fatty acid compositions of sphingomyelins from liver and erythrocytes (Table 2). While the effects were elicited here in quaking (qk.qk) mice, the fatty acid compositions of erythrocyte and liver sphingomyelins of these mice were not significantly different from those of their nonquaking (qk.+) counterparts. Thus, the quaking mutation does not affect the sphingomyelin of extra neural tissue which points to the biosynthesis of C_{24} fatty acids proceeding normally in extra neural tissues including the liver of quaking mice. This is supported by the abundance of 24:0 and 24:1 in milk sphingomyelin from quaking mice fed the control diet (Table 4). Therefore, the results obtained here for liver, erythrocytes, and milk may be directly compared with the various studies already published for the effects of dietary fatty acids in influencing the fatty acid compositions of tissue sphingomyelins in various species and especially the levels of 24:0 and 24:1 in sphingomyelin (15–22). Of these studies, the most definitive is that by Bettger and Blackadar (21) who fed rats 21 different oils, ranging in percentages of 18:1n-9 from 5–78%, 22:1n-9 from 0–23% and 24:1n-9 from 0–1%, and examined the effects of the diets on the ratios of 24:1/24:0 in liver sphingomyelin. These authors determined empirically that the ratio (r) of 24:1n-9/24:0 in rat liver sphingomyelin was determined by the dietary fatty acid composition according to the following relationship:

$$r = 1.88[24{:}1n\text{-}9] - 1.48[24{:}0] + 0.21[22{:}1n\text{-}9] + 0.01[18{:}1n\text{-}9] + 0.26 \quad [1]$$

where the figures in square brackets are the percentage of an individual fatty acid in the dietary oil. This relationship establishes the importance of chain elongation of both 18:1n-9 and 22:1n-9 in determining the final level of 24:1n-9 in liver sphingomyelin, and it equally demonstrates the importance of even small percentages of both 24:1n-9 and 24:0 in establishing the final levels of these fatty acids in liver sphingomyelin.

It is instructive to consider our results in terms of the Bettger and Blackadar (21) relationship. The predicted value of r = 24:1/24:0 for the control diet in this study is 0.65. The measured values of r = 24:1/24:0 for sphingomyelins are 0.59 and 0.62 for erythrocytes and liver, respectively, in qk.+ mice; and 0.75, 0.58, and 0.63 for erythrocytes, liver, and milk, respectively, in qk.qk mice. We conclude that the Bettger and Blackadar (21) relationship holds for sphingomyelins in extra neural tissues of both normal (qk.+) and quaking (qk.qk) mice fed the control diet. We also conclude that, because the control diet has negligible levels of both 24:1n-9 and 24:0, the final levels of these fatty acids in the extra neural sphingomyelins are determined largely by chain elongation reactions operating on shorter-chain fatty acid precursors.

In contrast, the predicted value of r = 24:1/24:0 for the test diet fed to qk.qk mice is 16.16. Here the measured values for sphingomyelins are 4.6, 8.6, and 36.6 for erythrocytes, liver, and milk, respectively. We conclude that the Bettger and Blackadar (21) relationship does not hold for extraneural tissues for the test diet and propose that this reflects (i) the high level of 6.8% of 24:1n-9 in the test diet, and (ii) the probability that such a high dietary input of 24:1n-9 inhibits not only the formation of 24:1n-9 from shorter-chain monoenoic acid precursors but also the formation of 24:0 from shorter-chain saturated acid precursors, because the microsomal fatty acid elongase forming C_{24} from C_{18} and C_{20} precursors does not discriminate between saturated and n-9 monounsaturated substrates (22,23). Thus, the observed substantial deviation of the predicted from the measured value for the test diet is proof of a direct and strong influence of elevated dietary 24:1n-9 in determining the levels of both 24:1n-9 and 24:0 in extra neural sphingolipids in qk.qk mice, not only by direct incorporation into sphingomyelin but probably also by inhibition of the biosynthesis of 24:0 from shorter-chain precursors.

Neither does the Bettger and Blackadar (21) relationship apply to brain for either diet (Table 5). Nonetheless, it is clear that the test diet has consistently increased the ratio of 24:1n-9/24:0 in brain sphingomyelin of qk.qk mice relative to that observed with the control diet. Further, the ratio in 2-wk-old qk.qk mice fed the test diet exceeds that in qk.+ mice fed the control diet. This results mainly from an elevation of 24:1n-9 (Fig. 6) and, to a lesser extent, from a depression of 24:0 (Fig. 5). Clearly, maternally provided 24:1n-9 has substantially influenced brain sphingomyelin in 2-wk-old qk.qk mice. Again we propose that dietary-administered 24:1n-9 is directly incorporated into brain sphingomyelin up to 2 wk postbirth, during which time it also inhibits the biosynthesis in brain of longer-chain fatty acids from shorter-chain precursors, seen here in the depressed levels of 22:0 and 24:0 in brain sphingomyelin of qk.qk mice fed the diet rich in 24:1n-9 at 2-wk postbirth. However, after this time the effects of dietary-administered 24:1n-9 fall off rapidly.

Sphingomyelin in brain is distributed approximately equally between grey matter (nonmyelinated) and white matter (myelinated), whereas cerebrosides and sulfatides are present predominantly in white matter (24). Myelination in mice only becomes extensive from 9–10 d postbirth (2,25). Therefore, the observed effects of the *Lunaria* oil on brain sphingomyelin 2-wk postbirth in qk.qk mice must involve largely nonmyelinated cells. Presumably these cells, mainly neurons and astrocytes, have sphingomyelin that has been influenced

TABLE 5
The Ratios of 24:1n-9/24:0 in Brain Sphingomyelin in the Present Study

	Postbirth (2 wk)	Postbirth (8 wk)	Postbirth (26 wk)
qk.+ Control (predicted 0.65)	1.86	2.56	—
qk.qk Control (predicted 0.65)	1.30	0.67	0.97
qk.qk Test (predicted 16.16)	2.27	1.03	1.41

by the dietary *Lunaria* oil in the present study, by preformed 24:1n-9 delivered from the mother, either *in utero* or after birth during lactation, increasing the percentage of 24:1n-9 by direct incorporation into sphingomyelin and also decreasing the percentage of 20:0, 22:0, and 24:0 in sphingomyelin by further inhibiting the already depressed formation of C_{22} and C_{24} fatty acids from shorter-chain precursors in the quaking mouse brain. Thus, (1-^{14}C)lignoceric acid injected into 15-d-old mice has a specific radioactivity in the total lipid of neurons and astrocytes that is essentially the same as in serum, consistent with its direct incorporation into these cells' sphingolipids (25). However, the specific activity of the injected (1-^{14}C)lignoceric acid in myelin is much less than in serum (26), consistent with C_{24} fatty acids in myelin sphingolipids being biosynthesized extensively if not solely from chain elongation of shorter-chain fatty acids. This is supported by radioactivity from subcutaneously administered (1-^{14}C) stearic acid being readily incorporated into C_{24} fatty acids of mice brain myelin during myelination (27). In contrast, high density serum lipoprotein, whose sphingomyelin contains labeled lignoceric acid, labels the lignoceric acid of brain sphingomyelin of either 20-d-old (rapidly myelinating) or adult (nonmyelinating) rats to only the same very low level (28), i.e., serum lignoceric acid is not incorporated directly into the sphingomyelin of myelin nor, once myelination has been initiated, is it incorporated into the sphingomyelin of neurons or astrocytes. Thus, the marked reduction of the percentage of 24:1n-9 in total brain sphingomyelin observed between 2–8 wk postbirth in the present dietary study reflects the accretion of sphingomyelin resulting from nerve myelination; this myelin-specific sphingomyelin is deficient in 24:1n-9 and elevated in 18:0 and 20:0 due first, to the blockage in converting C_{20} to C_{22} and C_{24} fatty acids in quaking mice brains and second, to the inability of serum 24:1n-9 to contribute to or influence brain sphingomyelin biosynthesis once myelination has been initiated. This presumably reflects development of the blood–brain barrier.

In summary, the results here establish that a dietary oil rich in 22:1n-9 and 24:1n-9 can effectively normalize the level of 24:1n-9 in brain sphingomyelin in the quaking mouse mutant, but only up to 2-wk postbirth. Thereafter, there is a progressive fall in the level of 24:1n-9 in brain sphingomyelin, despite a continuing high dietary input of 24:1n-9. We propose that the positive dietary effect up to 2-wk postbirth is confined largely to neurons and astrocytes, whose sphingomyelin can be biosynthesized from extraneural 24:1n-9 derived from the mother either *in utero* or early postbirth. The present data do not allow us to distinguish between these two possibilities. In contrast, during the 2–8-wk period postbirth, dietary 24:1n-9 is not available to the brain *via* the blood. Rather, the C_{24} fatty acids required for the biosynthesis of sphingomyelin for the rapid phase of myelination in the period starting *ca.* 2 wk postbirth are synthesized, presumably in oligodendrocytes, from C_{18} fatty acids by chain elongation reactions which are defective in quaking mice. Fatty acid chain elongation reactions may only assume true importance within the brain coincident with the initiation of rapid myelination of nerves, closure of the blood–brain barrier, and the imminent removal of maternally derived nutrients as the newborn approaches weaning.

ACKNOWLEDGMENT

We thank Croda Chemicals Ltd., Hull, United Kingdom, for the generous grant that made this research possible.

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[Received April, 9, 1998, and in final revised form August 20, 1998; revision accepted August 24, 1998]

Comparison of *RRR*-α- and all-*rac*-α-Tocopherol Uptake by Permanent Rat Skeletal Muscle Myoblasts (L6 cells): Effects of Exogenous Lipoprotein Lipase

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ABSTRACT: The purpose of the present investigation was to test whether permanent skeletal muscle cells (rat L6 cells) could serve as an *in vitro* model for α-tocopherol (αTocH) biodiscrimination studies. L6 cells were incubated in the presence of high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) labeled in the lipid moiety with either all-*rac*- or *RRR*-[^{14}C]αTocH. These incubations were performed either in the absence or in the presence of exogenously added bovine lipoprotein lipase (LPL) since skeletal muscle is one of the major expression sites of LPL *in vivo*. Time-dependent uptake studies (up to 24 h) in the absence of LPL have shown that equipotent doses of all-*rac*- and *RRR*-[^{14}C]αTocH (1.36:1) led to almost identical accumulation of the tracer, independent of the lipoprotein class used as αTocH carrier. With regard to αTocH donor capacity, it appeared that HDL is the most potent αTocH donor, followed by LDL and VLDL. In the presence of LPL, all-*rac*- and *RRR*-[^{14}C]αTocH uptake was significantly enhanced (between two- and tenfold). Biodiscrimination studies using chiral high-performance liquid chromatographic analysis with radiometric detection of the corresponding methyl ether derivatives on a Chiralcel OD column have demonstrated that the 2*S*-and 2*R*-isomers of αTocH were taken up in a 1:1 ratio by L6 cells independent of the absence or presence of LPL. In addition, we have not observed biodiscrimination between the four 2*R*-isomers, i.e., there was no preferential accumulation of the *RRR*-isomer. These data suggest that L6 cells do not discriminate between different αTocH isomers and that the addition of endogenous LPL significantly enhances the uptake of *RRR*- and all-*rac*-αTocH.
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Synthetic all-*rac*-α-tocopherol (2*RS*-, 4′*RS*-, 8′*RS*-αTocH) is an equimolar mixture of eight different stereoisomers and is used as a vitamin E supplement and as a food additive. The biopotencies of the eight αTocH isomers have been determined in the rat resorption and gestation assay. These data led to the officially established activity ratios of 1:1.36 [synthetic all-*rac*-/*RRR*-α-tocopheryl-acetate (αToc-acetate)] and 1:1.49 (synthetic all-*rac*-αToc-acetate/*RRR*-αTocH). For αToc-acetate relative biopotencies of 100 (*RRR*), 90 (*RRS*), 73 (*RSS*), 57 (*RSR*), 60 (*SSS*), 37 (*SRS*), 31 (*SRR*), and 21% (*SSR*) were established (1). Ingold and colleagues (2) investigated the structural necessities for optimal "biopotency" that give these lipid-soluble phenolic antioxidants Vitamin E activity. The most important determinants were a fully methylated aromatic ring, 2*R*-configuration, and the size of the heterocyclic ring (2).

Dietary vitamin E is taken up by intestinal cells and resecreted in chylomicrons in proportion to the concentration of the naturally occurring tocopherols in the diet (3–5). *In vivo* studies have demonstrated preferential enrichment of very low density lipoproteins (VLDL) with αTocH over γ-tocopherol, a process facilitated by a hepatic αTocH-binding/transfer protein (6–9). Traber *et al.* (3,4) demonstrated that the liver is where the preferential enrichment of newly synthesized VLDL with *RRR*-αTocH occurs. The organs with the highest capabilities for biodiscrimination of αTocH isomers (i.e., uptake of one isomer in excess of the others) are brain (10), adrenal glands (11), and also erythrocytes are capable of pronounced preferential uptake of *RRR*- over all-*rac*-αTocH (11,12). With regard to αTocH-isomer biodiscrimination in the liver, contradictory results were reported: Whereas some authors observed preferential enrichment with 2*R*-isomers (11,12), others demonstrated enrichment with 2*S*-isomers (13). Skeletal muscle—a major storage site of αTocH besides the liver and adipose tissue in the rat (14)—is not able to discriminate between different αTocH isomers (13).

In the fasting state, αTocH is distributed between the VLDL, low and high density lipoprotein (LDL and HDL, respectively) fractions in a 1:1.9:1.4 ratio (15) in humans, but in rats the vast majority of αTocH is transported in the HDL fraction. αTocH uptake by peripheral tissues may occur by different pathways: One of the first steps contributing to tissue supply with αTocH is lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride-rich lipoproteins (16). Second, tissues may acquire LDL-associated αTocH partially *via* the LDL receptor pathway. However, the quantitative importance

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Abbreviations: CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; αToc-acetate, α-tocopheryl-acetate; αTocH, α-tocopherol; αToc-ME, α-tocopheryl-methyl ether; VLDL, very low density lipoproteins.

of this pathway is not entirely clear since Watanabe heritable hyperlipidemic rabbits (lacking functional LDL receptors) have normal tissue αTocH concentrations (17). Finally, αTocH transfer between lipoproteins and tissues might contribute to the maintenance of tissue αTocH levels (17,18).

During the present study we compared the uptake of lipoprotein-associated all-*rac*- and *RRR*-αTocH by rat skeletal muscle myoblasts (L6 cells) in the absence or presence of LPL. HDL, LDL, and VLDL were labeled with either all-*rac*- or *RRR*-[^{14}C]-αTocH, and uptake and biodiscrimination were determined by chiral high-performance liquid chromatography (HPLC) with radiometric detection.

EXPERIMENTAL PROCEDURES

Materials. Methanol, 2-propanol, hexane, and potassium bromide were obtained from Merck (Vienna, Austria). LC-18 reversed-phase HPLC columns were obtained from Supelco (250 × 4.6 mm i.d.; Vienna, Austria) or from Chrompack (200 × 2 mm i.d.; Vienna, Austria). The chiral phase (Chiralcel OD; 250 × 4.6 mm i.d.) used for the separation of 2*R*- and 2*S*-αTocH isomers as their methyl ether derivatives was obtained from P.H. Stehelin Co. (Basel, Switzerland). Ready Safe scintillation cocktail was obtained from Beckman (Vienna, Austria). Synthetic all-*rac*- (specific activity: 2.07 GBq/mmol, radiochemical purity 99.1%, by HPLC) and natural *RRR*-[^{14}C]αToc-acetate (specific activity: 2.07 GBq/mmol, radiochemical purity 99.8%, by HPLC) was prepared following the procedure described in Reference 19. Consumables used during cell culture studies were obtained from BioWhitaker or Costar (Vienna, Austria). Dulbecco's modified Eagle's medium (DMEM) was obtained from Boehringer Ingelheim (Vienna, Austria). PD-10 size exclusion columns were from Pharmacia (Uppsala, Sweden). All other materials were purchased from Merck (Darmstadt, Germany) as analytical grade reagents. L6 cells (permanent rat skeletal muscle myoblasts) were obtained from American Type Culture Collection (Rockville, MD).

Hydrolysis of all-rac- *and* RRR-*αToc-acetate*. Free αTocH was prepared from all-*rac*- and *RRR*-αToc-acetate essentially as described by Mayer and Isler (20). In a conical vial containing 10 mg $LiAlH_4$ in 100 µL dry diethyl ether and a stirrer, 400 µCi (14.8 MBq) all-*rac*-αToc-acetate or 200 µCi (7.4 MBq) *RRR*-αToc-acetate in ether (1000 µL) were added dropwise at room temperature and stirred for 45 min, followed by the addition of moist ether (2 mL) and H_2SO_4 (1 N, 500 µL). The mixture was vortexed and then centrifuged at 3000 rpm. The upper ether layer was removed, and the remaining aqueous layer was extracted three more times. The ether extracts were combined and dried under argon. αTocH yields were ≈ 95–100%. Analysis of the products was performed on an LC-18 column with radiometric detection as described below.

Lipoprotein preparation. Human VLDL, LDL, and apoE-free HDL_3 (termed HDL throughout this report) were prepared by density gradient ultracentrifugation of plasma, obtained from normolipemic donors, in a TL120 tabletop ultracentrifuge (at $r_{av.}$ 350,000 × g) as described in Reference 21 or by vertical single-spin ultracentrifugation (22). Lipoproteins were recovered from the tubes, and excess KBr was removed by size exclusion chromatography on PD-10 columns. Lipoprotein-deficient serum (LPDS) was prepared by standard techniques (23).

Lipoprotein labeling. Lipoproteins were labeled with [^{14}C]αTocH as follows: 1.5 mg HDL-, 0.8 mg LDL-, and 0.08 mg VLDL-protein were labeled with 7.5, 4, and 0.8 µCi *RRR*-αTocH or with 10.2, 5.4, and 1.1 µCi all-*rac*-αTocH by direct syringe-injection of the corresponding tracer dissolved in ethanol (<5.5% of the final volume), followed by dilution with phosphate-buffered saline (PBS) (final volume 2 mL) and incubation at 37°C for 3 h. Free activity was removed on PD-10 size exclusion columns. This labeling procedure resulted in the following specific activities: 13 (all-*rac*-αTocH-HDL), 13 (all-*rac*-αTocH-LDL), 61 (all-*rac*-αTocH-VLDL), 9 (*RRR*-αTocH-HDL), 9 (*RRR*-αTocH-LDL), and 40 (*RRR*-αTocH-VLDL) cpm/ng protein. The intended differences in the specific activities of lipoprotein preparations labeled with either all-*rac*- or *RRR*-αTocH were 1.36 (USP ratio) according to the differences in biopotencies of the corresponding isomer (mixtures).

Isolation of bovine LPL. Bovine LPL was isolated as described (24). Fresh, unpasteurized bovine milk (1 L) was centrifuged (9000 rpm, 4°C, 30 min) to separate cream. The floating cake was discarded, and NaCl was added to a final concentration of 350 mM to the remaining skim milk. After filtration the enzyme was purified by affinity chromatography on heparin- and phenyl-sepharose. The indicated concentrations were added together with the labeled lipoprotein preparations and incubated for the indicated times in DMEM containing 10% (vol/vol) LPDS. LPL activity was measured with a synthetic triglyceride emulsion as described earlier (25).

Analysis of αTocH and αToc-acetate. Reversed-phase HPLC of radio labeled αTocH and αToc-acetate was performed on a Chrompack LC-18 column (200 × 2 mm) with methanol/water (98:2; vol/vol) as the mobile phase (200 µL/min), essentially as described in Reference 26. Radiolabeled compounds were detected with a Radiomatic Flo-One radiometric HPLC detector (Packard-Canberra, Vienna, Austria; channel set at 0–156 keV) which was preceded by an ultraviolet (UV) detector to allow simultaneous detection of labeled and unlabeled compounds. Liquid scintillation fluid flow rate was set to 1 mL/min. The concentration of endogenous αTocH in the lipoprotein preparations used was assessed as described in Reference 27.

Cell culture studies. Rat L6 skeletal muscle myoblasts were seeded in 35-mm multiwell culture plates and cultured under standard conditions (37°C, 5% CO_2, 95% humidity) in DMEM containing fetal calf serum (10% vol/vol), streptomycin (40 mg/mL), and penicillin (40 units/mL). When the cells were 70–80% confluent the monolayers were washed twice with PBS (pH 7.4) and conditioned in fresh DMEM containing LPDS (10%, vol/vol) for 24 h to deplete the intra-

cellular αTocH stores. The cells were then washed again (DMEM, 2×), and the uptake assays were initiated in DMEM containing LPDS and the correspondingly labeled lipoprotein preparation. Lipoproteins were added at concentrations of 50 (HDL), 25 (LDL), and 2.5 (VLDL) μg protein/mL medium, reflecting an approximation of the lipoprotein mass ratios present in plasma of normolipemic donors. Where indicated, the medium was supplemented with bovine LPL (50 μg/mL).

After 2, 6, and 24 h the tissue culture plates were placed on ice, the medium was removed, and the cells were washed twice with ice-cold PBS containing bovine serum albumin (2 mg/mL) followed by another two washes with ice-cold PBS and extracted with hexane/2-propanol (3:2, vol/vol; 1 mL, room temperature, 20 min). The organic extracts were used to analyze uptake and isomer distribution of the αTocH labels. The cellular residue was lysed in NaOH (1 mL, 0.3 N) and the cellular protein content measured according to the method of Lowry *et al.* (28).

Preparation of αToc-methylether derivatives for chiral HPLC. The cellular lipid extracts were dried under argon and converted to the corresponding methyl ether derivatives as described in Reference 20. Briefly, to the dry extracts (kept under argon) 50 μL monoglyme, 25 μL KOH (25%, wt/vol; dropwise addition; vortex after each addition) and 30 μL dimethylsulfate (dropwise addition, vortex after each addition) were added. The incubation mixture was then kept at 50°C for 30 min. The ether phase was removed with nitrogen and to the remaining mixture 100 μL water and 500 μL hexane were added and vortexed for 1 min. To achieve phase separation, the samples were centrifuged at 3000 rpm (4°C, 10 min). The hexane phase was removed, the samples were extracted a second time, and the combined hexane layers containing the αToc-methyl ether (αToc-ME) derivatives were dried under argon and resuspended in 150 μL hexane and analyzed by chiral phase HPLC with radiometric detection as described below.

Chiral phase HPLC of αToc-ME derivatives. αToc-ME derivatives were separated on a 250 × 4.6 mm Chiralcel OD column. This chiral phase separates all-*rac*-αToc-ME derivatives into five peaks, the first peak containing the four 2*S*-isomers and separating the four 2*R*-isomers [*RSS, RRS, RRR,* and *RSR* (29)]. The peak assignment of αToc-ME isomers was based on Reference 29. Forty microliters of the cellular lipid extracts in hexane was injected into the chromatographic system, which consisted of a Waters 510 pump, a Waters 717 plus autosampler, a Packard/Canberra radiometric FlowOne beta detector that was preceded by a Waters 490E programmable multiwavelength ultraviolet-visible detector set at 283 nm to allow simultaneous detection of labeled and unlabeled αToc-ME isomers. The mobile phase was hexane at 1 mL/min, and the scintillation fluid flow was set at 2 mL/min. Total analysis time for isomer separation was 80 min.

RESULTS

Analytical procedures. Prior to lipoprotein labeling, the αToc-acetate labels were converted to the corresponding αTocH tracers as described in the Experimental Procedures section. The αToc-acetate tracers (eluting at 5.6 min under the chromatographic conditions described in the Experimental Procedures section) were converted quantitatively to free αTocH (eluting at 4.8 min) under the conditions described in the Experimental Procedures section (data not shown).

The chiral separation of all-*rac*-αToc-ME derivatives is shown in Figure 1 (A: ultraviolet detection of unlabeled derivatives; B: radiometric detection of [^{14}C]-labeled derivatives). Derivatization and chiral phase HPLC was performed as described in the Experimental Procedures section. The separation of the αToc-ME derivatives results in the occurrence of five peaks, with the first one containing the four 2*S*-isomers (eluting at 25 min) and the remaining four peaks resembling the four 2*R*-isomers which were baseline-separated (29). Peak 1 contained 51.6% (2*S* isoforms) of the total peak

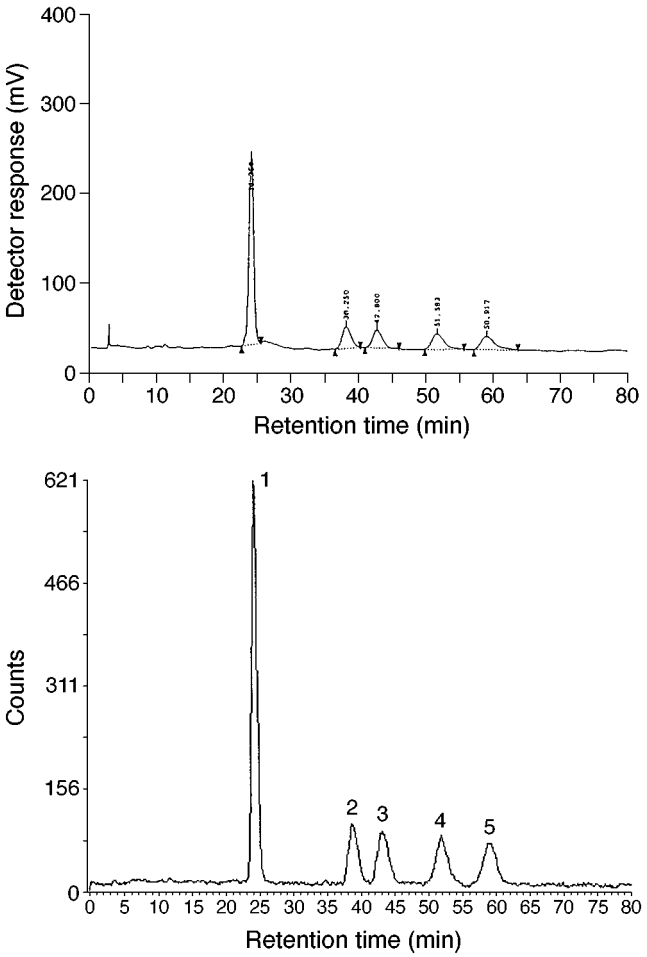

FIG. 1. Chiral high-performance liquid chromatographic separation of α-tocopheryl methyl ester (αToc-ME) derivatives. (A) Unlabeled αToc-ME derivatives (50 ng) and (B) approximately 100,000 cpm of all-*rac*-[^{14}C]αToc-ME derivatives were separated on a Chiracel OD column by the methods described in the Experimental Procedures section. Peak 1 eluted at 25 min and contained the four 2*S*-isomers. The four 2*R*-isomers were separated as follows: peak 2, *RSS,* 38 min; peak 3, *RRS,* 43 min; peak 4, *RRR,* 52 min; and peak 5, *RSR,* 61 min; peak assignment as in Reference 29.

area (or radioactivity) and the remaining four peaks (*RSS*-, *RRS*-, *RRR*- and *RSR*-αToc-ME) contained 12.2, 10.7, 12.6, and 12.8% of the activity as expected for the four 2*R*-αToc-ME isomers. The absolute limit of detection with radiometric detection was approximately 300 cpm/peak corresponding to $\approx 4 \times 10^{-12}$ mol per isomer. Owing to the good miscibility of the mobile phase and the liquid scintillation fluid the ratio was kept as low as possible, i.e., at 1:2 (mobile phase/scintillator). These chromatographic conditions were used to analyze the uptake of all-*rac*- and *RRR*-[^{14}C]αTocH by L6 rat skeletal muscle myoblasts throughout the study described here.

Uptake of HDL-, LDL- and VLDL-associated all-rac- *and* RRR-*[^{14}C]-αTocH by L6 cells in the absence or presence of LPL.* Lipoproteins were labeled with all-*rac*- or *RRR*-[^{14}C]-αTocH as described in the Experimental Procedures section. We intended to label the different lipoprotein classes with all-*rac*- and *RRR*-αTocH in a ratio of 1.36 (to account for the USP ratio of 1:1.36) and achieved differences in the specific activities of 1.4 (HDL and LDL) and a slightly higher value of 1.5 in case of VLDL. This experimental approach (instead of similar specific activities and addition of higher concentrations of the all-*rac*-labeled lipoproteins) was chosen to have equipotent [^{14}C]αTocH concentrations and similar protein and lipid concentrations on the cells. Prior to the uptake experiments, the L6 cells were preincubated in DMEM supplemented with LPDS to deplete the intracellular αTocH pool. The endogenous αTocH content of the lipoprotein preparations used was 1.9 ± 0.2, 3.7 ± 0.1, and 2.5 ± 0.08 nMol/mg total lipoprotein mass for HDL, LDL, and VLDL (mean ± SD, three different preparations), respectively, similar to previously published results (30–32).

The time-dependent uptake of all-*rac*- and *RRR*-[^{14}C]-αTocH labeled HDL, LDL, and VLDL, respectively, in the absence or presence of LPL is shown in Figure 2. From these data it is evident that αTocH concentrations in L6 cells increased in a time-dependent manner and, in the absence of LPL, this increase was more or less linear when HDL and LDL were used as αTocH donors. In case of VLDL, αTocH uptake tended to level off at the 24 h point. The resulting αTocH concentrations in L6 rat skeletal muscle myoblasts were not significantly different when the cells were either incubated in the presence of all-*rac*- or *RRR*-[^{14}C]-αTocH labeled lipoproteins, i.e., equipotent doses of all-*rac*- or *RRR*-αTocH led to a comparable increase of the cellular αTocH content (Fig. 2).

There was, however, a significant difference in the αTocH donor capacity of the different lipoprotein classes used during these experiments. The lipoprotein concentrations chosen during the present study reflect the approximate concentration ratio of VLDL, LDL, and HDL occurring in plasma of normolipemic donors. Although we have not analyzed the mass transfer of unlabeled, endogenous αTocH, the apparent donor capacity for [^{14}C]αTocH appears to be highest for HDL (1.5% of *RRR*- and 1.4% h^{-1} of all-*rac*-[^{14}C]-αTocH), followed by LDL (0.9% of *RRR*- and 0.8% h^{-1} of all-*rac*-[^{14}C]-αTocH) and VLDL (0.4% of *RRR*- and 0.4% h^{-1} of all-*rac*-[^{14}C]-αTocH). The addition of LPL to the cell incubations in the presence of HDL, LDL, or VLDL led to a significant increase of αTocH uptake by L6 myoblasts as compared with experiments performed in the absence of LPL, independent of which lipoprotein class was used as αTocH vehicle (Fig. 2). At the 24 h point, all-*rac*-αTocH uptake in the presence of

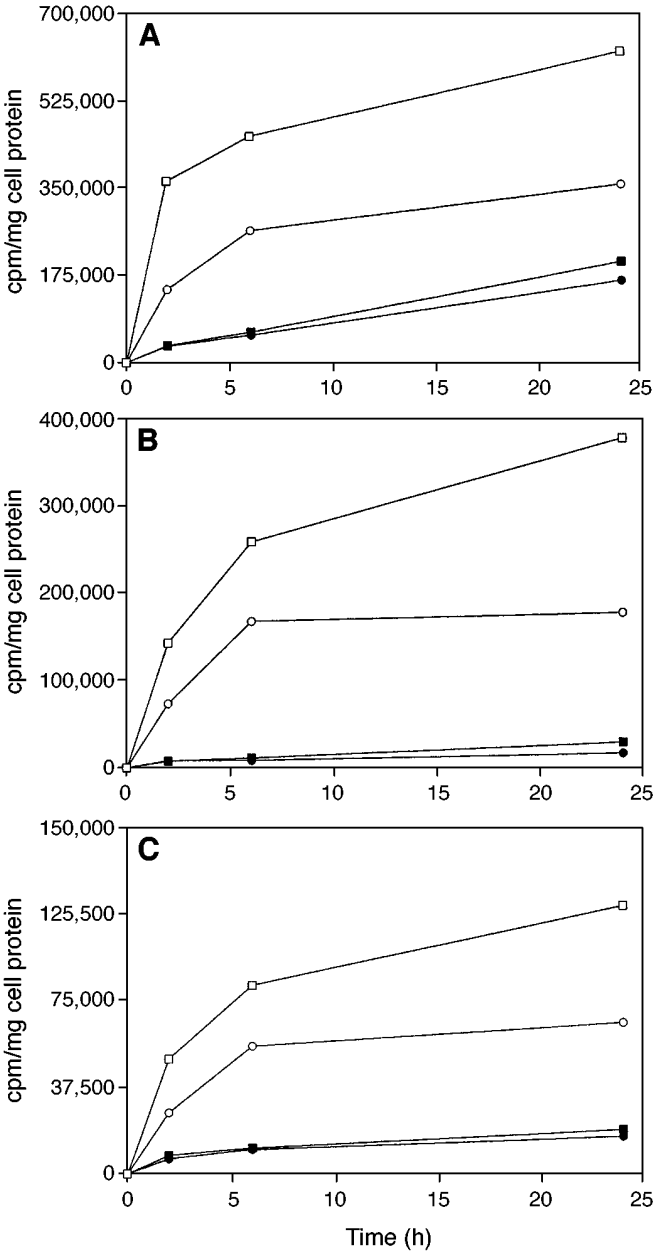

FIG. 2. Time-dependent accumulation of all-*rac*-[^{14}C]α-tocopherol (αTocH) and *RRR*-[^{14}C]αTocH incorporated into high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) in the presence or absence of lipoprotein lipase (LPL). According to the procedures described in the paragraph headed "Cell culture studies," lipoproteins labeled with [^{14}C]all-*rac*- (○,●) or [^{14}C]*RRR*-αTocH (□,■) were added at concentrations of (A) 50 HDL, (B) 25 LDL, and (C) 2.5 VLDL μg protein/mL medium in the absence (●,■) or presence (○,□) of bovine LPL (50 μg/mL) which was added together with the labeled lipoprotein preparations.

TABLE 1
α-Tocopherol (αTocH) Activity Ratios (all-*rac*-:*RRR*-) in L6 Cells After Coincubation with Labeled VLDL, LDL, or HDL in the Absence or Presence of LPL[a]

	Mean αTocH ratios (all-*rac*-:*RRR*-)					
	–LPL			+LPL		
Vehicle	2 h	6 h	24 h	2 h	6 h	24 h
HDL	1.03	1.1	1.23	2.47	1.72	1.74
LDL	0.97	1.32	1.62	1.94	1.54	2.1
VLDL	1.25	1.05	1.17	1.87	1.47	1.75

[a]VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPL, lipoprotein lipase.

LPL (50 µg/mL medium) increased by 3.1-, 11.9-, and 5.7-fold (HDL, LDL, and VLDL, respectively; Fig. 2) whereas *RRR*-αTocH uptake was increased by 2.2-, 9.2-, and 3.8-fold (HDL, LDL, and VLDL; Fig. 2).

Coincubation of L6 cells with all-*rac*- or *RRR*-[^{14}C]-αTocH verified the expectation that equipotent doses of the tracers incorporated into HDL, LDL, or VLDL will lead to comparable (intra)cellular accumulation of the tracers. This assumption is further substantiated by data shown in Table 1 where the cellular all-*rac*-:*RRR*-αTocH ratios are shown. In the absence of LPL the all-*rac*-:RRR-αTocH ratios were between 0.97 and 1.25 except for LDL at the 24 h point, where the ratio was 1.6 (Table 2). However, the situation was different when the cells were incubated with the respective lipoprotein preparations labeled with either all-*rac*- or *RRR*-[^{14}C]αTocH in the presence of LPL. During these experiments the all-*rac*-:*RRR*-αTocH ratios were much higher, i.e., between 1.5 and 2.5. Obviously the addition of LPL resulted in the preferential accumulation of all-*rac*-[^{14}C]-αTocH in L6 cells.

αTocH stereoisomer distribution in L6 cells. In general the αTocH isomer distribution in L6 cells after coincubation with all-*rac*-[^{14}C]αTocH-labeled HDL, LDL, or VLDL in the absence or presence of LPL led to almost similar patterns (Table 2). In line with the results shown in Table 1, we have not observed preferential enrichment of the myoblasts with one of the 2*R*-isoforms; rather, the 2*S*- and 2*R*-isoforms accumulated in a 1:1 ratio (Σ 2*S* = 46.2–52.7% and Σ 2*R* = 47.3–53.8%). This was observed for all the donor–lipoprotein preparations and all time points analyzed during the present study (with the exception of LDL at the 24 h point; 2*S*:2*R* = 0.72). Also within the 2*R*-isomer group we have not observed preferential uptake of one of the isomers. From these data it appears reasonable to conclude that (under the conditions described here) no biodiscrimination of αTocH isomer uptake occurs in L6 cells.

DISCUSSION

We investigated the relative bioavailability of *RRR*-αTocH and all-*rac*-αTocH and the changes in the concentration of the 2*S*- and 2*R*-isomers in permanent rat skeletal muscle myoblasts (L6 cells). To deliver αTocH to these cells we used HDL, LDL, and VLDL in the presence and absence of LPL as donor vehicles. In contrast to the liver and heart (33,34) other organs/tissues (including skeletal muscle) are devoid of αTocH transfer/bind-

TABLE 2
αTocH Isomer Distribution in L6 Cells After Coincubation in the Presence of All-*rac*-[^{14}C]-αTocH-labeled VLDL, LDL, and HDL in the Absence or Presence of LPL[a]

		Percentage 2*R*-isomer distribution				Percentage of all isomers	
	Time (h)	*RSS*	*RRS*	*RRR*	*RSR*	2*S*	2*R*
LPL absent							
HDL	2	12.3	11.9	13.5	12.4	49.9	50.1
	6	12.0	12.6	12.9	12.3	50.2	49.8
	24	9.6	12.2	16.3	15.7	46.2	53.8
LDL	2	12.2	11.6	12.5	12.2	51.3	48.8
	6	12.1	11.4	12.7	12.1	51.7	48.3
	24	16.4	13.3	15.4	13.0	41.9	58.1
VLDL	2	12.5	12.5	12.4	12.7	50.0	50.0
	6	11.7	10.9	12.8	11.9	52.7	47.3
	24	11.9	11.7	12.1	12.4	51.9	48.1
LPL present							
HDL	2	10.4	15.1	12.9	13.9	47.7	52.2
	6	12.1	13.3	15.3	11.8	47.5	52.5
	24	11.8	11.8	13.4	13.0	50.0	50.0
LDL	2	13.4	12.0	15.0	10.3	49.3	50.7
	6	12.0	11.7	13.0	12.6	50.7	49.3
	24	12.0	12.3	12.9	12.6	50.2	49.8
VLDL	2	12.2	11.8	13.9	12.7	49.9	50.1
	6	12.6	12.6	13.0	12.9	48.9	51.1
	24	11.9	12.3	12.7	12.3	50.7	49.3

[a]Experimental conditions as described in Table 1. For abbreviations see Table 1.

ing protein. Despite the absence of αTocH transfer/binding protein, *in vivo* studies have demonstrated that skeletal muscle acquires *RRR*-αTocH in excess of all-*rac*-αTocH during longer supplementation times (35) as a result of plasma enrichment with the *RRR*-isomer (13,35). In addition this apparent "biodiscrimination" was attributed to different αTocH uptake pathways, i.e., the contribution of chylomicrons (delivering all forms of αTocH) and the other lipoprotein classes (preferentially enriched in *RRR*-αTocH) (35). Our short-term studies with permanent rat myoblasts showed that the L6 cells do not discriminate between different stereoisomers of αTocH, independent of the donor lipoprotein used. We have not observed substantial enrichment of the myoblasts with one of the 2*R*′-isoforms, rather the 2*S*- and 2*R*-isoforms accumulated in a 1:1 ratio independent of the lipoprotein fraction used as αTocH donor (Table 2). From these data it is evident that the myoblasts show no enhancement of the 2*R*-/2*S*-isomer ratio relative to the isomer distribution present in the corresponding donor lipoprotein fractions. These findings are compatible with an *in vivo* study in rats (13), where it was demonstrated that three different types of skeletal muscle did not show an enhanced *RRR*/*SRR* ratio relative to plasma.

During the present study we have obtained evidence (on the basis of transfer rates) that HDL is a more efficient αTocH donor than LDL or VLDL. This is especially noteworthy as the experiments were performed under LPDS conditions where the expression of LDL receptors is upregulated (23). One interpretation is that under "normal" conditions the contribution of HDL to tissue supply with αTocH could be even more pronounced. The higher donor capacity of HDL is in line with findings obtained with permanent hepatocytes where we (31) demonstrated that the transfer of αTocH from HDL was approximately twofold higher than from LDL. The transfer of HDL- and LDL-associated αTocH occurred independent from lipoprotein particle uptake and was regulated by the intracellular cholesterol content (31), compatible with the concept of "selective" uptake as described for HDL cholesteryl esters (CE) (36). This assumption is further substantiated by the finding that the liver and the adrenals—the sites with the highest capacity for selective HDL-lipid uptake (14)—contain the highest αTocH concentrations. In addition, it was shown that interlipoprotein transfer of αTocH occurs with the highest efficacy from labeled HDL particles (18,37). These good αTocH donor properties of HDL might (at least in part) be a result of the high curvature of the particle, an important physical property affecting the efficacy of lipoprotein-independent lipid transfer (38). A different distribution of αTocH between the polar surface and the hydrophobic core in different lipoprotein classes could offer another explanation for this observation (39). Therefore, it appears reasonable to assume that *in vivo* the HDL fraction is an important (and somewhat underestimated) αTocH donor, contributing to the maintenance of αTocH levels in organs and tissues.

Significantly increased αTocH uptake by L6 cells in the presence of LPL was an interesting finding from the present study. *In vivo* studies (40) have demonstrated that the uptake of αTocH in skeletal muscle depends on the level of LPL expression. The enhancement of αTocH uptake in the presence of LPL is most probably due to higher lipolysis rates (41) and, in addition, to the proposed bridging function of the enzyme (40). This bridging function was attributed to the presence of a heparin- and a lipid-binding site in the LPL molecule (42) resulting in increased lipoprotein binding to the surface of a variety of cells (43–45). As a result LPL increases binding (bridging), internalization, and degradation of lipoproteins (46,47) by mechanisms involving the LDL receptor-related protein and the VLDL receptor (48–50). In one of our earlier studies we demonstrated that the addition of exogenous bovine LPL significantly increased selective HDL-CE uptake by mouse peritoneal macrophages (51). The effects of LPL on selective HDL-CE uptake could be attributed to enhanced HDL-particle internalization and a second pool of HDL particles delivering CE without concomitant particle internalization (51). Whether LPL-generated lipolysis products enhance cell permeability [as demonstrated for endothelial layer permeability (52)] remains to be elucidated. Along this line it is noteworthy that αTocH-uptake by skeletal (thigh) muscle of LPL-overexpressing mice was dependent on the level of LPL (over)expression and was highly correlated with the amount of free fatty acids in the tissue (40). A combination of three effects mediated by LPL—enhanced lipoprotein binding, enhanced selective uptake, and enhanced permeability caused by lipolysis products—could ultimately result in increased αTocH uptake as observed in the present study. From the chiral HPLC data shown in Table 2 it is evident that the addition of LPL did not affect the isomer distribution in L6 cells incubated in the presence of all-*rac*-αTocH.

The present study showed that permanent L6 rat myoblasts do not acquire 2*R*-isomers in excess of 2*S*-αTocH isomers, in line with findings obtained *in vivo* (13). From these results one might anticipate that for the treatment of, e.g., muscle myopathies equipotent doses of natural *RRR*- and synthetic all-*rac*-αTocH would be equally effective. In addition we would emphasize L6 cells as a valuable *in vitro* model reflecting comparable αTocH uptake mechanisms as observed for rat and mouse skeletal muscle *in vivo*. We expect verification of our *in vitro* findings during *in vivo* experiments with transgenic mice overexpressing LPL in skeletal and cardiac muscle (40).

ACKNOWLEDGMENT

This work was supported by the Austrian Research Foundation (P12000 Med to WS) and by Eisai Co., Ltd.

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[Received July 9, 1998, and in final revised form and accepted August 28, 1998]

Metal-Ion Stimulation and Inhibition of Lysophospholipase D Which Generates Bioactive Lysophosphatidic Acid in Rat Plasma

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ABSTRACT: We found that lysophospholipase D (LPLD) in rat plasma prefers unsaturated to saturated lysophosphatidylcholines as substrates, generating a biologically active lipid, lysophosphatidic acid, but it does not hydrolyze diacyl-phospholipids. In this study, this LPLD required a metal ion for activity, Co^{2+} being the most effective, followed in order by Zn^{2+}, Mn^{2+}, and Ni^{2+}. This metal-ion-stimulated LPLD with unique substrate specificity, which has not been described previously, was susceptible to thiol-blocking reagents and serine esterase inhibitors, but not to a histidine-modifying reagent. Consistent with results using thiol-modifying agents, short-chain fatty aldehydes, secondary products of lipid peroxidation, were found to inhibit LPLD. Addition of dibutylhydroxytoluene or butylhydroxyanisole to the plasma increased the activity of this enzyme, probably in a manner independent of its antioxidant activity, since another antioxidant, propyl gallate, was rather inhibitory. These results suggest that rat plasma contains an active LPLD that differs in some properties from other members of the known phospholipase D family detected in animal tissues and body fluids.

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Lysophosphatidic acid (LPA) has the simplest structure of all phospholipids, but has a variety of biological activities (1–3), among which its effect in inducing proliferation received much attention (4). Recent reports on cloning of genes for LPA receptors from various sources (5–7) support the idea that LPA functions as a first messenger through its specific seven transmembrane-spanning proteins (8). LPA was originally identified as a vasoactive principle with platelet-aggregating activity in the incubated serum of rats (9). Later, several groups identified LPA as an active component in serum or ascites, using *in vitro* assay systems with fibroblast cells (10), neuronal cells (11), glioma cells (12), tumor cells (13,14), and oocytes (11). LPA was found to be present at micromolar concentrations in serum, by its chemical measurement after thin-layer chromatography (TLC) separation of lipid extracts of serum (15,16). Two mechanisms participated in the accumulation of LPA in serum or plasma: (i) its release from activated human platelets (15), possibly *via* the action of phospholipase A_2 on phosphatidic acid in membranes of platelets (17) and (ii) lysophospholipase D (LPLD), which hydrolyzes unsaturated lysophosphatidylcholines (LPC) preferentially over saturated LPC in rat plasma (18). We report here some properties of this LPLD. Our results suggest that plasma LPLD is a novel type of LPLD requiring a metal ion for optimal activity.

EXPERIMENTAL PROCEDURES

Materials. 1-[1-^{14}C]Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (16:0-LPC, 1.48–2.22 GBq/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). l-Oleoyl-2-lyso-*sn*-glycerol-3-phosphate (18:1-LPA), acetaldehyde, 1,1,3,3-tetramethoxypropane, and *p*-bromophenacyl bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Acrolein was a product of Tokyo Kasei Kogyo Co. (Tokyo, Japan). Propyl gallate and 3-*tert*-butyl-4-hydroxyanisole were from Kanto Chemical Co. (Tokyo, Japan). 2,6-Di-*tert*-butyl-4-hydroxy-methylphenol (BHT), *N*-ethylmaleimide (NEM), *o*-phenanthroline, diisopropyl fluorophosphate (DFP), EGTA, and EDTA were from Wako Pure Chemical Industries (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF) and dithio-bis-(2-nitrobenzoic acid) (DTNB) were obtained from Nacalai Tesque (Kyoto, Japan). Other chemicals were reagent grade, and solvents were double-distilled.

Rat plasma. Blood was withdrawn through an abdominal artery from fasting (8–10 h) male Wistar rats (8–11 wk old; Japan SLC, Shizuoka, Japan) anesthetized with sodium pentobarbital (40 mg/kg, i. p.), and immediately mixed with 0.01 vol of heparin solution (final concentration, 2 I.U./mL) in sterilized plastic centrifugation tubes, unless otherwise de-

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Abbreviations: BHT, 2,6-di-*tert*-butyl-4-hydroxy-methylphenol; DFP, diisopropyl fluorophosphate; DTNB, dithio-bis-(2-nitrobenzoic acid); LAT, lysolecithin acyltransferase; LCAT, lecithin:cholesterol acyltransferase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPLD, lysophospholipase D; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

scribed. In some experiments, rat blood was anticoagulated with 0.15 vol of acid–citrate–dextrose solution, EDTA solution (6.7 mg/mL), or EGTA (6.7 mg/mL) solution. Serum was prepared by allowing rat blood to stand in the absence of anticoagulant at 37°C for 1 h and then overnight at 4°C.

Measurement of LPLD activity. The standard procedure for measurement of LPLD activity was as follows: First, rat plasma or serum was mixed with 0.05 vol of a solution of [^{14}C]16:0-LPC in saline containing 0.25% bovine serum albumin. The final concentration of radioactive LPC was 3.5 nmol/mL (0.0074 MBq/mL). Immediately after the addition of [^{14}C]16:0-LPC, a 0.2-mL aliquot of plasma or serum was withdrawn as the 0 h sample. The remaining plasma or serum was incubated at 37°C for 6 h, and 0.2-mL samples were withdrawn after 2, 4, and 6 h and diluted with 2% KCl solution to 2 mL. Lipids were extracted from these diluted samples by the method of Bligh and Dyer (19) after adjusting the samples to pH 2.5 with 1 N HCl, except in experiments on the metal-ion requirement of LPLD, in which lipids were only extracted after 6-h incubation. The lipid extracts were fractionated together with 18:1-LPA (0.1 μmol) as a carrier on a Merck Silica gel 60 TLC plate (Darmstadt, Germany) in a solvent system of chloroform/methanol/20% ammonium hydroxide (60:35:8, by vol). Lipid bands were visualized under an ultraviolet lamp after spraying with a 1-mM solution of *p*-toluidino-2-naphthalenesulfonic acid in 50 mM of Tris-HCl (pH 7.4). Bands of individual phospholipids were scraped off the gel, mixed with 5 mL of Sintisol EX-H (Wako), and counted in a liquid scintillation counter. LPLD and lysolecithin acyltransferase (LAT) activities were calculated from the radioactivities of the bands of the LPA and phosphatidylcholine (PC), respectively, against the sum of the radioactivities of the PC, LPC and LPA bands, and expressed as percentage conversion/h.

In experiments with various agents that modified amino-acid residues of proteins, plasma from heparinized rat blood was preincubated with test reagents at 37°C for 15 min, and then incubated with [^{14}C]16:0-LPC (0.0074 MBq), as described. All test reagents were dissolved in ethanol. The final concentration of ethanol in rat plasma was 1%. In control experiments, the plasma was preincubated with ethanol alone at a final concentration of 1%, followed by the addition of [^{14}C]16:0-LPC.

For experiments with chelators, the plasma obtained from heparinized blood was mixed with 0.05 vol of EDTA solution at final concentrations of 1, 2.5, 5, 10, 20, and 50 mM or *o*-phenanthroline at final concentrations of 0.25, 0.5, and 1 mM or distilled water as a control. The mixtures were then incubated with 0.05 vol of a solution of [^{14}C]LPC for 6 h.

For studies on the metal-ion requirement for LPLD activity, the plasma from heparinized blood was first treated with 0.04 vol of a solution of EDTA, and the pH of the mixture was adjusted to pH 7.4. The plasma was then mixed with 0.01 vol of a solution of $CaCl_2$, $MgCl_2$, $BaCl_2$, $CoCl_2$, $NiCl_2$, $FeCl_3$, $CdCl_2$, $HgCl_2$, $ZnSO_4$, $CuSO_4$, or $MnCl_2$ at final concentrations of 5 or 10 mM or distilled water as a control (EDTA–plasma). The final concentration of EDTA was 5 mM in these experiments. As another control (heparin–plasma), the plasma was mixed with 0.05 vol of distilled water. These samples were then incubated with 0.05 vol of a solution of [^{14}C]LPC for 6 h. In several experiments, the heparinized plasma was mixed with 0.01 vol of a solution of a metal ion such as $CoCl_2$ or $CuSO_4$ without preaddition of EDTA or *o*-phenanthroline. In such cases, control plasma was mixed with 0.01 vol of distilled water.

In experiments with short-chain fatty aldehydes, the plasma (0.9 mL) was first treated with 0.05 mL of [^{14}C]LPC solution and then promptly treated with 0.05 mL of a solution of freshly prepared acetaldehyde or acrolein in saline or saline alone (as a control). Malondialdehyde was generated from 1,1,3,3-tetramethoxypropane as described previously (20). The plasma was mixed with 0.05 mL of [^{14}C]LPC solution and 0.05 mL of a solution of malondialdehyde in 0.1 M sodium phosphate buffer, pH 6.4, or vehicle alone.

The antioxidants used in this study were poorly water-soluble, so desired amounts of these chemicals were dried in tubes, to which the plasma was added directly. The mixtures were then shaken vigorously and incubated with [^{14}C]LPC with or without preaddition of Cu^{2+} or Fe^{3+}.

Statistics. Experimental values are means ± SEM, and differences between the means were compared by unpaired Student's *t*-test. A P value of <0.05 was considered statistically significant.

RESULTS

Cation requirement for LPLD activity. For measurement of LPLD activity, rat plasma was incubated with [^{14}C]16:0-LPC for up to 6 h. As shown in Figure 1, the radioactive LPC was almost linearly decreased with time, concomitant with the in-

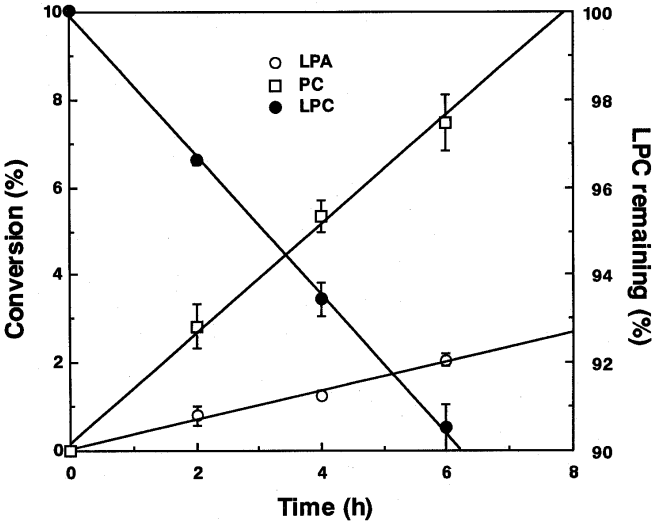

FIG. 1. Metabolic conversions of [^{14}C]lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) and phosphatidylcholine (PC) during incubation of rat plasma at 37°C. Values are mean ± SEM of four independent experiments.

creased radioactivities in two bands of PC and carrier LPA during the incubation period. The radioactivity comigrating with carrier LPA is actually due to LPA degraded by the action of LPLD, since this radioactive lipid shows the same chromatographic behavior as that of standard LPA on a TLC plate developed with a mixture of chloroform/methanol/water (65:35:5), chloroform/methanol/28% ammonium hydroxide (65:35:5), or chloroform/methanol/20% ammonium hydroxide (60:35:8). The conversion of LPC to PC would be due to the LAT activity of lecithin:cholesterol acyltransferase (LCAT; E.C. 2.3.1.43), as first demonstrated with human plasma (21). The rates of both reactions occurring during incubation of rat plasma could be calculated from the slopes of the straight lines obtained.

Incubation of the plasma in conditions which prevent bacterial contamination (100 U/mL penicillin + 100 µg/mL streptomycin) does not modify the results described above. Furthermore, we confirmed that incubation of [^{14}C]LPC with heat-denatured plasma (60°C, 1 h) or without plasma at 37°C for 6 h did not lead to its conversion to LPA.

Using this convenient method, we first examined the effect of anticoagulant on the plasma LPLD activity. Because EDTA and EGTA, but not acid–citrate–dextrose, inhibited the LPLD activity (Fig. 2), metal ions may be required for optimal activity. The LPLD activity in the serum was almost the same as that of the heparinized plasma. Next, we examined effect of metal-cation chelators on the LPLD activity of the heparinized plasma. Figure 3A and B shows the concentration-dependent inhibitions of LPLD by additions of EDTA and *o*-phenanthroline, respectively. The transition metal chelator *o*-phenanthroline was more effective than the bivalent cation chelator EDTA. These results suggest that at least one transition metal cation is required for optimal activity of LPLD. Consistent with previous reports on LAT activity of human plasma (22),

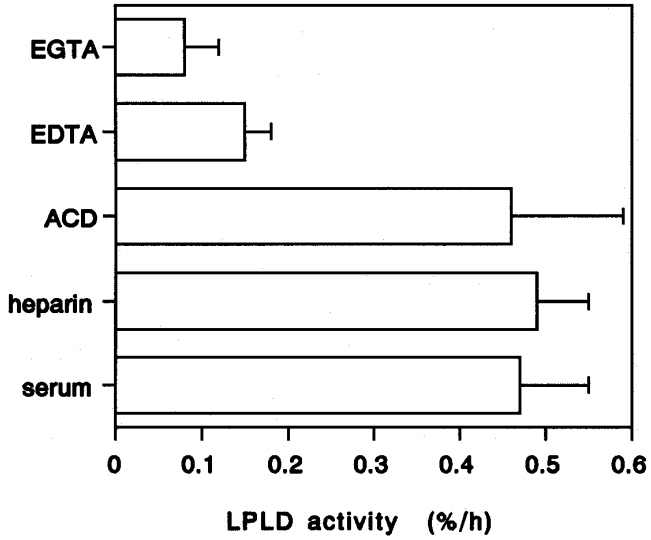

FIG. 2. Effect of anticoagulant on lysophospholipase D (LPLD) activity. Values are mean ± SEM of three independent experiments. ACD, acid–citrate–dextrose.

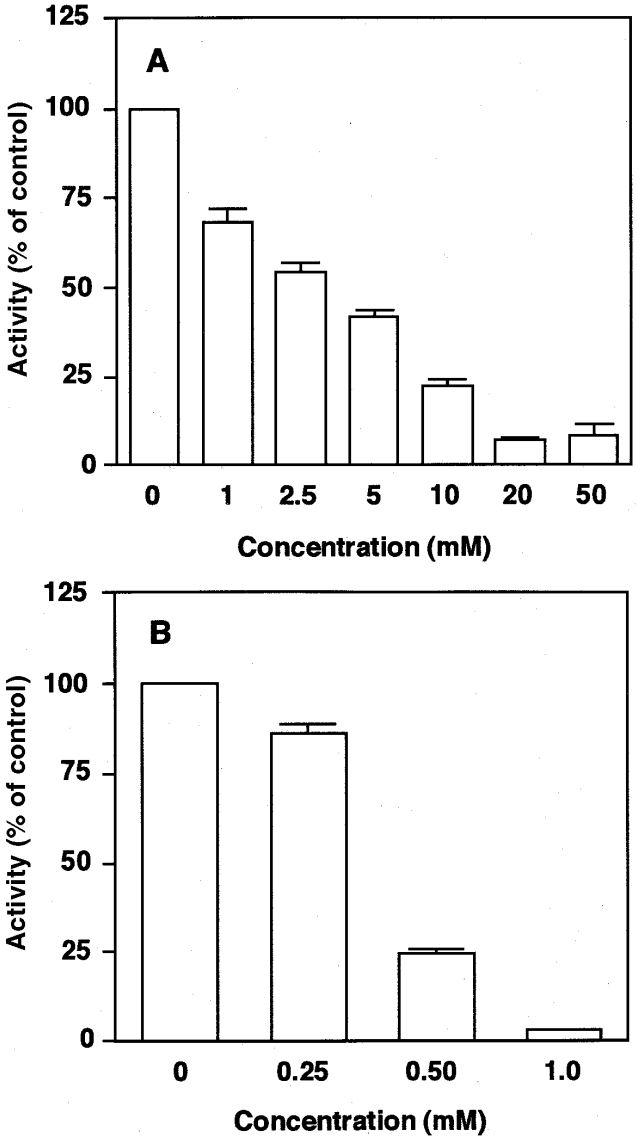

FIG. 3. Effects of EDTA (A) and *o*-phenanthroline (B) on LPLD activity. Values are expressed as percentages of the control (heparinized plasma mixed with 0.15 vol of saline), and shown as mean ± SEM of three independent experiments. See Figure 2 for abbreviation.

LAT activity in rat plasma was insensitive to EDTA (Table 1) and *o*-phenanthroline (data not shown).

To examine the metal-ion requirement of the LPLD in detail, we added various metal ions to plasma pretreated with 5 mM EDTA. Table 1 summarizes the results of these experiments and the effects of metal ions on LAT activity, which could be simultaneously measured with our assay system. LPLD activity was restored to approximately the normal level by addition of 5 mM Mn^{2+}, 5 mM Zn^{2+}, or 10 mM Co^{2+}. Ni^{2+} at 5 mM and Mg^{2+} at 10 mM were less effective, and Ca^{2+}, Ba^{2+}, Fe^{3+} and Cu^{2+} at 5 mM were all inactive. Consistent with a previous finding that LAT in human plasma does not require any metal ion (22), the LAT activity in rat plasma was not stimulated by any metal ion used (Table 1), except for a slight but significant increase in the activity on addition of 5

TABLE 1
Effect of Metal Ions on the Activities of Lysophospholipase D (LPLD) and Lysolecithin Acyltransferase (LAT) in Incubated Rat Plasma[a]

Metal	Concentration (mM)	*n*	Activity (% of control) LPLD	LAT
Heparin–plasma	—		100.0	100.0
EDTA–plasma	—	14	36.8 ± 2.7	102.5 ± 2.0
Mg^{2+}	5	3	47.1 ± 2.2	99.4 ± 4.3
	10	4	57.4 ± 2.3**	99.4 ± 3.9
Ca^{2+}	5	3	45.8 ± 2.1	108.2 ± 6.3
	10	3	40.2 ± 2.4	110.9 ± 5.4
Ba^{2+}	5	3	40.6 ± 1.8	101.6 ± 1.2
	10	3	47.2 ± 3.6	100.4 ± 1.5
Fe^{3+}	5	4	43.5 ± 5.9	71.8 ± 1.4**
Cu^{2+}	5	3	39.7 ± 2.0	54.7 ± 2.5**
Cd^{2+}	10	3	54.1 ± 1.1**	68.4 ± 3.0**
Hg^{2+}	5	3	40.5 ± 0.8	51.2 ± 0.4**
	10	3	23.7 ± 2.8*	33.1 ± 0.2**
Mn^{2+}	5	3	92.5 ± 5.1**	100.6 ± 5.4
	10	3	79.3 ± 2.4**	92.9 ± 2.0
Co^{2+}	5	3	153.6 ± 11.2**	84.7 ± 8.3**
	10	3	105.1 ± 13.4**	69.7 ± 5.3**
Ni^{2+}	5	3	78.8 ± 6.4**	116.1 ± 8.1*
Zn^{2+}	5	3	90.7 ± 5.9**	74.7 ± 2.6**

[a]Values are mean ± SEM. *$P < 0.05$ vs. EDTA–plasma, **$P < 0.01$ vs. EDTA–plasma.

mM Ni^{2+}. Since the addition of 5 mM Co^{2+} to EDTA-treated plasma resulted in increase in LPLD activity to 1.5-fold that of heparinized plasma without preaddition of EDTA, we examined the concentration-dependent augmentation of the LPLD activity by Co^{2+}: a maximal value of about 2.5-fold higher than the control was attained at concentrations below 1 mM, as shown in Figure 4.

In order to confirm the metal-cation requirement of plasma LPLD, we performed two types of experiments. First, heparinized rat plasma was dialyzed against saline, pH 7.4, at 4°C for 2 d, resulting in reduction of LPLD activity to 17.2 ± 1.6% ($n = 5$) of controls before dialysis. Addition of 2 mM Zn^{2+} after dialysis increased the LPLD activity to 64.8 ± 14.2% ($n = 5$) of the original activity, but supplement of Ca^{2+} was not as effective (29.4 ± 5.6%, $n = 5$). The second experiment was undertaken to examine reversal of EGTA inhibition of LPLD activity by Zn^{2+} or Ca^{2+}. Addition of 2.5 mM Zn^{2+} to rat plasma anticoagulated with EGTA restored the LPLD activity (81.9 ± 9.9% of the activity of heparinized plasma, $n = 4$), but no reactivation was observed upon addition of 2.5 mM Ca^{2+} (0.2 ± 0.2% of the activity of heparinized plasma, $n = 4$).

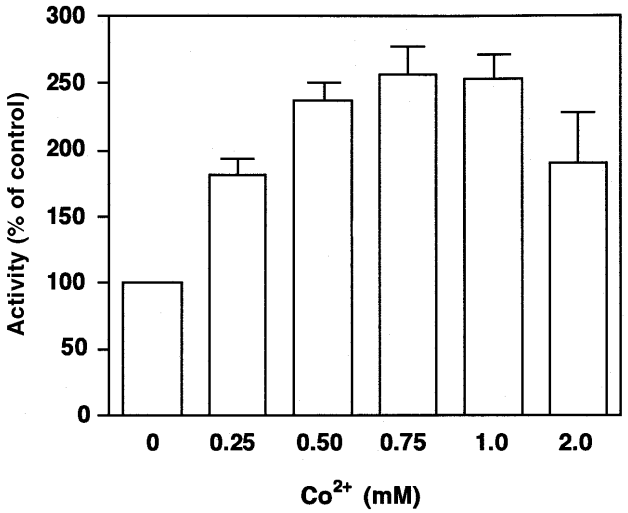

FIG. 4. Potentiation of LPLD activity by cobalt ion. Values are expressed as percentages of the control (heparinized plasma mixed with 0.1 vol of saline) and shown as mean ± SEM of three independent experiments. See Figure 2 for abbreviation.

Effects of short-chain fatty aldehydes and antioxidants on LPLD activity. Several heavy metal ions such as Cu^{2+} induce lipid peroxidation in plasma lipoproteins (23), and short-chain fatty aldehydes, secondary products of lipid peroxidation, inactivate human plasma LCAT (24,25). Therefore, we examined the effects of acetaldehyde, acrolein, and malondialdehyde on the LPLD and LAT activities in rat plasma. As shown in Table 2, both acetaldehyde and acrolein reduced the LPLD activity, whereas a slight increase in the LPLD activity was seen after treatment of the plasma with malondialdehyde. On the other hand, the LAT activity was inhibited by all three aldehydes, consistent with previous results on LCAT in human plasma (25). Acrolein had a stronger inhibitory effect than acetaldehyde on both LPLD and LAT activity.

To confirm that metal-ion-induced lipid peroxidation is involved in the observed inhibitions of LPLD activity by some metal ions, we tested the protective effects of several antioxidants on metal-ion-induced inhibition of LPLD activity. Typical results of this experiment are shown in Figure 5. As expected, incubation of rat plasma with 2 mM of Cu^{2+} resulted in considerable reduction in LPLD activity, and BHT seemed to restore this reduced LPLD activity. However, by increasing the concentration of BHT by 2 mM, the activity increased further, reaching nearly twice the normal level, indicating that the effect of BHT is not related to its activity in preventing lipid peroxidation in the plasma, but rather to its specific stimulatory action on the enzyme. To confirm this idea, we examined the effect of BHT on LPLD activity without preaddition

TABLE 2
Effects of Fatty Aldehydes on LPLD and LAT Activities in Incubated Rat Plasma[a]

Aldehyde	Concentration (mM)	Activity (% of control) LPLD	LAT
Acetaldehyde	5	87.8 ± 1.2**	72.5 ± 11.8
	10	72.5 ± 5.1*	63.9 ± 7.7*
Acrolein	5	72.9 ± 5.1*	48.5 ± 2.0**
	10	65.4 ± 5.7*	37.7 ± 2.8**
Malondialdehyde	5	112.1 ± 6.5	73.7 ± 7.8
	10	116.7 ± 5.2	62.3 ± 5.5*

[a]Values are mean ± SEM of three independent experiments. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control. See Table 1 for abbreviations.

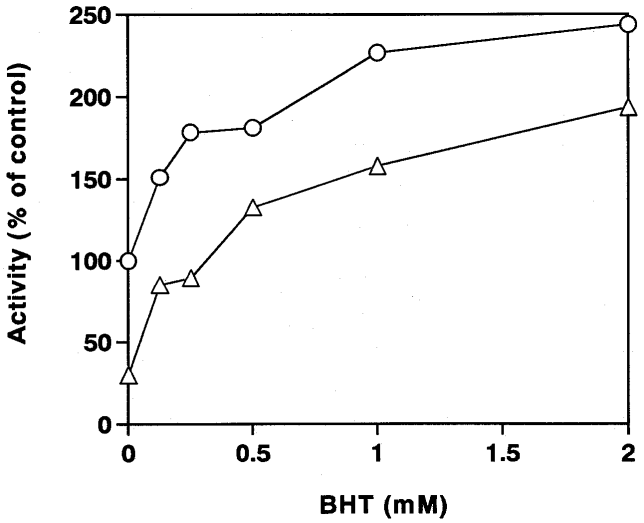

FIG. 5. Effect of butylated hydroxytoluene (BHT) on LPLD activity. Heparinized rat plasma was incubated with various concentrations of BHT in the presence (△) and absence (○) of 2 mM Cu^{2+} for 6 h.

of Cu^{2+}, and found that BHT increased the LPLD activity in a concentration-dependent manner to a similar extent to that observed in the presence of Cu^{2+} (Fig. 5). The above idea was further supported by the results shown in Figure 6, where the effects of three antioxidants at different concentrations on LPLD activity are compared: butylhydroxyanisole increased the LPLD activity to a lesser extent than BHT, and propyl gallate reduced the activity slightly.

Inhibition of LPLD activity by chemicals that modify amino acid residues of proteins. Table 3 shows the inhibitory effects of various compounds that react with specific amino acid residues of proteins on the LPLD activity in rat plasma and on LAT activity for comparison. LPLD was resistant to 1

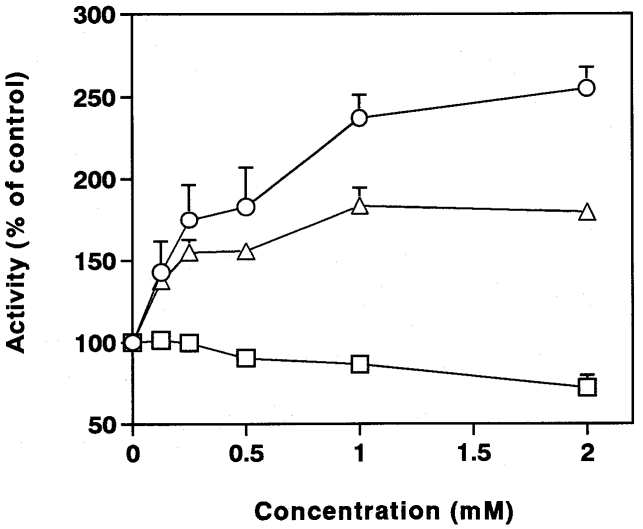

FIG. 6. Effect of antioxidants on LPLD activity. Values are expressed as percentages of that of heparinized plasma and shown as mean ± SEM of three independent experiments. Small error bars are masked by symbols for mean values. Butylated hydroxytoluene (○), butylhydroxyanisole (△), propyl gallate (□). See Figure 2 for abbreviation.

TABLE 3
Effects of Various Amino Acid-Modifying Agents on LPLD and LAT Activities[a]

Inhibitor	Concentration (mM)	Activity (% of control) LPLD	LAT
pBPB	1	103.4	2.7
PMSF	1	99.0	75.7
	3	83.3	63.7
DFP	1	97.5	53.2
	3	88.0	34.8
	10	40.6	1.5
NEM	3	90.7	64.0
	10	75.9	44.9
DTNB	1	73.6	53.3
	3	72.3	36.5
	10	52.4	5.5

[a]pBPB, *p*-bromophenacyl bromide; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide; DTNB, dithio-bis-(2-nitrobenzoic acid). See Table 1 for other abbreviations.

mM *p*-bromophenacyl bromide, unlike LAT in the plasma. Both PMSF and DFP, serine esterase inhibitors, reduced the LPLD activity less than the LAT activity. The results from the effect of serine esterase inhibitors on LAT activity are consistent with previous reports on their effects on rat LCAT (26) and human LAT (22,27). Similar results were obtained with DTNB, a thiol-modifying agent. NEM was less effective than DTNB in inhibiting the LPLD and LAT activities in rat plasma. In this case also, LPLD was more resistant to this thiol-modifying chemical than LAT. These results are consistent with previous results (28).

DISCUSSION

Our previous study (18) showed that 16:0-LPC was the most abundant molecular species in rat plasma (166 ± 9 nmol/mL). Approximately only 2% of the radioactivity of exogenous 16:0-LPC (3.5 nmol/mL) was recovered in LPA during 6-h incubation in the current study as a result of dilution of exogenous 16:0-LPC into large pools of endogenous 16:0-LPC in rat plasma. Based on this assumption, the total amount of 16:0-LPA generated from the endogenous 16:0-LPC during 6-h incubation is estimated to be about 3.7 nmol/mL. If the LPLD activity were not inactivated during further incubation for up to 48 h, 16:0-LPA would amount to 29.6 nmol/mL, which is higher than 17 nmol/mL quantified by gas–liquid chromatography (18). This difference can be explained by a finding that the rate of LPA accumulation was gradually decreased 12 h after incubation of rat plasma at 37°C (Tokumura, A., Miyake, M., Nishioka, Y., Yamano, S., Aono, T., and Fukuzawa, K., unpublished data). Thus, it is likely that our method with radioactive 16:0-LPC could reflect the metabolic conversion of endogenous 16:0-LPC to 16:0-LPA in rat plasma, prompting us to examine some properties of LPLD in rat plasma by this convenient method.

Our results clearly show that LPLD in rat plasma requires a metal ion for optimal activity. Several lipolytic enzymes had Zn^{2+} in their active site: i.e., sphingomyelinase in fetal bovine

serum (29), phospholipase C from *Bacillus cereus* (30), glycosylphosphatidylinositol-specific phospholipase D in plasma (31), and leukotriene A_4 hydrolase in mammalian tissues (32). Therefore, Zn^{2+} is likely to be an endogenous metal ion associated with LPLD. This metal ion may be included in the active center of LPLD, because the reduced activity in the presence of EDTA or EGTA was restored on its addition to the plasma. However, the additional role of stabilization of protein structure by metal ions cannot be completely excluded at this stage.

The LPLD has a relatively broad specific requirement for metal ions, but the strong stimulatory effect of Co^{2+} on LPLD activity in rat plasma is noteworthy. In this context, a broader spectrum of cation requirement (Zn^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}, and Ca^{2+}) was recently reported for phospholipase D activity in mitochondria isolated from rat small intestine (33), and equipotent stimulations by Co^{2+} and Zn^{2+} were reported for purified leukotriene A_4 hydrolase (32) . Like LPLD in rat plasma, the activity of plasma glycosylphosphatidylinositol-specific phospholipase D reduced by EGTA and *o*-phenanthroline can be restored by several metal ions such as Co^{2+}, Ni^{2+} and Mn^{2+}, although these were less effective than Zn^{2+} (31).

The present investigation showed that Cu^{2+} inhibited LPLD and LAT activities in rat plasma, although the mechanism of their inhibition is unknown. A previous report suggested that several short-chain fatty aldehydes inactivated LCAT in human plasma by reacting with a cysteine residue of the enzyme (25). Consistent with this report, both acrolein and acetaldehyde, secondary products of lipid peroxidation, inhibited LPLD activity, suggesting that an essential cysteine residue is near the active center, as in the case of LCAT. However, the Cu^{2+}-induced inhibition of LPLD activity observed in this study cannot likely be attributed to its induction of lipid peroxidation in rat plasma, since incubation with the antioxidant propyl gallate did not inhibit Cu^{2+}-induced inhibition of the LPLD, and BHT prevented the inhibitory effect of Cu^{2+}, but potentiated LPLD activity in the absence of Cu^{2+}.

As noted above, plasma LPLD may have a cysteine residue(s) with an essential catalytic role, because both DTNB and NEM inhibited the LPLD activity in a concentration-dependent manner. Alternatively, the cysteine residue may have a steric effect near the site of substrate binding, as in the case of LCAT (34), although the susceptibility of the cysteine residue of LPLD in rat plasma to these thiol-modifying reagents was less than that of plasma LAT. Similarly, LPLD activity seems to be less sensitive to serine esterse inhibitors such as DFP and PMSF than LAT activity, although no direct evidence exists of a serine residue located in or near LPLD, as reported for LCAT (35). LPLD does not appear to have a histidine residue that plays a catalytic role, unlike that in LCAT (35).

LPLD in rat plasma differs from intracellular LPLD reported in various mammalian tissues (36–39), in both substrate specificity and cation requirement. The existence in plasma of a phospholipase D with Zn^{2+} in its active site was reported (31), but its metal-ion requirement differs from that of rat plasma LPLD. Furthermore, the enzyme has strict substrate specificity: it hydrolyzes glycosylphosphatidylinositol selectively in *in vitro* conditions (40). In conclusion, the LPLD activity in rat plasma is due to an undescribed metalloenzyme. The biological role of plasma LPLD is to generate LPA with growth factor- and hormone-like activities continuously in the circulating blood and to provide it to various tissues. Thus, modulation of plasma LPLD is an interesting subject for further study. Our results suggest that the accumulation of LPA in blood vessels may be affected by various metal deficiencies, cigarette smoking, and *in vivo* lipid peroxidation in the circulation.

ACKNOWLEDGMENTS

This study was supported in part by a grant (07672363) from The Japanese Ministry of Education, Science and Culture. We thank Sizuka Kawano and Yuko Kajiwara for technical assistance.

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[Received September 26, 1997, and in final revised form August 17, 1998; revision accepted August 24, 1998]

Lipid and Fatty Acid Composition of Different Fractions from Rat Urinary Transitional Epithelium[1]

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ABSTRACT: The phospholipid composition of rat urinary transitional epithelium (TE) and the fatty acid composition of microsomal, mitochondrial, cytosolic, and plasma membrane (PM) subcellular fractions were investigated. PM marker enzymes and electron microscopy analysis were used to characterize the PM fraction, which showed a distinctive lipid composition compared to the general profile of PM from different sources. The levels of cholesterol and sphingomyelin were not enriched in the PM fraction; on the other hand, the increased amounts of glycosphingolipids and phosphatidylserine, and the decreased level of phosphatidylcholine followed the general features of a PM profile. This differential PM lipid composition may reflect the unique morphology of this mammal TE, consisting of concave plaques with an asymmetrical membrane unit. The distribution of the double bond across the PM indicated a higher unsaturation of the inner relative to the outer part of the PM hemileaflet. In addition, the presence of 20:3n-9 nonessential fatty acid in a normal TE may represent a characteristic fatty acid metabolism of this epithelium.

Lipids 33, 1017–1022 (1998).

The transitional epithelium (TE) covering the innermost surface of the organs of the mammal urinary tracts consists of several layers of cells. The luminal surface of this epithelium has a thick asymmetric plasma membrane (PM) which is in contact with hypertonic urine (1). As a consequence, this epithelium exhibits a strikingly low permeability to water and solutes of small molecular size (2,3). In spite of the relatively large amount of data available on the protein components of this membrane in normal (4–6) or pathological (7,8) conditions, the molecular bases responsible for this lack of permeability remain unknown. Previous reports on the lipid composition of the luminal surface of bladder epithelial cells (9,10) indicated that phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylinositol, and cerebroside (CB) are the major components of the lipid fraction. Ketterer *et al.* (9) reported the presence of saturated and polyunsaturated fatty acids, mainly n-6 derivatives, and found high levels of one eicosatrienoic acid, an unusual fatty acid for a normal tissue since it is a hallmark of essential fatty acid (EFA) deficiency (11). As far as we know, no further studies on the fatty acid composition of this epithelium have been done. Thus, it is important to know whether the peculiar asymmetry of the PM proteins and lipids (including their fatty acid composition) may play a role in barrier function (12). Urothelial PM possesses the same lipid classes as all biological membranes; however, the ratio among these classes and the molecular species are unique for each membrane. Most lipids are synthesized in the endoplasmic reticulum (ER); consequently, the finding of a particular ratio in each organelle implies that lipid sorting may occur in the traffic route from the biosynthesis site to the final localization. The question is how the interplay between the biosynthetic site and all the processes during the lipid sorting and traffic may result in unique urothelial PM characteristics. Even more interesting is how this interplay is modulated by different cellular conditions. Following this reasoning, we have investigated in this work the lipid and fatty acid composition of microsome (Mic), mitochondrial (Mit), cytosol (Cyt), and PM fractions from rat urinary bladder epithelium as a preliminary approach to understanding the morphological, functional, and biochemical changes of those organelles resulting from dietary lipid manipulation.

MATERIALS AND METHODS

Animals. Sprague-Dawley adult rats of both sexes and weighing 250–350 g were used.

Isolation of TE. TE cells from urinary bladders of 30 rats were obtained using a procedure developed in our laboratory for exfoliating urinary epithelium (13). Histological controls were done in order to ensure that basal membrane remained undamaged and attached to the underlying connective tissue.

Subcellular fractionation and PM purification. The procedure was essentially as described by Stubbs *et al.* (14). Briefly, the cell pellet containing the exfoliated and washed TE was homogenized in 10 mM Tris buffer (pH 7.4) contain-

[1]The authors wish to dedicate this work to the memory of the late Professor Benito Monis, who participated in the generation of this research and whose working hypothesis remains a source of fruitful inspiration.

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Abbreviations: CM, crude membranes; Cyt, cytosol; D-BI, double-bond index; EFA, essential fatty acid; ER, endoplasmic reticulum; GSL, glycosphingolipids; Mic, microsome; Mit, mitochondria; PC, phosphatidylcholine; PM, plasma membrane; PS, phosphatidylserine; SPH, sphingomyelin; TE, transitional epithelium.

ing 1 mM each of EDTA and EGTA. The same buffer was used during the entire procedure. After centrifugation at 63,600 × *g* for 30 min, the supernatant was kept as the Cyt fraction, and the pellet was resuspended in the buffer and layered on a support of 1.6 M sucrose (in the same buffer). This two-layer sucrose gradient was centrifuged for 30 min at 10,200 × *g*. The pellet, consisting of nuclear and cell debris, was discarded. The upper layer was then centrifuged at 63,000 × *g* for 15 min. The resulting pellet, or crude membrane (CM), was resuspended in buffer and layered over a discontinuous sucrose density gradient consisting of steps of 1.3, 1.1, and 0.75 M sucrose in buffer. The centrifugations were performed in an SW 25.1 (Beckman) rotor at 26,000 rpm for 2 h. Membranes at each interface, named P1, P2 and P3 from the lower to the higher density, were collected, diluted with 10 vol of buffer, and centrifuged at 10,000 × *g* for 30 min. The purity of the gradient-separated fractions was assayed by determination of enzyme marker activities and subsequent electron microscopy examination.

Electron microscopy procedure. Membrane fractions were prepared as described (15). Briefly, pellets were fixed in 2.5% glutaraldehyde in 0.02 M sodium cacodylate, pH 7.3, and postfixed in osmium tetroxide. Thin sections from three different blocks of each pellet were obtained and stained with uranyl acetate and lead citrate and examined in a Siemens 101 electron microscope.

Membrane enzyme assay. 5′-Nucleotidase (E.C. 3.1.3.5), glucose-6-phosphatase (E.C. 3.1.3.9), succinic-INT-reductase [INT: 2-(*p*-indophenyl)-3(*p*-nitrophenyl)-5-phenyltetrazolium], and thiamine pyrophosphatase were assayed as described by Morre (16). Na-K-ATPase was determined by the procedure of Post and Sen (17). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Phospholipid determination. Total lipids were extracted and fractionated into neutral and acidic lipids as described by Macala *et al.* (18). Phospholipid was measured in lipid extracts by the modified microprocedure of Bartlett (19). For phospholipid class analysis, acidic or neutral phospholipids were fractionated by high-performance thin-layer chromatography, developed, and quantified by densitometry (Shimadzu CS-9000 scanning densitometer) as described by Macala *et al.* (18).

Fatty acid determination. The total lipid fraction was saponified in 10% KOH containing 50% MeOH as described by Bridges and Coniglio (20). Digests were acidified (pH 2.0) with concentrated HCl and extracted three times with petroleum ether. Combined extracts were taken to dryness under N_2 and methylated at 95°C with 3% methanolic HCl. Methyl esters were extracted with petroleum ether and quantitated by gas–liquid chromatography as previously described (21).

RESULTS

Several membrane markers were determined in subcellular fractions of urinary TE. As shown in Table 1, the specific activities of enzymes associated with the PM (5′-nucleotidase, Na-K-ATPase) were enriched in P2 fraction relative to the specific activities of the enzymes in the CM fraction. On the other hand, the specific activities of the enzymes associated with Mit [succinic-INT-reductase, where INT is 2-(*p*-indophenyl)-3(*p*-nitrophenyl)-5-phenyltetrazolium] and Golgi (thiamine pyrophosphatase) were decreased relative to their specific activities in CM fraction. The P2 fraction (PM) was somewhat contaminated with ER as indicated by the specific activity of glucose-6-phosphatase. Overall, the enzymatic profile of P2 as well as the ultrastructural studies (Fig. 1), showing a great profusion of profiles with the characteristic concave plaques of the asymmetric unit membrane of this epithelium (22), were consistent with a luminal PM origin of the TE cells.

Table 2 shows the lipid composition of TE. The cholesterol/phospholipid molar ratio (C/P: 0.64) was similar to that reported for luminal PM of TE (9,10,23). This may represent a characteristic of luminal PM of TE since most PM are enriched in cholesterol with respect to the levels found in the total homogenate. The only report showing a cholesterol/phospholipid (molar ratio) for PM higher than the value reported in this work for total homogenate is that from Stubbs *et al.* (14). The lipid and phospholipid contents (as the percentage of dry weight) were lower in TE than those reported for purified luminal PM (Table 2). Table 3 shows that the major phospholipids of TE were PC, phosphatidylethanolamine, phosphatidylserine, and cerebroside. Table 4 gives the fatty acid composition of total lipids of different subcellular fractions from TE. All fatty acids were identified according to their retention time on gas chromatography, as compared with known pure standards. The major fatty acids in PM were 16:0, 18:0, 18:1, 18:2, 20:3n-9, and 20:4n-6. Intriguingly, the nonessential 20:3n-9

TABLE 1
Specific Activities of Marker Enzymes in Subcellular Fractions of TE[a]

Fraction	CM	P1	P2	P3	Cyt
5′-Nucleotidase	1.4 ± 0.3	3.0 ± 1.1	25.0 ± 4.0	7.0 ± 1.0	7.0 ± 0.9
Na-K-ATPase	5.0 ± 0.9	12.0 ± 1.3	130.0 ± 1.3	40.0 ± 5.2	3.0 ± 1.3
Glucose-6-phosphatase	3.0 ± 0.8	49.0 ± 4.4	8.0 ± 1.4	15.0 ± 1.2	27.0 ± 1.9
Thiamine pyrophosphatase	12.0 ± 1.0	26.0 ± 1.3	6.0 ± 0.36	36.0 ± 1.8	96.0 ± 4.8
Succinic-INT- reductase	26.0 ± 2.1	1.8 ± 0.6	5.2 ± 1.5	330.0 ± 13.2	12.0 ± 0.1

[a]Cellular fractions were isolated as described in the Materials and Methods section. All values are 10^3 times the specific activities ± SEM. Succinic-INT-reductase is expressed in absorbance/mg prot/min; all others are expressed as µg Pi/mg prot/min. Values are the average of three experiments. Abbreviations: TE, transitional epithelium; CM, crude membranes; P1, microsome; P2, plasma membrane; P3, mitochondria; Cyt, cytosol; INT, 2-(*p*-indophenyl)-3(*p*-nitrophenyl)-5-phenyltetrazolium.

TABLE 2
Lipid Composition of Rat Urinary TE[a]

Reference	Lipid (μg/μg dry weight)[b]	Protein (μg/μ/g dry weight)[b]	Phospholipid (μg/μg protein)	C/P (molar ratio)
This report[c]	0.28	0.78	0.17	0.64
Caruthers and Bonneville (23)[d]	0.54	—	0.93	0.58
Ketterer *et al.* (9)[d]	—	—	—	0.6
Stubbs *et al.* (14)[d]	0.59	0.41	0.26	1.89
Vergara *et al.* (10)[d]	0.65	0.32	1.1	0.47

[a]Lipid analyses were performed as indicated in the Materials and Methods section.
[b]Dry weight represents the sum of the total lipids and total protein determinations.
[c]Values correspond to total TE.
[d]Values correspond to PM fraction. All values are the average of three experiments ± SEM. Maximal SEM values for all determinations were below ± 6% of the mean. C/P, cholesterol/phospholipid molar ratio. For other abbreviation see Table 1.

TABLE 3
Phospholipid Composition of Rat Urinary TE[a]

Lipid class	Percentage of total phospholipids	Percentage of total lipids
Total phospholipids	100.0	59.3 ± 3.8
SPH	6.5	3.9 ± 0.2
PC	59.7	35.0 ± 3.5
PE	24.5	14.5 ± 1.3
PI	2.3	1.3 ± 0.1
PS	7.6	4.5 ± 0.5
CB	7.1	4.2 ± 0.3
Sulf	0.6	0.3 ± 0.02
FA	—	16.9 ± 1.0
C	—	19.2 ± 2.4

[a]Lipid analyses were performed as indicated in the Materials and Methods section. Values represent the average of three experiments ± SEM. SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CB, cerebroside; Sulf, cerebroside sulfatide; FA, fatty acid; C, cholesterol. For other abbreviation see Table 1.

fatty acid appeared in a high proportion (14%) in the Mic fraction. Nervonic acid (24:1n-9) was detected only in CM and PM fractions (results not shown). Our data show that the double-bond index (D-BI) of PM (0.9) was lower than that of the Mic fraction (1.9). Similar differences in the D-BI between both PM and Mic were observed in hepatocyte membranes (24). Major changes in PM relative to Mic fraction were increased values for palmitic (16:0), stearic (18:0), and oleic (18:1) acids and decreases in 20:3n-9 and arachidonic acid (20:4). Accordingly, the ratios 16:0/18:0, 18:0/18:1, and 18:0/20:4 were increased in PM. The Mit showed the lowest D-BI (0.3), whereas the Cyt fraction, containing probably very small vesicles, showed the same value of PM D-BI (0.94).

The introduction of multiple double bonds creates structurally different zones within the bilayer. The structural gradient arising from different concentration of double bonds at different depths of the bilayer membrane may influence membrane function. Thus, it is important to analyze the double-bond profile across the bilayer. Table 5 illustrates the degree of unsaturation along the hydrocarbon chain from C_5 to C_{12}. Clearly, the largest difference between PM and Mic corresponded to the C8 and C11 depths.

TABLE 4
Fatty Acid Composition of Subcellular Fractions from Rat TE[a]

Fatty acid	TH	PM	Mic	CM	Mit	Cyt
16:0	13.8	19.3	1.9	20.7	19.6	19.6
16:1	2.3	3.4	7.3	7.7	n.d.	3.4
18:0	16.7	19.5	7.8	9.0	50.0	20.6
18:1	17.0	21.7	14.7	31.2	21.4	21.0
18:2	1.6	6.0	15.7	14.3	1.6	n.d.
20:1n-9	0.4	1.9	14.3	0.3	n.d.	0.3
20:2n-9	3.7	2.0	n.d.	0.8	n.d.	0.3
20:3n-9	18.0	1.2	14.3	0.7	n.d.	0.3
20:4n-6	11.4	6.3	10.8	1.7	n.d.	14.3
20:4n-3	0.6	3.8	9.4	n.d.	n.d.	2.1
22:1n-9	0.4	3.9	0.8	n.d.	n.d.	0.2
D-BI	1.3	0.9	1.9	0.8	0.3	0.9
$D\text{-}BI_{EFA}/D\text{-}BI_{non\text{-}EFA}$	0.62	1.36	1.4	0.82	0.15	2.48

[a]Cellular fractions were isolated as described in the Materials and Methods section. Only the more representative fatty acids are shown. Values represent percentage of total. The SEM for all values (three separate determinations) were not above 10% of the mean shown. D-BI is the sum of the fraction of each fatty acid times the number of double bonds in that acid. TH, total homogenate; PM, plasma membrane; Mic, microsome; CM, crude membranes; Mit, mitochondria; Cyt, cytosol. For other abbreviation see Table 1.

TABLE 5
Degree of Unsaturation at Different Membrane Depths[a]

Carbon number	5	6	7	8	9	10	11	12
PM	8.6	1.2	1.6	14.3	32.6	0.1	16.4	6.9
Mic	26.0	0.0	4.7	35.4	35.4	3.2	49.7	15.7

[a]Values are the sum of the percentages of each fatty acid from PM and Mic fractions containing a double bond at the carbon indicated. For abbreviations see Table 4.

DISCUSSION

This work reports the fatty acid composition of several subcellular fractions and additional data about phospholipid content of rat urinary bladder TE. It is important to try to relate the striking plasticity and recycling of luminal surface membrane and their discoidal vesicles of TE cells (25,26) to the lipid composition of different membrane compartments. Moreover, luminal PM is part of a complex membranous system where interrelationships among all components may be modulated by different factors. The biology, biochemistry, biophysics, and pathology of this epithelium are particularly interesting. Table 1 summarizes the enzymatic activities of several subcellular fractions from TE, showing that the P2 fraction resembles a PM profile with low enzyme activity of markers other than those in PM organelles, indicating an acceptable degree of purification. This was corroborated by ultrastructural observations (Fig. 1).

Table 2 indicates that total lipid and phospholipid contents (as percentage of dry weight) were lower in TE than those reported for purified luminal PM (4). This is not an unexpected result since loss of soluble Cyt proteins occurs after PM purification.

It is known that PM are typically enriched in cholesterol, sphingomyelin (SPH), and phosphatidylserine (PS), whereas PC is decreased in comparison to those values in ER (27). This implies that cholesterol and SPH are sorted and concentrated together in their pathway to the trans-Golgi and further out (27). Interestingly, this does not seem to be the case for urothelial TE since cholesterol and SPH contents in total TE (37 and 6.5, respectively, Table 3) were about the same as those in PM (40 and 8.5, respectively; Refs. 9,14). Alternatively, the relative lack of concentration at the luminal surface of these two typically PM lipids may also imply a poorly developed ER. If this is the case, PM-like organelles such as Golgi, endocytotic vesicles (endosomes), and the PM itself would be the major components of the total membrane fraction of this epithelium. By contrast, PS content was lower in total TE (7.6%) than that reported for PM (26.3%; Refs. 9,14), in agreement with the general concept that PS is enriched in PM. The high amount of PS in PM may contribute to maintaining the integrity of the rigid plaques of the urinary bladder luminal surface through the interaction of PS with the cytoskeleton (28). It has been shown that anionic phospholipids (i.e., PS) in urothelial membrane greatly increase calcium oxalate–crystal membrane interactions (29). The amount of PC was higher in total TE (59.7%) than in PM (22.0%, Refs. 9,14). This result agrees with the general idea that the enrichment of glycosphingolipids (GSL) at the apical surface is at the expense of PC. GSL content was previously reported (9,13,14); and nervonic acid (24:1n-9), a characteristic compound of monohexosyl GSL (cerebrosides), was detected in Cyt (enriched in Golgi marker enzyme, see Table 1) and PM fractions. The enrichment of GSL and the lack of concentration of SPH in PM fraction would indicate, contrary to the concept on their intracellular traffic to the surface (27), that these two lipids are sorted in different domains. This may represent another particular characteristic of the lipid transport of this mammal's TE.

The high percentage of the nonessential fatty acid 20:3n-9 (Table 4) was not unexpected since an eicosatrienoic acid was first described by Ketterer *et al.* (9,14) in luminal surface of rat urinary bladder epithelium. The presence of this non-EFA in ER (Mic), the site where the acyl desaturase activity is localized, has no explanation at the present time. Nevertheless,

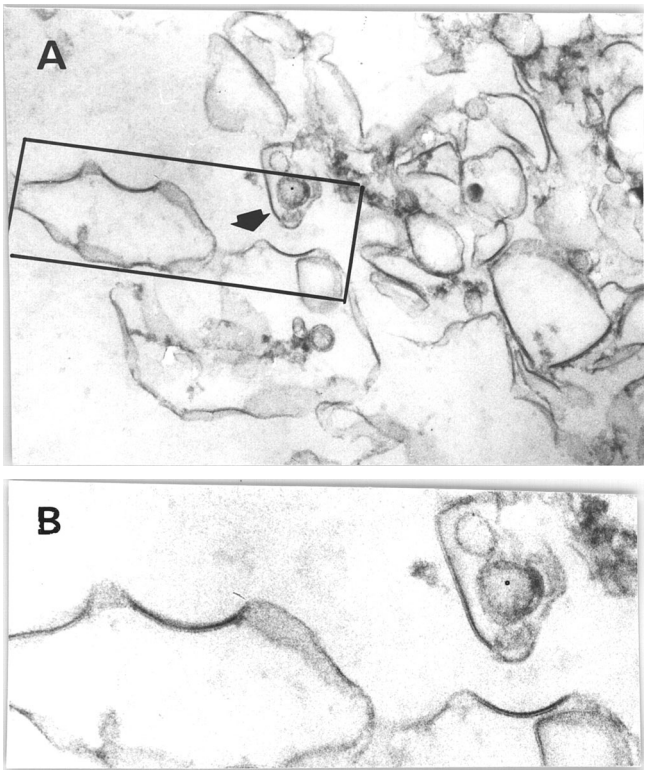

FIG. 1. Both panels correspond to pellets of plasma membrane fraction (P2) from rat urinary transitional epithelium. The subcellular fraction was obtained and prepared for electron microscopy analysis as described in the Materials and Methods section. Typically scalloped membrane profiles showing the thick, asymmetrical plasma membrane (arrow) are seen. (A): 20,000 ×, (B): 60,000 ×.

the accumulation of 20:3n-9 in TE, also described in normal human cartilage (30), may suggest a metabolic shift of the elongation and desaturation enzyme activities to the precursors of the nonessential n-9 fatty acid family (31) in response to a shortage of n-6 EFA precursors. Testing this hypothesis will require further metabolic and dynamic studies. On the other hand, exogenous 20:3n-9 behaves as a down-regulator of certain antimetastatic cell adhesion molecules (E-cadherin and desmoglein) in human epidermoid cells (32).

Contrary to the difference observed in the D-BI of PM and Mic fractions, no significant change in the values of the D-BI_{EFA}/D-$BI_{non\text{-}EFA}$ ratios of those fractions was observed. These assessments emphasize the need to study unsaturation in the cross-sectional direction of the bilayer. The results (Table 5) show that the maximal differences between PM and Mic fractions were localized at the depths of C8 and C11. Table 4 indicates that all fatty acids containing double bonds at C8 and C11 (20:3n-9, 20:4n-6, 20:4n-3, and 20:1n-9) were decreased in the PM fraction relative to those values in Mic fraction. Nevertheless, the fatty acids showing the greatest decline were 20:1n-9 and 20:3n-9. According to the representation in Leger *et al.* (33), the first double bond starting from the methyl group in 20:1n-9 and 20:3 n-9 causes a shortening of one C-C bond; this results in a shift of the double bond in the 11-position from the inner to the outer half of the leaflet defined by the C10 depth. Thus, the decreased amounts of these fatty acids in PM may represent a lesser degree of unsaturation of the outer part of the leaflet with a concomitant higher unsaturation of the inner part, relative to those of the Mic membrane. It is tempting to consider that the different shape of the double-bond distribution in urethelial PM may affect the structural order and function of this membrane, leading to the unique properties and morphology of the urinary luminal surface.

In summary, from the comparison of the phospholipid content of total TE (this report) and PM fraction (14), some evidence is presented that cholesterol, SPH, and GSL do not follow the general concept that they are transported in the same microdomain from the ER to the PM. This also suggests that differential regulatory mechanisms may govern the intracellular traffic of those lipids. The presence of the nonessential 20:3n-9 fatty acid in normal TE is an important point in future metabolic studies of this epithelium. The study of the unsaturation across the membrane from different fractions revealed that plasma membrane C11 depth is different from that of the other fractions, and this may represent important functional consequences for PM. Finally, lipid quantitation of different subcellular membrane fractions may be useful as a complementary approach for future assessments to how compositional distribution may affect or reflect cellular function, particularly compartmental interrelationships within the cell.

ACKNOWLEDGMENTS

This work was supported in part by SECYT-UNC, CONICET, and CONICOR, Argentina. We thank Nelida Ramonda and Lucia Artino for skillfull technical assistance.

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[Received April 28, 1998, and in final revised form and accepted September 1, 1998]

Alterations in Heart and Kidney Membrane Phospholipids in Hypertension as Observed by ^{31}P Nuclear Magnetic Resonance

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ABSTRACT: Abnormalities of phospholipids in hypertension have previously been described in human erythrocyte, platelet, and plasma lipoproteins. Since the heart and kidney are adversely affected by hypertension, we investigated possible alterations in their membrane phospholipids, which could play a role in the derangement of intracellular ion balance widely observed in hypertension. The phospholipid compositions of heart and kidney from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were determined by using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Absolute contents of all phospholipids in hypertensive hearts and kidneys were significantly higher than in normotensive hearts and kidneys. Expressed as a fraction of total phospholipid, cardiolipin (CL) and phosphatidylethanolamine plasmalogen (PEp) were significantly increased in SHR hearts compared to WKY hearts (CL and PEp were 7.95 ± 0.22% and 13.16 ± 0.35% in SHR vs. 7.01 ± 0.20% and 11.19 ± 0.42% in WKY rats, $P \leq 0.05$), but phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were significantly decreased in SHR (PE and PC were 22.46 ± 0.37% and 44.81 ± 0.43% in SHR vs. 24.02 ± 0.44% and 46.01 ± 0.50% in WKY rats, $p \leq 0.05$). In the phospholipids extracted from rat kidneys, the percentage of PE was significantly higher for SHR than for WKY rats (20.37 ± 0.60% vs. 18.43 ± 0.37%, $P \leq 0.05$), while PEp and phosphatidylserine (PS) were significantly lower for SHR (PEp and PS were 10.22 ± 0.36% and 8.42 ± 0.28% in SHRs vs. 11.29 ± 0.36% and 9.71 ± 0.40% in WKY rats, $P \leq 0.05$). The above alterations in phospholipid composition might contribute to the higher oxygen consumption in the hypertensive heart and abnormal intracellular ion concentrations and ion transport in the heart and the kidney in hypertension.

Lipids 33, 1023–1030 (1998)

Abnormalities in phospholipids of erythrocyte membranes from essential hypertensive patients have been reported in the literature (1). Phospholipid composition of human red blood cell membranes for hypertensive patients was different from that for normal individuals, and these differences could be correlated to alterations in Na^+–Li^+ exchange activity across the erythrocyte membrane in hypertension. Altered phospholipids were also found in plasma lipoproteins (2) and in platelet membranes (3) in hypertensive patients. In animal models, abnormal phospholipids have been reported in the jejunal brush-border membranes from spontaneously hypertensive rats (SHR) (4), in left and right ventricles of aorta-constricted rats (5), and in pressure-overload hypertrophied rat hearts (6). Changes in phospholipids in the kidney were also observed during ischemia (7) and other pathological conditions (8). Depletion of dietary magnesium and/or phosphorus also alters membrane lipids (9,10). However, to our knowledge, there are no reports of alterations in phospholipid compositions of heart and kidney membranes in hypertension. Since abnormalities in cardiac and renal functions often are associated with hypertension, we investigated possible alterations in phospholipid compositions of the heart and kidney membranes in hypertension. Alterations in membrane phospholipids in the heart and the kidney might contribute to the abnormal intracellular ion concentrations and ion transport observed in these organs in hypertension.

We utilized the SHR as a model of human hypertension (11) with the Wistar-Kyoto (WKY) rat serving as the normotensive control. The phospholipid compositions of heart and kidneys from SHR and WKY rats were determined by using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. This technique has been successfully used to measure phospholipid composition of biological membranes (1,12–14).

MATERIALS AND METHODS

Materials. Anhydrous methanol (MeOH), deuterated chloroform ($CDCl_3$), and deuterium oxide (99.8% D_2O) were supplied by Aldrich (Milwaukee, WI). Potassium chloride (KCl), chloroform ($CHCl_3$), ethylenediaminetetraacetic acid (EDTA), cardiolipin (CL) sodium salt from bovine heart, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from frozen egg yolk, phosphatidylserine (PS) sodium salt and sphingomyelin (Sph) from bovine brain, and phosphatidylinositol (PI) ammonium salt from bovine liver were purchased from Sigma Chemical Company (St. Louis, MO).

Phospholipid preparation. Male SHR (250–400 g) were used as hypertensive models. Male WKY rats (250–400 g) were used as the normotensive controls. Pentobarbital anesthesia (65 mg/kg i.p.) was administered; laparotomy was per-

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Abbreviations: CL, cardiolipin; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEp, phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; PS, phosphatidylserine; SHR, spontaneously hypertensive rat; Sph, sphingomyelin; WKY, Wistar-Kyoto.

formed. Fresh kidneys and hearts were taken out and frozen immediately by using liquid nitrogen or dry ice. Total lipid was extracted by a modification of the methods of Meneses and Glonek (15), and Bligh and Dyer (16). Frozen tissues were weighed and then homogenized, using a homogenizer, in 5 mL of a mixture of chloroform/methanol (2:1) for 2 min. The homogenates were transferred to a flask, and more chloroform/methanol (2:1) mixture was added up to 20 vol per unit tissue wet weight (20 mL/g). The mixture was stirred for 20 min and filtered through a sintered glass funnel. The same volume of solution was used to rinse the residuals through the funnel. The filtrate was mixed with 0.2 times its total volume with 0.74% KCl to remove all nonlipid impurities and allowed to separate overnight. The bottom (chloroform) layer was collected and dried in a rotary evaporator at 30°C (13,15–17). The purified dry phospholipids were suspended in 3 mL of the solvent mixture solution [$CDCl_3$/MeOH (anhydrous)/0.2 M EDTA (in D_2O) = 118:8:3] and used for NMR experiments (1).

NMR measurements. The phospholipid extract was placed in a 10-mm NMR tube and allowed to stand for a few minutes until the aqueous phase had separated. The spinning turbine was adjusted along the NMR tube so that only the chloroform phase was in the region of signal detection. ^{31}P NMR spectra for analyzing phospholipids in the extracts of kidney and heart membranes were obtained at 202.3 MHZ on a Varian VXR-500 NMR spectrometer using a spectral width of 10,000 Hz, 16 K data points, a flip angle of 60° (pulse width, 30 μs), an acquisition time of 0.82 s, a relaxation delay of 4.2 s, and 512 transients, at room temperature. Chemical shifts are referenced to PC set at −0.84 ppm (1,12,13). Each resonance in the ^{31}P NMR spectra of phospholipid extract samples was assigned by spiking the samples with pure known phospholipids (PC, PE, PS, PI, Sph, and CL) purchased from Sigma. Our assignments are generally consistent with those in the literature (1,12,13), although ^{31}P peak positions are in some cases (e.g., PS and Sph) somewhat displaced relative to those published in the literature owing to differences in solvent used. Resonance intensities were measured as peak areas. Relative intensities remained unchanged upon doubling the pulse recycle time, showing that they were unaffected by relaxation times. The areas under the ^{31}P NMR resonances in the spectra of phospholipid extracts were integrated to obtain concentrations that are expressed as absolute concentrations and also as percentage of total phospholipid measured as area under all resonances for the seven major classes of phospholipids. Only half of the area under the CL resonance was included in the concentration and percentage calculations, since this phospholipid contains two phosphorus atoms. The resonance of a methyl phosphonate sample of known concentration was used to quantitate individual phospholipids in extracts as μmol/g wet tissue weight.

Statistical analysis. All data are reported as the mean ± standard error. The statistical significance of the differences between SHR and WKY rats was analyzed by the nonpaired *t*-test. $P \leq 0.05$ was considered significantly different.

RESULTS

Figures 1 and 2 show typical ^{31}P NMR spectra of phospholipids extracted from heart and kidney membranes of SHR and WKY rats. The assignments of the phospholipid resonances are indicated in the spectra and were obtained as described in experimental procedures. Two types of PE are present in heart and kidney membranes, and they can be discriminated by ^{31}P NMR spectroscopy: diacyl PE and alkenylacyl PE (PE plasmalogen, PEp).

As shown in Table 1, the absolute amounts of all the major phospholipid components were significantly higher in extracts from hearts of hypertensive rats than of normotensive rats. The percentages of CL and PEp in total phospholipid were also significantly higher for SHR than for WKY rats (CL and PEp were 7.95 ± 0.22% and 13.16 ± 0.35% in SHR heart membranes vs.7.01 ± 0.20% and 11.19 ± 0.42% in WKY heart membranes, $P \leq 0.05$), but the percentages of PE and PC were significantly lower for SHR than for WKY rats (PE and PC were 22.46 ± 0.37% and 44.81 ± 0.42% in SHR heart membranes vs. 24.02 ± 0.44% and 46.01 ± 0.50% in WKY heart membranes, $P \leq 0.05$).

Table 2 shows data on phospholipids extracted from rat kidneys. As in hearts, the absolute amounts of all ofthe phospholipid components in kidneys were significantly higher in hypertensive rats than in normotensive rats. The percentage of PE in total phospholipid was significantly higher for SHR than for WKY rats (20.37 ± 0.60% in SHR kidney membranes vs. 18.43 ± 0.37% in WKY kidney membranes, $P \leq 0.05$), but the percentages of PEp and PS were significantly lower for SHR than for WKY rats (PEp and PS were 10.22 ± 0.36% and 8.42 ± 0.28% in SHR kidney membranes vs. 11.29 ± 0.36% and 9.71 ± 0.40% in WKY kidney membranes, $P \leq 0.05$).

DISCUSSION

Biological membranes are composed of protein, phospholipid, and cholesterol and are organized as the structural framework for a wide variety of membrane functions including membrane permeability barrier and appropriate environment for the optimal function of transmembrane proteins such as ion channels and ion pumps (18). Phospholipids comprise the predominant chemical components present in the membranes. In heart and kidney the main phospholipid components are PC, PE, PS, PI, Sph, CL. It is well known that phospholipid bilayers are asymmetrical (19). The head groups of the phospholipids PI and PS are negatively charged, and these two anionic phospholipids reside primarily in the inner leaflet of the plasma membrane. PC, PE, and Sph are neutral. PC and Sph are predominantly present in the outer leaflet, whereas PE is predominantly restricted to the inner leaflet. CL contains four fatty acyl chains and a backbone of three glycerol groups chained together by two negatively charged phosphate groups (20,21). In mammalian tissues, CL is almost exclusively localized in mitochondria where it is biosynthesized (22–24). Extensive research on phospholipids suggests that alterations in phospholipid compo-

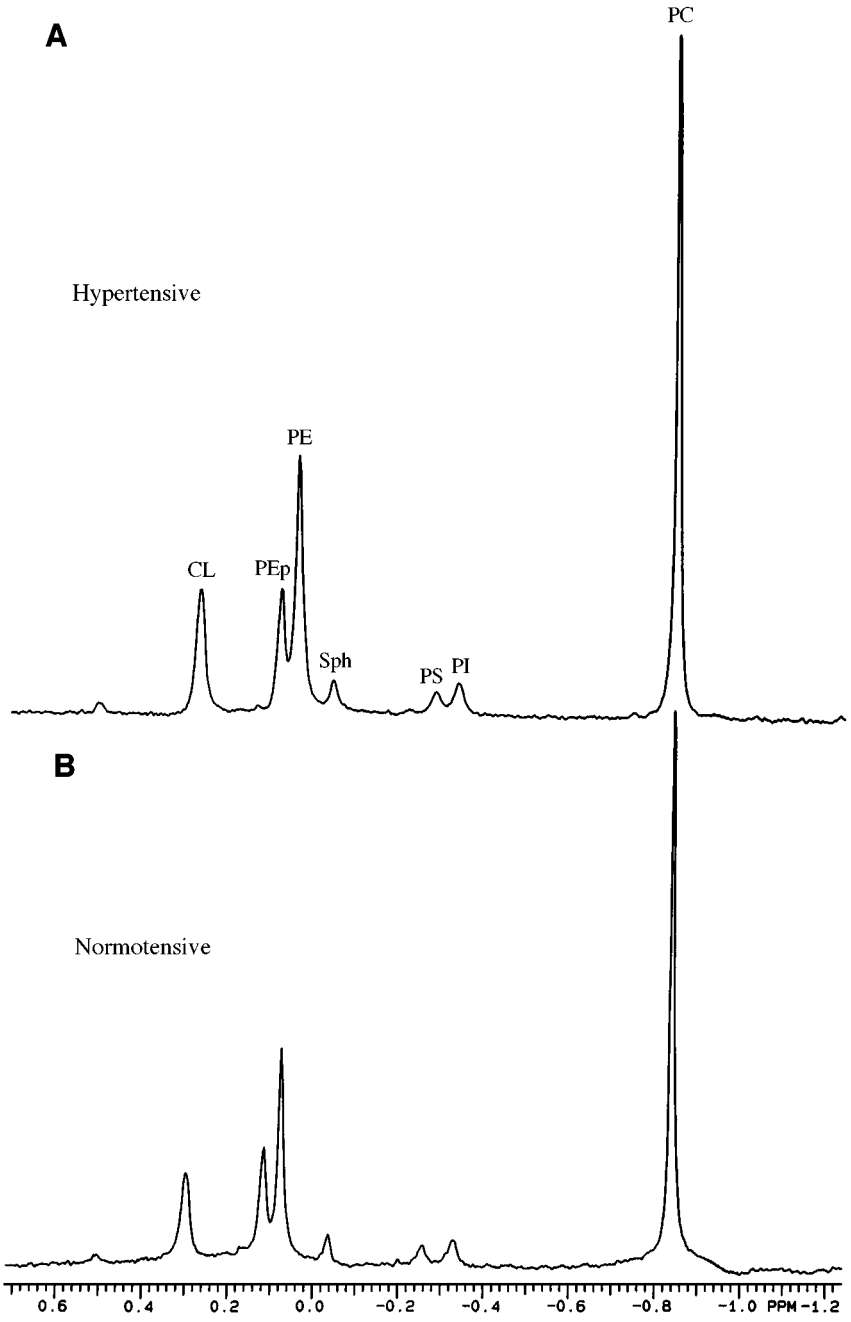

FIG. 1. Comparison of ^{31}P nuclear magnetic resonance (NMR) spectra of phospholipids from a spontaneously hypertensive rat heart (A) and a normotensive rat heart (B). Assignments of various resonances to individual phospholipids are shown. Chemical shifts are referenced to PC set at –0.84 ppm. Vertical scales have been adjusted to take into account small differences in tissue weights. Abbreviations: CL, cardiolipin; PEp, phosphatidylethanolamine plasmalogen; PE, phosphatidylethanolamine; Sph, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.

sition may cause significant modification of membrane functions. For example, Lijnen *et al.* (25) reported a significant correlation between total phospholipid content of erythrocyte membranes and the activities of Na^+–Li^+ exchange, Na^+–K^+ cotransport, and Na^+ pump. Engelmann *et al.* (26,27) reported that a change in composition of only 3% of total membrane lipids could cause up to 40% variation in sodium–lithium countertransport activity. They also showed that small changes in membrane PC or Sph (~5% of total phospholipid) resulted in doubling or halving of the rate of Na^+–Li^+ exchange (28). Thus

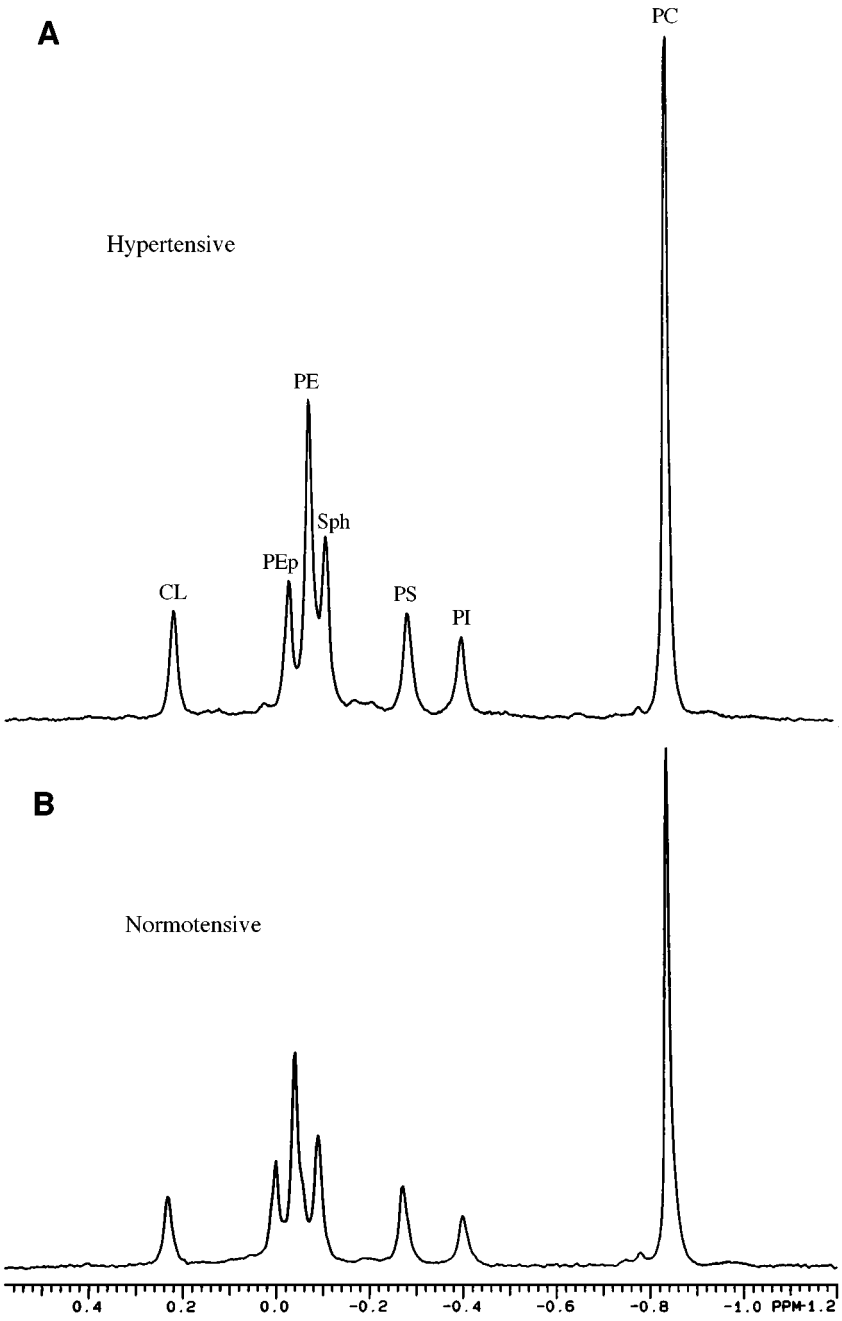

FIG. 2. Comparison of ^{31}P NMR spectra of phospholipids from a spontaneously hypertensive rat kidney (A) and a normotensive rat kidney (B). Assignments of various resonances to individual phospholipids are shown. Chemical shifts are referenced to PC set at –0.84 ppm. Vertical scales have been adjusted to take into account small differences in tissue weights. For abbreviations see Figure 1.

small changes in membrane lipids could, in principle, be responsible for significant alterations in ion transport activities.

In this study, the experimental data in Tables 1 and 2 and Figures 1 and 2 show a sizable increase in all of the membrane phospholipid components in the heart and the kidneys from the SHR in comparison to a normotensive rat. Earlier work also showed altered phospholipids in the heart and kidneys under pathological conditions. Foudin *et al.* (29) reported that PE, PEp and PI were significantly increased in heart mitochondria from rats after chronic ethanol administration, which often results in development of refractory hypertension (30–32). Consistent with this, we found that the absolute contents of PE, PEp, and PI from the heart membranes of hypertensive rats were significantly increased. PEp,

TABLE 1
Phospholipid Components of Hearts from Normotensive and Spontaneously Hypertensive Rats

	WKY rats (n = 13) (μmol/g wet weight)[a]	%[b]	SHR (n = 9) (μmol/g wet weight)[a]	%[b]
PC	22.32 ± 0.26	46.01 ± 0.50	27.85 ± 0.28[c]	44.81 ± 0.43[c]
PI	2.13 ± 0.07	4.39 ± 0.14	2.61 ± 0.10[c]	4.20 ± 0.15
PS	1.55 ± 0.07	3.20 ± 0.13	1.96 ± 0.08[c]	3.15 ± 0.12
Sph	2.03 ± 0.08	4.18 ± 0.22	2.65 ± 0.10[c]	4.26 ± 0.20
PE	11.65 ± 0.28	24.02 ± 0.44	13.96 ± 0.35[c]	22.46 ± 0.37[c]
PEp	5.43 ± 0.32	11.19 ± 0.42	8.18 ± 0.24[c]	13.16 ± 0.35[c]
CL	3.40 ± 0.10	7.01 ± 0.20	4.94 ± 0.22[c]	7.95 ± 0.22[c]

[a]Absolute content of phospholipids; values are mean ± SEM, calculated assuming total tissue phospholipid was fully extracted.
[b]Percentage of total phospholipids; values are mean ± SEM.
[c]Significantly different ($P \leq .05$) from the WKY control. Statistical analysis performed with t-test. Abbreviations: WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rat; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; PE, phosphatidylethanolamine; PEp, phosphatidylethanolamine plasmalogen; CL, cardiolipin.

TABLE 2
Phospholipid Components of Kidneys from Normotensive and Spontaneously Hypertensive Rats

	WKY rats (n = 9) (μmol/g wet weight)[a]	%[b]	SHR (n = 11) (μmol/g wet weight)[a]	%[b]
PC	17.16 ± 0.17	37.04 ± 0.35	25.12 ± 0.41[c]	37.25 ± 0.58
PI	2.81 ± 0.08	6.06 ± 0.17	4.14 ± 0.11[c]	6.14 ± 0.16
PS	4.50 ± 0.14	9.71 ± 0.40	5.68 ± 0.30[c]	8.42 ± 0.28[c]
Sph	6.20 ± 0.29	13.38 ± 0.61	9.01 ± 0.46[c]	13.36 ± 0.63
PE	8.54 ± 0.17	18.43 ± 0.37	13.74 ± 0.42[c]	20.37 ± 0.60[c]
PEp	5.23 ± 0.17	11.29 ± 0.36	6.89 ± 0.25[c]	10.22 ± 0.36[c]
CL	1.89 ± 0.08	4.08 ± 0.17	2.86 ± 0.10[c]	4.24 ± 0.18

[a]Absolute content of phospholipids; values are mean ± SEM, calculated assuming total tissue phospholipid was fully extracted.
[b]Percentage of total phospholipids; values are mean ± SEM.
[c]Significantly different ($P \leq .05$) from the WKY control. Statistical analysis performed with t-test. For abbreviations see Table 1.

expressed as a percentage of total phospholipid, was also significantly increased. Interestingly, we also found that CL, a special dimeric phospholipid with unique structural features, was significantly increased in both absolute content and as a percentage of total phospholipid in hearts from hypertensive rats relative to normotensive controls. It has been suggested that CL participates in movement of Ca^{2+} across the inner mitochondrial membrane (33–35). Increased CL may contribute to alterations in cardiac myocyte calcium homeostasis in hypertension (36). To get an idea of the affinity of Ca^{2+} to CL, we measured the binding constants of Ca^{2+} to CL and other phospholipids. Of all the phospholipids (PC, PE, PS, CL) measured, the interaction of Ca^{2+} and CL was the strongest. The binding constant for Ca^{2+} and CL was measured as 4.32 ± 0.14 mM^{-1}, while the interaction between Ca^{2+} and PC was undetectable ($K_a \leq 0.1$ mM^{-1}). These results are in agreement with the work of others (33). Alterations in fatty acid components of CL may also affect the affinity of CL for Ca^{2+} (37). We have previously reported that the unsaturation and chain length of fatty acids decreased in heart and kidney membrane phospholipids from hypenensive rats (38). These alterations might affect the ability of CL to participate in Ca^{2+} translocation. A decreased percentage of PC in heart membrames from hypertensive rats would also facilitate greater interaction of Ca^{2+} with membranes in hypertensive cardiac cells since an increase in PC inhibits the interaction of Ca^{2+} with liposomes containing PC (33).

Although the binding constant of Ca^{2+} to CL is relatively low in relation to intracellular free Ca^{2+} concentration (~100–200 nM), the total concentration of CL in mitochondrial membrane of ~4 mM (33,35) is over an order of magnitude higher than calcium–CL dissociation constant which would still result in significant binding of Ca^{2+} to CL.

The precise functional role of membrane CL remains unknown. However, it appears absolutely required for maximal mitochondrial cytochrome c oxidase activity (22,39,40) and for mitochondrial carnitine/acylcarnitine translocase activity (41). Creatine kinase and ATP/ADP carrier are also specifically bound to CL in the inner mitochondrial membrane (42–47). Interestingly, increased CL content has been found to correlate width increased cytochrome oxidase activity in rat heart mitochondria (48). Therefore, increased CL in hypertensive heart observed in our study might in part be responsible for increased oxygen consumption in the hypertensive heart reported previously (49–51).

Our results show that absolute concentrations of all of the major phospholipid components are significantly increased in kidney membranes from hypertensive rats. Previous work of our group showed that intracellular free calcium concentration was significantly increased in perfused hypertensive kid-

ney (52). Significantly increased contents of CL, PI, and PS are consistent with the elevated level of free calcium in the hypertensive kidney since Tyson *et al.* (53,54) have demonstrated that CL is one of the most active ionophores for translocating calcium. Also, PS is required to activate protein kinase C, which is involved in calcium translocation; and inositol 1,4,5-trisphosphate, which is derived from a phosphorylated form of PI, mediates calcium mobilization (54,55); and both are increased in the hypertensive kidney.

The bilayer structure of biological membrane as originally proposed by Gortel and Grendel in 1925 is still an attractive way to explain many properties of cell membranes (56). Luzatti and coworkers (57) established that every biological membrane consists of large amounts of nonbilayer lipids next to bilayer-forming lipids. The ability of lipids to adopt nonbilayer structures is believed to be important for membrane fluidity (58). PE is typical nonbilayer-preferring lipid (58). It may regulate the fluidity of membranes (56,58–60) and modify the Ca^{2+} transport processes by enhancing a slow sustained uptake component that strongly depends on temperature (35). An increase in PE content in kidney membranes from SHR would contribute to nonbilayer characteristics in the lipid bilayer. Furthermore, increased CL content, together with increased PE, in kidney membrane from hypertensive rats would lead to more Ca^{2+} sequestered in the mitochondrial membranes as well as more Ca^{2+} translocated into mitochondria.

To our knowledge, this study is the first report in the literature on alterations in phospholipids of the hypertensive heart and kidney, the two organs well known to be adversely affected in essential hypertension. It appears possible that these abnormalities may at least in part account for abnormal ion balance and ion transport observed in hypertensive heart and kidney cells (25–28,51,52,55,61–70).

ACKNOWLEDGMENT

Research supported by NIH Research Grant DK 32030.

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[Received March 13, 1998, and in final revised form August 10, 1998; revision accepted September 30, 1998]

Inhibition of 2,2′-Azobis(2,4-dimethylvaleronitrile)-Induced Lipid Peroxidation by Sesaminols

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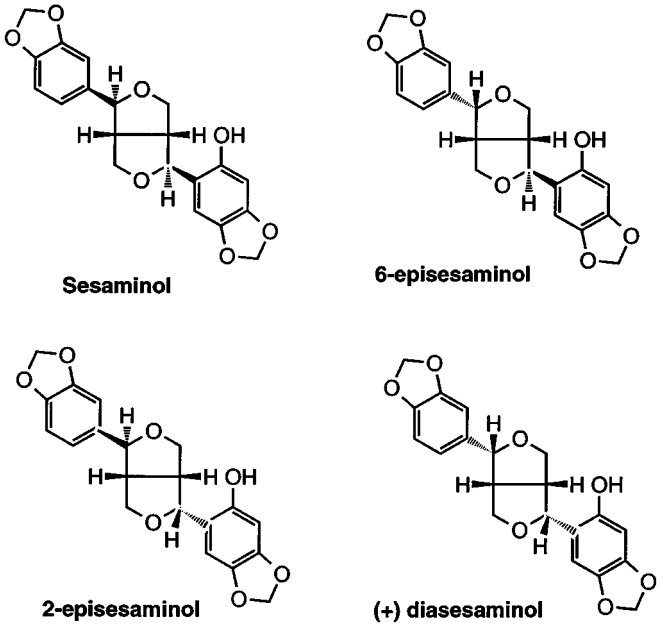
ABSTRACT: We found that sesaminols, a mixture of sesaminol and its stereoisomers, are potent inhibitors of the oxidation of low-density lipoprotein induced by 2,2′-azobis(2,4-dimethylvaleronitrile). Although sesaminols strongly inhibit lipid peroxidation related to their ability to scavenge free radicals, their antioxidant effects have not been investigated. To confirm the involvement of the phenolic moiety in the sesaminol structure in antioxidant activity, sesaminols were reacted with 2,2′-azobis(2,4-dimethylvaleronitrile). The reaction products were isolated by high-performance liquid chromatography and found to have a 1-cyano-1,3-dimethyl-butyl-peroxyl group in their structures. These chemical structures suggest that the sesaminols reacted with the alkylperoxyl radicals to form four major reaction products that are stereoisomers of each other, although the stereochemistry of each isomer has not yet been confirmed. Further instrumental analyses of the reaction products may increase our understanding of the antioxidant activity of sesaminols.
Lipids 33, 1031–1036 (1998).



SCHEME 1

Sesame (*Sesamum indicum* L.) is one of the world's most important oil seed crops. Not only is it a source of edible oil but also the seed itself provides a nutritious food for humans. Sesame seed oil resists oxidative deterioration (1,2). Its remarkable stability is thought to be due to the presence of the endogenous antioxidants sesaminols and sesamol, together with tocopherols (3). Quantification using high-performance liquid chromatography (HPLC) showed that the amount of sesaminols dramatically increased during the manufacturing process, particularly during bleaching; we previously found that sesaminol and three stereoisomers including 2-episesaminol, 6-episesaminol, and (+) diasesaminol (Scheme 1) are produced by intermolecular transformation from sesamolin (3,4), a minor component of sesame oil. Sesamolin is readily converted to sesamol under such conditions as hydrolytic scission at the C_2 position (5). We recently reported the absorption, distribution, and metabolism of sesamolin after having fed it to rats (6). In that study, we found that sesamolin is transformed to sesamol and sesamolinol during absorption from the gastrointestinal tract (6). We suggested therefore that the metabolism of sesamolin differs *in vivo* from that observed *in vitro*. We also found that the chelation of sesaminol with ADP-Fe^{3+}/NADPH inhibits microsome peroxidation (6). Various studies have investigated the therapeutic potential of several synthetic and natural antioxidants as inhibitors of low density lipoprotein (LDL) oxidation (7,8). We found that sesaminol from sesame seed exhibits the strongest antioxidant properties (9–15), and is a potent inhibitor of LDL oxidation induced by $CuSO_4$ or 2,2′-azobis(2-amidinopropane) dihydrochloride *in vitro* (Kang, M.-H., Naito, M., Sakai, K., Uchida, K., and Osawa, T., submitted for publication).

The products formed by the reaction of several natural antioxidants with the peroxyl radical have recently been isolated and identified, and the reaction mechanisms have been studied (16–20). α-Tocopherol is an important natural antioxidant that is present in food as well as in living cells. The reaction products of α-tocopherol with alkylperoxyl radicals have been elucidated, and the antioxidant mechanism of tocopherol has been proposed (16–18). Sugiyama *et al.* (19) reported that

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; NMR, nuclear magnetic resonance; TBARS, 2-thiobarbituric acid-reactive substances.

tetrahydrocurcuminoids, which are major metabolites of curcumin, form 3,4-dimethoxybenzoic acid, 3′,4′-dimethoxy acetophenone, and 3-(3,4-dimethoxyphenyl)-propionic acid *via* the reaction of peroxyl radicals generated by thermolysis. The two reaction products of an anthocyanin pigment with 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were identified as 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (20). However, the oxidation products of sesaminols with alkylperoxyl radical have not been elucidated.

The present study evaluated the inhibitory effects of sesaminols on the oxidation of LDL induced by AMVN. To investigate the antioxidant mechanism of sesaminols, we identified the oxidation products of sesaminol and its stereoisomers with the alkylperoxyl radicals, generated in the reaction with AMVN, which is an initiator of free radicals.

MATERIALS AND METHODS

Materials. Scum from the commercial refining process for unroasted sesame oil was esterified and extracted as previously reported (3,4). Sesaminol and 2-episesaminol were found and identified as the major products by comparing the results of analysis with data from authentic samples. The ratio of sesaminol/2-episesaminol/6-episesaminol/(+) diasesaminol was found to be 37:41:6:16 by wt. Because these stereoisomers isomerize each other at room temperature (3,4), we used the mixture of sesaminol and its stereoisomers for further experiments. AMVN and acetonitrile were purchased from Wako Pure Chemical (Osaka, Japan). Tetramethylsilane and $CDCl_3$ were purchased from Aldrich Chemical (Milwaukee, WI).

Lipoprotein preparation. LDL (d = 1.019 to 1.063 g/mL) was isolated from the serum of healthy humans by stepwise ultracentrifugation at 150,000 × g for 20 h, followed by extensive dialysis against phosphate-buffered saline containing 0.01% EDTA at 4°C for 48 h. To remove EDTA, LDL was dialyzed against a 2,000-fold vol of phosphate-buffered saline without EDTA for 48 h at 4°C. LDL was sterilized by passage through a 0.22 μm filter and then used within 7 d. Protein concentrations were determined with a bicinchonic acid protein assay kit (Pierce Chemical, Rockford, IL), using bovine serum albumin as the standard.

Oxidation of LDL. The mixture of sesaminol and its stereoisomers was dissolved in dimethyl sulfoxide with sonication. The final content of the solvent (DMSO) was <1%. Sesaminols were added to a tube containing 2 mM AMVN and LDL (0.2 mg protein/mL, diluted with PBS) to a final concentration of 1 μM (21). The tubes were incubated in a thermostatically controlled water bath for the indicated time. The reaction was stopped by the addition of 1 mM EDTA and 10 μM butylated hydroxytoluene. The lipid peroxidation of LDL was evaluated by measuring the formation of 2-thiobarbituric acid-reactive substances (TBARS). TBARS were calculated as malondialdehyde equivalents, using as the standard freshly diluted malondialdehyde bis(dimethyl acetal), i.e., 1,1,3,3-tetraethoxypropane. Malondialdehyde was prepared by hydrolysis of 1,1,3,3-tetraethoxy-propane (Aldrich Chemical) with 1 N HCl (22).

Procedure for reacting sesaminols with AMVN. For the oxidation reaction, a mixture of 10 mM sesaminols and 18 mM AMVN was dissolved in 18 mL oxygen-saturated acetonitrile and incubated in a screw-cap test tube at 37°C in air for 4 h. The oxidation products were separated by reverse-phase HPLC on a Develosil ODS-5 column (4.6 mm i.d. × 250 mm, Nomura Chemical, Aichi, Japan) using MeOH/H_2O (70:30, vol/vol) as the solvent. These were repurified by normal-phase HPLC on a Develosil SI-60-5 column (4.6 mm i.d. × 250 mm, Nomura Chemical) using *n*-hexane/ethyl acetate (70:30, vol/vol) as the solvent. The elution profile was monitored by measuring the absorbance at 280 nm and a flow rate of 2.5 mL/min. The peak area was determined with a *C*-R3A Chromatopac (Shimadzu, Kyoto, Japan).

Spectroscopy. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on an NMR JNM-EX-270 spectrometer (JEOL, Tokyo, Japan) using $CDCl_3$ as the solvent and tetramethylsilane as the internal standard (tetramethylsilane δ = 0.00). ^{13}C NMR spectra were measured at 67.5 MHz, and ^1H NMR at 270 MHz. Mass spectra were obtained with a gas chromatograph mass spectrometer system (JMS-DX-300, JEOL). Electron impact spectra were obtained with an ionizing energy of 70 eV. The infrared spectra of samples in liquid film were determined with an FT/IR-8300 device (Japan Spectroscopic, Tokyo, Japan). Ultraviolet spectra were determined with a U-best 50 (Japan Spectroscopic). Wavelengths of maximal light absorption and specific rotations were determined using a DIP-370 spectrometer (Japan Spectroscopic).

Statistical analysis. Results are presented as means ± SD. Data were analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

Antioxidant effect of sesaminols. Reactive oxygen species are involved in the oxidative attack on polyunsaturated fatty acids leading to lipid peroxidation of such biological systems as biological membranes (23,24) or lipoproteins (25). The process of lipid peroxidation is thought to be involved in various types of vascular disease. This observation is consistent with the fact that biological membranes as well as lipoproteins contain high concentrations of polyunsaturated fatty acids which are unstable and susceptible to oxidation. Oxidative modification of LDL may be of particular importance in the pathogenesis of atherosclerosis (26). Therefore, agents that protect LDL from oxidation are of interest as possible antiatherogenic agents. The oxidative modification of LDL by transitional metal ions *ex vivo* appears to be prevented by the addition of antioxidants such as vitamin E (27), probucol (28), butylated hydroxytoluene (29), and flavonoids (30). As shown in Figure 1, we evaluated the inhibitory effects of sesaminols in the oxidative modification of LDL induced by AMVN. Sesaminols (1 μM) exhibited a strong antioxidant effect in terms of decreasing TBARS formation. Sesaminols

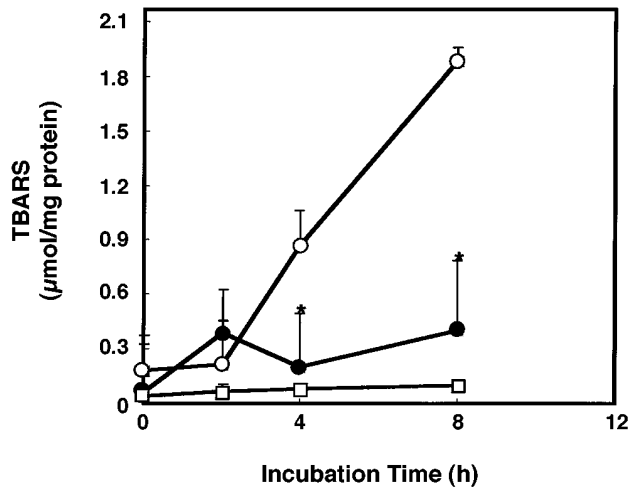


FIG. 1. Time-dependent effect of sesaminols in 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced low-density lipoprotein (LDL) peroxidation. LDL (0.2 mg protein/mL) was incubated in the absence (native LDL, □) or presence (oxidized LDL, ○) of 2 mM AMVN without or with sesaminols (●) for 2 h at 37°C. The reaction was terminated by the addition of EDTA and butylated hydroxytoluene. Oxidation was monitored by the formation of TBARS. *Significantly different from oxidized-LDL ($P < 0.05$).

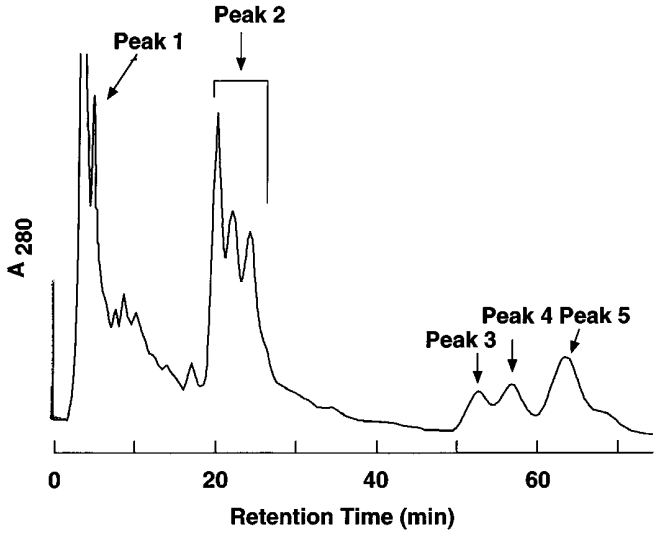


FIG. 2. Representative chromatogram of the high-performance liquid chromatography (HPLC) separation of reaction products of sesaminol and its stereoisomers with AMVN, as determined by absorbance at 280 nm. Sesaminol and its stereoisomers (10 mM) were incubated with AMVN (200 mM) in oxygen-saturated acetonitrile for 4 h at 37°C. An aliquot of the solution was subjected to reverse-phase HPLC on a Develosil ODS-5 column (Nomura Chemical, Aichi, Japan). For abbreviation see Figure 1.

exist as a mixture of sesaminol and its stereoisomers, which are inter-convertible, and we found that there is no significant difference between sesaminol and its stereoisomers in their antioxidant activity. The potent inhibitory effect of sesaminols against lipid peroxidation could be attributed to their ability to scavenge free radicals (3,4).

As suggested by Osawa *et al.* (9,13,19,31,32), dietary antioxidants such as the β-diketone type, including tetrahydrocurcuminoids, curcumin, and eucalyptus leaf waxes must play an important role in protecting against oxidative damages.

Time course of the formation of reaction products. As shown in Figure 2, we isolated five main peaks through HPLC separation. Peaks 1, 3, 4, and 5 increased with incubation time. However, peak 2 decreased gradually with incubation time (Fig. 3) and finally reached a constant peak area. Because the formation of such products reached a maximum after 4 h, we stopped the reaction at 4 h and isolated and identified the reaction products.

Isolation of reaction products. The oxidation products were separated by reverse-phase HPLC using MeOH/H_2O (70:30, vol/vol) as the solvent. Purification of peaks 3, 4, and 5 yielded the following amounts of each product: product 2; 5.9 mg (peak 3); product 3; 7.3 mg (peak 4); and product 4; 14.5 mg (peak 5). Peak 2 was found to be a mixture of sesaminols plus the reaction product, which was further repurified by normal-phase HPLC using *n*-hexane/ethyl acetate (70:30, vol/vol) as the solvent. Pure reaction product or product 1, 12.1 mg (peak 2), was obtained.

Structure determination of the reaction products. Peak 1 was confirmed to contain only the degradation products, because it was found not to possess the sesaminol structure in ^1H NMR spectra. All of the other products named, or products 1–4, which were contained in peaks 2–5, respectively, were confirmed as the adducts of sesaminol structure with AOO$^\bullet$ by NMR and mass spectra. Products 1–4 have essentially the

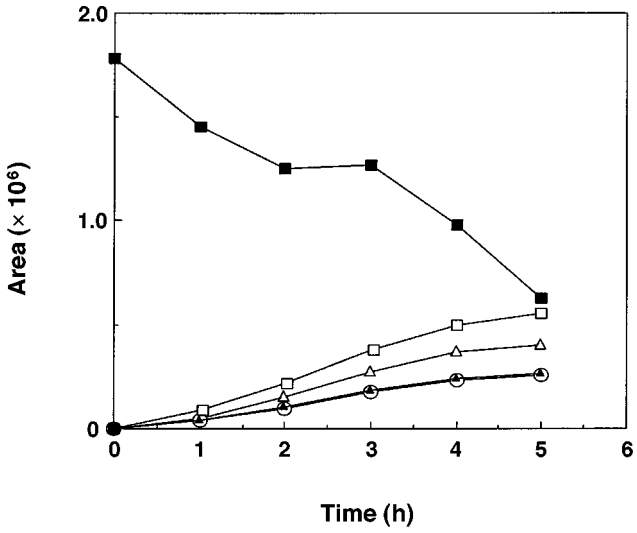


FIG. 3. Time-dependent effect for the oxidation products of sesaminol and its stereoisomers induced by AMVN. Sesaminol and its stereoisomers (10 mM) were incubated with AMVN (200 mM) in oxygen-saturated acetonitrile at 37°C. At the indicated time, aliquots of the solution were subjected to reverse-phase HPLC on a Develosil ODS-5 column [△, peak 1; ■, peak 2 (sesaminol and its stereoisomers plus product); ○, peak 3; ▲, peak 4; □, peak 5]. Peak numbers refer to peaks in Figure 2. For abbreviations see Figures 1 and 2. For manufacturer see Figure 2.

TABLE 1
Summary of the Spectroscopic Analyses of Oxidation Products of Sesaminols[a]

Product	1	2	3	4
Mass spectrum ([M$^+$])	511	511	511	511
Ultraviolet absorbance				
λ_{max}(nm)	288	287	287	287
(ε_{max})	(5280)	(7560)	(5060)	(4680)
	226	229	228	225
	(8050)	(13400)	(9200)	(9060)
$[\alpha]^{25}_{D}$	−31.0	+85.8	+94.3	−64.7

[a]As determined in this laboratory.

same spectral data except for $[\alpha]^{25}_{D}$ as shown in Table 1. NMR data are summarized in Tables 2 and 3. By comparision with the infrared spectrum of sesaminol, a new absorption was found at 1686 cm^{-1} that was due to the α,β-unsaturated carbonyl. During ^{13}C NMR analysis, the signal of the phenolic carbon at C-2′ of sesaminol (δ 150.7 ppm) shifted to δ 186.6 ppm as a result of the conversion into a carbonyl carbon in the oxidation products. In addition, a large shift in the signal of the C-5′ from δ 140.8 ppm in sesaminol to δ 101.4 ppm in the oxidation products suggested that a 1-cyano-1,3-dimethyl-butyl-peroxyl group derived from AMVN attached to the C-5′ position of sesaminol. Further detailed examination of the ^{1}H NMR and ^{13}C NMR spectra as well as other analyses of the oxidation products using instrumentation suggested that the chemical structures of the four reaction products are essentially the same. As described in the Materials and Methods section, sesaminol and 2-episesaminol exist as the major components in sesame seed oil, and 6-episesaminol and (+) diasesaminol exist as the minor components (3,4). Although it is possible that each of four stereoisomers of sesaminols reacts with AOO$^\bullet$ (the alkoxyl radical generated by AMVN) to produce four stereoisomers which have different stereochemistry at C-5′ and C-1[a] (Scheme 2), only four stereoisomers were found to be produced as the main reaction products. By instrumental analyses, we cannot confirm the stereochemistry of four reaction products; therefore, we can only propose the chemical structure for all of the reaction products without stereochemistry as shown in Scheme 2. This process may represent the antioxidant mechanism of sesaminols with AMVN. The first step may be the abstraction of hydrogen. The resulting sesaminol radicals can then react with the peroxyl radicals. We therefore suggest that sesaminols are chain-breaking antioxidants that react with peroxyl radicals to stop the autoxidation of polyunsaturated fatty acids. Peroxyl radicals are known to be chain-carrying radicals in lipid peroxidation (18). Our group has elucidated the reactions of peroxyl radicals with such phenolic-type antioxidants as tetrahydrocurcurminoids and cyanidin 3-*O*-D-glucoside (19,20). α-Tocopherol is highly reactive with free radicals and reactive oxygen species and forms a variety of products. For example, it traps free radicals generated from such radical initiators as benzoyl peroxide (33), AMVN (17), and *tert*-butyl hydroperoxide (16) and reacts with alkyl and alkoxyl radicals to produce alkylated derivatives (16,17,33–36). Free radical oxidation of α-tocopherol was reported to take place *via* the tocopheroxyl radical as an intermediate (37).

Sesame seed and oil are used for pharmaceutical purposes. These substances are also important in traditional Indian medicine, where sesame oil is used to prepare extracts of herbs and medicinal plants (38). Sesame oil is also believed to be suitable for application to the skin because of its spreading and antioxidant properties (38). Our studies have mainly fo-

TABLE 2
^{1}H Nuclear Magnetic Resonance (NMR) Chemical Shifts[a]

	Sesaminol	Product 1	Product 2	Product 3	Product 4
1	3.14, *m*	2.85, *m*	2.62, *m*	2.64, *m*	2.85, *m*
2	4.76, *d*, *J* = 4	4.46, *m*	4.65, *dd*, *J* = 2,8	4.64, *dd*, *J* = 2,6	4.46, *m*
4a	3.84, *dd*, *J* = 4,10	3.94, *m*	3.86, *dd*, *J* = 7,10	3.86, *dd*, *J* = 7,10	3.93, *dd*, *J* = 7,10
4e	4.12, *dd*, *J* = 7,10	4.53, *dd*, *J* = 1,10	4.45, *dd*, *J* = 2,9	4.45, *dd*, *J* = 1,9	4.53, *dd*, *J* = 1,7
5	3.14, *m*	3.16, *m*	3.10, *d*, *m*	3.10, *m*	3.16, *m*
6	4.76, *d*, *J* = 4	4.80, *d*, *J* = 6	4.77, *d*, *J* = 6	4.77, *d*, *J* = 6	4.80, *d*, *J* = 6
8a	3.87, *dd*, *J* = 2,9	3.32, *m*	3.35, *dd*, *J* = 8,9	3.34, *dd*, *J* = 8,9	3.34, *m*
8e	4.34, *dd*, *J* = 7,9	3.76, *m*	3.73, *dd*, *J* = 8,9	3.74, *m*	3.75, *m*
3′	6.46, *s*	7.02, *dd*, *J* = 2,9	6.98, *d*, *J* = 2	6.93, *d*, *J* = 2	7.02, *dd*, *J* = 2,9
6′	6.53, *s*	5.88, *s*	5.95, *brs*	5.88, *s*	5.88, *s*
2″	6.80–6.90, *m*	6.86, *brs*	6.86, *brs*	6.86, *brs*	6.86, *brs*
5″	6.80–6.90, *m*	6.80, *m*	6.78, *m*	6.79, *m*	6.78, *m*
6″	6.80–6.90, *m*	6.80, *m*	6.78, *m*	6.79, *m*	6.78, *m*
-O-CH$_2$-O-a	5.90, *s*	5.64, *d*, *J* = 2	5.64, *d*, *J* = 2	5.64, *brs*	5.64, *d*, *J* = 2
-O-CH$_2$-O-b	5.90, *s*	5.69, *d*, *J* = 2	5.71, *d*, *J* = 2	5.71, *brs*	5.69, *d*, *J* = 2
-O-CH$_2$-O-′	5.97, *s*	5.96, *s*	5.95, *brs*	5.95, *s*	5.95, *s*
1b[a]		1.61, *s*	1.58, *s*	1.58, *s*	1.61, *s*
2[a]		1.73, *d*, *J* = 7	1.70, *d*, *J* = 7	1.71, *m*	1.73, *m*
3a		1.93, *m*	1.95, *m*	1.85, *m*	1.93, *m*
3a[a]		0.99 or 1.03, *d*, *J* = 2	0.99 or 1.01, *d*, *J* = 3	0.96 or 0.98, *s*, *J* = 2	1.00 or 1.03, *d*, *J* = 2
4[a]		0.99 or 1.03, *d*, *J* = 2	0.99 or 1.01, *d*, *J* = 3	0.96 or 0.98, *s*, *J* = 2	1.00 or 1.03, *d*, *J* = 2

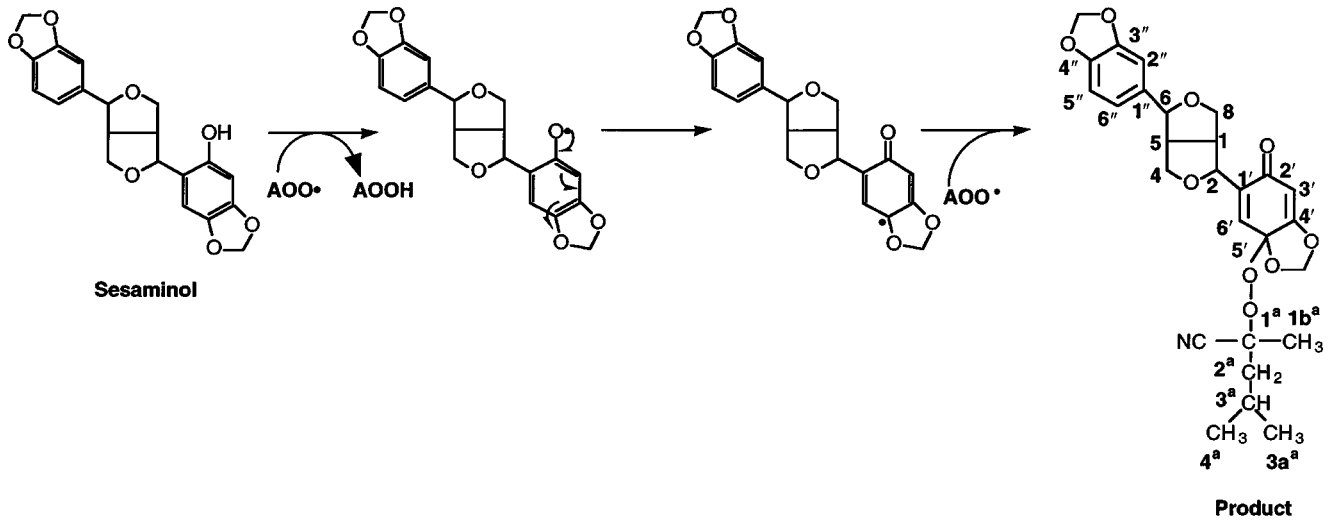
[a]Positions are identified in the Product molecule in Scheme 2.

TABLE 3
^{13}C NMR Chemical Shifts[a]

	Sesaminol	Product 1	Product 2	Product 3	Product 4
1	53.3	52.7	52.1	52.1	52.6
2	85.3	83.1	83.1	83.2	83.1
4	70.5	70.1	69.5	69.4	69.5
5	52.9	50.2	50.1	50.1	50.2
6	86.6	81.5	81.7	81.7	81.5
8	72.3	72.7	72.6	72.6	72.7
1′	119.6	144.0	144.0	144.9	144.0
2′	150.7	186.6	186.6	186.6	186.6
3′	99.4	125.3	125.4	124.9	125.3
4′	148.9	165.7	165.8	165.8	165.8
5′	148.8	101.6	101.4	101.4	101.5
6′	106.0	100.4	100.6	100.6	100.4
1″	134.4	132.5	132.4	132.4	132.4
2″	106.4	106.3	106.5	106.5	106.5
3″	150.6	147.5	147.6	147.6	147.6
4″	147.1	144.2	146.6	145.0	144.2
5″	108.1	108.1	108.1	108.1	108.1
6″	119.2	118.6	118.8	118.8	118.8
-O-CH_2-O-	101.1	100.1	100.3	100.3	100.1
-O-CH_2-O-′	101.1	101.1	101.0	100.9	100.9
1[a]		79.4	79.8	79.5	79.4
1b[a]		24.0	24.3	23.7	24.1
2[a]		46.3	46.3	46.1	46.3
3[a]		24.7	25.0	24.5	24.8
3a[a]		23.5 or 23.6	23.4 or 23.6	23.5 or 23.6	23.5 or 23.6
4[a]		23.5 or 23.6	23.4 or 23.6	23.5 or 23.6	23.5 or 23.6
-CN		119.4	119.4	119.4	119.4

[a]Positions are identified in the Product molecule in Scheme 2. For abbreviation see Table 2.

cused on the physiological and pharmacological roles of sesaminols because this substance is readily available and is widely used. It is thus important to investigate the inhibitory effects of sesaminols against oxidative damage and its mechanism of action in biological systems. We are now conducting detailed studies on the effects of sesaminols in preventing oxidative damage in an animal model to expand our present findings to clinical applications.



SCHEME 2

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[Received April 28, 1998, and in final revised form September 10, 1998; revision accepted October 5, 1998]

S-Methyl Derivatives from Thiol Compounds by the Pyrolytic Reaction with Trimethylsulfonium Hydroxide

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ABSTRACT: Base-catalyzed transesterification of acyl lipids with methanol in the presence of trimethylsulfonium hydroxide (TMSH) is an easy and convenient method for the preparation of fatty acid methyl esters for gas chromatography (GC) analyses. Free fatty acids are converted to fatty acid methyl esters by the pyrolytic reaction with TMSH as well. We have found that lipids and other compounds containing thiol groups are also converted easily to the corresponding methyl sulfides (methyl thioethers) by the pyrolytic reaction with TMSH occurring in the injector of the gas chromatograph. For example, alkane thiols such as dodecane thiol and octadecane thiol are converted to the corresponding alkyl methyl sulfides, whereas bis(methylthio) derivatives are formed from α,ω-alkane dithiols, e.g., 1,8-octanedithiol, and from 2,3-dimercaptopropan-1-ol (dimercaprol). Furthermore, 3β-mercaptocholest-5-ene (thiocholesterol) is converted to 3-methylthiocholest-5-ene by the same reaction. The *S*-methylation reactions which finally lead to the corresponding methylthio derivatives of lipids and other compounds with thiol groups may be of diagnostic value for the structural analysis of such compounds by GC and GC/mass spectrometry.
Lipids 33, 1037–1041 (1998).

Trimethylsulfonium hydroxide (TMSH) is a very useful reagent for the preparation of fatty acid methyl esters for gas chromatography (GC) analysis by base-catalyzed transmethylation of acyl lipids (1–6) or by the pyrolytic decomposition of the TMSH salts of fatty acids in the GC injector (7). Recently, we showed that various lipids having acidic substituents such as amino, amide, or hydroxy groups form *N*- and *O*-methylated derivatives, in varying proportions, by the pyrolytic reaction with TMSH (8–10). Thiol compounds are versatile intermediates for various technical applications such as rubber substitutes (11–14), stabilizers for polyvinyl chloride (15), antioxidants (16,17), lubricant additives (11–13,16,18,19), wetting agents and surface-active chemicals (20,21) as well as coatings (11,18,22). They are also used for the preparation of pharmaceuticals (17,23), cosmetic formulations (17,19,21), insecticides and insect repellents (12,24) as well as compounds with antimicrobial activity (25). Thiols are a further series of compounds having acidic properties which might be converted to the corresponding *S*-methyl derivatives by the pyrolytic reaction with TMSH. This study demonstrates the use of TMSH in the *S*-methylation of lipids and other compounds containing thiol groups for GC and GC/mass spectrometry (MS) analyses.

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Abbreviations: GC, gas chromatography; MS, mass spectrometry; TMSH, trimethylsulfonium hydroxide.

MATERIALS AND METHODS

Chemicals. Dodecanethiol and octadecanethiol, didodecyl sulfide, 1,8-dimercaptooctane, 2,3-dimercaptopropan-1-ol (dimercaprol; BAL), *threo*-1,4-dimercapto-2,3-butandiol (dithiothreitol, Cleland's reagent), mercaptoacetic acid (thioglycolic acid) as well as its methyl- and 2′-ethylhexyl esters, 3,3′-thiodipropionic acid methyl ester, 2-mercaptopropionic acid (thiolactic acid) and 3-mercaptopropionic acid, *N*-(2-mercaptopropionyl)-glycine (tiopronin), 2-amino-3-mercapto-3-methylbutanoic acid (DL-penicillamine), cholest-5-ene-3β-thiol (thiocholesterol), and 2-ethylhexanol were purchased from Sigma-Aldrich-Fluka (Deisenhofen, Germany). TMSH reagent (0.2 M TMSH in methanol) was a product of Macherey-Nagel (Düren, Germany).

Preparation of standards. Methyl esters of carboxylic acids as well as methyl thioethers were obtained by the reaction of the original compounds having carboxy and/or thiol groups with diazomethane (26). Derivatives with methyl ester and/or methylthio groups were purified by thin-layer chromatography on 0.3 mm Silica Gel H (E. Merck, Darmstadt, Germany) layers using hexane/diethyl ether (99:1, vol/vol) as the solvent system for alkyl methyl sulfides, hexane/diethyl ether (95:5, vol/vol) for cholesteryl methyl sulfide and mercaptoacetic acid derivatives, hexane/diethyl ether (1:1, vol/vol) or diethyl ether for 2,3-dimercaptopropan-1-ol and dithiothreitol as well as their methylthio derivatives.

Derivatization for GC. Under standard conditions, 30–60 μL TMSH reagent was added to 0.4 mg of a compound containing thiol groups (molar ratio TMSH/thiol and/or carboxy equivalents, *ca.* 4:1). The derivatization mixture, 1–2 μL, was directly injected into the gas chromatograph.

GC. GC of compounds containing thiol groups as well as of their derivatives formed by the pyrolytic reaction with TMSH was carried out in a Hewlett-Packard (Böblingen,

Germany) HP-5890 Series II gas chromatograph fitted with a flame-ionization detector. *S*-Methyl derivatives (methyl thioethers) of various thiol compounds were chromatographed on a 0.12 μm DB-23 fused-silica capillary column (Macherey-Nagel), 40 m × 0.18 mm i.d using hydrogen as the carrier gas. *S*-Methyl derivatives of short-chain thiol compounds were separated at 60°C for 5 min, followed by linear programming from 60 to 240°C at 10°C per min. The long-chain alkyl methyl sulfides were separated initially at 200°C for 5 min, followed by linear programming from 200 to 240°C at 10°C per min. The final temperature was kept constant for 20 min. Cholesteryl methyl sulfide was separated isothermally at 270°C on a 0.52 μm HP-1 fused-silica column (Hewlett-Packard), 25 m × 0.32 mm i.d. The split ratio was 1:10; the injector as well as flame-ionization detector temperatures were 280°C. GC peaks were assigned by comparison of their retention times with those of known standards.

GC/MS. GC/MS of methylthio derivatives of compounds containing thiol groups was performed at electron impact mode (70 eV) on a Hewlett-Packard HP-5890 Series II/5989 A gas chromatograph/mass spectrometer as described recently (8,9). The GC instrument was equipped with a 0.23 μm Permabond OV-1 fused-silica capillary column (Macherey-Nagel) 25 m × 0.32 mm i.d. The carrier gas was He at a flow rate of 1.0 mL/min. The column temperature was initially kept at 50 or 70°C for 5 min and then programmed from 50 or 70°C to 240°C at 12°C/min; the final temperature was held for 5 min. Other operating conditions were split/splitless injector in splitless mode (temperature 300°C), interface temperature 280°C, and ion source temperature 200°C.

Structural analysis. GC peaks were assigned by comparison of their retention times and mass spectral data with those of commercial or synthetic standards. Similarly, structural assignments were made for standards containing thiol groups or their methylthio derivatives which were prepared by the reaction with diazomethane. Peaks of *S*-methylated compounds obtained by the pyrolytic reaction with TMSH (7) were assigned a particular structure if ions corresponding to all the important mass fragments were present.

RESULTS AND DISCUSSION

TMSH is involved in two very different types of chemical reactions. The first is as a base catalyst for the mild transesterification of acyl lipids. Commercial TMSH reagent is a 0.2 M methanolic TMSH solution which transmethylates acyl lipids such as triglycerides to the corresponding fatty acid methyl esters at room temperature within a few minutes (1–6). Other functional groups of lipids such as hydroxy, amino, or amide groups do not react under these conditions with TMSH reagent either at room temperature or under reflux conditions (8–10).

The second type of reaction is the pyrolytic reaction of TMSH with various acidic groups of compounds occurring in the injector of the gas chromatograph at high temperatures. Acidic groups of compounds such as carboxylic functions and phenolic hydroxy groups (2,7) were transformed by the pyrolytic reaction of TMSH almost completely to the corresponding *O*-methyl derivatives. In contrast, this reaction leads to partial methylation if less acidic groups are present, as observed for hydroxy, amino, and amide groups (8–10).

Because of the higher acidity of the SH-group, conversion to the corresponding methylthio derivatives by the pyrolytic reaction of thiol compounds with TMSH might be expected to be as high as that of free fatty acids. Therefore, we have reacted various thiol compounds with TMSH reagent at different temperatures and checked the formation of reaction products by thin-layer chromatography. At room temperature incomplete derivatization was found even after 2 h, whereas almost complete *S*-methylation was observed within 30 min in a closed tube at 70°C. For GC derivatization the pyrolytic reaction of TMSH in the GC injector at 280°C was tested for its usefulness in *S*-methylation of various thiol compounds.

$$R - SH \xrightarrow[250°C]{TMSH} R - S - CH_3$$

Under standard conditions, long-chain alkanethiols such as 1-dodecanethiol and 1-octadecanethiol were converted almost completely (>99%) to methyl dodecyl sulfide (methyl dodecyl thioether; *m/z* (rel%) 216 (48), $[M]^+$) and methyl octadecyl sulfide (methyl octadecyl thioether; *m/z* (rel%) 300 (34), $[M]^+$), respectively, as was checked by GC/MS (Table 1). In addition to the molecular ion peaks, the mass spectra of both *S*-methylated derivatives showed typical fragments that were formed by loss of the *S*-methyl group {*m/z* (rel%) 201 (100) $[M - CH_3]^+$ and *m/z* (rel%) 285 (100) $[M - CH_3]^+$, respectively}, while the tendency for the loss of methyl thiol was not strongly pronounced. The fragment *m/z* 61 was formed by α-cleavage of the C-C bond next to the *S*-atom (Table 1). These data agree with those given in the literature (27,28).

Studies concerning the optimal molar ratio between alkyl thiol substrates and TMSH reagent revealed that the ratio of pyrolytic conversion of thiol compounds to the corresponding *S*-methylated products in the presence of TMSH depended on the molar excess of the methylating reagent (Fig. 1). Extensive conversion was reached already at a molar ratio of about 2:1 (TMSH/thiol equivalents). Under these conditions, dodecanethiol was converted to an extent of 96% to methyl dodecylsulfide. In contrast, lipids containing hydroxy, amine, and amide groups were not found to be converted completely to the corresponding *O*-methyl or *N*-methyl derivatives, even if a 20-fold excess of TMSH was used for derivatization (8–10).

In vulcanization, bifunctional thiol compounds such as 1,6-dimercaptohexane and 1,8-dimercaptooctane are used for the formation of polythioethers by addition reaction onto α,ω-diene monomers (12). Under standard conditions (molar ratio TMSH/thiol equivalent = 4:1), the product of the pyrolytic reaction of 1,8-dimercaptooctane with TMSH was 1,8-

TABLE 1
Important Fragments of Electron Impact Mass Spectra of *S*-Methyl Derivatives of Various Thiol Groups Containing Compounds Formed by the Reaction with Trimethylsulfonium Hydroxide

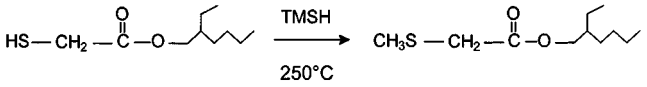
S-Methyl derivatives of lipids and other compounds containing thiol groups	Important mass fragments, *m/z* (rel%)[a]			
Alkane thiol derivatives				
Methyl dodecyl sulfide	216 (51.9) $[M]^+$	201 (100) $[M - CH_3]^+$	168 (4.0) $[M - CH_4S]^+$	61 (59.0) $[C_2H_5S]^+$
Methyl octadecyl sulfide	300 (33.5) $[M]^+$	285 (100) $[M - CH_3]^+$	252 (1.7) $[M - CH_4S]^+$	61 (34.4) $[C_2H_5S]^+$
1-Mercapto-8-methylthiooctane	192 (15.1) $[M]^+$	159 (52.9) $[M - SH]^+$	143 (8.3) $[M - CH_5S]^+$	61 (100) $[C_2H_5S]^+$
1,8-Bis(methylthio)-octane	206 (35.9) $[M]^+$	159 (26.1) $[M - CH_3S]^+$	143 (34.4) $[M - C_2H_7S]^+$	61 (100) $[C_2H_5S]^+$
Mercaptoacetic acid (thioglycolic acid) and mercaptopropionic acid derivatives				
Methylthioacetic acid methyl ester	120 (48.3) $[M]^+$		74 (61.1) $[M - CH_2S]^+$	61 (100) $[C_2H_5S]^+$
Methylthioacetic acid 2′-ethylhexyl ester[b]	218 (11.6) $[M]^+$	157 (28.8) $[M - C_2H_5S]^+$	112 (42.2)	61 (80.8) $[C_2H_5S]^+$
2-Methylthiopropionic acid methyl ester	134 (39.6) $[M]^+$		88 (28.4)	75 (100) $[C_2H_7S]^+$
3-Methylthiopropionic acid methyl ester	134 (57.7) $[M]^+$	87 (19.4) $[M - CH_3S]^+$	74 (100) $[M - C_2H_4S]^+$	61 (91.9) $[C_2H_5S]^+$
Mercaptoalkanol derivatives				
2(3)-Mercapto-3(2)-methylthiopropan-1-ol	138 (36.9) $[M]^+$	104 (37.1) $[M - H_2S]^+$	91 (53.6) $[M - CH_3S]^+$	61 (100) $[C_2H_5S]^+$
2,3-Bis(methylthio)-propan-1-ol	152 (33.8) $[M]^+$	104 (58.0) $[M - CH_4S]^+$	91 (53.6) $[M - C_2H_5S]^+$	61 (100) $[C_2H_5S]^+$
threo-1(4)-Methylthio-4(1)-mercaptobutan-2,3-diol	168 (1.6) $[M]^+$	134 (8.6) $[M - H_2S]^+$	91 (38.3) $[C_3H_7OS]^+$	61 (100) $[C_2H_5S]^+$
threo-1,4-Bis-(methylthio)-butan-2,3-diol	182 (5.6) $[M]^+$	134 (11.0) $[M - CH_4S]^+$	91 (33.9) $[C_3H_7OS]^+$	61 (100) $[C_2H_5S]^+$
Cholest-5-ene 3β-thiol (thiocholesterol) derivatives				
3-Methylthiocholest-5-ene	416 (16.2) $[M]^+$	401 (8.1) $[M - CH_3]^+$	368 (100) $[M - CH_4S]^+$	255 (16.0) $[M - (CH_4S + C_8H_{17})]^+$

[a]Intensities relative to base peak.
[b]Base peak: *m/z* 57.

bis(methylthio)octane as was checked by gas chromatographic comparison with synthetic standards as well as by GC/MS (Table 1). The dominant fragment peak at *m/z* 61 (base peak) for both compounds is formed by α-cleavage as mentioned above. By using a suboptimal TMSH concentration (molar ratio TMSH/thiol equivalent = 2:1), two reaction products, i.e., 1-mercapto-8-methylthiooctane and 1,8-bis(methylthio)octane, were observed in the gas chromatogram in addition to small proportions of nonderivatized 1,8-dimercaptooctane (data not shown).

$$HS-[CH_2]_8-SH \xrightarrow[250°C]{TMSH} CH_3S-[CH_2]_8-SH + CH_3S-[CH_2]_8-SCH_3$$

Mercaptoacetic acid 2′-ethylhexylester (thioglycolic acid 2′-ethylhexylester) and other mercaptoacetic acid esters are used as stabilizers for polymers (15). Under standard conditions, mercaptoacetic acid 2′-ethylhexylester was *S*-methylated to an extent of (>94%) to the corresponding methyl sulfide by the pyrolytic reaction with TMSH, whereas other possible reaction products such as mercaptoacetic acid methylester and its methylthio derivative as well as 2-ethylhexan-1-ol and its methoxy derivative were not detected by gas chromatographic analysis. These data suggest that the formation of methylthio acetic acid 2′-ethylhexylester is by far the preferred reaction when mercaptoacetic acid 2′-ethylhexylester is reacted with TMSH under pyrolytic conditions.



Retention time of methylthio acetic acid 2′-ethylhexylester was compared with that of a synthetic standard that had been prepared by the reaction of mercaptoacetic acid 2′-ethylhexylester with diazomethane. In addition, the reaction product was analyzed by MS (Table 1). The typical fragmentation of this thioether led to two important fragments at *m/z* 157 and 61 because the ester group stabilizes the positive ions formed by α-cleavage in contrast to other thioethers which do not have further stabilizing groups.

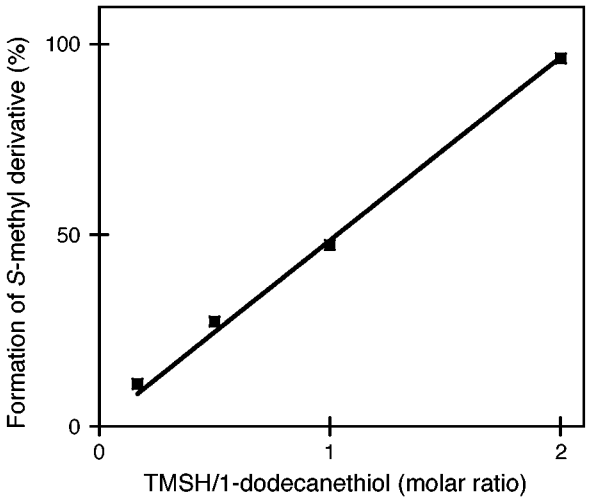


FIG. 1. Formation of methyl dodecyl sulfide (methyl dodecyl thioether) in relation to the molar ratio of trimethylsulfonium hydroxide (TMSH) (values are mean of two determinations).

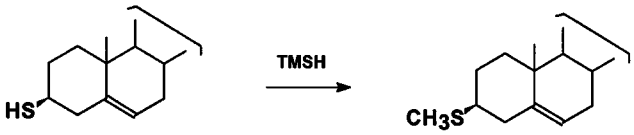
Similar results were obtained for the pyrolytic reaction of mercaptoacetic and mercaptopropionic acids with TMSH. Obviously, these thiol compounds were almost completely (>99%) converted to methylthioacetic acid methyl ester and methylthiopropionic acid methyl ester, respectively, which were compared by GC and GC/MS with the corresponding derivatives prepared by the reaction of mercaptoacetic or mercaptopropionic acids with diazomethane (Table 1).

However, the pyrolytic reaction of compounds having additional amino groups, e.g., penicillamine and tiopronin, mainly led to partially methylated derivatives even in the presence of high molar excess of TMSH. For example, 2-amino-3-mercapto-3-methylbutyric acid (penicillamine) was predominantly derivatized to 2-*N*-methylamino-3-methylthio-3-methylbutyric acid methyl ester {*m/z* (rel%): 191 (0.7; $[M]^+$), 103 (25.4; $[CH_3\text{-}NH\text{-}CH_2\text{-}COOCH_3]^+$), 89 (100; $[(CH_3)_2\text{-}C\text{-}S\text{-}CH_3]^+$}, whereas 2-*N,N*-dimethylamino-3-methylthio-3-methyl-butyric acid methyl ester {*m/z* (rel%): 205 (0.9; $[M]^+$), 116 (100; $[(CH_3)_2N\text{-}CH\text{-}COOCH_3]^+$), 89 (26.9; $[(CH_3)_2C\text{-}S\text{-}CH_3]^+$} was formed in small proportions only. Similarly, *N*-(2-mercaptopropionyl)-glycine (tiopronin) which is used as an antidote against heavy metal poisoning was mainly derivatized to *N*-(2-methylthiopropionyl)-glycine methyl ester {*m/z* (rel%): 191 (9.8; $[M]^+$), 145 (100; $[M - S\text{-}CH_2]^+$), 88 (8.3; $NH\text{-}CH_2\text{-}COOCH_3]^+$), 75 (57.6; $[CH_3\text{-}CH\text{-}S\text{-}CH_3]^+$)}. In addition, small proportions of *N*-methyl-*N*-(2-methylthiopropionyl)-glycine methyl ester {*m/z* (rel%): 205 (9.6; $[M]^+$), 159 (100; $[M - S\text{-}CH_2]^+$), 102 (31.2; $CH_3N\text{-}CH_2\text{-}COOCH_3]^+$), 75 (45.1; $[CH_3\text{-}CH\text{-}S\text{-}CH_3]^+$} were formed. These results agree with our previous observations on the incomplete derivatization by TMSH of lipids containing amino and amide groups (9,10). Therefore, derivatization by the pyrolytic reaction with TMSH of thiol compounds having additional amino or amide groups is not recommended without limitation.

The alkyl sulfides (dialkyl sulfides, alkyl thioethers), prepared from the corresponding thiol compounds, are mainly used as stabilizers and antioxidants for technical products such as polymers and lubricants (15–17). Cleavage of the sulfide (thioether) bond by the pyrolytic reaction with TMSH was not observed for dialkyl sulfides, e.g., didodecyl sulfide, but for 3,3′-thiodipropionic acid methyl ester suggesting an activation of the sulfide bond by the two neighboring β-carboxy groups (data not shown).

2,3-Dimercaptopropan-1-ol (dimercaprol, BAL) is a highly effective antidote against arsenic and heavy metal compounds (23). By using a molar ratio of TMSH/thiol equivalent (≈3:1), dimercaprol was converted almost completely (>96%) to the corresponding 2,3-bis(methylthio)-propan-1-ol as is obvious from GC comparison with synthetic standards and GC/MS studies (Table 1). Typical mass fragments were found at *m/z* 61 (α-cleavage) and 91. However, using a suboptimal molar ratio of 1:1 (TMSH/thiol equivalent) partially *S*-methylated isomers of dimercaprol, i.e., 2-methylthio dimercaprol and 3-methylthio dimercaprol, were detected together with the nonderivatized compound and the 2,3-bis(methylthio) derivative (data not shown). Under the conditions described, *O*-methylation of the hydroxy group of dimercaprol was observed in trace amounts only. Similar results were obtained for dithiothreitol (*threo*-1,4-dimercapto-2,3-butandiol; Table 1).

Cholest-5-ene 3β-thiol (thiocholesterol), i.e., a thioanalog of cholesterol, is used in biochemical studies (29,30). Under standard conditions, more than 90% of cholest-5-ene 3β-thiol was converted to the cholest-5-enyl 3-methyl sulfide (3-methylthiocholest-5-ene) by the pyrolytic reaction with TMSH.



The 3-methylthio derivative was identified by comparison with a synthetic standard as well as by GC/MS. Typical mass fragments are given in Table 1. The mass spectrum is very similar to that of 3-*O*-methylcholesterol. The only difference was observed for the molecular ion at *m/z* 416 instead of *m/z* 400 and the fragment at *m/z* 401 (loss of CH_3 group) instead of *m/z* 385 due to the higher molecular mass of *S* (32 amu) as compared to *O* (16 amu).

In summary, our data show that *S*-methylated derivatives are formed, almost quantitatively, by the pyrolytic reaction of thiol compounds with TMSH in the GC injector, whereas thiol groups react slowly with the TMSH reagent at room temperature. The extent of the formation of *S*-methylated products depends particularly on the molar excess of the TMSH reagent. However, the pyrolytic reaction of compounds containing both thiol and amino groups with TMSH leads to incomplete derivatization of the NH_2 group. The use of TMSH is therefore recommended for the analysis of thiol compounds that do not contain additional amino groups. The

pyrolytic formation of *S*-methyl derivatives with TMSH can be of diagnostic value for the structural analysis of thiol compounds in samples by GC and GC/MS. It is worth noting that long-chain dialkyl sulfides (dialkyl thioethers) are neither cleaved nor converted to the corresponding methylthio derivatives at all.

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[Received May 26, 1998, and in final revised form and accepted August 24, 1998]

A Semiautomated Enzymatic Method for Determination of Nonesterified Fatty Acid Concentration in Milk and Plasma

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ABSTRACT: An enzymatic assay for the determination of nonesterified fatty acid concentrations in milk and plasma is described. The procedure is semiautomated for use with a plate luminometer or plate spectrophotometer and enables routine batch processing of large numbers of small samples (≤5 μL). Following the activation of nonesterified fatty acids (NEFA) by acyl-CoA synthetase, the current assay utilizes UDP-glucose pyrophosphorylase to link inorganic pyrophosphate to the production of NADH through the reactions catalyzed by phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase. With this assay sequence the formation of NADH from NEFA is complete within 50 min at 37°C. Enzymatic spectrophotometric techniques were unsuitable for NEFA determination in human milk due to the opacity of the sample. The use of the NADH-luciferase system has overcome this problem, allowing the enzymatic determination of NEFA in human milk. Sample collection and treatment procedures for milk and plasma have been developed to prevent enzymatic lipolysis and to limit interference from enzymes present in milk. The recovery of palmitic acid added to milk and plasma samples was 94.9 ± 2.9 and 100 ± 4.5%, respectively. There was no difference (P = 0.13) in plasma NEFA concentrations determined by the current method and a commercially available enzymatic spectrophotometric technique (Wako NEFA-C kit). Plasma NEFA concentrations determined by gas chromatography were 28% higher compared to both the Wako NEFA-C kit and the current method.

Lipids 33, 1043–1049 (1998).

Determination of the nonesterified fatty acid (NEFA) concentration in biological fluids is of importance in a wide variety of clinical and experimental conditions. As a consequence, considerable effort has been applied to the development of accurate methods for NEFA determination that are sufficiently convenient for routine analysis. Techniques for analysis of NEFA include titrimetry with alkali after extraction (1), colorimetry of NEFA-copper or -cobalt soaps (2), and gas chromatography (GC; [3]). Titrimetric and colorimetric methods enable quantification of NEFA, whereas GC also provides identification of individual NEFA. Nevertheless, these methods involve the use of hazardous chemicals and are complex and labor-intensive.

More recently, enzymatic techniques based on the specificity of acyl-CoA synthetase have been established (4). These methods, although unable to identify individual NEFA, do not require extraction of the sample with organic solvents and hence have enabled the development of relatively simple and convenient procedures. However, current enzymatic methods are costly (Wako NEFA-C kit; Wako Pure Chemical Industries Ltd., Osaka, Japan) and have not previously been reported for the determination of NEFA in milk.

A further limitation of enzymatic techniques is the possibility of interference from biologically active substances in the sample. Reducing agents (ascorbic acid, bilirubin) and alkaline phosphatase inhibit enzymatic colorimetric (5) and luminometric (6) methods, respectively. Alternatively, the action of lipases present in the sample may result in overestimation of NEFA concentration (7). Several methods for collection and treatment of samples for NEFA determination have been described (6–9). However, the appropriate method of choice is dependent on the analytical technique, assay sequence, and nature of the sample. In this context, a sample collection and treatment method suitable for enzymatic determination of NEFA concentration in milk has not previously been described.

Our research on (i) the role of NEFA in the control of human milk synthesis and (ii) NEFA metabolism during exercise involves the collection of large numbers (≥600) of small samples (≤5 μL). To facilitate batch processing of these samples, a suitable, semiautomated method was required. Accordingly, we have developed an enzymatic assay which has been linked to plate luminometry to enable the routine determination of NEFA in milk and plasma. In addition, collection and treatment procedures for milk and plasma, suitable for enzymatic determination of NEFA, have been established.

MATERIALS AND METHODS

Subjects. Human milk samples were obtained from volunteers recruited from the Nursing Mothers' Association of Australia (Western Australian Branch). Human plasma was obtained by finger prick from nonfasting volunteers. Volunteers provided written, informed consent and all procedures were approved by

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GC, gas chromatography; MOPS, 3-[*N*-morpholino]propanesulfonic acid hemisodium salt; NEFA, nonesterified fatty acids; TEA, triethanolamine; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid hemisodium salt.

the Human Rights Committee of The University of Western Australia.

Materials. Bacterial luciferase E.C. 1.14.14.3 (from *Vibrio harveyi*), bovine serum albumin (BSA; essentially fatty acid-free, 0.005%), *n*-caproic acid (6:0, Na salt, 99% pure), *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid hemisodium salt (TES; free acid, 99.5% pure), 3-[*N*-morpholino] propanesulfonic acid hemisodium salt (MOPS, 99% pure), oleic acid (18:1n-9, Na salt, 95% pure), palmitic acid (16:0, Na salt), and tetradecylaldehyde (80% pure) were obtained from Sigma Chemical Company (Castle Hill, Australia). Dipotassium hydrogen orthophosphate 3-hydrate ($K_2HPO_4 \cdot 3H_2O$, 99.0% pure) and potassium dihydrogen orthophosphate (KH_2PO_4, 99% pure) were obtained from BDH Chemicals (Port Fairy, Australia). Ethylenediaminetetraacetic acid (EDTA, tetra-Na salt, 99% pure), hydrochloric acid (HCl, 31.5% w/w), and sodium hydroxide (NaOH, 97% pure) were obtained from Ajax Chemicals (Sydney, Australia). Acyl-CoA synthetase E.C. 6.2.1.3 (from *Pseudomonas fragi*), adenosine triphosphate (ATP; di-Na salt, 99% pure, from yeast), coenzyme A (CoA; lyophilized free acid, grade 1), dithiothreitol, flavin mononucleotide (FMN, 95% pure), glucose-6-phosphate 1-dehydrogenase E.C. 1.1.1.49 (from *Leuconostoc mesenteroides*), nicotinamide adenine dinucleotide (NAD, 98% pure, from yeast), reduced nicotinamide adenine dinucleotide (NADH, di-Na salt, 98% pure), phosphoglucomutase E.C. 5.4.2.2 (from rabbit muscle), triethanolamine (TEA), uridine-5′-diphosphoglucose (UDP-glc, di-Na salt, 98% pure, from yeast), and uridine-5′-diphosphoglucose pyrophosphorylase E.C. 2.7.7.9 (from beef liver) were obtained from Boehringer Mannheim (North Ryde, Australia). Lauric acid (12:0, A grade, 99.5% pure) was obtained from California Corporation for Biochemical Research (Los Angeles, CA). The Wako NEFA-C kit was obtained from Wako Pure Chemical Industries Ltd. Materials used for GC determination have been listed previously (10).

Assay principles. (i) Nonesterified fatty acids. Following the activation of NEFA by acyl-CoA synthetase (4), the current assay sequence utilizes UDP-glucose pyrophosphorylase, phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase to link inorganic pyrophosphate to the reduction of NAD to NADH as outlined below:

$$\text{ATP} + \text{NEFA} + \text{CoA} \xrightarrow{\text{acyl-CoA synthetase}} \text{acyl}-\text{CoA} + \text{AMP} + \text{PPi} \quad [1]$$

$$\text{PPi} + \text{UDP}-\text{glc} \xrightarrow{\text{UDP-glucose pyrophosphorylase}} \text{glucose-1-phosphate} + \text{UTP} \quad [2]$$

$$\text{glucose-1-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-phosphate} \quad [3]$$

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{glucose-6-phosphate 1-dehydrogenase}} \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADH} + \text{H}^+ \quad [4]$$

The amount of NADH present is directly proportional to the concentration of NEFA in this reaction sequence.

(ii) Luminescence detection. The principle of the luminescent method, outlined below, has been described previously (11):

$$\text{NADH} + \text{H}^+ + \text{FMN} \xrightarrow{\text{oxidoreductase}} \text{NAD}^+ + \text{FMNH}_2 \quad [5]$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{bacterial luciferase}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light} \quad [6]$$

This process is metabolite-independent and involves the kinetic measurement of NADH. The amount of light output measured is directly proportional to the NADH present.

The technique of luminescence detection (11) has been modified and semiautomated for the measurement of lactose, galactose, β-hydroxybutyrate, and D-lactate (12) with the ML2250 Microtiter Plate Luminometer (Dynatech Laboratories, Chantilly, VA). In the current study, this technique (12) has been adapted for the determination of NEFA in milk and plasma.

Methods. (i) Sample collection and treatment. Milk samples were collected from mothers at the Department of Biochemistry, The University of Western Australia. Mothers expressed a small amount of milk (<1 mL) before and after each breast feed. Subsamples (250 µL) for NEFA determination were taken immediately and 400 mM EDTA (20 µL) was added according to Chappell *et al.* (13). Samples were frozen at −80°C until required for assay. Prior to assay, milk samples were thawed and then incubated at 80°C in a water bath for 5 min. Following incubation, samples were sonicated (Branson Sonifer B-12; Branson Ultrasonics Corp., Danbury, CT) for 20 s and a subsample (50 µL) was acid-denatured. The method of acid denaturation was modified from Kather and Weiland (6) and involved the addition of 1 M HCl (50 µL) to the 50 µL subsample which was mixed and left to stand at room temperature for 1 min. Samples were neutralized with an equal volume of 1 M NaOH in 100 mM TES.

Finger-prick blood samples were collected in heparinized hematocrit tubes (Chase Instruments, Glen Falls, VT) and centrifuged (H.I. Clements, North Ryde, Australia) for 5 min at $8000 \times g$. Plasma was treated at collection according to a modification of the acid denaturation procedure used for milk. Five microliters of plasma was added to the same volume of 200 mM HCl, mixed, and allowed to stand for 1 min at room temperature. The sample was neutralized by addition of an equal volume of 200 mM NaOH in 100 mM TES and stored at −80°C until required for assay. For GC analysis plasma was obtained from the Blood Transfusion Service of Western Australia.

(ii) Working solutions: reagents. Assay reagent: ATP (4 mM), $MgCl_2$ (10 mM), CoA (30 mM in 25 mM MOPS buffer, pH 5), UDP-glc (3 mM), NAD^+ (8.5 mM), acyl-CoA synthetase (1.2 U/mL), UDP-glucose pyrophosphorylase (0.8 U/mL in 50 mM TEA and 200 µM dithiothreitol), phosphoglucomutase (13.4 U/mL), glucose-6-phosphate 1-dehydro-

genase (6 U/ml), Triton X-100 (0.03%, vol/vol) in 100 mM TEA, pH 8.4. Control reagent: assay reagent excluding acyl-CoA synthetase. Components for the assay/control reagent (except phosphoglucomutase, glucose-6-phosphate 1-dehydrogenase) were stored as stock solutions and were stable for at least 3 mon at −80°C. The complete assay and control reagents were aliquoted and stored at −80°C and were stable for 1 mon. The bioluminescent reagent was prepared according to Thompson *et al.* (12): potassium phosphate (100 mM), BSA (1.6%), EDTA (4 mM), FMN (8 μM), tetradecylaldehyde (0.1%), and bacterial luciferase (0.4 mg/mL) in TES (300 mM, pH 6.8) was aliquoted and stored at −80°C and was stable for at least 4 mon (13). Reagents for the Wako NEFA-C kit were prepared according to manufacturers' instructions. Reagents prepared for modification of the Wako NEFA-C kit have been outlined previously (14).

(ii) Working solutions: standards. A 1 mM NEFA standard was prepared by adding 16:0 to a heated solution of 4% BSA and 0.1% Triton X-100 (50°C). This solution was briefly sonicated and diluted with the BSA/Triton solution to prepare individual standards (range 0–500 μM) which were stored at −20°C. Standards for the Wako NEFA-C kit were prepared according to manufacturers' instructions.

Procedures. Standards were heated to 37°C and vortexed. Two microliters (plasma) and 5 μL (milk) of each standard, sample, blank, and quality control were pipetted in triplicate into a 96-well V-bottom microtiter plate (Flow Laboratories, McLean, VA). Two or 5 μL of either assay or control reagent was added to each well. The plate was mixed (Denley Welltech Wellmixx2, Cytosystems, Castle Hill, Australia), sealed (plate sealer, ICN Biomedicals, Aurora, CA), and incubated at 37°C for 60 min. The procedure for plate luminometry was modified from Thompson *et al.* (12). The reaction was stopped with the addition of 200 μL NaOH (20 mM). Five-microliter aliquots from each well were transferred to a Microlite® 96-well flat-bottom plate (Dynatech Laboratories), diluted with 150 μL NaOH (10 mM), and reacted with 15 μL of bioluminescent reagent in a ML2250 Microtiter Plate Luminometer (Dynatech Laboratories). The luminometer was programmed for a 5-s delay after addition of the bioluminescent reagent which was followed by a 5-s read period. The peak and integrated light response during the read period were automatically calculated. The sample concentration was determined by calculation relative to a standard curve.

The current method for plasma was adapted for use with a plate spectrophotometer (Titertek Multiskan® MCC/340 plate reader; Flow Laboratories) according to the following modifications: 50 μL of each sample, standard, blank, and quality control was plated in duplicate and 50 μL of either assay or control reagent was added to each well. Following incubation, 50 μL of double-deionized water was added to each well and absorbance was measured at 340 nm on the plate spectrophotometer.

Samples were analyzed using the Wako NEFA-C kit according to either manufacturers' instructions or Johnson and Peters (14) using a plate spectrophotometer (Titertek Multiskan®). Assay procedures for NEFA analysis by GC have been previously described (10). Briefly, lipids were extracted from plasma into methanol/chloroform (1:1, vol/vol) with internal standard (heptadecanoic acid). Lipid extracts, evaporated to dryness under N_2 at 40°C, were dissolved in hexane/*tert*-butyl methyl ether (200:3, vol/vol). Nonesterified fatty acids were separated (porasil silica column) and evaporated to dryness under N_2. Dried NEFA samples were methylated by the addition of H_2SO_4 in methanol and incubated at room temperature for 40 min. Methyl esters were extracted into hexane and evaporated to dryness under N_2. Toluene containing internal standard (methyl pentadecanoic acid) was added and methyl esters were separated and quantified by GC at 150°C [(Shimadzu GC-8A (Kyoto, Japan), glass column, 2 m length and 2.6 mm internal diameter packed with 10% SP-2340 on 100/120 Chromosorb (Supelco, Bellefonte, PA)].

Statistics. Paired and unpaired *t*-tests were used to determine the significance of differences between plasma sample collection and treatment procedures and differences in NEFA concentrations determined between methods. Statistical analysis was performed using Statview SE + Graphics® (Abacus Concepts, Inc., Berkeley, CA). All results presented are means ± SE unless stated otherwise.

RESULTS AND DISCUSSION

Our aim was to develop an assay for determination of NEFA concentration in large numbers (≥600) of small (≤5 μL) human milk and plasma samples. An appropriate technique was required to enable routine processing of these samples. In this context the current method has been developed as a semiautomated procedure utilizing either a Microtiter plate luminometer or a Titertek multiscan plate spectrophotometer and 96-well microtiter plates.

Standards. To avoid underestimation of NEFA concentration, standards were prepared with 4% BSA based on the method of Miles *et al.* (15). Light responses increased linearly for 16:0 standards over the range 0–500 μM ($r = 0.998$) and showed close agreement with known concentrations of NADH ($P < 0.001$, $r = 0.999$; $y = -5.06 \times 10^{-3} + 0.978x$, y = NADH, x = 16:0). The plate spectrophotometric procedure showed a linear increase in absorbance for 16:0 standards across the range 0–500 μM ($r = 0.999$). Standards were stable for approximately 1 mon at −20°C.

Sample collection and treatment. (i) Milk. Chemical colorimetric (16,17) and GC (18,19) methods are predominantly used to determine NEFA concentration in milk. These methods are costly, labor-intensive, and require relatively large sample volumes (13). The development of enzymatic spectrophotometric (4) and luminometric procedures (6) for NEFA determination in plasma has overcome many of these problems. Determination of NEFA requires sample treatment to prevent lipolysis during storage and analysis. However, enzymatic determination of NEFA in milk requires additional sample treatment to limit interference from enzymes present in milk.

In vitro enzymatic lipolysis due to lipases in milk can be prevented by the addition of EDTA to milk samples at collection (13). Accordingly, NEFA concentration in samples collected without EDTA increased by 102 ± 28% during 60 min at room temperature (361 ± 17 to 714 ± 64 μM, $n = 5$; $P < 0.001$) whereas samples collected with EDTA increased by 5 ± 5% (361 ± 17 to 375 ± 3 μM, $n = 5$; $P = 0.81$). Production of NADH beyond the incubation period (drift) was observed in milk samples collected with EDTA. This outcome is likely to be a consequence of thioesterase II (20) as well as nondivalent cation-dependent lipases present in milk (13). This drift was abolished by incorporating an incubation step (80°C for 5 min) and acid denaturation after the addition of EDTA during treatment of milk samples.

(ii) Plasma. Heparin is known to activate plasma lipoprotein lipase (21), however, collection of blood samples in heparinized containers is often desirable to prevent clot formation. Despite the potential for *in vitro* enzymatic lipolysis (7), available literature reveals a lack of consistency in the approach to limit this effect (9,22). The efficacy of acid denaturation to prevent enzymatic lipolysis in plasma samples collected with heparinized tubes was examined in the current study. Duplicate plasma samples were either acid-denatured immediately upon collection and stored at −80°C or stored at −80°C and diluted with water prior to assay. Immediate acid denaturation resulted in lower values (17.1 ± 8.5%) for plasma NEFA when compared to samples untreated before storage ($P = 0.059$; $n = 9$). There was no difference when samples were untreated before storage and either acid-denatured or diluted with water prior to assay ($P = 0.979$; $n = 4$). These findings indicate that plasma collected in heparinized containers should be acid-denatured immediately at collection.

The potential for variation in sample final pH following treatment with acid and base during acid denaturation can result in chemical hydrolysis of plasma triacylglycerol and hence overestimation of NEFA concentration. When plasma samples (5 μL) were acid-denatured with HCl (1 M) and NaOH (1 M) according to Kather and Weiland (6), a large range for plasma sample final pH (5–9) was observed. Hydrochloric acid (1 M; 1:1, vol/vol) added to the sample (pH 0–1) and allowed to stand at room temperature for 0–4 min had no effect on NEFA concentration ($P = 0.521$; $n = 4$). However, alkaline conditions are known to cause saponification of triacylglycerol. Duplicate plasma samples were acid-denatured and NaOH was added to the sample/HCl mixture resulting in either an alkaline (range, 9–13) or a neutral pH. Plasma NEFA concentrations were higher in alkaline compared to neutral pH samples immediately following sample treatment ($P = 0.015$; 91 ± 29 μM neutral, 333 ± 53 μM alkaline) and after 60 min at room temperature ($P = 0.001$; 124 ± 33 μM neutral, 539 ± 40 μM alkaline). There was no change in NEFA levels across the 60 min at room temperature in neutral pH samples ($P = 0.203$). In contrast, a 67 ± 14% increase in NEFA was observed during the same period in alkaline samples ($P = 0.009$). Alkaline final pH following plasma sample treatment with acid and base has been avoided by reducing the HCl and NaOH concentrations from 1 M to 200 mM and including a TES buffer (100 mM, pH 7) with the NaOH.

Reagent. All enzymatic methods for determination of NEFA require activation by acyl-CoA synthetase (4). Following this initial reaction, the current assay sequence differs from existing enzymatic methods (4–6,15,23,24) by utilizing UDP-glucose pyrophosphorylase to convert inorganic pyrophosphate to glucose-1-phosphate in the presence of UDP-glc. Glucose-1-phosphate is linked to the production of NADH by way of the reactions catalyzed by phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase. The control reagent (assay reagent excluding acyl-CoA synthetase) was used to account for the presence of glucose-1-phosphate, glucose-6-phosphate, and inorganic pyrophosphate in milk (<5%) and plasma (≤10%).

This assay sequence has enabled a reduction in completion time compared to previous luminometric methods (6). The formation of NADH from NEFA in a 500 μM 16:0 standard and in samples of milk and plasma was complete within 50 min at 37°C and was stable from 60–120 min (Fig. 1). Furthermore, changes in absorbances from a 500 μM 16:0 standard and a human plasma sample showed a similar response for the plate spectrophotometric method (Fig. 1). Accordingly, an incubation period of 60 min at 37°C for both methods was chosen to ensure complete formation of NADH. The concentrations of acyl-CoA synthetase and UDP-glucose pyrophosphorylase, the rate-limiting enzymes for the assay sequence, were optimized for these incubation conditions and to reduce background reagent contamination.

The reactions involved in luminescence detection can be affected by several components present in either the reagent mixture or the sample. Inhibition or stimulation of the light

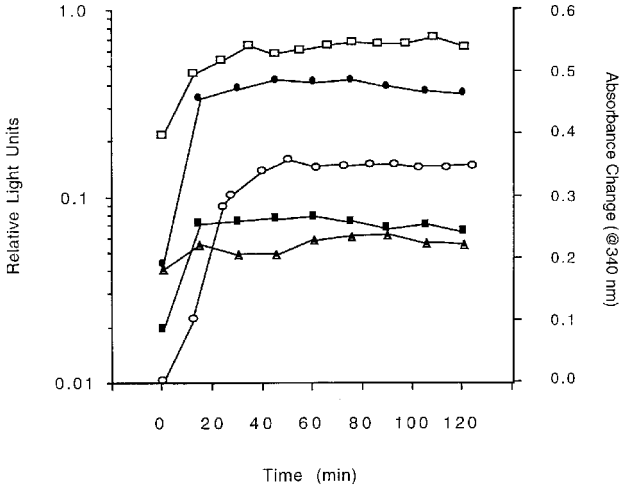

FIG. 1. Time course of nonesterified fatty acid (NEFA) conversion from a 500 μM 16:0 (●) standard and from samples of (▲) human milk and (■) plasma (plate luminometric method, closed symbols; plate spectrophotometric method, open symbols). NADH from NEFA is converted to relative light units as described in the Materials and Methods section of the text. Values are means of duplicate determinations.

TABLE 1
Effect of Reagent and Sample Components on Light Response

Component	Dilution factor[a]	
	10% change[b]	50% change[b]
Milk	65.9	11.5
Plasma	111	4.4
Reagent	520	92.9

[a]Final dilution factor for component in the luminometer well which provided either a 10 or 50% change in relative light units.
[b]Light response normally present in diluted luminescent reagent was taken as 100%.

response by these components was minimized by the high final dilution (1428-fold in the luminometer well) of the reaction mixture. The effects of specific components on the light response are outlined in Table 1.

We found enzymatic spectrophotometric techniques unsuitable for NEFA determination in whole milk and milk whey fractions owing to the opacity of the sample. This problem has been overcome by the use of the NADH-luciferase system. In addition, the combination of the current assay sequence with this system resulted in considerably lower reagent costs when compared to a commercially available spectrophotometric method (Wako NEFA-C kit).

Validation. Recovery, precision, and sensitivity. The recovery of a range of NEFA added to different milk and plasma samples, the interassay variation, and the detection limit (defined as three times the standard deviation of the mean blank) and sensitivity (defined as the detection limit concentration in the luminometer well volume) for the luminometric and spectrophotometric assays are listed in Table 2.

Method comparisons. Human plasma NEFA concentrations determined by the plate luminometric method and the plate spectrophotometric procedure were highly correlated ($r = 0.985$; $P < 0.02$) and showed close agreement ($P = 0.53$; Fig. 2). The plate luminometric method was also compared to a commercially available spectrophotometric method (Wako NEFA-C kit) and a modification of this method for use with 10-µL sample volumes (14). The concentration of NEFA in human plasma samples determined by the Wako NEFA-C kit and the plate luminometric method were highly correlated ($r = 0.998$; $P < 0.001$) and differed by less than 6% ($P = 0.13$; Fig. 2). In contrast, NEFA concentrations determined by the method of Johnson and Peters (14), although highly correlated with the plate luminometric method ($r = 0.989$; $P < 0.001$), were approximately 50% lower ($P = 0.01$; Fig. 2). This difference was found to be due to the effect of sample treatment procedures. Specifically, NEFA concentrations in untreated plasma were lower compared to both acid-denatured ($P = 0.009$) and water-diluted ($P = 0.04$) plasma although all treatments were highly correlated ($P < 0.001$, $r = 0.998$, $y = 11.6 + 1.51x$, y = water-diluted, x = untreated; $P < 0.001$, $r = 0.975$, $y = 112.5 + 1.41x$, y = acid-denatured, x = untreated).

Considerable differences in NEFA concentrations have been reported for blood samples collected under similar conditions, in unrelated studies, when determined by either GC (25) or enzymatic methods (22). When plasma samples were subjected to parallel analysis, we found NEFA concentrations in plasma measured by GC were highly correlated with those determined by the Wako NEFA-C kit and the plate luminometric method ($r = 0.986$, $P < 0.02$; and $r = 0.992$, $P < 0.01$, respectively). However, values derived from GC were 28% higher when compared to those determined by the enzymatic methods ($P = 0.02$, Wako NEFA-C kit; $P = 0.03$, plate luminometry; Fig. 2). These results support the findings of previous direct comparisons between GC and enzymatic methods (26,27). Differences in NEFA–protein interactions between standards and samples have been avoided by the inclusion of albumin with standards (15). This discrepancy is therefore not entirely due to the analytical behavior of the standards. Alternatively, the lower values determined by enzymatic methods compared to GC may be explained by the inability of acyl-CoA synthetase to activate fatty acids tightly bound to albumin (28) or those containing more than 20 carbon atoms (5). As a consequence of the difference in NEFA levels determined by enzymatic procedures when compared to GC, the method of determination should be taken into account when comparing values reported in the literature.

TABLE 2
Recovery of Known Concentrations of NEFA Added to Human Milk and Plasma, the Interassay Variation, and the Detection Limit and the Sensitivity of the Plate Luminometric and Spectrophotometric Methods

NEFA	Recovery (%)	Interassay variation (%)	Detection limit (µM)[a]	Sensitivity (nM)[b]
6:0[c]	97.5 ± 1.52 $n = 8$	7.4 $n = 5$	8.0 ± 0.6 $n = 8$	5.6 ± 0.4 $n = 8$
12:0[c]	96.4 ± 1.64 $n = 8$			
16:0[c]	94.9 ± 0.91 $n = 10$			
18:1n-9[c]	97.4 ± 2.55 $n = 7$			
16:0[d]	100 ± 4.50 $n = 8$	6.8 $n = 7$	9.0 ± 1.7 $n = 10$	2.6 ± 0.5 $n = 10$
16:0[e]	95.8 ± 1.80 $n = 6$	4.9 $n = 5$	13.2 ± 2.7 $n = 5$	4393 ± 916 $n = 5$

[a]Defined as three times the standard deviation of the mean blank.
[b]Defined as the detection limit concentration in the final well volume.
[c]Luminometry for milk.
[d]Luminometry for plasma.
[e]Spectrophotometry for plasma. For abbreviation see Table 1.

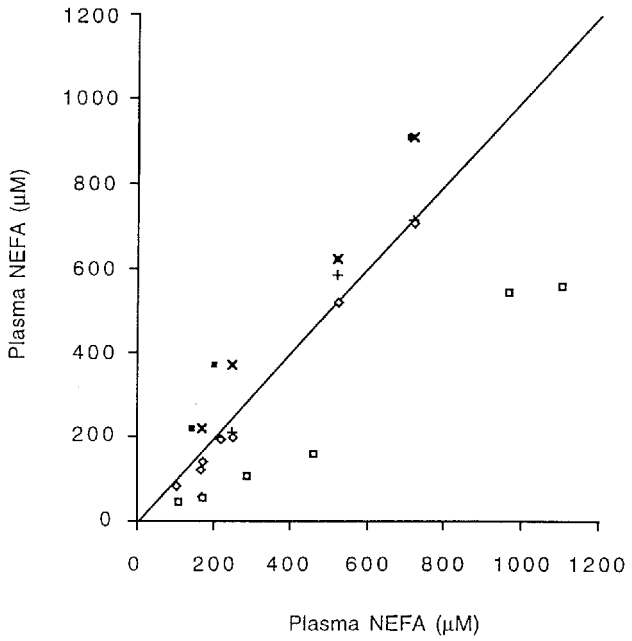

FIG. 2. Comparison of methods for determination of NEFA in human plasma. (+) Plate luminometric method vs. plate spectrophotometric method, $y = -40.67 + 1.09x$; (◇) plate luminometric method vs. Wako NEFA-C kit, $y = -32.23 + 1.04x$; (×) plate luminometric method vs. gas chromatography (10), $y = 37.80 + 1.19x$; (□) plate luminometric method vs. Johnson & Peters (14), $y = -44.23 + 0.57x$; (■) Wako NEFA-C kit vs. gas chromatography (10), $y = 90.73 + 1.12x$; — line of unity, $y = x$ (i.e., slope = 1, intercept = 0). Values are in micromoles and are means of triplicate determinations. For abbreviation see Figure 1.

ACKNOWLEDGMENTS

This project was supported by the Grains Research and Development Corporation of Australia, Meadow Lea Foods Ltd., the Pig Research and Development Corporation of Australia, and the Australian Research Council. The authors thank Stephen Smith and Associate Professor Michael Guppy for the gas chromatographic determination of NEFA, and Dijana Mihic and Christine Wakeford for technical assistance.

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[Received January 6, 1998, and in final revised form and accepted October 5, 1998]

Obesity as a Risk Factor for Certain Types of Cancer[1]

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A survey of body weight, smoking, and cancer mortality involving 750,000 Americans over the period from 1959 to 1972 showed a 33% increase in cancer deaths for men and a 55% increase for women whose body weight was more than 40% above average. Overweight men had significantly higher rates of colorectal cancer, whereas women had higher rates of cancers of the breast, cervix, endometrium, gallbladder and biliary passages, ovary, and uterus (1). It should be noted, however, that being overweight is not necessarily synonymous with obesity (2).

Other more recent studies, such as the Danish record-linkage study (3), have provided additional evidence of an association between obesity and cancer (4–6). This is a matter for concern because of the high incidence of obesity in many countries and because of the increasing incidence of obesity in many parts of the world (5–15).

The aim of this review is to summarize information on obesity in relation to cancer at the various sites for which there is evidence of a positive association.

ENDOMETRIAL CANCER

The link between obesity and endometrial cancer is well-established (4,16–18), particularly for postmenopausal women (19). The association has been observed in both case–control and follow-up studies and in many cases was confined to very obese women (18). The relationship is stronger in older women (19–21). A positive association between endometrial cancer and upper body fat, as measured by waist-to-hip ratio or waist-to-thigh ratio, has been observed in some studies (22–24), but not in others (25). In some cases, this positive association disappeared after adjustment for body mass index (BMI) (26–28).

The association between obesity and endometrial cancer probably has a hormonal basis. Endometrial epithelial cells are stimulated by elevated estrogen levels; and this stimulation, unopposed by progesterone, is thought to be conducive to development of cancer (29,30). Obese women have higher levels of serum estrone and estradiol (27,28,31), presumably as a result of the production of estrogen in adipose tissue by aromatization of androstenedione (32). Obese women also have decreased levels of sex hormone-binding globulin, thus increasing the amount of bioavailable estrogen (32–34).

Steroid measurements in urine collected from women in a cohort study provided further evidence in favor of the hypothesis that unopposed estrogen is a risk factor for endometrial cancer (35). In a recent case–control study, high circulating levels of androstenedione were associated with increased risk of endometrial cancer in both pre- and postmenopausal women, but high levels of estrone and estradiol were only associated with increased risk in postmenopausal women (36). In premenopausal women, obesity may influence endometrial cancer through its tendency to cause amenorrhea and luteal phase progesterone deficiency (21).

BREAST CANCER

A number of studies have shown that obesity increases the risk of breast cancer in postmenopausal women (4,5,17, 37–43). In contrast, a meta-analysis showed a modest inverse association between BMI and premenopausal breast cancer (44). An inverse association between adiposity and breast cancer has also been observed in a number of other studies (43). This may not apply in all cases, however, since a study of women in seven different countries showed that incidence of breast cancer in premenopausal women increased with body mass in low- and moderate-risk countries but decreased with increasing body mass in high-risk countries. In postmenopausal women, the rates increased with body mass in all risk groups (45,46). Cohort studies have been less supportive of the positive correlation between body mass and breast cancer in postmenopausal women than case–control studies (39,41; but see 42).

Breast cancer may also be influenced by the distribution of body fat. An increase in central adiposity has been reported to increase the risk of postmenopausal breast cancer, independent of relative weight, particularly when there is a family history of breast cancer (41). Breast cancer patients were also found to have more visceral fat compared to subcutaneous fat than controls, as measured by computed tomography (47).

Breast cancer risk was not found to be related to body fat distribution in women under 45 yr of age (48). In agreement with earlier studies, Swanson *et al.* (48) found an inverse correlation between body weight and breast cancer in young

[1]This paper was prepared as one of the background documents for the WHO Consultation on Obesity (Geneva, Switzerland, 3-5 June 1997). For further details, please see the report of the Consultation, Obesity: preventing and managing the global epidemic (WHO/NUT/NCD/97.2).

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Abbreviation: BMI, body mass index.

women. It was concluded that this probably can not be explained by increased frequency of anovulatory cycles or by detection bias (45).

Adult weight gain has consistently been associated with increased risk of breast cancer, even in cohort studies that showed no association between baseline relative weight and subsequent risk of breast cancer (17,43). In most studies, increased body mass was associated with poorer survival and increased likelihood of recurrence, and adverse outcomes were more pronounced among postmenopausal women and women with less advanced disease (41,49,50). In this connection, it is of interest that most women gain weight during the menopause (51). Weight loss has been associated with reduced risk in some studies but not in others (17,43,52).

Breast cancer, like endometrial cancer, is strongly influenced by hormonal factors, and the differing effects of adiposity on pre- and postmenopausal breast cancer may be explainable on the basis of differences in steroid hormone metabolism (41). Before the menopause, the ovaries are the main source of estrogen, whereas after the menopause production from the ovaries decreases and the estrogen produced in adipose tissue by aromatization of androstenedione assumes greater importance, particularly in obese individuals (53,54). Other factors, such as increased levels of androstenedione and decreased levels of sex hormone-binding globulin, may enhance the risk of breast cancer as well as endometrial cancer after the menopause.

In a recent brief communication, Potischman *et al.* (55) reported that serum total estradiol levels decreased with increasing BMI in premenopausal women, whereas they increased in postmenopausal women. This could be a reason for the differing relationships between obesity and breast cancer in premenopausal compared to postmenopausal women. They suggested that the lower levels of estrogen in obese premenopausal women may be due to uptake by adipocytes and a higher metabolic clearance rate. Gerber (56) suggested, on the other hand, that high estrogen levels may be a cause of leanness in premenopausal women. This idea was based on the observation of an inverse relationship between BMI and high density lipoprotein cholesterol levels in premenopausal women. It was suggested that stimulation of hepatic lipoprotein lipase by estrogen results in high low density lipoprotein cholesterol and lower serum triglycerides, leading to lower BMI.

Breast cancer normally originates in mammary epithelial tissue (57) and the adipose tissue of the mammary gland may have a particularly important influence on the epithelial tissue because of their close association with one another (58). This influence may be exerted through estrogens produced in the adipose tissue or through other fat-soluble compounds, such as eicosanoids formed from polyunsaturated fatty acids present in the lipids of adipose tissue (59). An experiment showed a positive correlation between the fat content of the mammary gland in rats on different diets and the number of mammary tumors induced by 7,12-dimethylbenz(a)anthracene in rats on those diets (60). The possibility that mammary gland mass is positively associated with risk of breast cancer has been discussed by Trichopoulos and Lipman (61).

Hyperinsulinemia related to obesity and insulin resistance may pose a risk for breast cancer (62,63). In a case–control study, serum levels of C-peptide, a marker of hyperinsulinemia, were significantly higher in patients with early breast cancer compared to controls, but the study indicated that hyperinsulinemia with insulin resistance was a risk factor for breast cancer independent of general adiposity or body fat distribution (64).

PROSTATE CANCER

Obesity does not appear to be an important risk factor for prostate cancer (4). Although some cohort studies have shown a positive association with overweight, others have not, nor have most case–control studies (65). In a recent report on a large cohort of Swedish construction workers, various aspects of body size, including adult weight and BMI, were positively associated with prostate cancer, and the risk was more strongly related to mortality than to incidence (66). A high BMI may be due to greater muscle mass rather than adipose tissue, and a prospective study in Japanese-American men showed a positive association between prostate cancer and muscle area, but not fat area (67). In fact, obese men with prostate cancer have been reported to have a better prognosis than those with more normal weight (68). This could be related to inhibition of prostate cancer growth and metastasis by increased endogenous estrogen and decreased endogenous testosterone, associated with obesity.

PANCREATIC CANCER

A positive association between body weight or obesity and pancreatic cancer has been reported in some studies but not others (3,69). It has been suggested that effects of obesity on pancreatic cancer may be mediated through sex hormones, since the pancreas contains sex hormone receptors and the anti-estrogen, tamoxifen, inhibits the growth of pancreatic tumors (69).

COLORECTAL CANCER

Although the relationship of BMI to colon cancer has been investigated rather extensively, there does not appear to be a clear consensus (4,70,71). Obese men have been reported to be at higher risk, whereas obese women are not (5). In contrast, the authors of a recent report on a large cohort study in Denmark concluded that the least obese men had the highest risk of colon cancer (72). In the Framingham study, obesity in men combined with low serum cholesterol was associated with four times greater risk compared to people with average values (73). Other studies indicated that obesity in adolescence or early adulthood poses a risk of colon cancer for men (71,74,75).

A report on Harvard alumni provided evidence that higher

levels of Quetelet's index were associated with higher colon cancer risk only among those who were less active (76). The results of a recent large case–control study by Slattery *et al.* (77) also indicated that a large BMI was more strongly associated with increased risk of colon cancer in individuals who were physically inactive and had a high energy intake. There was some evidence that men may be at higher risk than women, especially older women, as a result of an unfavorable energy balance.

In other recent studies, higher BMI was related to higher risk of adenomas in the distal colon in both men and women (78,79). This relationship was stronger for larger adenomas. It was also observed that waist circumference and waist-to-hip ratio are positively related to higher risk of large colon adenomas or cancer in men (78). Slattery *et al.* (77) reported that those at greatest risk of colon cancer had the most unfavorable energy balance, were physically inactive, had high energy intakes and large BMI.

Insulin is a growth factor for colonic mucosal cells and a mitogen for colonic carcinoma cells *in vitro,* and insulin or related growth factors may mediate the influence of obesity on colon cancer (80). Men have a greater tendency than women to accumulate fat tissue in the abdomen, and this is associated with higher insulin levels, which could help to account for the stronger association between obesity and colon cancer in men (80).

Although cancers of the colon and rectum are frequently considered together as colorectal cancer, the rectum differs from the colon in a number of respects (70). Colon cancer and rectal cancer should therefore probably be considered separately in relation to factors that affect them. In a study of Seventh-Day Adventists, a 25% excess in body weight was associated with increased rectal cancer in both men and women, but with increased colon cancer in men only (81). Whereas BMI was positively correlated with higher risk of colonic adenomas in both men and women, it was not apparently related to rectal adenomas in women (78,79).

RENAL CELL CANCER

Renal cell cancer has been consistently associated with overweight and obesity (4,82). The results of case–control studies and a large multicenter study indicated that body weight has a stronger impact on renal cell cancer in women than on men, and in the multicenter study, rate of weight change appeared to be an independent risk factor for women (82). The stronger association in women suggests a role for increased levels of endogenous estrogens (4). These may affect renal cell proliferation and growth by means of receptors present in renal cells or through paracrine growth factors (82).

CANCER AT OTHER SITES

In the large American study cited in the introduction, obese women had higher rates of cancer at a number of sites other than those discussed above (1). The Danish record-linkage study of obese subjects also showed increased incidence of cancer at some additional sites, including the esophagus, liver, and ovary (3). The excess cancers of the esophagus and liver may have been related to higher alcohol intake in the obese cohort. Obese women tend to have more anovulatory cycles, which might affect their susceptibility to ovarian cancer (3). The risk of epithelial ovarian cancer has been observed to decrease with increasing numbers of full-term pregnancies (83). One of the more surprising observations in the Danish study was an excess of brain cancer in the year following discharge from hospital, for which there was no good explanation (3).

SUMMARY AND CONCLUSIONS

In conclusion, obesity has been associated with increased risk for a number of different types of cancer. The evidence has been most consistent for endometrial cancer, breast cancer in postmenopausal women, and renal cell cancer. More variable results have been reported for colorectal, prostate and pancreatic cancer.

Possible mechanisms by which obesity may influence cancer risk include alteration in hormonal patterns, including sex hormones and insulin, and factors such as the distribution of body fat and changes in adiposity at different ages. The increasing prevalence of obesity in many parts of the world emphasizes the importance of learning more about the relationship between obesity and cancer and the mechanisms involved in their interaction.

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[Received August 26, 1997, and in revised form July 24, 1998; revision accepted October 16, 1998]

ARTICLE

A Pathway for Biosynthesis of Divinyl Ether Fatty Acids in Green Leaves

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ABSTRACT: [1-^{14}C]α-Linolenic acid was incubated with a particulate fraction of homogenate of leaves of the meadow buttercup (*Ranunculus acris* L.). The main product was a divinyl ether fatty acid, which was identified as 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid. Addition of glutathione peroxidase and reduced glutathione to incubations of α-linolenic acid almost completely suppressed formation of the divinyl ether acid and resulted in the appearance of 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid as the main product. This result, together with the finding that 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid served as an efficient precursor of the divinyl ether fatty acid, indicated that divinyl ether biosynthesis in leaves of *R. acris* occurred by a two-step pathway involving an ω6-lipoxygenase and a divinyl ether synthase. Incubations of isomeric hydroperoxides derived from α-linolenic and linoleic acids with the enzyme preparation from *R. acris* showed that 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid was transformed into the divinyl ether 12-[1′(*Z*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid. In contrast, neither the 9(*S*)-hydroperoxides of linoleic or α-linolenic acids nor the 13(*R*)-hydroperoxide of α-linolenic acid served as precursors of divinyl ethers.

Lipids 33, 1061–1071 (1998).

Fatty acid hydroperoxides are generated in higher plants by lipoxygenase-catalyzed dioxygenation of linoleic and α-linolenic acids. Further metabolism of α-linolenic acid 13(*S*)-hydroperoxide catalyzed by allene oxide synthase, allene oxide cyclase, reductase, and β-oxidation enzymes results in the formation of 12-oxo-10,15(*Z*)-phytodienoic acid (12-oxo-PDA) and jasmonic acid (1,2). This pathway is of biological importance in plants because it produces compounds which are involved in defense reactions against insects and other phytopathogens (3), in mechanical responses such as tendril coiling (4), and in pollen development (5). Other pathways of hydroperoxide metabolism in higher plants result in the formation of fatty acid epoxides and epoxy alcohols (6,7), short-chain aldehydes (8), and divinyl ethers (9). The last-mentioned family of compounds includes colneleic and colnelenic acids {9-[1′(*E*),3′(*Z*)-nonadienyloxy]-8(*E*)-nonenoic acid and 9-[1′(*E*),3′(*Z*),6′(*Z*)-nonatrienyloxy]-8(*E*)-nonenoic acid, respectively}, which are generated from the 9(*S*)-hydroperoxide derivatives of linoleic acid and α-linolenic acid, respectively, in potato tubers (9) and tomato roots (10). Etheroleic and etherolenic acids {12-[1′(*E*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid and 12-[1′(*E*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid, respectively} constitute another set of divinyl ether fatty acids, which are produced from the 13(*S*)-hydroperoxides of linoleic acid and α-linolenic acids, respectively, in bulbs of garlic (11). In addition, small amounts of divinyl ethers have been isolated from marine algae. Thus, divinyl ethers biogenetically related to 6,9,12,15-octadecatetraenoic acid, 5,8,11,14,17-eicosapentaenoic acid, and α-linolenic acid have been obtained from the brown alga *Laminaria sinclairii* (12), whereas the red alga *Polyneura latissima* was found to contain a divinyl ether, polyneuric acid, related to the 9(*S*)-hydroperoxide derivative of arachidonic acid (13).

Divinyl ethers of higher plants have been generated in various nonphotosynthetic tissues and have not been detected in preparations of green leaves (10,14). Colneleic acid has been reported to be a potent inhibitor of potato lipoxygenase (15); however, so far no specific biological function has been ascribed to colneleic acid or other divinyl ethers. The present study is concerned with a pathway for biosynthesis of divinyl ether fatty acids in green leaves of certain plants.

EXPERIMENTAL PROCEDURES

Plant materials. Specimens of the meadow buttercup (*Ranunculus acris* L.) and other plants used in this study were collected in June–September 1998 at a large number of locations outside Stockholm.

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Abbreviations and trivial names: 9-H(P)OD, 9-hydro(pero)xy-10(*E*),12(*Z*)-octadecadienoic acid; 9-H(P)OT, 9-hydro(pero)xy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 12-oxo-PDA, 12-oxo-10,15(*Z*)-phytodienoic acid; 13-H(P)OD, 13-hydro(pero)xy-9(*Z*),11(*E*)-octadecadienoic acid; 13-H(P)OT, 13-hydro(pero)xy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; colneleic acid, 9-[1′(*E*),3′(*Z*)-nonadienyloxy]-8(*E*)-nonenoic acid; colnelenic acid, 9-[1′(*E*),3′(*Z*),6′(*Z*)-nonatrienyloxy]-8(*E*)-nonenoic acid; etheroleic acid, 12-[1′(*E*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; etherolenic acid, 12-[1′(*E*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; FT-IR, Fourier transform infrared; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; GSH, glutathione (reduced form); GSH-px, glutathione peroxidase; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; SP-HPLC, straight-phase high-performance liquid chromatography; SPE, solid-phase extraction; UV, ultraviolet.

Fatty acids and fatty acid hydro(pero)xides. Linoleic and α-linolenic acids were purchased from Nu-Chek-Prep (Elysian, MN). [1-^{14}C]α-Linolenic acid (DuPont NEN, Boston, MA) was mixed with unlabeled material and purified by SiO_2 chromatography to afford a specimen having a specific radioactivity of 4.0 kBq/μmol. 9(*S*)-Hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid [9(*S*)-HPOT] and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*)-HPOT] were prepared by incubation of α-linolenic acid with tomato lipoxygenase (16) and soybean lipoxygenase (17), respectively. 9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPOD] and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD] were obtained in an analogous way from linoleic acid. 13(*R*)-HPOT was prepared by incubation of α-linolenic acid with manganese lipoxygenase followed by isolation by reversed-phase high-performance liquid chromatography (RP-HPLC) (18). Hydroxy fatty acids were prepared from the corresponding hydroperoxides by reduction with sodium borohydride in methanol. 12-Oxo-PDA and its 10,11,15,16-tetrahydro derivative were prepared as described (19).

Divinyl ether fatty acids. Colneleic and colnelenic acids were prepared by incubation of 9(*S*)-HPOD and 9(*S*)-HPOT, respectively, with the 9300 × *g* supernatant fraction of homogenate of potato tuber followed by purification by RP-HPLC (20). Etheroleic and etherolenic acids were obtained by incubations of 13(*S*)-HPOD and 13(*S*)-HPOT, respectively, with whole homogenates of bulbs of garlic (*Allium sativum* L.) followed by isolation by RP-HPLC (11,21).

Enzyme preparations. Leaves of *R. acris* were minced and homogenized at 0°C in 0.1 M potassium phosphate buffer pH 6.7 (1:5, vol/wt) using an Ultra-Turrax. The homogenate was filtered through gauze, and the filtrate (protein, 3.8 mg/mL) was centrifuged for 15 min at 1100 × *g*. Further centrifugation of the supernatant (protein, 2.7 mg/mL) for 60 min at 105,000 × *g* provided a particulate fraction and a particle-free supernatant (protein, 1.6 mg/mL). The particulate fractions obtained by centrifugations at 1100 × *g* and 105,000 × *g* were resuspended in volumes of phosphate buffer equal to those of the corresponding supernatants (protein in 1100 × *g* sediment fraction, 1.1 mg/mL; protein in 105,000 × *g* particulate fraction, 0.7 mg/mL). Most of the incubations were carried out using the 105,000 × *g* particulate fraction; however, in large-scale preparations, use was also made of the 1100 × *g* particulate fraction which contained appreciable divinyl ether synthase activity. Although fresh leaves of *R. acris* were used for most of the experiments, it was also possible to use leaves which had been shock-frozen in liquid nitrogen and subsequently stored at −80°C. Glutathione peroxidase (GSH-px) and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO).

Chemical methods. Catalytic hydrogenation, reduction with sodium borohydride, and oxidative ozonolysis were performed as described (18,22). Short-chain fragments formed upon oxidative ozonolysis were identified by gas chromatography–mass spectrometry (GC–MS) analysis. Configurational determination of hydroxy acids and 12-oxo-PDA were carried out by gas–liquid chromatography (GLC) analysis of diastereomeric (−)-menthoxycarbonyl derivatives (22,19).

Chromatographic and instrumental methods. Analytical RP–HPLC was performed with a column of Nucleosil 100-5 C_{18} (250 × 4.6 mm) purchased from Macherey-Nagel (Düren, Germany). The solvent systems used were mixtures of acetonitrile/water/acetic acid in the proportions 50:50:0.02, by vol (system A), 60:40:0.02, by vol (system B), and 70:30:0.02, by vol (system C). For straight-phase high-performance chromatography (SP–HPLC), a column of Nucleosil 50-5 (250 × 4.0 mm) and a solvent system of ethyl acetate/hexane (8:992, vol/vol) were used. The absorbance (210 nm) and radioactivity of HPLC effluents were determined on-line using a Spectromonitor III ultraviolet (UV) detector (Laboratory Data Control, Riviera Beach, FL) and a liquid scintillation counter (IN/US Systems, Tampa, FL), respectively. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. Retention times found on GLC were converted into C-values as described (23). GC-MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. UV spectra were recorded with a Hitachi (Tokyo, Japan) model U-2000 UV/VIS spectrophotometer. Fourier transform infrared (FT-IR) spectrometry was carried out using a Perkin-Elmer (Norwalk, CT) model 1650 FT-IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-EX270 instrument. Deuteriochloroform containing 0.03% tetramethylsilane was used as the solvent. Radioactivity was determined with a Packard Tri-Carb model 4450 liquid scintillation counter (Packard Instruments, Downers Grove, IL).

Incubations and treatments. [1-^{14}C]α-Linolenic acid (300 μM) or hydroperoxides (200 or 300 μM as indicated) were stirred with enzyme preparation (2–10 mL) at 23°C for 20 min. The mixtures were acidified to pH 4 and rapidly extracted with 2 vol of diethyl ether. Material obtained after evaporation of the solvent was dissolved in 3 mL of 2-propanol/chloroform (1:2, vol/vol) and subjected to solid-phase extraction (SPE) using an aminopropyl column (0.5 g; Supelco, Bellefonte, PA). After rinsing with 5 mL of the same solvent mixture, acidic components were eluted with 10 mL of diethyl ether/acetic acid (98:2, vol/vol) (*cf.* Ref. 24). The material obtained was subjected to RP-radio-HPLC using solvent systems A and C.

Preparation of compound **4**. Batches of 5–10 mg of crystalline compound **4** were prepared in the following way. Leaves of *R. acris* (24 g) were homogenized in potassium phosphate buffer (120 mL), and the fractions sedimenting at 1100 × *g* and 105,000 × *g* were collected. They were suspended and homogenized in buffer (130 and 120 mL, respectively) and separately stirred at 23°C for 30 min with unlabeled 13(*S*)-HPOT (300 μM). The incubation mixtures were

pooled, acidified to pH 4, and extracted with diethyl ether. The residue remaining after evaporation of the solvent was dissolved in 12 mL of 2-propanol/chloroform (1:2, vol/vol) and subjected to SPE (4 columns) as described above. The material obtained was dissolved in HPLC mobile phase (6 mL) and aliquots of 1 mL were subjected to semipreparative RP-HPLC using a column of Nucleosil C_{18} 100-7 (250 × 10 mm) and solvent system B at a flow rate of 5 mL/min. This procedure afforded 10–12 mg of pure Compound **4** (yield from 13(*S*)-HPOT, 45–54%). Crystallization from hexane at –25°C afforded white crystals, m.p. 49–50°C.

RESULTS

Isolation of oxidation products of α-linolenic acid. [1-^{14}C]α-Linolenic acid (300 μM) was stirred at 23°C for 20 min with a suspension (10 mL) of the 105,000 × *g* particle fraction of a homogenate of *R. acris*. Material obtained following extraction with diethyl ether and preliminary purification by SPE was subjected to RP-HPLC radiochromatography. As seen in Figure 1, a major labeled compound (compound **4**; 77% of the recovered radioactivity) as well as three minor compounds (compounds **1–3**; 4, 1, and 2%, respectively, of the recovered radioactivity) appeared. A small peak (9%) due to residual unconverted α-linolenic acid was also observed. Larger amounts of compound **4** needed for identification were prepared as described in the Experimental Procedures section. Compound **4** obtained in this way was a crystalline solid melting at 49–50°C. The methyl ester prepared by means of diazomethane was a colorless oil which solidified on cooling and remelted at 25°C. When an aliquot of compound **4** was placed on a hot plate and heated above its melting point, a distinct odor of cut apple became apparent, probably due to cleavage of the divinyl ether structure and liberation of 3-hexenal.

Identification of compound **4**. Compound **4** showed strong UV absorption with λ_{max} (EtOH) = 267 nm (ε = 41,200) (Fig. 2, Table 1). The fact that the absorption band was smooth with no sign of splitting excluded the possibility of a conjugated triene chromophore but was in agreement with, e.g., a conjugated dienone or a bathochromically shifted diene. The FT-IR spectrum (film) of the methyl ester of compound **4** is given in Figure 3. Bands were observed at 1740

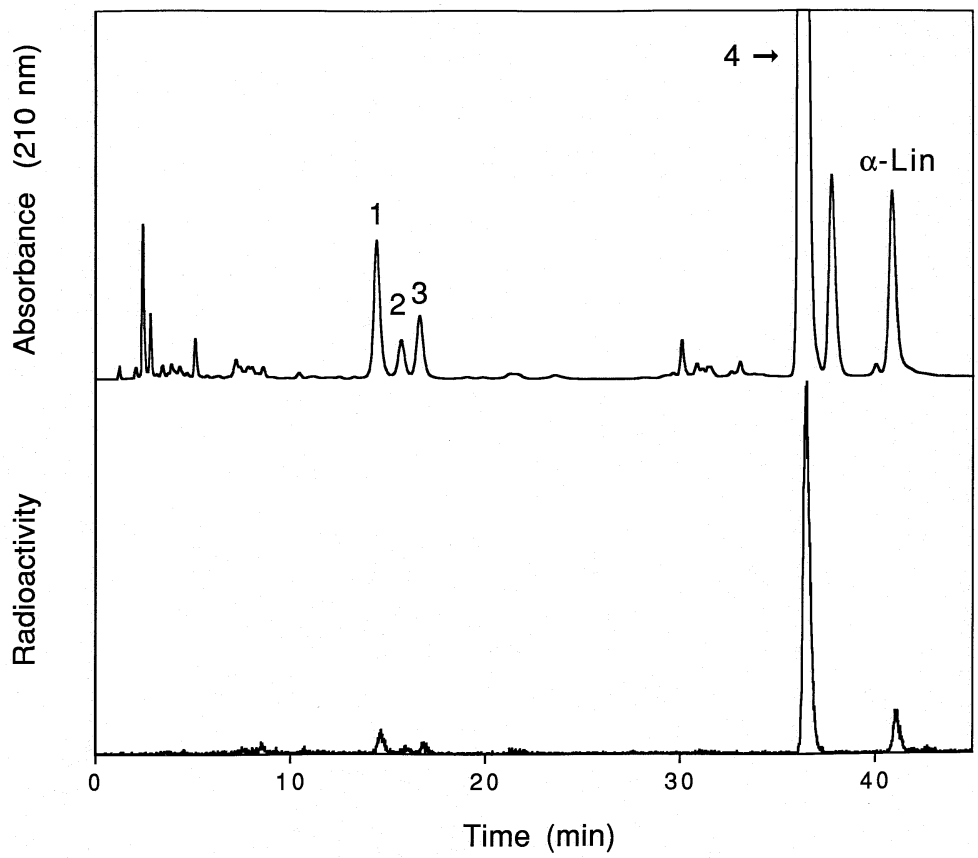


FIG. 1. Analysis by reversed-phase-radio-high-performance liquid chromatography of reaction product obtained following incubation of [1-^{14}C]α-linolenic acid (300 μM) with the 105,000 × *g* particulate fraction of homogenate of *Ranunculus acris*. The column was eluted at a flow rate of 1.5 mL/min with solvent system A (0–25 min) followed by solvent system C (25–45 min). Peak 1, compound **1**; peak 2, compound **2**; peak 3, compound **3**; peak 4, compound **4**; α-Lin, α-linolenic acid. The peak of ultraviolet absorption appearing between compound **4** and α-linolenic acid was due to 2,6-di-*tert*-butyl-4-methylphenol (BHT), an antioxidant present at a level of 10 ppm in the diethyl ether used.

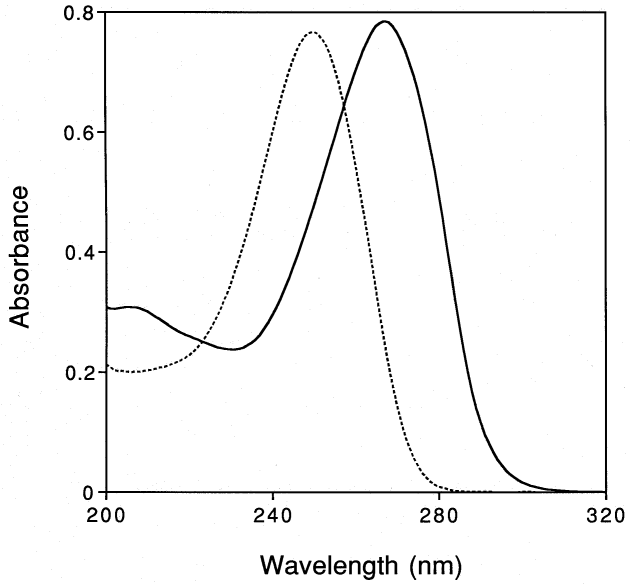


FIG. 2. Ultraviolet spectra of compound **4** (solid line) and compound **6** (dashed line). Solvent, 99.5% ethanol.

TABLE 1
Ultraviolet and Chromatographic Data of Divinyl Ethers[a]

Compound	λ_{max}[b] (nm)	Retention volume[c] (mL)	C-value[d]
Compound **6**	250	20.8	18.83
Compound **4**	267	27.2	19.74
Etheroleic acid	250	21.4	19.33
Etherolenic acid	268	25.8	19.74[e]
Colneleic acid	250	25.5	19.41
Colnelenic acid	253	30.2	19.45[e]

[a]Abbreviations/trivial names: compound **6**, 12-[1′(*Z*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; compound **4**, 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; etheroleic acid, 12-[1′(*E*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; etherolenic acid, 12-[1′(*E*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid; colneleic acid, 9-[1′(*E*),3′(*Z*)-nonadienyloxy]-8(*E*)-nonenoic acid; colnelenic acid, 9-[1′(*E*),3′(*Z*),6′(*Z*)-nonatrienyloxy]-8(*E*)-nonenoic acid.
[b]Ultraviolet spectra were recorded on the free acids dissolved in 99.5% ethanol.
[c]Straight-phase high-performance liquid chromatography was performed on the methyl esters using a column of Nucleosil 50-5 and a solvent system of ethyl acetate/hexane (8:992) at a flow rate of 1.0 mL/min. The detector was set at 250 nm.
[d]Gas–liquid chromatography was performed on the methyl esters using a methyl silicone capillary column (25 m) at 230°C.
[e]Partial degradation on the capillary column.

(ester C=O), 1646 (C=C), 1595 (C=C), 1165 (C–O–C), and 911 cm^{-1} (*trans*-substituted vinyl ether). The mass spectrum of the methyl ester of compound **4** showed prominent ions at *m/z* 306 (26%; M^+), 277 (2; $M^+ - 29$; loss of $\cdot C_2H_5$), 275 (3; $M^+ - 31$; loss of $\cdot OCH_3$), 245 [4; $M^+ - (29 + 32)$], 217 (3), 199 (3), 163 [10; $M^+ - 143$; loss of $\cdot(CH_2)_6$-$COOCH_3$], 149 (25), 131 (27), 107 (31), 81 (100), and 55 (98). Proof for the presence of four double bonds in compound **4** was provided by the mass spectrum of the derivative formed upon catalytic hydrogenation of the methyl ester of compound **4**. The molecular ion of this derivative was shifted to *m/z* 314 (0.1%) and the ion due to loss of $\cdot OCH_3$ appeared at *m/z* 283 (2%). Further ions were observed at *m/z* 229 (18%; $M^+ - 85$; loss of $\cdot(CH_2)_5$-CH_3), 214 (15, $M^+ - 100$; loss of OHC-$(CH_2)_4$-CH_3); 197 [49; $M^+ - (85 + 32)$], 143 {27; $[(CH_2)_6$-$COOCH_3]^+$}, 129 {11; $[(CH_2)_5$-$COOCH_3]^+$}, 97 {31; $[(CH_2)_4$-$CH{=}C{=}O]^+$}, 87 {59; $[(CH_2)_2$-$COOCH_3]^+$}, 74 {69; $[CH_2{=}C(OH)$-$OCH_3]^{\cdot+}$}, and 55 (100). Oxidative ozonolysis performed on the methyl ester of compound **4** produced monomethyl nonanedioate as

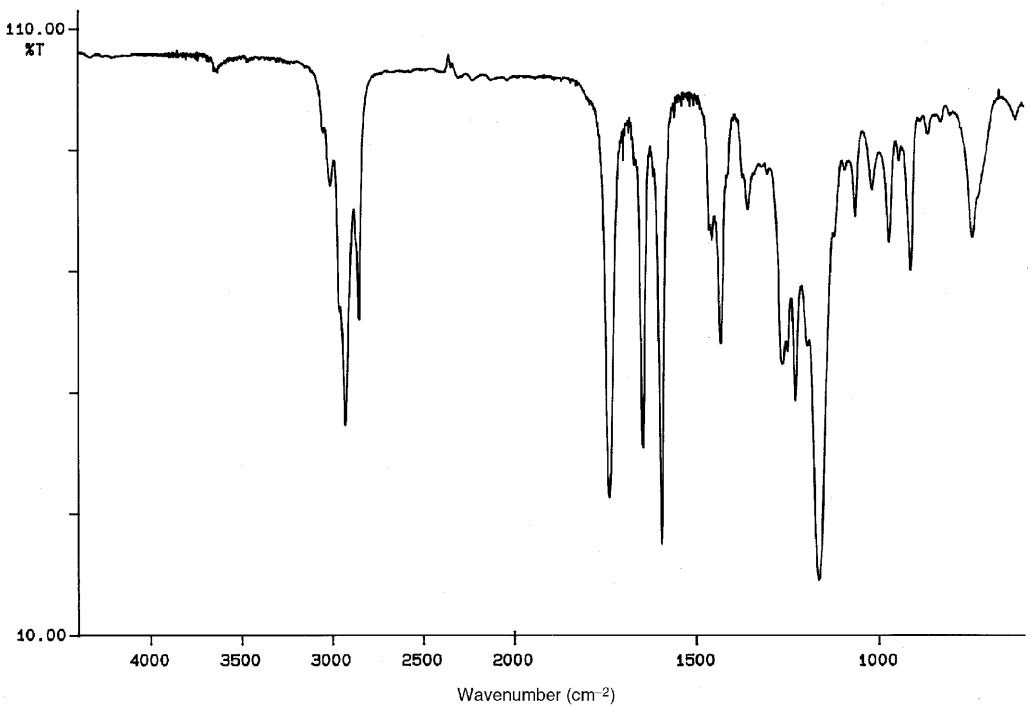
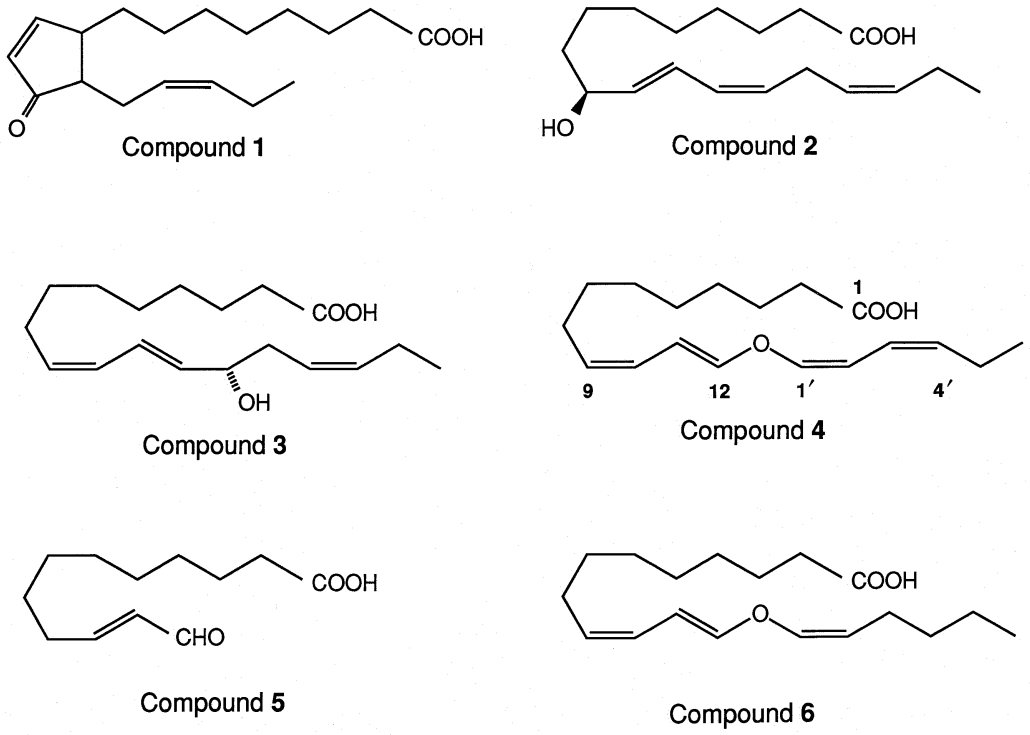


FIG. 3. Fourier transform infrared spectrum (film) of the methyl ester of compound **4**.

the major nonvolatile fragment. The data mentioned indicated that compound **4** possessed a tetraunsaturated ether structure located in a position corresponding to carbons 9–16 of the parent fatty acid. Support for the presence of such a partial structure was provided by an experiment in which compound **4** (400 μg) in acetone (7.5 mL) was treated with 20% hydrochloric acid (2.5 mL) at 23°C for 1 h (*cf.* Ref. 11). The product was subjected to RP-radio-HPLC using solvent system A. A major peak of radioactivity appeared (compound **5**; 8.3–9.1 mL effluent). Compound **5** was identified as 12-oxo-10-dodecenoic acid by its UV spectrum, which showed λ_{max} (EtOH) = 220 nm, and by its mass spectrum (methyl ester), which showed prominent ions at *m/z* 194 (5%; $M^+ - 32$; loss of CH_3OH), 166 (4), 149 (4), 123 (8), 112 (12), 98 (65), 83 (42), 74 (43), and 55 (100). Oxidative ozonolysis performed on the methyl ester of **5** produced monomethyl decanedioate, thus localizing the double bond to the Δ^{10} position. Identification of the acid hydrolysis product **5** as 12-oxo-10-dodecenoic acid confirmed the presence in compound **4** of an unsaturated ether structure with its oxygen attached to C-12 (Scheme 1). NMR spectroscopy was used to determine the geometrical configurations of the double bonds. Eight groups of signals due to the eight olefinic protons of compound **4** were observed (Fig. 4A and Table 2). Correlation of the signals with the different protons was achieved by spin-spin decoupling experiments and by using the NMR spectrum of methyl colneleate (Fig. 4C) as a reference (15,25). The signals centered at 5.30 and 5.84 ppm were due to the protons at C-9 and C-10, respectively, whereas the signals centered at 6.32 and 5.41 ppm were due to the protons at C-3′ and C-4′, respectively. As shown by the values of $J_{9,10}$ and $J_{3',4'}$ (10.6 and 11.1 Hz, respectively), both the Δ^9 and $\Delta^{3'}$ double bonds had the *Z* geometrical configuration. Earlier work on divinyl ether fatty acids (11–13,15,21,25) and on other vinyl ether compounds, such as ethyl propenyl ether (26), have shown that the coupling constants of protons across *Z* and *E* vinyl ether double bonds have values of 6–7 and 11–12 Hz, respectively. As seen in Table 2, $J_{11,12}$ determined for the signals of the vinylic protons at C-11 (6.05 ppm) and C-12 (6.58 ppm) was 11.9 Hz, thus demonstrating that the Δ^{11} double bond had the *E* configuration. In the same way, the coupling constant of the protons of the $\Delta^{1'}$ double bond (signals centered at 6.20 and 5.50 ppm) had the value 6.2 Hz. This showed that the $\Delta^{1'}$ double bond had the *Z* geometrical configuration. On the basis of the data presented, compound **4** was identified as 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid (Scheme 1). This compound had previously been isolated from *L. sinclairii*, a brown alga (12). The spectral data of compound **4** (Figs. 2–4, Table 2) were almost identical to those of the algal divinyl ether, although the value of the extinction coefficient at 267 nm found in the present work (ε 41,200) was considerably higher than that previously reported (ε 30,000) (12). Furthermore, compound **4** was structurally closely related to etherolenic acid (11), differing from this compound only with respect to the geometrical configuration of the double bond introduced in the divinyl ether synthase reaction [1′(*Z*) in compound **4**, 1′(*E*) in etherolenic acid].

Identification of 12-oxo-PDA. The UV spectrum of compound **1** showed λ_{max} (EtOH) = 220 nm (ε = 12,500), and the mass spectrum of the methyl ester showed prominent ions at



SCHEME 1

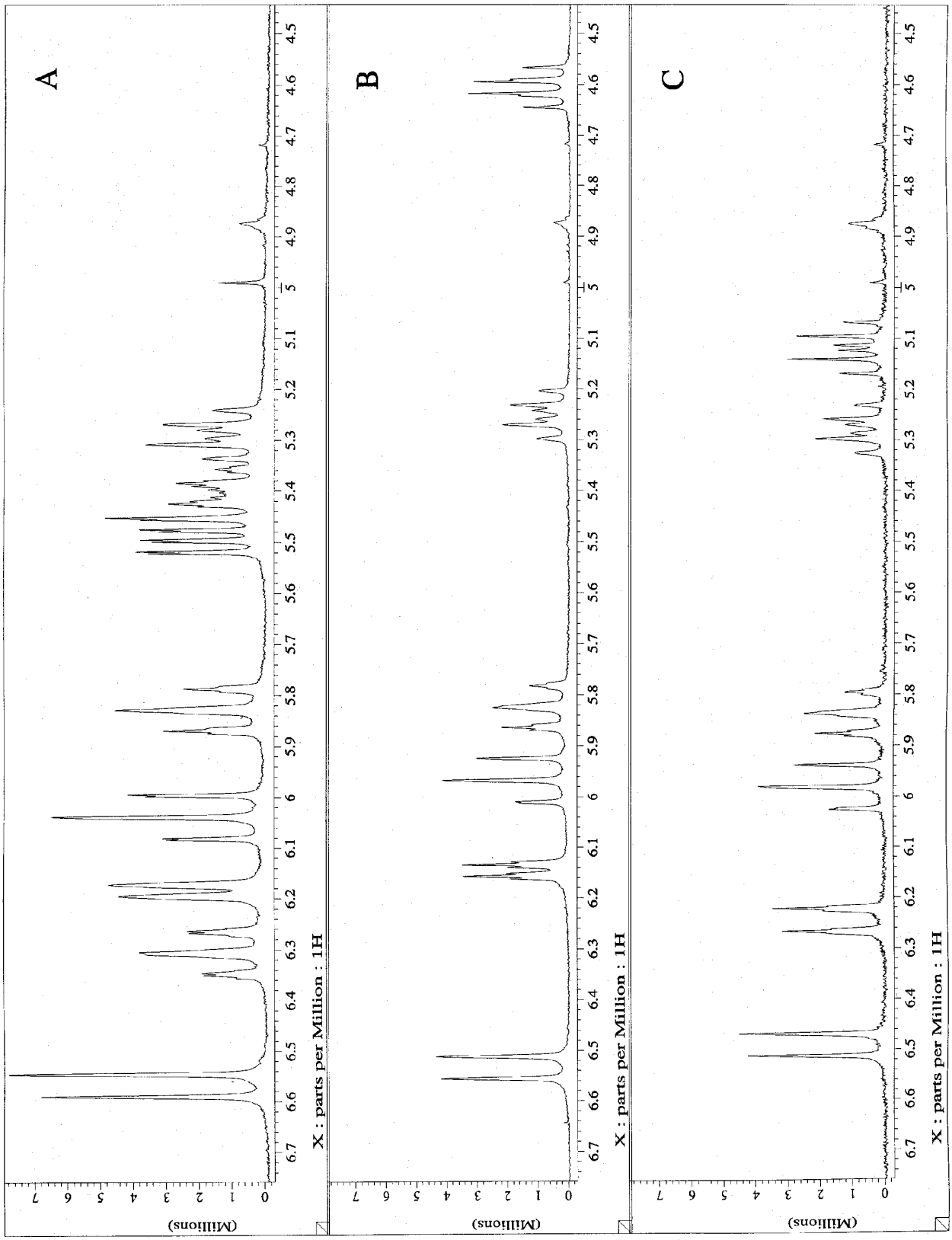


FIG. 4. Partial nuclear magnetic resonance spectra of the methyl esters of compound **4** (A), compound **6** (B), and of methyl colneleate (C). The region δ 4.5–6.7 ppm containing signals due to the olefinic protons are shown. Solvent, deuteriochloroform.

TABLE 2
Proton Nuclear Magnetic Resonance Data for the Methyl Esters of Compounds 4 and 6[a]

	Methyl ester of Compound **4**			Methyl ester of Compound **6**		
Carbon #	δ (ppm)	Multiplicity	*J* (Hz)	δ (ppm)	Multiplicity	*J* (Hz)
2	2.30	*t*	7.5	2.30	*t*	7.5
3	1.62	*m*		1.62	*m*	
4	1.31	*m*		1.31	*m*	
5	1.31	*m*		1.31	*m*	
6	1.31	*m*		1.31	*m*	
7	1.31	*m*		1.31	*m*	
8	2.13	*m*		2.11	*m*	
9	5.30	*dt*	7.4, 10.6	5.27	*dt*	7.4, 10.6
10	5.84	*m*	10.6, 11.4	5.84	*m*	10.6, 11.4
11	6.05	*ddd*	0.7, 11.4, 11.9	5.98	*dd*	11.4, 11.8
12	6.58	*d*	11.9	6.55	*d*	11.8
1′	6.20	*d*	6.2	6.16	*dt*	1.4, 6.2
2′	5.50	*ddd*	1.0, 6.2, 11.4	4.62	*dt*	6.2, 7.4
3′	6.32	*ddd*	1.0, 11.1, 11.4	2.11	*m*	
4′	5.41	*m*		1.31	*m*	
5′	2.13	*m*		1.31	*m*	
6′	1.00	*t*	7.5	0.90	*t*	6.9
OCH_3	3.66	*s*		3.67	*s*	

[a]Proton nuclear magnetic resonance spectra were recorded at 270 MHz in $CDCl_3$ with tetramethylsilane as internal chemical shift reference.

m/z 306 (15%; M^+), 275 (16; $M^+ - 31$; loss of $\cdot OCH_3$), 238 (18; $M^+ - 68$; β-cleavage with loss of C_5H_8); 206 [11; $M^+ - (68 + 32)$], 177 [19; $M^+ - 129$; loss of $\cdot(CH_2)_5$-$COOCH_3$], 163 [32; $M^+ - 143$; loss of $\cdot(CH_2)_6$-$COOCH_3$], 149 [25; $M^+ - 157$; loss of $\cdot(CH_2)_7$-$COOCH_3$], 109 (53), 107 (54), 95 (96), 82 (54), and 55 (100). These spectral data were identical to those of authentic 12-oxo-PDA. The identity of compound **1** with 12-oxo-PDA was confirmed by the findings that compound **1** and authentic 12-oxo-PDA cochromatographed on RP-HPLC (solvent system A; effluent volume, 21.6 mL) and on GLC (methyl ester; C-value, 20.11), and that catalytic hydrogenation performed on **1** produced 10,11,15,16-tetrahydro-12-oxo-PDA.

Identification of hydroxy acids. Compounds **2** and **3** were identified as 9(*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid [9(*S*)-HOT] and 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*)-HOT], respectively, by UV spectrometry, mass spectrometry (methyl ester–trimethylsilyl ether derivatives), and by their chromatographic behavior on RP-HPLC and GLC. The authentic compounds were used as references.

Incubation in the presence of GSH-px. [1-^{14}C]α-Linolenic acid (300 μM) was stirred at 23°C for 20 min with a suspension of the 105,000 × *g* particulate fraction in the presence of GSH-px (8 units/mL) and GSH (2 mM). Analysis of the product by RP-radio-HPLC showed the presence of a main peak of radioactivity, i.e., compound **3** (90%), and a minor peak due to compound **4** (2%). Although the extent of oxygenation of α-linolenic acid (92%) was close to that found in a control incubation carried out in the absence of GSH-px and GSH (93%), it was apparent that inclusion of GSH-px/GSH resulted in strong inhibition of the formation of compound **4** and a correspondingly increased formation of compound **3**.

*Incubation of 13(*S*)-HPOT*. [1-^{14}C]13(*S*)-HPOT (200 μM) was stirred with a suspension of the 105,000 × *g* particulate fraction at 23°C for 20 min, and the product was analyzed by RP-radio-HPLC. A main peak of radioactivity comigrating with compound **4** (85%) appeared. Examination of this material by UV spectroscopy and GC–MS (methyl ester) gave results identical to those found for compound **4** biosynthesized from α-linolenic acid (see above). This experiment thus showed that 13(*S*)-HPOT served as an efficient precursor of compound **4** and, together with the results of the incubation carried out in the presence of GSH-px (see above), indicated that biosynthesis of **4** from α-linolenic acid occurred by a two-step pathway involving 13(*S*)-HPOT as a free intermediate.

*Incubation of 13(*S*)-HPOD and structure of compound* **6**. Incubation of [1-^{14}C]13(*S*)-HPOD (200 μM) as described above led to the formation of a major radioactive product, i.e., compound **6** (61% of the recovered radioactivity). Peaks due to 13(*S*)-HPOD remaining nonconverted (27%) and 13(*S*)-HOD (7%) were also observed. The UV spectrum of **6** showed λ_{max} (EtOH) = 250 nm (Fig. 2, Table 1), and the mass spectrum of its methyl ester showed prominent ions at *m/z* 308 (39%; M^+), 277 (3; $M^+ - 31$; loss of $\cdot OCH_3$), 251 (4; $M^+ - 57$; loss of $\cdot(CH_2)_3$-CH_3), 219 [3; $M^+ - (57 + 32)$], 177 (16), 165 [21; $M^+ - 143$; loss of $\cdot(CH_2)_6$-$COOCH_3$], 159 (15), 135 (26), 81 (63), 67 (79), and 55 (100). Catalytic hydrogenation of **6** produced a derivative identical to that formed from **4** upon hydrogenation (see above). Oxidative ozonolysis performed on the methyl ester of **6** yielded monomethyl nonanedioate as the main product. The data mentioned indicated the presence in **6** of a triunsaturated ether partial structure located

TABLE 3
Biosynthesis of Compound 4 and 12-oxo-PDA in Green Leaves[a]

Plant	Compound **4**[b] (%)	12-Oxo-PDA[b] (%)	Plant	Compound **4** (%)	12-Oxo-PDA (%)
Urtica dioica	0	1	*Melilotus officinalis*	0	0
Stellaria media	0	5	*Vicia cracca*	0	13
Ranunculus acris	59	19	*Geranium sylvaticum*	0	0
R. repens	39	5	*Hypericum perforatum*	0	0
R. polyanthemos	73	15	*Anthriscus sylvestris*	0	30
R. peltatus	67[c]	16	*Lamium album*	0	1
R. lingua	11[c]	6	*Lycopersicum esculentum*	0	33
Aquilegia vulgaris	0	1	*Plantago major*	0	25
Chelidonium majus	0	40	*Achillea millefolium*	0	13
Bunias orientalis	0	10	*Senecio vulgaris*	0	23
Filipendula ulmaria	0	0	*Taraxacum vulgare*	0	2
F. vulgaris	0	0	*Tanacetum vulgare*	0	5
Trifolium pratense	0	7	*Cirsium arvense*	0	10

[a]Fresh leaves (3 g) were minced and homogenized in 0.1 M potassium phosphate buffer pH 6.7. Ten milliliters of the filtered homogenate were stirred at 23°C for 20 min with 300 μM [1-^{14}C]α-linolenic acid. The product obtained by extraction with diethyl ether and solid-phase extraction was subjected to reversed-phase-radio-high-performance liquid chromatography using the conditions given in the legend to Figure 1.
[b]The percentages of the recovered radioactivity associated with compound **4** and 12-oxo-PDA are given.
[c]Mixture of compound **4** and an unknown isomer as described in the text.

with its oxygen attached to C-12. NMR spectroscopy showed six groups of signals due to olefinic protons (Fig. 4B, Table 2). The protons at the Δ^9 double bonds gave rise to signals at 5.27 and 5.84 ppm. The value of $J_{9,10}$ (10.6 Hz) demonstrated that this double bond had the *Z* geometrical configuration. Signals at 5.98 and 6.55 ppm were due to the protons at the Δ^{11} double bond. The observed $J_{11,12}$ (11.8 Hz) was in agreement with a vinyl ether double bond having the *E* configuration. In the same way, the $\Delta^{1'}$ double bond was assigned the *Z* configuration based on the value of $J_{1',2'}$ (6.2 Hz) calculated from the signals at 4.62 and 6.16 ppm (protons at C-2′ and C-1′, respectively). The NMR spectrum of the methyl ester of compound **6** was closely similar to that earlier reported for the methyl ester of synthetic 8(*Z*)-colneleic acid (15). On the basis of the results described, **6** was assigned the structure 12-[1′(*Z*)-hexenyloxy]-9(*Z*),11(*E*)-dodecadienoic acid, a new member of the oxylipin family of compounds.

*Incubations of 9(*S*)-HPOD, 9(*S*)-HPOT, and 13(*R*)-HPOT*. The hydroperoxides [1-^{14}C]9(*S*)-HPOD, [1-^{14}C]9(*S*)-HPOT, and [1-^{14}C]13(*R*)-HPOT (200 μM) were stirred with the enzyme preparation, and the products were analyzed by RP-radio-HPLC. In each case, a main peak of radioactivity appeared corresponding to unchanged hydroperoxide. In addition, a small peak due to the corresponding hydroxy acid (11–13% as compared to the hydroperoxide) was also observed. Compounds having the same polarity as divinyl ether fatty acids were undetectable.

Biosynthesis of divinyl ethers in other plants. Whole homogenates of leaves of 26 plants were incubated with [1-^{14}C]α-linolenic acid, and the product profiles were determined by RP-radio-HPLC (*cf.* Fig. 1). As seen in Table 3, formation of compound **4** was observed in leaves of *R. acris*, *R. repens*, *R. polyanthemos*, *R. peltatus*, and *R. lingua* but not in any of the other 21 plants tested. Interestingly, leaves of *R. peltatus* and *R. lingua*, two aquatic plants, produced compound **4** as well as another, slightly less polar compound. This compound appeared to be an isomer of compound **4** as suggested by its UV spectrum, which showed λ_{max} (EtOH) = 267 nm, and its mass spectrum (methyl ester), which was similar to but not identical to that of the methyl ester of compound **4**. Work is in progress to determine the full structure of this isomer.

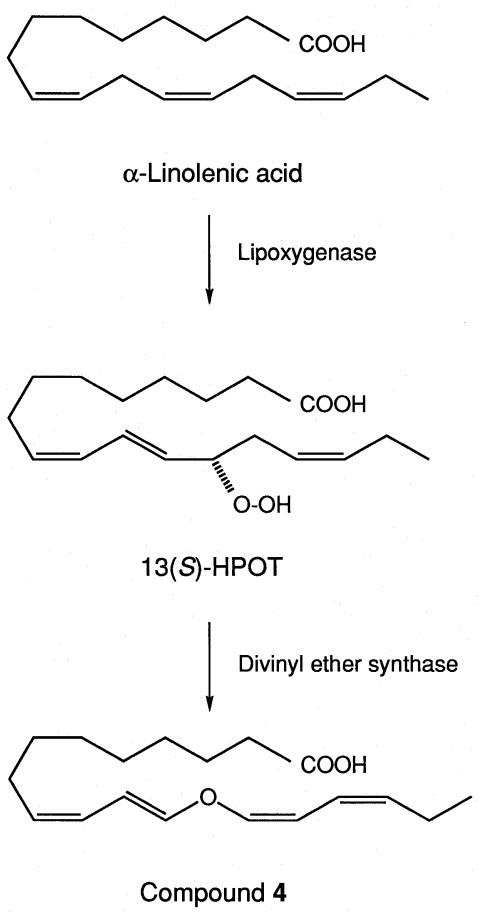
Chromatographic separation of divinyl ether fatty acids. For analytical purposes, it was of interest to examine the chromatographic separation of regio- and stereoisomeric divinyl ethers. As expected, RP-HPLC readily separated compounds **4** and **6** (effluent volumes, 34.2 and 46.2 mL, respectively, using solvent system B). On the other hand, RP-HPLC did not allow separation of compounds **4** or **6** from their corresponding 1′(*E*) isomers (etherolenic and etheroleic acids). SP-HPLC was useful for separation of the methyl esters of the three groups of C_{18} divinyl ethers fatty acids presently known. As seen in Table 1, within each group, the trienoic ester was easily separated from the corresponding tetraenoic ester. The three trienoates as well as the three tetraenoates were also separable; however, the separation between the trienoate methyl colneleate and the tetraenoate methyl etherolenate was small. Also GLC was useful for identification of the divinyl ethers, although the methyl esters of compound **4** and etherolenic acid were inseparable (Table 1). In fact, apart from separation by SP-HPLC, the only reliable way of distinguishing compound **4** and etherolenic acid was by NMR.

DISCUSSION

Compound **4** was isolated in crystalline form as a product of metabolism of α-linolenic acid in a preparation of leaves of the meadow buttercup (*R. acris* L.). By means of spectral and

chemical methods, the structure assigned to compound **4** was 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid. This compound had previously been isolated from a brown alga, *L. sinclairii* and characterized in form of its methyl ester derivative (12). Formation of compound **4** in *R. acris* occurred by a two-step process involving the hydroperoxide 13(*S*)-HPOT as an intermediate (Scheme 2). The enzymes involved, i.e., ω6-lipoxygenase and divinyl ether synthase, were both particle-bound. Linoleic acid 13(*S*)-hydroperoxide was metabolized analogously to 13(*S*)-HPOT and produced compound **6**. The transformation catalyzed by divinyl ether synthase of *R. acris* proceeded in a regio- and stereospecific way as judged by the finding that neither the 9-hydroperoxides of linoleic or α-linolenic acids nor the 13(*R*)-hydroperoxide of α-linolenic acid was convertible into divinyl ethers. As would be expected, 11(*S*)-hydroperoxy-7(*Z*),9(*E*),13(*Z*)-hexadecatrienoic acid, an ω6 hydroperoxide having the "*S*" absolute configuration, was efficiently converted by the enzyme to provide the dinor homolog of compound **4** (data not shown).

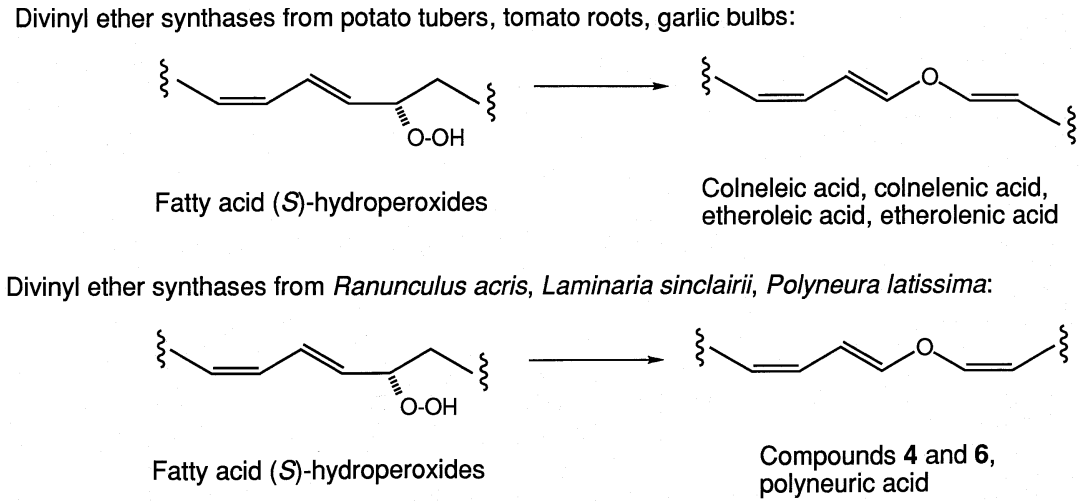
Profiles of oxylipins formed in fresh leaves were determined by incubation of [1-^{14}C]α-linolenic acid with leaf whole homogenates followed by analysis of the products by RP-radio-HPLC. The radio-HPLC method, which allowed separation of *inter alia* α- and γ-ketols, 12-oxo-PDA, and epoxyoctadecadienoates and of regioisomeric hydroxy-, hydroperoxy-, and oxooctadecatrienoates, appeared to be advantageous to the radio-thin-layer chromatography method recently described (10). By means of the RP-radio-HPLC technique, biosynthesis of compound **4** was consistently observed in leaves of plants belonging to the genus *Ranunculus*, family *Ranunculaceae* (Table 3). It was also noted that most of the plants were capable of biosynthesis of 12-oxo-PDA, thus giving support to the notion that compounds produced by the allene oxide synthase/allene oxide cyclase pathway are of general importance in plants (27,28).



SCHEME 2

In previous work, colneleic acid was isolated as a product of enzymatic dehydration of 9(*S*)-HPOD in preparations of potato tuber (9) and tomato roots (10). Isotopic studies of the conversion showed that one of the two hydroperoxide oxygens of the parent hydroperoxide was retained in the product (29), and that the *pro-R* hydrogen at C-8 of the hydroperoxide was selectively eliminated during formation of the Δ^8 double bond (20). Proposed intermediates in the conversion are an epoxide carbocation (20,29) or an oxonium ion, formed in a Bayer-Villiger-type reaction (15). Another divinyl ether, etheroleic acid, was recently obtained by incubation of 13(*S*)-HPOD with a membrane-bound divinyl ether synthase from bulbs of garlic (11,21). Etheroleic and colneleic acids may be regarded as regioisomers having identical configurations of their double bond systems, i.e., *Z,E* for the conjugated diene retained from the parent hydroperoxide, and *E* for the double bond introduced in the divinyl ether synthase reaction. Enzymatic dehydration catalyzed by extracts of potato and garlic also took place with α-linolenic acid 9- and 13-hydroperoxides, respectively. Thus, 9(*S*)-HPOT was converted into colnelenic acid by potato tuber (25), and 13(*S*)-HPOT was converted into etherolenic acid by preparations of garlic (11). These transformations appeared to proceed analogously to those of the corresponding linoleic acid hydroperoxides and involved introduction of a vinyl ether double bond having the *E* geometrical configuration (Scheme 3). Biosynthesis of compounds **6** and **4** from 13(*S*)-HPOD and 13(*S*)-HPOT, respectively, also proceeded with retention of the positions and configurations of the *Z,E*-conjugated double bonds of the parent hydroperoxides; however, in this case the newly introduced double bond ($\Delta^{1'}$) had the *Z* rather than the *E* geometrical configuration (Scheme 3). Introduction of a vinyl ether double bond having the *Z* configuration was similarly involved in the proposed biosynthesis of divinyl ethers from fatty acid hydroperoxides in marine algae (12,13).

Divinyl ether fatty acids emerge as a growing and interesting family of compounds produced by enzymatic dehydration of fatty acid hydroperoxides. The present study demonstrates that divinyl ethers can be produced in green leaves of certain higher plants. This makes it of interest to explore whether divinyl ethers, like jasmonic acid and other oxylipins of the allene oxide synthase–allene oxide cyclase pathway, are involved as mediators in defense reactions against pathogens or in other physiological or pathological processes in plants.



SCHEME 3

ACKNOWLEDGMENTS

Mrs. G. Hamberg is thanked for expert technical assistance and for collection and identification of the plant materials used. The author is greatly indebted to Prof. Per-Erik Jansson and Mr. Reine Eserstam (Clinical Research Center, Huddinge University Hospital, Stockholm) for their help with the NMR analyses. This work was supported by the Swedish Medical Research Council (Proj. No. 03X-5170) and by Vesical Co. (Stockholm).

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[Received September 23, 1998, and in final revised form October 20, 1998; revision accepted October 22, 1998]

Acid-Catalyzed Isomerization of Fucosterol and Δ^5-Avenasterol

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ABSTRACT: This work shows that fucosterol, Δ^5-avenasterol, and similar ethylidene-side chain sterols can undergo acid-catalyzed isomerization to give a mixture of five isomers. Four isomers formed from fucosterol were analyzed, using gas chromatography–mass spectrometry, and were characterized as Δ^5-avenasterol, two $\Delta^{5,23}$-stigmastadienols, and $\Delta^{5,24(25)}$-stigmastadienol. When the unsaponifiables fraction from oat oil was subjected to acid hydrolysis, the two $\Delta^{5,23}$-stigmastadienol isomers and $\Delta^{5,24(25)}$-stigmastadienol were detected while fucosterol coeluted with sitosterol. Interisomerization of ethylidene-side chain sterols represents a limitation to the use of the acid hydrolysis method in the determination of sterols in food and other plant materials rich in these sterols, e.g., oat lipids.
Lipids 33, 1073–1077 (1998).

Currently, the analysis of phytosterol content in foods is of interest because of their cholesterol-lowering properties (1). Plant sterols can be found in four forms: free sterols, sterol esters of fatty or phenolic acids, sterylglycosides, and acylated sterylglycosides (2,3). The major dietary sources for phytosterols are vegetable oils (4) and cereals (2). Although methodology for the analysis of sterols in oils is well established (5), methods for the analysis of sterols in cereals are yet to be developed and validated (6).

Campesterol, stigmasterol, sitosterol, and Δ^5-avenasterol (Table 1) are the most widespread phytosterols in plant foods. Analysis of the free and esterified forms of these sterols is possible by gas chromatography (GC) or by high-performance liquid chromatography after alkaline hydrolysis of whole foods or their lipid extracts (7), whereas analysis of these sterols in glycosylated forms requires hydrolysis of the glycosidic bonds (6,7). Furthermore, sterols may also be bound, together with other lipids, to the carbohydrate matrix of the cereal which makes them unavailable for straight extraction with lipophilic solvents. Thus, hydrolysis may also be needed to release starch-bound lipids including sterols (8). Jonker *et al.* (9) recommended the use of acid hydrolysis for the breakdown of the glycosidic bonds, but acid hydrolysis was previously reported to decompose labile sterol glycosides, namely Δ^5- and Δ^7-avenasterolglycosides in oats (7).

During our work on the analysis of oat sterols, we found that the conditions of strong acid and high temperature caused a marked decrease in the amounts of Δ^5-avenasterol and the emergence of an unknown peak (Määtä, K., Lampi, A., Petterson, J., Fogelfors, B.M., Piironen, V., and Kamal-Eldin, A., submitted for publication). In this paper, we investigate the effect of acid hydrolysis on fucosterol and other commercially available sterols (i.e., campesterol, stigmasterol, and sitosterol) (Table 1). We also study the nature of the degradation products resulting from fucosterol and compare them to those generated from Δ^5-avenasterol present in oat kernel unsaponifiables.

EXPERIMENTAL PROCEDURES

Materials. Lipids were extracted from oat kernels in a Soxhlet apparatus using *n*-hexane. Reference sterols were obtained from the following sources: sitosterol (Research Plus Inc., Bayonne and Denville, NJ), campesterol (C-5157), stigmasterol (S-2424), and fucosterol (F-5379) from Sigma Chemical Co. (St. Louis, MO). Cholestane (C-8003, Sigma) was used as a reference standard in GC analyses. Tri-Sil®, the reagent used to derivatize the sterols to trimethylsilyl ethers (TMS ethers), was purchased from Pierce Chemical Co. (Rockford, IL). All other solvents and reagents used were of analytical grade (E. Merck, Darmstadt, Germany) and were used without further purification.

Preparation of the sterol samples. Oat lipids were saponified by reaction with saturated aqueous KOH/ethanol (1:8, vol/vol) at 85°C for 10 min, and the unsaponifiables were extracted with cyclohexane and deionized water. The unsaponifiables obtained from oat lipids and different sterol standards (*viz.,* fucosterol, sitosterol, campesterol, and stigmasterol) were

TABLE 1
Trivial and Systematic Names of Sterols Mentioned in the Text

Trivial name	Systematic name
Campesterol	Δ^5-24α-methyl-cholesten-3β-ol
Stigmasterol	$\Delta^{5,E\text{-}22}$-24α-ethyl-cholestadien-3β-ol
Sitosterol	Δ^5-24α-ethyl-cholesten-3β-ol
Fucosterol	Δ^5-24*Z*-ethylidene-cholestadien-3β-ol
Δ^5-Avenasterol	Δ^5-24*E*-ethylidene-cholestadien-3β-ol
Δ^7-Avenasterol	Δ^7-24*E*-ethylidene-cholestadien-3β-ol

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; RRT, relative retention time; TMS, trimethylsilyl.

analyzed for their sterol contents by GC with and without acid hydrolysis. Cholestane was added as a reference standard in amounts approximately equal to those of the sterol in the samples. Acid hydrolysis was performed by a modification of the AOAC method (8) using 6 M HCl for 30 min at 80°C, and sterols were then extracted with cyclohexane/diethylether (1:1, vol/vol) (Toivo, J., unpublished). Most of the solvents were evaporated at 40°C by a rotary evaporator, and sterols were dissolved in chloroform, transferred to glass-stoppered tubes, and stored at –20°C until further analysis.

Derivatization. TMS ether derivatives of the sterols were prepared by adding 100–120 μL of the Tri-Sil reagent to sterols (50–200 μg) in glass-stoppered tubes. After shaking to dissolve the sterols in the reagent, the tubes were heated at 60°C for 45 min. Excess reagent was then removed under nitrogen, and the residue was dissolved in hexane.

GC and GC–mass spectrometry (MS). Analysis of sterol TMS derivatives was performed using a DB-5ms fused-silica capillary column (5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d. and 0.25 μm film thickness; J&W Scientific, Folsom, CA) fitted in an HP 5890 II series gas chromatograph (Hewlett-Packard, Avondale, PA) fitted with a flame-ionization detector, a splitless injector, and an autosampler (7673; Hewlett-Packard). The separation was performed under the following temperature program: 70°C (1 min), 25°C/min, 275°C (21 min). The injector and detector temperatures were 250 and 330°C, respectively, and the helium (carrier gas) was used at an inlet pressure of 18 psi. Peaks were recorded and peak areas were computed using a GyncoSoft version 5.3 software (Gynkotek GmbH, Germering, Germany).

GC–MS was performed on an HP 5890 series II gas chromatograph coupled to a TRIO-1000 mass spectrometer with a LAB-BASE™ data system (version R2.10; Fisons Instruments, VG MASSLAB, Manchester, United Kingdom). The separation was performed on the same column as above under the following temperature program: 70°C (1 min), 25°C/min, 245°C (1 min), 3°C/min, 275°C (20 min). Helium was used as a carrier gas at an inlet pressure of 12 psi. Electron ionization (EI) mass spectra (*m/z* 100–500) were obtained at a scan rate of 10 scans/min, a 70 eV ionization energy, 280°C interface temperature, and 200°C ion source temperature.

RESULTS AND DISCUSSION

Kesselmeier *et al.* (7) showed that Δ^5- and Δ^7-avenasterols present in oat lipids are destroyed by acid hydrolysis. Figure 1 presents gas chromatograms of the unsaponifiable fractions of oat lipids analyzed, in this study, with and without acid hydrolysis. Samples analyzed before acid hydrolyis showed peaks for campesterol, stigmasterol, sitosterol, Δ^5-avenasterol, and Δ^7-avenasterol. The chromatogram of samples analyzed after acid hydrolysis showed a significant reduction in the level of Δ^5-avenasterol accompanied by the emergence of a number of unknown peaks. Tisconia and Bertini (10) previously reported that a remarkable change in the contents of Δ^5-avenasterol occurred in olive oil during bleaching and that the effect was magnified by increasing the level of bleaching earth. Touche *et al.* (11) made similar observations for bleached coconut oil, where about 50% of Δ^5-avenasterol was destroyed. In both studies, the decrease in Δ^5-avenasterol content was accompanied by the appearance of some unidentified components, one of which was then identified as its geometrical isomer, fucosterol.

The unknown peaks in acid-hydrolyzed oat unsaponifiables (Fig. 1) had retention times and mass spectra similar to the isomerization products of fucosterol (Fig. 2). The two sterols eluting in the double peak before sitosterol in Figures 1 and 2 [isomers a and b, relative retention times (RRT) 1.70 and 1.71, respectively] as well as the last isomer (RRT = 1.85) had the same retention times and mass spectra as those resulting from fucosterol (*vide infra*). However, fucosterol coeluted with sitosterol (as judged by injection of standards) under the chromatographic conditions used. The presence of fucosterol in the acid-hydrolyzed oat unsaponifiables was confirmed by the detection of the ions at *m/z* 296 and *m/z* 386 in the mass spectrum of a mixed sitosterol/fucosterol peak (RRT = 1.75) and their absence in the unhydrolyzed oat unsaponifiables (Fig. 3).

Commercially available campesterol, stigmasterol, and sitosterol were subjected to acid hydrolysis and analyzed by GC–MS. Results showed that acid hydrolysis had no effect on these sterols since chromatograms of untreated and acid-treated sterols were similar (data not shown). Owing to the lack of a commercial standard of Δ^5-avenasterol, its geometrical isomer fucosterol was used for acid hydrolysis. The GC–MS chromatogram of the TMS derivatives of acid-treated fucosterol (Fig. 2) showed that fucosterol was partially transformed into Δ^5-avenasterol and three other peaks. Examination of the mass spectra of all five peaks (Fig. 4) revealed that all peaks have similar molecular masses (484 for TMS derivatives), suggesting that they are all isomers. Acid-catalyzed isomerization of fucosterol can be expected to affect only the side chain double bond since it is generally known that the sterol Δ^5 double bond is insensitive to acids, in contrast to Δ^7 double bonds which isomerize to Δ^8- under acid conditions (Parish, E.J., personal communication). The mass spectra of fucosterol (RRT = 1.75, relative to cholestane which elutes at 16.7 min) and Δ^5-avenasterol (RRT = 1.79) are nearly identical and are similar to the mass spectrum of Δ^5-avenasterol presented elsewhere (Määtä, K., Lampi, A., Petterson, J. Fogelfors, B.M., Piironen, V., and Kamal-Eldin, A., submitted for publication). The characteristic ions in these spectra are the *m/z* 386 due to McLafferty-type cleavage of the C22-C23 bond together with a one-hydrogen transfer from C-20 (12) and the ion at *m/z* 296 (386 – trimethylsilanol). The spectrum of the last isomer (RRT = 1.85) is similar to those of fucosterol and Δ^5-avenasterol and may be attributed to $\Delta^{5,24(25)}$-stigmastadienol (13). The mass spectra of the two compounds eluting in the double peak (RRT 1.70 and 1.71) just before sitosterol (RRT = 1.76) are nearly identical and suggest a $\Delta^{5,23}$ structure based on the assignment of the characteristic peak at *m/z* 283 to an allylic cleavage of the C20-C22 bond coupled with loss of the silanol group (12,14). The assignment of a $\Delta^{5,23}$ to these compounds may

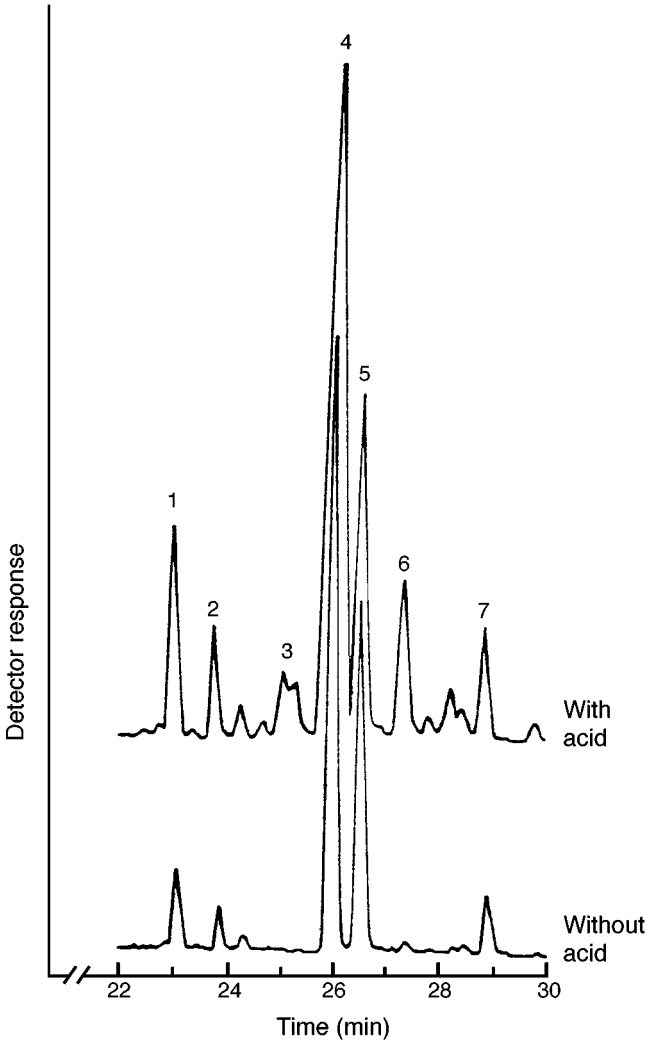

FIG. 1. Gas chromatography–mass spectrometry chromatogram of sterol extracts obtained from oat lipids with and without acid hydrolysis. Peaks: (1) campesterol, (2) stigmasterol, (3) unknowns, (4) sitosterol + fucosterol (with acid) and only sitosterol (without acid), (5) Δ^5-avenasterol, (6) unknown sterol having similar mass spectrum as Δ^5-avenasterol, and (7) Δ^7-avenasterol.

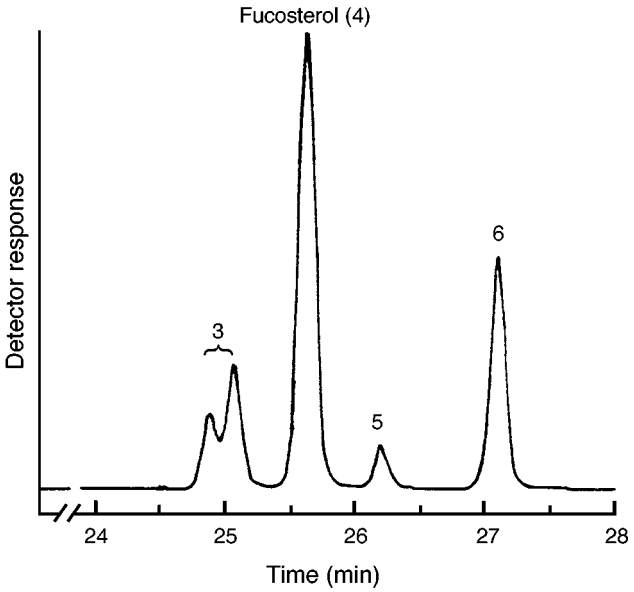

FIG. 2. Gas chromatography–mass spectrometry chromatogram showing acid-catalyzed isomerization products of fucosterol. Peaks: (3) sterols (a) and (b), (4) fucosterol, (5) Δ^5-avenasterol, and (6) sterol (c) or $\Delta^{5,24(25)}$-stigmastadienol. For identifications of (a), (b), and (c) see Figure 4.

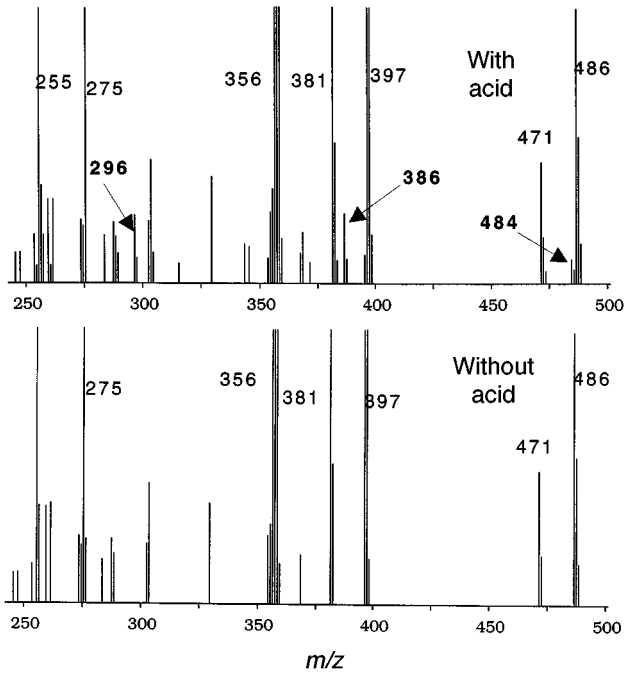

FIG. 3. Magnified part (*m/z* 250–500) of the mass spectra of peak 4 in the sterol extract from oat lipids with and without acid hydrolysis (see Figure 1). Peaks at *m/z* 296, 386, and 484 (upper panel) are typical for fucosterol and their intensities can be compared to the peak at *m/z* 471 (M-15, relative abundance to an *m/z* 129 base peak = 3.4%), one of the typical peaks for sitosterol

be argued since this structure has been reported not to occur naturally. This structure is supported by knowledge related to carbonium ion stabilities and by mass fragmentation patterns. Two other structural possibilities, *viz.* $\Delta^{25(27)}$ and $\Delta^{28(29)}$, were ruled out since an ion at *m/z* 290 (due to allylic cleavage at the C23-C24 bond together with loss of the silanol group) was absent in their mass spectra.

The isomerization of fucosterol, and probably other ethylidene-side chain sterols, can be explained by considering the stability of the carbonium ion resulting from reactions of acids with alkenes. It is known that the stability of carbonium ions determines the relative reactivities of different alkenes toward acids. The side chain double bond in these sterols is labile owing to the possibility of formation of a stable tertiary carbonium ion, whereas the double bond in stigmasterol is stable toward acid hydrolysis possibly because of a relatively

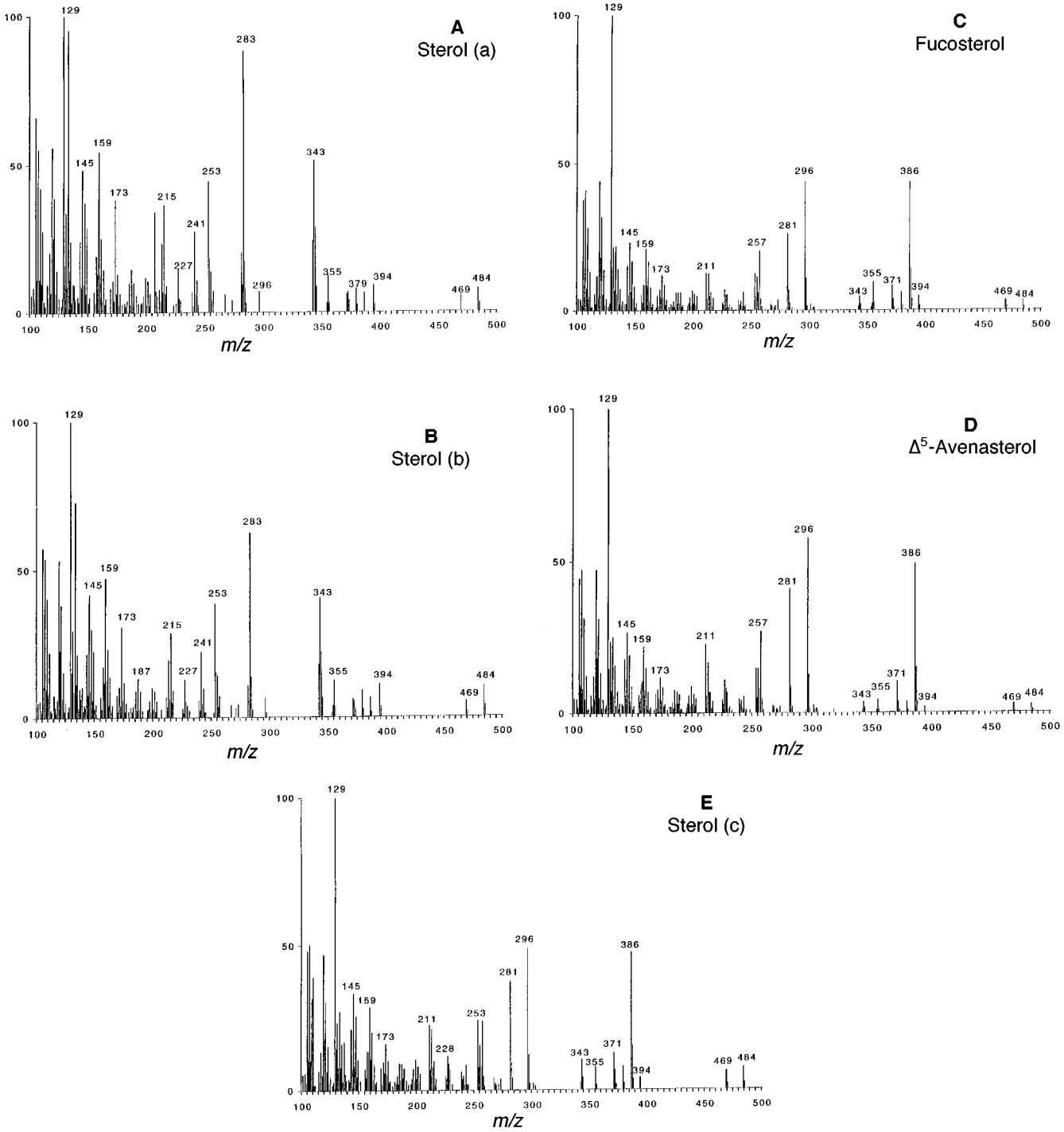

FIG. 4. Mass spectra of the isomerization products of fucosterol. See chromatogram in Figure 2 for peak positions.

lower stability of the secondary carbonium ion resulting in that case. The stability of the carbonium ions determines not only the reactivity of double bonds toward acids but also the type of products resulting from the return of the carbonium ion to an alkene *via* the loss of the acidic proton. After the carbonium ion is formed, it can lose a proton to produce a number of possible isomeric alkenes, the more highly substituted alkenes being the most preferable products (15). Application of this basic notation to the fucosterol/Δ^5-avenasterol case (Fig. 5) explains the findings obtained in this study.

The isomerization of Δ^5-avenasterol and other ethylidene-side chain sterols needs to be considered when analyzing sterol levels in foods derived from plants and other plant products. Fortunately, these sterols are abundant only in a few foods (e.g., oats) which restricts the analytical problem only to those foods. Ways to overcome this problem, including the

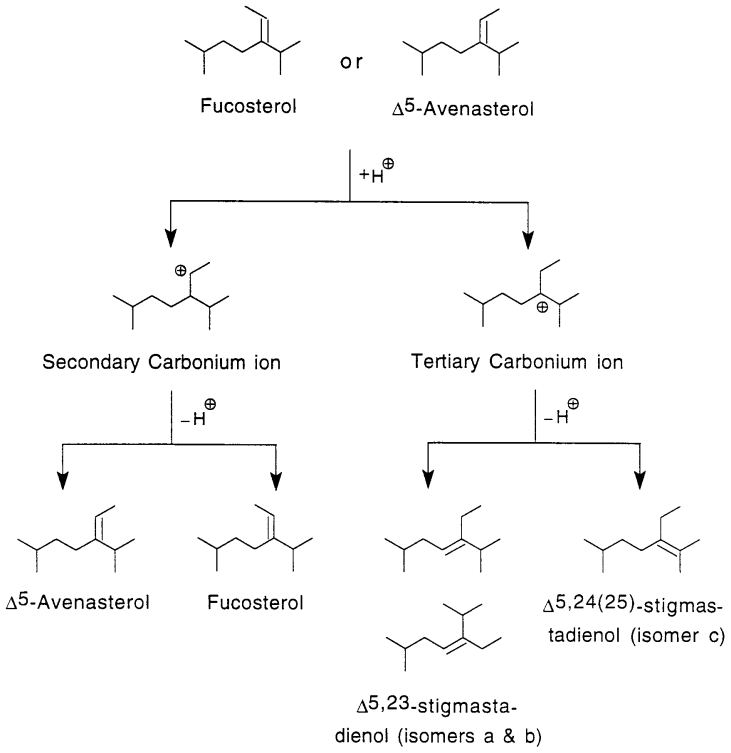

FIG. 5. Representation of the hypothetical acid-catalyzed isomerization of fucosterol and Δ^5-avenasterol.

enzymatic hydrolysis suggested by Kesselmeier *et al.* (7) need to be investigated, and alternative or complementary analytical methods need to be developed and validated.

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[Received June 2, 1998, and in final revised form October 19, 1998; revision accepted October 20, 1998]

Surface Pressure-Dependent Cross-Modulation of Sphingomyelinase and Phospholipase A_2 in Monolayers

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ABSTRACT: We investigated the ways in which phospholipase A_2 and sphingomyelinase are mutually modulated at lipid interfaces. The activity of one enzyme is affected by its own reaction products and by substrates and products of the other enzyme; all this depends differently on the lateral surface pressure. Ceramide inhibits both the sphingomyelinase activity rate and the extent of degradation, and decreases the lag time at all surface pressures. Dilauroyl- and dipalmitoylphosphatidylcholine, the substrates of phospholipase A_2 (PLA_2), do not affect sphingomyelinase activity. The products of PLA_2, palmitic acid and lysopalmitoylphosphatidylcholine, strongly enhance and shift to high surface pressures the activity optimum and the cut-off point of sphingomyelinase. Palmitic acid also shifts to high surface pressures the cut-off point of PLA_2 activity. Sphingomyelin strongly inhibits PLA_2 at surface pressures above 5 mN/m, while ceramide shifts the cut-off point and the activity optimum to high surface pressures. The sphingolipids increase the lag time of PLA_2 at low surface pressures. Both phosphohydrolytic pathways involve different levels of control on precatalytic steps and on the rate of activity that appear independent on specific alterations of molecular packing and surface potential. The mutual lipid-mediated interfacial modulation between both phosphohydrolytic pathways indicates that phospholipid degradation may be self-amplified or dampened depending on subtle changes of surface pressure and composition.
Lipids 33, 1079–1087 (1998).

Lipid substrates organized in well-controlled simple model membrane systems have been invaluable for unraveling on a molecular basis the generic surface determinants of lipolytic activity in relation to the physical properties of the lipid substrate. Lipid monolayers in particular, the model on which the very concept of a lipid bilayer as the basic constitutive structure of biomembranes was derived, clearly demonstrated that the activity of several phospholipases from different sources is markedly affected by both the long-range physical state and the fine intermolecular organization of the lipid interface (1,2). The unique possibility of lipid monolayers maintaining a control and precise knowledge of several surface parameters in real time during a catalytic reaction showed that, besides the influence of structural defects and curvature (3–5), the lateral surface pressure and electrostatic interfacial potential (6,7) can markedly affect the activity of phospholipase A_2 (PLA_2), phosphatidylinositol-specific and nonspecific phospholipase C, and sphingomyelinase (Sphmase) (8–14). Despite the fact that phosphohydrolytic enzymes constitute a very heterogeneous group of proteins, all activities studied to date have revealed a profound dependence on a few fundamental supramolecular surface parameters of the lipid interface on which they act (1–9,13–15). This is extremely important because it means that biophysical studies in model systems, usually requiring relatively large amounts of well-purified enzymes and lipids, can provide valuable information using readily available secretory or bacterial phosphohydrolytic enzymes when the aim is, as in the present work, to understand the general basic physicochemical parameters regulating these activities.

On the other hand, several lipid mediators and biomodulators of membrane function can either be common intermediates or participate indirectly as parent or derived compounds that relate different signaling pathways (16–19). Several of these lipids cause profound local and long-range effects on the molecular organization and overall topology of the membrane interface (5,20) that can markedly affect lipolytic activity (2,5,10,21). Nonsubstrate lipids such as gangliosides, neutral glycosphingolipids, ceramide (Cer), sphingosine, and semisynthetic derivatives of sphingosine in mixed phospholipid monolayers, unilamellar vesicles, and micelles can modulate the activity of PLA_2 and phospholipase C (2,7,21–26). Recent studies indicate that lipid mediators such as diacylglycerols and Cer, formed by *Bacillus cereus* phospholipase C and Sphmase degradation of bilayer vesicles, affect markedly and in different manners the membrane topology, stability, and susceptibility to bilayer recombination (27,28).

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Abbreviations: Cer, ceramide (*N*-stearoylsphingosine); Cer*P*Cho, brain sphingomyelin; dlPC, (didodecanoyl-*sn*-glycero-3-phosphocholine); dpPC, (dihexadecanoyl-*sn*-glycero-3-phosphocholine); llPC, lysolauroylphosphatidylcholine, (1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine); lpPC, lysopalmitoylphosphatidylcholine, (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine); PLA_2, porcine pancreatic phospholipase A_2 (E.C. 3.1.1.4); Sphmase, sphingomyelinase, *Bacillus cereus* sphingomyelin phosphodiesterase (E.C. 3.1.4.12).

In previous work we showed that it is possible to follow the degradation of brain sphingomyelin (Cer*P*Cho) to Cer by *B. cereus* Sphmase using lipid monolayers to precisely control the intermolecular organization of the interface while the catalytic reaction is taking place (10). An independent study with a bacterial Sphmase from *Staphylococcus aureus* (11) published almost simultaneously to ours also showed that this is a valid technique. These results are in general agreement regarding the use of the technique, the catalytic rate and presence of lag time, while the surface pressure dependence of the activity of the two bacterial Sphmases reveals some differences (10,11).

We showed that the activities of *B. cereus* Sphmase and porcine pancreatic PLA_2 can be mutually modulated by the presence of their respective substrates and products (10). The importance of this finding resides mostly in that it clearly indicated under well-controlled molecular organization and in real time that the rate of phospholipid degradation by one enzymatic pathway contains information that can be sensed by, transduced to, and markedly modulate the catalytic rate of another pathway with which no common intermediates are shared (10). In continuation of our studies and with the objectives of understanding the generic biophysical determinants of the mutual modulation of both enzymes under well-controlled molecular conditions, within the context given at the beginning of this section, we investigated the possible dependence of the lipid-mediated changes of enzymatic activities on the lateral surface pressure and on whether these could be correlated to alterations of the lipid organization according to film composition and surface pressure. The results obtained provide evidence, at least from this simplified model system, which supports the concept of a surface-regulated interfacial "cross-talk" between Sphmase- and PLA_2-catalyzed pathways which selectively depends on which lipid intermediate of either pathway is present. In addition, the mutual modulation is controlled dramatically by the lateral surface pressure and in different manners depending on the surface composition; the lipids participating in both pathways affect differently the rate of activity, the lag time, or the extent of the catalytic reactions mediated by Sphmase and PLA_2, but the effects are independent of molecular packing expansion–condensation, or alterations of the local electrostatic dipole potential caused by specific lipid–lipid interactions.

EXPERIMENTAL PROCEDURES

Porcine pancreatic PLA_2 and *B. cereus* Sphmase were from Sigma-Aldrich, Inc. (St. Louis, MO). Didodecanoyl-*sn*-glycero-3-phosphocholine (dlPC), dihexadecanoyl-*sn*-glycero-3-phosphocholine (dpPC), palmitic acid, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lpPC), Cer*P*Cho, and Cer were from Avanti Polar Lipids (Alabaster, AL). Organic solvents and chemicals were of the highest purity available, inorganic salts were roasted at 400°C, and water was double-distilled in an all-glass apparatus. Absence of surface-active impurities in aqueous solution and spreading solvents was controlled as described (28,29). Details of the equipment used were given elsewhere (30): a specially designed thermostated circular Teflon-coated trough with two adjacent compartments separated from each other by shallow and narrow slits. Monolayers of pure or mixed lipids (containing, unless otherwise stated, 10 mol% of a nonsubstrate lipid) were prepared as described previously at the desired surface pressure (10), which was kept constant by an automatic surface barostat (31). An aliquot of a concentrated enzyme solution was injected into the subphase (final concentration for Sphmase and PLA_2 was 83 and 105 ng protein/mL buffer, respectively) of the reaction compartment (18 mL, 17 cm^2 of surface area) under continuous stirring with a miniature Teflon-coated rod. The adjacent compartment served as a monolayer reservoir that automatically supplied lipid to the surface of the reaction compartment in order to maintain constant the surface pressure during the enzymatic reaction. The time course of the reaction was followed at 25 ± 1°C by the decrease of surface area as a function of time (10). The rates of enzymatic activities reported in the ordinate of the figures and in Table 1 are calculated as the number of lipid molecules degraded per minute per unit of surface area as reported elsewhere (1,7,10,11,22–25) and correspond to the slope of the linear portion of the time-course curves (Fig. 1) obtained in duplicate or triplicate for the different systems studied. The lag time (1) is taken by extrapolation of the linear part of the curve of phospholipid degradation as a function of time

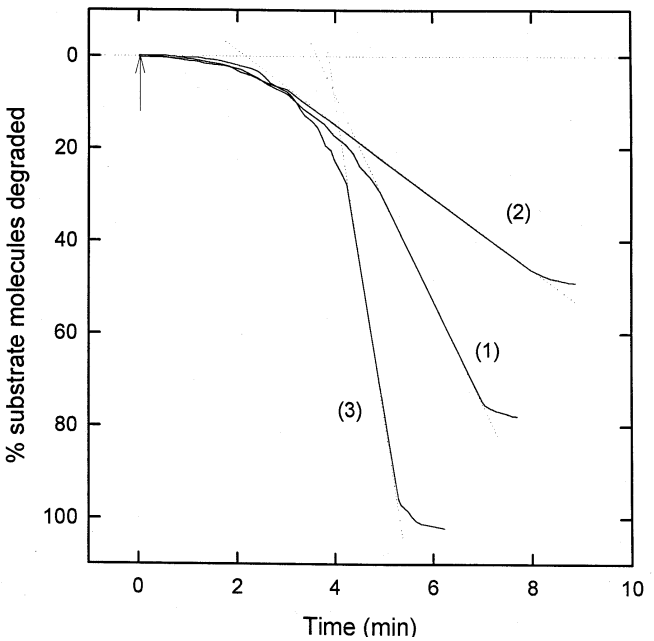

FIG. 1. Sphingomyelinase, *Bacillus cereus* sphingomyelin phosphodiesterase (E.C. 3.1.4.12) (Sphmase) activity against mixed lipid monolayers. Representative real time experiments of the degradation by Sphmase of a lipid monolayer of pure brain sphingomyelin (Cer*P*Cho) at 15 mN/m (curve 1), Cer*P*Cho/ceramide (Cer) (9:1) at 15 mN/m (curve 2), and Cer*P*Cho/palm (9:1) at 20 mN/m (curve 3) are shown. The arrow represents injection of 83 ng/mL Sphmase (final concentration) in the subphase.

TABLE 1
Comparison Between the Deviation from Ideality of the Molecular Parameters and Enzymatic Activities and Lag Times in Mixed Monolayers[a]

		10 mN/m				20 mN/m			
Enzyme	Lipid mixture (9:1)	Percentage deviation of mean molecular area	Percentage deviation of surface potential/ molecule	Enzymatic activity (mol/min·cm^2) $\times 10^{-13}$ ± SEM	Lag time (min)	Percentage deviation of mean molecular area	Percentage deviation of surface potential/ molecule	Enzymatic activity (mol/min·cm^2) $\times 10^{-13}$ ± SEM	Lag time (min)
PLA_2	Pure dlPC	0	0	2.39 ± 0.01	2.0 ± 0.4	0	0	0[b]	—[b]
	dlPC/Cer*P*Cho	3.6	4.9	1.01 ± 0.15	2.5 ± 0.3	7.3	8.5	0[b]	—[b]
	dlPC/Cer	8.5	13.8	2.05 ± 0.15	5.8 ± 0.2	11.8	15.5	2.12 ± 0.82	9.4 ± 2.7
	dlPC/palm	2.3	–0.7	1.99 ± 0.11	3.8 ± 0.6	3.6	0.7	0[b]	—[b]
Sphmase	Pure Cer*P*Cho	0	0	1.82 ± 0.02	4.9 ± 0.9	0	0	0.71 ± 0.02	16.3 ± 1.6
	Cer*P*Cho/Cer	3.3	–10.8	1.14 ± 0.22	1.9 ± 0.5	4.9	–3.4	0.38 ± 0.18	1.5 ± 0.4
	Cer*P*Cho/palm	–8.3	–19.7	1.01 ± 0.09	2.2 ± 0.2	–5.9	–12.8	6.06 ± 1.41	3.3 ± 0.4
	Cer*P*Cho/lpPC	–7.1	–30.7	1.69 ± 0.05	2.8 ± 0.1	–7.6	–27.6	7.97 ± 0.24	4.6 ± 0.7
	Cer*P*Cho/dlPC	3.2	–29.8	1.78 ± 0.01	3.5 ± 0.4	6.1	–23.7	1.44 ± 0.20	2.9 ± 0.2

[a]The table shows the percentage deviation from the ideal behavior of the mean molecular area and surface potential/molecule and the porcine pancreatic phospholipase A_2 (PLA_2) or sphingomyelinase, *Bacillus cereus* sphingomyelin phosphodiesterase (E.C. 3.1.4.12) (Sphmase) activities and lag times in mixed monolayers of didodecanoyl-*sn*-glycerol-3-phosphocholine (dlPC) or brain sphingomyelin (Cer*P*Cho) with 10 mol% of the nonsubstrate lipid indicated at 10 and 20 mN/m. Similar lack of correlation of molecular packing or surface potential/molecule with the rate of enzyme activity or lag times was found at other surface pressures; Cer, ceramide; lpPC, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine.
[b]These mixed lipid monolayers at 20 mN/m are above the surface pressure cut-off point for PLA_2.

(Fig. 1) to the abscissa at zero substrate degradation (10). The number of molecules is obtained from the known area per molecule at each surface pressure, using the respective lipid surface pressure–area compression isotherm for each mixture (data not shown). The extent of degradation is expressed as the percentage of substrate molecules hydrolyzed at the end of the reaction (Fig. 1), with respect to those initially present on the surface of the reaction compartment. The halting of the reaction occurs at different times for the different mixtures and surface pressures employed, and the final extent of degradation is independent of the reaction rate.

For PLA_2 we used dlPC as substrate (7,22). Its degradation produces lysolauroylphosphatidylcholine (llPC) and lauric acid, both of which are immediately desorbed from the monolayer into the subphase, resulting in a true zero-order kinetic regime when pure substrate is continuously supplied from the monolayer reservoir (1,22, 23). In the case of Sphmase, the hydrolysis of Cer*P*Cho produces Cer which remains in the monolayer. Because of the marked difference between the molecular area of Cer*P*Cho and Cer, the catalytic activity can be measured by the decrease of monolayer area at a specified surface pressure (10,11). The portion of linear decrease of area as a function of time represents a pseudo zero-order kinetics (10,22,23). After this, the velocity of the enzymatic reaction gradually decreases to zero due to the increased mole fraction of product remaining at the interface.

RESULTS

In order to properly determine the rate of degradation of Cer*P*Cho and dlPC by Sphmase or PLA_2, respectively, in lipid monolayers containing 10 mol% of other lipids, it is necessary to know the possible alteration of the molecular packing areas due to lipid–lipid interactions. To this end, we carried out surface pressure– and surface potential–area isotherms at 25 ± 1°C for mixed films of Cer*P*Cho with 10 mol% of Cer, dlPC, lpPC and palmitic acid, as well as of dlPC with 10 mol% of palmitic acid, lpPC, Cer, and Cer*P*Cho. Table 1 shows the percentage deviations from the ideal mixing behavior for the mean molecular area and mean surface potential per unit of molecular surface density of these films [usually denominated surface potential/molecule (30)] at two different representative surface pressures together with the rate of activity of Sphmase or PLA_2 and the lag time, at the indicated film composition and surface pressure. The deviations in mean molecular area were taken into account for the calculation of the number of molecules of Cer*P*Cho or dlPC degraded by the respective enzymes acting on each mixed film (22). All the films studied showed small deviations of the mean molecular area, while relatively low negative deviations of the surface potential per molecule were observed in some of the films. In any case, in spite of similar or different behavior regarding the alteration of molecular parameters, the activation or inhibition of the rate of enzymatic activities, or variation of the lag times, showed no consistent correlation (i.e., always increases or decreases enzyme activity or lag times for molecular expansions or condensations, or depolarizations or hyperpolarizations) with changes of intermolecular packing or alterations of the surface electrostatic potential per molecule across the interface (Table 1).

Activity of Sphmase against mixed monolayers: *(i) Effect of Cer.* At all surface pressures studied, and in all the films, after a time that depends on the surface pressure and on the film composition, the reaction reaches a constant rate of degradation (pseudo zero-order kinetic regime); after this it decreases

gradually to zero (see Fig. 1) owing to the increasing proportion of product remaining at the interface (10). The existence of the linear pseudo zero-order regime indicates that the enzymatic catalysis is proceeding in temporary steady state in the film due to the initial substrate excess with respect to the enzyme at the interface. The rate of activity is altered by the presence of Cer, the product of the Sphmase reaction. The inhibition exerted by the initial presence of Cer that was described before at 15 mN/m (10) was also found over the whole range of surface pressures studied (Fig. 2A). The inhibition is less marked at surface pressures beyond 18 mN/m largely because the very Sphmase activity against a pure film of Cer*P*Cho decreases dramatically at 18 mN/m and above (10). In films containing Cer, the lag time consistently remains slightly shorter than that found against pure Cer*P*Cho (between surface pressures of 5 and 20 mN/m), and it does not show the dramatic increase above 18 mN/m (Fig. 2B). Cer decreases the extent of degradation of Cer*P*Cho by Sphmase over the whole range of surface pressure studied (Fig. 2C). The initial presence of product stops the reaction at a lower ratio of Cer/Cer*P*Cho than that necessary to end the reaction against a pure film of Cer*P*Cho. A defined threshold of relative proportions for the action of Cer appears to be present. Similar results were obtained in films containing 10 or 5 mol% of Cer; with proportions of 2.5 mol% or less, Cer induces no alteration of the lag time or the rate of activity compared to a film of pure Cer*P*-Cho, an effect of Cer exerted directly at the interface and not on the enzyme in the bulk subphase (10).

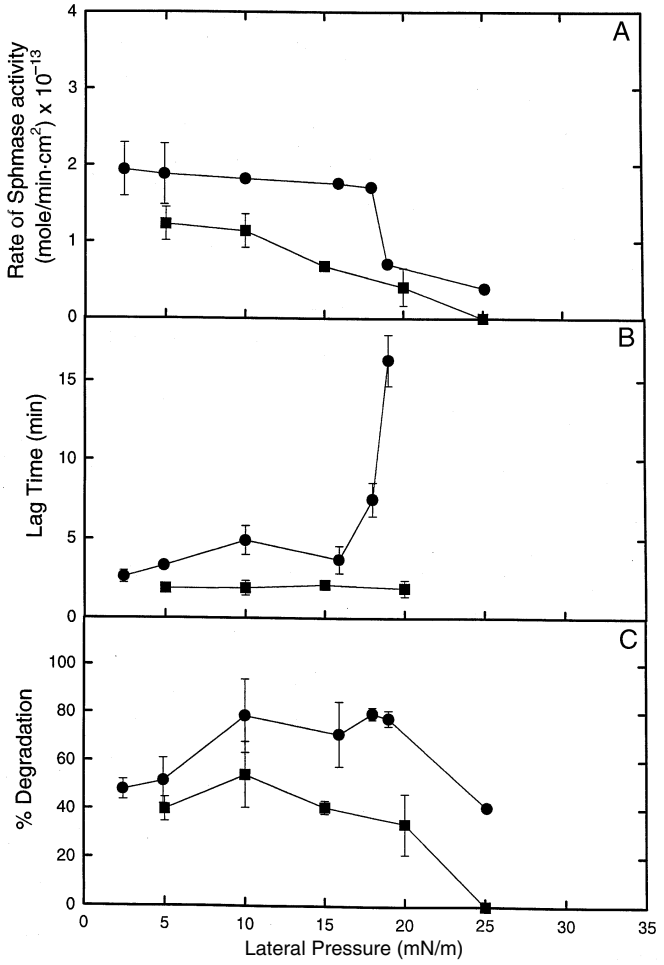

FIG. 2. Surface pressure dependence of sphingomyelinase activity against monolayers containing Cer. The rate of sphingomyelinase activity (A), lag time (B), and percentage substrate degradation (C) against monolayers of pure Cer*P*Cho (●) and Cer*P*Cho/Cer (9:1) (■) are shown at different constant surface pressures. The results are average values ± SEM; where no error bars can be seen these are within the size of the point. See Figure 1 for abbreviations.

(ii) Effect of substrates and products of PLA$_2$. We previously reported that dlPC or dpPC, substrates of PLA$_2$, had no significant influence on the Sphmase activity at 15 mN/m when present at a mole fraction of 0.1 in mixed monolayers with Cer*P*Cho (10). Figure 3 shows that this is generally the case also at all the other surface pressures studied. For simplicity, only the results for dpPC are shown in Figure 3 since the effects of dlPC on Sphmase activity were generally similar. Experiments using mixed films of Cer*P*Cho with the products of degradation of dlPC cannot be done because the short-chain products lauric acid and llPC desorb immediately from the interface into the subphase. Because of this and since, different from dlPC, the products of degradation of dpPC, namely palmitic acid and lpPC, remain at the interface where we performed the experiments with the latter lipids.

Figure 3A shows that both palmitic acid and lpPC have a similar influence on the pattern of surface pressure dependence of Sphmase activity, compared to that found for pure Cer*P*Cho at surface pressures below 15 mN/m (although the dependence on pressure is similar, palmitic acid is actually inhibitory of the activity by about 50–70% over the range 5–15 mN/m while lpPC has no significant effect). At 18 mN/m and above, either palmitic acid or lpPC strongly enhances the hydrolysis of Cer*P*Cho by Sphmase exhibiting a peak of activation at 20 mN/m. Also, either lipid markedly shifts to higher values the cut-off surface pressure point for activity. As mentioned before (10), an equimolar mixture of lpPC and palmitic acid has no significant effect on the activity of Sphmase over the whole range of surface pressure studied (data not shown).

The lag times for the Sphmase reaction against the mixed films with palmitic acid or lpPC are similar over the range of surface pressures studied (Fig. 3B). Again, both products of the reaction catalyzed by PLA$_2$ abolished the lag-time increase of the Sphmase reaction observed in films of pure Cer*P*Cho above 18 mN/m. This is similar to the effect of Cer, but this lipid inhibits the Sphmase activity while the products derived from the PLA$_2$ hydrolysis activate Sphmase. The extent of degradation of Cer*P*Cho in films containing lpPC or palmitic acid reaches about 80–100% over the surface pressure range where the enhanced activation in these films is observed (beyond 18 mN/m).

Activity of PLA$_2$ against mixed monolayers: *(i) Effect of palmitic acid and lpPC.* The variation of the PLA$_2$ activity with the surface pressure is similar to that reported previously

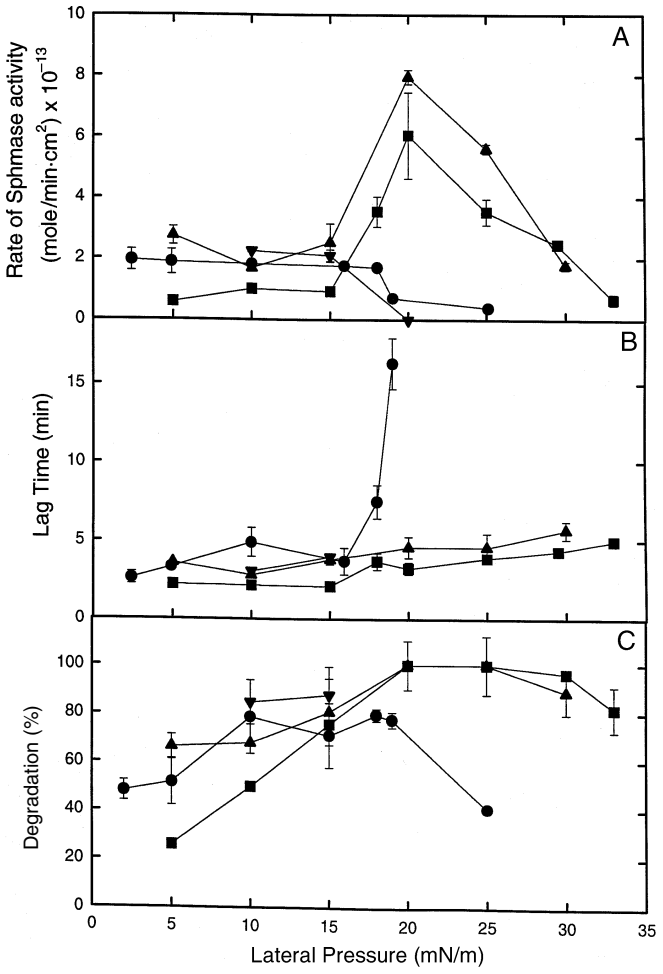

FIG. 3. Surface pressure dependence of sphingomyelinase activity against monolayers containing substrates and product of porcine pancreatic phospholipase A_2 (E.C. 3.1.1.4) (PLA_2). The rate of sphingomyelinase activity (A), lag time (B) and percentage substrate degradation (C) against monolayers of pure Cer*P*Cho (●), Cer*P*Cho/palmitic acid (9:1) (■), Cer*P*Cho/1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lpPC) (9:1) (▲), and Cer*P*Cho/dihexadecanoyl-*sn*-glycerol-3-phosphocholine (9:1) (▼) are shown at different constant surface pressures. The results are average values ± SEM; where no error bars can be seen these are within the size of the point. See Figure 1 for other abbreviation.

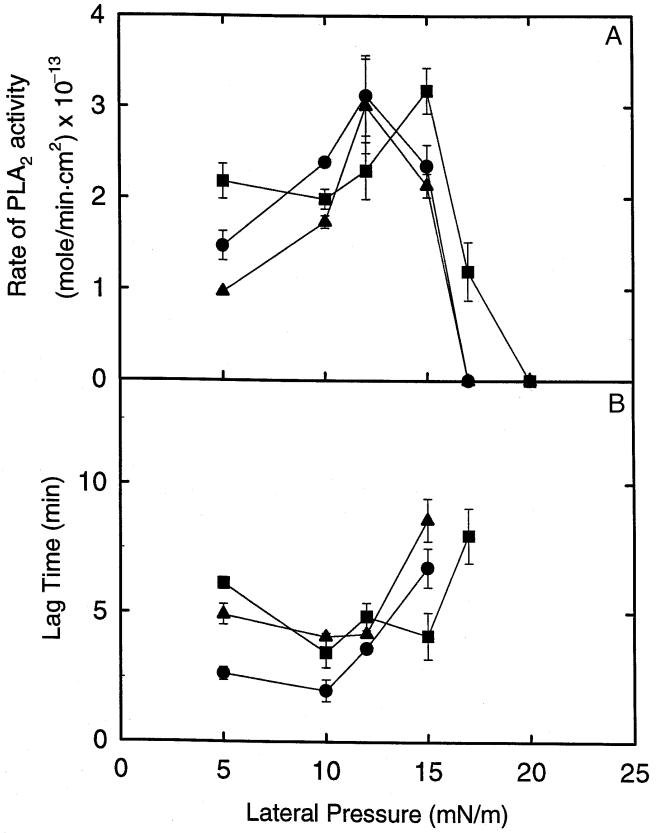

FIG. 4. Surface pressure dependence of PLA_2 activity against monolayers containing its own reaction products. The rate of PLA_2 activity (A) and lag time (B) against monolayers of pure didodecanoyl-*sn*-glycero-3-phosphocholine (dlPC) (●), dlPC/palmitic acid (9:1) (■), and dlPC/lpPC (9:1) (▲) are shown at different constant surface pressures. The results are average values ± SEM; where no error bars can be seen these are within the size of the point. See Figure 3 for other abbreviations.

(23,24). The pattern of variation is not altered significantly by the presence of 10 mol% of lpPC in the mixed film with dlPC (Fig. 4), except for a small inhibition at surface pressures below 12 mN/m. In mixed monolayers with palmitic acid, the optimal surface pressure for activity is slightly shifted from 12 to 15 mN/m, and the enzyme exhibits enhanced hydrolytic activity at high surface pressures; also, the cut-off pressure point for activity shifts from 17 to 20 mN/m (Fig. 4A). The pattern of dependence of the reaction lag time on the surface pressure (Fig. 4B) is generally similar; the lag times tend to be increased in films containing palmitic acid or lpPC at surface pressures below 12 mN/m, and the changes are not significant above this pressure. The products of hydrolysis of dlPC by PLA_2 immediately desorb from the interface, and the reaction in all film exhibits zero-order kinetics (22–24); thus, in all cases the degradation can reach 100% and comparisons of the extent of substrate degradation for different films are not possible.

(ii) Effect of substrate and product of Sphmase. The presence of Cer*P*Cho or Cer at 10 mol% in mixed films with dlPC induces marked changes in the surface pressure dependence of the PLA_2 activity (Fig. 5A). Cer*P*Cho markedly inhibits the PLA_2 activity except at the lowest surface pressure used in our study (5 mN/m). On the other hand, the presence of Cer induces slight decreases of PLA_2 activity at surface pressures below 15 mN/m but shifts the cut-off pressure point for activity well above 20 mN/m. The lag times in the mixed films with Cer*P*Cho are not significantly different except at 5 mN/m where the latency period is more than twice the value observed in the pure film of dlPC. The presence of Cer induces longer lag times at surface pressures below 12 mN/m and shifts the marked increase of the lag time to above 15 mN/m (Fig. 5B).

DISCUSSION

As stated in the introductory section, our objectives were to investigate, under well-controlled conditions of intermolecu-

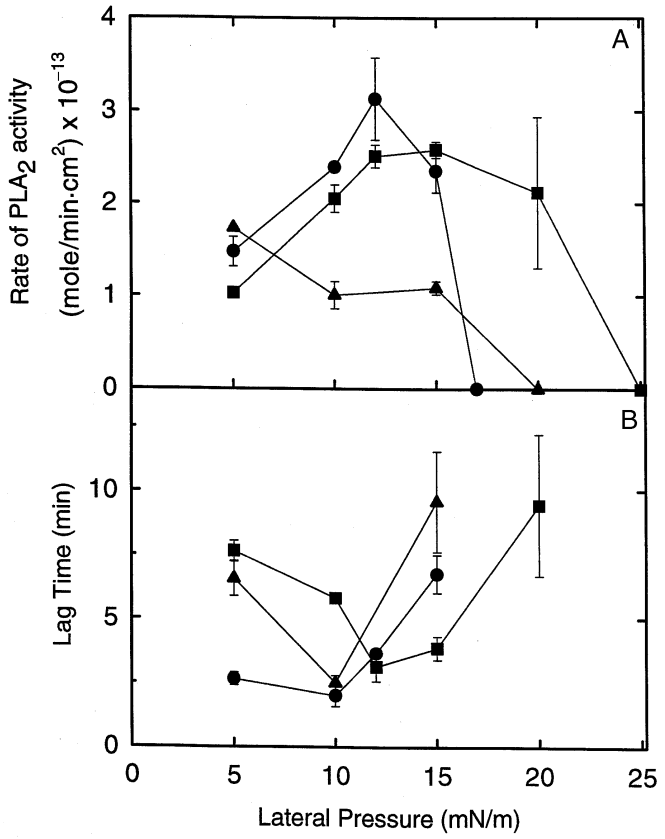

FIG. 5. Surface pressure dependence of PLA_2 activity against monolayers containing the substrate and product of Sphmase. The rates of PLA_2 activity (A) and lag time (B) against monolayers of pure dlPC (●), dlPC/Cer (9:1) (■), and dlPC/Cer*P*Cho (9:1) (▲) are shown at different constant surface pressures. The results are average values ± SEM; where no error bars can be seen these are within the size of the point. See Figures 1 and 4 for abbreviations.

lar organization in real time, the generic physicochemical factors that may modulate the surface activity of two enzymes catalyzing different phosphohydrolytic pathways as mediated by their noncommon lipid substrates and products. Two well-characterized enzymes were used, namely porcine pancreatic PLA_2 and *B. cereus* Sphmase. The reason for this choice, apart from the fact that both enzymes are readily available, was that published information already exists on several aspects of the interfacial regulation by different lipids of porcine pancreatic PLA_2 (1–3,6,7,22–24,32–34) and Sphmase (10,11,27,28). This is an invaluable advantage for further exploring the new aspects described in this work on the complex factors regulating phosphohydrolytic surface catalysis. Moreover, all phosphohydrolytic enzymes studied so far in model systems have revealed very similar generic interfacial factors for their surface regulation (1–9,12–15). Obviously, the specific critical values of the regulatory surface parameters (i.e., cut-off and optimal surface pressure points, composition-dependent lateral microheterogeneity, electrostatic surface potential) may certainly be expected to vary for different enzymes, including the physiologically relevant mammalian enzymes such as Group II or V secretory PLA_s, the various forms of membrane or cytosolic PLA_2 (35) that might work in conjunction with membrane Sphmase. Except for both brain phosphatidylinositol phosphodiesterase (8) and more recently for phosphoinositide-specific phospholipase C (12,13) and phospholipase Cβ (14) for which surface pressure-dependent activity was shown, biophysical studies have not yet been performed with most membrane phospholipases.

Our results show that the generic interfacial molecular factors regulating the cross-modulation of Sphmase and PLA_2 by their respective substrates and products are complex and appear to act at several independent levels. The effects of the different lipids are all exerted at the interface itself and are not the consequence of a direct action on the enzymes in the bulk subphase. As described before (10), the enzymatic activities against monolayers of the corresponding pure substrates are unaltered if the enzymes are injected into the subphase after preincubation for 10 min with the lipids studied in molar ratio 10:1 with respect to the enzyme (but still keeping a subphase lipid concentration below their respective critical micellar concentration values in order to prevent increases of surface pressure due to lipid adsorption).

The activity of one enzyme is affected by the presence of its own reaction products and by the substrate or products of the other enzyme; all this depends differently on the lateral surface pressure. The effects on the enzymatic rates of activity and on the lag times are not directly correlated to alterations of mean molecular area and average surface potential/molecule induced on each substrate by specific interactions with the lipids belonging to either hydrolytic pathways (Table 1). Sphmase from *S. aureus* degrades unsaturated (liquid-expanded) Cer*P*Cho substrates with a higher rate of activity than saturated (liquid-condensed) Cer*P*Cho (11). Our results on the surface pressure dependence of the activity of *B. cereus* Sphmase are in general agreement with these observations and also indicate that the rate of activity against monolayers of pure Cer*P*Cho decreases dramatically at 18 mN/m and above, with a marked increase of the lag time. This surface pressure approximately coincides with the surface pressure at which Cer*P*Cho monolayers undergo a liquid-expanded to a liquid-condensed two-dimensional phase transition. The lipids belonging to the hydrolytic pathway catalyzed by PLA_2, at 10 mol% in mixed films with Cer*P*Cho, induce very little alteration of the mean molecular area of Cer*P*Cho (deviations from the ideal mixing behavior, see Table 1). Furthermore, the isotherms of the mixtures are only slightly displaced along the axis of area/molecule, but their compressibility, and thus the surface-phase state, is not altered (not shown). However, the presence of these lipids can markedly activate or inhibit Sphmase (depending on surface pressure) as well as modify the lag time of the reaction. This indicates that an increase of Sphmase activity or a shortening of the reaction lag time is not occurring merely as a consequence of the lipid interface simply acquiring a more liquid-expanded character. The modulation appears to act at least at two different levels affecting independently the rate of catalysis and

the lag time of the reaction [which involves enzyme adsorption and precatalytic steps (2,23,24)]. This suggests that the lipids studied in this work influence the phosphohydrolytic reactions at the level of interfacial enzyme activation and/or the catalytic reaction itself, either specifically or simultaneously, rather than at the level of intermolecular packing or dipolar properties of the substrates (at least in mixed films with the proportions of lipids used). Film-transfer experiments with PLA_2 (2,10,23,25) and with Sphmase (10; Fanani, M.L., and Maggio, B., unpublished data) showed that phospholipid degradation starts immediately (without lag time) in films that were transferred at constant surface pressure over an enzyme-free subphase, indicating that the precatalytic steps of adsorption and interfacial activation of Sphmase and PLA_2 (2,10,23–25) proceed separately from those required for effective catalysis.

The expected correlation between lower rate of activity and increased lag time or vice versa (2,7,32,33) observed for both enzymes acting against monolayers of their pure substrates is lacking in the mixed films studied. This is similar to what we found previously for the modulation by gangliosides of PLA_2 and *B. cereus* phospholipase C activities (7). For Sphmase, Cer inhibits the rate of activity but exhibits shorter lag times and abolishes the increase of lag time at high surface pressures (Fig. 2) that is observed against monolayers of pure Cer*P*Cho, without extending the cut-off pressure point for activity. Analysis of the precise step at which nonsubstrate lipids affect interfacial catalysis is of paramount importance but very complex due to the multiple molecular and supramolecular levels of regulation of membrane-associated enzymes (1–9,36,37). In work related to phosphohydrolytic inhibition by nonsubstrate lipids, it has recently been proposed that the observed Cer*P*Cho inhibition of phospholipase Cδ1 activity may be due to a tightening and dehydration of the lipid interface region resulting in poor penetration of the enzyme (38). However, Cer (having the same hydrogen-bonding donor–acceptor interfacial amide group as Cer*P*Cho) induces intermolecular expansion rather than condensation (Table 1) which should have facilitated enzyme activity in mixed films, contrary to the effects observed. Experiments undertaken in our laboratory using labeled ^{125}I-Sphmase are showing that films containing Cer induce the adsorption of 3.8 ± 0.1 ng protein/cm^2 instead of the 2.1 ± 0.2 ng protein/cm^2 adsorbed to a pure film of Cer*P*Cho. The dual and apparently opposite effects of Cer on the rate of catalytic activity and on the lag time are probably due to a facilitation by Cer of enzyme adsorption to the interface, together with the induction of decreased specific enzymatic activity at all surface pressures studied. This and the marked decrease of the lag time in mixed films with Cer (Fig. 2) indicate that this lipid favors, as yet in unknown molecular terms, the precatalytic steps of the Sphmase reaction but subsequently inhibits the effective rate of hydrolysis. No significant effect on Sphmase activity and on the lag time was induced by dlPC or dpPC. On the other hand, contrary to Cer, lpPC and palmitic acid activate the enzyme at high surface pressures and, similar to Cer, abolish the increase of lag time above 18 mN/m (Fig. 3). This suggests that the products of dpPC hydrolysis by PLA_2, lpPC, and palmitic acid favor both the precatalytic steps and the rate of hydrolysis of Cer*P*Cho by Sphmase at high pressure by mechanisms independent of the changes of intermolecular packing or the mean surface potential per molecule in mixed monolayers (Table 1).

The products of the PLA_2 reaction have only a moderate effect on PLA_2 activity except for films containing palmitic acid in which a slight displacement to high surface pressures of the cut-off point for activity is found (Fig. 4). It has been known for a long time that a small amount of fatty acid products enhances the activity of PLA_2 (33,34). The presence of Cer*P*Cho in the film with dlPC generally inhibits (except at 5 mN/m) the activity of PLA_2 (Fig. 5), but the lag times at the surface pressure where the largest inhibition is observed are not altered, indirectly suggesting little alteration (or complex compensation) of overall precatalytic steps. On the other hand, compared to films of pure dpPC, the presence of Cer increases the PLA_2 activity above 15 mN/m but the corresponding lag times are longer, even compared to those found in films containing Cer below 15 mN/m and vice versa. Thus, also in the case of the effects on PLA_2, Cer exhibits a dual effect and appears to act on precatalytic steps in a way that is not simply correlated to its subsequent effect on the rate of enzymatic activity.

Direct biological correlations of our results are not yet possible, but they open possibilities for generic implications of some of the biophysical factors required for the kinetic regulation and interfacial cross-modulation of phosphohydrolytic pathways by nonsubstrate and nonproduct lipid intermediates. Figure 6 summarizes in a simplified sketch the general combined effects observed in monolayers (as controlled by low or high surface pressures) illustrating the possible mutual "communication" of both pathways if they were evolving si-

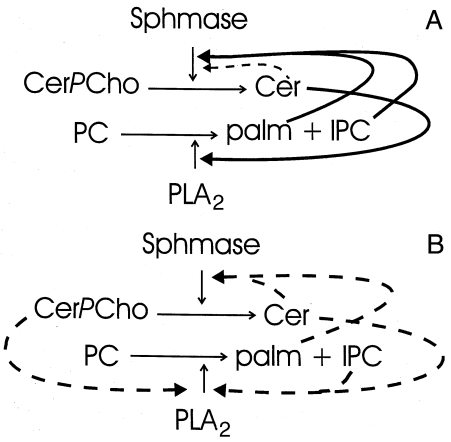

FIG. 6. Simplified scheme summarizing the interfacial cross-talk between Sphmase and PLA_2 activities at representative high and low surface pressures. The influences of substrates and products of both phosphohydrolytic pathways on the Sphmase and PLA_2 activities are illustrated at 20 (A) and 10 (B) mN/m. Solid lines represent activation, and dashed lines correspond to inhibition. PC, phosphatidylcholine. See Figures 1 and 3 for other abbreviations.

multaneously within the limits of a same surface microregion [i.e., over an interfacial two-dimensional space region covering the size of physically correlated microheterogeneous lipid domains (36,39)]. At 20 mN/m, no degradation of phosphatidylcholine by PLA_2 occurs since this pressure is above the cut-off point for activity against the pure substrate (Fig. 4). On the other hand, Sphmase exhibits a low activity against pure Cer*P*Cho monolayers (Fig. 2) and, according to the rate observed against a film of pure Cer*P*Cho, a film composition containing 10 mol% of Cer can be reached in about 30 min (in the conditions of trough surface area used in our experiments, local domain concentrations could be reached much faster). The presence of Cer inhibits the Sphmase activity while, at the same time, decreasing the lag time, allowing a slow continuous activity and, in conditions in which the amount of Cer*P*Cho does not become rate-limiting, Cer can be slowly produced. The presence of 10 mol% Cer should induce a shift to high pressure of the cut-off point for PLA_2 activity (Fig. 5), and this enzyme could become active after the reaction catalyzed by Sphmase started. Our results on the rate of activity of PLA_2 against films containing Cer show that about 10 mol% of fatty acid and lysoderivative can be produced in about 14 min. The accumulation of either product of PLA_2 markedly activates Sphmase at 20 mN/m, accelerating by about five times the production of Cer (Fig. 3). Increased production of Cer can again activate PLA_2 to produce more products which should further activate Sphmase and so on, in an amplified effect of mutual activation between both phosphohydrolytic pathways.

The kinetic pattern and its implications change dramatically at 10 mN/m. At this surface pressure, both enzymes can degrade their own substrates, with the PLA_2 reaction being slightly more active than that mediated by Sphmase. At 10 mN/m, either Cer*P*Cho or Cer can inhibit the rate of activity of PLA_2 (Fig. 5). Also, the products of this enzyme formed at a slow rate have a further inhibitory effect on PLA_2 (Fig. 4) so that this enzyme would become inhibited at 10 mN/m. Sphmase is likewise inhibited by Cer and palmitic acid (Figs. 2 and 3) which would lead to a decreased production of Cer and a relative accumulation of Cer*P*Cho. This latter lipid, in turn, further inhibits PLA_2 and so on. Thus, at 10 mN/m, the simultaneous influence of both phosphohydrolytic pathways should lead to a synergic and large decrease of the activity of both enzymes, causing a mutual dampening and cooperative shutdown of their reaction pathways, compared to when either enzyme is acting in the absence of the lipid intermediates produced by the other.

Several thermodynamic, mechano-elastic, partitioning, and enzymatic studies are consistent in defining the range of 30–35 mN/m as a reasonable figure for the average half-bilayer-equivalent surface pressure (40). However, clearly, the average value can fluctuate by more than ±15 mN/m depending on the compressibility of the lipid–protein interface at each temperature (41); in addition, the fluctuations are enhanced in mixtures exhibiting lateral phase separation and domain microheterogeneity (40). Considering these facts, fluctuations of surface pressure between 10–40 mN/m are likely in bilayer and cell membranes (40,41). Taken together, our observations suggest that a cross-modulation by the substrates and products of the activities of Sphmase and PLA_2 may be regulated by lipid composition and overall lateral surface pressure altering the catalytic properties of the enzymes associated with the interface through lipid–enzyme interactions in the film while local changes of lipid packing or dipolar properties caused by specific lipid–lipid interactions are less likely. Probable levels at which the regulation may occur involve precatalytic adsorption and activation steps, conformational changes of the enzyme associated to the interface, or alterations involved directly in the effective catalytic rate (i.e., enzyme–substrate recognition, enzyme accessibility to the substrate cleavage sites, and/or rate of product release) that are currently under investigation.

ACKNOWLEDGMENTS

This work was supported in part by Secretaría de Ciencia y Tecnología (SECyT-UNC), Agencia Nacional de Promoción Cientifica y Technológica (FONCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Consejo de Investigaciones Científicas de la Provincia de Córdoba (CONICOR). M.L.F. is a Fellow and B.M. is Principal Investigator of CONICET, Argentina.

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[Received June 22, 1998, and in final revised form September 22, 1998; revision accepted October 19, 1998]

GD_3 and GM_2 Synthase Activities in Rat Testes During the Period of Sexual Development

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ABSTRACT: Activities of two key enzymes of gangliosides biosynthesis were determined in rat testes during development. GD_3 synthase activity was low and showed small variations with age. GM_2 synthase activity increased 10-fold in testes from 10- to 30-d-old animals, showing a maximum activity at 30 d, followed by a small decrease until 45 d and then a constant activity up to adulthood. These developmental changes in the activity of both glycosyltransferases were related to the increasing complexity in the ganglioside pattern observed in rats testes during the period of sexual development.

Lipids 33, 1089–1092 (1998).

Gangliosides are synthesized in a stepwise manner by transfer of carbohydrates from sugar nucleotide donors to glycolipid acceptors by specific glycosyltransferases. This process occurs in the endoplasmic reticulum and Golgi apparatus (1). The mechanisms of intracellular traffic of gangliosides have been elucidated, particularly in the nervous system (2–4). Modifications in the ganglioside pattern during development may be associated with correspoding changes in the activities of specific glycosyltransferases. In the chick embryo retina system and the mammalian brain developmental changes in ganglioside composition correlate with variations in the activities of the key ganglioside glycosyltransferases: GD_3 and GM_2 synthases (5–8).

Gamallo and Bernard (9) demonstrated that rat testes gangliosides show a pattern of increasing complexity with aging. GM_3 is the major ganglioside present in rat testes of 10-d-old animals. With sexual development, the content of GM_3 decreases and the complexity of gangliosides increases, with the appearance of longer-chain gangliosides as GM_1, GD_{1a} (series "a"), and GT_{1b} (series "b") (9).

The aim of this study was to investigate a possible correlation between the developmental changes in ganglioside composition observed in rat testes and the variations in the activity of the two key ganglioside glycosyltransferases: GD_3 (E.C. 2.4.99.8) and GM_2 (E.C. 2.4.1.92) synthases.

MATERIALS AND METHODS

Chemicals. UDP-*N*-acetylgalactosamine (UDP-GalNAc), CMP-*N*-acetylneuraminic (CMP-NeuAc), Triton CF54, sodium cacodylate, CDP-choline, octylglucoside, bovine serum albumin, mercaptoethanol, Sephadex A-25, and gangliosides GD_3, GM_2 and GM_3 were from Sigma Chemical Co. (St. Louis, MO). CMP-[4,5,6,7,8,9-^{14}C]*N*-acetylneuraminic acid (CMP-[^{14}C]NeuNAc), 256 mCi/mmol, was from Amersham International plc (Amersham, United Kingdom). UDP-*N*-acetyl-D-[6-^{3}H]galactosamine (UDP-[^{3}H]GalNAc), 10 Ci/mmol, was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Ultima Gold was from Packard B.V. Chemical Operators (Groningen, The Netherlands). All other chemicals and solvents used were of analytical grade.

Animals. Male Wistar rats from our breeding stock, aged between 10 and 90 d, were used. The size of the litters was adjusted to eight pups per mother on the first day after birth. The animals were weaned at 21 d of age and kept in an animal room under controlled conditions of temperature, with alternating periods of light (12 h) and dark (12 h). Standard dry pellets and water were available *ad libitum.*

Sertoli cell-enriched testes (SCE). SCE were obtained by *in utero* irradiation with 100 rad of ^{60}Co at day 20 of pregnancy (11).

Enzyme preparation. The total particulate fraction, used as the enzyme source, was prepared as described by Bushway *et al*. (12). Pools of testes from unequal numbers of animals (according with their ages) were homogenized in 3 vol of cold 320 mM sucrose/14 mM 2-mercaptoethanol. Homogenates were centrifuged at $1,000 \times g$ (Sorvall centrifuge, RC-2B, SS-34 rotor; Du Pont Instruments, Newton, CT) for 10 min to remove debris. The resulting supernatant was centrifuged at $176,000 \times g$ (Model L5-75B, SW 50.1 rotor, Beckman Instruments, Palo Alto, CA) for 1 h. The resulting pellet, designated total particulate fraction, was resuspended in cold 0.32 M sucrose/14 mM 2-mercaptoethanol and was used as the enzyme source.

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The terminology used for gangliosides in this paper is that recommended by Svennerholm (Ref. 10): GD_{1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide; GD_3, (*N*-acetylneuraminyl)$_2$-galactosylglucosyl ceramide; GM_1, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide; GM_2, acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide; GM_3, *N*-acetylneuraminylgalactosylglucosyl ceramide; GT_{1b}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)$_2$-galactosylglucosyl ceramide. Abbreviation: SCE, Sertoli cell-enriched.

GM_3 isolation. The lipid acceptor GM_3 was extracted from dog spleen and purified as described by Nores and Caputto (13). Identity and purity of GM_3 were monitored by thin-layer chromatography (9) with an authentic GM_3 standard.

Enzyme activity determination. The compositions of the incubation systems were those described by Klein *et al*. (14) with minor modifications according to the method of Bushway *et al*. (12) and Senn *et al*. (15) for GD_3 synthase and GM_2 synthase activities, respectively.

GD_3 synthase activity was determined in an incubation system containing 500 µM glycolipid acceptor GM_3, 500 µM CMP-[^{14}C]-NeuAc (25,000 cpm), 150 mM cacodylate/HCl buffer (pH 6.35), 10 mM $MgCl_2$, 120 µg Triton CF54, and 150 µg total particulate protein as enzyme source, in a final volume of 20 µL.

GM_2 synthase activity was assayed in a similar incubation mixture containing 500 µM glycolipid acceptor GM_3, 100 µM UDP-[^{3}H]-GalNAc (400,000 cpm), 160 µg octylglucoside, 150 mM cacodylate/HCl (pH 7.3), 5 mM CDP-choline, 20 mM $MnCl_2$, and 120 µg total particulate protein in a final volume of 20 µL.

The systems were incubated at 34°C for 150 min for GD_3 synthase and 60 min for GM_2 synthase. In both cases an assay was run in the absence of exogenous glycolipid acceptor, in order to discount the incorporation into endogenous acceptors. Reactions were begun by the addition of particulate protein fraction and were stopped by addition of 0.5 mL 5% (wt/vol) trichloroacetic acid/0.5% phosphotungstic acid. The products of the two enzymatic activities were extracted as described by Maccioni *et al*. (6). Radioactivity was measured with an LKB Scintillation Spectrometer 1215 Rack Beta using Ultima Gold.

Enzyme specific activities were expressed as pmol of NeuAc or GalNAc transferred/mg protein/h for GD_3 or GM_2 synthase activities, respectively, and were calculated from the difference in the average radioactivity of the exogenous acceptor assay minus the radioactivity of the corresponding one without acceptor.

The identification of the reaction products was performed in a system with twice the volume of the standard assay, which was interrupted by adding chloroform/methanol (2:1, vol/vol), purified as described by Wells and Dittner (16), and analyzed chromatographically on high-performance thin-layer chromatographic plates and developed in chloroform/methanol/0.25% aqueous $CaCl_2$ (60:35:8, by vol) (9). Labeled reaction products with the same R_f as the ganglioside standards were determined as reaction products.

Protein determination. Protein contents were estimated by the method of Lowry *et al.* (17). Bovine serum albumin (Sigma Chemical) was used as the standard.

RESULTS AND DISCUSSION

Gangliosides are sialic acid-containing glycosphingolipids that have a function as structural membrane components *per se* and that, possibly, play an important role in receptor-mediated signal transduction (18) as well as direct cell/cell and cell/extracellular matrix interactions (19).

The present work studied the activities of two key enzymes of the biosynthesis of gangliosides: GD_3 and GM_2 synthases. These enzymes, acting in the commom precursor GM_3, are responsible for the biosynthesis of the gangliosides of the series "b" and "a," respectively, and are considered regulatory enzymes (20).

GD_3 synthase activity was low and showed small variations with age. It diminished about 30% from 10- to 30-d-old rats (Fig. 1). This result agrees with that of Bushway *et al*. (12) in adult bovine testis, where various sialyltransferase activities have been measured, and GD_3 synthase likewise showed low activity. SCE animals (deprived of germ cells) also showed this result (Table 1).

GM_2 synthase activity increased 10-fold from 10- to 30-d-old rats, showing a maximum enzymatic activity at 30 d of age, followed by a small decrease until 45 d of age, at which time activity remained the same until adulthood (Fig. 1).

As mentioned previously (9), adult rat testes contain a complex pattern of gangliosides that is different from the one frequently observed in most extraneural tissues where gangliosides of the hematoside series (GM_3 and GD_3) predominate (21–23). Short-chain gangliosides are present in testes of 10-d-old animals (GM_3 and GD_3) (9). From this age on, other longer-chain gangliosides appear (GM_1, GD_{1a}, GT_{1b}). This increase in complexity is already evident in 20-d-old animals, when more than one trisialoganglioside is present (9). This pattern correlates with the increasing activity of GM_2 synthase, which is coincident with the appearance of more complex gangliosides. Fishman (21) previously referred to participation of this enzyme in ganglioside chain complexity.

In testes of SCE rats (deprived of germ cells), the activity of GM_2 synthase in adult animals (90 d old) was similar to that of 20-d-old normal animals (compare Table 1 to Fig. 1). By 20 d of age, as we reported (9), complex gangliosides were already present in rat testes, as well as in rat testes of SCE animals. These observations allowed us to state that the activity of GM_2 synthase observed in SCE animals is enough for the appearance of complex gangliosides in rat testes. Therefore, it seems that the variations in testicular gangliosides during sexual maturation cannot be correlated with the appearance of advanced germinal cells (12).

Developmental changes in ganglioside glycosyltransferases have been extensively demonstrated in central nervous system (6–8,24). In embryonic rat brain, major GD_3 synthase activity has been observed during early embryonic stages, while an increase in GM_2 synthase activity has been shown to occur during later embryogenesis and adulthood (6,7). Similar results have been observed in embryonic chick retina and embryonic human brain (5,8).

As far as we know, there are no published data about GM_2 synthase activity variations in extraneural tissues during development. In bovine thyroid of adult animals GM_2 synthase activity is considered high when compared to other glycosyltransferases measured in this tissue (22). This activ-

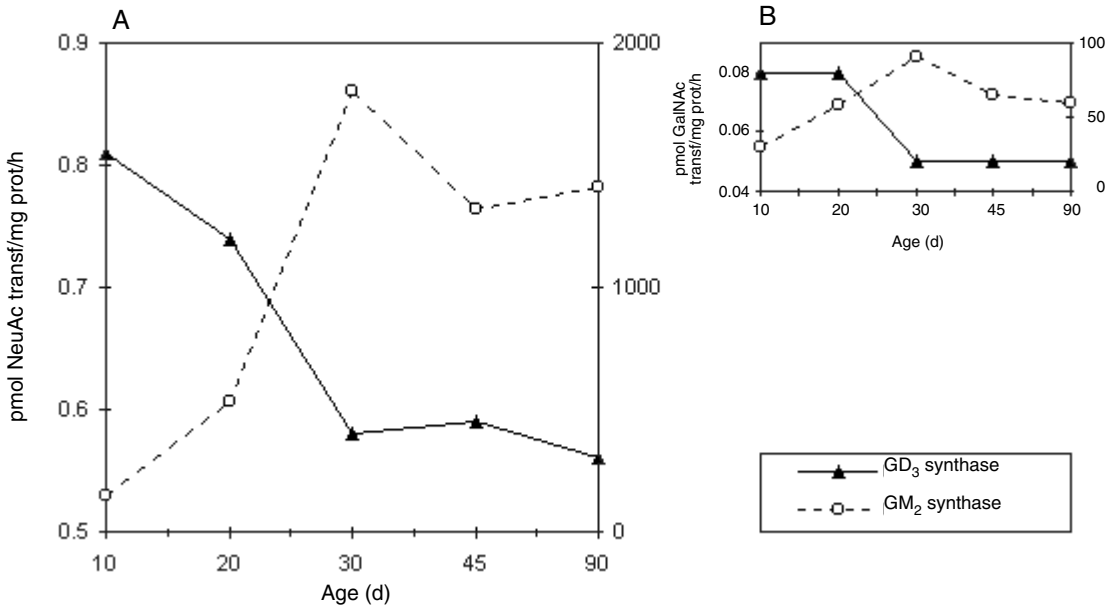

FIG. 1. GD_3 (▲) and GM_2 (□) synthase activities in rat testes during the period of sexual development. (A) Representative experiment of three determinations with different enzymatic preparations, in which each point corresponds to an assay carried out in triplicate; (B) enzymatic activities obtained without exogenous acceptor addition.

ity is correlated with the high abundance of GD_{1a} in this organ.

The present study described, for the first time, the variations of GD_3 and GM_2 synthase activities in rat testes during development. According to the literature relating to the central nervous system (6–8,24), developmental changes observed in rat testicular gangliosides (9) may be related to variations of the activities of these key ganglioside glycosyltransferases during the period of sexual development. We suggest, in agreement with Maccioni *et al*. (6), Yu *et al*. (7), and Percy *et al*. (8) for the central nervous system, that the simple ganglioside predominance and major GD_3 synthase activity in testes of 10-d-old animals might be correlated with the proliferation phase of Sertoli cells (25). The later decrease of GD_3 synthase activity and simultaneous increase of GM_2 synthase activity with consequent complex ganglioside synthesis may be correlated with the maturation of the Sertoli cells.

Recent experiments with knockout mice of β-1,4-GalNac transferase (GD_2/GM_2 synthase) showed that complex gangliosides are not essential in the morphogenesis and organogenesis of the brain, but they are required in neural functions (26). Perhaps these observations cannot be applied to testis development since spermatogenesis is arrested in knockout mice (27). This blockade may be due to an alteration in spermatogeneis regulation as modulated by the hypothalamic-hypophyseal unit (28) or by Sertoli cells (29).

TABLE 1
GD_3 and GM_2 Synthase Activities of Normal and of SCE Rat Testes of Adult Animals (90 d old)

	GD_3 synthase[a]	GM_2 synthase[b]
Normal testes	0.6 ± 0.15	1406 ± 140
SCE testes	1.28 ± 0.04	666 ± 10

[a]Results are expressed as pmol NeuAc transferred/mg prot/h ± standard error of the mean.
[b]Results are expressed as pmols GalNAc transferred/mg prot/h ± standard error of the mean. The data of the normal and of the SCE rat testes represent the mean of three and two determinations, respectively, with different enzymatic preparations, each one carried out in triplicate. SCE, Sertoli cell-enriched.

ACKNOWLEDGMENTS

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Financiadora de Estudos e Projetos (FINEP). We are grateful to Dr. Guilhermo F. Wassermann for irradiation of the pregnant females and to Dra. F.T.R. Guma for helpful discussions.

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[Received May 1, 1998, and in final revised form September 2, 1998; revision accepted October 16, 1998]

Lipid and Fatty Acid Composition of Brush Border Membrane of Rat Intestine During Starvation

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ABSTRACT: Alterations in the lipid and fatty acid composition of brush border membrane (BBM) of small intestine were studied in well-fed, starved, and refed rats. The ratios of cholesterol/phospholipid (mol/mol), sphingomyelin/phosphatidylcholine (mol/mol), protein/lipid (w/w), and free fatty acids (w/w) decreased whereas the total phospholipid (w/w) ratio and the double-bond index increased in BBM of the intestine of the starved rat compared to that of the well-fed rat. Analyses of fatty acids showed higher percentage of stearic and arachidonic acids whereas oleic and linoleic acids decreased under starvation. The acyl chain of starved rat BBM was less ordered compared with that of well-fed rat BBM. On refeeding, these changes were restored to well-fed levels. The change in membrane state under starvation is associated with alterations in the lipid and fatty acid composition of BBM and may be responsible for functional changes that occur under nutritional stress.
Lipids 33, 1093–1097 (1998).

The structure and function of the brush border membrane (BBM) of enterocytes are sufficiently differentiated to absorb a wide variety of luminal nutrients (1,2). Several physiological and/or pathological conditions such as dietary composition, postnatal maturation, diabetes and intestinal resection are associated with selective alterations in the percentage composition of lipids in BBM (3–6), with alterations in the biophysical characteristics of the BBM, and with transport of the nutrients and activity of certain enzymes in the membrane (4,5,7,8). Starvation is one of the physiological stresses that an animal often undergoes in its lifetime; however, not much is known about the structural and compositional changes of BBM under this stress.

Studies from our laboratory showed that starvation increases transport of glucose and amino acids such as lysine, proline, glycine, and glutamic acid across the membrane of intestinal epithelial cells (IEC) of the rat (9–11). The increase in the transport of these substances may be due to the increase in surface area of microvilli and fluidity of BBM of IEC (9–11). However, changes in the lipid composition of BBM that may be associated with changes in membrane state have not been investigated in detail. In this communication, we report the effect of starvation on the composition and acyl chain order of rat intestinal BBM, which in turn might affect its function.

MATERIALS AND METHODS

Materials. Fatty acids, methyl esters, lipid standards, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Sigma Chemical Company (St. Louis, MO). The gas–liquid chromatographic column was purchased from Shimadzu (Tokyo, Japan). High-performance thin-layer chromatographic plates were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and purchased locally.

Preparation of animals. Adult male (90 ± 5 d) Wistar rats weighing 300 ± 20 g each were used for the present study. The well-fed, starved and refed rats were prepared as described earlier (9,11). The protocol of animal preparations for the present experiment was approved by the Ethics Committee of our institute (Centre for Cellular and Molecular Biology). A standard rat diet containing 5% fat (Hundusthan Liver Limited, Mumbai, India) was provided to the rats. The rats belonging to well-fed, starved and refed groups were killed by cervical dislocation. After dissecting away 10 cm from the pyloric end of the stomach, about 15 cm of the anterior portion of the small intestine from each animal was used for the present study.

Preparation of BBM. The proximal region of each intestine was washed throughly with ice-cold 0.15 M NaCl, the enterocytes were collected by scraping the everted intestine with a glass slide into buffer I (50 mM mannitol, 2 mM Tris/HCl, pH 7.1), and BBM was prepared according to the method of Kessler *et al.* (12). Briefly, epithelial cells were homogenized in a polytran homogenizer (Kinematica, Lucern, Switzerland) for 1 min at maximum speed. $CaCl_2$ was added to a final concentration of 10 mM, and after 15 min the homogenate was centrifuged at $3000 \times g$ for 15 min. The supernatant was then spun down at $27{,}000 \times g$ for 30 min. The pellet was resuspended in buffer II (50 mM mannitol, 10 mM Tris/HCl, pH 7.1) and centrifuged once more at $27{,}000 \times g$ for 30 min. Purity and comparability of the BBM were assessed using various criteria as described earlier (9–11).

BBM composition. Total lipids were extracted from BBM

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Abbreviations: BBM, brush border membrane; DPH, 1,6-diphenyl-1,3,5-hexatriene; IEC, intestinal epithelial cells.

by the method of Folch *et al*. (13). Cholesterol and phospholipids were estimated by the methods of Zak *et al*. (14) and Meclare (15), respectively. Total lipid containing 1 µg of cholesterol was applied to 10 cm × 10 cm high-performance thin-layer chromatography plates. The plates were developed first with chloroform/methanol/ammonia (65:25:6, by vol) to 4 cm and then with hexane/ether/acetic acid (70:30:1.5, by vol) to full length. The plates were immersed in phosphoric acid/33% acetic acid/sulfuric acid/0.5% copper sulfate (5:5:0.5:90, by vol) for 40 s and then heated at 130°C for 12 min (16). The chromatogram was scanned by photodensitometer (Molecular Dynamics) at 650 nm. Percentage composition of the total lipid was determined based on integration of the peaks for the lipid fractions.

To determine the acyl chain composition, fatty acids of the total lipid extract were derivatized as described by Gartner and Vahouny (17), and the fatty acid methyl esters were quantified on a Shimadzu-17A gas chromatograph (DB-Wax capillary column) equipped with a flame-ionization detector and interfaced with an integretor. Authentic fatty acid methyl esters were used to identify retention times. Proteins were estimated by the method of Waheed and Gupta (18) using bovine serum albumin as the standard.

Fluorescence polarization. Steady-state fluorescence polarization experiments were performed using the lipid-soluble fluorophore DPH in a Hitachi F-4000 spectrophotometer. A stock solution of 2 mM DPH in tetrahydrofuran was added directly to the membrane preparations such that the final concentration of the probe was 1 µM; the resultant solution was mixed thoroughly and then incubated at 25°C for 30 min. Measurements were made by using the ratio of the intensity of excitation (355 nm) to the intensity of emission (430 nm) (19). Correction for light scattering was routinely made. Fluorescence polarization was expressed as the fluorescence anisotropy, *r*. The results were obtained according to the modified Perrin relationship (20): $r = r_\alpha + (r_o - r_\alpha)\,[T_c/(T_c + T_f)]$, where *r* is the fluorescence anisotropy; r_o is the maximal limiting anisotropy, taken as 0.365 for DPH; r_α is the limiting hindered anisotropy, T_c is the correlation time; and T_f is the mean lifetime of the excited state. Values for r_α for DPH were calculated from steady-state fluorescence anisotropy, r_s values ($r_\alpha = 4/3r_s - 0.10$) as described by van Blitterswijk *et al*. (21). Values of r_s were calculated from fluorescence emission intensities recorded parallel and perpendicular to the excitation plane of the probe as described (21). The static component of membrane fluidity was assessed by an order parameter, *S*, which was obtained from the equation $S = (r_\alpha/r_o)^{1/2}$ (21).

Statistical analyses. Statistical analyses were carried out by one-way analysis of variance with linear contrasts for comparison of the subgroups. Results are expressed as means ± S.E. $P < 0.05$ was considered significant.

RESULTS

Composition of lipids in BBM. Lipids extracted from starved-rat BBM showed higher amounts of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine compared with that of well-fed rats (Table 1). As a result, the total phospholipid content was higher in starved-rat BBM. Sphingomyelin was the only phospholipid which did not change

TABLE 1
Effect of Starvation and Refeeding on Small Intestinal BBM Lipid Composition (wt% of total lipids) and Compositional Analysis[a]

Lipid classes	Well-fed	2-d Starved	4-d Starved	6-d Starved	Refed
Chol	24.6 ± 1.5	22.9 ± 3.2[b]	21.8 ± 1.2[b]	20.1 ± 1.5[b]	23.9 ± 1.4[e]
Chol est	6.0 ± 0.8	3.8 ± 0.3[d]	4.0 ± 0.3[d]	4.0 ± 0.4[d]	6.1 ± 0.1[g]
FFA	21.6 ± 1.3	13.5 ± 2.1[d]	9.4 ± 1.5[d]	10.9 ± 2.0[d]	22.0 ± 3.2[f]
TPL	47.8 ± 1.6	59.6 ± 4.2[b]	64.7 ± 1.9[d]	65.0 ± 3.2[d]	48.2 ± 5.1[g]
PC	20.2 ± 1.1	28.7 ± 1.4[d]	28.8 ± 1.2[d]	28.8 ± 1.7[d]	20.6 ± 1.8[g]
LPC	3.4 ± 0.6	4.1 ± 0.7	5.1 ± 1.3[b]	4.9 ± 0.5	3.5 ± 0.9[e]
PE	14.1 ± 0.8	17.8 ± 2.4	21.3 ± 2.0[c]	20.7 ± 3.2[b]	14.5 ± 2.2[e]
SP	10.1 ± 0.7	9.1 ± 0.5	9.5 ± 1.9	10.5 ± 0.6	9.5 ± 0.6
Protein/lipid (w/w)	2.60 ± 0.20	2.28 ± 0.10[b]	2.34 ± 0.08[b]	2.34 ± 0.08[b]	2.38 ± 0.06
Chol/TPL (mol/mol)	1.21 ± 0.07	0.82 ± 0.09[b]	0.77 ± 0.05[c]	0.70 ± 0.04[d]	1.16 ± 0.15[e]
SP/PC (mol/mol)	0.52 ± 0.05	0.34 ± 0.05[b]	0.34 ± 0.03[b]	0.36 ± 0.03[b]	0.48 ± 0.05[e]
PE/PC (mol/mol)	0.71 ± 0.05	0.65 ± 0.08	0.75 ± 0.07	0.72 ± 0.09	0.70 ± 0.07

[a]Values are means ± SEM for seven determinations from four rats per group. Values of composition are obtained by peak integration of the lipid fractions and are given as percentage (w/w). Mole quantity of an individual lipid is obtained by dividing the weight of each lipid by its molecular weight.
[b–d]A significant difference from the values of the well-fed group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
[e–g]Values statistically differ from 6-d starved group, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
Abbreviations: Chol, cholesterol; Chol est, cholesterol esters; FFA, free fatty acids; TPL, total phospholipid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; SP, sphingomyelin; BBM, brush border membrane.

TABLE 2
Fatty Acid Composition (wt% of total fatty acids) in BBM of Rat Enterocytes of Well-Fed, Starved and Refed Rats[a]

Fatty acids	Well-fed	2-d Starved	4-d Starved	6-d Starved	Refed
12:0	0.9 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
14:0	2.2 ± 0.2	2.1 ± 0.2	2.5 ± 0.4	2.5 ± 0.4	2.9 ± 0.2
16:0	21.7 ± 0.9	20.7 ± 0.7	21.6 ± 1.5	22.7 ± 0.8	23.9 ± 1.0
18:0	18.7 ± 1.4	24.9 ± 1.2[e]	25.4 ± 0.6[e]	24.3 ± 1.5[d]	15.8 ± 0.5[h]
18:1	17.5 ± 1.4	11.3 ± 0.4[e]	9.4 ± 0.5[f]	9.8 ± 0.5[f]	20.2 ± 1.2[i]
18:2	24.3 ± 1.2	10.8 ± 0.8[f]	10.5 ± 0.7[f]	12.9 ± 1.0[f]	19.9 ± 1.2[i]
20:0	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.1
20:4	13.3 ± 1.0	27.8 ± 1.0[f]	28.1 ± 1.8[f]	25.1 ± 1.0[f]	14.9 ± 0.9[i]
Saturated	44.9 ± 2.6	50.1 ± 1.3[d]	52.0 ± 1.8[d]	52.2 ± 2.0[d]	45.0 ± 1.2[g]
Monoene	17.5 ± 1.4	11.3 ± 0.8[e]	9.4 ± 0.5[f]	9.8 ± 0.5[f]	20.2 ± 1.2[i]
Polyene	37.6 ± 0.9	38.6 ± 0.7	38.6 ± 1.2	38.0 ± 1.0	34.8 ± 1.7
Saturation index[b]	0.36 ± 0.01	0.35 ± 0.02	0.37 ± 0.02	0.37 ± 0.02	0.38 ± 0.02
Double-bond index[c]	1.19 ± 0.02	1.44 ± 0.04[e]	1.43 ± 0.04[e]	1.38 ± 0.04[e]	1.17 ± 0.01[h]

[a]Values are means ± SEM for seven determinations from four rats per group. Values of composition are given as percentage (w/w) of total fatty acids.
[b]Saturation index was calculated by dividing the number of saturated acyl chains by the sum of each unsaturated chain multiplied by number of double bonds.
[c]Double-bond index is the sum of each unsaturated fatty acid chain multiplied by number of double bonds/100.
[d–f]A significant difference from the values of well-fed group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
[g–i]Values statistically differ from 6-d starved group, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

significantly during starvation. In contrast to phospholipids, neutral lipids decreased during starvation, with the decrease in fatty acid and cholesterol esters being highly significant compared with cholesterol. The ratios of protein/lipid (w/w), cholesterol/phospholipid (mol/mol), and sphingomyelin/phosphatidylcholine (mol/mol) decreased in starved-rat BBM. The decrease in the molar ratio of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine was not due to a decrease in the contents of cholesterol or sphingomyelin but to an increase in the quantities of phosphatidylcholine and phosphatidylethanolamine.

Compositional analyses of fatty acids showed greater variation under starvation (Table 2). There was an increase in the content of stearic acid (18:0) and arachidonic acid (20:4) to 138 and 200%, respectively, in starved-rat BBM compared with that of well-fed rats. Decreases of about 50% in oleic acid (18:1) and linoleic acid (18:2) were observed in starved-rat BBM. No significant changes in the composition of lauric (12:0), myristic (14:0), palmitic (16:0), and arachidic (20:0) acids were observed. The double-bond index was higher in starved-rat BBM whereas the change observed in saturation index was not significant. The changes in lipid and fatty acid composition were reversable since the levels in the refed group were comparable to those of the well-fed group (Tables 1 and 2).

Fluorescence polarization. Steady-state fluorescence polarization studies using DPH showed that fluorescent anisotropy, *r*, of DPH decreased in starved rats' BBM, indicating decreased acyl chain order of these membranes (Table 3). The decrease in *r* values was 4% for rats starved for 2 d, 6.6% for 4 d, and 11.3% for 6 d compared with that of well-fed rats. The static component of membrane order as assessed by r_α and *S* values of DPH was found to be less in membranes prepared from the starved rat, suggesting that the lipid order parameter decreased during starvation. The decrease in order parameter was progressive with days of starvation, i.e., 2.2% in rats starved for 2 d, 3.8% for 4 d, and 7.0% for 6 d compared with the well-fed group. In refed rats, the fluorescent anisotropy *r*, limiting hindered anisotropy r_α, and order parameter *S* were restored to levels found in well-fed animals.

DISCUSSION

The present data demonstrate that during starvation the acyl chains of BBM become less ordered, and also that significant alterations occur in the lipid and fatty acid composition of the membrane. The quantitative changes in cholesterol and proteins have profound effects on packing of phospholipids in the membrane (6). In starved-rat BBM the molar ratios of

TABLE 3
Fluorescence Polarization Studies of DPH in BBM of Rat Small Intestine from Well-fed, Starved and Refed Rats[a]

Experimental condition	Fluorescent anisotropy (*r*) of DPH	Limiting hindered anisotropy (r_α)	Order parameter (*S*)
Well-fed	0.256 ± 0.001	0.245 ± 0.001	0.819 ± 0.002
2-d Starved	0.246 ± 0.001[b]	0.235 ± 0.001[b]	0.801 ± 0.002[b]
4-d Starved	0.239 ± 0.001[b,c]	0.226 ± 0.001[b,c]	0.788 ± 0.002[b,c]
6-d Starved	0.227 ± 0.004[b,d]	0.212 ± 0.004[b,d]	0.762 ± 0.007[b,d]
Refed	0.251 ± 0.001[e]	0.239 ± 0.001[e]	0.809 ± 0.002[e]

[a]Values are means ± SEM for seven determinations from four rats per group. A significant difference [b]from well-fed ($P < 0.001$), [c]from 2-d or 6-d starved ($P < 0.01$), [d]from 2-d starved ($P < 0.001$), and [e]from 6-d starved ($P < 0.001$).
DPH, 1,6-diphenyl-1,3,5-hexatriene

cholesterol/phospholipid and protein/lipid decrease; this may be one of the factors for the less-ordered acyl chains of these membranes. The decrease in sphingomyelin/phosphatidylcholine ratio also accounts for the decreased lipid order of BBM as suggested by Dudeja *et al*. (20). Our earlier studies on freeze-fracture replica of BBM showed that the levels of intramembranous particles and filipin-sterol complexes which represent protein and cholesterol, respectively, decreased during starvation (22). The ratio of phosphatidylethanolamine/phosphatidylcholine does not change because the levels of both these phospholipids increase. This increase is likely to counterbalance the distribution of these phospholipids in inner and outer leaflets of the lipid bilayer. The present data indicate that, under starvation, the content of free fatty acids decreases, which may be utilized in synthesis of phospholipids; therefore, there is a net increase in the content of total phospholipids in starved-rat BBM.

Brasitus *et al*. (19) reported that the lipid fluidity of intestinal microvillus membranes is associated with an increase in double-bond index. During starvation there is a decrease in the levels of oleic and linoleic acids; however, because of an increase in the levels of arachidonic acid there is a net increase in the double-bond index by about 25% in starved-rat BBM. This is in agreement with observations of Garg *et al*. (23), who showed that after 24 h of fasting the arachidonic acid content of the microsomal membrane of IEC increased with a concomitant decrease in linoleic acid. A decrease in linoleic acid accounts for increased amounts of arachidonic acid, as linoleate is the sole precursor for arachidonate (23). In model bilayer membrane *cis* unsaturation of phospholipid acyl chains increases the molecular packing area; as a result, the membrane acyl chain becomes less ordered (24). The percentage composition of saturated fatty acids does not change significantly during starvation. The unaltered saturation index and increased double-bond index in BBM of rats fed a diet containing unsaturated fats (corn oil) may be responsible for enhanced fluidity of these membranes compared to rats fed a saturated fat diet (butter fat) (24). Others have reported that the increased fluidity of cell membranes of the large intestine of small bowel-resected rats is due to an increased double-bond index (6). The changes observed in lipid and fatty acid composition are rapid by 2 d of starvation and do not change much on further starvation. Refeeding experiments in which the lipid order, composition of lipids, and fatty acids revert back to well-fed levels suggest that the structural and compositional changes in starved-rat BBM are likely to be physiological and not pathological.

Transport through BBM is correlated to its membrane state in various physiological and pathological conditions (1,2,5, 7,9,11,25). Under starvation there is an increase in surface area of microvilli (9–11), which is likely due to loose packing of phospholipids; however, the levels of glucose transporter proteins decrease and cannot account for the increased transport of glucose (unpublished data from this laboratory). Hence, it is likely that the increase in transport is due to greater transporter activity, which is facilitated in more fluid membrane (1,2,7,25). In the present study we have shown that the change in membrane state of starved-rat intestinal BBM is associated with alterations in lipid and fatty acid compositions.

ACKNOWLEDGMENTS

The authors are grateful to Drs. Sajid Husain and P.N. Sarma of the Indian Institute of Chemical Technology, Hyderabad, for their help in lipid analyses. AAW is a RONPAKU Fellow of the Japan Society for the Promotion of Science, Japan, under the Indo-Japan collaborative scientific exchange program.

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[Received January 27, 1998, and in final revised form and accepted October 27, 1998]

Metabolism of Dietary α-Linolenic Acid vs. Eicosapentaenoic Acid in Rat Immune Cell Phospholipids During Endotoxemia

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ABSTRACT: Short-term (i.e., 3 d) continuous enteral feeding of diets containing eicosapentaenoic (EPA) and γ-linolenic (GLA) polyunsaturated fatty acids (PUFA) to endotoxemic rats reduces the levels of arachidonic acid (AA) and linoleic acid (LA) in alveolar macrophage (AM) and liver Kupffer and endothelial (K&E) cell phospholipids with attendant decreases in prostaglandin formation by these cells *in vitro*. Diets that contain α-linolenic acid (LNA) as a substrate for endogenous formation of EPA may not be as effective in facilitating these immune cell modifications given the limited activity of Δ6 desaturase. In the present study we compared the effectiveness of an LNA-enriched diet vs. an (EPA + GLA)-enriched diet to displace phospholipid AA from AM and liver K&E cells *in vivo* in endotoxemic rats fed enterally for 3 or 6 d. We determined the fatty acid composition of AM and K&E cell phospholipids by gas chromatography. We found that AM and K&E cells from rats that had received the EPA + GLA diet for 3 d had significantly ($P < 0.001$) higher mole percentage of EPA and the GLA metabolite, dihomoGLA, than corresponding cells from rats given the LNA diet or a control diet enriched with LA. Rats given the LNA diet had relatively low levels of stearidonic acid, EPA and other n-3 PUFA, while rats given the LA diet had low levels of GLA and dihomoGLA. We conclude that diets enriched with LNA or LA may not be as effective as those enriched with EPA + GLA for purposes of fostering incorporation of EPA or dihomoGLA into and displacement of AA from macrophage phospholipids under pathophysiologic conditions commonly found in acutely septic patients.

Lipids 33, 1099–1105 (1998).

Alveolar macrophages (AM) and liver sinusoidal Kupffer and endothelial cells (K&E) play a prominent role in the host immune response to infection. When activated during sepsis or trauma, these immune cells are capable of altering the intermediary metabolism and physiologic function of proximal parenchymal cells within their respective organs by releasing vasoactive eicosanoids derived from arachidonic acid (AA, 20:4n-6) and cytokines that promote neutrophil chemotaxis, platelet aggregation, and inflammation (1–3). Overexpression of these and other mediators fosters a persistent systemic inflammatory response, increasing the likelihood of organ dysfunction in the critically ill patient (4–6). For this reason, newer therapeutic modalities are being directed toward the modulation of immune cell AA metabolism to attenuate disregulated inflammatory processes.

Experimental and clinical studies showed that the fatty acid composition and inflammatory responses of host immune cells can be modulated by providing diets enriched with eicosapentaenoic acid (EPA, 20:5n-3) from marine oil either alone (7–11) or in combination with γ-linolenic acid (GLA, 18:3n-6) from borage oil (12–15). We demonstrated that EPA and the GLA metabolite, dihomoGLA (DHGLA, 20:3n-6), is rapidly incorporated into immune cell membranes *in vivo* after short-term enteral feeding with EPA- and GLA-enriched diets (16) irrespective of concurrent endotoxemia (17). EPA and DHGLA displace both AA and linoleic acid (LA, 18:2n-6), respectively, from macrophage phospholipids and effectively compete with AA for cyclooxygenase and lipoxygenase binding sites to foster formation of eicosanoids with relatively lower inflammatory properties (18), e.g., thromboxane A_3, leukotriene B_5 and prostaglandin E_1 (PGE_1), than those generated from AA (e.g., thromboxane A_2 and leukotriene B_4)(Figs. 1 and 2). In our recent study, rats that had been given an (EPA + GLA)-enriched diet had lower ratios of thromboxane A_2/A_3 and leukotriene B_4/B_5 measured in the supernatants of AM exposed to endotoxin *in vitro* than corresponding levels measured from rats given a standard diet enriched with LA (19). The AM from the rats given the (EPA + GLA)-supplemented diet also released greater quantities of PGE_1 than AM from the rats receiving the LA-supplemented diet. PGE_1 is a metabolite of DHGLA that exhibits vasodilatory and antiaggregatory properties in the lung, effects that appear to be beneficial to patients with the acute respiratory distress syndrome (20,21).

One concern in the design of enteral formulations enriched with n-3 polyunsaturated fatty acids (PUFA) is whether α-linolenic acid (LNA, 18:3n-3) present in canola oil could

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Abbreviations: AA, arachidonic acid; AM, alveolar macrophage; DHA, docosahexaenoic acid; DHGLA, dihomo-γ-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; K&E, Kupffer and endothelial; LA, linoleic acid; LNA, α-linolenic acid; PBS, phosphate buffered saline; PGE_1, prostaglandin E_1, PUFA, polyunsaturated fatty acids.

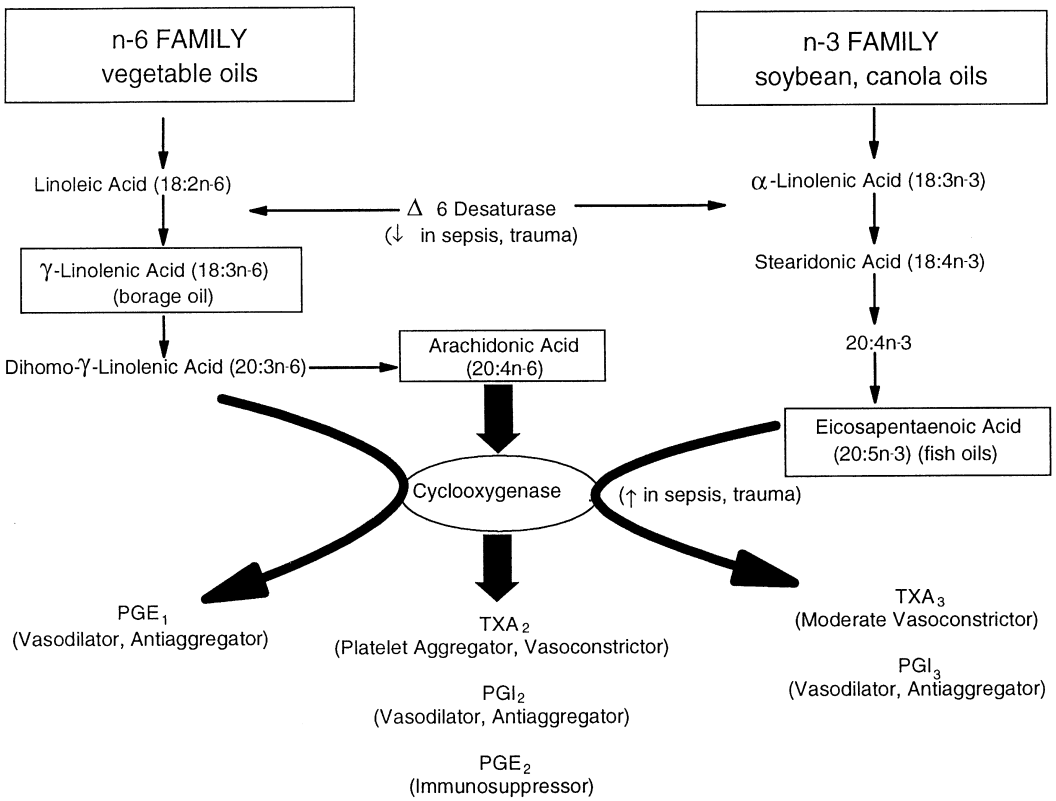

FIG. 1. Metabolic pathways for conversion of dietary polyunsaturated fatty acids to prostaglandins and thromboxanes. PGE_1, PGE_2, PGI_2, and PGI_3 are prostaglandins E_1, E_2, I_2 and I_3, respectively. TXA_2 and TXA_3 are thromboxanes A_2 and A_3, respectively.

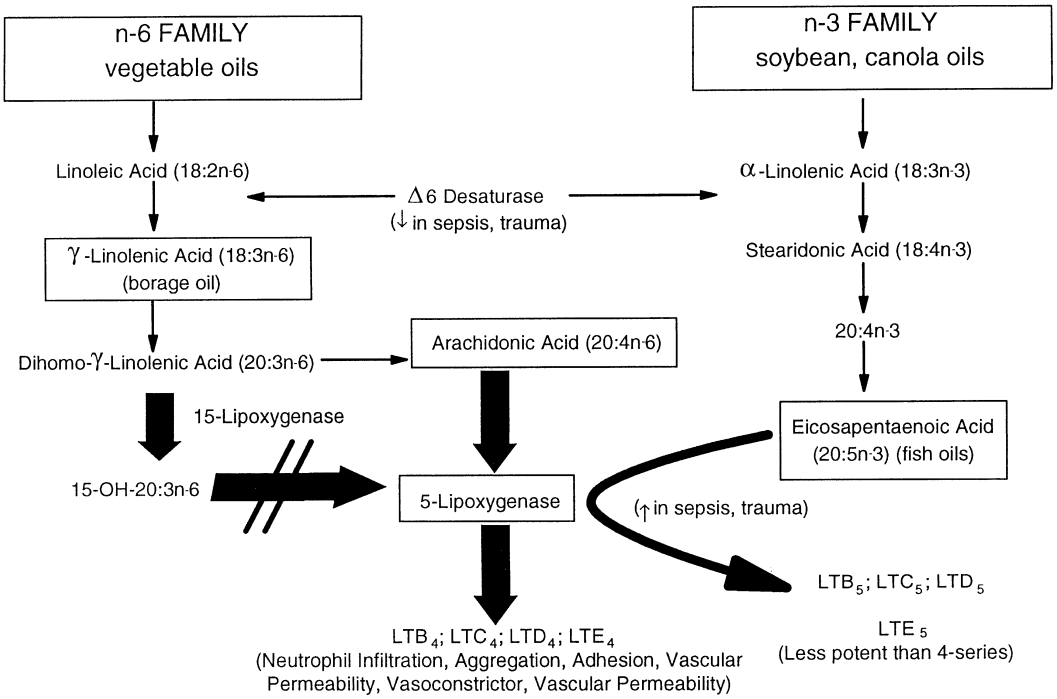

FIG. 2. Metabolic pathways for conversion of dietary polyunsaturated fatty acids to leukotrienes. LTB_4, LTC_4, LTD_4, LTE_4, LTB_5, LTC_5, LTD_5, and LTE_5 are the leukotrienes B_4, C_4, D_4, E_4, B_5, C_5, D_5, and E_5, respectively.

serve as a metabolic precursor of EPA and its longer-chain derivatives, docosapentaenoic (DPA, 22:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids (Fig. 1). It is not known whether dietary LNA can be rapidly elongated and desaturated within mammalian immune cells to form EPA, DPA, and DHA within a clinically relevant time frame, i.e., 3 to 6 d. Experimental studies in rodents orally fed LNA-enriched diets over a 3- (22) or 12-wk period (23) showed that both the amount of EPA present in and the amount of AA displaced from plasma or cell phospholipids were lower than those measured in animals supplemented with dietary EPA. Recent clinical studies in healthy subjects consuming diets enriched in LNA and low levels of LA demonstrated that the percentage of EPA appearing in plasma (24) and mononuclear cell (25) phospholipids had increased slowly over an 8-wk period, with little change occurring in AA levels. This in part may have been due to the postabsorptive release of LA from adipose stores, fostering restoration of AA displaced by LNA or newly formed EPA (16,26). Moreover, formation of EPA from LNA is subject to the activity of Δ6 desaturase, a rate-limiting enzyme that catalyzes the initial desaturation of LNA to stearidonic acid (18:4n-3) (27,28). Compounding this quandary are reports that the stress hormones, i.e., epinephrine (29,30), ACTH (30) or glucocorticoids (31), have an inhibitory effect on both the Δ6 and Δ5 desaturases involved in LNA desaturation to EPA. Reduced activity of Δ6 desaturase may also limit GLA formation from dietary LA, potentially retarding formation of DHGLA and its metabolite, PGE_1. Thus, stressed or septic patients may not be able to effectively convert dietary LNA to EPA and other long-chain n-3 PUFA in sufficient quantities to promote displacement of endogenous AA from immune cell membrane phospholipids (32,33). For this reason, provision of diets containing long-chain PUFA, such as EPA and GLA, that are metabolized regardless of existing alterations in Δ6-desaturase activity, may prove beneficial to critically ill patients with ongoing inflammatory processes.

Our objective in the present study was to assess whether short-term continuous enteral feeding with an LNA-enriched formulation could effectively displace AA and its precursor LA from rat hepatic and lung immune cell phospholipids under septic conditions *in vivo* to the same extent as that achieved with an (EPA + GLA)-enriched formulation. Comparisons were made with immune cells procured from rats given either a standard control diet enriched with LA or a diet enriched with EPA and GLA. We also assessed whether cell phospholipid DHGLA increased in rats given the high-LA diet relative to that in rats given the EPA + GLA formulation.

MATERIALS AND METHODS

The study design was approved by the Institutional Animal Care and Use Committee. The care of the animals was in accordance with the guidelines set forth by the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. The surgical catheterization, treatments, and analytic methods were similar to those described recently (11,17).

Pathogen-free male (225–275 g) Sprague-Dawley rats (Harlan, Altamont, NY) were allowed free access to a low-fat nonpurified diet devoid of fish meal (Teklad 7001; Harlan) for 5 d prior to study entry. All rats were randomized to a dietary treatment prior to study entry. After food was withheld overnight, each rat was anesthetized at 0900 h of day −1 with diethyl ether. A 1.0-mm i.d. Tygon® (Norton Plastics, Wayne, NJ) feeding catheter was inserted aseptically though a fundal gastrostomy, advanced 2.5 cm into the duodenum, and anchored to the stomach wall with a purse-string suture. A 0.64-mm i.d. silastic catheter was inserted in the left jugular vein for intravenous infusion of endotoxin. The proximal ends of the catheters were tunneled subcutaneously, exteriorized at the midscapular region, and attached to a dual channel flow-through swivel to permit freedom of movement during infusion. All incisions were closed with surgical-grade silk sutures. The rats were allowed to recover and were housed in individual wire-bottomed cages for the study duration. The rats were allowed free access to water.

All rats were infused enterally with a low-fat basal diet (Vital HN; Ross Laboratories, Columbus, OH) from 1500 h of day −1 until 0900 h of day 0 to facilitate rehydration and adaptation to enteral feeding as described (17). At 0900 h of day 0, subsets (n = 6–8/group) of rats were infused in continuous fashion with one of three diets (Table 1) @ 250 kcal/kg/d until 0900 h of either day 3 or day 6 using a programmable syringe pump (Model 42; Harvard Apparatus, Natick, MA). Under these conditions, we have found that the body weights and health of rats receiving similar test diets remain stable for at least 1 wk (17). All rats received fresh daily portions of their assigned diet to minimize the potential for bacterial growth during infusion.

Enteral diets. The rats were randomized to receive one of three high-fat, low-carbohydrate diets which have been utilized in the clinical setting (Table 1): an LA-enriched diet (59% of total fatty acids as LA) served as a standard control diet derived principally from corn oil; an LNA-enriched diet

TABLE 1
Principal Triglyceride Fatty Acids of Enteral Diets[a]

	mol% of Total Fatty Acids		
Fatty acid	LA Diet	LNA Diet	EPA + GLA Diet
Caprylic (8:0)	0.0	12.9	13.0
Capric (10:0)	0.0	9.0	9.9
Lauric (12:0)	0.1	0.2	0.2
Myristic (14:0)	0.2	0.2	1.2
Palmitic (16:0)	11.3	4.9	6.0
Palmitoleic (16:1n-7)	0.0	0.2	1.7
Stearic (18:0)	2.3	2.1	1.9
Oleic (18:1n-9)	25.0	42.1	26.0
Linoleic (18:2n-6)	59.4	21.1	16.7
γ-Linolenic (18:3n-6)	0.0	0.0	4.6
α-Linolenic (18:3n-3)	1.0	5.3	3.9
Eicosapentaenoic (20:5n-3)	0.0	0.0	5.3
Docosapentaenoic (22:5n-3)	0.0	0.0	0.5
Docosahexaenoic (22:6n-3)	0.0	0.0	2.5

[a]LA, linoleic acid; LNA, α-linolenic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

(5.3% LNA) represented an enteral diet containing canola oil, medium-chain triglycerides, and high-oleic safflower oil to reduce the dietary level of LA; the third diet also contained medium-chain triglycerides but was enriched with EPA (5.3%), LNA (3.9%), and GLA (4.6%) from sardine oil, canola oil, and borage oil, respectively. The fatty acid compositions of these diets were determined by gas chromatography. Each of these diets provided 16.7% of total energy as protein, 28.1% as carbohydrate, and 55.2% as lipid, and all were fortified with minerals and antioxidant vitamins (C, E, and β-carotene). Each diet was manufactured (Ross Products Division, Abbott Laboratories, Columbus, OH) in 237-mL portions and sealed under nitrogen.

Endotoxin infusion. We utilized a nonlethal model of endotoxicosis (17) which consistently produced the following clinical signs: piloerection, conjunctival hemorrhage, ear flattening, and general lethargy. The intravenous infusion of endotoxin lipopolysaccharide B (*Escherichia coli* 026:B6; Difco, Detroit, MI) in 1 g albumin/L of saline was started at 1500 h on day −1. The endotoxin solution was delivered continuously by syringe pump at 0.4 mg/kg for as long as the rats were undergoing enteral feeding.

Isolation of sinusoidal K&E cells. The method for collection and isolation of AM and sinusoidal K&E cells has been described (17). Briefly, each rat was anesthetized with 50 mg/kg pentobarbital administered by intraperitoneal injection at 0900 h of either day 0 (Baseline), day 3 (Day 3) or day 6 (Day 6), depending on the randomization schedule. After laparotomy, 0.5 mL heparin was injected into the vena cava and allowed to circulate. A 16-gauge angiocatheter was inserted in the exposed hepatic veins, and the liver lobes were flushed with HEPES buffer (5 mmol/L, pH = 7.2) in retrograde fashion. The liver was then flushed *in situ* with collagenase (2.5 g/L type II and 2.5 g/L type IV; Sigma, St. Louis, MO) in HEPES (pH = 7.6). The liver was then excised and incubated at 37°C for 15 min before separation of the cells from the supporting connective tissue. The sinusoidal cells were separated from the hepatocytes and other contaminating cells by differential centrifugation (2 min at $50 \times g$, three times) followed by isolation on a 17.5% metrizamide discontinuous gradient. The K&E cells removed from the gradient interface were resuspended in Grey's balanced salt solution prior to assessment of cell number and viability (>90%) by trypan blue exclusion under light microscopy. The K&E cells were then pelleted by centrifugation ($500 \times g$, 10 min) and stored at a density of 2×10^7 cells/mL at −20°C under nitrogen.

AM collection and isolation. A 16-gauge angiocatheter was inserted through a laparotomy into the superior vena cava, and the lung vasculature was flushed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) to remove blood components. The vena cava was severed and the rats expired by exsanguination. A 14-gauge angiocatheter was inserted through a tracheostomy, and the lungs were lavaged eight times with 5 mL cold PBS containing 0.6 mmol/L EDTA; gentle chest massage facilitated fluid recovery (>90%). The cumulative lavage fluid was kept on ice during this procedure. The lavage fluid was spun at $400 \times g$ for 10 min at 4ºC, and the cell pellet was then resuspended in PBS and centrifuged at $110 \times g$ for 10 min at 4ºC. The cell pellet was resuspended in PBS, layered on Ficoll Paque (Pharmacia, Piscataway, NJ), and centrifuged at $500 \times g$ at 22°C for 35 min to isolate the AM. Purity and viability by trypan blue exclusion were >95%. The AM were stored at a density of 5×10^6 cells/mL PBS under nitrogen in PBS at −20ºC for analysis of the fatty acid composition of the total phospholipid fraction.

Analysis of macrophage phospholipid fatty acids. Quantitation of 16 identifiable fatty acids (12–24 carbons in length) in the phospholipid fraction of AM and K&E cells was carried out (17). The lipid component of the cells was extracted in chloroform/methanol (2:1, vol/vol), and the phospholipid fraction was isolated on silica gel H (Analtech, Newark, DE) thin-layer plates using a mobile phase of petroleum ether/diethyl ether/glacial acetic acid (80:20:1, by vol), and identified relative to the migration of standards using dichlorofluorescein spray. The phospholipid band was isolated, hydrolyzed, and methylated with 140 g/L boron trifluoride in methanol. The fatty acid methyl esters were separated on a 50-m fused-silica capillary column containing SP-2330 (Supelco, Bellefonte, PA), using a Hewlett-Packard 5890 Series II gas chomatograph (Hewlett-Packard, Palo Alto, CA) equipped with a 7673 Autosampler. The relative mol% of individual fatty acids was identified and quantified using Chem Station software (Hewlett-Packard) based upon the relative responses of an external standard of pure fatty acid methyl esters (Nu-Chek-Prep, Elysian, MN).

Statistical analyses. Descriptive and inferential statistics were generated using computer-based programs. Generalized linear models for two-way analysis of variance were employed to examine the effects of diet and time for each cell type and fatty acid. Contrasts were included in the models to compare levels of each effect while holding levels of other effects fixed. Sensitivity analyses of model assumptions were robust under most data conditions, e.g., normality assumption. Statistical significance was set at $P < 0.05$.

RESULTS

The control (i.e., LA-enriched) and experimental [LNA- or (EPA + GLA)-enriched] diets were well tolerated during enteral infusion for the 3- and 6-d periods. All rats showed persistent signs of endotoxicosis, as described above, during endotoxin infusion. No significant differences occurred in initial or final body weights or changes in body weight among the dietary groups for a given time period.

Overall, the majority of dietary effects on the composition of phospholipid fatty acids of the alveolar (Table 2) and hepatic (Table 3) immune cells occurred rapidly within the first 3 d of enteral feeding. These changes generally reflected the differences in the composition of fatty acids present within each diet, with notable exceptions as discussed below. For example, after 3 d of continuous enteral feeding, the AM and K&E cells from the rats fed the LA diet had significantly

TABLE 2
Principal Long-Chain Fatty Acids of Alveolar Macrophage Phospholipids[a]

Fatty acid	Day 3			Day 6		
	LA diet	LNA diet	EPA + GLA diet	LA diet	LNA diet	EPA + GLA diet
Palmitic (16:0)	39.4 ± 2.9[a]	45.8 ± 1.3[b]	41.1 ± 2.6[a,b]	38.5 ± 1.5[a]	47.6 ± 1.3[b]	39.5 ± 1.6[a]
Palmitoleic (16:1n-7)	2.6 ± 0.6[a]	0.9 ± 0.2[b]	1.6 ± 0.3[a,b]	1.6 ± 0.5[a,b]	0.8 ± 0.03[b]	2.2 ± 0.4[a]
Stearic (18:0)	17.5 ± 1.0[a]	12.8 ± 0.7[b]	16.3 ± 1.0[a]	16.9 ± 0.9[a]	13.8 ± 0.7[b]	16.5 ± 0.5[a]
Oleic (18:1n-9)	6.8 ± 0.2[a]	14.7 ± 0.8[b]	9.3 ± 0.3[c]	7.8 ± 0.7[a]	13.8 ± 0.4[b]	9.1 ± 0.3[a]
Linoleic (18:2n-6)	11.6 ± 0.3[a]	6.2 ± 0.4[b]	6.0 ± 0.2[b]	11.9 ± 0.4[a]	5.7 ± 0.2[b]	5.3 ± 0.2[b]
Dihomo-γ-linolenic (20:3n-6)	0.06 ± 0.06[a]	0.08 ± 0.04[a]	0.72 ± 0.17[b]	0.11 ± 0.07[a]	0.00 ± 0.00[b]	0.30 ± 0.19[a]
Arachidonic (20:4n-6)	14.2 ± 0.9[a]	14.6 ± 0.7[a]	10.5 ± 0.8[b]	14.6 ± 0.8[a]	12.8 ± 0.6[a,b]	11.7 ± 1.0[b]
Eicosapentaenoic (20:5n-3)	0.00 ± 0.00[a]	0.02 ± 0.02[a]	2.5 ± 0.7[b]	0.04 ± 0.04[a]	0.00 ± 0.00[a]	3.7 ± 0.5[b,b]
Docosapentaenoic (22:5n-3)	0.6 ± 0.3[a]	1.0 ± 0.4[a]	2.8 ± 0.3[b]	0.2 ± 0.1[a]	1.5 ± 0.3[b]	2.8 ± 0.3[c]
Docosahexaenoic (22:6n-3)	1.9 ± 0.5[a]	1.6 ± 0.3[a]	4.7 ± 0.4[b]	1.8 ± 0.3[a]	1.7 ± 0.2[a]	4.8 ± 0.6[b]
Total saturates	59.4 ± 2.2	61.0 ± 1.1	60.4 ± 2.5	58.1 ± 1.1	63.5 ± 0.9	59.0 ± 2.0
Total monounsaturates	12.0 ± 1.4[a]	15.3 ± 0.6[b]	12.0 ± 0.8[a]	11.8 ± 1.0[a]	14.8 ± 0.5[b]	12.5 ± 0.4[a,b]
Total n-3 polyunsaturates	2.5 ± 0.6[a]	2.8 ± 0.6[a]	10.1 ± 1.2[b]	2.5 ± 0.7[a]	3.2 ± 0.4[a]	11.3 ± 1.4[b]
Total n-6 polyunsaturates	26.0 ± 1.1[a]	21.0 ± 0.8[b]	17.2 ± 0.9[c]	27.0 ± 0.9[a]	18.5 ± 0.6[b,b]	17.6 ± 0.9[b]

[a]Mean mol% + SEM; n = 6–8/group. Saturates = 14:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0. Monounsaturates = 16:1n-7 + 18:1n-9 + 20:1n-9 + 22:1n-9 + 24:1n-9. n-3 Polyunsaturates = 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3. n-6 Polyunsaturates = 18:2n-6 + 20:2n-6 + 20:3n-6 + 20:4n-6. Values with different roman superscript letters within each time interval are significantly different, $P < 0.05$ by analysis of variance.

[b]Significantly different from corresponding diet group on day 3, $P < 0.05$. See Table 1 for abbreviations.

TABLE 3
Principal Long-Chain Fatty Acids of Liver Kupffer and Endothelial Cell Phospholipids[a]

Fatty acid	Day 3			Day 6		
	LA diet	LNA diet	EPA + GLA diet	LA diet	LNA diet	EPA + GLA diet
Palmitic (16:0)	24.4 ± 0.8	22.4 ± 0.3	24.4 ± 0.9	23.5 ± 1.0[a,b]	21.3 ± 0.6[b]	24.8 ± 1.7[a]
Palmitoleic (16:1n-7)	0.3 ± 0.1	0.3 ± 0.04	0.4 ± 0.1	0.1 ± 0.1[a]	0.2 ± 0.1[a]	0.6 ± 0.1[b]
Stearic (18:0)	23.5 ± 0.4	22.4 ± 0.5	22.0 ± 0.9	22.6 ± 0.2	21.4 ± 0.7	22.0 ± 0.8
Oleic (18:1n-9)	7.8 ± 0.3[a]	14.7 ± 1.1[b]	9.7 ± 0.2[a]	8.6 ± 1.1[a]	14.7 ± 1.3[b]	9.3 ± 0.7[a]
Linoleic (18:2n-6)	13.9 ± 1.0[a]	11.5 ± 0.4[b]	9.2 ± 0.3[c]	15.9 ± 0.8[a,b]	12.7 ± 0.4[b]	9.5 ± 0.5[c]
Dihomo-γ-linolenic (20:3n-6)	0.8 ± 0.2[a]	0.8 ± 0.03[a]	2.6 ± 0.1[b]	1.1 ± 0.08[a]	0.9 ± 0.04[a]	2.3 ± 0.1[b]
Arachidonic (20:4n-6)	21.7 ± 1.0[a]	19.2 ± 0.7[b]	14.9 ± 0.7[c]	20.8 ± 0.5[a]	20.8 ± 0.3[a]	14.7 ± 1.0[b]
Eicosapentaenoic (20:5n-3)	0.20 ± 0.11[a]	0.29 ± 0.04[a]	5.36 ± 0.73[b]	0.18 ± 0.18[a]	0.26 ± 0.07[a]	5.51 ± 1.14[b]
Docosapentaenoic (22:5n-3)	0.5 ± 0.2[a]	1.6 ± 0.1[b]	3.7 ± 0.3[c]	0.3 ± 0.2[a]	1.7 ± 0.4[b]	3.1 ± 0.3[c]
Docosahexaenoic (22:6n-3)	2.1 ± 0.3[a]	2.7 ± 0.3[a]	4.0 ± 0.5[b]	3.2 ± 0.3[a,b,b]	2.8 ± 0.3[b]	3.6 ± 0.3[a]
Total saturates	49.3 ± 1.2	46.4 ± 0.4	47.5 ± 1.6	48.2 ± 0.9[a]	44.1 ± 0.6[b]	48.8 ± 2.3[a]
Total monounsaturates	9.9 ± 0.4[a]	16.7 ± 1.1[b]	11.8 ± 0.7[a]	9.8 ± 1.2[a]	16.2 ± 1.1[b]	12.6 ± 1.0[a]
Total n-3 polyunsaturates	2.8 ± 0.3[a]	4.8 ± 0.3[a]	13.5 ± 1.2[b]	3.6 ± 0.2[a]	4.8 ± 0.5[a]	11.4 ± 1.7[b]
Total n-6 polyunsaturates	37.6 ± 1.2[a]	32.2 ± 0.9[b]	27.1 ± 1.0[c]	39.2 ± 1.3[a]	34.9 ± 0.4[b,b]	26.9 ± 0.9[c]

[a]See Table 2 for footnote information.

higher mole percentage of LA and total n-6 PUFA than corresponding cells from the LNA or (EPA + GLA)-supplemented rats. However, AA levels in these immune cells were relatively similar across the LA and LNA diet groups after 6 d. AM and K&E cells from rats given the LNA diet which contained high-oleic safflower oil had higher levels of 18:1n-9 and total monounsaturates than cells from rats infused with the LA or EPA + GLA diet after 3 or 6 d of feeding. Levels of LNA, stearidonic acid, and GLA were either negligible or not detectable in most cell phospholipids (data not shown). As observed in previous studies (16,17), the mole percentage of DHGLA was significantly higher in both the AM (Table 2) and K&E cell (Table 3) phospholipids from rats that had received the EPA + GLA diet relative to rats given either the LA or LNA diet after either 3 or 6 d.

With regard to the long-chain n-3 PUFA (i.e., EPA, DPA, or DHA), the immune cells from rats that received the EPA + GLA for 3 d had significantly higher ($P < 0.001$) enrichment of each of these n-3 PUFA than that observed in corresponding cells from either the LA or LNA rats. Moreover, the percentage of EPA present in the AM after 6 d of feeding the EPA + GLA diet was higher (3.7 vs. 2.5%, $P < 0.05$) than that observed after 3 d (Table 2), whereas K&E cell EPA remained unchanged after an additional 3 d of feeding (Table 3). Given that the levels of EPA, DHA, and total n-3 PUFA present in either the AM or K&E cells from the rats given the LNA-supplemented diet for 3 or 6 d were similar to those measured in the corresponding cells from rats given the LA diet, these data indicate that dietary LNA was not effectively elongated and desaturated to the longer-chain n-3 PUFA under existing endotoxemic conditions. Interestingly, a small but significantly higher mole percentage of DPA was measured in the K&E cells (Table 3) on both day 3 and day 6, but only on day 6 in the AM (Table 2) from rats given the LNA diet vs. the LA diet.

DISCUSSION

The data from this study indicated that provision of a diet enriched with LNA to endotoxemic rats by continuous enteral infusion for either 3 or 6 d did not promote EPA or DHA formation or displacement of AA from AM or K&E phospholipids *in vivo*. In addition, rats which had received the LA-enriched diet for 6 d did not have increased levels of DHGLA or AA in the AM or K&E cell phospholipids relative to the rats given the LNA-enriched diet. As discussed above, these outcomes may be attributable to several compounding factors. While Δ6 desaturation of both LNA to stearidonic acid (18:4n-3) and LA to GLA is a rate-limiting step under normal conditions, evidence exists to suggest that Δ6-desaturase activity is further reduced under conditions that foster release of epinephrine and other stress-associated hormones (29–31). This hormonal milieu can be found in traumatized or acutely septic patients. The finding that the mole percentage of DPA was increased in immune cells from rats consuming the LNA diet (Tables 2 and 3) attests to the probability that Δ6-desaturase activity was a primary factor influencing fatty acid metabolism and incorporation into these immune cells. In this regard, other investigators have proposed that the conversion of DPA to DHA occurs independently of Δ4 desaturase, i.e., DPA is instead initially elongated to 24:5n-3 prior to Δ6 desaturation to form 24:6n-3, which in turn undergoes β-oxidation to form 22:6n-3 (34). Thus, inhibition of Δ6-desaturase activity would foster a buildup of both DPA and 24:5n-3, with preferential β-oxidation of 24:5n-3 back to DPA under these conditions (34).

The limitations imposed by Δ6 desaturase on the metabolism of LNA or LA under endotoxemic conditions can be circumvented by providing dietary fatty acids that bypass the Δ6 desaturation step (Figs. 1 and 2). As shown in the present study, dietary EPA was readily incorporated into the AM and K&E cell membrane phospholipids irrespective of concurrent endotoxemia (Tables 2 and 3). Similarly, the significant increase in immune cell DHGLA content in rats given the EPA + GLA diet demonstrated that GLA metabolism was not inhibited during endotoxicosis. In this regard, EPA enhances accumulation of DHGLA by decreasing Δ5-desaturation activity and conversion of DHGLA to AA (35). As observed previously (16,17), the incorporation of EPA and DHGLA promoted the rapid displacement of AA and its precursor, LA, from AM and K&E cell phospholipids. As we and others have shown, this exchange of PUFA favors the formation of eicosanoids derived from EPA and DHGLA, which have reduced inflammatory properties, as opposed to eicosanoids derived from AA which are more vasoactive and proinflammatory (8,13,14,19).

It is not known whether the observed modulation of immune cell phospholipid fatty acids is due primarily to exchange with plasma lipoprotein fatty acids or to elongation/desaturation processes within the macrophages (36). Our previous studies in endotoxemic rats enterally fed with either an EPA (11) or EPA + GLA diet (17) revealed that plasma phospholipids are enriched with EPA or EPA and DHGLA, respectively. We did observe in the present study that the mole percentage of EPA in AM increased significantly between day 3 and day 6 (Table 2). Given the relative isolation of these cells from the liver and intestine as well as the circulating plasma within the lung capillaries, the latter finding suggests that exchange mechanisms could play an important role for incorporation of EPA into AM phospholipids over time. However, the concurrent findings that the mole percentage of the GLA and EPA metabolites, i.e., DHGLA, and DPA and DHA, respectively, did not increase further after day 3 (Table 2) and that GLA was not detected in these cells would suggest that elongation and desaturation processes also may have facilitated fatty acid modification (36). With regard to the K&E cells, we did not observe any significant modifications in fatty acid composition between day 3 and day 6 (Table 3). The proximity of the K&E cells to hepatocytes as well as to dietary fatty acids present in the portal circulation (37) would favor exchange processes as a mechanism for rapidly modulating the composition of the phospholipid fatty acids within these immune cells.

In summary, short-term enteral feeding of a diet enriched with LNA and a reduced level of LA under endotoxemic conditions did not promote AA displacement or formation of EPA in lung and liver immune cell phospholipids *in vivo*. The results of this study did demonstrate that provision of dietary EPA and GLA to circumvent the rate-limiting step of Δ6 desaturation, while also reducing dietary LA, rapidly decreased the mole percentage of AA and LA present in lung and liver immune cell phospholipids within a clinically relevant time frame irrespective of concurrent endotoxemia. In addition, displacement of AA and LA occurred in conjunction with the incorporation of EPA and DHGLA into the cell phospholipids. The exchange of AA and LA with EPA and DHGLA can foster generation of eicosanoids with reduced inflammatory properties, which should prove beneficial to critically ill patients with excessive systemic inflammation due in part to persistent generation of vasoactive eicosanoids derived from AA.

ACKNOWLEDGMENTS

This study was supported in part by a grant from Ross Products Division, Abbott Laboratories, Columbus, OH. The authors gratefully acknowledge the technical assistance of Anne Rudick and Vicky Huang.

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[Received July 13, 1998, and in final revised form and accepted October 2, 1998]

Effect of Tetracosahexaenoic Acid on the Content and Release of Histamine, and Eicosanoid Production in MC/9 Mouse Mast Cell

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ABSTRACT: 6,9,12,15,18,21-Tetracosahexaenoic acid (24:6n-3) was isolated from a brittle star, *Ophiura sarsi* Lütken, at >95% purity to evaluate its physiological functions. The effects of 24:6n-3 on the production of leukotriene (LT)-related compounds such as LTB_4, LTC_4 and 5-hydroxyeicosatetraenoic acid, and the accumulation and release of histamine in an MC/9 mouse mast cell line were studied. We found that 24:6n-3 could inhibit the antigen-stimulated production of LT-related compounds as well as other n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), which are major n-3 PUFA in fish oils; 24:6n-3 was also shown to reduce the histamine content in MC/9 cells at 25 μM (27% reduction from the control), and the effect was diminished with increase of the fatty acid concentration (up to 100 μM). These two n-3 PUFA, 20:5n-3 and 22:6n-3, also reduced the histamine content (16 and 20% reduction at 25 μM, respectively), whereas arachidonic acid (20:4n-6) increased it (18% increase at 25 μM). Spontaneous- and antigen-induced release of histamine was not influenced with these PUFA (at 25 μM). Ionophore-stimulated release of histamine was suppressed by the PUFA (13, 9, 15, and 11% reduction with 20:4n-6, 20:5n-3, 22:6n-3, and 24:6n-3, respectively). The patterns of the effects of 24:6n-3 on the synthesis of eicosanoids and histamine content were more similar to those of 22:6n-3 than 20:5n-3. From these results, 24:6n-3 can be expected to have anti-inflammatory activity and antiallergic activities similar to those of 22:6n-3.
Lipids 33, 1107–1114 (1998)

Since the discovery by Dyerberg *et al.* (1–3) of a low incidence of heart disease in Eskimos, the nutritional, physiological, and pharmacological effects of fish oil on humans have been recognized, and several studies on the beneficial effects of fish oil have been carried out. As a result of these studies, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), which are major n-3 polyunsaturated fatty acids (PUFA) rich in fish oil, were demonstrated to have beneficial effects on pathophysiological states like coronary heart disease, hyperlipidemia, and inflammation (4).

Fish oil contains various n-3 PUFA besides 20:5n-3 and 22:6n-3. 6,9,12,15,18,21-Tetracosahexaenoic acid (24:6n-3) is one of the n-3 PUFA and is a very long chain fatty acid (5). Distribution of 24:6n-3 in marine organisms was investigated by several researchers. Takagi *et al.* (6) reported relatively high contents of 24:6n-3 in sea lilies and brittle stars (4–10% of total fatty acids). High 24:6n-3 content was also found in marine coelenterates (7). In some edible fishes, 24:6n-3 was detected at significant levels (0–10% of total fatty acids) (8,9).

The existence of 24:6n-3 in mammalian tissues was reported with other very long chain fatty acids in the spermatozoa (10), the retina (11), and the brain (12). Voss *et al.* (13) reported that 24:6n-3 is formed as an intermediate in the metabolic pathway from 20:5n-3 to 22:6n-3 in rat liver.

Even though 24:6n-3 is a PUFA existing in fish and mammalian species, physiological functions of 24:6n-3 have not been studied. As functions to be studied, anti-inflammatory and antiallergic effects of 24:6n-3 are noteworthy because these events are known to be closely related to the unsaturated fatty acid metabolism such as in the arachidonic acid cascade, and 20:5n-3 and 22:6n-3 were reported to suppress inflammatory actions by influencing arachidonic acid metabolism (4,14–16).

In this paper, we isolated 24:6n-3 from brittle stars, *Ophiura sarsi* Lütken, and examined the effects on the production of eicosanoids and the accumulation and release of histamine, using an MC/9 mouse mast cell line (17). Histamine and eicosanoids are produced and released by mast cells and act as chemical mediators in allergic reactions (18). MC/9 cells accumulate histamine and synthesize lipoxygenase pathway metabolites such as leukotriene (LT) B_4, LTC_4, and 5-hydroxyeicosatetraenoic acid (5-HETE) from arachidonic acid (20:4n-6)(19,20).

EXPERIMENTAL PROCEDURES

Materials. Linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) were purchased from Wako Pure Chemical Indus-

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Abbreviations: DNP-KLH, dinitrophenylated keyhole limpet hemocyanine; FAME, fatty acid methyl ester; FFA, free fatty acid; GC, gas chromatography; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatograph; IgE, immunoglobulin E; LT, leukotriene; PUFA, polyunsaturated fatty acid.

try (Osaka, Japan), and 20:4n-6 and 22:6n-3 from Sigma Chemical Company (St. Louis, MO); all of these fatty acids were obtained as methyl esters. The 20:5n-3 ethyl ester was a kind gift from Nissui Pharmaceuticals (Tokyo, Japan). Prostaglandin B_2, LTB_4, LTC_4, LTD_4, LTE_4, 5-HETE, 12-HETE, and 15-HETE were obtained from Cascade Biochem Ltd. (Reading, United Kingdom). RPMI-1640 medium, a culture medium for MC/9, was purchased from Nissui Pharmaceuticals; fetal calf serum, from Biological Industry Inc. (Kibbutz, Israel); dinitrophenylated keyhole limpet hemocyanine (DNP-KLH), from LSL (Tokyo, Japan); and murine anti-DNP-KLH immunoglobulin E (IgE) and calcium ionophore A23187, from Sigma. All other chemicals used in this report were of analytical grade or higher.

The MC/9 mouse mast cell line was purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10 μM β-mercaptoethanol (Wako), 10% fetal calf serum, and 20% conditioned medium of WEHI-3 cell (Riken Gene Bank, Saitama, Japan), which is a murine monocyte-like cell and produces growth factor(s) to support the growth of MC/9. WEHI-3-conditioned medium was prepared as described by Razin *et al.* (21)

Preparation and purification of 24:6n-3 from brittle star. Brittle stars, *O. sarsi* Lütken, were caught at Wakasa Bay, Fukui Prefecture, Japan. Total lipid of the brittle stars was extracted by Bligh and Dyer's method (22). Fatty acid methyl esters (FAME) were prepared by refluxing total lipid in 3% HCl-methanol for 3 h. The methyl esters were fractionated by silver-nitrate impregnated thin-layer chromatography in toluene/acetic acid (19:4, vol/vol). The chromatogram was visualized under ultraviolet light (360 nm) following spraying with 0.005% primuline in acetone/water (1:1, vol/vol). FAME retarded at the origin (including 20:5n-3, docosahexaenoic acid and 24:6n-3 as major components) were scraped from the thin-layer chromatography plate and extracted by the method of Folch *et al.* (23). The extracted FAME were saponified in a mixture of 10 N NaOH and methanol (1:9, vol/vol) for 3 h at 4°C on a magnetic stirrer. Free fatty acids (FFA) were then extracted with hexane after acidification with 6 N HCl. FFA were dissolved in 90% methanol and applied to the high-performance liquid chromatograph (HPLC) (Waters 600 system) using a μ-Bondapak ODS column (Waters; 20 × 150 mm) and methanol/water (9:1, vol/vol) as the mobile phase (5 mL/min). Elution of FFA was monitored with a Waters Multiwavelength ultraviolet/visible detector Model 490 at 217 nm. A peak of 24:6n-3 was collected and was dried with a rotary evaporator. The residue was dissolved in argon-bubbled ethanol at a concentration of 50 mM and stored at −80°C. Small portions of it were applied for gas chromatography (GC) and GC–mass spectrometry analyses after derivatization (methyl esterification and pyrrolidination, respectively) (24). A GC analysis of FAME was carried out at 205°C with a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a flame-ionization detector and Omegawax 320 capillary column (0.32 mm × 30 m; Supelco Inc., Bellefonte, PA). The GC–mass spectrometric analysis was performed using JEOL JMS-DX303 (column: Omegawax 320, 260°C, EI 70eV; JEOL, Tokyo, Japan). Other fatty acids used in this report were saponified and purified by HPLC in the same manner as the case for 24:6n-3.

Stimulation of MC/9 cells. MC/9 cells grown as described in the preceding section were collected and washed with Ca^{2+}-, Mg^{2+}-free phosphate buffered saline twice and suspended in Tyrode solution (10^7 cells/mL). Cell suspension was dispensed into 1.5 mL micro test tubes (0.25 mL for histamine assay, 0.5 mL for eicosanoid assay). For stimulation with ionophore A23187, cell suspensions were added with A23187 in ethanol (final conc. 5 μM) and incubated for 10 min. For stimulation with antigen-IgE complex, cells were sensitized by incubating with murine monoclonal anti-DNP-KLH IgE (100 ng/10^6 cells) for 1 h. The cells were pelleted by centrifugation. The supernatant was discarded and the cells were resuspended in Tyrode. The sensitized cells were stimulated by the addition of the antigen, DNP-KLH (100 ng/10^6 cells), and incubated for 10 min. In case of eicosanoid production assay, the cell suspensions contained 30 μM arachidonic acid as a substrate for lipoxygenase pathway unless otherwise indicated in figure legends.

Assay of the production of eicosanoids. To stop the LT production of stimulated cells, 1 mL of acetonitrile containing 1 nmol of prostaglandin B_2 (internal standard) was added to the cell suspension. The mixture was stored at −20°C overnight and centrifuged at 10,000 × *g* to precipitate proteins. The supernatant was treated as described by Eskra *et al.* (25) on a Sep-Pak C18 cartridge (Waters) to extract the LT and HETE. The samples were dried with a centrifuge evaporator (Iwaki Glass Co., Tokyo, Japan) and dissolved in 200 μL of 66% methanol/ water. Of the sample, 100 μL was injected onto an HPLC with a Novapak ODS column (4 × 150 mm; Waters), equipped with a Waters 717 plus autosampler connected to a Waters model 600 pump system. Elution was carried out using a nonlinear gradient program as follows: 0–18 min, methanol/water/trifluoroacetic acid/triethylamine (65:35:0.09:0.05, by vol), 18–36 min; methanol/water/trifluoroacetic acid/triethylamine (75:25:0.09:0.05, by vol), 36–40 min; 100% acetonitrile (solvent flow: 1 mL/min). Elution was monitored with a photodiode array detector (Model 996; Waters). LT and HETE were quantified at 280 and 235 nm, respectively, with Waters Millennium software. With this procedure, LT (LTB_4, LTC_4, LTD_4, LTE_4) and HETE (5-HETE, 12-HETE, 15-HETE) were completely separated.

Assay of histamine accumulation and release. MC/9 cells were incubated in the medium containing various amounts of FFA (0–100 μM) for 24 h at a density of 2×10^6 cells/mL. The cells were collected by centrifugation at 100 × *g* for 5 min, washed with phosphate buffered saline twice, and suspended in Tyrode solution (10^7 cells/mL). For measurement of total histamine content, the cell suspension was added with 2.5% perchloric acid to achieve the final concentration at 1%. For histamine release assay, the cell suspension was stimu-

lated with ionophore or antigen as described above. Histamine release was stopped by the addition of 125 μL of ice-cold 4 mM EDTA in Ca^{2+}-, Mg^{2+}-free Tyrode. The cells were pelleted by centrifugation at 100 × *g* for 10 min at 4°C. An aliquot of the supernatant (200 μl) was mixed with 133 μL of 2.5% perchloric acid. The cell pellet was washed once with ice-cold 4 mM EDTA/Tyrode, resuspended in 200 μL of the same solution, and mixed with 133 μL of 2.5% perchloric acid. Each mixture was incubated in boiling water for 5 min to extract histamine and centrifuged at 10,000 × *g* for 5 min. The supernatants were used to measure the histamine concentration by the on-column HPLC method described by Saito *et al.* (26) with a Waters 600 pump connected with Waters 717 plus autosampler and model 474 fluorescence detector. Histamine release was expressed as a percentage of the histamine of the supernatant in the sum of the histamine of the supernatant and the cell suspension.

Statistical analysis. Data obtained in this report were analyzed by one-way analysis of variance. If significant, data were further analyzed by Duncan's multiple range test (27).

RESULTS

Isolation of 24:6n-3 from brittle stars. The purity of the fatty acid isolated from the brittle stars was >95% estimated by using GC analysis (data not shown). From the mass spectrum of the pyrrolidine derivatives of the fatty acid, double-bond locations were confirmed at the 6-, 9-, 12-, 15-, 18-, and 21-positions from the fragmentation pattern showing a molecular ion at *m/z* 409 and fragments at 394, 380, 366, 354, 340, 326, 314, 300, 286, 274, 260, 246, 234, 220, 206, 194, 180, and 168 *m/z*. Thus, the fatty acid purified from the brittle stars was identified as 24:6n-3. Identification of 24:6n-3 was confirmed by comparison of the equivalent chain length value on a Silar 5CP column (Supelco) with the value reported (9).

Effect of substrate concentration on eicosanoid production in MC/9 cells. In the absence of exogenously added 20:4n-6 as a substrate for eicosanoid production, neither LT nor HETE was observed in both ionophore- and antigen-stimulated MC/9 cells (Fig. 1). The production of LTB_4, LTC_4, and 5-HETE increased with increasing concentration of 20:4n-6. The synthesis of LTB_4 and LTC_4 reached almost a plateau

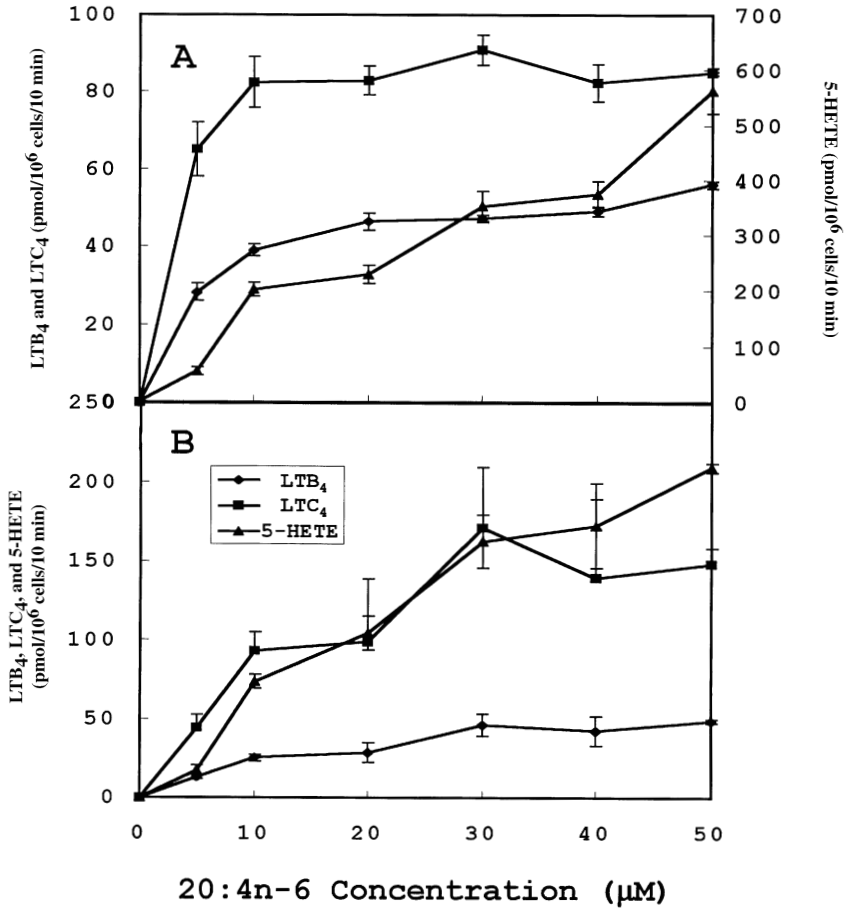

FIG. 1. Dose dependence of 20:4n-6 on the production of eicosanoids in: (A) ionophore- and (B) antigen-stimulated MC/9 cells. MC/9 cells were stimulated with (A) ionophore and (B) antigen in the presence of various concentrations of 20:4n-6 (0–50 μM). Leukotriene B_4 (LTB_4), LTC_4, and 5-hydroxyeicosatetraenoic acid (5-HETE) were analyzed as described in the Experimental Procedures section. Values are the mean of 3–4 samples. Vertical bars indicate the standard error of the mean.

from a concentration of 30 μM of 20:4n-6 although 5-HETE production increased with increasing concentrations of the substrate (Fig. 1A and B). Thus, we determined the concentration of 20:4n-6 as a substrate for the following assay of eicosanoid production in MC/9 at 30 μM.

Inhibition of eicosanoid production by 24:6n-3 in comparison with other n-3 PUFA. In the ionophore-stimulated production of eicosanoids, LTB_4 production tended to be suppressed by 20:5n-3, 22:6n-3, and 24:6n-3 (Fig. 2). However a significant difference was observed only in the presence of 20:5n-3 (30, 45 μM). Concerning the production of LTC_4 and 5-HETE, no significant inhibition by PUFA was observed in the ionophore-activated cells, although n-3 PUFA tended to suppress weakly the production of the two eicosanoids (Fig. 2B and C). In antigen-stimulated cells, inhibition of eicosanoid production by n-3 PUFA appeared more clearly (Fig. 3). LTB_4 production was inhibited strongly by 20:5n-3 and less strongly by 22:6n-3 (Fig. 3A); 24:6n-3 only exhibited a slight inhibitory effect on LTB_4 production. As for LTC_4 synthesis, the inhibitory effect of n-3 PUFA was more evident (Fig. 3B). All of n-3 PUFA significantly suppressed LTC_4 production. The inhibitory activity of 24:6n-3 was weaker than that of the other two PUFA (20:5n-3 and 22:6n-3), as a significant inhibition by 24:6n-3 was not found at the lowest concentration (15 μM). The production of 5-HETE was also significantly suppressed by all PUFA (Fig. 3C).

Concentration dependence of the inhibition of eicosanoid production by 24:6n-3. Antigen-stimulated eicosanoid production was inhibited by 24:6n-3, the inhibitory effect increasing with 24:6n-3 concentration (Fig. 4B). In the ionophore-activated MC/9 cells, LTB_4 synthesis tended to be suppressed with increase in the concentration of 24:6n-3 (Fig.

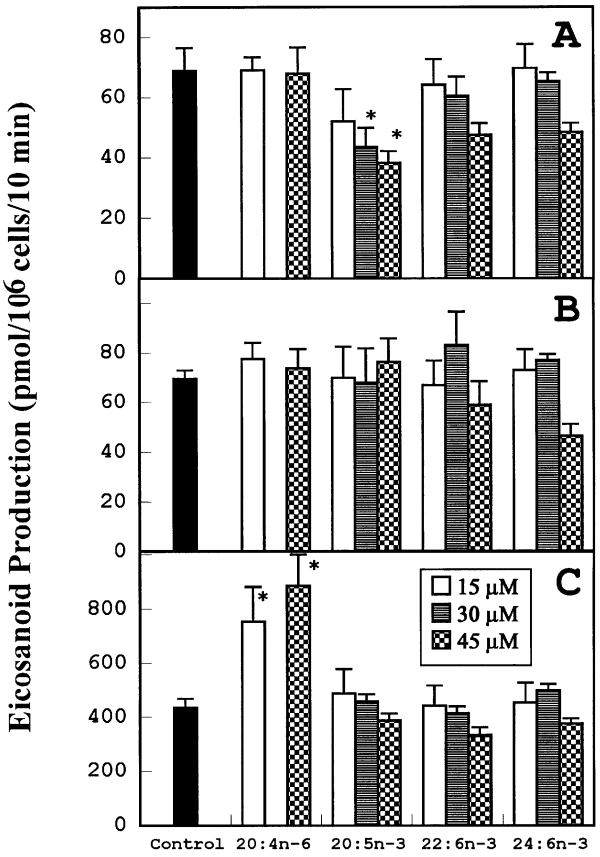

FIG. 2. Effect of 24:6n-3 on the production of lipoxygenase pathway metabolites in ionophore-stimulated MC/9 cells. MC/9 cells were stimulated by the addition of calcium ionophore A23187 and 20:4n-6 (30 μM as a substrate). The fatty acids were added simultaneously with the stimulants at 15, 30, and 45 μM (20:4n-6 was added additionally to the substrate 20:4n-6). (A) LTB_4, (B) LTC_4, and (C) 5-HETE were analyzed as described in the Experimental Procedures section. Values are the means of 3–4 samples. Vertical bars indicate the standard error of the mean. *Significantly different from the control at $P < 0.05$. See Figure 1 for abbreviations.

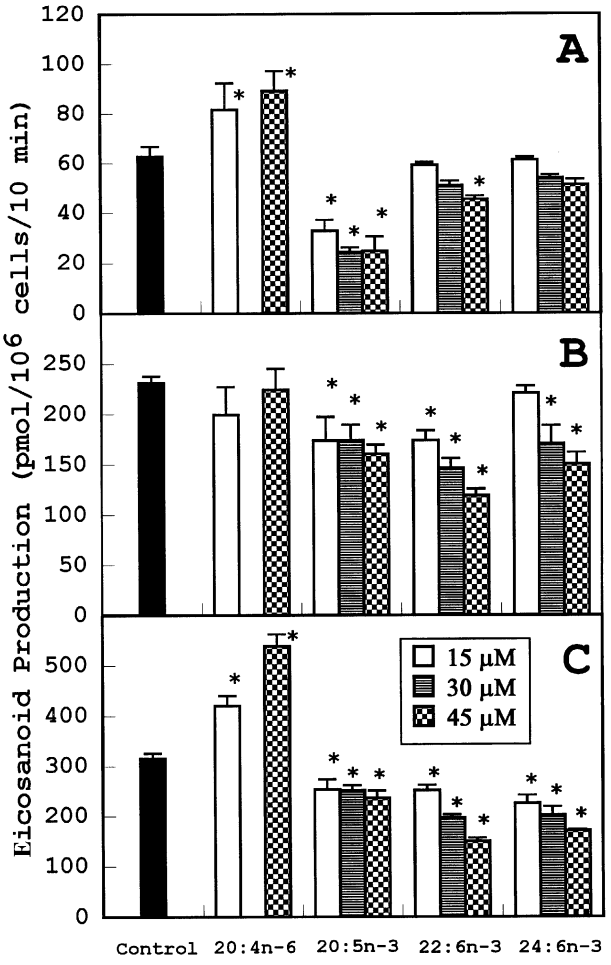

FIG. 3. Effect of 24:6n-3 on the production of eicosanoids in antigen-stimulated MC/9 cells. Immunoglobulin E-sensitized MC/9 cells were stimulated by the addition of antigen, dinitrophenylated keyhole limpet hemocyanine, and 20:4n-6 (30 μM as a substrate). The fatty acids were added simultaneously with the stimulants at 15, 30, and 45 μM (20:4n-6 was added additionally to the substrate 20:4n-6). (A) LTB_4, (B) LTC_4, and (C) 5-HETE were analyzed as described in the Experimental Procedures section. Values are the means of 3–4 samples. Vertical bars indicate the standard error of the mean. *Significantly different from the control at $P < 0.05$. See Figure 1 for abbreviations.

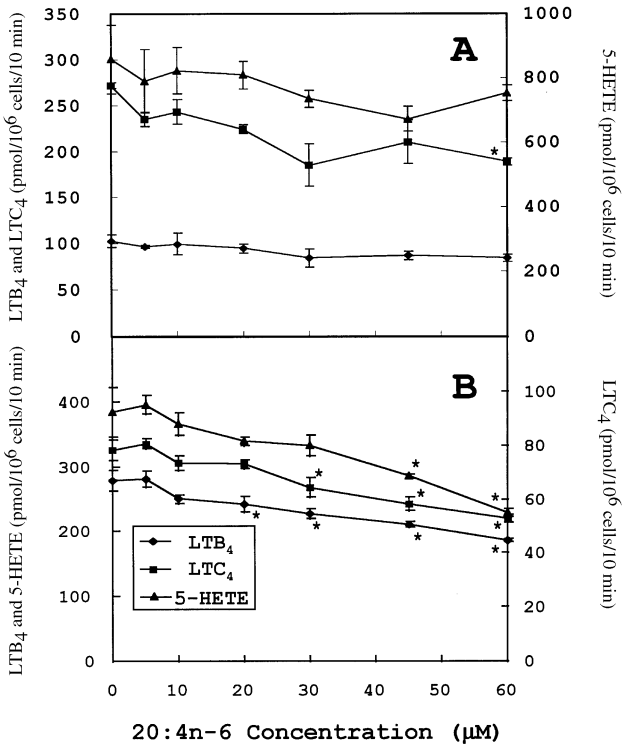

FIG. 4. Effect of the concentration of 24:6n-3 on the production of eicosanoids in (A) ionophore- and (B) antigen-stimulated MC/9 cells. MC/9 cells were stimulated with (A) ionophore and (B) antigen in the presence of various concentrations of 24:6n-3 (0–60 μM). All samples contained 30 μM of 20:4n-6 as a substrate. LTB_4, LTC_4, and 5-HETE were analyzed as described in the Experimental Procedures section. Values are the mean of 3–4 samples. Vertical bars indicate the standard error of the mean. *Significantly different from the control at $P < 0.05$. See Figure 1 for abbreviations.

4A). Significant suppression of LTC_4 production was observed at 60 μM of 24:6n-3 (Fig. 4A); however, significant inhibition of 5-HETE production was not observed. In the antigen-stimulated production of eicosanoids, a significant inhibition appeared at 20 μM and higher concentrations of 24:6n-3 for LTB_4 synthesis, 30 μM and higher for LTC_4 production, and 45 μM and higher concentrations for 5-HETE production, respectively (Fig. 4B).

Histamine content in MC/9 cells incubated with PUFA. MC/9 cells were incubated with PUFA (20:4n-6, 20:5n-3, 22:6n-3, and 24:6n-3) at various concentrations (0–100 μM) for 24 h, and the histamine content of the cells was measured (Fig. 5). A dose-dependent relationship was observed in MC/9 cells treated with 20:4n-6. On the other hand, with 20:5n-3, histamine levels decreased with increase of substrate concentration. Cellular histamine content did not decrease with increase in the concentrations of 22:6n-3 and 24:6n-3. At lower concentrations of 22:6n-3 and 24:6n-3, the histamine content was lower than the control. But at higher concentrations (100 μm) of these two PUFA, histamine content was not significantly different from the control.

Effect of PUFA on spontaneous, ionophore-induced, and antigen-stimulated release of histamine. Spontaneous, ionophore-induced, and antigen-stimulated release of histamine was observed in MC/9 cells incubated with 25 μM of various PUFA for 24 h (Fig. 6). All PUFA used did not influence the spontaneous and antigen-induced release of histamine in MC/9 cells (Fig. 6A and C). In the ionophore-stimulated cells, PUFA except for 18:3n-3 exerted a suppressive effect on the histamine release (Fig. 6B).

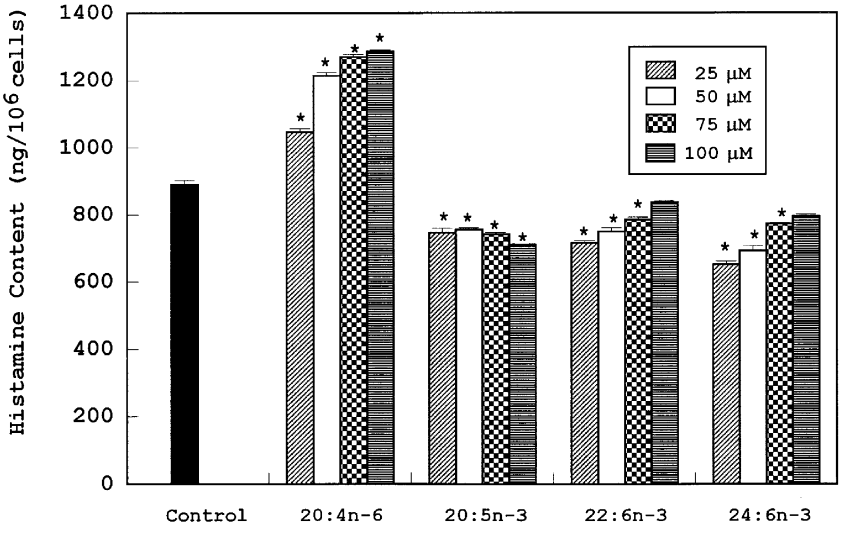

FIG. 5. Effect of 24:6n-3 on the accumulation of histamine in MC/9 cells. MC/9 cells were incubated with polyunsaturated fatty acids at various concentrations (0–100 μM) for 24 h. Cellular histamine content was measured as described in the Experimental Procedures section. Values are the mean of four samples. Vertical bars indicate the standard error of the mean. *Significantly different from the control at $P < 0.05$.

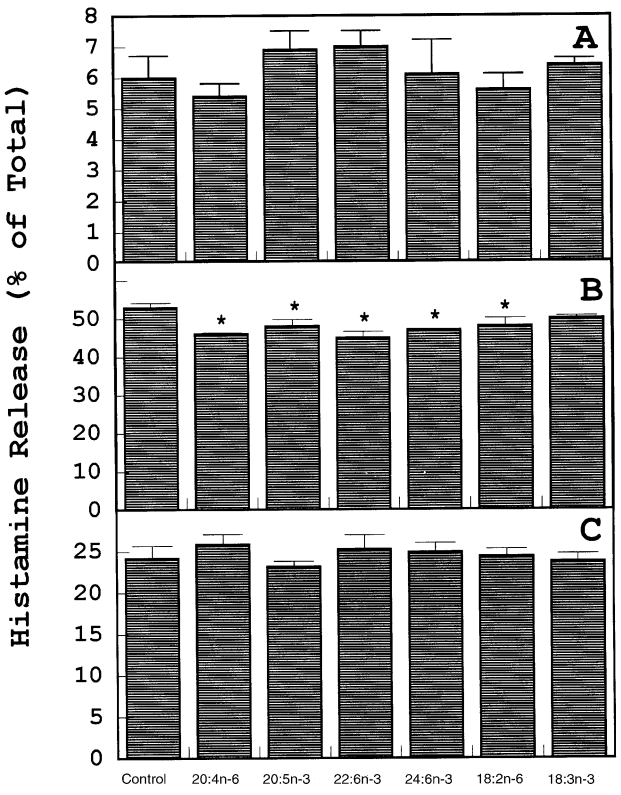

FIG. 6. Effect of 24:6n-3 on histamine mobilization in MC/9 cells. MC/9 cells were incubated with 25 μM of polyunsaturated fatty acids for 24 h. (A) Spontaneous, (B) ionophore-stimulated, and (C) antigen-activated releases of histamine were measured as described in the Experimental Procedures section. Values are the mean of four samples. Vertical bars indicate the standard error of the mean. *Significantly different from the control at $P < 0.05$.

DISCUSSION

In the present study, we isolated 24:6n-3 from brittle stars, *O. sarsi* Lütken, and investigated the effects of 24:6n-3 on the production of eicosanoids in the lipoxygenase pathway and the accumulation and release of histamine in MC/9 mouse mast cells in comparison with the effects of other PUFA including 20:5n-3 and 22:6n-3. The MC/9 mouse mast cell clone was isolated from mouse fetal liver and resembles mucosal mast cells (17,20), which accumulate histamine and release it in response to stimulation of the IgE-antigen complex or calcium ionophore (19,20). MC/9 cells also produce eicosanoids such as PGD_2, thromboxane B_2, LTB_4, LTC_4, and 5-HETE from exogenous and endogenous 20:4n-6 (19,20). Histamine- and lipoxygenase-pathway metabolites are known to be produced by mast cells and are involved in inflammatory and allergic reactions as chemical mediators (18). Therefore, in using MC/9 cells the effect of 24:6n-3 on the accumulation and release of histamine and eicosanoid production must be assessed.

In the absence of exogenously added 20:4n-6, no eicosanoid was detected in our experiment (Fig. 1). Thus, in our experimental system, we assessed the synthesis of eicosanoids from exogenously added 20:4n-6. LT (LTB_4 and LTC_4) synthesis reached a plateau at 30 μM of 20:4n-6 in both ionophore- and antigen-stimulated MC/9 cells (Fig. 1). Thus, we set the concentration of 20:4n-6 at 30 μM as a substrate for the assay of the inhibitory activity of 24:6n-3 on eicosanoid production. Reportedly, the plasma FFA level is about 0.3 mM (20). Thus, the concentration of 20:4n-6 seemed to be adequate for physiological concentration of individual fatty acids. For the same reason, the concentration of PUFA (25 μM) that we used for observations on their effect on histamine release also seemed to be physiological (Fig. 6).

The suppressive effect of 20:5n-3 and 22:6n-3 on the synthesis of lipoxygenase pathway metabolites *in vitro* has previously been described (14,16,29–34). Most of the reports studied the effects of PUFA on LT and HETE production from endogenous 20:4n-6 (16,29–32), although this study examined LT and HETE formation from exogenous 20:4n-6. As for LTB_4 formation, the suppression by 20:5n-3 has been demonstrated (16,29–32,34). The fatty acid 22:6n-3 was reported to have both no suppressive activity on LTB_4 generation (14,16) and a stronger inhibitory activity of 22:6n-3 than 20:5n-3 (31). In our present results, the effects of PUFA on LT and HETE synthesis in ionophore-activated cells (Fig. 2) roughly agreed with previous findings (suppression of LTB_4 synthesis by 20:5n-3 and little or no effect by 22:6n-3). The inhibition pattern of PUFA on antigen-stimulated production of LT and HETE was significantly different from the above-mentioned reports and the present result in the ionophore-stimulated cells (Fig. 3). In antigen-stimulated cells, LTB_4 production was inhibited most strongly by 20:5n-3 (Fig. 3A). LTC_4 and 5-HETE generation was suppressed more strongly by 22:6n-3 than by 20:5n-3 (Fig. 3B, C). Differences in the suppression pattern by PUFA in antigen-stimulated cells from ionophore-stimulated cells suggest that the processes of antigen-induced release of LT and HETE contain step(s) that could be inhibited by n-3 PUFA other than processes in ionophore-induced LT and HETE production.

In the dose-dependence study of 24:6n-3 on the synthesis of LT and HETE, 24:6n-3 showed minimal inhibition of the production of LT and HETE in ionophore-stimulated cells (Fig. 4A). In antigen-activated cells, the production of LT and HETE was inhibited dose dependently by 24:6n-3 (Fig. 4B).

Histamine content of MC/9 cells was increased by incubating with 20:4n-6, whereas incubation with n-3 PUFA decreased the histamine content (Fig. 5). Yamada *et al.* (31) examined the effects of fatty acids on histamine accumulation in RBL-2H3 rat basophilic leukemia cells and found the enhancing effect of 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3 on histamine accumulation in the cells. In the report of Kawasaki *et al.* (35), histamine content of RBL-2H3 cells was decreased by the addition of 18:3n-3. Engels *et al.* (36) demonstrated by an *in vivo* study that dietary modulation of fatty acid composition of mast cells did not affect histamine content and release. The reasons for the inconsistency of the results of these reports are not clear. Our present results show that the effects of PUFA on histamine accumulation are complex (Fig. 5).

Thus, it seems that further detailed investigations on the effect of fatty acids on histamine accumulation in mast cells are necessary to resolve this quandary.

The effects of PUFA on histamine release in intact, ionophore-stimulated, and antigen-activated cells were observed (Fig. 6). Since spontaneous release of histamine was not altered (Fig. 6A), it was unlikely that reduced histamine content in the cells incubated with n-3 PUFA was due to increased leakage of histamine from the cells. Ionophore-induced release of histamine was suppressed by PUFA, excluding 18:3n-3 (Fig. 6B). Antigen-induced histamine release was not influenced (Fig. 6C). A contradiction exists in the effect of PUFA on the release of histamine. Data indicate an augmentative (31), suppressive (Ref. 35 and Fig. 6B), and noneffective (Ref. 36 and Fig. 6C) effect of PUFA on histamine release. Clarification of the net effects of PUFA on histamine release examination under well-defined conditions is necessary.

In the present study, we showed that 24:6n-3 has an inhibitory effect on the antigen-stimulated production of lipoxygenase pathway metabolites in MC/9 mouse mast cells. Also 24:6n-3 reduced the content of histamine in the cells. These effects of 24:6n-3 closely resemble those of 22:6n-3, a fatty acid prominent in fish oils and well stated to have an anti-inflammatory effect *via* the modulation of arachidonate metabolism (14,15), than of 20:5n-3 (Figs. 2, 3, 5). Therefore, 24:6n-3 has an anti-inflammatory activity similar to 22:6n-3. Further studies about the incorporation, metabolism, and action of 24:6n-3 are necessary to evaluate its nutritional and physiological functions.

ACKNOWLEDGMENTS

We thank Yumiko Miyauchi for her excellent technical assistance and Nissui Pharmaceuticals (Tokyo, Japan) for the gift of 20:5n-3 ethyl ester. This work was granted in part by The Agriculture, Forestry and Fisheries Research Council, Ministry of Agriculture, Forestry and Fisheries, Japan.

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[Received July 10, 1998; and in final revised form and accepted October 7, 1998]

Low Density Lipoprotein of Synovial Fluid in Inflammatory Joint Disease Is Mildly Oxidized

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ABSTRACT: Oxidatively modified low density lipoprotein (LDL) has many biological activities which could contribute to the pathology of the atherosclerotic lesion. Because atherosclerosis has an inflammatory component, there has been much interest in the extent to which LDL could be oxidatively modified *in vivo* by inflammation. The present study examined LDL present in an accessible inflammatory site, the inflamed synovial joint, for evidence of compositional change and oxidative modification. LDL was isolated from knee joint synovial fluid (SF) from subjects with inflammatory arthropathies and also from matched plasma samples. SF and plasma LDL had similar free cholesterol and α-tocopherol content, but SF LDL had a lower content of esterified cholesterol. On electrophoresis, SF LDL was slightly more electronegative than LDL from matched plasma samples, but the changes were much less than those resulting from Cu^{2+}-treatment of LDL. Oxidized cholesterol was not detected in any samples, but cholesterol ester hydroperoxide levels were greater in SF than in plasma LDL. When samples from three subjects were incubated with macrophages, the SF LDL did not cause significant loading of the cells with cholesterol or cholesterol esters, in contrast to the situation with acetylated LDL. Overall, the SF LDL displayed evidence of slightly increased oxidation by comparison with matched plasma samples. Despite their isolation from an environment with active inflammation, changes were modest compared with those resulting from Cu^{2+} treatment. Thus, extensive LDL oxidation is not a necessary correlate of location in a chronic inflammatory site, even though it is characteristic of atherosclerotic lesions.

Lipids 33, 1115–1121 (1998).

Oxidized low density lipoproteins (LDL) have multiple biological effects which are potentially pro-atherogenic. These include stimulation of monocyte recruitment *via* enhanced chemokine production (1), unregulated uptake by macrophages resulting in foam cell formation (2), cytotoxicity (3), and T lymphocyte activation (4).

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; RA, rheumatoid arthritis; SF, synovial fluid.

Oxidized LDL can be generated *in vitro* by incubation of native LDL with transition metal ions. In this form, it is recognized by a range of receptors, including the scavenger receptor, and the membrane glycoprotein CD36. CD36 is a highly conserved protein which is expressed by human and murine monocytes/macrophages (5) and also by synovial microvascular endothelium in rats (Zhang, X., Cleland, L.G., and Mayrhofer, G., unpublished). Oxidized LDL undergoes accelerated and unregulated uptake by macrophages, and there is decreased intracellular degradation of its apoprotein B-100 (2,6). Both of these processes could contribute to formation of the lipid-laden "foam cells" characteristic of early and advanced atherosclerotic lesions.

Furthermore, metal-oxidized LDL injected intravenously in hamsters promoted generalized leukocyte adhesion to endothelium (7), and *in vitro* it caused induction of heat-shock protein in monocytic cells (8), increased expression of MHC Class II molecules on human mononuclear cells (9), and activation of T-lymphocytes manifest as upregulation of IL-2 receptors (4). All these events are important features of early stages of atherogenesis. LDL can be oxidatively modified to high-uptake forms also by incubation with neutrophils and macrophages in the presence of transition metal ions (10,11).

In mildly oxidized forms, LDL is not specifically recognized by cellular receptors with the possible exception of CD36, nor usually subject to accelerated endocytosis, but it has other activities which may be directly relevant to the generation of foam cells in the vessel wall. For example, minimally modified LDL can stimulate formation of monocyte chemotactic protein-1 and monocyte transmigration into the subendothelium (1).

Whereas most of the information on oxidatively modified LDL and foam cell formation derives from investigations into the cellular pathology of atheroscelerosis, foam cells have been observed also in the synovial membranes of subjects with rheumatoid arthritis (RA) (12). In addition, the lesions in both RA and atherosclerosis have in common monocyte and lymphocyte infiltrates, cellular hyperplasia, and fibrosis. Lipids from human atherosclerotic plaques are heavily oxidized, in spite of the maintenance of at least normal levels of several antioxidants including both vitamin E and ascorbate

(13). In addition, oxidized proteins and lipids are present in lipid–protein fractions isolated from such plaques at the density of LDL (and containing its characteristic apoprotein) (14; Dean, R.T., and Fu, S., unpublished). The commonality between inflammatory and atherosclerotic sites suggests that it is important to establish whether LDL present at nonatherosclerotic inflammatory sites is also oxidized, as might be the case if its oxidation were dependent on the inflammatory events *per se*. Conversely, it would be of interest if LDL oxidation were not characteristic of inflammatory sites in general, but rather, selective for atherosclerotic lesions.

Inflammatory sites contain appropriate cells and mechanisms for the induction of oxidative stress. In inflammatory joint disease, the synovium and joint space contain leukocytes that are capable of producing reactive oxygen species, and there are also suggestions of the production of reactive oxygen species in inflamed joints with effusions as a consequence of cycles of ischemia and reperfusion (15,16). Accordingly, it has been claimed that oxidized lipids and proteins are present in synovial fluid (SF) from subjects with RA (15,16). However, these studies (15,16) used assays of limited discrimination [thiobarbituric acid reactive substances (TBARS), IgG protein fluorescence]. In the more detailed of these studies (16), there were changes in SF TBARS during or independent of exercise, but no changes in overall conjugated dienes; although second-derivative spectra were used to gain further information about the conjugated dienes, no quantitative data were presented on the comparison between the exercised and nonexercised groups. The interpretation of these data, and particularly of whether lipid and protein oxidation products are accumulated more in rheumatoid SF, remains difficult.

Thus, it is of great interest to use SF LDL from inflamed joints to assess the extent of oxidative LDL modification *in vivo* in an inflammatory, oxidative environment. In the present study, we have examined human SF LDL for alterations in composition and for markers of oxidative modification. The results indicate that modest oxidative changes occurred, but they are minor by comparison with those present in LDL oxidized *in vitro* by exposure to Cu^{2+} ions. Also, the changes are not sufficient to promote accelerated LDL uptake by macrophages leading to foam cell formation.

METHODS

Plasma and SF. All SF samples were aspirated from knee effusions from patients with inflammatory arthropathies. In some instances where bilateral effusions were present, both knees were aspirated and the samples were examined individually as indicated. The clinical diagnoses are listed in Table 1. A peripheral venous blood sample was collected at the same time as joint aspiration and both SF and blood samples were collected into EDTA-containing tubes (final concentration 1 mg/mL). Aprotinin and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) (10 μg/mL) were added to all of the samples and bovine testicular hyaluronidase (Sigma Chemical Co.) (10 μg/mL) was added also to the SF samples.

TABLE 1
Diagnoses Related to the Inflammatory Arthropathies

Patient no.	Diagnosis
1	Spondylitis with psoriasis
2	RA[a]
3	Psoriasis
4	RA
5	RA
6	RA
7	RA
8	RA
9	RA
10	RA
11	Arthropathy associated with inflammatory bowel disease
12	RA
13	RA
14	RA
15	Reiter's syndrome

[a]RA, rheumatoid arthritis.

Blood and SF were centrifuged ($350 \times g$, 10 min) at room temperature, after which the supernatants were stored at 4°C.

Isolation of lipoproteins. KBr was used for density adjustments of SF and plasma which were subjected to ultracentrifugation at 4°C in a Beckman TL-100 Tabletop ultracentrifuge as follows. LDL were isolated in the density range $1.019 < d < 1.055$ g/mL with a single 18 h spin at the lower densities followed by two 18 h spins at the higher densities. The lipoprotein preparations were dialyzed against 3×1 L Tris-buffered saline (0.01 M, pH 7.4) containing NaCl (0.15 M), Na_2-EDTA (0.005% wt/vol), and NaN_3 (0.006% wt/vol).

Lipid analyses. LDL (approximately 20 μg protein) was added to 1 mL phosphate-buffered saline and extracted with 2 mL methanol (containing 0.02% acetic acid) and 10 mL water-washed hexane. All samples and reagents were kept on ice. Samples were centrifuged, and the hexane phases were stored at −80°C. High-performance liquid chromatography (HPLC) separation with ultraviolet (UV) detection at 210/234 nm was used for estimation of cholesterol, cholesteryl esters, and 7-keto cholesterol (17); electrochemical detection was used for estimation of α-tocopherol (18). Cholesteryl ester hydroperoxides were estimated by HPLC with postcolumn luminol/microperoxidase chemiluminescence detection (18).

Protein oxidation products. Evidence for protein oxidation in intact LDL was sought by measurement of valine hydroxides and hydroperoxides (19), protein-bound 3,4-dihydroxyphenylalanine (DOPA) (20), and hypochlorite modification of apo B protein (21).

Macrophage uptake of LDL. Murine peritoneal macrophages were harvested from QS mice by lavage (6), resuspended in culture medium (Dulbecco's modified Eagle's medium, DMEM) and plated at a density of 2×10^6 cells per 22-mm diameter well. After 2 h, nonadherent cells were removed. The remaining macrophages (>95% pure) were incubated with DMEM containing lipoprotein-deficient human serum (10% vol/vol) and 25 μg/mL of the indicated lipopro-

teins, dialyzed against phosphate-buffered saline before use. After 24 h at 37°C, cells were washed, lysed in NaOH (0.2 M), and lipids were extracted in hexane/methanol as described above. Cholesterol and cholesteryl esters were determined by HPLC as described above.

Statistical analysis. Except where specified, the Wilcoxon signed rank test was used to examine differences between values for the SF and matched plasma samples (22).

RESULTS

SF LDL lipids. The free cholesterol content in LDL isolated from SF was similar to that in LDL isolated from matched plasma samples (Table 2) [mean ± SD; plasma, 1163 ± 296; SF, 1026 ± 195 nmol/mg LDL protein; difference not significant (n.s.)]. However, the cholesterol ester content of SF LDL was significantly less than that of plasma LDL (Table 2) (plasma, 2765 ± 689; SF, 2077 ± 331 nmol/mg LDL protein; $P < 0.01$, *t*-test). A parallel difference was also observed for the individual cholesteryl esters linoleate, arachidonate, oleate, and palmitate (results not shown). There was no difference in the α-tocopherol content of SF and plasma LDL (Table 2) (plasma, 18.8 ± 3.7; SF, 18.6 ± 5.2 nmol/mg LDL protein; n.s.).

Lipid oxidation products in SF LDL. In samples where cholesterol ester hydroperoxides were detected in SF LDL, there were consistently higher levels compared with those in matched plasma LDL samples (Table 3). On average, plasma LDL contained 0.42 ± 0.27 (mean ± SD), whereas SF LDL contained 1.65 ± 1.11 nmol cholesteryl linoleate hydroperoxide/mg protein. These amounts were significantly different ($P < 0.01$). 7-Keto-cholesterol was not detectable in any of the plasma or SF LDL samples. Overall, these levels of oxidized lipids were considerably less than those generated in Cu^{2+}-treated plasma LDL, which after 24 h of oxidation with Cu:LDL particle ratios of 10:1 typically contain 150 nmol 7-keto cholesterol/mg protein (23). Furthermore, our reported values for atherosclerotic plaque homogenate are also substantially higher than those we observed for SF LDL (13).

Protein oxidation products in SF LDL. Three markers of protein oxidation were measured in LDL samples from patient nos. 9 and 14. SF LDL levels of valine hydroxides and protein-bound DOPA were within the normal range we determine for plasma LDL (approximately 10 pmol valine hydroxide/mg LDL protein and a DOPA/tyrosine ratio of approximately 50×10^{-5}). Also, there was no evidence of hypochlorite epitopes of apoB in these samples.

Charge alterations in SF LDL. When subjected to agarose gel electrophoresis, SF LDL was slightly more electronegative than matched plasma LDL samples (Table 4, Fig. 1). Relative to plasma samples, SF LDL had a mean (± SD) relative mobility of 1.13 ± 0.14 ($n = 13$). This value is statistically significantly different from 1.0 (*t*-test, $P < 0.05$), indicating that SF LDL was slightly more electronegative by comparison with matched plasma LDL. A comparable modest enhanced electronegativity has been observed for human aortic intimal LDL in comparison with matched plasmas (24), although comparison of LDL isolated from normal intima vs. atheromatous lesions indicated that both were comparably affected (25). However, these alterations are much less than those generated by Cu^{2+} treatment of plasma LDL samples (Table 4 and Ref. 23).

Macrophage uptake of SF LDL. Samples from three patients (3 plasma, 5 SF) were examined for uptake by murine peritoneal macrophages. There was no evidence for altered cholesterol levels or of cholesteryl ester accumulation in cells incubated with SF LDL (Fig. 2). By contrast, lipids from acetylated LDL caused greatly increased accumulation in

TABLE 2
Lipids [nmol/mg LDL protein (mean ± SD[a]) of Synovial Fluid LDL and Matched Plasma LDL

	Plasma			Synovial fluid[b]		
Patient	Free cholesterol	Cholesterol esters	α-Tocopherol	Free cholesterol	Cholesterol esters	α-Tocopherol
3	1139 ± 160	2676 ± 274	17	1066	2357	21 ± 1
				1188 ± 101	2144 ± 92	17 ± 1
5	874 ± 31	1832 ± 199	23 ± 1	863 ± 69	1568 ± 101	19 ± 3
				968 ± 44	1909 ± 98	26 ± 3
6	1173 ± 33	2654 ± 122	12 ± 0.5	1050 ± 53	2077 ± 95	10 ± 0.5
9	908 ± 34	2477 ± 81	19 ± 1	1395 ± 53	2266 ± 77	25 ± 1
10	1207	2693	18 ± 0.1	1331	2524	20 ± 1
12	1164	2891 ± 139	17 ± 3	1049 ± 3	2340 ± 869	17 ± 2
13	1061 ± 32	3115 ± 185	17 ± 1	891 ± 45	2473 ± 21	24 ± 1
14	1056 ± 66	2236 ± 130	22 ± 2	771 ± 30	1571 ± 91	15 ± 1
				925 ± 50	1922 ± 80	19 ± 1
15	1889 ± 125	4316 ± 251	24 ± 1	826 ± 62	1772 ± 135	10 ± 1
Mean ± SD	1163 ± 296	2765 ± 689[c]	18.8 ± 3.7	1026 ± 195	2077 ± 331	18.6 ± 5.2

[a]Mean ± SD of three determinations.
[b]Where two values appear, synovial fluid samples were taken from the left knee (top row) and right knee (bottom row) of the same individual.
[c]Superscript indicates statistically significant difference between plasma and synovial fluid values (Wilcoxon, $P < 0.01$). LDL, low density lipoprotein.

TABLE 3
Oxidized Lipids of Synovial Fluid LDL and Matched Plasma LDL

Patient	Plasma	Synovial fluid[a]
	Cholesterol linoleate hydroperoxide (nmol/mg LDL protein) mean ± SD[b]	
3	0.3	1.5 ± 0.1 2.0 ± 0.23
5	0.8 ± 0.22	4.1 ± 0.61 2.4 ± 0.17
6	n.d.[c]	n.d.
9	0.1 ± 0.12	0.8 ± 0.1
10	0.7 ± 0.06	2.5 ± 0.26
12	n.d.	1 ± 0.32
13	n.d.	n.d.
14	0.3 ± 0.03	0.9 ± 0.34 0.5 ± 0.09
15	0.3 ± 0.02	0.8 ± 0.04
Mean ± SD	0.42 ± 0.27[d]	1.65 ± 1.11

[a]Where two values appear, synovial fluid samples were taken from the left knee (top row) and right knee (bottom row) of the same individual.
[b]Mean ± SD of three determinations; where no SD is given, the mean is based on <3 replicates.
[c]n.d., not detected.
[d]Statistically significant difference between plasma and synovial fluid values (Wilcoxon, $P < 0.01$). For abbreviation see Table 2.

macrophages, indicating that functional endocytic receptor pathways were present (Fig. 2).

DISCUSSION

In subjects with inflammatory joint disease, the synovium and joint space become a potentially pro-oxidative environment.

TABLE 4
Relative Mobility of Synovial Fluid LDL[a]

Patient	Knee	Contralateral knee	Cu^{2+}-oxidized plasma LDL[b]
1	1.19		
2	1.21		
3	1.06	1.07	3.28
4	1.17		2.89
5	1.03		
6	1.06		
7	1.5	1.17	
8	1.03		
9	0.98		
10	1.03		
11	1.21		
Mean ± SD	1.13 ± 0.14[c,d]		3.08 ± 0.27[d]

[a]Mobilities are expressed as migration of synovial fluid LDL samples relative to the migration of matched plasma LDL samples.
[b]Selected plasma LDL samples were treated with $CuSO_4$ (10 μM) for 12 h at 37°C. The protein concentrations of the LDL used were 0.9 mg/mL (patient 3) and 1.7 mg/mL (patient 4).
[c]Mean ± SD for all synovial fluid samples, i.e., $n = 13$.
[d]Statistically significantly different from 1.0 (*t*-test, $P < 0.05$).

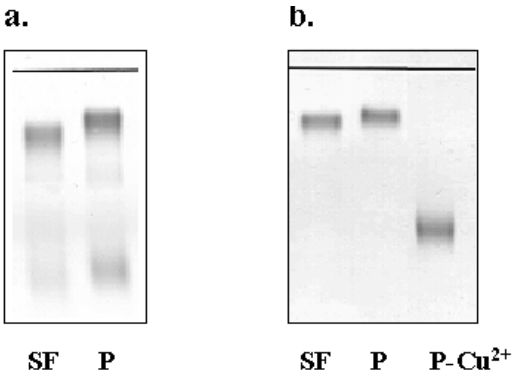

FIG. 1. Agarose gel electrophoresis of LDL. The horizontal line represents the origin and migration is toward the anode. (a) Patient #1; (b) patient #3. SF, synovial fluid sample; P, plasma sample; P-Cu^{2+}, Cu^{2+}-oxidized plasma sample (see Table 4 for details).

The leucocyte infiltrates comprise cells capable of producing reactive oxygen species when stimulated and there is evidence that, in joints with effusions, exercise causes cycles of synovial ischemia and reperfusion which result in production of reactive oxygen species (15,16). The evidence for production of reactive oxygen species is based on claims of detection of oxidized lipid and protein in SF of subjects with RA (15,16), and caveats about these data have been outlined above. In addition, there are suggestions of the presence of oxidatively modified LDL in rheumatoid joints. Thus, by

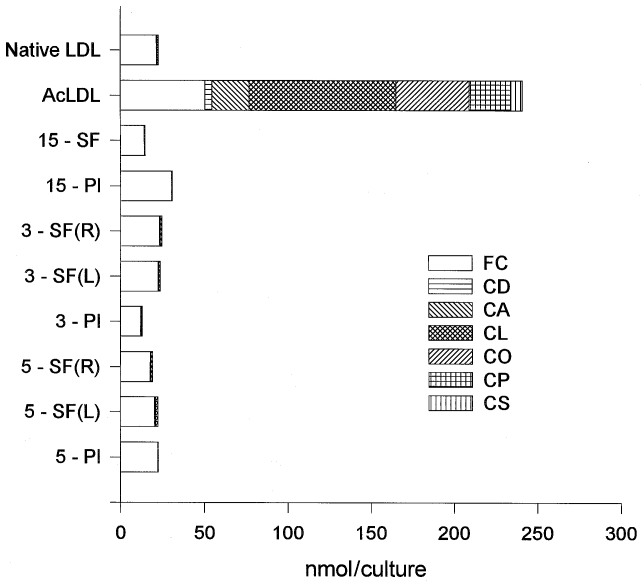

FIG. 2. Macrophage content of free and esterified cholesterol. Murine macrophages (2×10^6/culture) were incubated for 24 h with 25 μg/mL of the indicated low density lipoprotein (LDL) samples, as described in the text. Lipids were extracted and determined by reversed-phase high-performance liquid chromatography. Numbers refer to specific patients whose diagnoses are given in Table 1. Key: AcLDL, acetylated LDL; SF, synovial fluid LDL; Pl, plasma LDL. Letters in parentheses specify the right (R) or left (L) knee synovial fluid from which LDL was isolated. FC, free cholesterol; CD, cholesteryl docosahexaenoate; CA, cholesteryl arachidonate; CL, cholesteryl linoleate; CO, cholesteryl oleate; CP, cholesteryl palmitate; CS, cholesteryl stearate.

using antisera raised against Cu^{2+}-oxidized LDL, positive staining of perivascular macrophages was observed in some rheumatoid synovial samples (12). However, it has become increasingly apparent that such antibodies recognize a range of epitopes present on proteins other than apo B, and so the interpretation of such observations is very difficult (26).

In the present study, we have demonstrated the presence of mildly oxidized LDL in inflammatory synovial fluid. Mobility on agarose gel was slightly increased compared with matched plasma samples, suggesting modification of apo B lysines which is usually a consequence of derivatization by aldehydic products of lipid peroxidation (27). However, the mobility shift was considerably less than that observed in atherosclerotic plaque LDL. There was also evidence of increased fatty acid hydroperoxides, a marker of limited LDL oxidation, but no evidence of cholesterol oxidation, a later process (17,28). The mobility of LDL is also changed only late during oxidation (17,23), largely consequent on reactions of lipid peroxidation end-products with apo B lysines; again there were only slight changes in LDL mobility in the synovial samples, further suggesting that oxidation had proceeded only to a very limited degree. In agreement, there was no evidence of protein oxidation, including hypochlorite modification, and the SF LDL was not capable of loading macrophages with lipid. Thus, the SF LDL analyzed in the present study is not sufficiently modified to support foam cell formation.

The lack of oxysterols and oxidized protein in the synovial LDL samples suggests that the oxidation in the RA synovium is limited in extent. This may be due to limited oxidant generation, effective inhibition, and perhaps a contribution from removal of oxidation products. For example, LDL oxidation *in vitro* is inhibited by the presence of serum (29) and, in particular, by HDL (30). Several of the components in serum which suppress LDL oxidation are present in inflammatory SF. We observed that high density lipoprotein (HDL) (apo A-I) is present in inflammatory SF at approximately 50% of the concentration in plasma (James, M.J., Rye, K.A., Cleland, L.G., and Barter, P.J., unpublished observations). We observed also that inflammatory SF contains cholesterol ester transfer protein (CETP) activity at approximately 40% of the concentration in plasma (James, M.J., Rye, K.A., Cleland, L.G., and Barter, P.J., unpublished observations). This could be significant in explaining the low levels of cholesterol ester hydroperoxides in SF LDL because CETP can transfer cholesterol ester hydroperoxides from LDL to HDL where they are converted to hydroxides (31). Nevertheless, the same inhibitory factors are present in atherosclerotic plaque where oxidized lipids and proteins are present (13).

An additional contribution to the low levels of oxidation products in synovial LDL may be the residence time of the particles at this site, and this needs to be considered. Although residence time of LDL has not been measured, estimates can be made. The SF/plasma ratios of macromolecules which are plasma-derived reflect the net interactions of synovial vascular permeability, synovial blood flow, and the extent of lymphatic removal from the synovial space (proteins are removed *via* the lymphatics rather than the microcirculation (32,33). In the present study the synovial fluid/plasma ratios of LDL protein averaged 0.57 (data not shown). This value is similar to those reported for other plasma proteins such as albumin, ceruloplasmin and α_2-macroglobulin (33), suggesting that the rates of delivery and removal of lipoproteins are similar to those of the plasma-derived proteins. The rate of clearance of albumin from rheumatoid synovial effusions is about 30-fold less than the rate of clearance of small molecules such as NaI (34). Thus, the transit time of LDL in the synovial space is expected to be substantial. Although measurements of the transit time of lipoproteins have not been made, estimates can be made based on reported clearance rates of similar-sized proteins. α_2-Macroglobulin has a radius of 9.1 nm, similar to that of HDL but smaller than that of LDL, and was reported to be cleared from rheumatoid synovial effusions at the rate of 0.09 $mg \cdot min^{-1}$ (33). An effusion of 50 mL would have approximately 50 mg HDL and 120 mg LDL protein, and, if cleared with rates similar to that of α_2-macroglobulin, the fractional clearance would be 0.0018 and 0.00075 min^{-1}, respectively. Whereas this estimated clearance rate for LDL indicates a substantial transit time, it may not be sufficient to permit the extensive oxidation required to promote macrophage uptake.

Myeloperoxidase, a product of neutrophils which can catalyze the production of hypochlorite, has been detected in human atherosclerotic lesions (35) and in inflammatory SF (36). Hypochlorite can modify the apo B protein component of LDL to produce a high-uptake form of LDL (37). The lack of evidence for hypochlorite modification of LDL protein in the present study may result from the presence of an inhibitor of myeloperoxidase which has been detected in SF and in serum (38).

A role for localized inflammation in atheroma formation has been postulated because the atherosclerotic lesion has many elements commonly associated with chronic inflammation such as a macrophage and lymphocyte infiltrate, smooth muscle cell hyperplasia, and fibrosis (39–43). Thus, many cell types present in an atherosclerotic lesion, including endothelial cells and the leukocytes, can potentially oxidatively modify LDL. These elements and conditions are also present in the synovium and joint space of patients with inflammatory joint disease. However, the lack of substantial oxidative modification of LDL in inflammatory SF indicates either that appropriate oxidants do not reach the LDL particles there or that some of the many antioxidant defenses present even in an inflammatory focus are successful in limiting oxidation. As we have shown, however (13), a barrage of antioxidant molecules are present in advanced atherosclerotic lesions even though highly oxidized lipid–protein particles are present. A deficiency of co-antioxidation may account for the oxidation in plaque. However, an alternative explanation is that after oxidation occurs, antioxidants are repleted in plaque.

The observations here suggest that, although lipoprotein oxidation is pronounced in atherosclerotic lesions, extensive lipoprotein oxidation is not general in inflammatory sites. Since pro-inflammatory factors are present in both cases, it is

possible that other factors are critical in the causation of lipoprotein oxidation in plaque. An important implication of these findings is that markers of lipoprotein oxidation may be markers of atherosclerosis, and not simply of inflammation.

ACKNOWLEDGMENTS

The authors acknowledge the expert technical assistance of Cindy Hall; hypochlorite epitope determinations were done by Dr. Linda Hazell (HRI) and protein oxidation measurements by Dr. Shanlin Fu (HRI). W. Jessup is supported by NH&MRC (Grant G950459).

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[Received April 21, 1998, and in final revised form October 6, 1998; revision accepted October 10, 1998]

Lipid Peroxidation of Isolated Chylomicrons and Oxidative Status in Plasma After Intake of Highly Purified Eicosapentaenoic or Docosahexaenoic Acids

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ABSTRACT: Fourteen healthy male volunteers were given two separate high-saturated-fat meals with and without the addition of 4 g highly purified ethyl esters of either eicosapentaenoic acid (EPA) (95% pure, $n = 7$) or docosahexaenoic acid (DHA) (90% pure, $n = 7$) supplied as 1-g capsules each containing 3.4 mg vitamin E. The chylomicrons were isolated 6 h after the meals, at peak concentrations of n-3 fatty acids (FA). Addition of n-3 FA with the meal caused a 10.4-fold increase in the concentration of n-3 FA in chylomicrons compared to the saturated fat meal without addition of n-3 FA. After the saturated-fat meal, the concentration of thiobarbituric acid-reactive substances (TBARS) was 327.6 ± 34.6 nmol/mmol triacylglycerol (TAG), which increased to 1015.8 ± 212.0 nmol/mmol TAG ($P < 0.0001$, $n = 14$) after EPA and DHA were added to the meal. There was no significant correlation between the concentrations of TBARS and vitamin E in the chylomicrons collected 6 h after the test meal. The present findings demonstrate an immediate increase in chylomicron peroxidation *ex vivo* provided by intake of highly purified n-3 FA. The capsular content of vitamin E was absorbed into chylomicrons, but the amount of vitamin E was apparently not sufficient to protect chylomicrons against lipid peroxidation *ex vivo*. Daily intake of 4 g n-3 FA either as EPA or DHA for 5 wk did not change the plasma concentration of TBARS. Although not significantly different between groups, DHA supplementation decreased total glutathione in plasma ($P < 0.05$) and EPA supplementation increased plasma concentration of vitamin E ($P < 0.05$). The other lipid-soluble and polar antioxidants in plasma remained unchanged during 5 wk of intervention with highly purified n-3 FA.

Lipids 33, 1123–1129 (1998).

Fish oils, rich in n-3 polyunsaturated eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have antithrombotic, antiinflammatory, and hypolipemic properties (1,2), indicating that these fatty acids (FA) may be used in the prevention and treatment of atherothrombotic diseases.

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Abbreviations: AUC, area under the curve; CM, chylomicrons; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid(s); HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; TAG, triacylglycerol; TBA, thiobarbituric acid; TBARS, TBA-reactive substance.

Lipid peroxidation, especially of low density lipoproteins (LDL), may be important in the pathogenesis of atherosclerosis (3). Oxidatively modified LDL promotes atherosclerosis *in vitro* by increasing the expression of adhesion molecules at the endothelial surface (4,5), by inhibiting repair of wounded endothelial monolayers (6), by inducing foam cell formation through uptake of oxidized LDL *via* the scavenger receptor, which is resistant to intracellular proteolysis (7,8), and by inducing release of cytokine and eicosanoids with chemotactic and proliferative properties (9,10). In general, autooxidation of polyunsaturated FA increases with the extent of unsaturation. Thus, dietary intervention with highly polyunsaturated n-3 FA may render blood lipids and cells more susceptible to oxidation and thereby be potentially harmful (7). However, inconsistent data have been reported regarding the impact of n-3 polyunsaturated FA on the susceptibility of LDL to lipid peroxidation in humans (11–13).

Postprandial triglyceridemia may have an important role in atherogenesis (14). Patients with coronary artery disease (CAD) have increased plasma concentrations of chylomicron (CM) remnants and higher postprandial lipemia (15,16). Dietary oxidized lipids are absorbed and incorporated into CM and may thereby contribute to the oxidative stress of the vascular system (17). Long-term intake of n-3 FA reduces postprandial triglyceridemia (18,19), but it is unknown whether incorporation of n-3 FA into the CM particle affects its susceptibility to peroxidation. The purpose of the present study was to investigate the susceptibility of CM to peroxidation after intake of a standard high-saturated-fat meal with and without supplement of n-3 FA. Furthermore, we assessed the effect of daily intake of highly purified EPA and DHA for 5 wk on pro- and antioxidative systems in plasma.

MATERIALS AND METHODS

Participants. Fourteen healthy normolipemic male employees at the University of Tromsø (mean age 36.2 yr, range 28–49), who were consuming a traditional Western diet, were recruited into the study. The subjects were evaluated by medical history, physical examination, and measurements of blood pressure, hematological variables, blood glucose,

serum lipids, and routine biochemistry. One participant smoked cigarettes. The exclusion criteria were regular use of drugs, history of peptic ulcer, gastrointestinal disturbances likely to influence absorption, alcohol or drug abuse, and mental illness. The subjects were asked to maintain their regular physical activity and dietary habits throughout the study. None of the participants was taking supplements containing antioxidants (i.e., vitamin E and vitamin C) before and during the study. The study was approved by the regional board of research ethics, and informed, written consent was obtained.

Experimental design. The study was carried out as a randomized double-blind, parallel trial. After a 2-wk run-in phase with weekly physical examinations and blood collections (visits 1 and 2), the participants were randomly selected at visit 3 to receive 4 g/d of EPA (95% pure) or 4 g/d of DHA (90% pure), both prepared as ethyl esters (Pronova Biocare AS, Oslo, Norway), for 5 wk. The n-3 FA were given in soft gelatin capsules each containing 1.0 g of FA. The content of vitamin E (α-tocopherol) was 3.4 mg per capsule, the peroxide value was <0.020 meq/g, and *p*-anisidine value was <35.

Blood was collected weekly during the 5-wk intervention period (visits 4–8). A final examination and blood sampling were performed 15 wk after the intervention period (visit 9). Height was measured at visit 1, and body weight was recorded at visits 1, 3 and 8 using a digital scales with the subjects wearing light indoor clothing. Compliance was assessed by capsule counts and by measurements of serum phospholipid FA.

Blood sampling. Blood was drawn at 8 A.M. from an antecubital vein on the right arm after 12 h of overnight fasting by using a 19-gauge needle in a vacutainer system with minimal stasis. Serum was prepared by clotting whole blood in a glass tube at room temperature for 1 h and then centrifuging at 2000 × *g* for 15 min. Aliquots of 1 mL were transferred into sterile cryovials (Greiner Laboratechnik, Frickenhausen, Germany), flushed with nitrogen, and frozen at −70°C until further analysis. Blood for plasma preparation was collected into vacutainers (Becton Dickinson, Meylan Cedex, France) containing disodium EDTA as anticoagulant (EDTA K_3 0.12 mL, 0.34 M per tube). Plasma was prepared by centrifugation at 2000 × *g* for 15 min at 22°C, transfer into sterile cryovials in aliquots of 1 mL, flushing with nitrogen, and storage at −70°C until further analysis.

Fat-tolerance test. A fat-tolerance test was performed before intervention (visit 3) and under basal conditions 15 wk after completion of the dietary intervention period (visit 9). A standard 2.64 MJ (631 kcal) test meal was prepared as a 375-mL liquid formula containing 28.6 g carbohydrates, 22.6 g protein, and 47.0 g fat. The fat was mainly obtained from cream and milk and comprised 65.4% saturated, 30.9% monounsaturated, and 3.8% polyunsaturated FA. The test meals were freshly prepared each morning, served at 8.15 A.M. and consumed over a 10-min period. The subjects were given 100 mL orange juice with the test meal. At visit 3 the EPA and DHA groups received 4 capsules (1 g each) of EPA and DHA, respectively, with the test meal. At visit 9 the EPA and DHA capsules were replaced by similar amounts of fat from cream. The participants were allowed to drink 350 mL calorie-free beverages and eat a 200-g apple during the following 8 h. Taken together, the test meal, apple, and the orange juice contained 2.2 mg vitamin E (α-tocopherol) as calculated from standard food tables using a specially designed computer program (20). Blood samples for CM isolation and plasma preparation were collected prior to the meal and every second hour during the next 8 h.

CM isolation. CM were isolated by overlayering 8 mL plasma with 4 mL of buffer (150 mmol/L NaCl and 1 mmol/L EDTA) in a cellulose nitrate tube and centrifuged in a Beckman SW40 Ti swinging bucket rotor at 20,000 rpm for 1 h at 4°C (21). The CM were carefully removed by aspiration, divided into three aliquots in cryovials, flushed with nitrogen, and frozen at −70°C until further analysis.

Fatty acid and lipid analysis. Total fatty acids were extracted from 500 μL CM according to Folch *et al.* (22) with heptadecaenoic acid (17:0) as internal standard according to previously described procedures (23). The concentration of FA in CM were reported as μmol/L plasma. Serum lipids were analyzed on a Hitachi 737 Automatic Analyzer (Boehringer Mannheim, Mannheim, Germany) with reagents from the manufacturer. Total cholesterol was measured with an enzymatic colorimetric method (CHOD-PAP) and high density lipoprotein cholesterol was assayed by the same procedure after precipitation of LDL with heparin and manganese chloride. Triacylglycerol (TAG) concentration was determined with an enzymatic colorimetric test (GPO-PAP).

Prooxidants and antioxidants. Plasma and CM were analyzed for lipid peroxidation by the high-performance liquid chromatography (HPLC)-based thiobarbituric acid (TBA) method (24–26). Plasma and CM samples were processed for colorimetric reaction of TBA as described by Yagi (24) and Ohkawa *et al.* (25), respectively. Ten microliters of the samples was then injected onto a Spectral Physics SP 8800 HPLC system with a 5 μ Supelcosil LC18 column (25 cm × 4.6 mm). Malondialdehyde-TBA adducts were quantified by a Shimadzu RF 535 Spectrofluorimeter (excitation wave length 515 nm, emission wavelength 553 nm). Peak analysis was performed using a Spectral Physics Chromjet SP 4400 integrator. The mobile phase was initially 25 mM potassium phosphate buffer, pH 7.0/methanol, 65:35, vol/vol, at a flow rate of 1.0 mL/min. The methanol content was increased to 80% from 4.6–10 min. The typical retention time of the adduct was 4.6–4.8 min.

Plasma samples were analyzed for vitamin E (α-tocopherol), vitamin A (retinol), and β-carotene levels by HPLC as previously described (27–29). In brief, 200 μL plasma was mixed with an equal volume of 300 μM standard in ethanol. To these solutions 500 μL hexane was added, shaken vigorously for 2 min, centrifuged, and partly evaporated (250 μL under a stream of N_2; 125 μL ethanol was added, and then the sample was analyzed by HPLC. The analysis was performed with Supelcosil LC-8 column (25 cm × 4.6 mm i.d.) with LC-8 guard column packed with PERISORB RP-8 (2 cm × 2 mm i.d.). Methanol/H_2O (95:5, vol/vol and 100% methanol

were used as mobile phases. The flow was set to 1.0 mL/min, and the injection volume was 40 µL. Detection was achieved at 292 nm using a Shimadzu RF-535 detector with multiplicator (Hamamatsu R928-08).

The total and reduced forms of glutathione, cysteine, and cysteinylglycine were quantified after derivatization with the fluorescent agent monobromobimane by reversed-phase HPLC as previously described in detail (26,30).

Statistics. Changes in variables were calculated as the value obtained after minus the value obtained before intervention. Examination of data frequencies revealed normal distribution of the data. A paired *t*-test was applied for changes in variables within a treatment group, whereas an unpaired *t*-test was used for comparison of changes between treatment groups. Repeated measures analysis of variance was performed to assess changes in thiobarbituric acid-reactive substances (TBARS) and antioxidants over time. The postprandial increase in CM TAG concentrations was assessed in two ways. First, we calculated the areas under the curve (AUC) for the CM TAG concentration after intake of a standardized fat meal as the deviation from the basal value integrated over the sampling time (8 h). Second, the CM response was defined as the average of the two highest postprandial CM TAG concentrations minus the baseline value. Values are means ± SD if not otherwise stated. Two-sided *P*-values < 0.05 were regarded as significant. The SAS statistical software package was used (31).

RESULTS

There were no statistically significant differences between the EPA and DHA groups with regard to age, body weight, blood lipid concentrations, and n-3 FA levels in serum phospholipids (Table 1). None of the participants experienced discomfort, nausea, or diarrhea after ingestion of the liquid test meal with or without the addition of the highly purified n-3 FA. The participants did not experience significant side effects while taking EPA and DHA. The body weights remained stable during the intervention in both groups. All participants completed the study per protocol. The percentage of capsules taken was 98 (range 96–100%) in the EPA group and 91 (69–100%) in the DHA group.

Fat tolerance tests and CM peroxidation. The increase in plasma CM TAG induced by the liquid fat meals are shown in Figure 1A. After intake of the standard fat meal with addition of cream, the CM concentrations increased 7.6-fold, peaked after 4 h, then declined but still remained higher than baseline values 8 h after the meal. The CM response was 255.0 ± 125.4 µmol/L, and the AUC was 615.7 ± 253.3 µmol/L. A similar response was observed when 4 g of fat from cream was replaced by n-3 FA (either EPA or DHA); the CM response and the AUC were 224.3 ± 81.1 µmol/L and 520.0 ± 201.2 µmol/L, respectively. The concentration of n-3 FA in total lipids extracted from CM is shown in Figure 1B. Ingestion of EPA or DHA with the meal induced a similar maximal increase in CM n-3 FA (data not shown) which peaked 6 h after the meal (10.4-fold increase) (Fig. 1B). The

TABLE 1
Age, Body Weight, Serum Lipids, and Concentrations of the Main n-3 Polyunsaturated Fatty Acids in Serum Phospholipids at Baseline of Subjects Randomized to Receive Either 3.8 g EPA or 3.6 g DHA Daily for 5 wk[a]

	EPA (n = 7)	DHA (n = 7)
Age (yr)	32.4 (5.0)	40.0 (8.4)
Body weight (kg)	82.4 (5.0)	79.8 (7.5)
Total cholesterol (mmol/L)	5.80 (1.16)	5.34 (0.53)
HDL cholesterol (mmol/L)	1.28 (0.12)	1.38 (0.26)
TAG (mmol/L)	1.19 (0.53)	1.04 (0.41)
EPA (µmol/L)	34.7 (16.9)	40.2 (14.4)
DPA (µmol/L)	24.8 (10.3)	26.6 (4.8)
DHA (µmol/L)	125.2 (42.8)	133.1 (28.8)

[a]Values are means (SD). Abbreviations: HDL, high density lipoproteins; EPA, eicosapentaenoic acid; TAG, triacylglycerol; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

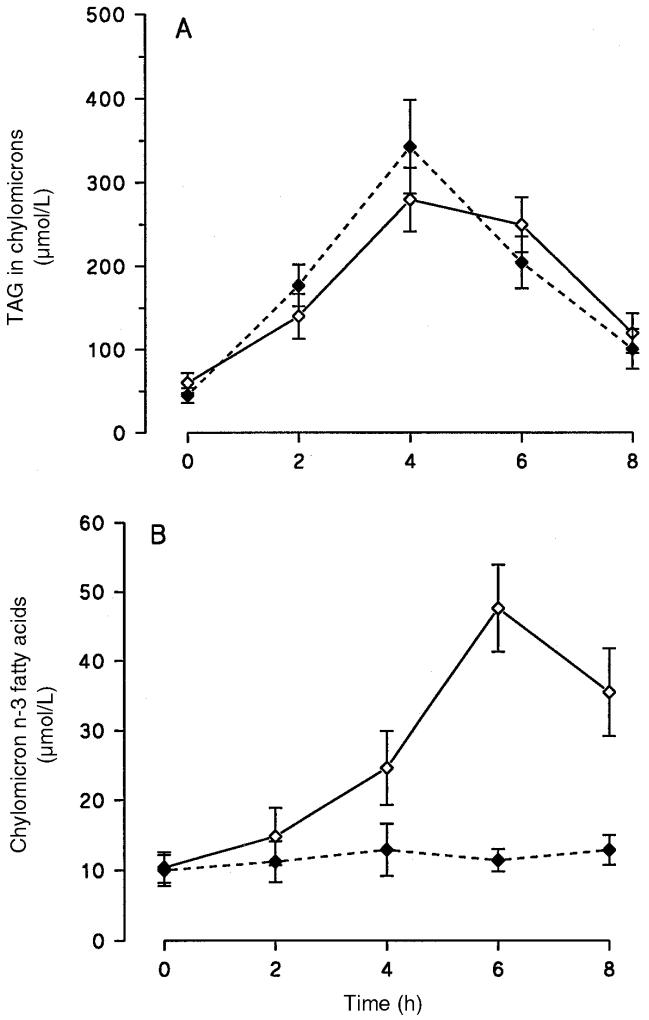

FIG. 1. Concentration of (A) triacylglycerols (TAG) and (B) total n-3 fatty acids in chylomicrons isolated from plasma after ingestion of a standard high-fat meal (solid symbol, n = 14) or a standard high-fat meal with 4 g n-3 fatty acids (open symbols, n = 14). Values are means ± SD.

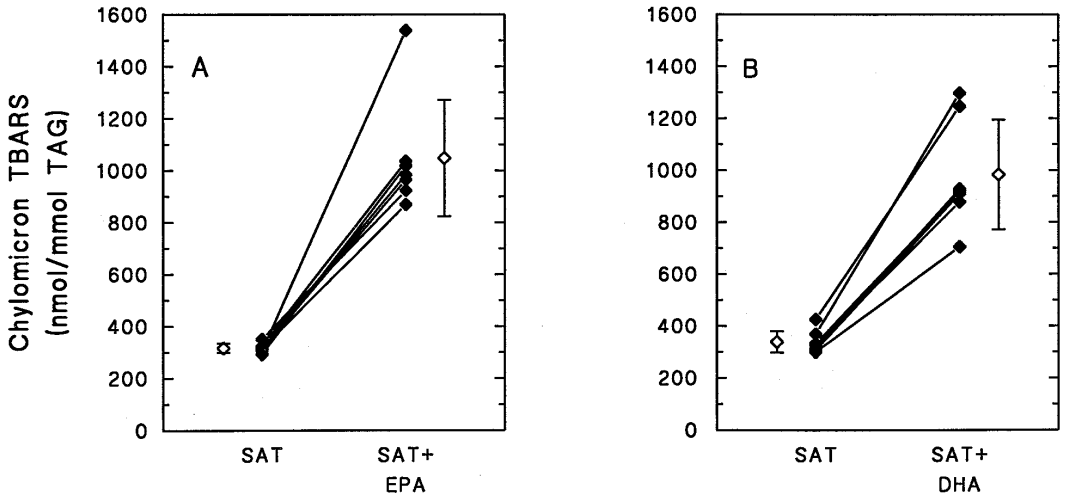

FIG. 2. Graphs showing individual values and means ± 1 SD of thiobarbituric acid-reactive substances (TBARS) in chylomicrons 6 h after a standard high-fat meal (SAT) or a standard high-fat meal with the addition of either 4 g (A) eicosapentaenoic acid (EPA; $n = 7$) or (B) docosahexaenoic acid (DHA; $n = 7$).

absorption of n-3 FA was delayed and prolonged compared to the CM curve. The concentration of n-3 FA in CM lipids did not change after the saturated-fat meal (Fig. 1B).

The CM fractions were isolated 6 h after the meal, at peak concentrations of n-3 FA, and TBARS were measured after both fat tolerance tests. After the saturated-fat meal the TBARS concentration was 327.6 ± 34.6 nmol/mmol TAG, which increased to 1015.8 ± 212.0 nmol/mmol TAG ($P < 0.0001$) when n-3 FA were added to the meal. The increase in TBARS was similar whether EPA or DHA was given as supplement to the meals (1048.1 ± 223.8 nmol/mmol TAG vs. 983.4 ± 211.8 nmol/mmol TAG, respectively, $P = 0.59$) (Fig. 2). The mean concentration of vitamin E in CM was 3.03 ± 1.18 µmol/L 6 h after meal containing 4 g highly purified n-3 Fa had been consumed. There was no significant correlation between the concentrations of TBARS and vitamin E in the CM ($r = 0.04$, $P = 0.87$).

Oxidative status after intake of EPA or DHA for 5 wk. Intake of highly purified EPA or DHA for 5 wk did not alter plasma levels of TBARS significantly (Fig. 3A). The antioxidant status in plasma at baseline and change after the intervention are shown in Table 2. The plasma concentration of vitamin E increased significantly ($P < 0.05$) within the EPA group, but the increase did not differ from that seen in the DHA group (Table 2 and Fig. 3B). The concentration of total glutathione was modestly decreased (−12%, $P < 0.05$) in the DHA group, whereas the reduced form was unchanged, indicating an actual decrease in its oxidized and/or protein-bound form. However, no statistical difference from the EPA group was observed. There was no significant change in the other antioxidants during dietary intervention with purified n-3 FA.

DISCUSSION

We observed a substantial increase in the amount of TBARS in plasma CM when highly purified EPA and DHA were supplied with a high-fat meal. Despite recommended amounts of vitamin E added to the capsules containing highly purified n-3 FA, the amount of vitamin E was apparently insufficient to

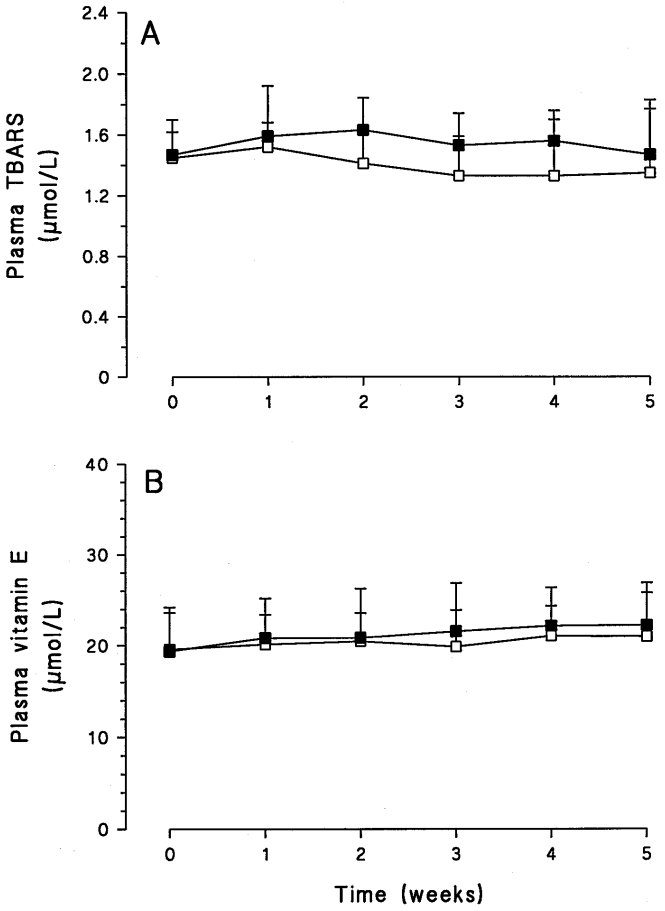

FIG. 3. Graphs showing plasma levels of (A) TBARS and (B) vitamin E during dietary intake of 4 g/d for 5 wk of EPA (■) or DHA (□). Values are means ± SD. For abbreviations see Figure 2.

TABLE 2
Antioxidants (μmol/L) in Plasma at Baseline and Changes from Baseline After Intake of 4 g/d for 5 wk of EPA or DHA[a]

	EPA (n = 7)		DHA (n = 7)		EPA vs. DHA
	Baseline	Change	Baseline	Change	Change during intervention
Cysteine					
Total	1.85 (0.41)	0.05 (0.61)	2.34 (1.05)	0.22 (0.79)	NS
Reduced	0.67 (0.42)	–0.10 (0.49)	1.05 (0.55)	–0.19 (0.66)	
Cysteinylglycine					
Total	0.78 (0.27)	–0.03 (0.05)	0.96 (0.23)	–0.12 (0.28)	NS
Reduced	0.23 (0.19)	0.01 (0.14)	0.17 (0.22)	–0.02 (0.18)	NS
Glutathione					
Total	3.03 (0.33)	–0.07 (0.76)	2.98 (0.42)	–0.39 (0.38)[a]	NS
Reduced	2.64 (0.15)	0.05 (0.26)	2.70 (0.33)	0.00 (0.29)	NS
Vitamin A	1.69 (0.23)	0.08 (0.17)	1.63 (0.13)	0.03 (0.19)	NS
β-Carotene	1.24 (0.57)	0.10 (0.66)	1.85 (1.75)	–0.81 (1.58)	NS
Vitamin E	19.37 (4.84)	2.88 (2.58)[a]	19.58 (4.02)	1.42 (1.73)	NS

[a]Values are mean (SD). Superscript letter a indicates statistical significant changes ($P < 0.05$) within each group by paired t-test. NS, no significant difference in change during intervention between groups (unpaired t-test). See Table 1 for abbreviations.

protect the CM particle against lipid peroxidation *ex vivo*. However, the oxidative status in plasma remained unchanged during 5 wk of daily supplementation with 4 g EPA or DHA, even though DHA tended to stress both lipid-soluble and polar antioxidants.

Postprandial triglyceridemia is thought to play an import role in atherogenesis (14). Oxidized lipids consumed in the diet are absorbed and incorporated into CM where they can contribute to the total body pool of oxidized lipids (17). Oxidized lipids may be involved in injury of the arterial wall and thereby provide a link between postprandial lipemia and atherothrombotic disease. Long-term supplementation with highly purified n-3 FA is believed to be antiatherogenic owing to its pronounced lowering effect on postprandial triglyceridemia (18,19). Recently, Mabile *et al*. (32) demonstrated that CM isolated after a meal rich in n-6 polyunsaturated FA exhibited a high oxidizability compared to CM enriched with monounsaturated and saturated FA. In our study, human CM enriched with n-3 polyunsaturated FA were more susceptible to lipid peroxidation than CM enriched with saturated fat. The TBARS values measured in isolated CM may be augmented, in part, as a result of removal of antioxidants present in plasma, thereby rendering the isolated CM more susceptible to oxidation. The observed difference in TBARS of CM isolated after the two meals, however, could not be explained by the methodological conditions since equal isolation procedures was performed.

In humans, vitamin E is an important lipid-soluble antioxidant, which is absorbed *via* the lymphatic pathway and transported in association with CM (33). Kontush *et al*. (34) recently reported that vitamin E could play a triggering role in lipoprotein oxidation by metal ions. The highly purified n-3 FA capsules administered in the present study contained 3.4 mg vitamin E each to avoid lipid oxidation. If vitamin E served as an efficient antioxidant in CM, one might have expected an inverse association between lipid peroxidation and vitamin E concentration in CM particles. However, no such association was observed in our study. This may indicate that vitamin E was inefficient as an antioxidant, that the amount of vitamin E in the capsules was insufficient to protect the CM particles against lipid peroxidation, or that peroxidation occurred in the gastrointestinal tract prior to CM assembly.

The investigation of the pathophysiological role of lipid peroxides in atherogenesis has mainly been concerned with oxidized LDL (3). However, oxidized CM exerts cytotoxic effects on endothelial cells (32) and promotes leukocyte adhesion through mechanisms involving the superoxide anion, platelet-activating factor (PAF), and adhesion molecules on leukocytes and endothelial cells (35). Thus, oxidized CM may be involved in the injury of the artery wall and provide a link between postprandial lipemia and atherogenesis (32).

Long-term supplementation with DHA was accompanied by a decrease in the total plasma glutathione content, but no such changes were observed after supplementation with EPA. In the DHA group, the level of the reduced/total ratio for the precursor aminothiol in glutathione synthesis, cysteine, tended to be lower, while the breakdown product of glutathione remained unchanged. These findings may indicate that the redox status of the aminothiols is essentially maintained during EPA supplementation, whereas aminothiols may undergo redox cycling and thiol–disulfide exchange in the DHA group. Likewise, the EPA and DHA capsules were equally enriched with vitamin E (3.4 mg/capsule), but the plasma concentration of vitamin E increased significantly only after long-term EPA supplementation. This may further indicate that DHA ingestion accompanied by consumption of supplied vitamin E implied more oxidative stress to blood lipids. As there were no changes in the aminothiols in the EPA group, this indicates that supplied lipid-soluble vitamin E renders the system efficient to manage free-radical attack, as increased lipid peroxidation was not observed.

Harmful lipid peroxidation is, however, dependent on the balance between cellular prooxidative processes and the level of antioxidants. For both the EPA and DHA groups, lipid per-

oxidation remained unchanged even though DHA supplementation tended to stress both lipid-soluble and polar antioxidants. In principal, these observations are in agreement with observations in rats (26) and mice (36). Surprisingly, EPA has been reported to attenuate lipid peroxidation both in animals (26,36) and under *in vitro* conditions (37).

In conclusion, enrichment of CM with polyunsaturated n-3 FA during a high-fat meal was accompanied by an immediate increase in lipid peroxides of isolated CM, whereas the oxidative status in plasma remained unchanged during 5 wk of supplementation with highly purified EPA and DHA.

ACKNOWLEDGMENT

This study was supported in part by grants from Pronova Biocare a.s., Oslo, Norway, the Norwegian Council on Cardiovascular Diseases, and the Norwegian Research Council.

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[Received March 2, 1998, and in final revised form and accepted October 20, 1998]

Low Doses of Eicosapentaenoic Acid, Docosahexaenoic Acid, and Hypolipidemic Eicosapentaenoic Acid Derivatives Have No Effect on Lipid Peroxidation in Plasma

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ABSTRACT: It was of interest to investigate the influence of both high doses of eicosapentaenoic acid (EPA) and low doses of 2- or 3-methylated EPA on the antioxidant status, as they all cause hypolipidemia, but the dose required is quite different. We fed low doses (250 mg/d/kg body wt) of different EPA derivatives or high doses (1500 mg/d/kg body wt) of EPA and DHA to rats for 5 and 7 d, respectively. The most potent hypolipidemic EPA derivative, 2,2-dimethyl-EPA, did not change the malondialdehyde content in liver or plasma. Plasma vitamin E decreased only after supplementation of those EPA derivatives that caused the greatest increase in the fatty acyl-CoA oxidase activity. Fatty acyl-CoA oxidase activity increased after administration of both EPA and DHA at high doses. High doses of EPA and DHA decreased plasma vitamin E content, whereas only DHA elevated lipid peroxidation. In liver, however, both EPA and DHA increased lipid peroxidation, but the hepatic level of vitamin E was unchanged. The glutathione-requiring enzymes and the glutathione level were unaffected, and no significant changes in the activities of xanthine oxidase and superoxide dismutase were observed in either low- or high-dose experiments. In conclusion, increased peroxisomal β-oxidation in combination with high amounts of polyunsaturated fatty acids caused elevated lipid peroxidation. At low doses of polyunsaturated fatty acids, lipid peroxidation was unchanged, in spite of increased peroxisomal β-oxidation, indicating that polyunsaturation is the most important factor for lipid peroxidation.

Lipids 33, 1131–1137 (1998).

Administration of peroxisome-proliferating hypolipidemic compounds produces marked increases in the enzyme activities of the peroxisomal fatty acid β-oxidation cycle, including the hydrogen peroxide-generating enzyme fatty acyl-CoA oxidase (FAO, E.C. 1.3.3.6) (1). In contrast, hydrogen peroxide-degrading enzymes such as catalase (E.C. 1.11.1.6) are not proportionally induced by peroxisome proliferators (1). This imbalance between hydrogen peroxide-generating and -degrading enzymes is an undesirable effect of the peroxisome-proliferating compounds as it may create cellular oxidative stress.

Membrane fatty acid composition can be rapidly modified by changing the source of dietary lipids (2). When polyunsaturated oils are increased in the diet, tissues become enriched with polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Membranes containing increased levels of these fatty acids are more easily oxidized (3), and the susceptibility of liver lipids to peroxidation is increased after ingestion of diets high in fish oil (4). Increasing polyunsaturation has been reported to be antagonistic to vitamin E status (5). A fish oil-containing diet may thus have an unfavorable effect on the antioxidant/prooxidant balance. In contrast, we showed that hypolipidemic doses of EPA enhance the hepatic antioxidant defense (6). PUFA have a hypolipidemic effect when administered to rats. However, high doses of EPA are necessary to cause hypolipidemia, whereas high doses of DHA have no lipid-lowering effect (7). The hypolipidemic effect of EPA was potentiated by branching, and the resulting 2- and 3-methylated EPA molecules caused hypolipidemic effects at low doses (Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., submitted for publication). It was of interest to investigate the influence of the above-mentioned fatty acids on the antioxidant status in rats, as they all cause hypolipidemic effect, but the doses of PUFA required to achieve this effect are different. For this purpose we measured the content of vitamins E, A and C, thiols, and lipid peroxidation, in addition to the enzyme activities of several enzymes participating in the antioxidant defense system.

MATERIALS AND METHODS

Chemicals. Ethyl esters of EPA (97.0% pure), DHA (91.2% pure), and the ethyl esters of the EPA derivatives 2-methyl-EPA (93.8% pure), 2-ethyl-EPA (96.1% pure), 2,2-dimethyl-EPA (95.3% pure), and 3-methyl-EPA (>90% pure) were obtained from Norsk Hydro AS, Research Centre (Porsgrunn, Norway). [α-^{32}P]deoxycytidine triphosphate (3000 Ci/mmol) was from Amersham (Buckinghamshire, England). Restriction enzymes were from Promega (Madison, WI). Nylon membranes (NY 13N) were from Schleicher & Schuell (Das-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAO, fatty acyl-CoA oxidase; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutase; XDH, xanthine dehydrogenase; XOX, xanthine oxidase.

sel, Germany). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Möllegaard breeding Laboratory (Ejby, Denmark), weighing 160–180 g, were housed as pairs in plastic cages in a room maintained at 12 h light-dark cycles and a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 1 wk under these conditions before the start of the experiment. The different fatty acids (all as ethyl esters) were suspended in 0.1% carboxymethylcellulose, and 0.5% (wt/vol) α-tocopherol acetate was added to the PUFA to prevent autooxidation. In the low-dose experiment EPA, DHA, and the EPA derivatives were administered at a dose of 250 mg/d/kg body wt for 5 d by gastric intubation in a final volume of 0.5 mL once a day. In the high-dose experiment, EPA, DHA, and oleic acid were administered at a dose of 1500 mg/d/kg body wt for 7 d by gastric intubation in a final volume of 1.0 mL once a day. In both experiments the control animals received only sodium carboxymethylcellulose. All animals had free access to water and food (rat and mouse standard diet, from B&K Universal, Sollentuna, Sweden). The food contained 61.0 mg/kg DL-α-tocopherol acetate and 7.0 mg/kg retinol. At the end of the feeding period, after overnight fasting, the animals were anesthetized by Hypnorm (Janssen Pharmaceutical Ltd., Oxford, England) Dormicum® (F. Hoffmann-La Roche AG, Basel, Switzerland) (Fentanyl/fluanisone-Midazolam), 0.2 mL/100 g body wt. Cardiac puncture was performed to obtain blood samples, and the liver was removed. Parts of the liver were immediately frozen in liquid nitrogen, and the rest of the liver was chilled on ice for homogenization.

Preparation of subcellular fractions. The livers were homogenized in ice-cold sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA) using a Potter-Elvehjem homogenizer. The subcellular fractions were isolated as previously described (8). Briefly, the homogenate was centrifuged at 1,000 × *g* for 10 min to separate the postnuclear from the nuclear fraction. A mitochondrial-enriched fraction was prepared from the postnuclear fraction at 10,000 × *g* for 10 min. A peroxisome-enriched fraction was prepared by centrifugation of the postmitochondrial fraction at 23,500 × *g* for 30 min. A microsomal-enriched fraction was isolated from the postperoxisomal fraction at 100,000 × *g* for 1 h 15 min. The remaining supernatant was collected as the cytosolic fraction. The procedure was performed at 0–4°C, and the fractions were stored at −80°C. Protein was assayed using the BioRad protein assay kit (BioRad, Richmond, CA) using bovine serum albumin as a standard.

*Vitamins E and A***.** Both liver and plasma were analyzed for vitamin E (α-tocopherol) and vitamin A (retinol) by high-performance liquid chromatography (HPLC). Plasma (200 μL) was mixed with an equal volume of 300 μM standard (α-tocopherol acetate) in ethanol, or 200 μL 10% (wt/vol) liver homogenate with 300 μM standard was added to an equal volume of water. To these solutions 500 μL hexane was added. The resultant mixture was shaken vigorously for 2 min, centrifuged, and partly evaporated (250 μL) under a stream of N_2. Then 125 μL ethanol was added, and the mixture was analyzed by HPLC. The analysis was performed with Supelcosil LC-8 column (25 cm × 4.6 mm i.d.) with LC-8 guard column packed with Perisorb RP-8 (2 cm × 2 mm i.d.). Methanol/H_2O, 95:5, and methanol were used as mobile phases. The flow was set to 1.0 mL/min and the injection volume was 40 μL. Detection was achieved at 292 nm using a Shimadzu RF-535 detector with multiplicator (Hamamatsu R928-08).

Vitamin C. Vitamin C (ascorbic acid) was measured in liver by HPLC. Two hundred microliters 10% (wt/vol) liver homogenate in 50 mM perchloric acid was added to 400 μL deproteinization solution [7% (vol/vol) perchloric acid, 1% (vol/vol) metaphosphoric acid], shaken vigorously for 20 s, frozen at −80°C, and centrifuged at 12,000 × *g* for 5 min. Supernatant was added 1:1 to the mobile phase [20 mM $NH_4H_2PO_4$, 0.015% (vol/vol) metaphosphoric acid, pH 3.0], and 20 μL was injected onto a Supelcosil LC-8 column (25 cm × 4.6 mm i.d.) with LC-8 guard column packed with Perisorb RP-8 (2 cm × 2 mm i.d.). Detection was achieved at 245 nm using a Spectra FOCUS detector.

Malondialdehyde (MDA). MDA was measured in plasma and liver by HPLC. As stated by Halliwell and Chirico (9), the HPLC-based MDA test can be used as a preliminary general measurement of lipid peroxidation. Briefly, 250 μL 20% (wt/vol) acetic acid and 250 μL 0.8% (wt/vol) 2-thiobarbituric acid in 0.1 N NaOH was added to 100 μL liver homogenate [10% (wt/vol) in sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA)] or plasma. The sample was then heated at 95°C for 1 h and then chilled, an equal volume of 1-butanol was added, and the resultant mixture was shaken vigorously for 2 min. After centrifugation at 1,700 × *g* for 5 min, the supernatant was analyzed by HPLC. The analysis was performed with Supelcosil LC-18 column (5 μm, 25 cm × 4.6 mm i.d.). KH_2PO_4 (25 mM, pH 7)/methanol, 65:35, was used as mobile phase. The flow was set to 1.0 mL/min, and the injection volume was 20 μL. Detection was achieved using a Shimadzu RF-535 detector with multiplicator (Hamamatsu R928-08), with excitation at 532 and emission at 553 nm.

Enzyme activities. Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured spectrophotometrically by monitoring the decay of superoxide ($O_2^{\bullet -}$) (10). FAO activity was determined in the peroxisome-enriched fraction of rat livers by the coupled assay (11). The production of hydrogen peroxide was measured by monitoring the increase in dichlorofluorescein absorbance in the presence of palmitoyl-CoA.

Preparation of hybridization probes. DNA fragments were labeled by random priming using the oligolabeling technique of Feinberg and Vogelstein (12). The DNA probes were purified fragments of cloned rat genes. Copper zinc (CuZn)-SOD: 600 bp *Eco*RI fragment of pCuSOD (13), and manganese (Mn)-SOD: 1400 bp *Eco*RI fragment of pMnSOD (13). As control we used rat P0 rRNA: 1046 bp *Bam*HI/*Xho*I fragment in pBluescript II SK (z29530, provided by A. Molven, University of Bergen, Norway) or human 28S rRNA: 1400 bp *Bam*HI fragment of pA (Gonzalez, I.L., personal communication).

RNA purification and analysis. Total cellular RNA was isolated by the guanidinium-thiocyanate method described by

Chomczynski and Sacchi (14). The RNA concentrations were determined spectrophotometrically. RNA was blotted onto nylon (15). Hybridization to immobilized RNA was performed in the presence of 50% formamide, 5 × saline-sodium citrate, 200 μg/mL heat-denatured herring sperm DNA, 0.1% (wt/vol) sodium dodecyl sulfate, 25 mM sodium phosphate pH 6.5, 8.25% dextran sulfate at 42°C for 24 to 48 h (16). Filters were washed to high stringency [0.2 × saline-sodium citrate, 0.1% (wt/vol) sodium pyrophosphate, 0.1% (wt/vol) sodium dodecyl sulfate at 65°C] and Kodak XAR-5 X-ray films were exposed in the presence of intensifying screens at −80°C. Densitometric scanning of autoradiograms was performed using the LKB Ultrogel laser–densitometer (Bromma, Sweden). When the filters were to be rehybridized, the bound probe was first stripped off in 0.1% (wt/vol) sodium dodecyl sulfate at 90–100°C for 7 min. The hybridization results were normalized to the signal of a cDNA probe for a ribosomal protein used as control or 28S rRNA hybridization in the individual samples. Relative mRNA inductions of the different genes were then calculated.

Thiols. Glutathione, cysteine, cysteinylglycine, and homocysteine were quantified in plasma and liver after derivatization with the fluorescent agent monobromobimane by reversed-phase HPLC (17).

Presentation of results. The results are reported as means ± SD from 3 to 8 animals. Statistical analysis was by one-way analysis of variance.

RESULTS

Effects on vitamins. Table 1 shows the changes in vitamin E, vitamin A and vitamin C in the different dietary groups. At low doses the plasma vitamin E content decreased 35 and 22%, respectively, in rats fed 2,2-dimethyl- and 3-methyl-EPA compared to EPA, while the plasma level of vitamin A was unchanged. In liver, the vitamin E content was unchanged, while vitamin A increased in rats fed EPA. At high doses, plasma vitamin E decreased 43% in rats fed EPA and DHA, but the hepatic content of vitamin E was unchanged. Plasma vitamin A, hepatic vitamin A and hepatic vitamin C were unaffected by the different feeding groups of the high-dose experiment (Table 1).

Effect on MDA. MDA, a product of lipid peroxidation, was unchanged in plasma and liver of rats fed a low dose of EPA derivatives, EPA, or DHA, compared to controls (Table 2). In the high-dose experiment, DHA feeding caused a 1.5- and 1.6-fold elevation of plasma and hepatic MDA, respectively. EPA-feeding at high doses did not affect the plasma MDA, but the hepatic MDA increased 1.4-fold (Table 2).

Effect on plasma and hepatic thiols. When measuring the plasma levels of total homocysteine, cysteine (the rate-limiting precursor amino acid in glutathione synthesis), glutathione, and cysteinylglycine (the breakdown product of glutathione), we found that low doses of 3-methyl-EPA caused a small increase in homocysteine, while glutathione, cysteine and cysteinylglycine were unchanged (Table 3). In liver, the cysteine and glutathione levels were unchanged (Table 3). In the high-dose experiment, we found no significant changes of the hepatic thiols after treatment with EPA, DHA, or oleic acid compared to control (data not shown).

Effect on enzyme activities and mRNA levels. In the low-dose experiment 2,2-dimethyl-EPA tended to increase total SOD activity in the peroxisome-enriched fraction, although not significantly (Table 4). This was accompanied by a 1.9-fold elevated mRNA level of Mn-SOD compared to the control or EPA, and a small (1.3-fold) elevation of CuZn-SOD mRNA compared to EPA. At high doses, the enzyme activity

TABLE 1
Effects of Different Doses of n-3 Fatty Acids on Plasma and Hepatic Levels of Vitamins E and A, and Hepatic Level of Vitamin C[a]

	Vitamin E		Vitamin A		Vitamin C
	Plasma (μmol/L)	Tissue (nmol/g liver)	Plasma (μmol/L)	Tissue (nmol/g liver)	Tissue (μmoL/g liver)
Low-dose experiment (250 mg/d/kg body wt)					
2-Methyl-EPA	12.3 ± 1.7	110.2 ± 20.8	2.3 ± 0.5	25.7 ± 5.1	n.d.
2-Ethyl-EPA	11.2 ± 3.0	103.9 ± 14.2	2.4 ± 0.7	26.1 ± 7.3	n.d.
2,2-Dimethyl-EPA	9.0 ± 0.3[b]	100.7 ± 8.3	1.8 ± 0.8	23.6 ± 5.9	n.d.
3-Methyl-EPA	7.6 ± 0.3[b,c]	100.7 ± 10.5	2.5 ± 0.6	24.8 ± 2.8	n.d.
EPA	11.6 ± 1.0	112.1 ± 9.3	2.4 ± 0.3	33.0 ± 4.4[c]	n.d.
DHA	12.1 ± 1.8	101.5 ± 6.6	2.5 ± 0.8	25.3 ± 5.6	n.d.
Control	10.9 ± 1.0	110.1 ± 11.6	2.3 ± 0.5	22.4 ± 3.7[b]	n.d.
High-dose experiment (1500 mg/d/kg body wt)					
EPA	8.8 ± 1.2[c]	81.5 ± 15.8	2.1 ± 0.0	13.6 ± 1.8	1.4 ± 0.1
DHA	8.7 ± 1.4[c]	73.7 ± 7.4	2.2 ± 0.3	13.1 ± 1.9	1.6 ± 0.1
Oleic acid	13.2 ± 1.7[b]	70.1 ± 25.1	2.2 ± 0.3	13.0 ± 3.4	1.7 ± 0.3
Control	15.3 ± 1.1[b]	80.7 ± 5.4	2.1 ± 0.2	14.0 ± 2.6	1.6 ± 0.3

[a]The values are means ± SD for 4–8 animals. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n.d., not determined.
[b]Significantly different from control (sodium carboxymethylcellulose).
[c]Significantly different from EPA ($P < 0.05$).

TABLE 2
Plasma and Hepatic Levels of MDA in Rats Fed Different Fatty Acids at Different Doses[a]

	Plasma (μmol MDA/L)	Tissue (nmol MDA/g liver)
Low-dose experiment (250 mg/d/kg body wt)		
2,2-Dimethyl-EPA	1.6 ± 0.1	44.8 ± 3.1
3-Methyl-EPA	1.6 ± 0.2	45.3 ± 6.0
EPA	1.5 ± 0.2	40.4 ± 0.8
DHA	1.4 ± 0.1	43.6 ± 3.2
Control	1.4 ± 0.1	43.6 ± 4.4
High-dose experiment (1500 mg/d/kg body wt)		
EPA	1.5 ± 0.3	112.5 ± 9.1[b]
DHA	1.9 ± 0.2[b]	127.7 ± 15.0[b]
Oleic acid	1.3 ± 0.1	84.1 ± 4.2[c]
Control	1.3 ± 0.1	81.2 ± 4.4[c]

[a]Values are means ± SD for 3–7 animals.
[b]Significantly different from control.
[c]Significantly difference from EPA ($P < 0.05$). MDA, malondialdehyde; for other abbreviations see Table 1.

of total SOD was unchanged in rats fed EPA and DHA. However, the SOD activity tended to decrease in rats fed oleic acid and there was a reduced expression of CuZn-SOD and Mn-SOD mRNA by oleic acid (Table 4). The SOD activity was unchanged in the mitochondrial-enriched and the cytosolic fractions in both the high- and low-dose experiments (data not shown). Low doses of 2,2-dimethyl- and 3-methyl-EPA or high doses of EPA and DHA did not affect the enzyme activities of xanthine oxidase (XOX, E.C. 1.1.3.22) and xanthine dehydrogenase (XDH, E.C. 1.1.1.204) compared to the control in the postnuclear or the cytosolic fractions (data not shown). Also, the activities of glutathione reductase (E.C. 1.6.4.2), glutathione peroxidase (E.C. 1.11.1.9), and glutathione-*S*-transferase (E.C. 2.5.1.18) were unchanged in the postnuclear, cytosolic or peroxisomal fractions (data not shown), as was the mRNA level of glutathione peroxidase (data not shown) in both experiments.

The enzyme activity of FAO was measured both in the low-dose (Fig. 1A) and the high-dose (Fig. 1B) experiments. Low doses of 2,2-dimethyl- and 3-methyl-EPA increased the FAO activity 2.7- and 2.2-fold, respectively, whereas 2-ethyl-EPA increased the FAO activity 1.7-fold compared to control.

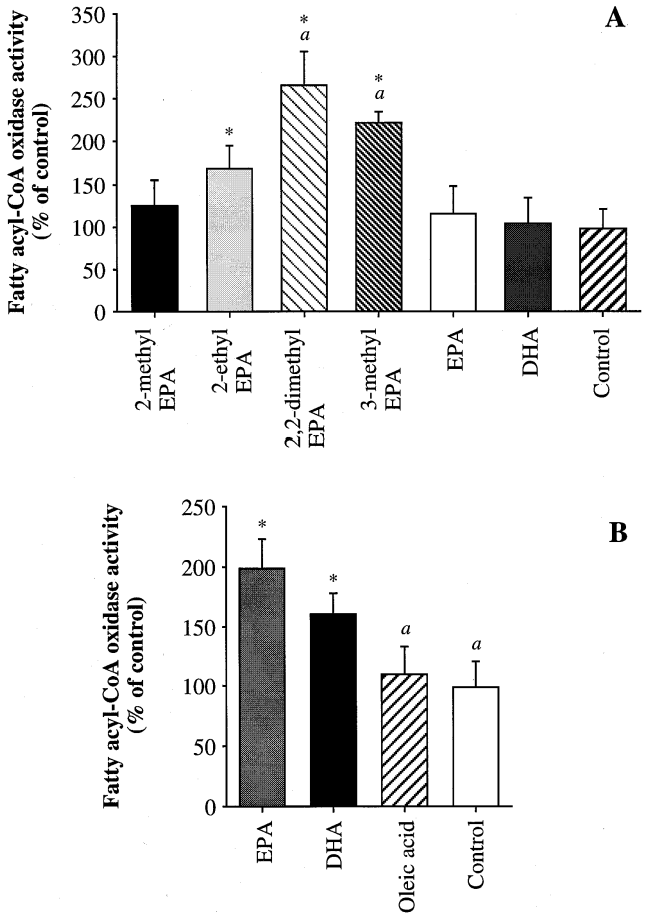

FIG. 1. Enzyme activity of fatty acyl-CoA oxidase in rats fed (A) low doses (250 mg/d/kg body wt) of the EPA derivatives, EPA or DHA, and (B) high doses (1500 mg/d/kg body wt) of EPA, DHA, or oleic acid. CMC-fed animals were used as control. Fatty acyl-CoA oxidase activity was measured in the peroxisome-enriched fraction. Data are relative percentage change from the means of the control values of 20.05 ± 4.08 and 16.41 ± 3.46 nmol/min/mg protein for (A) and (B), respectively. Data are given as means ± SD for 4–5 rats. *Significantly different from control. [a]Significantly different from EPA ($P < 0.05$). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CMC, sodium carboxymethylcellulose.

In the high-dose experiment EPA and DHA increased the FAO activity 2.0- and 1.6-fold, respectively.

TABLE 3
Plasma and Hepatic Thiols in Rats Fed Different n-3 Fatty Acids at Low Doses[a]

	Cysteine		Glutathione		Cys-Gly	Homocysteine
	Plasma (μmol/L)	Liver (nmol/g)	Plasma (μmol/L)	Liver (μmol/g)	Plasma (μmol/L)	Plasma (μmol/L)
2,2-Dimethyl-EPA	58.8 ± 9.1	100.1 ± 3.3	13.2 ± 1.7	4.5 ± 1.1	0.9 ± 0.1	5.6 ± 0.6
3-Methyl-EPA	71.0 ± 5.4	108.9 ± 0.6	15.1 ± 5.4	5.0 ± 1.0	0.9 ± 0.0	6.3 ± 0.3[b]
EPA	64.7 ± 4.5	108.1 ± 4.9	12.2 ± 1.8	4.7 ± 1.5	0.8 ± 0.1	5.6 ± 0.6
DHA	63.8 ± 6.9	115.6 ± 2.8	12.1 ± 2.2	5.9 ± 0.6	1.0 ± 0.1	4.9 ± 0.8
Control	62.6 ± 13.8	107.2 ± 3.7	11.8 ± 0.9	5.3 ± 1.2	1.0 ± 0.1	4.8 ± 0.5

[a]Values are means ± SD for 3–6 animals. EPA, DHA, and EPA derivatives were fed at a dose of 250 mg/d/kg body weight for 5 d.
[b]Significantly different from control ($P < 0.05$). Cys-Gly, cysteinylglycine; for other abbreviations see Table 1.

TABLE 4
Enzyme Activity and mRNA Level of Superoxide Dismutase (SOD) in Liver of Rats Fed n-3 Fatty Acids and EPA Derivatives[a]

	Total SOD activity (U/mg)	mRNA expression (relative levels)	
		CuZn SOD	Mn SOD
Low-dose experiment (250 mg/d/kg body wt)			
2,2-dimethyl EPA	1006 ± 481	1.3 ± 0.4[b]	1.9 ± 0.5[b,c]
3-Methyl-EPA	594 ± 74	0.8 ± 0.3	1.3 ± 0.3
EPA	671 ± 116	0.6 ± 0.2	1.0 ± 0.3
Control	684 ± 147	1.0 ± 0.4	1.0 ± 0.1
High-dose experiment (1500 mg/d/kg body wt)			
EPA	407.0 ± 74.9	0.7 ± 0.1	1.0 ± 0.2
DHA	378.3 ± 32.8	0.9 ± 0.2	1.2 ± 0.3
Oleic acid	370.7 ± 8.5	0.9 ± 0.1	1.0 ± 0.2
Control	460.3 ± 62.3	1.0 ± 0.3	1.0 ± 0.2

[a]The enzyme activity of SOD was measured in the peroxisomal-enriched fraction. RNA purification and hybridization experiments were performed as described in the Materials and Methods section, and the mean for the control from the mRNA data is set to 1. The values are means ± SD for 4–6 animals.
[b]Significantly different from control.
[c]Significantly different from EPA ($P < 0.05$). For other abbreviations see Table 1.

DISCUSSION

In the present experiments, the effects of hypolipidemic doses of different PUFA on the antioxidant status were investigated. Rats were fed EPA, DHA, and different EPA derivatives, that is, EPA methylated or ethylated at the 2- or 3-position, at doses known to cause hypolipidemia. Earlier our group found that high doses of EPA (1000 mg/d/kg body wt) were necessary to obtain hypolipidemic effects and elevate fatty acid oxidation in rats (7,18). The EPA derivatives used in this study have proved to reduce plasma lipids and increase the mitochondrial and peroxisomal β-oxidation in rats at low doses (Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., unpublished data). Low doses (250 mg/d/kg body wt) of the EPA derivatives also increased the activity of FAO. These EPA derivatives differed in their ability to increase the FAO activity, and the most potent derivatives were 2,2-dimethyl-EPA and 3-methyl-EPA (Fig. 1A). In this low-dose experiment, where rats were fed EPA derivatives, EPA or DHA, the plasma vitamin E content (Table 1) decreased only in the groups that caused the greatest induction of FAO activity, that is, 2,2-dimethyl-EPA and 3-methyl-EPA. At high doses (1500 mg/d/kg body wt) of EPA and DHA, the plasma vitamin E content decreased concomitantly with increased peroxisomal β-oxidation, measured as enzyme activity of FAO (Table 1, Fig. 1). Furthermore, plasma vitamin A was not changed by PUFA feeding in the two experiments. As both high doses of EPA and low doses of EPA derivatives are hypolipidemic (7; and Vaagenes, H., Madsen, L., Dyrøy, E., Elholm, M., Stray-Pedersen, A., Frøyland, L., Lie, Ø., and Berge, R.K., unpublished data) and the vitamin A content is unchanged, we can conclude that the plasma contents of the lipid-soluble vitamins are not a consequence of plasma lipid concentration. In the low-dose experiment, it is therefore reasonable to believe that the primary cause of decreased vitamin E content in plasma is upregulated peroxisomal β-oxidation, not polyunsaturation or reduced plasma lipids. In relation to this, it has recently been found that administration of low doses (360 mg/d/kg body wt) of EPA or DHA, which are known not to upregulate the peroxisomal fatty acid oxidation, to rats did not change the hepatic or plasma vitamin E level (19). Although the plasma vitamin E content decreased in the low-dose experiment, we observed no increase in lipid peroxidation, measured as MDA (Table 2). However, increasing the dose of PUFA administered to rats would increase the vulnerability of the cell to the hydrogen peroxide produced during elevated peroxisomal β-oxidation. Therefore, administration of DHA at high doses, which increases the peroxisomal β-oxidation, reduces plasma vitamin E content and consequently elevates lipid peroxidation. Increased vitamin E requirement with increasing polyunsaturation (5) may explain the difference between high doses of EPA and DHA on plasma lipid peroxidation. The difference in plasma MDA in rats fed equal doses of EPA and DHA could also be due to difference in the metabolism of these two n-3 fatty acids. It has recently been shown that EPA is mainly oxidized in the mitochondria, whereas DHA is most likely oxidized by the peroxisomes (20). Furthermore, DHA is a stronger peroxisome proliferator than EPA, whereas EPA causes mitochondrial proliferation. Unlike the mitochondria, the first enzyme in peroxisomal β-oxidation, FAO, produces hydrogen peroxide (1). Moreover, as EPA seems more easily oxidized than DHA (21), we expect to find a higher concentration of DHA than of EPA in plasma. Taken together, the higher amount of peroxisome-derived radicals and the higher concentration of DHA in plasma, as well as the fact that DHA contains one additional double bond, would increase the susceptibility of DHA to lipid peroxidation compared to EPA.

That the hepatic levels of vitamin E and A were unchanged in both the low- and the high-dose experiments shows that, although the plasma vitamin E level decreased, the rat was not depleted of vitamins. This observation fits with the assumption that parenchymal cells in the liver have the ability to conserve their content of vitamin E (22). Over a 3-mon period, however, administration of EPA or DHA to rats reduces the hepatic vitamin E content (6). In the low-dose experiment, there was no change in hepatic lipid peroxidation, measured as MDA. In the high-dose experiment, however, both EPA and DHA caused elevated lipid peroxidation in liver, but the hepatic content of vitamins E, A, and C were unaffected (Tables 2 and 1, respectively). Seemingly, these vitamins were not sufficient to prevent lipid peroxidation in the liver, and this might explain why EPA, with fewer double bonds than DHA, also increased hepatic lipid peroxidation. A small increase in the hepatic vitamin A content was observed in the low-dose experiment, which was not present in the high-dose experiment. The reason for this cannot be explained.

The total SOD activity tended to increase in the hepatic peroxisomal fraction of rats fed low doses of the EPA derivative

2,2-dimethyl-EPA. Significantly induced gene expression of Mn-SOD was observed (Table 3). These effects could be a kind of cellular adaptation, i.e., a secondary effect, to oxidative stress induced by 2,2-dimethyl-EPA. At high doses of EPA or DHA the activity and gene expression of SOD remained unchanged. Oleic acid, however, tended to decrease the SOD activity in the peroxisomal fraction concomitant with decreased gene expression of both Mn-SOD and CuZn-SOD. Reaven (23) has proposed that oleic acid has antioxidant properties, and these could possibly reduce the necessity of SOD activity in rats fed oleic acid, thereby downregulating this activity. Why low doses of 2,2-dimethyl-EPA, but not high doses of EPA and DHA, seemed to affect the activity of SOD remains unclear. Perhaps this effect is due to the production of hydrogen peroxide, as 2,2-dimethyl-EPA induces the FAO-activity 2.7-fold, whereas high doses of DHA or EPA only increase the FAO-activity by 1.7- and 2.0-fold, respectively. The enzyme activities of XOX and of XDH did not change in the low- or the high-dose experiments (data not shown). As the rate of conversion of XDH to XOX seemed to be unchanged compared to the control, it is likely that the amount of superoxide produced from XOX is constant. The superoxide produced from XOX would therefore not affect the SOD activity.

The enzyme activities of glutathione peroxidase, glutathione-*S*-transferase, and glutathione reductase did not change in the low- or the high-dose experiments (data not shown). The levels of glutathione and the glutathione metabolites were not affected in plasma or liver by any of the fatty acid administrations (Tables 4; additional data not shown). This indicates that the low- and high-dose experiments did not affect the glutathione metabolism to any great extent. Thus, EPA, DHA and the EPA derivatives did not seem to weaken the capacity of glutathione as an antioxidant.

The presence of homocysteine is an independent risk factor for cardiovascular diseases (24). The mechanism does not seem to involve lipid peroxidation (25,26). Our results in the low-dose experiment confirm this, since elevated plasma homocysteine in rats fed 3-methyl-EPA (Table 4) did not cause increased lipid peroxidation (Table 2).

To conclude, increased peroxisomal β-oxidation primarily seems to cause oxidative stress in rats fed PUFA. However, the amount of accumulated PUFA in the cell membranes is the critical parameter. Therefore, low doses of lipid-lowering EPA derivatives do not affect lipid peroxidation. High doses of EPA and DHA, however, overwhelm the antioxidant defense mechanisms, resulting in elevated levels of MDA in plasma and liver, even though the activity of the hydrogen peroxide-producing FAO was higher in rats fed low doses of lipid lowering EPA derivatives than high doses of EPA and DHA. The antioxidant enzymes were not affected to any great extent in these experiments.

ACKNOWLEDGMENTS

This work was supported by The Norwegian Council on Cardiovascular Disease and Norsk Hydro. The authors are grateful to Svein Krüger, Kari Williams, Bjørn Netteland, Liv-Kristine Øyseid, Randi Sandvik, and Kari Helland for excellent technical assistance. We thank the Medical Research Centre, University of Bergen, for providing equipment used in this study. We appreciate Drs. A. Molven, I.L. Gonzalez, C.-C. Lai, and J.L. Tilly for sharing probes with us.

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[Received May 26, 1998, and in final revised form and accepted October 27, 1998]

Quantitative Determination of Butylated Hydroxyanisole, Butylated Hydroxytoluene, and *tert*-Butyl Hydroquinone in Oils, Foods, and Biological Fluids by High-Performance Liquid Chromatography with Fluorometric Detection

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ABSTRACT: Concentrations of synthetic antioxidants butylated hydroxyanisole, butylated hydroxytoluene, and *tert*-butyl hydroquinone were quantified using a high-performance liquid chromatograph with spectrofluorometric detector. The antioxidants were separated and eluted on a reversed-phase column by gradient of a mixture of H_2O/acetonitrile/acetic acid (66.5:28.5:5, by vol) and a mixture of acetonitrile/acetic acid (95:5, vol/vol). The eluants were monitored at emission and excitation wavelengths of 310 and 280 nm, respectively. Calibration curves obtained using peak areas against concentration showed high coefficients of multiple determination ($R^2 > 0.99$) for all antioxidants. Known concentrations of added antioxidant standards were recoverable within 98–99% from oils and over 93% from mouse blood. This method requires minimum sample extraction and purification before analysis and provides a relatively high percentage recovery. The method has been applied successfully for the measurement of antioxidant concentrations in oils, dried foods, and biological fluids.

Lipids 33, 1139–1145 (1998).

Synthetic antioxidants are defined by the U.S. Food and Drug Administration (FDA) as food preservatives that specifically retard deterioration, rancidity, or discoloration due to lipid oxidation (1). When added beyond certain limits, however, antioxidants tend to be pro-oxidant (2,3), and rat experiments have revealed that a high dosage in the body causes various tumors and cancers (4). Thus, information on the amounts of antioxidants incorporated into foods is important for agencies controlling foods, for food processors, and for consumers. Many methods including colorimetry (5), spectrophotometry (6), fluorimetry (7,8), voltammetry (9), chromatography [paper (10), thin-layer chromatography (11,12), gas–liquid chromatography (13–16), or high-performance liquid chromatography (17–25)] have been used for qualitative and quantitative determination of antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (TBHQ) added to fats, oils, and foods. Almost every procedure requires considerable sample preparation such as saponification, extraction, and liquid–liquid separation prior to estimation of individual antioxidants (2,26). Recently, reversed-phase HPLC with direct injection and ultraviolet (UV) detection have been applied to phenolic antioxidants, tocopherols, and triglycerides in oils (25). However, possible interferences from other antioxidants and/or naturally occurring phenolic compounds were reported with the use of some UV procedures, such as spectrophotometry in antioxidant detection (26). Direct injection and fluorimetric detection improved the analysis of TBHQ in oils (19). Simultaneous detection with fluorescence, UV, and electrochemical detectors were applied for determination of phenolic antioxidants (21). However, these methods have not been applied to foods with possible interferences.

In this study, an HPLC method with fluorometric detector and simple extraction was applied to the determination of antioxidants including BHA, BHT, and TBHQ in oils and foods.

EXPERIMENTAL PROCEDURES

HPLC conditions. A gradient liquid chromatography system equipped with two Shimadzu LC 10 AS pumps, a Shimadzu C-R6A chromatographic integrator, and a 10 μL sample loop auto-injector was used for analysis. Eluants were detected with a Shimadzu RF-10 A spectrofluorometric detector with slit widths of 15 nm for excitation and emission and with a 12 μL flow cell. A reversed-phase column, Lichrosorb RP-18 (5 μm, 4 mm i.d. × 250 mm; Merck, Darmstadt, Germany), was used for separation of antioxidants.

Mobile phases and a solvent gradient program were used for elution of antioxidants according to the method of Page (18) with slight modifications. A mobile phase A, comprising water/acetonitrile/acetic acid (66.5:28.5:5, by vol), and mobile phase B, made up of acetonitrile/acetic acid (95:5, vol/vol) were used as eluants. The flow rate was 1 mL/min. The solvent gradient for elution was programmed as listed in Table 1.

The emission and excitation spectra of BHA, BHT, and

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; TBHQ, *tert*-butyl hydroquinone; TMS, trimethylsilyl; UV, ultraviolet.

TABLE 1
Solvent Gradient for Elution

Time (min)	Solvents[a] A (%)	B (%)
0	75	25
2	75	25
7	65	35
10	65	35
15	50	50
20	50	50
30	20	80
35	20	80
35.1	0	100
45	0	100
45.1	75	25

[a]Solvent A: water/acetonitrile/acetic acid (66.5:28.5:5, by vol); solvent B: acetonitrile/acetic acid (95:5, vol/vol).

TBHQ were obtained with a Shimadzu RF 1500 spectrofluorimeter equipped with stirring apparatus at slitwidths of 10 nm for excitation and emission. Solvent compositions were = 33:62:5 in water/acetonitrile/acetic acid by volume for BHA, 13:82:5 for BHT, and 50:45:5 for TBHQ, respectively. Stopped-flow analyses in the above HPLC system gave almost similar values for maximum excitation and emission wavelengths as listed in Table 2. The wavelengths of 280 and 310 nm were adopted as excitation and emission, respectively.

Calibration curves. Acetonitrile solutions of antioxidants within the concentration range of 0.01–50 μg/mL for BHA and TBHQ and of 0.1–200 μg/mL for BHT were prepared, filtered with a Millipore filter [polyvinylidene difluoride, 0.5 μm pore size, Millipore] and subjected to HPLC with an injection volume of 10 μL. Calibration curves of peak area against concentration were obtained. The limit of detection was calculated from the following equation:

$$L_d = a + 3\left\{\frac{\sum_i (y_i - \hat{y}_i)^2}{n - 2}\right\}^{1/2} \quad [1]$$

where L_d is the limit of detection, a is the y-axis intercept deduced from line of regression of area on concentration, y_i are the experimental values of area, $\hat{y}_i$ are the individual values of area in line of regression for area on concentration, and n is the number of samples.

Recovery. The percentage recovery of antioxidant was determined for the acetonitrile extraction for lipids, foods, and mouse blood, and also when antioxidants were extracted by the Bligh and Dyer procedure (27) prior to quantification. The following procedures were carried out at an ambient temperature of 25–29°C.

(i) Acetonitrile extraction from lipids. A known amount of antioxidant was added to soybean oil or total lipid extracted from horse mackerel (*Trachurus japonicus*) white muscle. An aliquot of 5 mL of acetonitrile was added to 2 g of lipids in a screw-capped vial. After a brief purge with a N_2 stream, the vial was tightly capped and shaken vigorously for 2 min to extract the added antioxidant. The resulting extract was then filtered with a Millipore filter (PVDF, 0.5 μm pore size) and loaded into the HPLC system. Antioxidant concentrations were determined by measurement of the peak areas and interpolation from calibration curves.

(ii) Acetonitrile extraction of smoke-flavored sausage. Smoke-flavored sausage without preservatives (sausage A) was obtained from a local supplier. A known amount of antioxidant in ethanol was added to 50 g minced sausage and mixed vigorously. A 5-g minced sample was homogenized in 5 mL acetonitrile and filtered through No. 5C filter paper (Advantec TOYO, Tokyo, Japan). The extraction and filtration procedure for the residue plus filter paper was repeated two times. The resulting filtrates were combined and made up to volume in a 25-mL volumetric flask. All extraction procedures were carried out within 30 min from addition of antioxidants. An aliquot of the solution was filtered through a Millipore filter (polyvinylidene difluoride, 0.5 μm pore size) and loaded into the HPLC system.

(iii) Acetonitrile extraction from biological fluid. A 10-μL ethanol solution containing 10 μg BHA, 10 μg TBHQ, and 100 μg BHT was added to 2 mL whole blood freshly obtained from mice. One milliliter of acetonitrile was added to 250 μL of the resulting blood, shaken vigorously for 2 min, and centrifuged for 10 min at 3,000 × *g*. The supernatant was collected and the same extraction procedure was repeated. The combined supernatant was made up to 5 mL with acetonitrile, then applied to an HPLC analysis as above.

(iv) Recovery after chloroform/methanol/water extraction. A known amount of antioxidant in chloroform solution was extracted using the Bligh and Dyer procedure for simulation of total lipids extraction. The concentration of extracted antioxidant was determined to estimate the losses that occurred during extraction and liquid–liquid separation.

Application to foods. The method was further applied to the determination of antioxidant concentrations in boiled and dried anchovy product and smoke-flavored sausage (sausage B) obtained from local suppliers. The sample was minced and crushed with a food cutter. Extraction from the samples and

TABLE 2
Maximum Excitation and Emission Wavelength (nm) of Antioxidants

Antioxidant[a]	Spectrophotometer Excitation	Emission	Stopped-flow analysis Excitation	Emission
BHA	290	320	285	320
BHT	276	304	280	310
TBHQ	293	322	295	328

[a]BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, *tert*-butyl hydroquinone.

the following procedures were carried out as described above. The concentration of antioxidants in the samples was estimated from the calibration curve.

Peak assignnment by gas chromatography–mass spectrometry (GC–MS). Peaks obtained in the above HPLC system were assigned by fragmentation patterns in GC–MS.

Samples were prepared for injection into the HPLC system as described above. The eluants from the HPLC column were fractionated and evaporated by flushing them to dryness with a N_2 stream; then they were immediately dissolved in *n*-hexane and subjected to GC–MS analysis as below. The trimethylsilyl (TMS) ether derivatives were also prepared and analyzed. TMS ether derivatives were prepared by addition of 0.5 mL distilled pyridine, 1 mL 1,1,1,3,3,3-hexamethyldisilazane, and 0.5 mL trimethylchlorosilane. After 1 h all solution was evaporated by flushing under a N_2 stream. The contents of the flask were dissolved in *n*-hexane and filtered with a Millipore filter (polyvinylidene difluoride, 0.5 µm pore size). Samples were analyzed by GC–MS using a Shimadzu QP 1000 or QP 5000 mass spectrometer fitted with an electron impact ionization source to which a Quadrex 65HT column (0.25 mm i.d. × 15 m) was directly connected.

RESULTS

Operating wavelengths and separation. The emission and excitation spectra for 0.01 µg/mL of BHA, BHT, and TBHQ are shown in Figure 1. The order of increasing sensitivity was BHT < TBHQ < BHA. The maximum excitation and emission wavelengths were similar for BHA and TBHQ—290 and 320 nm, respectively, for BHA, and 293 and 322 nm, respectively, for TBHQ—but different for BHT at excitation and emission of 276 and 304 nm, respectively, as listed in Table 2. These wavelengths were fairly consistent with the values obtained through stopped-flow analyses, where the wavelengths for maximum emission and excitation were 285 and 320 nm, respectively, for BHA, 280 and 310 nm, respectively, for BHT, and 295 and 328 nm, respectively, for TBHQ. The operating wavelengths favored the detection of BHT rather than BHA and TBHQ, because fluorescence intensity of BHT was much lower compared to those of the others mentioned below. Thus, antioxidants were analyzed at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. The wavelengths caused reduction of fluorescence intensity for BHA, about 60% of its maximal intensity, and TBHQ, about 40% of its maximal intensity. However, such intensities were satisfactory for detection of the two antioxidants, which exhibited very high emission yields.

Calibration and recovery. Peak area and concentration of BHA, BHT, and TBHQ correlated well (Fig. 2). Coefficients of determination were over 0.99 for all antioxidants, and the limits of detection were 93 ng for BHA, 810 ng for BHT, and 57 ng for TBHQ. Percentages recoverable from fish oil containing 0.5 µg/g BHA, 5 µg/g BHT, and 0.5 µg/g TBHQ by the acetonitrile extraction were as follows: 98.0% for BHA, 99.2% for BHT, and 98.2% for TBHQ (Table 3). These percentages were consistent with those of fish oil containing 20 µg/g BHA, 20 µg/g TBHQ, and 200 µg/g BHT, and with those in soybean oil. Recoveries of acetonitrile extraction from sausage A to which were added 0.5 µg/g BHA, 5 µg/g BHT, and 0.5 µg/g TBHQ were 87.7% for BHA, 85.9% for BHT, and 50.3% for TBHQ, whereas those for addition of 20 µg/g BHA, 200 µg/g BHT, and 20 µg/g TBHQ were 92.5, 89.9, and 62.3%, respectively. Recoveries from mouse blood were higher than those from sausage A; 97.5% for BHA, 93.6% for BHT, and 93.2% for TBHQ. With the Bligh and Dyer procedure, more losses were observed in the recoverable concentrations of TBHQ, but not in BHA and BHT; 86.6% for BHA, 99.5% for BHT, and 43.9% for TBHQ.

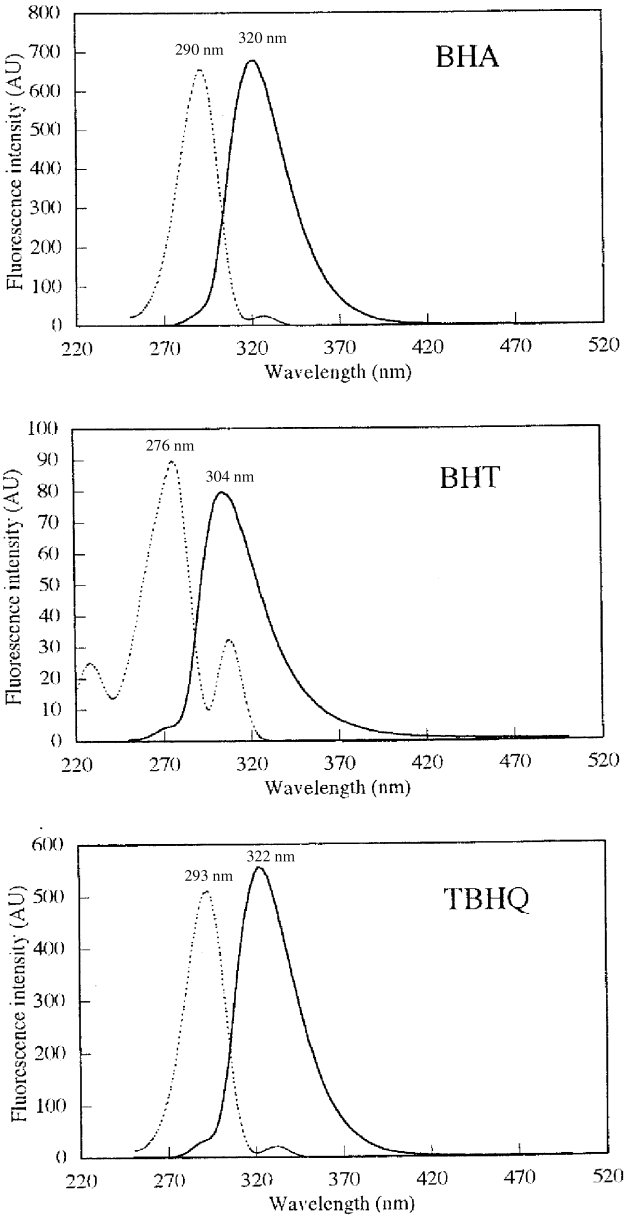

FIG. 1. Excitation (....) and emission (—) spectra of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (TBHQ).

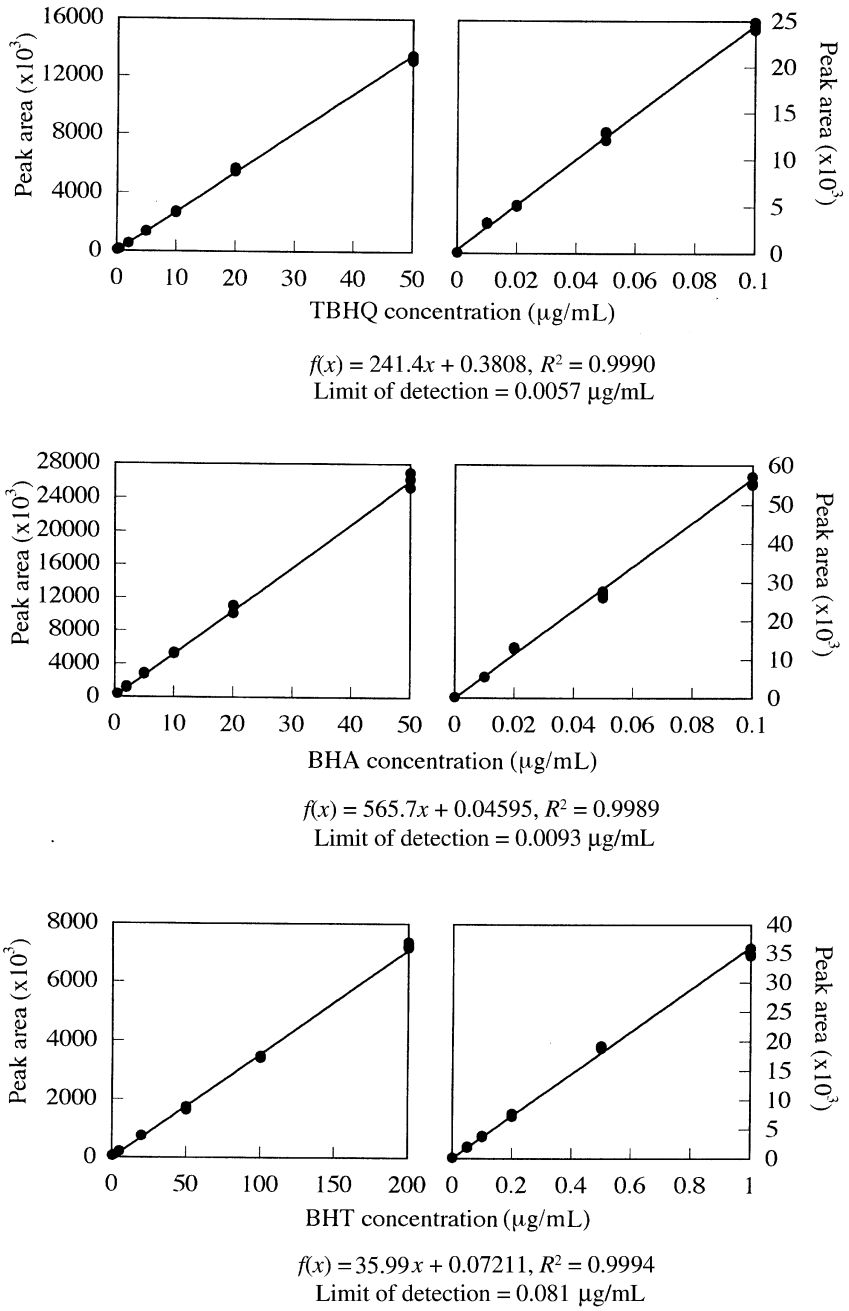

FIG. 2. Calibration curves of TBHQ, BHA, and BHT. The right-hand sides of each panel are calibration curves for the smaller range of the left-hand sides. For abbreviations see Figure 1.

Typical HPLC elution patterns of standards, foods, and blood extract are shown in Figure 3. Antioxidant peaks were well-isolated from interfering peaks in each sample, except for TBHQ with a small shoulder peak in sausage A plus standards.

Determination of antioxidant in foods. Peaks A and B collected from sausage B and anchovy eluants (Fig. 4) were subjected to GC–MS analysis. Because fragmentation patterns of both peaks were consistent with that of BHA, the corresponding peak was assigned to BHA. Neither BHT nor TBHQ was detected in both cases. BHA content of sausage B was calculated as 22.4 ± 1.8 mg/kg wet basis (n = 3). BHA concentration determined in commercial boiled and dried anchovy was 32.9 ± 1.9 mg/kg wet basis for BHA (n = 3).

DISCUSSION

The present study demonstrated that the reversed-phase HPLC with fluorescence detection enables one to detect BHA, BHT, and TBHQ at sufficiently low limits. It also prevents some interferences in oils, foods, and a biological fluid without the necessity of complicated pretreatments, such as

TABLE 3
Percentage Recovery of Antioxidants BHA, BHT, and TBHQ

Antioxidants	Amount	Fish oil	Soybean oil	Sausage A	Mouse blood	CH_3Cl/MeOH
BHA	0.5 μg/g[a]	97.8 ± 0.3[d]	98.0 ± 0.5	87.7 ± 2.1	—	—
	20 μg/g	98.1 ± 0.6	97.6 ± 0.7	92.5 ± 1.7	—	—
	5 μg/mL[b]	—[e]	—	—	97.5 ± 2.7	—
	5 μg/mL[c]	—	—	—	—	86.6 ± 1.2
BHT	5 μg/g	97.9 ± 0.6	99.2 ± 0.2	85.9 ± 1.5	—	—
	200 μg/g	98.8 ± 0.5	98.7 ± 0.5	89.9 ± 1.4	—	—
	50 μg/mL	—	—	—	93.6 ± 5.2	—
	5 μg/mL	—	—	—	—	99.5 ± 0.4
TBHQ	0.5 μg/g	98.4 ± 0.3	98.2 ± 0.4	50.3 ± 5.6	—	—
	20 μg/g	98.3 ± 0.5	98.5 ± 0.6	62.3 ± 3.4	—	—
	5 μg/mL	—	—	—	93.2 ± 2.6	—
	5 μg/mL	—	—	—	—	43.9 ± 1.1

[a]Micrograms of antioxidant per gram of lipid or wet tissue.
[b]Micrograms of antioxidant per milliliter of whole blood.
[c]Micrograms of antioxidant per milliliter of chloroform.
[d]Mean ± S.D. (n = 3).
[e]—, Not determined. For abbreviations see Table 2.

liquid–liquid separation. Although a much lower limit of detection for BHT, around 0.5 ng, was achieved by Mizutani *et al.* (22), the method needs the derivatization procedure of BHT.

One cause that elevated the limit of detection for BHT was a low yield in emission as reported by Latz and Hurtubise (7). They also reported maximal wavelengths for excitation and emission in ethanol as follows: 293 and 342 nm, respectively, for BHA, and 287 and 350 nm, respectively, for BHT. These values were inconsistent with the present results in water/acetonitrile/acetic acid, probably due to different solvents. However, we could not confirm their results, obtained in ethanol solutions, with our experimental instruments.

The recoveries of BHA, BHT, and TBHQ from oils, mouse blood, and sausages achieved practical determination levels for the most part, except for TBHQ from sausage. Remark-

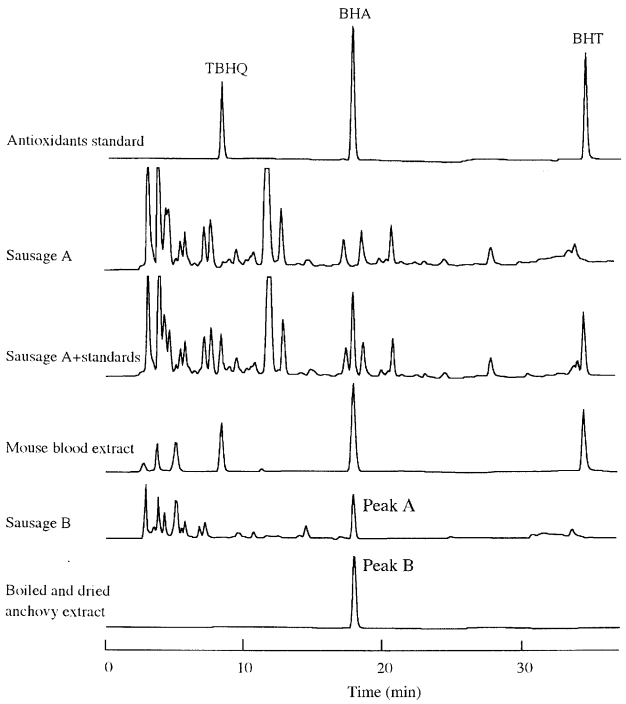

FIG. 3. Typical high-performance liquid chromatograms of TBHQ, BHA, and BHT in foods and mouse blood. For abbreviations see Figure 2.

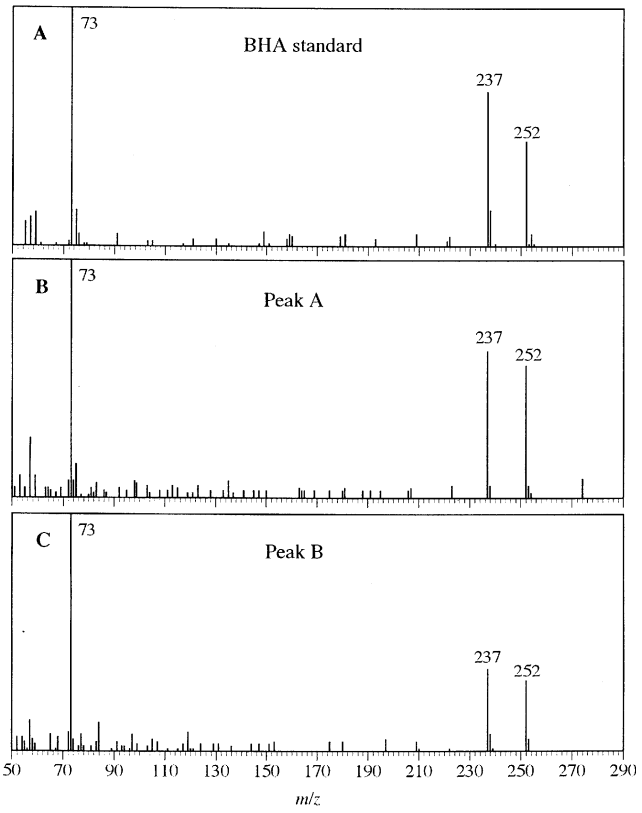

FIG. 4. Mass spectra of (A) BHA standard, (B) Peak A, and (C) Peak B from Figure 3. For abbreviation see Figure 2.

able losses of TBHQ during extraction and analysis procedures were reported by other investigators (17–19,25,28). Stijve and Diserens (28) carried out direct extraction from milk fat with acetonitrile to improve recovery of the antioxidant. However, a rapid extraction procedure with acetonitrile and cooling could not curtail the recovery loss for sausage. The latter is thought due to loss in extraction and breakdown in the procedure.

Initially extracting the total lipid fraction from the food sample before analysis, using the chloroform/methanol/water method, achieved high recoveries for BHA and BHT and resulted in high losses for TBHQ. Causes of that loss are not clearly known but may be due to degradation of TBHQ, and also to losses to the more polar methanol/water layer during extraction procedures as discussed above.

With the AOCS recommended method for antioxidant analysis (24), a problem especially with the analysis of TBHQ was the loss of antioxidant during sample extraction and preparation, and also the incomplete separation of hexane and acetonitrile solution due to formation of emulsion. Andrikopoulos *et al.* (25) improved on the AOCS method and the direct oil injection method of Anderson and Van Niekerk (23) using UV detection. The application of this extraction method described in this paper is convenient for extraction of the antioxidants before detection, to eliminate interferences from other naturally occurring phenolic compounds in the sample.

The BHA concentration in a boiled and dried anchovy sample obtained commercially did not exceed the permitted level of BHA in Japanese fish products, which is restricted to 200 mg/kg wet basis (29). Although the use of antioxidant is not allowed in meat products, sausage B contained a detectable amount of BHA. This observation is supposedly due to the facts that the sausage was imported and/or that the antioxidant may migrate from wrapping materials into the sausage (11,26).

In conclusion, antioxidants BHA, BHT, and TBHQ in foods can be extracted with acetonitrile and measured using HPLC with a spectrofluorometric detector. Sample extraction is simple and reproducible with minimal losses.

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[Received February 7, 1996, and in final revised form June 9, 1997; revision accepted May 8, 1998]

ARTICLE

Assessment of the Arachidonic Acid Content in Foods Commonly Consumed in the American Diet

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ABSTRACT: Arachidonic acid (AA) is an extremely important fatty acid involved in cell regulation. When provided in the diet, it is cogently incorporated in membrane phospholipids and enhances eicosanoid biosynthesis *in vivo* and *in vitro*; however, controversy exists as to the levels of AA in food and in the diet. This study determined the amount of AA in cooked and raw portions of beef (rib eye), chicken (breast and thigh), eggs, pork (loin), turkey (breast), and tuna; it compared these results to values published in Agriculture Handbook No. 8 (HB-8). The cooked portions were prepared as described in HB-8. With the exception of chicken thigh and tuna, the levels of AA (w/w) in the selected foods analyzed were significantly higher, in general, than those values published in HB-8. The greatest differences were observed in beef (raw and cooked), turkey breast (raw and cooked), and pork (cooked) where AA levels were twice that of the values in HB-8. In contrast, the AA and n-3 fatty acid contents in tuna were almost half the HB-8 values. The present data indicate that HB-8 tends to underreport the amounts of AA in a number of foods commonly consumed in the American diet, and new initiatives should be considered to validate and update the current database for fatty acid composition of foods.
Lipids 33, 1151–1157 (1998).

Arachidonic acid (AA) is among the most important fatty acids associated with membrane phospholipids, and its diverse biological functions are unraveling with increased scrutiny (1). Intracellular AA can modulate cell differentiation (2–4), cell proliferation (5–7), gene expression (8–12), and signal transduction (13–17). The best-described functions of AA are mediated through eicosanoids. Increasingly, AA and its metabolites are being linked to chronic diseases, such as cancer; and antagonism of AA and its metabolism, i.e., eicosanoid formation, reduce risk (18,19).

AA is derived metabolically from linoleic acid (LA: 9,12 octadecadienoic acid, 18:2n-6), the major polyunsaturated fatty acid (PUFA) in the diet. Although dietary LA is the major source of tissue AA, this metabolic pathway is regulated such that variations in the LA content of the Western diet do not significantly alter AA content of tissue phospholipids (20). In addition to LA, dietary AA also can contribute to tissue AA levels. Even though dietary LA is the primary metabolic source of AA in tissues, AA from food can significantly impact tissue levels. Both animal (21–23) and human (24,25) studies demonstrated that consuming AA increases plasma and tissue levels of AA and significantly augments eicosanoid biosynthesis *in vivo* and *in vitro*.

Compared to the daily intake of LA (10–20 g/day), the estimated intake of AA (100–500 mg/day) is a minor contributor to the total daily dietary intake of PUFA (22,26–29). But even at these levels, AA is able to alter tissue AA content. Phinney *et al.* (29) reported that AA content in plasma lipids was significantly higher in subjects whose diets contained meat and eggs as compared to vegetarians. Sinclair *et al.* (30) reported that increasing dietary AA intakes from 70 to 490 mg/d significantly enriched plasma phospholipids and cholesteryl esters with AA. Diets high in lean beef (500 g lean beef/d; ~230 mg AA/d) significantly increased the concentration of AA in plasma phospholipids compared to diets containing 30–100 g of lean beef per day (31). Increasing dietary intakes of AA to 1.7 g/d in 50 volunteers, from a baseline intake of 200 mg/d, increased the levels of urinary metabolites of thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) (25) and resulted in an enrichment of AA in platelet phospholipids (32). However, increasing AA consumption from 76–78 to 137–140 mg/d in Australian subjects in the form of various meats did not alter platelet AA content, *ex vivo* platelet TXB_2 production, nor levels of the urinary metabolites of TXA_2 and PGI_2 (33).

These data suggest that the impact of dietary AA on tissue AA content is dose-dependent and that these doses are within reasonable dietary ranges. However, conflicting information exists as to how much AA is actually consumed and how much is in the foods we eat (22,26–29,31,34). A series of comprehensive studies on the Australian diet suggest the amount of AA in their foods and in their diet may be lower than anticipated, but these numbers are influenced by the lean and visible fat portions of the food samples (26,34). A reanalysis of meats from the U.S. diet has yet to be done.

Agriculture Handbook No. 8 (HB-8), as published by the United States Department of Agriculture (USDA), is a comprehensive compilation of the nutrient composition of virtu-

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Abbreviations: AA, arachidonic acid; BRE, beef rib eye; CB, chicken breast; CT, chicken thigh; HB-8, Agricultural Handbook No. 8; LA, linoleic acid; MUFA, monounsaturated fatty acids; PL, pork loin; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TB, turkey breast; TX, thromboxane; USDA, United States Department of Agriculture; WE, whole egg.

ally all foods consumed in the American diet (35). Many software programs assessing human nutrient intakes rely on this database. Understandably, it is difficult for this database to remain current by keeping pace with technological advances in analytical procedures and changes associated with animal feeding and breeding practices. Therefore, despite volumes of nutrient composition tables, direct empirical data of the fatty acid composition, of many foods, in particular AA, is lacking. Preliminary data from our laboratory suggested that HB-8 was underreporting the amount of AA in a number of U.S. food products (36). Therefore, the purposes of this study were to determine the amount of AA in foods known to be relatively high in AA and commonly consumed in the American diet and to compare these results to those published in HB-8.

MATERIALS AND METHODS

Samples. The selection criteria for samples were based on foods known to be relatively rich in AA that are commonly consumed in the American diet, including beef, chicken, eggs, pork, and turkey. White tuna canned in water was included as a food known to be rich in n-3 fatty acids.

With the exception of the tuna and crackers, all samples were obtained fresh from local outlets of a large supermarket chain. They were refrigerated at 4°C after purchase and processed on the same day, usually within 1 h of purchase. Beef rib eye (BRE, $n = 4$), chicken breast (CB, $n = 5$), chicken thigh (CT, $n = 5$), pork loin (PL, $n = 4$), and turkey breast (TB, $n = 4$) samples were trimmed of excess fat, cut in half, and weighed. Poultry samples were skinned. One-half of each BRE sample was broiled, and one-half of each sample of CB, CT, PL, and TB was roasted according to protocol established in HB-8 (37–39). All meat samples were cooled for 1 h after cooking prior to further processing and analysis. Approximately 77-g samples of commercially canned and processed (in water) tuna were analyzed. The fatty acid composition of single raw whole eggs (WE) weighing approximately 50 g was determined. In addition, four eggs were hard-boiled for 10 min, cooled for 20 min, shelled, weighed, and analyzed.

All samples were finely chopped in a Cuisinart food processor (Model DLC-10E; Greenwich, CT) for 2 min in 0.9% NaCl at a concentration of 380 g sample/L buffer. The average weight of the samples prior to processing was approximately 70 g (before or after cooking). Of the internal standard nonadecanoic acid methyl ester (19:0) 50 mg was added to each sample prior to processing. The chopped samples then were finely pureed in a Waring blender (New Hartford, CT) for 3 min at a final concentration of 210 g sample/L buffer. The pureed homogenates were filtered through cheesecloth (crude mesh) to remove connective tissue, and under constant stirring three aliquots were removed for fatty acid analysis. Four to five individual food samples were prepared and analyzed as indicated above. [Note: Follow-up experiments indicate if food samples are homogenized in a 20% saline solution of methanol/chloroform (2:1, vol/vol), the recovery of the internal standard is improved by approximately 5%.]

Lipid extraction and fatty acid analysis. Lipids were extracted by a modified method of Bligh and Dyer (40). Briefly, one part sample was suspended in three parts methanol/chloroform (2:1, vol/vol), and lipids were extracted following the addition of chloroform and 200 μL saturated NaCl solution. The chloroform extraction was repeated (× 2). Following complete evaporation of the pooled chloroform fractions under a stream of nitrogen, the lipids were solubilized with toluene and saponified with KOH (0.5 N) in methanol for 8 min at 86°C. The samples were cooled and acidified with HCl (0.7 N) in methanol, extracted with 2 vol of hexane (× 2), evaporated under an atmosphere of nitrogen, and methylated with ethereal diazomethane. The fatty acid methyl esters were resuspended in hexane and separated by gas chromatography [Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA) equipped with a flame-ionization detector] using a DB-23 capillary column (J&W Chromatography, Folsom, CA) with hydrogen as the carrier gas. The fatty acid methyl esters were identified by comparison of retention times with those of known standards (Nu-Chek-Prep, Elysian, MN) and quantitated based on the internal standard. As previously established, each sample did not contain an appreciable amount of the internal standard.

Statistical analysis. The Statistical Analysis System (SAS Institute, Inc., Cary, NC) was used to analyze the data. Data were expressed as means ± SEM of 4–5 samples, each of which was expressed as the means of three aliquots. Differences between experimental values and those published in HB-8 were analyzed using the one-sample *t*-test. Data were considered significant at $P < 0.05$.

RESULTS

PUFA composition of raw samples. Levels of AA varied in raw samples from a low of 46 mg/100 g in raw BRE to a high of 156 mg/100 g in raw WE, significantly different from those values reported in HB-8 (Table 1). AA content was significantly higher in BRE (46 vs. 20 mg/100 g), CB (64 vs. 40 mg/100 g), and TB (59 vs. 30 mg/100 g) compared with the HB-8 values. Overall, AA content was significantly higher in half of the raw foods analyzed (Fig. 1). LA content was significantly lower in PL (262 vs. 440 mg/100 g) and higher in WE (1272 vs. 1148 mg/100 g) compared to HB-8 (Table 1). Similar inconsistencies among the experimental and HB-8 values were observed with the n-3 PUFA. HB-8 reports significantly higher levels of 22:6n-3 in CB and CT, and significantly lower levels in TB. They failed to report the presence of a number of n-3 and n-6 PUFA that we observed, including 20:3n-6, 22:4n-6, 22:5n-6, 20:5n-3, 22:5n-3, and 22:6n-3, in many of the food items.

PUFA composition of cooked samples. Values of AA in cooked samples varied from 33 mg/100 g in white tuna packed in water to 239 mg/100 g in WE hard-boiled (in the shell) (Table 2). In general, AA content (per 100 g cooked sample) was found to be significantly higher in the experimental samples compared to the HB-8 values, i.e., BRE (77 vs. 30 mg), CB (83 vs. 60 mg), WE (239 vs. 149 mg), PL (74

TABLE 1
Fatty Acid Concentration (mg/100 g sample) of Raw Samples

Fatty Acid	Beef rib eye[a]	Chicken breast	Chicken thigh	Egg whole	Pork loin	Turkey breast
Saturated fatty acids (SFA)						
10:0	21 ± 4[b]	3.5 ± 1.2	11 ± 4	5.1 ± 2.0	16 ± 4	8.0 ± 1.8
10:0 USDA	—	—	—	3	—	—
12:0	3.7 ± 0.3	0.9 ± 0.2	2.3 ± 0.2[c]	0.6 ± 0.1[c]	2.4 ± 0.4[c]	3.6 ± 0.9
12:0 USDA	—	—	20	3	10	—
14:0	122 ± 17[c]	7.1 ± 1.2	22 ± 2	28 ± 1[c]	27 ±7[c]	22 ± 8
14:0 USDA	240	10	20	34	60	—
16:0	1122 ± 191[c]	283 ± 45	779 ± 66	2092 ± 35[c]	552 ± 132	413 ± 163
16:0 USDA	1930	210	670	2226	1110	90
18:0	697 ± 158	109 ± 10	267 ± 17	778 ± 16	308 ± 66[c]	215 ± 57
18:0 USDA	1060	100	260	784	550	60
Total SFA	2064 ± 369	406 ± 57	1091 ± 88	2922 ± 33[c]	913 ± 208[c]	672 ± 233
Total SFA USDA	3230	320	970	3096	1730	150
Monounsaturated fatty acids (MUFA)						
14:1	21 ± 3	2.3 ± 0.5	8.6 ± 0.8	6.4 ± 0.6	0.4 ± 0.2	3.0 ± 1.8
14:1 USDA	—	—	—	8	—	—
16:1	108 ± 13[c]	72 ± 16	254 ± 25[c]	229 ± 13[c]	62 ± 18[c]	51 ± 30
16:1 USDA	290	30	180	298	160	10
18:1n-9	1565 ± 327[c]	404 ± 77	1257 ± 112	3192 ± 66[c]	883 ± 237[c]	567 ± 249
18:1n-9 USDA	3260	250	1010	3473	2070	90
18:1n-7	71 ± 11	41 ± 6	93 ± 10	165 ± 9	97 ± 26	48 ± 15
18:1n-7 USDA	—	—	—	—	—	—
Total MUFA	1772 ± 351[c]	523 ± 99	1625 ± 147[c]	3614 ± 78	1059 ± 285[c]	675 ± 298
Total MUFA USDA	3560	280	1200	3810	2270	100
Polyunsaturated fatty acid (PUFA)						
18:2n-6	178 ± 20	222 ± 31	698 ± 58	1272 ± 38[c]	262 ± 46[c]	399 ± 130
18:2n-6 USDA	240	170	750	1148	440	110
18:3n-3	9.5 ± 1.5	7.5 ± 1.6	28 ± 3	31 ± 2	12 ± 5	25 ± 11
18:3n-3 USDA	10	10	30	33	20	—
20:3n-6	16 ± 2	13 ± 1	18 ± 1	18 ± 1	8.7 ± 1.0	4.9 ± 1.2
20:3n-6 USDA	—	—	—	—	—	—
20:4n-6	46 ± 3[c]	64 ± 5[c]	106 ± 7	156 ± 7	53 ± 5	59 ± 2[c]
20:4n-6 USDA	20	40	90	142	60	30
20:5n-3	5.1 ± 0.4	2.6 ± 0.3	3.2 ± 0.7[c]	0.4 ± 0.2[c]	2.8 ± 1.5	5.1 ± 1.0
20:5n-3 USDA	—	—	10	4	—	—
22:4n-6	7.3 ± 0.4	16 ± 1	27 ± 2	14 ± 2	12 ± 3	13 ± 1
22:4n-6 USDA	—	—	—	—	—	—
22:5n-6	1.5 ± 0.4	5.5 ± 0.6	8.4 ± 0.8	40 ± 3	1.3 ± 0.4	2.5 ± 0.4
22:5n-6 USDA	—	—	—	—	—	—
22:5n-3	11.9 ± 0.6	6.4 ± 0.8	9.0 ± 0.4[c]	6.3 ± 1.2	7.2 ± 1.8	10.7 ± 0.4
22:5n-3 USDA	—	10	20	—	—	10
22:6n-3	1.7 ± 0.5	6.0 ± 0.7[c]	7.5 ± 0.7[c]	44 ± 2	2.1 ± 0.8	16 ± 1[c]
22:6n-3 USDA	—	20	40	37	—	10
Total PUFA	282 ± 25	352 ± 34	922 ± 69	1603 ± 53[c]	373 ± 59	541 ± 144
Total PUFA USDA	270	250	940	1364	520	160
Total fatty acids	4118 ± 742[c]	1281 ± 185	3637 ± 293	8138 ± 87	2345 ± 546[c]	1889 ± 675
Total fatty acids USDA	7060	850	3110	8270	4520	410

[a]United States Department of Agriculture (USDA) Nutrient Data Bank (NDB) numbers: beef: #13097; chicken breast: #05062; chicken thigh: #05096; whole egg: #01123; pork loin: #10040; turkey breast: #05219.
[b]Values are means ±SEM of 4–5 independent samples, each analyzed in triplicate.
[c]$P < 0.05$ significantly different compared with USDA value.

vs. 30 mg), and TB (72 vs. 40 mg); and significantly lower in white tuna packed in water (33 vs. 51 mg). No detectable levels of AA are present in saltine crackers. Overall, AA content was significantly different in six out of the seven foods analyzed as compared to those values published in HB-8 (Fig. 1).

Significant differences also were observed in the 18:2n-6 content of seven of the eight experimental foods as compared to HB-8. For example, HB-8 reports more than twice the levels of 18:2n-6 in CT, but one-third the levels in TB. In addition, HB-8 reports that 18:2n-6 content increases threefold in CT following cooking (Tables 1 and 2). Similar discrepancies were observed with the n-3 PUFA. For example, in tuna, the experimental value (per 100 g sample) for 20:5n-3 was significantly lower compared to HB-8 (94 vs. 233 mg). In addi-

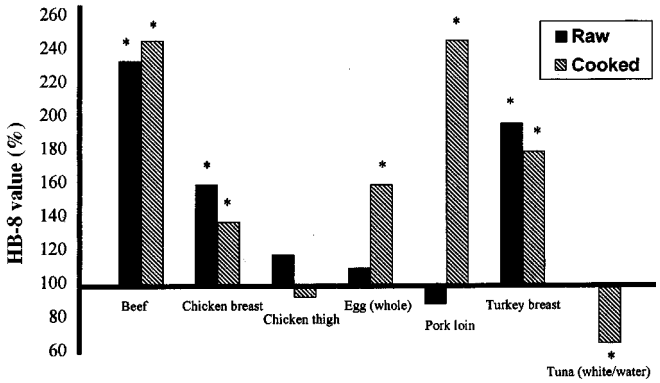

FIG. 1. Relative amounts of AA (based on g/100 g sample) in selected foods as compared to values published in Agricultural Handbook No. 8 by the United States Department of Agriculture (USDA). Asterisks denote values that were significantly different from the USDA values at $P < 0.05$.

tion, HB-8 failed to report the presence of a number of n-3 and n-6 PUFA, including 20:3n-6, 22:4n-6, 22:5n-6, 20:5n-3, 22:5n-3 and 22:6n-3, in many of the food items. Some of the data were missing as indicated by blanks in the data for some select fatty acids.

Saturated fatty acid (SFA) composition of raw samples. Of the major SFA, 16:0 was significantly lower in BRE and WE, and 16:0 and 18:0 were significantly lower (per 100 g of sample) in PL compared to HB-8 (Table 1). In many of the food items, HB-8 failed to report the presence of a number of SFA, i.e., 10:0, 12:0, 15:0, and 17:0.

SFA composition of cooked samples. The 12:0, 14:0, 16:0, and 18:0 content of CB, CT, and PL, and 14:0 and 16:0 of white tuna were significantly lower in the experimental foods compared to HB-8, while the 16:0 and 18:0 content of TB were significantly higher (Table 2).

Monounsaturated fatty acid (MUFA) composition of raw samples. MUFA values for 16:1 and 18:1n-9 were significantly lower in BRE, WE and PL, and 16:1 was higher in CT compared to HB-8 (Table 1).

MUFA composition of cooked samples. MUFA values for 16:1 and 18:1n-9 were significantly lower in CB, CT, PL, and tuna compared to HB-8. The 18:1n-9 levels in TB were significantly higher than the HB-8 values (Table 2).

Total fatty acid composition of raw and cooked samples. For the most part, total fatty acid, SFA, MUFA, and PUFA content in the raw samples reported in HB-8 were similar in the experimental foods with the exception of BRE and PL (Table 1).

Total fatty acid, SFA, MUFA, and PUFA content were significantly lower in the cooked samples of CB, CT, PL, and tuna compared to HB-8 (Table 1). In contrast, overall fatty acid content was higher in WE (hard-boiled) and TB compared to HB-8. Saltine crackers, the only nonanimal product included in the analyses, contained significantly lower amounts of PUFA, in the form of 18:2n-6, compared to the value reported in HB-8.

DISCUSSION

Fatty acid analysis of eggs and a variety of meats revealed that the values reported in HB-8 for AA content were significantly different ($P < 0.05$), in general, from those observed in our experimental foods (Fig. 1). AA content in land-based meats tended to be underreported in HB-8, while AA content in the marine source meat (tuna) was significantly lower than the HB-8 value. In a number of instances, the HB-8 fatty acid values for raw and cooked portions of the same food were inconsistent. For example, the HB-8 reports that the total PUFA content (w/w) of pork loin increased 31% following cooking, but the AA content decreased by 50%. We did not observe a decrease in AA content in any of the samples tested following cooking. In general, we and others (41,42) observed consistent increases in fatty acid content (w/w), including AA, in cooked portions compared to raw samples. Consistent with HB-8 values, we did not observe reductions in the PUFA content following cooking. Sinclair *et al*. (31) report that cooking lean beef results in significant reductions in PUFA content, in particular the 20-carbon PUFA. Possibly the incongruity of these data may be related to different methods of cooking (i.e., roasting vs. grilling and frying) and how the cooked data were reported (i.e., mg/100 g cooked sample vs. mg/100 g original raw sample weight). Loss of moisture due to cooking resulted in a higher fatty acid content on a w/w basis, and as such, the relative abundance of AA to the total fatty acid content remained fairly similar (43). Cooking can result in 30% moisture loss and can enrich the amount (w/w) of extractable lipids in the muscle component (44,45). However, Chang and Watts (46) report that they did not consistently observe an enrichment of fatty acids in meats following cooking, including AA, suggesting nonuniform distribution of triglycerides rather than the destruction of unsaturated fatty acids (46) contributed to these results. More recently, though, Li *et al.* (34) report that the amount of AA (w/w) in the visible fat of animal meats (beef, lamb, turkey, pork, chicken, and duck) is equal to or greater than that of comparable lean portions even though the relative amounts of AA (wt% of total fatty acids) were 10–100 times higher in the lean portions. These data indicate that the triglyceride portion of meat can have a significant impact on AA content, and care must be taken when selecting and comparing meat portions for fatty acid analysis.

Several explanations are possible for the discrepancies between the HB-8 values and the experimental values of this study. Studies conducted for the USDA report the relative contributions of AA to the total fatty acid profile of the triglyceride portion (separable fat) of meats (i.e., beef) were considered quantitatively insignificant (42), while more recent data indicate this is not the case (34).

Many of the values in HB-8 are estimates. When analytical data were not available, calculation or imputing of nutrient values (35) was sometimes necessary. The USDA routinely generated fatty acid data by using mathematical formulas based on the weight percentage of the total lipid in a food,

TABLE 2
Fatty Acid Concentration of Cooked Samples

Fatty Acid	Beef rib eye[a]	Chicken breast	Chicken thigh	Egg whole	Pork loin	Turkey breast	Tuna white
SFA							
10:0	29 ± 6[b]	4.3 ± 2.0	8.9 ± 3.3	8.6 ± 0.6[c]	21 ± 3[c]	11 ± 3	3.4 ± 1.0
10:0 USDA	10	—	—	3	10	—	—
12:0	7 ± 1	1.1 ± 0.1[c]	2.5 ± 0.3[c]	0.5 ± 0.2[c]	3.4 ± 0.5[c]	3.3 ± 0.6	1.3 ± 0.2
12:0 USDA	10	10	30	3	10	—	—
14:0	254 ± 48	7.9 ± 0.8[c]	27 ± 1[c]	43 ± 4	48 ± 6[c]	21 ± 5	26 ± 6[c]
14:0 USDA	370	30	80	35	120	—	82
16:0	2193 ± 401	334 ± 33[c]	940 ± 16[c]	2927 ± 138[c]	930 ± 121[c]	420 ± 93[c]	273 ± 40[c]
16:0 USDA	2800	690	2120	2349	2060	110	592
18:0	1365 ± 297	139 ± 6[c]	313 ± 5[c]	1168 ± 107	505 ± 75[c]	237 ± 33[c]	97 ± 13
18:0 USDA	1540	250	690	828	1080	70	118
Total SFA	4025 ± 752	490 ± 38[c]	1301 ± 19[c]	4173 ± 236[c]	1518 ± 206[c]	704 ± 131[c]	420 ± 61[c]
Total SFA USDA	4730	980	2920	3218	3280	180	792
MUFA							
14:1	47 ± 12	2.7 ± 0.4	10.1 ± 0.3	8.6 ± 0.6	1.2 ± 0.1	3.3 ± 1.1	0.6 ± 0.2
14:1 USDA	—	—	—	—	—	—	—
16:1	222 ± 41[c]	80 ± 13[c]	311 ± 11[c]	303 ± 27	118 ± 15[c]	47 ± 18	36 ± 7[c]
16:1 USDA	400	150	560	310	300	20	144
18:1n-9	3094 ± 623	471 ± 52[c]	1499 ± 40[c]	4447 ± 120[c]	1582 ± 173[c]	551 ± 126[c]	211 ± 37[c]
18:1n-9 USDA	4540	1040	3460	3725	3570	110	518
18:1n-7	160 ± 32	47 ± 4	114 ± 4	217 ± 13	166 ± 17	51 ± 8	34 ± 7
18:1n-7 USDA	—	—	—	—	—	—	—
Total MUFA	3537 ± 692	607 ± 69[c]	1949 ± 51[c]	5006 ± 154[c]	1897 ± 208[c]	659 ± 153[c]	314 ± 58[c]
Total MUFA USDA	4940	1220	4090	4068	3950	130	784
PUFA							
18:2n-6	320 ± 51	272 ± 17[c]	838 ± 27[c]	1884 ± 183[c]	341 ± 33[c]	415 ± 71[c]	25 ± 2[c]
18:2n-6 USDA	280	590	2100	1188	630	130	55
18:3n-3	17 ± 3	8.8 ± 0.9[c]	34 ± 1[c]	41 ± 1[c]	16 ± 6	23 ± 5	5.8 ± 0.9[c]
18:3n-3 USDA	20	30	100	35	20	—	71
20:3n-6	27 ± 5	16 ± 1	22 ± 2	30 ± 3	12 ± 1	7.0 ± 0.7	1.5 ± 0.3
20:3n-6 USDA	—	—	—	—	—	—	—
20:4n-6	77 ± 14[c]	83 ± 8[c]	121 ± 6	239 ± 21[c]	74 ± 4[c]	72 ± 4[c]	33 ± 4[c]
20:4n-6 USDA	30	60	130	149	30	40	51
20:5n-3	6.9 ± 0.7	3.1 ± 0.3[c]	4.1 ± 0.9[c]	—	3.3 ± 1.7	4.9 ± 1.1	94 ± 20[c]
20:5n-3 USDA	—	10	10	5	—	—	233
22:4n-6	13 ± 2	20 ± 2	30 ± 2	14 ± 8	15 ± 3	16 ± 1	3.4 ± 0.4
22:4n-6 USDA	—	—	—	—	—	—	—
22:5n-6	1.9 ± 0.9	7.2 ± 0.9	9.5 ± 0.7	92 ± 11	2.0 ± 0.4	4.2 ± 0.6	18 ± 2
22:5n-6 USDA	—	—	—	—	—	—	—
22:5n-3	20 ± 2	8.0 ± 0.8	10 ± 1[c]	3.6 ± 1.6	10 ± 3	13 ± 1	20 ± 3
22:5n-3 USDA	—	10	30	—	—	10	18
22:6n-3	2.4 ± 0.6	8.0 ± 1.0[c]	9.0 ± 0.6[c]	68 ± 9[c]	3.4 ± 1.0	19 ± 2[c]	417 ± 67
22:6n-3 USDA	—	20	50	38	—	10	629
Total PUFA	494 ± 73	437 ± 22[c]	1096 ± 29[c]	2409 ± 233[c]	492 ± 46[c]	582 ± 84[c]	623 ± 100[c]
Total PUFA USDA	330	720	2420	1415	680	190	1057
Total fatty acids	8056 ± 1500	1534 ± 119[c]	4345 ± 72[c]	11,587 ± 570[c]	3907 ± 444[c]	1945 ± 367[c]	1357 ± 219[c]
Total fatty acids USDA	10,000	2920	9430	8701	7910	500	2633

[a]USDA NDB numbers: beef: #13098; chicken breast: #05064; chicken thigh #05098; whole egg #01129; pork loin #10043; turkey breast #05220; tuna #15126.
[b]Values are means ±SEM of 4–5 independent samples, each analyzed in triplicate.
[c]$P < 0.05$ significantly different compared with USDA value. See Table 1 for abbreviations.

multiplied by a set of conversion factors (42,47–49). The conversion factors varying among different foods, raw and cooked samples (47), were primarily based on the relative abundance of triglycerides and phospholipids and their fatty acid contents (42). For a given sample, the fatty acid composition (w/w) was determined by first determining the amount of total lipid gravimetrically and by multiplying this value by its conversion factor and the weight percentage (sometimes assumed) of the individual fatty acid methyl esters. Based on preliminary experiments, fatty acid profiles for related anatomical portions of meat from a beef carcass, for example, were assumed to be similar (47). When necessary, the

fatty acid data were adjusted so that the amount of fatty acid was consistent with the total amount of extracted lipid.

Scientific advances in breeding, feeding practices, and analytical techniques may influence fatty acid composition data. Over the last 30 yr, feeding practices for beef cattle have increasingly shifted from forage-fed to grain-fed animals resulting in significantly higher levels of AA in the muscle of grain-fed animals (50). Also, it is assumed that cuts of meat presented in HB-8 reflect retail cuts of meat. But, retail beef is leaner than the values published in HB-8 (possibly reflecting the health-conscious trend of the public), and retail cuts may have multiple muscle groups associated with them (51).

The breed and age of an animal and an interaction of these two variables (52) also may affect fatty acid composition. For example, PUFA content of muscle phospholipids increase and SFA and MUFA decrease with age in Jersey and Limousin cows, and the level of AA in muscle phospholipids in mature Limousin cows is 28% higher compared to Jersey cows. In addition, the AA content in muscle phospholipids in heifers is almost 2.5 times higher than that of steers (52).

Most of the food analyses for HB-8 were performed in the 1970s and 1980s (35). For example, the HB-8 Series for poultry, pork, and beef products were issued in 1979, 1992, and 1990, respectively, but some of the data, such as poultry, originated from the mid 1960s and 1970s and have not been updated. The food analysis data published in HB-8 are derived from multiple sources including contracts with universities and commercial laboratories, contacts with the food industry, trade groups, academia, and other government agencies (35). Previously, fatty acid data were generated using packed-column gas chromatography, gas–liquid chromatography (43–45), and spectrophotometric methods (46); this may explain many of the missing values for the PUFA (see Tables 1 and 2). Recent advances in analytical techniques using internal standards and capillary gas chromatography improved the separation, identification, and measurement of fatty acids, in particular, longer-chain unsaturated fatty acids.

Finally, the inconsistency between our results and those in HB-8 could be related to the sample size used in this study, source of the samples, and the limited variation in the analyzed samples. We selected samples, as the public would, from a regional supermarket chain and analyzed 4–5 samples in each of the food categories. The sample size and the limited variation in the samples could account for the observed differences. The breeding and feeding practices of the animals from which the samples came are unknown. Similarly, this information is unavailable from the USDA, but the data published in HB-8 is based on a compilation of data from a variety of sources, seasons, and geographic locations as indicated previously.

In summary, our results suggest wide differences exist in the fatty acid content of individual foods when compared with the HB-8 values; these differences may be related to scientific advances in breeding, feeding practices, and analytical techniques. The content of AA in foods analyzed in this study was, for the most part, significantly higher than that reported in HB-8. Because of the importance of HB-8 as a reliable source of nutrient content in foods and for determining average daily intakes, a reevaluation of the fatty acid data may be warranted.

ACKNOWLEDGMENT

This research was supported in part by the Agricultural Experiment Station of Tennessee.

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[Received August 25, 1998, and in final revised form and accepted November 23, 1998]

Involvement of Phospholipids in Apolipoprotein B Modification During Low Density Lipoprotein Oxidation

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ABSTRACT: An increased amount of phospholipids remained attached on delipidated apolipoprotein B originated from oxidized low density lipoprotein (LDL). ^{31}P nuclear magnetic resonance analysis of such apolipoprotein showed an organic phosphorus peak at –0.55 ppm, which suggests the formation of adducts (most probably Schiff bases) of oxidized phospholipids with apolipoprotein B. The above reaction occurs in parallel with the hydrolysis of oxidized phospholipids, catalyzed by the LDL-attached platelet-activating factor acetylhydrolase, and may contribute to the proatherogenic effect of oxidatively modified low density lipoprotein.
Lipids 33, 1159–1162 (1998).

Recent studies have shown that oxidative modification of low density lipoprotein (LDL) plays an important role in the initiation and progression of atherosclerosis (1). Oxidation of LDL is a free radical, autocatalytic process. The polyunsaturated fatty acids (PUFA), which are bound in the different lipid classes of the LDL molecule, are the major targets of the active forms of oxygen. The initial products of phospholipid (PL) oxidation are their hydroperoxy derivatives which, after chain fragmentation, give rise to a variety of aldehydic lipid peroxidation products (2–4). This fragmentation also leads to polar PL, containing short-chain acyl groups at the *sn*-2 position (5). The oxidized PL (oxPL) serve as substrates for platelet-activating factor-acetylhydrolase (PAF-AH), which hydrolyzes them toward lysophospholipids (lyso-PL) (6). PAF-AH is an enzyme that is associated, in plasma, with LDL and high density lipoproteins (7,8), and that plays a major role in the regulation of the pathophysiologic effects of PAF (9).

The aldehydic peroxidation products react with ε-amino groups of lysine residues in apolipoprotein B (apoB), forming Schiff bases and thus contributing to the modification of apoB (3). Oxidized LDL (oxLDL) do not bind to LDL receptors, but instead are recognized by other types of receptors, namely, the acetylated LDL (10) or scavenger receptors (11,12), leading to foam cell formation, an early step of atherosclerosis (1). After delipidation of oxLDL, solubilized fractions of apoB are responsible for the recognition of oxLDL by the scavenger receptor (13). The aim of the present study was to investigate the possible involvement of the oxPL, generated during the oxidation of LDL, in the modification of apoB.

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Abbreviations: ApoB, apolipoprotein B; LDL, low density lipoprotein; lyso-PC, lysophosphatidylcholine; nLDL, native LDL; NMR, nuclear magnetic resonance; oxLDL, oxidized LDL; oxLDLi, oxidized LDL treated with pefabloc; oxPL, oxidized phospholipids; PAF-AH, platelet activating factor-acetylhydrolase; PC, phosphatidylcholine; pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride; PL, phospholipids; PLA_2, phospholipase A_2; PL-apoB, phospholipids bound to apoB; SDS, sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURES

LDL preparation. LDL (d = 1.019–1.063) was prepared from freshly isolated, pooled, normolipidemic, human plasma containing 0.01% EDTA (wt/vol) and 5 μg/mL gentamicin sulfate (Garamycin, Schering-Plough, produced by Famar, Athens, Greece) by sequential ultracentrifugation (14) in a Beckman L7-65 ultracentrifuge (Fullerton, CA) at 40,000 rpm, 14°C for 10 h with a Type NVT65 rotor. The LDL preparation was dialyzed against 10 mM Tris buffer containing 0.9% NaCl (wt/vol) (pH = 7.4) for 24 h at 4°C. Then it was filter-sterilized (0.22 mM, Millipore, Bedford, MA) and stored in the dark at 4°C under nitrogen for up to 2 wk. LDL protein was determined using the Bicinchoninic Acid assay (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL).

LDL oxidation. OxLDL was prepared by incubating LDL (1 mg of protein/mL) with 50 μM $CuSO_4$ in Tris buffer (pH = 7.4) at 37°C for 6 h. Oxidation was terminated by the addition of 0.01% EDTA and subsequent refrigeration. In some experiments 4-[2-aminoethyl]benzenesulfonyl fluoride (pefabloc) (Pefabloc SC, Pierce) (1 mM), an irreversible inhibitor of PAF-degrading AH, was added to the LDL preparation (oxLDLi) (15). The LDL oxidation was followed by the thiobarbituric acid-reactive substances assay and the relative electrophoretic mobility value on agarose gel electrophoresis.

Measurements. Total lipids of native LDL (nLDL) or oxLDL (treated or not with serine esterase inhibitor), corresponding to 0.5 mg of protein, were extracted according to Bligh and Dyer (16) and subjected to thin-layer chromatography on silica gel G plates using chloroform/methanol/water (65:35:6, by vol) as solvent system. Lipids were identified after brief exposure to iodine vapors, and the bands corresponding to the R_f of standard sphingomyelin, phosphatidylcholine (PC), lysophosphatidylcholine (lyso-PC), and phos-

phatidylethanolamine were scraped off the plate and submitted to phosphorus assay according to Bartlett (17) as modified by Marinetti (18). (Standard lipids were purchased from Sigma (St. Louis, MO) and solvents, analytical grade, from LAB-SCAN, Dublin, Ireland). The phosphorus assay was not affected by 100 mM sodium dodecyl sulfate (SDS) or by lipid peroxidation products formed during PL oxidation.

ApoB preparation. Delipidated, water-soluble apoB was prepared according to Cardin *et al.* (14). Briefly, LDL (nLDL, oxLDL, and oxLDLi) (4.5 mg/mL) was dialyzed for 24 h against 1 mM EDTA, pH = 8. The LDL was then lyophilized and subjected to four extractions with 12 mL of ether/ethanol (3:1, vol/vol). At each step of the extraction, the protein–solvent mixture was incubated at −20°C for 1 h and pelleted by low-speed centrifugation at −20°C; the solvent was then removed by aspiration. After the final extraction, the protein was washed once with anhydrous ether and pelleted, and the solvent was removed by aspiration while the protein was dried under nitrogen. The delipidated apoB was suspended in 2 mL of 10 mM Tris-HCl and 1 mM EDTA buffer (pH = 8.5) containing 100 mM SDS. Complete solubilization of apoB was achieved by gentle vortexing, followed by stirring at room temperature. ApoB was then dialyzed at 4°C against 10 mM Tris-HCl and 1 mM EDTA buffer (pH = 8.5) to remove the excess SDS. The apoB modification was tested by SDS-polyacrylamide gel electrophoresis (5–19% gradient) (20). Since the amount of phosphorus remaining attached to the delipidated apoB is a measure of PL bound to the apolipoprotein (PL-apoB), the apoB preparations were submitted to phosphorus assay.

^{31}P nuclear magnetic resonance (NMR) analysis. The ^{31}P NMR spectra were obtained at 161.9 MHz with a Bruker AMX-400 instrument (Rheinstetten-Forchheim, Germany). The chemical shifts were determined relative to the resonance position of 1% H_3PO_4 as an external standard. The resolution of spectra was enhanced by multiplication of the free-induction decay with a Gaussian exponential function (21).

RESULTS AND DISCUSSION

Table 1 shows the composition of plasma LDL PL which were separated by silica gel thin-layer chromatography, before and after oxidation of LDL, treated or not with the serine esterase inhibitor. In accordance with results reported previously by our laboratory (22) and other investigators (23), there was a marked loss of the PC content and an increase in the amount of lyso-PC after oxidation. No significant change was observed in the amount of the other PL, suggesting that PC and its oxidation and hydrolysis products may be involved in LDL modification. The increase in lyso-PC was higher when the LDL was oxidized in the absence of pefabloc, supporting the assumption (22) that PAF-AH is mainly responsible for the formation of lyso-PC on LDL, after PC oxidation.

Note that (Table 1) the sum of the amount PC + lyso-PC was significantly higher on nLDL than on oxLDL and oxLDLi. This finding suggests that PAF-AH hydrolyzes only a part of the oxidized PC products. As shown in Table 1, in accordance with Tertov *et al*. (24), an increased amount of phospholipids (PL), remained attached on oxidized apoB after delipidation (PL-apoB), suggesting that a part of oxidized PC might have formed adducts with apoB. The adducts of oxidized PL with apoB render the PL more hydrophilic, and thus they cannot be extracted in the chloroform phase of the Bligh and Dyer extraction mixture. The above can explain the decrease in the total amount of LDL PL after oxidation (Table 1), which was also reported by other investigators, working with oxidized human plasma (25).

To investigate the above suggestion further, we submitted delipidated apoB (1.5 mg/mL) before and after oxidation, with or without treatment with pefabloc, to ^{31}P NMR analysis. In some experiments apoB, from oxLDL, were treated with phospholipase A_2 (PLA_2) before the NMR analysis. The apoB preparation was incubated with 100 μg of PLA_2 (porcine pancreatic PLA_2; Boehringer, Mannheim, Germany) in the presence of 2 mM Ca^{2+} for 30 min, at room temperature.

TABLE 1
Composition of LDL Phospholipids Which Were Separated by Silica Gel Thin-Layer Chromatography, Before and After Oxidation of LDL[a]

PL	nLDL	oxLDL	oxLDLi	nLDL vs. oxLDL	nLDL vs. oxLDLi	oxLDL vs. oxLDLi
	(nmol PL/mg of protein)					
lyso-PC	24.3 ± 14.8	91.7 ± 24.7	46.5 ± 12.5	$P < 0.002$	$P < 0.02$	$P < 0.003$
Sph	272.8 ± 91.0	241.6 ± 105.0	271.5 ± 112.9	N.S.	N.S.	N.S.
PC	707.4 ± 163.1	403.1 ± 146.3	439.3 ± 202.1	$P < 0.02$	$P < 0.05$	N.S.
PE	32.3 ± 13.2	23.9 ± 12.9	28.2 ± 11.9	N.S.	N.S.	N.S.
Total PL	1034.7 ± 179.0	760.3 ± 196.8	785.5 ± 268.2	$P < 0.03$	N.S.	N.S.
lyso-PC + PC	731.7 ± 146.5	494.8 ± 117.5	485.8 ± 173.9	$P < 0.02$	$P < 0.03$	N.S.
PL-apoB	25.3 ± 9.6	68.0 ± 20.0	71.8 ± 18.4	$P < 0.01$	$P < 0.005$	N.S.

[a]Data represent mean ± SD from six independent LDL preparations and were compared by *t*-test for independent samples. $P < 0.05$ is considered significant. Abbreviations: LDL, low density lipoprotein; PL, phospholipid; nLDL, native LDL; oxLDL, oxidized LDL; oxLDLi, oxidized LDL treated with 4-[2-aminoethyl]benzenesulfonyl fluoride; lyso-PC, lysophosphatidylcholine; Sph, sphingomyelin; PE, phosphatidylethanolamine; PL-apoB, phospholipids bound to apolipoprotein B. N.S., not significant.

As shown in Figure 1A the ^{31}P NMR spectrum of delipidated apoB from nLDL shows one peak at –0.37 ppm corresponding to the PL remaining hydrophobically bound to the protein even after the delipidation process. This peak is observed in all the other preparations (Fig. 1B,C) and disappears after PLA_2 treatment, giving rise to two new peaks at –0.06 and –0.21 ppm, possibly corresponding to lyso PL (Fig. 1D). The ^{31}P NMR spectrum of PC in $CHCl_3$/MeOH/H_2O (50:50:15, by vol), showed two peaks at –0.40 and –0.37 ppm, whereas the spectrum of lyso-PC, in the same solvent mixture, showed one peak at –0.13 ppm (data not shown). In the apoB preparations originated from oxLDL (Fig. 1B,C) treated or not with pefabloc, a second peak of organic phosphorus at –0.55 ppm was observed, which could explain the higher amount of PL (Table 1) attached on apoB after oxidation. Moreover, the ratios of the integrals of the two peaks, 1:2 and 1:2.5, for apoB from oxLDL and oxLDLi are in agreement with the results from the PL attached on delipidated apoB observed in Table 1. The amount of PL that remained hydrophobically bound to apoB even after the delipidation procedure, calculated by the integrals of the two peaks of the ^{31}P NMR spectrum (Fig. 1B,C) and the total amount of PL bound to delipidated apoB (Table 1), did not exceed the 22–25 nmol per mg apoB. It corresponded to the amount of PL-apoB measured by the phosphorus assay on apoB derived from nLDL (Table 1). The second peak of organic phosphorus at –0.55 ppm was only slightly affected by the PLA_2 treatment(Fig. 1D). The observed magnetic shielding of phosphorus and the failure of PLA_2 to recognize and hydrolyze the *sn*-2 ester bond, presented in Figure 1B,C,D, support the suggestion that adducts of oxidized forms of PC with apoB are formed during LDL oxidation. The possibility that lyso-PC also accounts for the above adducts could not be excluded. However, in this case the second phosphorus peak in Figure 1B would be expected to be higher than that of Figure 1C, since lyso-PC formation on oxLDL without PAF-AH inhibition is twofold higher than that on oxLDLi (Table 1).

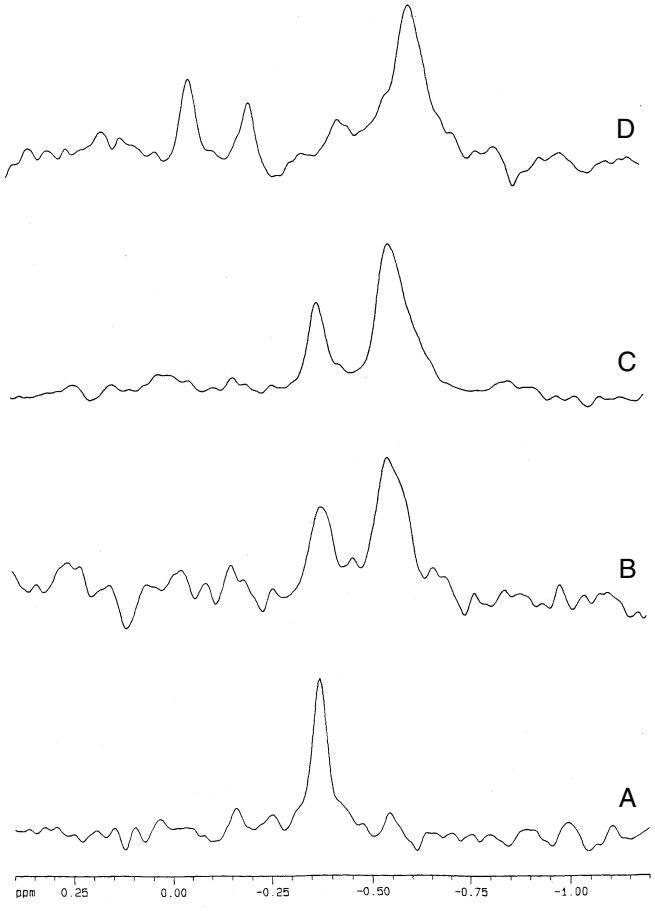

FIG. 1. Conventional ^{31}P nuclear magnetic resonance spectrum of apolipoprotein B. (A) From native low density lipoprotein (LDL), (B) from oxidized LDL, (C) from oxidized LDL treated with 4-[2-aminoethyl]benzenesulfonylfluoride (pefabloc), and (D) from oxidized LDL treated with pefabloc and phospholipase A_2. Solvent, temperature and acquisition parameters for A–D are as follows: 10 mM Tris-HCl and 1 mM EDTA, at 300 K, 5 mm sample tube, on a Bruker AMX-400 instrument (Rheinstetten-Forchheim, Germany). Spectral acquisition parameters: 0.74 s acquisition time, 4 hHz spectral width, 2 s relaxation delay time, 36000 scans.

The present work does not elucidate the chemical character of the adducts mentioned above. OxPL with semialdehydic oxidized short-chain fatty acid in the *sn*-2 position are formed during oxidation (26), and the aldehydic group esterified on the PL backbone may react with the proteins to form Schiff base adducts (27). The above evidence, together with our results, strongly suggests that Schiff base adducts of PL could be formed with the lysine residues of apoB. This reaction occurs in parallel with the hydrolysis of oxPL to lyso-PL, a reaction catalyzed by the LDL attached PAF-AH, and may have an important role in apoB modification during oxidation and the pathophysiological effects of oxLDL.

ACKNOWLEDGMENTS

This work was partially supported by the Research Committee of the University of Ioannina and the BIOMED 2 PL963791 Programme.

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[Received April 22, 1998, and in final revised form and accepted November 12, 1998]

Fish Oil Supplementation With and Without Added Vitamin E Differentially Modulates Plasma Antioxidant Concentrations in Healthy Women

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ABSTRACT: The purpose of this study was to assess the effect of fish oil with or without vitamin E on plasma vitamin antioxidants. Thirty-three apparently healthy women aged 18–28 yr were recruited from the university environs, and 30 completed the double-blinded, parallel design supplementation trial. Blood samples were collected at baseline (week 0) and following 28 d of supplementation with three capsules/d (0.8 g × 3) of either fish oil (FO) or FO with vitamin E (3 IU/g) (FOE). An additional blood sample was taken at day 91 (washout). Plasma antioxidant vitamins, fatty acid composition, and lipid peroxides were measured. Plasma α-tocopherol concentrations were increased significantly in both groups postsupplementation FO ($P = 0.018$) and FOE ($P = 0.003$) compared with baseline and washout values. Plasma retinol concentration was significantly increased ($P = 0.034$) compared with baseline and washout values following supplementation with FOE but not FO, while plasma β-carotene was significantly increased ($P = 0.036$), compared with baseline and washout values, following supplementation with FO but not FOE. There was a trend ($P = 0.059$) toward decreased plasma ascorbic acid following FO supplementation compared with baseline and washout. Plasma lipid peroxides did not change following either supplementation. Results suggest that low-dose FO feeding with and without vitamin E differentially modulates plasma antioxidant vitamins but has no significant effect on lipid peroxidation.

Lipids 33, 1163–1167 (1998).

Epidemiological studies linking fatty fish consumption with a low incidence of coronary heart disease have led to numerous clinical trials. Evidence that fish oils (FO) do not act on a single aspect, but rather modulate the entire cascade of factors involved in atherogenesis—curbing the excesses and correcting the deficiencies—continues to accumulate from these clinical trials.

Concurrent with the proposed beneficial effects, workers caution that potential side effects of fish oil supplementation are increased vitamin E requirements and increased oxidative stress *in vivo* (1). Vitamin E deficiency is induced with pharmacological doses of FO over prolonged periods of time in animal studies (2). Likewise in human trials, administration of 30 mL/d of FO results in decreased serum vitamin E (3). The importance of vitamin E in maintaining the biological integrity of cells and lipoproteins is well established (4). Epidemiological studies usually link high plasma α-tocopherol with low incidence of coronary heart disease (5), whereas decreased plasma α-tocopherol is associated with increased susceptibility to lipid peroxidation (6). In many animal and human studies, the decreases in plasma vitamin E are only a reflection of decreases in circulating lipoproteins following FO supplementation (7,8).

More subtle and perhaps more physiologically relevant effects of FO on vitamin E status have not been reported in detail, as most supplementation studies use FO with added vitamin E FOE and often use pharmacological doses of FO. The lack of a sensitive and accurate marker for *in vivo* lipid peroxidation and the presence of vitamin E in all commercial FO preparations have hampered progress toward resolving the effects of n-3 polyunsaturated fatty acids on antioxidant status *in vivo*.

Reported effects of high-dose FO supplementation decreasing plasma α-tocopherol and increasing lipid peroxidation, coupled with high-dose FO feeding being used to induce vitamin E deficiency in animals, have lead to a generalized acceptance that FO supplementation is liable to result in oxidant stress *in vivo*. This study, using 2.4 g of FO/d [equivalent to approximately 0.72 g of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], comparable to that used in the Diet and Reinfarction Trial (DART) which proved to be cardioprotective (9), focused on the effects of FO prepared without any vitamin E (FO) on plasma antioxidant vitamin status and lipid peroxide levels in healthy female volunteers, using FOE as a positive control.

MATERIALS AND METHODS

Subjects and protocol. A total of 33 healthy women volunteers (mean age 22; range 18–28 yr) were recruited from university staff and students. Informed consent was obtained and the study protocol was approved by the University of Ulster Research Ethical Committee. Eligibility for enrollment re-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; FOE, fish oil with vitamin E; MDA, malondialdehyde.

quired that subjects be healthy women; aged 18–30 yr; not pregnant; and not currently using aspirin, anti-inflammatory agents, vitamin, mineral or other nutritional supplements.

The study was conducted on a double-blind, parallel design with washout. FO supplementation studies are notoriously difficult to blind or to provide an appropriate placebo; therefore, FOE was used as a positive control. Subjects were randomly assigned to one of two groups and were required to take three capsules (0.8 g × 3) daily of either FO or FOE (3 mg/g) for 28 d and give three blood samples. The FO (a gift from Seven Seas Ltd., Hull England) provided approximately 18% EPA and 12% DHA. Sodium dodecyl gallate was present (100 ppm) as an antioxidant in both preparations. Compliance was assessed through close personal communication with the subjects, and changes in plasma total fatty acid were analyzed. Blood samples (25 mL) were taken, by venipuncture, at day 0 (baseline), day 28 (postsupplementation), and day 91 (washout). Subjects were requested to maintain their usual dietary habits and activity levels throughout the study.

Venous blood (25 mL) was drawn, carefully avoiding stasis into tubes containing lithium heparin. Samples were centrifuged at 2000 rpm for 15 min. The plasma was removed, aliquoted for each assay, and stored at −70°C. Plasma for ascorbate analysis was stabilized immediately with 50 g/L metaphosphoric acid before storage. Following the collection of the final washout blood samples, samples were batch-analyzed.

Ascorbate in plasma. Plasma ascorbate was measured by electrochemical detection following reverse-phase chromatography through a 10 cm Spherisorb-C18 3 μm ODS2 column and a 201-PSC guard column (5 × 0.4 cm) (Technicol Ltd., Manchester, United Kingdom). Homocysteine (100 mg/L) was added to the mobile phase to stabilize the ascorbate during chromatography. The mobile phase (pH 5.5) was run at a flow rate of 1 mL/min and contained 0.1 M sodium acetate (pH 5.0), 1 mM octylamine, and 200 mg/L Na_2EDTA (10).

Fat-soluble vitamins in plasma. Fat-soluble vitamins in plasma were assessed using reverse-phase high-performance liquid chromatography by the method of Thurnham *et al*. (11). The mobile phase was methanol/dichloromethane/acetonitrile (500:128:500, by vol, with 10 mg butylated hydroxytoluene), and samples were run on a Spherisorb 53-micron (ODS2) 10-cm column. In this method the samples were extracted with *n*-hexane, evaporated to dryness under nitrogen gas, and reconstituted in the mobile phase. Plasma lipid levels were measured on the Cobas Fara Automatic Analyser. Total cholesterol was assessed using a kit (Roche Diagnostic Systems, Basel, Switzerland).

Plasma EPA and DHA. Total lipids were extracted with chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene as an antioxidant and dried under nitrogen gas. Fatty acids were transmethylated with sulfuric acid and methanol. Fatty acid methyl esters were separated on a fused-silica capillary column (SGE; Foss Scientific, Belfast, United Kingdom) in a gas–liquid chromatograph equipped with a flame-ionization detector.

Lipid peroxide analysis. Lipid peroxides were measured using commercial kits (LPO-586 Biotech). A patented chromogenic agent reacts with malondialdehyde (MDA) and 4 hydroxynonenal at 45°C to give a stable chromophore with maximal absorbance at 586 nm. The detection threshold of the assay is quoted by the manufacturers as 0.5 μM; reproducibility determined over 10 d gave SEM values lower than 5%.

Assessment of food intake. Subjects' habitual food intake was assessed by diet history interview. The method established meal patterns in both working and weekend days and obtained details of meals and snacks in terms of a description of the food and an outline of portion sizes. Food portions were quantified using standard food portion sizes (12) and analyzed for composition using the computerized dietary analysis program, Food Base (The Institute of Brain Chemistry and Human Nutrition, version 1.2, 1992).

Statistical analysis. Differences between values at the baseline, postsupplementation, and washout were tested using multivariate analysis of variance for repeated measures with the least significant difference *post-hoc* test. All data are expressed as mean ± standard error (SE) with each subject acting as her own control.

RESULTS

Of the 33 subjects enrolled, 30 completed the study; the three withdrawals were for personal reasons unrelated to supplementation. Oral communication, between investigator and subjects, and the change in plasma total fatty acid profile (Fig. 1) suggested good compliance with the supplementation regime. EPA and DHA were undetectable in the plasma of this healthy population at baseline. EPA and DHA were still detectable following 63 d of washout. The supplements were generally well tolerated, although subjects did complain of experiencing a "fishy" aftertaste. Dietary intakes of the antioxidant vitamins E and C were within the normal range for this population group (Table 1). Group intakes were not significantly different.

Following FO supplementation (Table 2), plasma ascorbate was decreased ($P = 0.059$) when compared with baseline and washout. Plasma α-tocopherol increased ($P = 0.018$) postsupplementation. This effect, however, was nonsignificant when α-tocopherol was corrected for cholesterol concentration. Plasma β-carotene increased ($P = 0.036$) postsupplementation with FO when compared to baseline and washout.

The FOE supplementation (Table 2) resulted in a significant increase in plasma α-tocopherol ($P = 0.003$) and in α-tocopherol corrected for cholesterol ($P < 0.000$) postsupplementation compared with baseline and washout. Likewise, plasma retinol increased ($P = 0.034$) postsupplementation.

Neither supplementation regime showed any effects on plasma lycopene, lutein, α-carotene, and γ-tocopherol (data not shown) or on lipid peroxidation as assessed by plasma MDA and 4-hydroxynonenal levels. In the FO group ($n = 14$) the mean ± SE values for MDA and 4-hydroxynonenal (μmol/L) for baseline, postsupplementation, and washout were 10.0 ± 1.0, 10.6 ± 1.5, and 11.5 ± 1.0, respectively. For the FOE group ($n = 16$), the corresponding values were 10.6 ± 1.2, 10.6 ± 1.0, and 12.3 ± 1.0.

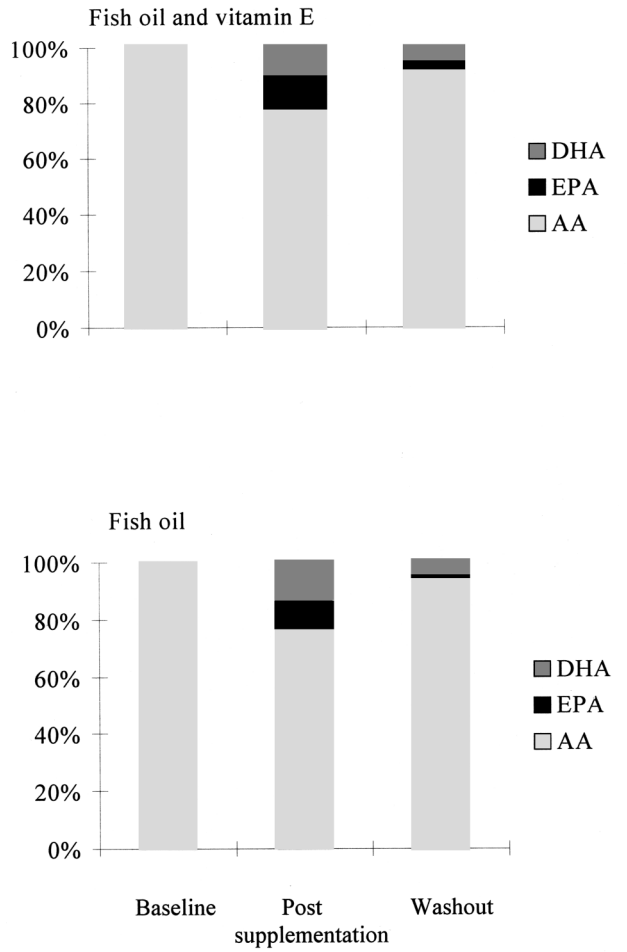

FIG. 1. Changes in percentage distribution of plasma long-chain C_{20} and C_{22} fatty acids following fish oil supplementation of healthy female volunteers. Only the long-chain polyunsaturated fatty acids were assessed. Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid.

When comparing the effect of the oils on plasma antioxidant status, the notable difference was the effect on plasma ascorbate, with FO producing a nonsignificant decrease and FOE a nonsignificant increase in plasma ascorbate concentration.

TABLE 1
Daily Intakes of Energy, Vitamin E, and Vitamin C by the Healthy Female Volunteers[a]

	Fish oil and vitamin E (FOE) (n = 16)	Fish oil (FO) (n = 14)
Energy (kJ)	8761 ± 367	9299 ± 433
Fat (% energy)	37%	35%
Protein (% energy)	15%	14%
Carbohydrate (% energy)	48%	51%
Vitamin E (mg)	9.8 ± 0.93	11.4 ± 1.30
Vitamin C (mg)	90.0 ± 11.3	83.2 ± 9.3

[a]Values reported as mean ± SE.

DISCUSSION

The essential paradox of FO supplementation is that, while many workers highlight that it is accompanied by an increased requirement for vitamin E, the purported health benefits accruing from FO are generally antioxidant in nature and show functional similarities with vitamin E *in vivo* (13).

This study was conducted to compare the effects of low doses of FO, with or without added vitamin E, on plasma antioxidant vitamins in healthy female volunteers. This low dose was found to modulate leukocyte function and cytokine production in these subjects (14).

The increases in plasma α-tocopherol and tocopherol/lipid ratio following both supplementation regimes are notable. The level of vitamin E in the FOE oil is relatively low (7.2 IU/d), supplementation with vitamin E usually is in the range of 50–1000 IU/d. It appears that low-dose FO, the only natural polyunsaturated fatty acid rich oil not loaded with vitamin E, has the capacity to mobilize α-tocopherol in plasma. Shapiro *et al.* (15) found that using MaxEPA, a commercial FO rich in EPA (18 g/d, approximately 5 g n-3 fatty acids), for 6 wk increased plasma α-tocopherol. Luostarinen *et al.*

TABLE 2
Effect of Supplementation on Plasma Antioxidant Vitamins in Healthy Female Volunteers

Variable	Baseline (mean ± SE)	Postsupplementation (mean ± SE)	Washout (mean ± SE)	Significance level[a]
FO (n = 14)				
Ascorbate (μmol/L)	99.9 ± 9.0	79.9 ± 6.9	94.4 ± 5.0	.059
α-Tocopherol (μmol/L)	23.2 ± 0.9	25.1[a] ± 1.0	21.8 ± 0.2	.018**
α-Tocopherol/total cholesterol ratio	5.44 ± 0.22	5.82 ± 0.27	5.29 ± 0.20	.080
β-Carotene (μmol/L)	0.40 ± 0.02	0.45[a] ± 0.06	0.34 ± 0.05	.036**
Retinol (μmol/L)	1.67 ± 0.11	1.80 ± 0.15	1.71 ± 0.78	.452
FOE (n = 16)				
Ascorbate (μmol/L)	98.3 ± 9.0	105.8 ± 11.0	107.2 ± 5.0	.682
α-Tocopherol (μmol/L)	24.2 ± 1.0	26.9[a] ± 1.4	24.9 ± 1.1	.003**
α-Tocopherol/total cholesterol ratio	5.30 ± 0.21	6. 00[a] ± 0.28	5.56 ± 0.25	.000***
β-Carotene (μmol/L)	0.44 ± 0.07	0.43 ± 0.06	0.39 ± 0.05	.536
Retinol (μmol/L)	1.69 ± 0.09	1.92[a] ± 0.12	1.78 ± 0.08	.034*

[a]Differences at each of the sample times were tested using multivariate analysis of variance for repeated measures with least significant differences. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; [a]denotes significantly different from baseline and washout. For abbreviations see Table 1.

(16), however, showed that supplementing with 30 mL/d FO (8.6 g n-3 fatty acids) produced no change in plasma α-tocopherol. Older populations are more susceptible to oxidative stress, and FO supplementation has been shown to decrease plasma α-tocopherol in these subjects (17). Moreover FO supplementation resulted in a significant decrease in plasma α-tocopherol in non-insulin-dependent diabetics (18).

FO may modulate vitamin E absorption, transport, storage and utilization. Absorption of vitamin E is dependent upon digestion and absorption of fat. Free tocopherols are absorbed by a nonsaturable passive process into the lymphatic circulation (19). The effects of differing fatty acids, ethyl esters, and triacylglycerols on this process are unclear. There have been relatively few investigations of how vitamin E can exchange between lipoproteins and red blood cells, and between plasma and red blood cells. Vitamin E is known to exchange more slowly than cholesterol but more rapidly than triacylglycerols. Although a transfer protein is not required for exchange to occur, transfer will be affected by low density lipoprotein receptor and lipoprotein lipase function (20,21).

Total plasma vitamin E concentration, however, is not considered a suitable predictor of cell vitamin E status (22). It has been shown that FO (15 g/d) given for 10 wk can lower plasma, red blood cell, and platelet α-tocopherol (23). Animal experiments have shown that immune cell vitamin E status seems particularly resistant to any decrease following FO supplementation despite significantly decreased plasma and liver α-tocopherol concentrations (24).

In the present study FO supplementation resulted in a significant increase in β-carotene. Mean plasma concentration of β-carotene shows seasonal variation with a twofold increase from July to September (25). It has been calculated that the critical difference for two serial results to be significantly different ($P < 0.05$) is such that, at a level of 0.5 μmol/L, an increase of 0.8 μmol (160%) is required (25). Therefore, the difference observed here of only 12.5% may reflect natural variation in plasma levels over the study period. FOE supplementation had no significant effect on plasma β-carotene but did significantly increase plasma retinol compared with baseline. This is in agreement with other studies (26,27) and may reflect the retinal esters content of commercial FO products (26) although FO did not show a similar effect. Others have suggested that FO modulates the conversion of β-carotene to retinol (23).

While the change in plasma ascorbate following either oil was insignificant, the contrasting effect of FO, which decreased plasma ascorbate, and FOE, which increased plasma ascorbate, is notable. Relatively little has been published concerning the effect of FO supplementation on plasma ascorbate. Weber *et al*. (27) reported FO supplementation had no effect on plasma ascorbate.

FO or FOE supplementation had no effect on plasma lipid peroxidation as indicated by the lack of change in hydroxyalkanes and MDA concentration. Higher doses of FO have been shown to increase MDA production, because MDA is a marker of peroxidation only of fatty acids with three or more double bonds. Increases, however, have been reported as transitory, with values returning to normal within the first 4 wk of supplementation (28).

In conclusion, this study showed that FO and FOE have modulatory effects on plasma α-tocopherol, retinol, and β-carotene concentrations and differential effects on plasma ascorbate. Plasma antioxidant status appeared to remain adequate among these healthy young women as there was no change in lipid peroxide concentrations in either group. Further work is required to examine the potential role of FO in modulating plasma and cellular antioxidant status.

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[Received June 26, 1998, and in final revised form November 3, 1998; revision accepted November 11, 1998]

Vitamin E Reduces Cholesterol Esterification and Uptake of Acetylated Low Density Lipoprotein in Macrophages

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ABSTRACT: The effects of vitamin E on cholesteryl ester (CE) metabolism in J774 cells were examined. Pretreatment of J774 cells with vitamin E at concentrations above 50 μM significantly decreased acetylated low density lipoprotein (LDL)-induced incorporation of [^{14}C]oleate into CE in cells in a dose-dependent manner. This was partly due to vitamin E also significantly inhibiting the uptake of [^{3}H]CE-labeled acetylated LDL by J774 cells. A trend existed toward suppression of acyl-CoA:cholesterol acyltransferase (ACAT) activity in the cell lysate at high vitamin E concentration, but there was no effect on hydrolysis of CE. These data indicate that vitamin E reduces the uptake of modified LDL and suppresses ACAT activity, resulting in less cholesterol esterification in macrophages: a novel mechanism underlying the antiatherogenic properties of vitamin E.
Lipids 33, 1169–1175 (1998).

Epidemiological studies demonstrated that antioxidant consumption, especially of vitamin E, is inversely correlated with the incidence of coronary heart disease (CHD) (1–6). Previous studies reported that vitamin E is one of the most effective antioxidants present in low density lipoprotein (LDL), and that its supplementation protects LDL from oxidative stress *in vitro* (7–9). Moreover, vitamin E is believed to exert additional effects that could result in antiatherogenic activities (10–15).

Formation of foam cells in arterial walls is an important event in the early stage of atherogenesis. Foam cells in atheromatous plaques are thought to be derived predominantly from monocyte-derived macrophages (16). The macrophages take up oxidized LDL through scavenger receptors, accumulate cholesteryl ester (CE), and are transformed into foam cells (17,18). CE accumulation in cells is mediated by the intracellular esterification of cholesterol by acyl-CoA:cholesterol acyltransferase (ACAT). Several studies addressed the effects of vitamin E on the formation of foam cells (19–21). Van der Schroeff *et al.* (19) reported that low doses of vitamin E failed to suppress the accumulation of CE in J774 cells that was induced by acetylated LDL. Suzukawa *et al.* (20) reported that enrichment of J774 cells with vitamin E inhibited macrophage-mediated oxidation of LDL and inhibited CE formation in J774 cells. In that study, however, no information was provided on whether these effects of vitamin E on foam cell formation were due to a decreased capacity of macrophages to oxidize LDL or to effects on cholesterol metabolism. Asmis *et al.* (21) reported that the enrichment of P388D macrophages with vitamin E reduced the cellular ratio of CE/free cholesterol after incubation with fetal bovine serum (FBS), but without significantly changing CE accumulation induced by modified LDL. Thus, the effects of vitamin E on CE accumulation induced by atherogenic lipoproteins in macrophages remain unclear.

In the present study, we examined the effects of cellular (J774 cells) enrichment with vitamin E on whole-cell cholesterol esterification, ACAT activity, lipoprotein uptake, and cellular CE hydrolysis.

METHODS

Materials. DL-α-Tocopherol and L-ascorbic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories (ICN Biochemical, Auroa, OH). Oleic acid, oleoyl CoA, phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Oleic acid and [1-^{14}C]oleoyl CoA were obtained from New England Nuclear (Boston, MA). [1a,2a(*n*) ^{3}H]Cholesteryl oleate and [1a,2a(*n*) ^{3}H]cholesteryl linoleate were obtained from Amersham (Arlington Heights, IL).

Cell culture. J774 macrophages (RIKEN Cell Bank, Tsukuba, Japan) were grown in DMEM containing 10% (vol/vol) FBS. For each experiment, the cells were cultured in 12-well Multiwell (Falcon, Franklin Lakes, NJ) cultured plates at 37°C in 5% CO_2 and 95% air.

Isolation, modification, and labeling of LDL. Blood was collected in vacutainer tubes containing 1 mg/mL of EDTA

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ANOVA, analysis of variance; CE, cholesteryl ester; CHD, coronary heart disease; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate buffer saline; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

from healthy male volunteers fasted overnight. LDL ($1.019 < d < 1.063$ g/mL) was isolated by sequential ultracentrifugation (22). LDL was acetylated by the method of Basu *et al*. (23). CE in LDL was labeled by exchange with a microemulsion containing [^{3}H]cholesteryl linoleate by the method of Zhang *et al*. (24). [^{3}H]CE-labeled LDL was acetylated by the method of Basu *et al*. (23). Specific activity of [^{3}H]CE-labeled LDL and acetylated LDL was 1027 ± 16 and 1235 ± 15 dpm/μg protein, respectively. LDL, acetylated LDL, [^{3}H]CE-labeled LDL, and [^{3}H]CE-labeled acetylated LDL were extensively dialyzed at 4°C against 0.15 M NaCl, 0.24 mM EDTA, pH 7.4. Each lipoprotein was sterilized by filtration (0.22 μm). Lipoprotein-deficient serum (LPDS) was prepared by the method of Goldstein *et al*. (25). The contents of protein in each lipoprotein and LPDS were determined by the method of Lowry *et al.* (26).

Enrichment of J774 macrophages with vitamin E. Monolayers of cells were incubated in DMEM with 10% FBS and with 10–200 μM of vitamin E. Cultured medium was supplemented with vitamin E dissolved in ethanol (10 μL ethanol/1 mL DMEM) and then incubated at 37°C for 24 h. The same amount of ethanol without vitamin E was added to control dishes.

Whole-cell cholesterol esterification assay in J774 cells. Whole-cell cholesterol esterification in J774 cells was assayed by the method of Goldstein *et al*. (25). Monolayers of J774 cells were incubated in DMEM containing 10% LPDS and 0.2 mM of [1-^{14}C]oleate-albumin (5,500 dpm/nmol) in the presence or absence of acetylated LDL for 6 h. After incubation, cellular lipids were extracted by hexane/isopropanol (3:2, vol/vol). [1,2-^{3}H]Cholesteryl oleate was added to the solvent as an internal standard. CE was isolated by thin-layer chromatography (TLC) developed in heptane/ethyl ether/acetic acid (90:30:1, by vol), and radioactivity assayed. After the lipids were extracted, the cells were dissolved, and cellular protein was determinated by the Lowry *et al.* procedure.

Uptake of [^{3}H]CE-labeled lipoprotein in J774 cells. Monolayers of J774 cells were incubated in DMEM containing 10% LPDS with 5 μg/mL [^{3}H]CE-labeled lipoproteins for 24 h. After incubation, cell monolayers were washed by phosphate buffer saline (PBS), and total lipids were extracted as described above. The organic solvent was collected and measured by scintillation counting. After the lipids were extracted, cellular protein was determined as described above.

Cell-free extract ACAT activity assay. ACAT activity was assayed in cell-free extracts by a modification of the method of Nagayoshi *et al*. (27). Cell-free extracts were prepared by suspending pellets of J774 macrophages from 60-mm dishes in 1 mL of sucrose buffer (10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 0.1 mM PMSF, and 0.1 mM EDTA), and then sonicated. The sonicated cells were then centrifuged for 15 min at $10{,}000 \times g$ at 4°C, and clear supernatants were assayed both for ACAT activity and for protein content using the Lowry *et al.* procedure. Twenty-five μL of extract was incubated in 225 μL of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.02 mM bovine serum albumin for 5 min, then 5 nmol [1-^{14}C]oleoyl CoA was added to each extract. After incubation for 5 min, the reaction was terminated, and [^{3}H]cholesteryl oleate was added as an internal standard. The cholesteryl [^{14}C]oleate in the chloroform/methanol extracts was isolated by TLC as described above.

Hydrolysis of cholesteryl [1-^{14}C]oleate in J774 cells. Hydrolysis of cholesteryl [^{14}C]oleate was assayed by the method of Tomita *et al*. (28). [^{14}C]Oleic acid was incorporated into CE in cells by incubating for 24 h with 0.2 mM [^{14}C]oleic acid–albumin complex and acetylated LDL. Cell monolayers were extensively washed with PBS and were further incubated for indicated times in fresh medium with 10% LPDS in the presence or absence of vitamin E. The radioactivity of remaining cholesteryl [^{14}C]oleate was extracted and counted as described above. The hydrolyzed amount of cholesteryl [^{14}C]oleate was calculated by subtracting the remaining cholesteryl [^{14}C]oleate radioactivity from that at time 0.

Vitamin E measurement. The vitamin E content of J774 cells was measured by the method of Bieri *et al*. (29).

Statistical analysis. Data are presented as means ± SE. Groups were compared by one-way analysis of variance (ANOVA). Bonferroni test was performed with $P < 0.005$ level of significance to adjust for multiple testing, when there were indications of significance by one-way ANOVA.

RESULTS

To investigate the effect of vitamin E on whole-cell cholesterol esterification, J774 cells were loaded with 10–200 μM vitamin E. Cytotoxicity, as assessed by trypan blue procedures and lactic dehydrogenase assay in the medium, was not apparent in cells incubated in DMEM containing 10% FBS and indicated concentrations of vitamin E. In the absence of vitamin E in the medium, cellular vitamin E could not be detected in J774 cells. After incubation of J774 cells with medium containing 10–200 μM vitamin E for 24 h, the vitamin E content of J774 cells increased up to 6.8 ± 1.1 nmol/mg protein in a dose-dependent manner.

Pretreatment of J774 cells with vitamin E at doses up to 200 μM inhibited whole-cell cholesterol esterification inducible with acetylated LDL (Fig. 1A). The content of cholesteryl [^{14}C]oleate in nonstimulated cells (no addition of acetylated LDL) was 1.25 nmol/mg/6 h cell protein. In the presence of 50 μg/mL of acetylated LDL, the content of cholesteryl [^{14}C]oleate in cells was 9.2-fold that without acetylated LDL (11.45 vs. 1.25 nmol/mg cell protein/6 h). Vitamin E significantly inhibited whole-cell cholesterol esterification in a dose-dependent manner ($P < 0.0001$, one-way factorial ANOVA). In the cells pretreated with 50 μM of vitamin E [which is found in plasma with moderate vitamin E supplementation (30)], whole-cell cholesterol esterification was 14% lower than that in cells without vitamin E (9.9 vs. 11.45 nmol/mg protein/6 h, $P < 0.0001$). In the cells pretreated with 200 μM of vitamin E, it was 55% lower than that in the absence of vitamin E (5.2 vs. 11.45 nmol/mg protein/6 h, $P < 0.0001$). Pretreatment of J774 cells with vitamin C over a

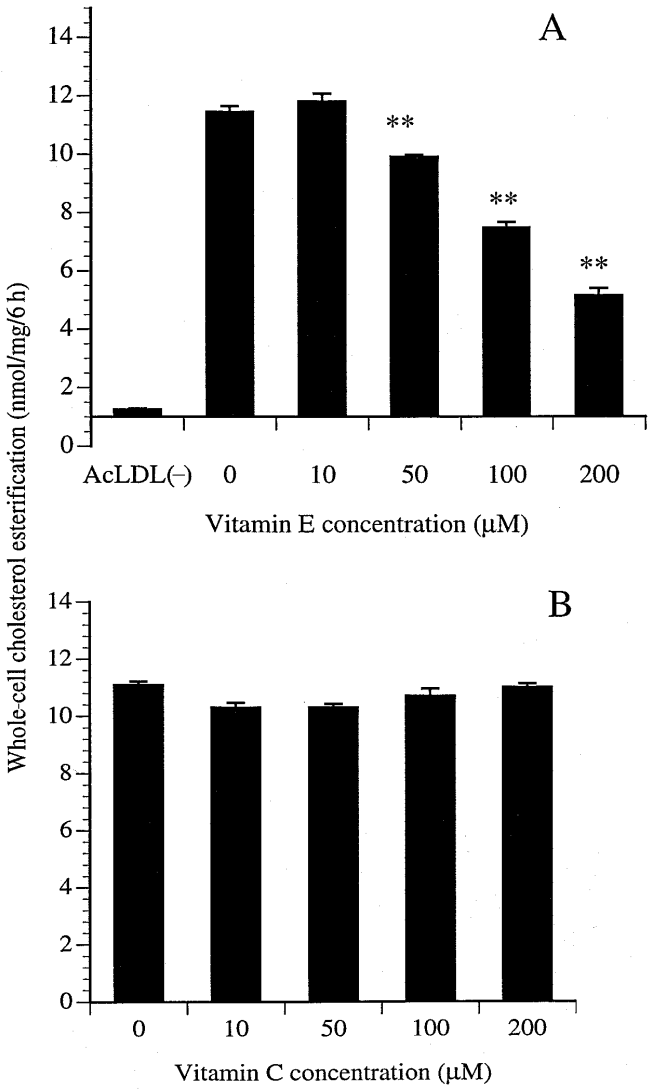

FIG. 1. Effects of vitamin E and vitamin C on whole-cell cholesterol esterification in J774 cells incubated with acetylated low density lipoprotein (AcLDL). J774 cell monolayers were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and indicated concentrations of vitamin E (A) or vitamin C (B) for 24 h. Thereafter cells were thoroughly washed with phosphate buffered saline (PBS) and further incubated in DMEM containing 10% lipoprotein-deficient serum (LPDS), 50 μg/mL AcLDL, and 0.2 mM [^{14}C]oleate-albumin for 6 h. After incubation, cells were washed thoroughly by PBS, and cellular lipids were extracted with 2 mL of hexane/isopropanol (3:2, vol/vol), and developed by thin-layer chromatography (TLC) with developing solvent [heptane/ethyl ether/acetic acid (90:30:1, by vol)]. Cholesteryl [^{14}C]oleate was quantified by liquid scintillation counting. Data represent means ± SE of 12 dishes. $^{**}P < 0.0001$ compared to control (without vitamin E).

range of concentrations failed to inhibit acetylated LDL-induced whole-cell cholesterol esterification (Fig. 1B).

The inhibition of acetylated LDL-inducible whole-cell cholesterol esterification by vitamin E could occur at several steps. First, we investigated whether this represented reduced substrate availability, thus measuring the effects of vitamin E on the lipoprotein uptake by J774 cells. After incubation of J774 cells with vitamin E for 24 h, cells were further incubated in DMEM containing 10% LPDS with 5 μg protein/mL of [^{3}H]CE-labeled acetylated LDL or [^{3}H]CE-labeled native LDL for 24 h. Supplementation of J774 cells with vitamin E significantly decreased the uptake of [^{3}H]CE-labeled acetylated LDL in a dose-dependent manner ($P < 0.001$, one-way ANOVA) (Fig. 2A). The uptake of acetylated LDL was 9% lower in cells pretreated with 50 μM of vitamin E than in the absence of vitamin E (5.9 vs. 6.5 μg protein/mg cell protein, $P < 0.005$), and was 17% lower in the cells pretreated with 200 μM of vitamin E compared with those without vitamin E (5.4 vs. 6.5 μg protein/mg cell protein, $P < 0.001$). On the other hand, vitamin E had no effect on the uptake of native LDL (Fig. 2B). These data indicate that enrichment of cells with vitamin E selectively inhibits the uptake of acetylated

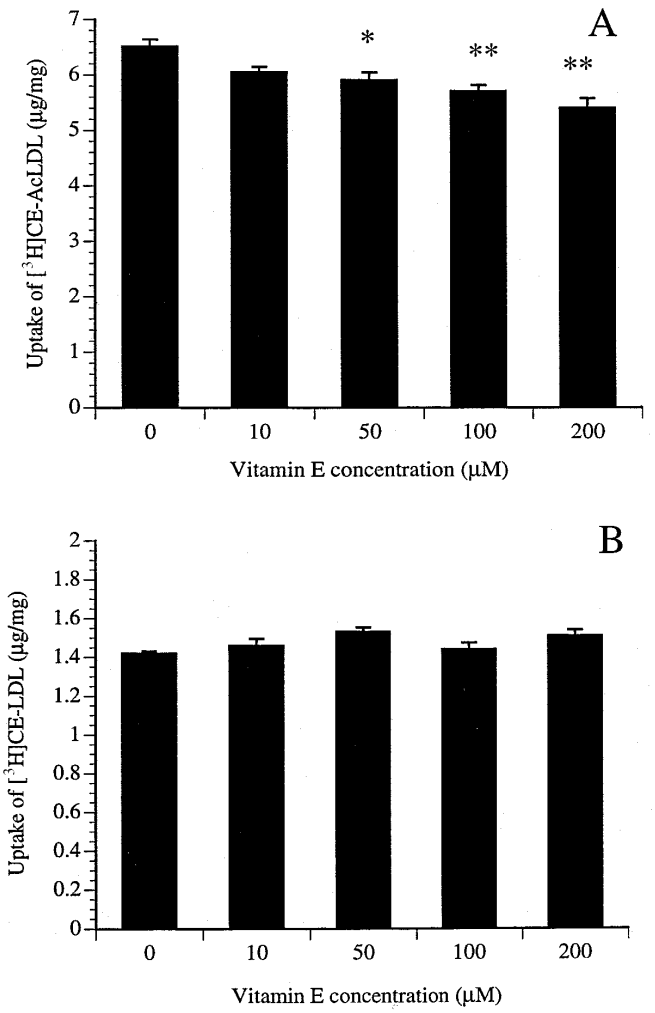

FIG. 2. Effects of vitamin E on the uptake of [^{3}H]cholesteryl ester (CE)-labeled AcLDL and [^{3}H]CE-labeled native LDL by J774 cells. J774 cells were incubated in DMEM containing 10% FBS and indicated concentrations of vitamin E for 24 h. Thereafter cells were washed with PBS and further incubated in DMEM containing 10% LPDS and 5 μg/mL of [^{3}H]CE-labeled AcLDL (A) or [^{3}H]CE-labeled native LDL (B) for 24 h. After incubation, cells were thoroughly washed with PBS, cellular lipids were extracted, and [^{3}H]CE was counted by scintillation counting. Data represents means ± SE of 12 dishes. $^{*}P < 0.005$, $^{**}P < 0.001$, compared to control (without vitamin E). See Figure 1 for other abbreviations.

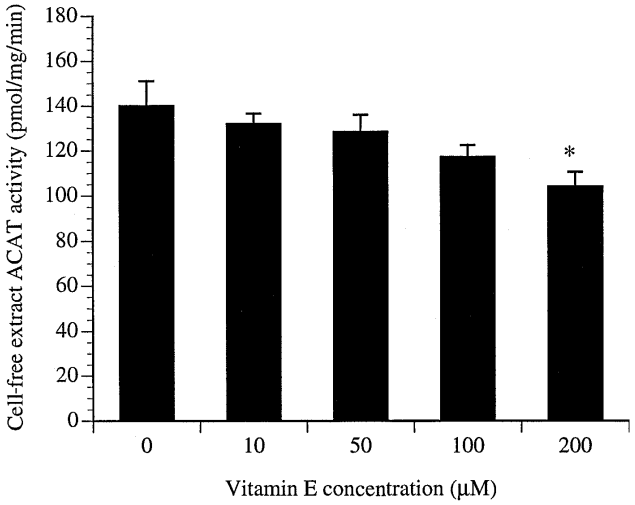

FIG. 3. Effects of vitamin E on cell-free extract acyl CoA:cholesterol acyltransferase (ACAT) activity in J774 cells. J774 cells were incubated in DMEM containing 10% FBS and indicated concentrations of vitamin E for 24 h. Thereafter cells were thoroughly washed with PBS and harvested. Cell-free extracts were assayed for ACAT activities. Data represent means ± SE of 16 dishes. *$P < 0.005$ compared to control (without vitamin E). See Figure 1 for other abbreviations.

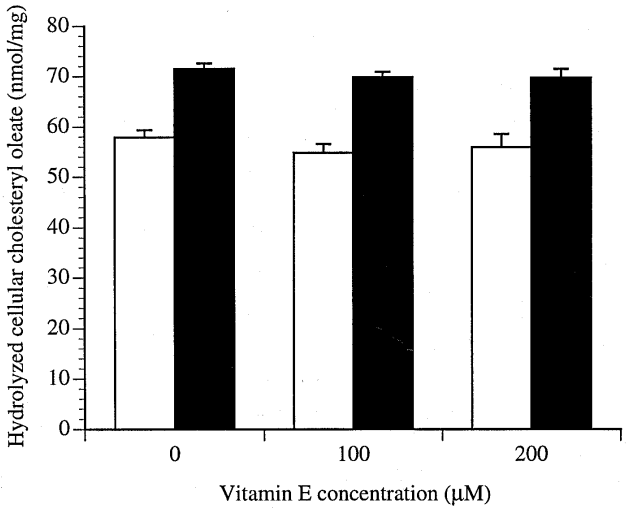

FIG. 4. Effects of vitamin E on hydrolysis of esterified cholesterol in J774 cells. J774 cells were loaded with cholesteryl [^{14}C]oleate as described in the Methods section and were then incubated in fresh DMEM containing 10% LPDS and indicated concentrations of vitamin E for 24 or 48 h. Thereafter cells were washed with PBS, cellular lipids were extracted, and cholesteryl [^{14}C]oleate was quantified as described in the legend to Figure 1. The cellular content of cholesteryl [^{14}C]oleate at time 0 was 95.45 ± 3.45 (mean ± SE of 12 dishes). Data represent means ± SE of six dishes. □, 24 h; ■, 48 h. See Figure 1 for abbreviations.

LDL, but not of native LDL. These data suggested that decreased uptake of acetylated LDL in vitamin E-treated cells may have at least partly accounted for decreased whole-cell cholesterol esterification.

Calculations based on data in Figures 1 and 2 showed that 50 μg/mL of acetylated LDL (3.0 nM total cholesterol/μg protein) delivered 49 nmol of cholesterol/mg protein/6 h, which was associated with an increment in whole-cell cholesterol esterification of 10.2 nmol cholesterol/mg protein/6 h. Therefore, at most, about 21% of cholesterol delivered within acetylated LDL was esterified into CE, assuming for the purpose of argument that all newly synthesized CE was derived from acetylated LDL cholesterol. Pretreatment with vitamin E at concentrations of 50, 100, and 200 μM decreased the acetylated LDL-delivered cholesterol to 44.8, 41.4, and 39.4 nmol/mg protein/6 h, and also reduced whole-cell cholesterol esterification by 1.5, 4.0, and 6.3 nmol cholesterol/mg protein/6 h, respectively. By assuming a constant proportion of delivered cholesterol becomes esterified, one would expect decrements of delivered cholesterol of 4.2, 7.6, and 9.6 nmol to decrease whole-cell cholesterol esterification by 0.9, 1.6, and 2.0 nmol, respectively. However, inhibition of whole-cell cholesterol esterification by vitamin E was substantially greater, with only 19, 15, and 10% of cholesterol delivered through acetylated LDL uptake esterified in cells treated with 50, 100, and 200 μM vitamin E, respectively. These calculations showed that the reduced acetylated LDL uptake was most unlikely to fully explain the reduction in whole-cell cholesterol esterification by vitamin E.

We next investigated the effects of vitamin E on ACAT activity in a cell-free extract. After preincubation of J774 cells with increasing amounts of vitamin E for 24 h, cells were harvested, and ACAT activity of the cell-free extract was assayed. A trend existed for reducing ACAT activity by pretreating cells with vitamin E ($P < 0.05$, one-way ANOVA) (Fig. 3). However, inhibition of ACAT activity in cells reached statistical significance only with 200 μM vitamin E, by 26% (104.5 vs. 140.0 pmol/mg/min, $P < 0.005$).

The effect of vitamin E on the hydrolysis of cellular CE was also examined since changes in hydrolysis would also affect substrate availability. Cells loaded with cholesteryl [^{14}C]oleate were incubated with fresh DMEM containing 10% LPDS in the presence or absence of vitamin E, and the remaining cellular cholesteryl [^{14}C]oleate in the extracted lipids was quantified by TLC. The content of cholesteryl [^{14}C]oleate was approximately 100 nmol/mg protein at the start of incubation and decreased with incubation time. Approximately 50% of cholesteryl [^{14}C]oleate was hydrolyzed after 24 h incubation in control cells. Hydrolysis of cholesteryl [^{14}C]oleate was not significantly altered by the presence of vitamin E (Fig. 4).

DISCUSSION

Accumulation of CE in macrophages in the subendothelial space plays a critical role in the initiation and progression of atherogenesis (31). Modified LDL is reportedly recognized and internalized by macrophages through scavenger receptors that stimulate CE accumulation which in turn transform macrophages into foam cells (17,32). Thus, reducing CE accumulation in macrophages would diminish the initiation and progression of atherogenesis. Both macrophages and endothelial cells can oxidize LDL that is transported into the artery through several pathways (33). Protecting LDL against

oxidation by antioxidants, such as vitamin E, would be expected to reduce CE accumulation in macrophages. In addition to the antioxidant protection of LDL, vitamin E has other beneficial effects on endothelial cells, platelets, and smooth muscle cells (10–15). Few studies exist of the effects of vitamin E on cholesterol metabolism in macrophages, and the effects on CE accumulation induced by an atherogenic lipoprotein are unclear. In this study, enrichment of J774 cells with vitamin E inhibited acetylated LDL-inducible whole-cell cholesterol esterification in a dose-dependent manner. Whole-cell cholesterol esterification after acetylated LDL uptake was significantly reduced by pretreating with 50 μM vitamin E, a concentration reported in human plasma after moderate vitamin E supplementation (30). This would be a novel mechanism whereby vitamin E might contribute to the prevention of atherogenesis.

Monocyte-derived macrophages take up and degrade lipoproteins by receptor-mediated endocytosis. Lysosomal acidic CE hydrolase releases free cholesterol from CE, and thereafter the free cholesterol is reesterified in the cytoplasm by ACAT. Accumulated CE is not inert, but undergoes hydrolysis by neutral CE hydrolase (32). The relative activities of ACAT and of two CE hydrolases are believed to be responsible for the regulation of cellular CE content (34). Therefore, the accumulation of CE in foam cells will depend on the uptake of atherogenic lipoprotein and the relative activities of ACAT and CE hydrolase. In the present study, enrichment of macrophages with vitamin E significantly inhibited also the uptake of acetylated LDL in addition to the reduction in whole-cell cholesterol esterification. In contrast, cellular hydrolysis of CE was not affected by vitamin E. These data indicate that vitamin E inhibits whole-cell cholesteryl esterification, which is mediated in part by interfering with the uptake of acetylated LDL and partly by suppressing ACAT activity. The lesser inhibition by vitamin E of ACAT activity in cell-free extracts is probably not contradictory as will be discussed later.

In this study, pretreatment of J774 cells with 50 μM or higher concentrations of vitamin E significantly decreased the uptake of acetylated LDL. We did not explore further mechanisms whereby this might have occurred. Acetylated LDL is taken up by macrophages through scavenger receptors (32). Previous studies reported that induction of scavenger receptors in macrophages or smooth muscle cells is regulated by protein kinase C (PKC) (35–37). For example, platelet-derived growth factor-mediated induction of the scavenger receptor in J774 cells is regulated by PKC, and this induction is inhibited by a PKC inhibitor (35). Many earlier studies reported that vitamin E suppressed PKC activity in various cells, including macrophages (10,13–15,38,39). In the present study, we cultured J774 cells in DMEM containing 10% heat-inactivated FBS and did not stimulate these cells further. Possibly vitamin E might limit the induction of scavenger receptors in J774 cells stimulated by some growth factors remaining in FBS.

As discussed previously, calculation of the data for whole-cell cholesterol esterification and acetylated LDL uptake showed that the diminished uptake of acetylated LDL could not fully explain the reduction in whole-cell cholesterol esterification by vitamin E. Changes in cellular hydrolysis of CE were excluded. Further, ACAT activity in cell-free extract was also reduced but only at the highest concentration of vitamin E. Since the aqueous cell-free extract of macrophages (used to measure ACAT activity) was likely to contain a much lower concentration of the lipid-soluble vitamin E than present in intact cells, the cell-free extract assay was probably not as sensitive a method to estimate the effect of vitamin E.

Another possibility is an alteration of cholesterol trafficking by vitamin E. Previous studies reported that whole-cell cholesterol esterification in J774 cells after uptake of acetylated LDL is regulated by the amount of cholesterol that comes into contact with ACAT, and not through the induction of ACAT itself (40–42).

Whole-cell cholesterol esterification is an index that could represent CE accumulation in cells (32). However, notably, others found no significant effect of vitamin E on CE mass accumulation in macrophages following incubation with modified lipoproteins (19,21). In one of the earlier studies, the final concentration of vitamin E was only 10 μM (19) which was ineffective also in our study. In the other study, P388D macrophages were used (21), and the difference in cell species might underlie the discrepancy.

In summary, enrichment of J774 macrophages with vitamin E significantly inhibited acetylated LDL-inducible whole-cell cholesterol esterification in J774 macrophages. Although this was associated with reduced acetylated LDL uptake, the latter could not fully account for diminished whole-cell cholesterol esterification. This effect would be a novel mechanism underlying the antiatherogenic properties of vitamin E.

ACKNOWLEDGMENT

We thank Emiko Miyajima for her technical assistance.

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[Received June 4, 1998, and in final revised form October 29, 1998; revision accepted November 25, 1998]

Effect of Dietary Cholesterol on Low Density Lipoprotein-Receptor, 3-Hydroxy-3-methylglutaryl-CoA Reductase, and Low Density Lipoprotein Receptor-Related Protein mRNA Expression in Healthy Humans

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ABSTRACT: We investigated the possibility that dietary cholesterol downregulates the expression of low density lipoprotein (LDL) receptor and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase genes of circulating mononuclear cells *in vivo* in healthy humans. We also studied the variations of the LDL receptor-related protein (LRP) gene in the same conditions. Dieters ($n = 5$) were submitted to a 4-d fat restriction (mean cholesterol intake: 6 ± 4 mg/d), followed by a 7-d cholesterol (a mean of 791 ± 150 mg/d) supplementation. Controls ($n = 3$) did not change their diet. During fat restriction, serum total and LDL cholesterol decreased significantly ($P < 0.05$), and LDL receptor and HMG-CoA reductase mRNA copy numbers in mononuclear cells increased by 57 and 147%, respectively ($P < 0.05$). After reintroducing cholesterol, serum cholesterol was stable whereas LDL receptor and HMG-CoA reductase mRNA decreased by 46 and 72% ($P < 0.05$) and LRP mRNA increased by 59% ($P < 0.005$). The changes in LDL receptor and HMG-CoA reductase mRNA abundance were correlated ($r = +0.79$, $P = 0.02$) during cholesterol reintroduction as were LDL receptor and LRP mRNA levels, but negatively ($r = -0.70$, $P = 0.05$). Also, 70% of the variability in LRP mRNA ($P < 0.005$) was explained by dietary cholesterol. Thus, the basic mechanisms regulating cellular cholesterol content, the coordinate feedback repression of genes governing the synthesis and uptake of cholesterol, are operating *in vivo* in humans. However, serum cholesterol did not increase in response to dietary cholesterol, suggesting that these mechanisms may not play as predominant a role as previously believed in the short-term control of serum cholesterol *in vivo* in humans. A new finding is that LRP gene is also sensitive to dietary cholesterol, suggesting that it may participate in the control of serum cholesterol. Further *in vivo* studies in humans are warranted to explore the molecular mechanisms of the physiological response to dietary cholesterol in humans.

Lipids 33, 1177–1186 (1998).

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Abbreviations: CHD, coronary heart disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LDL, low density lipoprotein; LRP, LDL-receptor-related protein; RT-PCR, reverse transcriptase-polymerase chain reaction; VLDL, very low density lipoprotein.

The exact molecular mechanisms by which dietary lipids (1,2) are risk factors for coronary heart disease (CHD) are unknown. Epidemiological studies show that dietary cholesterol is an independent predictor of CHD, apart from its effect on blood cholesterol (3,4), but how this deleterious effect is mediated was not determined. Another unresolved major question is why healthy humans show no or only a mild increase in their blood cholesterol in response to high cholesterol intake (5,6). Answers to these questions may have major clinical implications.

In humans, cholesterol metabolism is thought to be integrated and coordinated in the liver (7). Much of our knowledge about how the hepatocytes achieve this regulation comes from experiments conducted on cultured cells or animal models, with results varying considerably among species relative to sensitivity to dietary cholesterol. Cultured cells respond to the presence or absence of cholesterol in the medium by regulating the activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis, and the abundance of the low density lipoprotein (LDL) receptors. These processes act coordinately to maintain intracellular cholesterol levels within narrow limits (7,8). One commonly proposed mechanism for increases (in sensitive animals) in blood LDL cholesterol in response to increased dietary cholesterol is the suppression of LDL receptor-mediated uptake secondary to increased cholesterol content of hepatocytes (7–9). Promoters of the HMG-CoA reductase and LDL receptor genes contain 10 bp sterol regulatory elements (10,11) that are believed to be responsible for the coordinate nature of these regulatory processes. Thus, the primary regulatory mechanism to prevent the intracellular accumulation of cholesterol and the increase of blood cholesterol is thought to be the repression of the transcription of these two genes (7–9).

No studies however have examined these regulatory hypotheses in humans, and very little is known about the transcriptional regulation of cholesterol metabolism in healthy humans *in vivo,* in particular in response to dietary changes. Re-

cently, using deuterium incorporation and urinary mevalonic acid levels, Jones *et al.* (12) reported only modest suppression of cholesterol synthesis in humans in response to dietary cholesterol *in vivo,* suggesting a possible failure of the feedback repression of the genes regulating endogenous cholesterol synthesis. Also, it should be noted that other cholesterol-rich lipoprotein receptors, for instance the the LDL receptor-related proteins (LRP) (13–15) or the very low density lipoprotein receptor (16), may be implicated in humans in cholesterol homeostasis although few data are presently available.

In humans, the *in vivo* approach is especially difficult because of the lack of appropriate tissues from which repetitive samples may be made and also the lack of accurate methodologies for quantitative measurements of low abundant materials. Thus, detailed kinetic studies of *in vivo* cholesterol regulatory mechanisms within individuals were not conducted. Recent studies however have begun to focus on the *in vivo* regulation of the major genes involved in cholesterol homeostasis, primarily because in humans, basic regulatory mechanisms in hepatocytes and blood mononuclear cells are similar (17,18), allowing repetitive samplings and time-course evaluation. Also, competitive reverse transcriptase-polymerase chain reaction (RT-PCR) provides a new powerful tool for quantifying very low abundant species of mRNA (19–21).

The primary aim of the present study in humans therefore examines whether dietary cholesterol actually and significantly downregulates the expression of HMG-CoA reductase and LDL receptor genes *in vivo*. A two-step dietary protocol was specifically designed to control for dietary confoundings (fatty acids) when studying the specific effects of dietary cholesterol and to sensitize the responses of the genes. A secondary aim was to investigate the response of the LRP gene because this cholesterol-rich lipoprotein receptor was proposed for use in the regulation of blood lipoprotein levels (13–15), but no data are available presently in humans.

MATERIALS AND METHODS

Eight healthy men ages 24 to 45 years volunteered for this study. They were of average fitness levels and none was taking medication. No attempt was made to alter their usual lifestyle pattern.

The protocol was approved by the Ethics Committee of the Hospices Civils de Lyon, and informed consent was obtained from each subject. The protocol was designed to study the effects of dietary cholesterol on gene expression, independent of other dietary fats known to have a major impact on cholesterol metabolism (e.g., saturated fatty acids). The confounding effects of other fats were controlled by eliminating all fats from the diet during a first period followed by the reintroduction of cholesterol, and only cholesterol, in the diet. Statistically, the main endpoint of the study was to compare, between dieters and control subjects, the changes in mRNA levels between the period without fat and the period with cholesterol only. Practically, after a 6-d observation allowing baseline diet recording, the dieters modified their diet in two steps according to individual instructions of a dietitian. In a first step (a total fat restriction period of 4 d), they were instructed to eliminate from their diet all the foodstuffs containing fat. Then, during a second step of 7 d, a cholesterol reintroduction period, two eggs per day for 3 d (day 4 to day 6) and four eggs per day for 4 d (day 7 to day 10) were added to the previous fat-restricted diet. The two time intervals were chosen for practical purposes: not to disturb the daily life of the subjects (who were hospital workers) and to be ethically acceptable and nutritionally efficient. Therefore, the protocol started on Monday morning, lasted less than 2 wk, and ended on the Friday morning of the second week. Obviously, a third week of fat restriction would have been too difficult for the dieters along with the concern that prolonging such a diet might disturb nutrient balances (essential fatty acids, indispensable amino acids, liposoluble vitamins).

Control subjects maintained their usual dietary habits. Adherence to the experimental diet and evaluation of the diet of the controls were monitored daily by the dietitian and by measuring plasma fatty acids as biomarkers of the dietary modifications. The subjects weighed their foods, and information was translated into nutrients using nutrition software (2).

Isolation of blood mononuclear cells. Venous blood was collected for the isolation of mononuclear cells (15 mL) and the determination of serum lipids and lipoproteins (5 mL) at 8 A.M. after a 12-h overnight fast. Mononuclear cells were immediately isolated by centrifugation of whole blood on a Ficoll gradient at 4°C as described (22) and stored at −70°C. Cell population consisted of 85–95% lymphocytes.

Total RNA preparation. Total RNA was prepared from frozen samples by the RNAzol method (Bioprobe Systems), a variant of the guanidinium/chloroform procedure described by Chomczynski and Sacchi (23). To remove any contaminating DNA, total RNA was incubated with 20 units of DNase RNase-free for 1 h at 37°C. Then total RNA was again extracted and resuspended in water. Total RNA was quantified spectrophotometrically at 260 nm and by electrophoresis on agarose gel of serial dilutions compared with known amounts of standard RNA (Boehringer Mannheim, Indianapolis, IN). Gel electrophoresis showed clear undegraded 18S and 28S bands. Fifty nanograms of total RNA was used for each PCR.

Measurement of LDL receptor and HMG-CoA reductase mRNA levels. LDL receptor and HMG-CoA reductase mRNA copy numbers were determined by competitive RT-PCR according to methods described by Powell and Kroon (17) and Gebhard *et al*. (19), using a commercial kit (Perkin Elmer Cetus Instruments, Norwalk, CT). The unique advantage of including a synthetic RNA as an internal standard in the RT-PCR is that one can know the exact copy number per reaction. Total cellular RNA was thus reverse-transcribed into cDNA in the presence of dilution series of AW 109 cRNA (internal standard) which derives from the plasmid pAW 109 and contains primer sites for the LDL receptor and HMG-CoA reductase. Reactions were performed in a final volume of 100 µL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl,

2.5 mmol/L $MgCl_2$, 150 µmol/L of each of the four deoxyribonucleoside triphosphates (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden), and 0.65 µmol/L of LDL receptor or HMG-CoA reductase downstream primers. Primer sequences were identical to those described by Powell and Kroon (21). After initial denaturation, 10 units of reverse transcriptase were added to the reaction mixture, incubated at 42°C for 40 min and at 94°C for 2 min. PCR was performed by adding 5 units of Taq DNA polymerase (Perkin Elmer Cetus) and 0.65 µmol/L of LDL receptor or HMG-CoA reductase upstream primers to the entire cDNA product from the RT reaction. Thus, the competitor product was that from the AW 109 cRNA. All PCR included a negative control for each RNA, and each experiment was performed omitting reverse transcriptase in one control tube. The number of PCR cycles was 35 as amplification was shown to be exponential up to 37 cycles. Reaction products corresponding to the mRNA tests and the internal controls were analyzed on agarose gel stained with ethidium bromide and documented on Polaroid nº667 film. For quantitation, we used densitometric scanning of photographic images of the gels. The ratio of amplified RNA test to amplified competitor was determined for each sample, and the logarithm of the band density ratio was plotted against the logarithm of the initial amount of the competitor, giving linear titration curves. The initial amounts of each RNA test and competitor were assumed to be equal when the ratio competitor/RNA test was equal to one. The results were expressed as copy number per µg of total cellular RNA.

Measurement of LRP mRNA levels. LRP mRNA levels were evaluated according to a semiquantitative RT-PCR adapted from Dante *et al*. (24). Briefly, aliquots (50 ng) of total cellular RNA were reverse-transcribed into cDNA. The cDNA encoding for LRP and the ubiquitous glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were simultaneously amplified in each assay tube. Reactions were performed following the methods described for the LDL receptor and HMG-CoA reductase mRNA. LRP primer sequences were 5′-CTCAGGTACGTGGCCAAGAT-3′, located at position 1311–1330 and 5′-CGAGTTGGTGGACTAGAGAT-3′, located at position 1730–1750 according to the LRP gene sequence published by Herz *et al*. (25). GAPDH primers are those used by Dante *et al*. (24). PCR products were analyzed on agarose gel stained with ethidium bromide. For quantitation of relative band intensities, densitometric scanning of photographic images was performed, and the ratio of LRP to GAPDH was determined for each sample. Regarding the specificity of the RT-PCR assay for the LRP mRNA, the expected PCR product of 440 bp should contain Hinc II, Alu I, and Ava II restriction sites at positions 242, 236, 146, and 278, respectively. The restriction map of the RT-PCR products indicated that the fragments of predicted size were obtained using these enzymes and confirmed the authenticity of the PCR products. RT-PCR was also performed using a serial dilution of total RNA in sterile water which indicated that the ratio of LRP to GAPDH was not dependent on the total RNA concentration for amounts ranging from 10 to 200 ng. All PCR included a negative control, and the absence of genomic DNA contamination in the RNA samples was confirmed by the use of RT-negative RNA samples. The number of PCR cycles was also 35.

Plasma lipids and lipoprotein measurements. Cholesterol and triglyceride concentrations in plasma and in lipoprotein fractions were assayed by enzymatic procedure using commercial kits (Boehringer Mannheim). LDL-cholesterol was determined using the Friedewald formula (26). Apo B_{100} and A_1 were measured with a Beckman nephelometer (Fullerton, CA) (27).

Plasma fatty acid analysis. Plasma lipids were extracted by the method of Folch *et al.* (28) and methylated by boron fluoride fatty acids methanol reagent according to Morrison and Smith (29). Fatty acids were analyzed by gas–liquid chromatography with a Hewlett-Packard 6890 series gas chromatograph (Palo Alto, CA), a BPX 70 (SGE) capillary column, and nitrogen as the carrier gas as described (2).

Statistical methods. Statistical significance of the differences between dieters and controls in the changes in each mRNA, between day 0 and day 4 and between day 4 and day 11, respectively, was determined by two-tailed unpaired Student's *t*-test. The relationships between the changes in mRNA levels (between day O and day 4 and between day 4 and day 11, respectively) and between the changes in each mRNA and the changes in dietary cholesterol (between day 0 and day 4 and between day 4 and day 11) were evaluated by linear regressions performed by the least-squares method. A $P < 0.05$ was considered significant.

RESULTS

Dietary and plasma lipid results. Daily nutrient intakes of the dieters (mean ages 31.5 ± 4.7 yr, mean body mass index 23.2 ± 1.0 kg/m^2), and controls (30.5 ± 3.5 yr and 22.7 ± 0.6 kg/m^2) are shown in Table 1. The control subjects did not change their dietary habits. In particular, the slight increases in total lipid and cholesterol intake during the second period were not significant. In dieters at the end of the fat restriction period (day 4), total energy, proteins, total lipids, and cholesterol significantly ($P < 0.05$) decreased whereas carbohydrates increased ($P < 0.05$) compared to baseline (day 0). Lipid intake decreased from 36.9 to 2.6% of energy and cholesterol from 438 to 6 mg/day. During the cholesterol reintroduction period (from day 4 to day 11), energy intake did not change (8494 ± 565 and 8498 ± 1050 Joules), whereas the consumption of eggs provided a mean intake of 791 ± 150 mg cholesterol per day.

Analyses of plasma fatty acids (Table 2) indicated in dieters a significant decrease in the concentration of the major essential fatty acid, linoleic acid, observed already at day 4 (22.8 ± 6.4% of total fatty acids against 31.0 ± 4.6 at day 0, $P < 0.05$) and persisting at day 11 (22.6 ± 3.8), confirming (as the other changes between day 4 and day 11 were not significant) that dieters actually did not eat fat other than eggs during the study.

TABLE 1
Summary of Daily Nutrient Intake in Dieters and Controls[a]

	Baseline	Fat restriction	Cholesterol reintroduction
Dieters (n = 5)			
Total energy (Joules)	11556 ± 2556	8494 ± 565	8498 ± 1050
Proteins (% energy)	14.6 ± 2.0	10.8 ± 1.1	12.2 ± 1.1
Carbohydrates (% energy)	44.5 ± 7.0	85.1 ± 1.1	74.2 ± 3.8
Total fat (% energy)	36.9 ± 3.5	2.6 ± 0.4	10.8 ± 1.2
Cholesterol (mg/day)	438 ± 206	6 ± 4	791 ± 150
Controls (n = 3)			
Total energy (Joules)	11242 ± 3682	9858 ± 2695	10682 ± 1937
Proteins (% energy)	14.7 ± 3.0	15.2 ± 1.1	13.7 ± 2.2
Carbohydrates (% energy)	47.1 ± 4.6	46.4 ± 2.7	45.6 ± 2.3
Total fat (% energy)	37.0 ± 3.6	37.7 ± 1.9	39.3 ± 1.0
Cholesterol (mg/day)	374 ± 156	389 ± 193	507 ± 187

[a]Results are given as means ± SD. Baseline data correspond to the mean daily intake recorded during the 6 d prior to day 0; fat restriction data to the mean daily intake between day 0 and day 4 and cholesterol reintroduction data to the mean daily intake between day 5 and 10.

TABLE 3
Plasma Lipids and Lipoproteins[a]

	Baseline (day 0)	Fat restriction (day 4)	Cholesterol reintroduction (day 11)
Dieters (n = 5)			
Total cholesterol, mmol/L	4.69 ± 0.60	4.06 ± 0.65*	4.32 ± 0.55
LDL-cholesterol, mmol/L	3.02 ± 0.49	2.49 ± 0.45*	2.69 ± 0.46
HDL-cholesterol, mmol/L	1.16 ± 0.29	0.80 ± 0.30**	0.88 ± 0.18
Triglycerides, mmol/L	1.12 ± 0.35	1.70 ± 1.03	1.65 ± 0.73
ApoB100, g/L	0.94 ± 0.17	0.85 ± 0.21	0.89 ± 0.16
ApoA1, g/L	1.48 ± 0.23	1.07 ± 0.14**	1.14 ± 0.12
Controls (n = 3)			
Total cholesterol, mmol/L	4.95 ± 0.39	4.69 ± 0.09	4.73 ± 0.50
LDL-cholesterol, mmol/L	3.44 ± 0.31	3.20 ± 0.02	3.22 ± 0.37
HDL-cholesterol, mmol/L	1.12 ± 0.03	1.02 ± 0.08	1.15 ± 0.09
Triglycerides, mmol/L	0.86 ± 0.10	1.00 ± 0.33	0.76 ± 0.08
ApoB100, g/L	0.90 ± 0.09	0.91 ± 0.18	0.85 ± 0.06
ApoA1, g/L	1.50 ± 0.06	1.30 ± 0.07	1.37 ± 0.02

[a]Results are given as means ± SD. *P < 0.005; **P < 0.001vs. baseline. In parentheses are indicated the days when blood samples were collected.

Total cholesterol, high-density lipoprotein-cholesterol, LDL-cholesterol, and apo A_I in the dieter group decreased significantly ($P < 0.05$) by day 4 as compared with controls (Table 3). During the cholesterol reintroduction period, the changes in blood lipoproteins and lipids were not significant.

LDL receptor and HMG-CoA reductase mRNA measurements. Figure 1 shows the results of LDL receptor (A) and HMG-CoA reductase (B) mRNA measurements. During the 4-d dietary fat restriction period, mean LDL receptor and HMG-CoA reductase mRNA copy numbers increased respectively from $41.2 \pm 5.4 \times 10^4$ and $996 \pm 244 \times 10^4$ per µg of total RNA at day 0 to 64.8 ± 9.1 and 2462 ± 645 at day 4 (both $P < 0.05$ when compared with the changes in the control group). These changes corresponded to 57 and 147% increase for each gene, respectively. After cholesterol reintroduction, the mRNA levels of both genes decreased to 34.8 ± 3.3 and 678 ± 129 copy numbers ($P < 0.05$) corresponding to 46 and 72% decrease. When analyzing individual data, the responses of the two genes (amplitude and kinetics) to the fat restriction were not homogeneous as shown by the large standard deviations of the means at days 0, 2, and 4. Certain subjects responded immediately (+244% for HMG-CoA reductase in dieter 2 after 2 d), whereas others had a delayed response (+3% after 2 d but +72% after 4 d for the LDL receptor in dieter 4) or had an early paradoxical response with −12% after 2 d for the LDL receptor in dieter 1 followed by +37% after 4 d. In contrast, cholesterol reintroduction was associated with a more homogeneous response for both LDL receptor (reduction range, 28 to 60%) and HMG-CoA reductase (52 to 84%) at day 11, and standard deviations were small at days 9 and 11 (Fig. 1).

TABLE 2
Plasma Fatty Acids (in % of total fatty acids)[a]

	Baseline (day 0)	Fat restriction (day 4)	Cholesterol reintroduction (day 11)
Dieters (n = 5)			
Sum of saturated FA	29.8 ± 1.1	32.0 ± 4.5	33.4 ± 3.6
Oleic acid 18:1n-9	20.6 ± 3.7	24.0 ± 3.0	24.1 ± 0.7
Linoleic acid 18:2n-6	31.0 ± 4.6	22.8 ± 6.4*	22.6 ± 3.8*
α-Linolenic acid 18:3n-3	0.58 ± 0.2	0.56 ± 0.3	0.68 ± 0.5
Sum of n-6 FA	38.6 ± 5.9	30.7 ± 7.4	30.8 ± 3.4*
Sum of n-3 FA	4.8 ± 0.8	5.0 ± 1.1	4.8 ± 0.6
Controls (n = 3)			
Sum of saturated FA	29.7 ± 5.4	30.2 ± 1.2	30.6 ± 2.1
Oleic acid 18:1n-9	18.9 ± 1.1	18.3 ± 1.8	17.3 ± 0.9
Linoleic acid 18:2n-6	33.3 ± 2.9	31.4 ± 2.2	31.4 ± 1.8
α-Linolenic acid 18:3n-3	0.35 ± 0.1	0.45 ± 0.2	0.36 ± 0.1
Sum of n-6 FA	41.7 ± 4.6	40.9 ± 1.2	40.4 ± 1.8
Sum of n-3 FA	4.9 ± 0.3	5.4 ± 1.1	6.3 ± 1.7

[a]Results are given as means ± SD. In parentheses are indicated the days when blood samples were collected. FA, fatty acids; *P < 0.05 vs. baseline.

LRP mRNA measurements. The results are expressed as LRP to GAPDH ratio with day 0 taken as 100%. A significant decrease (from 100% to 76 ± 4%) of the LRP mRNA level was observed between day 0 and day 4 ($P < 0.05$, Fig. 2). After day 4, as cholesterol was reintroduced, LRP mRNA levels increased progressively, reaching about 100% at day 9 and 121 ± 10% at day 11 ($P < 0.05$), corresponding to a mean increase of 59% (range 13 to 80%). The influence of dietary lipids on LRP mRNA levels in a representative subject (dieter 4) is shown in Figure 3.

Relationship analyses. We principally addressed the question of whether the expression of the LDL receptor, HMG-CoA reductase, and LRP genes are coordinated in response to dietary cholesterol, when cholesterol was reintroduced (from day 4 to day 11). As a matter of fact, during fat restriction (between day 0 and day 4), the relationship between the changes in LDL receptor mRNA levels and either the changes in HMG-CoA reductase or LRP mRNA levels were not significant (r^2 = 0.25 and 0.20), whereas the relationship between the changes in HMG-CoA reductase and LRP mRNA levels

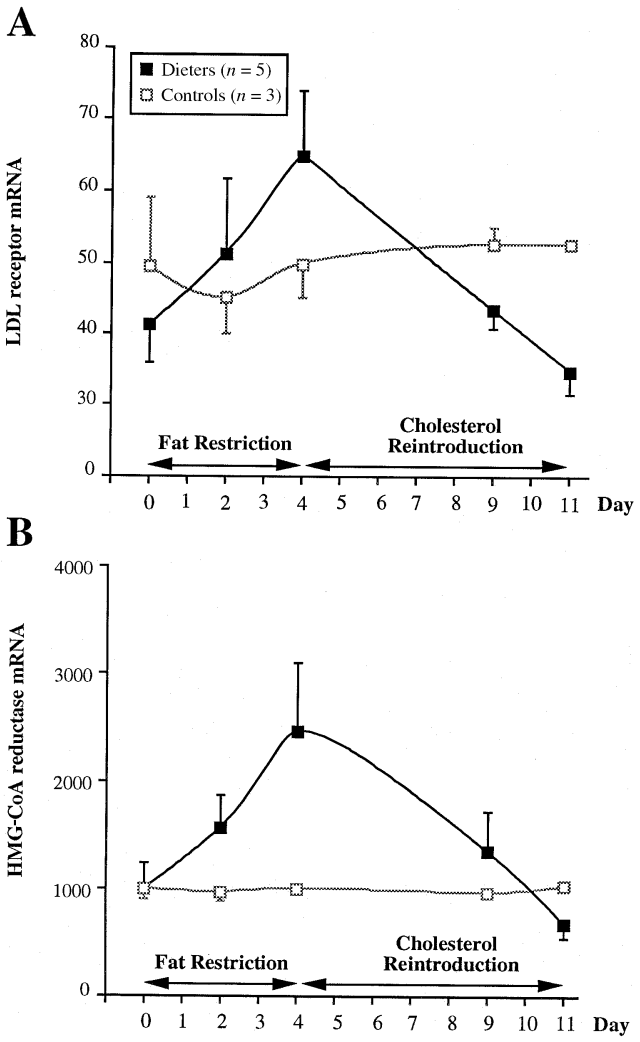

FIG. 1. Low density lipoprotein (LDL) receptor (A) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (B) mRNA measurements (means ± SD) at baseline (day 0) and during fat restriction (from day 0 to day 4 in the morning) and after reintroducing cholesterol (from day 4 at lunch to day 11 in the morning) in five dieters compared with three controls who did not change their diet during the same periods. The between-group differences in the changes (from day 0 to day 4 and from day 4 to day 11) were statistically significant ($P < 0.05$) for both genes.

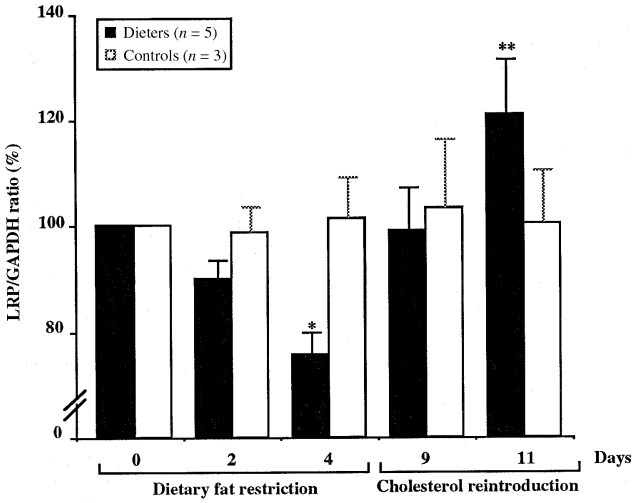

FIG. 2. Effect of the two-step dietary modifications on LDL-receptor-related protein (LRP) mRNA levels in mononuclear cells of five dieters and three control subjects. Evaluations were performed during a 4-d dietary fat restriction (from day 0 to day 4 in the morning with blood samplings at day 2 and day 4 in the morning) and during the cholesterol reintroduction period (from day 4 at lunch, after the day 4 blood sampling, to day 11 with blood samplings at day 9 and day 11). The levels of LRP mRNA at day 2, 4, 9, and 11 are expressed in percentage of the day 0 levels which are taken as 100%. Results are given as means ± SD. $^*P < 0.05$ vs. day 0 and $^{**}P < 0.005$ vs. day 4. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. See Figure 1 for other abbreviation.

was significant ($r = 0.69$, $r^2 = 0.48$, $P = 0.05$).

Figure 4 shows the relationship between the changes in LDL receptor and HMG-CoA reductase mRNA levels during cholesterol reintroduction (between days 4 and 11). These data are consistent with a coordinate regulation of LDL receptor and HMG-CoA reductase genes ($r = 0.79$, $r^2 = 0.62$, $P = 0.02$). The relationship between LDL receptor and LRP genes ($r = 0.70$, $r^2 = 0.49$, $P = 0.05$) is less significant, whereas it is not significant at all between HMG-CoA reductase and LRP ($r^2 = 0.17$, $P = 0.30$).

We also analyzed the relationship between blood lipids and mRNA level changes. We found no statistically significant correlation.

We finally examined the relationship between the changes in dietary cholesterol and the changes in mRNA. The strongest correlations (Fig. 5) were found between dietary cholesterol and LRP during both cholesterol reintroduction ($r = 0.81$, $r^2 = 0.65$, $P < 0.01$) and total fat deprivation ($r = 0.86$, $r^2 = 0.74$, $P < 0.005$), indicating that about 70% of the changes in LRP mRNA may be explained by the variations in dietary cholesterol. Regarding the relationship between dietary cholesterol changes and changes in LDL receptor and

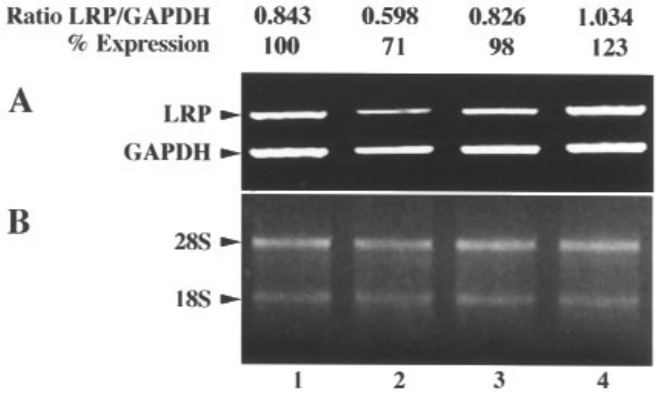

FIG. 3. Detection of LRP transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) in a representative subject dieter. A, RT-PCR was performed from 50 ng of total RNA, using GAPDH as an internal control. An aliquot of 15 µL of the reaction was analyzed on 2% agarose gel with: day 0 in lane 1, day 4 in lane 2, day 9 in lane 3, and day 11 in lane 4. B, 50 ng of total RNA extract was electrophoresed in 1% agarose gel at day 0 in lane 1, day 4 in lane 2, day 9 in lane 3, and day 11 in lane 4. Ethidium bromide staining was performed to confirm clear undegraded 18S and 28S bands and that the same amount of RNA was applied to each lane. See Figure 2 for other abbreviations.

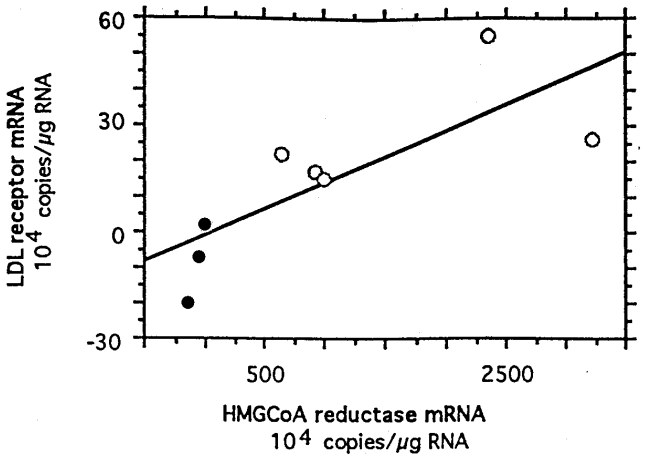

FIG. 4. Relationships between the changes (the differences between the values measured at day 4 and day 11) in LDL receptor and HMGCoA reductase mRNA levels ($r^2 = 0.62$, $P = 0.02$) during cholesterol supplementation, dieters (○), controls (●). See Figure 1 for abbreviations.

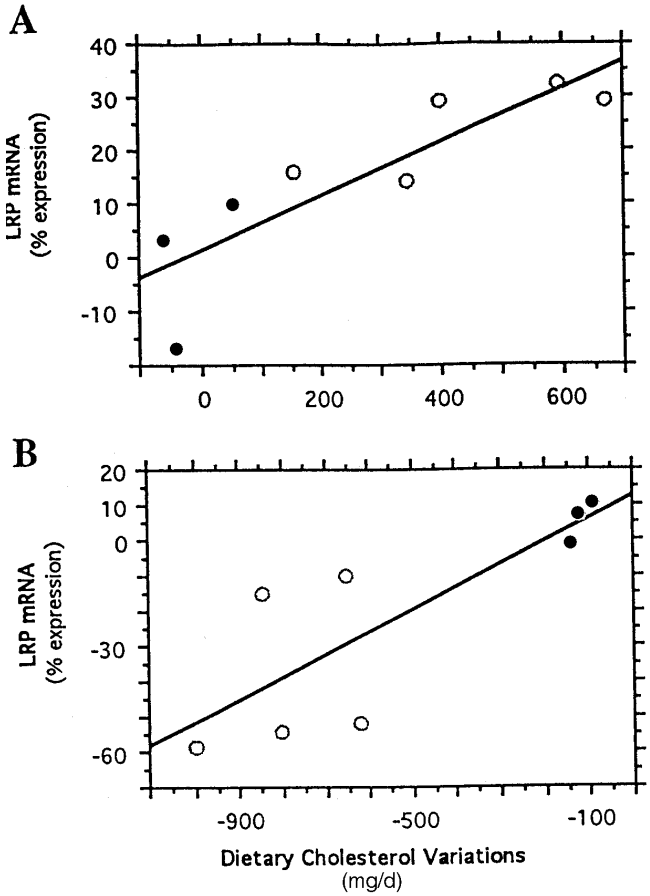

FIG. 5. Relationships between dietary cholesterol variations (the differences between the values measured at two different days) and changes in LRP mRNA levels during fat restriction, from day 0 to day 4 (A, $r^2 = 0.74$, $P < 0.005$), and during cholesterol supplementation, from day 4 to day 11 (B, $r^2 = 0.65$, $P < 0.01$). Dieters (○), controls (●). See Figure 2 for abbreviation.

HMG-CoA reductase mRNA levels, nonsignificant trends were observed during either fat restriction or cholesterol reintroduction.

DISCUSSION

Under physiological conditions, dietary lipids are incorporated into chylomicrons in the intestinal mucosa during their digestive absorption and are then processed by lipoprotein lipase in the peripheral organs. The resulting chylomicron remnants, enriched in dietary cholesterol, are rapidly cleared from the plasma, being taken up by the liver and other tissues (7). The LDL receptor is involved in the metabolism of cholesterol and the control of blood cholesterol not only by mediating the uptake of LDL from plasma into cells (8) but also by participating in the clearance of chylomicron remnants from the circulation (30–32). Another major and distinct receptor that binds apoprotein E, the LRP, is also involved in the clearance of chylomicron remnants and perhaps of LDL (13–15), whereas little is known about the factors which regulate its synthesis.

Although blood cholesterol is influenced by the amount and type of dietary fats, the response to dietary cholesterol varies widely among species and among individuals of the same species (5,6). For instance, 0.5% cholesterol feeding in the rabbit or the hamster results in a considerable increase in blood cholesterol (9,33), whereas in most healthy humans comparable high cholesterol intake in the absence of saturated fats has little effect and sometimes no effect at all (5,6). Low cholesterol absorption, high conversion to, and excretion of, bile acid (5), feedback inhibition of endogenous cholesterol synthesis, and reduced rate of the production of LDL (8,9) may explain the human resistance to dietary cholesterol. Another unexamined possibility is that the sterol-mediated feedback repression of LDL receptor gene transcription, the main mechanism regulating cellular cholesterol homeostasis and thought to aid in the control of blood cholesterol (7,8), is not operating *in vivo* in humans, possibly because of a lack of significant absorption of cholesterol. To investigate that point, we characterized, at the mRNA level, the response of circulating mononuclear cells known to reflect liver lipid metabolism in humans (17,18). For that purpose, we designed a dietary protocol in which the first period served to place the subjects in a "fat-deprived state" and the second period served to actually test the effect of reintroducing cholesterol (and only cholesterol) in the diet on the expression of the genes.

Summary of the results and interpretation. After 4 d of fat deprivation, blood cholesterol decreased significantly in association with an augmented expression of the LDL receptor gene. In contrast, after reintroducing cholesterol, serum total and LDL cholesterol did not change, whereas LDL receptor and HMG-CoA reductase mRNA levels decreased significantly, consistent with the current theory that in response to increased cholesterol availability, cells coordinately suppress endogenous cholesterol synthesis and repress the synthesis of LDL receptors. In other words, what is true for the isolated cultured cell is also true for circulating cells known to reflect

liver lipid metabolism in humans. On the other hand, these data cannot explain why the dieters were resistant to high cholesterol intake. The fact that the two genes were repressed indicates that a sufficient amount of dietary cholesterol was actually absorbed. Thus, low absorption of cholesterol cannot explain the resistance to dietary cholesterol in this study (5,6). While comparing the degree of gene repression in this study with those reported in animal species either resistant (rat, mouse) or sensitive (rabbit, hamster) to dietary cholesterol, we observed similar trends. For instance, in the mouse, Rudling (34) reported 43 and 66% reduction for hepatic LDL receptor and HMG-CoA reductase mRNA, whereas in the hamster Horton *et al.* (35) showed 33 and 55% reduction of hepatic LDL receptor mRNA in response to 0.06 and 0.12% cholesterol diets. Finally, in the cebus monkey, which is also very sensitive, Hennessy *et al.* (36) reported variations of less than 30% of hepatic LDL receptor mRNA.

Thus, despite major differences among species in the blood cholesterol responses to dietary cholesterol, roughly parallel gene suppressions were observed in both sensitive and nonsensitive species. This strongly suggests that neither low cholesterol absorption nor LDL receptor and HMG-CoA reductase gene repression can explain the human (or mouse) resistance to dietary cholesterol *in vivo*.

Which alternative explanations could be proposed? First, it is possible that increased cholesterol intake may induce increased conversion of cholesterol to bile acids through increased expression of the hepatic cholesterol 7-α-hydroxylase. Certain cholesterol-resistant animal species actually differ from the sensitive ones with respect to their ability to upregulate expression of hepatic 7-α-hydroxylase at both mRNA and protein levels in response to increasing levels of the substrate (34,37). In the hamster, transient overexpression of the enzyme was reported to reduce blood cholesterol levels (38). In the present study, however, we did not observe (in the few measurements we performed) significant changes in blood concentrations of total bile acids (data not shown), suggesting that recirculation of bile acids, and probably excretion, were not greatly modified. This is actually not surprising as the increase in 7-α-hydroxylase expression was shown to be accompanied by a parallel increase in HMG-CoA reductase gene expression (38). It was not the case in the present study where HMG-CoA reductase gene expression was actually reduced after cholesterol reintroduction. However, we cannot exclude the possibility that the excretion of bile acids was not augmented in these subjects, and further studies (requiring repetitive biliary catheterism) are warranted to clarify that point.

Another possibility is that, in parallel with a reduced uptake of LDL by hepatic LDL receptors, LDL production rate was reduced in our subjects, resulting in an absence of significant effect on blood LDL levels. This is, however, quite unlikely as in most studies in which LDL production rate and uptake were studied, the two parameters were shown to change in a reciprocal manner. For instance, when LDL receptor activity is reduced (by either genetic or environmental factors), LDL production invariably increases (39–41). One major reason for this reciprocal relationship is the specificity of the clearance process of LDL in the liver (42). When LDL receptor activity is high, a large proportion of VLDL remnants is taken up by the receptors and not converted to LDL, whereas when LDL receptor activity is low, VLDL remnants are metabolized to LDL and, consequently, LDL production rate increases (42).

Another type of regulation is thus probably operating to explain the human resistance to dietary cholesterol, possibly involving another form of LDL clearance that does not depend upon the LDL receptor. This LDL receptor-independent component of LDL clearance was identified in every species that was examined and may account for almost 50% of LDL clearance in humans (43). Whether this process involves LRP is currently unknown but may represent a realistic hypothesis given our present data on LRP regulation discussed below.

Methodological issues. Once the experimental use of pure cholesterol is excluded [as its effects were shown to be different from those observed when cholesterol is associated with substantial amounts of oil or fat as in natural foods (44)], an additional difficulty lies in the control for the possible dietary confoundings, chiefly fatty acids, that may influence cholesterol metabolism (45,46). Supplementation by egg yolk was used in many studies because the amount of fatty acids present in that aliment is considered negligible. Nonetheless, for a given amount of dietary cholesterol, other dietary modifications (consumption of meat, fish, butter, edible oils, etc.) induced by egg consumption may result in different, and maybe opposing, effects on blood cholesterol in different subjects. For instance, in one of the few studies investigating the effect of dietary cholesterol on LDL receptor and HMG-CoA reductase activities in blood mononuclear cells in man, Mistry *et al*. (47) reported a wide range of responses after daily addition of 1500 mg of cholesterol (which is quite considerable) in the diet in the form of eggs. Unfortunately, the authors did not control for the other lipid components of the diet, preventing a clear interpretation of the data.

To control for such confoundings in the present study, subjects were submitted to a period of total fat restriction prior to the period of cholesterol supplementation and, in the second period, only the effect of dietary cholesterol was tested. In fact, upon reintroducing egg yolk, a small amount of fatty acids is also reintroduced, as our evaluation indicated a mean intake of total fats of 2.6% of total energy during fat restriction and 10.8% when consuming eggs. This difference was however small and did not result in significant changes in plasma fatty acids after reintroducing cholesterol (Table 2).

It should be pointed out that the primary aim of this study was to investigate in humans the possibility that mechanisms of transcription regulation considered as basic for the survival of cells were also operating *in vivo*. We did not intend to study general metabolic regulations. Thus, post-transcriptional regulation and protein measurements were not within the scope of the present study. Nevertheless, notably, the pathways under study are essentially (but not exclusively) regulated at the transcriptional level (7,8,10,11,17,18,34–36,48–50) and,

in most studies, changes in LDL receptor mRNA correlate well with the changes in the corresponding protein mass or activity and also the relative rate of transcription (35,49,50), indicating that mRNA changes usually reflect fairly well metabolic regulations.

When comparing the copy numbers of LDL receptor and HMG-CoA reductase measured in this study with those reported by Powell and Kroon (17), who used very similar methodologies and the same synthetic internal standard in the RT-PCR, surprisingly, the data differ dramatically. The most plausible explanation is that the two populations under study are very different. Our subjects were young (mean age 35 yr), healthy males. Powell's subjects were quite old (mean age 53 yr), sick (all underwent major surgery for liver disease), and 70% were female. Despite the high percentage of women, their mean weight was 76 kg (against 69 kg in our study), and blood lipids (for instance, mean cholesterol was >6 mmol/L) were very different than those of our subjects (mean cholesterol <5 mmol/L). Thus, lipid metabolism in the two populations was very different, and not surprisingly to see differences existed in the expression of genes implicated in cholesterol homeostasis.

Coordinate gene regulation and the response of the LRP gene. The basic mechanisms regulating cellular cholesterol content, the feedback repression of genes governing the synthesis and uptake of cholesterol, are thus operating *in vivo* in humans. However, as expected from many previous data (summarized in Ref. 51), blood lipids were not modified by increasing cholesterol intake, suggesting that this regulation by itself does not play a predominant role in the control of blood lipids in healthy humans. These results are consistent with those reported in animal species known to be resistant to high cholesterol intake (34).

This study also documents that, in response to a dietary cholesterol challenge, the HMG-CoA reductase and LDL receptor genes are coordinated. This is in agreement with previous animal or human nonnutritional studies (17,34), whereas, to our knowledge, this is the first report showing in humans such a coordinated gene response to a dietary manipulation. We observed that after cholesterol reintroduction, the two genes were similarly downregulated, up to 62% of the variability of one gene being related to the variability of the other. Given the *in vivo* situation, the shortness of the study and the small sample size, this correlation is actually strong.

Yet, we found significant inverse correlations between the variations of LDL receptor and LRP mRNA levels and also between dietary cholesterol and LRP mRNA, demonstrating that LRP mRNA changes likely resulted from the dietary changes.

A 59% increase in LRP mRNA levels was observed after reintroducing cholesterol in the diet, demonstrating that the LRP gene is actually sensitive to dietary cholesterol. Although specific mechanisms of LRP gene regulation were not addressed in this study, these data raise questions as to whether LRP may be responsible, at least partly, for the lack of increase of serum cholesterol levels in response to high dietary cholesterol in humans.

LRP is a multifunctional and multiligand receptor expressed in a large variety of cells and tissues (52–54). In addition to its role in the cellular uptake of the remnant lipoproteins, LRP mediates the cellular internalization and catabolism of a number of molecules and molecular complexes potentially involved in inflammation (55), thrombogenesis (56,57) thrombolysis (58,59), and lipid metabolism (60,61). Therefore it may contribute to the formation of the atherosclerotic fatty streak lesion (54,62–64).

Recently it was established in the rat that LRP and the mannose receptor are the sole major receptors responsible for the clearance of tissue plasminogen activator, a serine protease that plays a central role in the fibrinolytic system (65). Speculatively, given its several possible interactions in the development of CHD, LRP may constitute a link between dietary cholesterol and CHD independent of blood cholesterol level, one mystery in the epidemiology of CHD (3,4).

Regarding the mechanism(s) by which dietary cholesterol increases LRP mRNA levels, notably the promoter of the LRP gene was reported either to contain (66) or not contain (52) a sterol regulatory element. The present data indicate an upregulation of LRP in response to cholesterol, which is in agreement with a previous study showing that cholesterol feeding enhances the expression of the LRP gene in the aorta of the rabbit (63). Also, Willnow *et al.* (14) showed in mice submitted to transient inactivation of LRP *in vivo* that not only chylomicron remnants but also LDL (although to a lesser degree) accumulated in the plasma, suggesting that LRP participates in the clearance of LDL and may contribute to the control of blood LDL level. Thus, theoretically, increased synthesis of LRP during high cholesterol intake is an adequate physiological response to ensure efficient clearance of chylomicron and VLDL remnants and also of LDL. Although these mechanisms are quite speculative, upregulation of LRP gene expression in response to high dietary cholesterol may be interpreted as either a protection against blood cholesterol increase or as a contribution to the accumulation of lipids within cells, in particular, vascular cells. Also, LRP may influence the occurrence of acute thrombotic complications by altering the anticoagulant properties of the vessel wall as this receptor mediates degradation of molecular complexes involved in thrombogenesis and fibrinolysis (56–59,65). Further basic and clinical studies are urgently warranted to examine whether LRP may be, in terms of CHD, a friend or a foe.

ACKNOWLEDGMENTS

This work was supported by grants from the Fondation de France, Hospices Civils de Lyon, Fédération Française de Cardiologie and Fondation pour la Recherche Médicale.

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[Received March 19, 1998, and in final revised form August 17, 1998; revision accepted October 29, 1998]

Effect of Diet on the Fatty Acid and Molecular Species Composition of Dog Retina Phospholipids

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ABSTRACT: Dogs were born to mothers fed commercial diets low or enriched in n-3 fatty acids and raised on those diets until they were about 50 d old. Retinas were removed, lipids were extracted, and total phospholipids were analyzed for fatty acid and molecular species composition. Animals from the low n-3 group had significantly lower retinal levels of 22:6n-3 and higher levels of n-6 fatty acids, especially 20:4n-6 and 22:5n-6. There was no difference in the retinal levels of 18:2n-6, and only small differences were found in saturated and monounsaturated fatty acids. The most dramatic differences in molecular species occurred in 22:6n-3–22:6n-3 (4.7 vs. 0.8%) and 18:0-22:6n-3 (27.6 vs. 14.4%); total molecular species containing 22:6n-3 were significantly lower in the low n-3 group (45.5 vs. 24.0%). Molecular species containing 20:4n-6 and 22:5n-6 were greater in the low n-3 animals (13.0 vs. 25.7%), as were molecular species containing only saturated and monounsaturated fatty acids (40.8 vs. 35.4%). These results show that modest differences in the amount of n-3 fatty acids in the diets of dogs can have profound effects on the fatty acid and molecular species composition of their retinas.
Lipids 33, 1187–1193 (1998).

Neural tissues, including the retina, can take up small amounts of n-3 fatty acids present in the circulation and conserve them during n-3 deficiency (1–4). In the retina, 22:6n-3, the most abundant n-3 fatty acid in neural tissues (5), is conserved by recycling from the retinal pigment epithelium (6–9). This pathway, called the "short loop" by Bazan *et al.* (10), is very effective in maintaining high retinal levels of n-3 fatty acids for glycerolipid synthesis. In the vertebrate retina, the distal 10% of rod outer segment (ROS) discs is shed daily and phagocytized by the retinal pigment epithelium (11). Without an efficient "short loop," the retina could easily become depleted of n-3 fatty acids during dietary n-3 deficiency.

Prolonged deprivation of n-3 fatty acids in adult or weanling rats leads to a reduction in retinal n-3 fatty acids, but the changes are relatively small and occur over a long period of time (1–4). However, if the dietary restrictions are begun during pregnancy and continued through the nursing period and thereafter, greater changes can be effected, as observed in rats (12) and monkeys (13). In the retina (1–4, 12,13) and brain (14–16), changes in n-3 fatty acids, primarily in 22:6n-3, are balanced by an increase in 22:5n-6. This occurs even in animals denied both n-3 and n-6 fatty acids, probably owing to the larger stores of 18:2n-6 than of 18:3n-3 in adipose tissue (3).

Our laboratories have studied inherited retinal degenerations in miniature poodles for a number of years and have reported that animals with progressive rod-cone degeneration (*prcd*), which is genetically inherited, have lower plasma (17) and ROS (18) levels of 22:6n-3 than unaffected dogs. In these studies, dog food was routinely analyzed to determine dietary levels of both n-3 and n-6 fatty acids. Recent analyses have revealed that the levels of polyunsaturated fatty acids (PUFA), particularly the n-3 family, are now higher in commercial dog food compared to feed analyzed several years ago. To determine if this increase in dietary n-3 fatty acids has any effect on plasma and retinal levels of PUFA we analyzed the plasma and retinal fatty acid and molecular species compositions of total phospholipids from unaffected dogs that had been born and raised in our dog colony and fed dog food low or enriched in n-3 fatty acids.

MATERIALS AND METHODS

Animals and diets. The unaffected dogs used in this study were made available as part of an National Eye Institute–National Institutes of Health-sponsored project (EY06855, "Canine Models for Hereditary Retinal Degenerations"). The low n-3 group had 5 females and 1 male, and the high n-3 had 6 males; there was no difference between lipid analyses in male and female animals. The study was done in accordance with the guidelines of the Association for Research in Vision and Ophthalmology and followed protocols approved by the animal protocol review committee of Cornell University and the University of Oklahoma Health Sciences Center. All dogs

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Abbreviations: DGBZ, diacylglyceroben Z Dates; PUFA, polyunsaturated fatty acid; ROS, rod outer segment; TLC, thin-layer chromatography.

TABLE 1
Fatty Acid Composition of Diets

	Low n-3 diets[a]			High n-3 diets		
Fatty acid	Adult chow	Pup chow	Canned food	Adult chow	Pup chow	Canned food
14:0	n.d.	3.8	5.3	1.2	1.2	2.1
15:0	n.d.	0.5	0.6	0.1	0.1	0.4
16:0	24.6	23.3	29.6	25.8	25.1	25.9
18:0	9.9	12.9	15.3	5.6	7.2	14.5
20:0	n.d.	n.d.	n.d.	0.1	0.2	0.1
Total saturate	34.5	40.5	50.8	32.8	33.8	43.0
16:1n-9 + 16:1n-7	n.d.	2.3	4.2	7.4	3.8	4.2
18:1n-9 + 18:1n-9	38.4	36.6	39.7	38.1	40.6	38.2
20:1n-9	n.d.	n.d.	n.d.	0.3	0.5	0.5
Total monoenoic	38.4	38.9	43.9	45.8	44.9	42.9
18:2n-6	25.5	19.6	4.6	17.8	19.6	11.6
18:3n-6	0.4	0.2	n.d.	0.2	0.1	0.1
20:3n-6	n.d.	0.1	0.1	0.2	0.1	0.3
20:4n-6	0.3	0.1	0.3	0.5	0.5	1.0
22:4n-6	0.1	n.d.	n.d.	0.1	0.1	0.1
22:5n-6	0.1	n.d.	n.d.	0.1	0.1	0.1
Total n-6	26.4	20.0	5.0	18.9	20.5	13.2
18:3 n-3	n.d.	0.9	0.5	1.3	0.8	0.6
22:5 n-3	0.1	n.d.	0.1	0.6	0.1	0.2
22:6 n-3	0.2	n.d.	0.1	0.5	0.1	0.3
Total n-3	0.3	0.9	0.7	2.4	1.0	1.1

[a]From Aguirre *et al.* (18). n.d. = not detectable. Only identified fatty acids are listed in the table.

were maintained in the same animal care facilities (Retinal Disease Studies Facility, Kennet Square, PA), in indoor runs with controlled cyclic illumination (12 h on–12 h off). In the low n-3 groups, adult dogs were fed Purina Lab chow (Ralston Purina, St. Louis, MO) and pups were fed Purina Puppy chow plus canned dog food (Hi Tor beef, Triumph Pet Industries, Warnick, NY). Adult dogs receiving the diets enriched in n-3 fatty acids were fed Eukanuba Premium Performance Adult Formula (IAMS Company, Dayton, OH) and pups received IAMS Puppy chow plus canned dog food (Beef Formula for Dogs, IAMS). The mothers of dogs were fed dry food *ad libitum*. One week prior to whelping, and continuing until pups were weaned, they received canned food to supplement dry food. For this period, in terms of weight ratio, canned food constituted about 25% of the diet. Mothers were always on the respective diets. The weaning age for the pups was 6–7 wk postnatal. The fatty acid compositions of the low n-3 diets, published previously (18), are presented in Table 1, along with the compositions of diets enriched in n-3 fatty acids. Dogs were killed between 47–66 d of age, and retinas were taken and kept frozen at –80°C.

Lipid extraction. The retinas were homogenized in 1 mL Tris-HCl 150 mM (pH 7.4) and centrifuged at 1000 × *g* for 15 min at 4°C. The supernatant was analyzed for protein (BCA reagents; Pierce, Rockford, IL), and aliquots containing 1 mg protein were taken in duplicate for lipid analysis. Retina and plasma samples were extracted with chloroform/methanol by the procedure of Bligh and Dyer (19) and separated by thin-layer chromatography (TLC) using the solvent system hexane/diethyl ether/acetic acid (70:30:1, by vol). Phospholipids were extracted from the silica gel according the procedure of Arvidson (20) and used for methyl ester or molecular species analysis. Diets were extracted by the method of Bligh and Dyer (19), and an aliquot was taken for total lipid analysis.

Fatty acid analysis. Total phospholipids and lipids were treated with 14% boron trifluoride-methanol (21), and the resulting fatty acid methyl esters were analyzed by gas–liquid chromatography using a DB-225 capillary column with helium as a carrier gas (22), and identified by comparison with appropriate standards.

Molecular species analysis. Total phospholipids were converted to their respective diradylglycerols with phospholipase C (23). Diradylglycerols were derivatized with benzoic anhydride (24) and resolved into diacylglycerobenzoates (DGBZ) and ether-linked glycerobenzoates by TLC on silica gel using a solvent system of toluene/hexane/diethyl ether (50:45:5, by vol). The DGBZ were visualized by spraying with 0.05% 2′,7′-dichlorofluorescein in 75% aqueous methanol, and the silica gel was scraped into 2 mL of ethanol and mixed vigorously. Two milliliters of water was added, and the suspension was extracted three times with 2 mL of hexane. The pooled hexane extracts were dried under nitrogen and redissolved in acetonitrile/isopropanol (80:20, vol/vol). The DGBZ were fractionated into molecular species by high-performance liquid chromatography (HPLC) on a Supelcosil LC-18 column (5 μm, 25 cm × 4.6 mm i.d.; Supelco, Bellefonte, PA) using acetonitrile/isopropanol (80:20, vol/vol) at a flow rate of 1.0 mL/min. Lipids were detected at 230 nm and identified by comparison with standard (25). The identities of the peaks of interest were confirmed by gas–liquid chromatography analysis of the fatty acid methyl esters.

TABLE 2
Effect of Diet on the Fatty Acid Composition of Phospholipids in Normal Dog Retina and Plasma

	Retina[a]		Plasma[a]	
Fatty acid	Low n-3 (n = 6)	High n-3 (n = 9)	Low n-3 (n = 5)	High n-3 (n = 6)
16:0 DMA	1.69 ± 0.20	1.62 ± 0.28	n.d.	n.d.
18:0 DMA	2.75 ± 0.42	3.27 ± 0.43[a]	n.d.	n.d.
16:0	25.62 ± 0.77	25.59 ± 1.61	18.63 ± 1.87	16.01 ± 0.79
18:0	20.17 ± 1.67	23.05 ± 1.83[a]	27.79 ± 1.21	31.47 ± 0.95[a]
20:0	0.28 ± 0.06	0.32 ± 0.05	n.d..	n.d.
Total saturate	50.45 ± 2.55	53.81 ± 2.90[a]	46.42 ± 1.61	47.48 ± 1.59
16:1n-9	0.88 ± 0.30	0.50 ± 0.11[a]	1.02 ± 0.17	0.35 ± 0.05[c]
16:1n-7	0.52 ± 0.13	0.35 ± 0.09	n.d.	n.d.
18:1n-9	8.58 ± 0.54	7.32 ± 0.43[c]	6.70 ± 0.78	5.37 ± 0.97
18:1n-7	3.32 ± 0.24	3.26 ± 0.19	2.46 ± 0.18	2.24 ± 0.15
20:1n-9	0.17 ± 0.04	0.14 ± 0.03	n.d.	n.d.
Total monoenoic	13.45 ± 1.05	11.48 ± 0.69[c]	10.17 ± 1.00	7.96 ± 0.89[a]
18:2n-6	1.21 ± 0.29	1.00 ± 0.11	14.84 ± 2.23	7.73 ± 1.15[c]
18:3n-6	n.d.	n.d.	n.d.	0.02 ± 0.01
20:3n-6	0.57 ± 0.16	0.79 ± 0.52	0.95 ± 0.16	0.94 ± 0.09
20:4n-6	8.92 ± 0.72	5.85 ± 0.39[c]	22.78 ± 2.27	27.98 ± 1.77[c]
22:4n-6	2.32 ± 0.07	1.52 ± 0.18[c]	1.17 ± 0.44	1.62 ± 0.39
22:5n-6	7.71 ± 1.00	1.93 ± 0.42[c]	0.22 ± 0.08	1.08 ± 0.36[c]
Total n-6	20.73 ± 1.55	11.10 ± 0.81[c]	39.95 ± 1.97	39.38 ± 1.57
18:3n-3	n.d.	n.d.	0.18 ± 0.02	0.14 ± 0.05
20:5n-3	n.d.	0.15 ± 0.04	0.36 ± 0.16	0.38 ± 0.54
22:5n-3	0.35 ± 0.10	0.57 ± 0.13[b]	1.92 ± 0.39	1.49 ± 0.39
22:6n-3	15.08 ± 1.65	23.00 ± 2.60[c]	1.07 ± 0.08	3.41 ± 0.80[c]
Total n-3	15.37 ± 1.64	23.61 ± 2.67[c]	3.45 ± 0.48	5.41 ± 1.16[c]
n-3/n-6	0.75	2.15	0.086	0.137

[a]Values are mean mol% ± SD of n retinas analyzed in duplicate; plasma values are from single analyses. The percentage distribution was calculated on the sum of identified fatty acids listed on the tables. n.d., not detected. Significant differences between the diets: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$.

RESULTS

Fatty acid composition of dog food. The fatty acid compositions of several types of commercial dog food fed over the past 8 yr at the Retinal Disease Studies Facility are given in Table 1. Since 1995, there has been an increase in the level of n-3 fatty acids in the food, due to the inclusion of fish products**.** This is especially evidenced by higher contents of 18:3n-3 and 22:6n-3 in the high n-3 lab chow diet, the average of total n-3 fatty acids (from lab chow, puppy plus canned food) being twice more in the high n-3 than in the low n-3 diets.

Effect of diet on the fatty acid composition of plasma phospholipids. Table 2 contains the fatty acid composition of total phospholipids from plasma of dogs fed commercial diets low and high in n-3 fatty acids. Several interesting differences are noteworthy. The total n-3 and 22:6n-3 levels are higher in the high n-3 group, as expected. However, the 22:5n-3 level was higher in the low n-3 group (but the difference is not significant by *t*-test). The total n-6 fatty acid levels were the same, although the low n-3 group had higher 18:2n-6 and 22:5n-6, and lower 20:4n-6 than the high n-3 group.

Effect of diet on the fatty acid composition of retinal phospholipids. Fatty acid compositions of total phospholipids from retina of each diet group are presented in Table 2. The major PUFA in both groups is 22:6n-3, which is significantly lower in the low n-3 group; the level of 22:5n-3 is also lower in this group. These lower levels of n-3 fatty acids are balanced by significantly higher proportions of 22:5n-6, 20:4n-6, and 22:4n-6. Overall, when dogs were fed the low n-3 diet, there was a reciprocal replacement of n-3 by n-6 fatty acids in the retina, especially evidenced in 22-carbon fatty acids. Interestingly, the percentage of 22-carbon PUFA remained remarkably similar in the two groups (25.5% in low n-3, and 27.0% in high n-3). However, the n-3/n-6 ratio was threefold higher in the high n-3 group. Apart from the differences in PUFA levels, only minor differences were noted between groups in the levels of saturated and monoenoic fatty acids.

Effect of diet on the molecular species composition of retinal phospholipids. The molecular species of retinal phospholipids from the two groups are shown in the representative high-performance liquid chromatographic tracings illustrated in Figure 1. The unidentified peaks between 22:6n-3–22:6n-3 and 18:1–22:6n-3 contain co-eluting molecular species composed of one 22:6n-3 and one 20-, 22-, or 24-carbon fatty acids having four, five, or six double bonds (23). The relative mole percentage of the identified molecular species are given in Table 3. Those molecular species not cleanly resolved are combined as "others." The data show that the fatty acid compositional changes are reflected in the molecular species compositions. The proportions of 22:6n-3-containing species are lower in the low n-3 group, and are compensated for by higher levels of 22:5n-6-containing species. The greatest effect is seen in 22:6n-3–22:6n-3, where the level is sixfold higher in the high n-3 group. The levels of the other 22:6n-3–containing

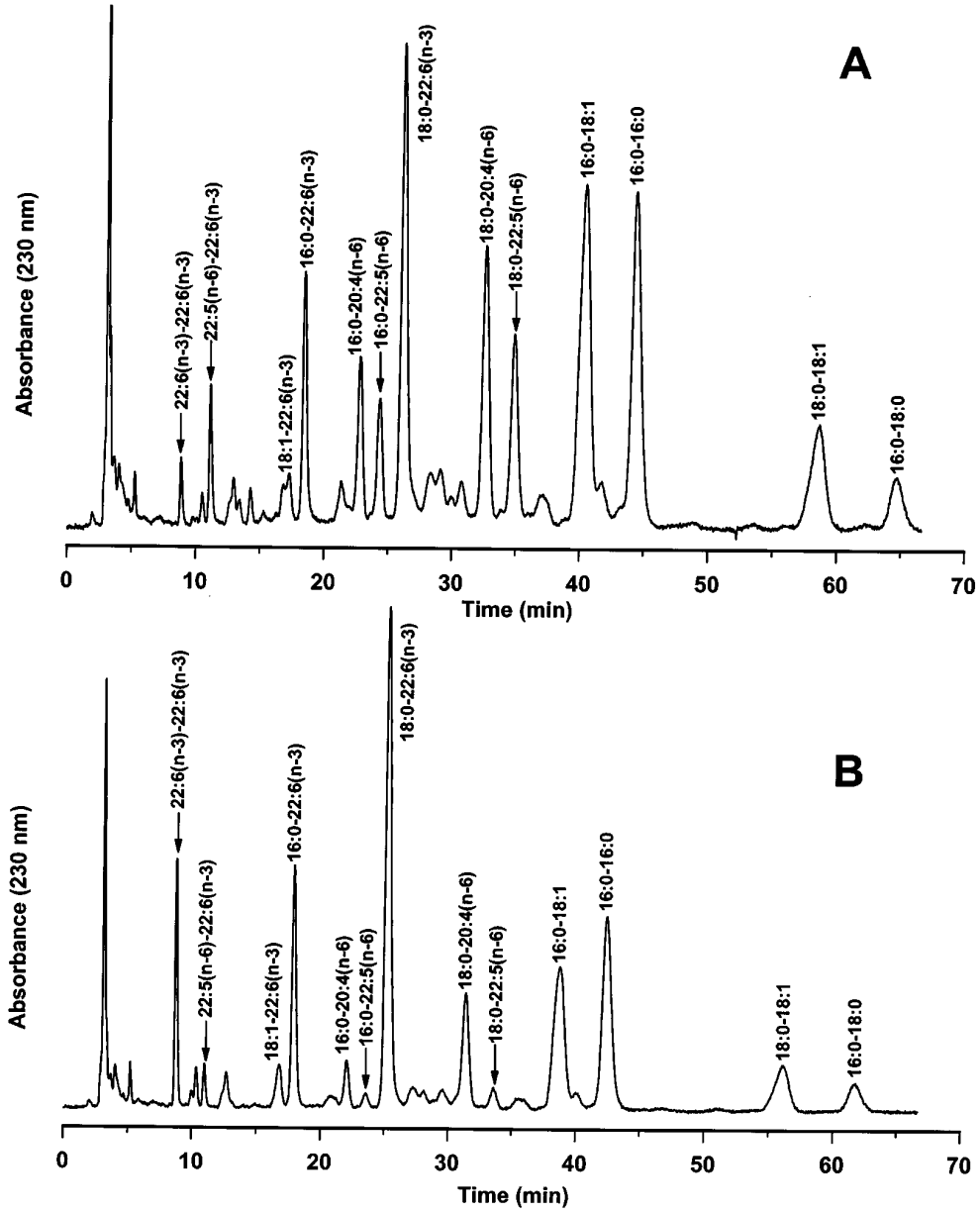

FIG. 1. Representative high-performance liquid chromatographic elution profiles of the molecular species of diacylglycerobenzoates derived from retinal phospholipids of dogs fed (A) low n-3 diet or (B) high n-3 diet. The separations were achieved on a C-18-phase column with acetonitrile/isopropanol (80:20, vol/vol) as solvent, and diacylglycerobenzoates were detected at 230 nm.

species are about two fold higher. The two major 22:5n-6-containing species, 16:0–22:5n-6 and 18:0–22:5n-6, are fourfold higher in the low n-3 group. The proportions of 20:4n-6-containing molecular species are also higher in the low n-3, although to a lesser extent. Only minor changes are seen in 18:1-containing species, while disaturated species are not affected by the diet.

DISCUSSION

PUFA of the n-3 family have been shown to be important in retinal function of experimental animals and human infants. Rats (26–29), guinea pigs (30,31), and monkeys (13,32) fed n-3 deficient diets undergo characteristic electroretinographic changes associated with decreased retinal ability to absorb light and generate a visual signal. Human infants, especially those born prematurely, show a slower development of their visual system if they do not receive n-3 fatty acids, especially 22:6n-3 (reviewed in Ref. 33). Therefore, maintenance of optimal visual function depends on a dietary source of n-3 fatty acids.

Dogs (17), cats (34), and humans (35–38) with inherited retinal degenerations have been shown to have lower plasma and red blood cell levels of PUFA, especially 22:6n-3, than

TABLE 3
Effect of Diet on the Molecular Species Composition of Phospholipids in Normal Dog Retina

Molecular species	Low n-3[a] (n = 6)	High n-3[a] (n = 9)
22:6n-3–22:6n-3	0.77 ± 0.25	4.73 ± 0.69
22:5n-6–22:6n-3	1.99 ± 0.35	1.40 ± 0.21
18:1–22:6n-3	1.31 ± 0.29	2.40 ± 0.16
16:0–22:6n-3	5.53 ± 0.30	9.35 ± 0.78
16:0–20:4n-6	4.10 ± 0.38	2.32 ± 0.22
16:0–22:5n-6	3.22 ± 0.27	0.92 ± 0.19
18:0–22:6n-3	14.37 ± 1.30	27.62 ± 1.61
18:0–20:4n-6	9.92 ± 0.91	6.56 ± 0.55
18:0–22:5n-6	6.54 ± 0.94	1.70 ± 0.54
16:0–18:1	16.70 ± 1.05	12.24 ± 0.64
16:0–16:0	13.78 ± 0.65	14.29 ± 1.61[ns]
18:0–18:1	7.31 ± 0.68	6.01 ± 0.35
16:0–18:0	3.03 ± 0.40	2.93 ± 0.45[ns]
Others	11.41 ± 0.71	7.53 ± 1.51
22:6n-3-containing species	23.98 ± 1.88	45.50 ± 1.51
22:5n-6-containing species	11.76 ± 1.42	4.01 ± 0.88

[a]Values are mean mol% ± SD of n retinas analyzed in duplicate. Others: sum of unidentified molecular species. Differences between diets are all significant ($P < 0.001$) except where *ns* (not significant) is indicated.

appropriate controls. Retinal photoreceptor membrane levels of 22:6n-3 are also reduced in affected dogs (18). We originally thought that the lower 22:6n-3 in the retina reflected a reduced synthetic capability (17); however, we subsequently demonstrated that the retina and retinal pigment epithelium from affected dogs could convert 22:5n-3 to 22:6n-3 as efficiently as tissues from normal animals (39). Since 22:6n-3 is the most abundant fatty acid in the retina, it has been suggested that the retinal degeneration may be causally related to the low 22:6n-3 levels (17,39,40). To address this question, we administered fish oil to affected and normal dogs for 5 mon and then measured retinal function and structure (18). Fish oil supplementation did not preserve either, but the retinal levels of 22:6n-3 were less in supplemented affected dogs compared to controls. Currently, we are considering two possibilities to explain the 22:6n-3 findings in affected dogs: (i) there is a defect in transport of 22:6n-3 from liver to target organ, and (ii) the mutation produces a metabolic stress on the animals that results in increased catabolism of 22:6n-3.

The results of the present study show that it is possible to alter the retinal fatty acid composition of dogs by feeding them diets with different levels of n-3 fatty acids. Surprisingly, profound effects were observed, especially a marked increase of 22:6n-3 content, with only small additional amounts of n-3 fatty acids to the diets. The magnitude of the changes was indeed higher that we had predicted from the diet composition. It thus appears imperative, in any dietary study where animals are fed commercial diets, that all diets be analyzed prior to use. To observe these changes in neural tissues at such a relatively young age was unexpected, and undoubtedly reflects the n-3 state of the mother. In studies with rats fed fat-free or n-3 deficient diets from weaning age (3 wk), only small changes in retinal membrane fatty acid compositions could be achieved (1–3). Similarly, only a small decrease of 22:6n-3 in retinal lipids was observed for neonatal piglets fed formula low in n-3 fatty acids (low 18:3n-3 and no 20C or 22C n-3) for 15 d after birth (41). However, if the dietary restrictions were started during pregnancy and continued through the nursing period, then much more profound changes in retinal fatty acids occurred (12). In the present study, the female dogs were fed diets containing the two levels of n-3 fatty acids prior to conception, and their blood levels of 22:6n-3, which were not determined, presumably were quite different. Therefore, the pups had access to different levels of 22:6n-3 *in utero* and during nursing, both periods of time when the most profound effect of dietary changes on retinal fatty acid composition can be achieved (12). Feeding neonatal piglets a formula enriched with only small amounts of 22:6n-3 for 25 d after birth resulted in an increase of 22:6n-3 in retinal lipids (42). Interestingly, the phosphatidylcholine fraction was more responsive to dietary supplementation than the phosphatidylethanolamine fraction (42). In another study comparing high α-linolenate formula or low α-linolenate plus fish oil formula to a low α-linolenate formula, and where retinal phosphatidylethanolamine was analyzed, only a minor increase of 22:6n-3 was observed (41).

The higher levels of n-3 fatty acids in the retina of animals fed the high n-3 diet are compensated for by lower levels of n-6 fatty acids. Such a compensatory effect between these two fatty acid series has been described in the retina of rats and monkeys fed diets specifically formulated to be low (1–4,12,13) or high (43) in n-3 fatty acids. However, this phenomenon has not been previously observed in dogs fed commercially available diets. As reported for the rat photoreceptor membranes (12), the variations in the fatty acid composition of dog retina were reflected in the molecular species. Indeed, the greatest differences were found for molecular species containing 22:6n-3 or 22:5n-6. Based upon the magnitude of the change of di-22:6n-3 species compared to 22:6n-3 levels, analyses of molecular species appear to be especially appropriate to detect some variations in fatty acid metabolism. Interestingly, the 22:6n-3–22:6n-3 species was the most increased, by sixfold, compared to a twofold increase (on a relative basis) of species containing 22:6n-3 and monounsaturated or saturated fatty acid. However, the increase of 18:0–22:6n-3 is also of interest as it has the most significant amplitude, resulting in a predominant proportion (about 30%) of 18:0–22:6n-3 over the other molecular species. This large increase may represent a large incorporation of 22:6n-3 in phosphatidylcholine as reported in the study by Craig-Schmidt *et al.* (42). The functional significance of the di-22:6n-3 molecular species is unknown, but it is noteworthy that, to our knowledge, it has only been described in the retina (44), and more recently in fish sperm phospholipids (45), another 22:6n-3 enriched tissue. Metabolic studies have shown that, in bovine ROS, the di-22:6n-3 molecular species selectively incorporates 22:6n-3 compared to other 22:6n-3-containing molecular species (46). In frog ROS, the same investigators have reported that the different 22:6n-3-containing molecular species show distinctive rates of biosynthesis and

turnover (25). In addition, these parameters vary for the same molecular species depending on the glycerophospholipid. These metabolic studies support a role for 22:6n-3-containing molecular species in visual function. Interestingly, in rat ROS, 22:6n-3-containing molecular species are selectively decreased during oxidative stress related to light (12).

In conclusion, we report here that the fatty acid and molecular species compositions of dog retina are very sensitive to the diet received by the mother during gestation and nursing, and then by the dog during the first months after birth. Whether such changes have any effect on retinal function of normal dogs or contribute to the progression of retinal degeneration in *prcd*-affected dogs remains to be determined.

ACKNOWLEDGMENTS

Supported in part by grants from NIH (EY00871, EY04149, EY01244, and EY06855); The Foundation Fighting Blindness; Research to Prevent Blindness, Inc.; Presbyterian Health Foundation (Oklahoma City, OK); and Samuel Roberts Noble Foundation (Ardmore, OK). REA is a Senior Scientific Investigator of Research to Prevent Blindness, Inc.

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[Received July 6, 1998, and in final revised form October 29, 1998; revision accepted October 30, 1998]

Regiospecific Analysis of Fractions of Bovine Milk Fat Triacylglycerols with the Same Partition Number

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ABSTRACT: Bovine milk fat was fractionated using preparative reversed-phase high-performance liquid chromatography. The conditions consisted of two successive linear gradients of acetonitrile and *tert*-butylmethylether, followed by a final isocratic mixture of the two eluants, leading to triacylglycerols grouped by their partition number (PN). Fractions corresponding to partition numbers 32 to 50 were isolated and analyzed for fatty acid distribution between *sn*-1,3 and *sn*-2 positions by Grignard degradation. Results showed that the fatty acid distribution in milk fat triacylglycerols is nonrandom. The distribution of short-chain fatty acids, stearic (predominantly at *sn*-1,3 position) and palmitic (predominantly *sn*-2 position), did not change with triacylglycerol size. Medium-chain fatty acids were predominantly located at *sn*-2 position, but their proportion at this position decreased with triacylglycerol size. Oleic acid distribution was also size-dependent in that it was located in high proportions at *sn*-2 position in smaller triacylglycerols and vice versa. Results also showed that the *sn*-2 position was more unsaturated than *sn*-1,3 position in the PN range from 32 to 40, but it was more saturated in triacylglycerols with higher PN.
Lipids 33, 1195–1201 (1998).

Stereospecific analyses have shown that the distribution of fatty acids in bovine milk fat triacylglycerols is nonrandom. In overall fatty acid composition, short-chain fatty acids are largely found in the *sn*-3 position, and unsaturated fatty acids tend to be in higher concentration in the *sn*-1 and *sn*-3 positions. Although many studies have been carried out to determine the positional distribution of fatty acids in milk fat, few studies have addressed the relationship between stereo- or regiospecific distribution of fatty acids and size or acyl carbon number (ACN) of triacylglycerols. The importance of this knowledge lies in its implication in both functional and nutritional properties of milk fat and its fractions. Kuksis and coworkers overcame the difficulties in the stereospecific analysis of milk fat, using pancreatic lipase because of its preference for short-chain triacylglycerols (1), by analyzing milk fat fractions which had been enriched in either short-chain triacylglycerols (ACN34 to 42) or long-chain triacylglycerols (ACN36 to 54) (2–5). Their results suggested that positional distribution of some fatty acids in bovine milk fat could vary with size or ACN of triacylglycerols.

In the present study, reversed-phase high-performance liquid chromatography (HPLC), which constitutes the most widely used method for triacylglycerol analysis, including identification (6–9), has been used to fractionate milk fat by partition number (PN = ACN – 2 × DB, where DB is the number of double bonds). A method for structural analysis of milk fat triacylglycerols was adapted from literature methods for vegetable oils and used a Grignard reagent to degrade triacylglycerols (10–12). Since milk fat triacylglycerols are composed of short-, medium-, and long-chain fatty acids, analysis presents certain difficulties at both the stages of hydrolysis and of separation of the degradation products (mono-, di-, and triacylglycerols). Milk fat fractions corresponding to PN 32 to 50 were obtained and analyzed for the regiospecific distribution of fatty acids between *sn*-1,3 and *sn*-2 positions.

MATERIALS AND METHODS

Bovine milk fat. The winter butterfat was a sample from Ault Food (Mitchell, Ontario, Canada). A 25-kg block was tempered and then divided into samples of 100 g, which were rapidly melted and washed with deionized water in an oven (60°C), dried over anhydrous sodium sulfate, filtered, and kept under nitrogen in closed vials, at –30°C, until utilization.

Fractionation of milk fat by HPLC. Fractionation of bovine milk fat was carried out by micro-scale preparative reversed-phase HPLC to yield enough material, in a limited number of runs, to proceed with regiospecific analysis of most fractions. Standard triacylglycerols covering an extensive range of ACN (i.e., ACN12 to 54) and degrees of unsaturation were eluted for determination of the temporal limits on PN, in order to fractionate milk fat triacylglycerols according to this parameter. HPLC conditions consisted of an elution in three steps—two linear gradients of a mixture of acetonitrile and methyl-*tert*-butyl ether (MTBE) followed by an isocratic period—that allowed an even separation of triacylglycerols grouped by PN (Angers, P., F. Cho, R. Lencki, A. Bondreau, and J. Arul., 1998, submitted for publication). This technique was used to fractionate milk fat triacylglycerols by PN, and

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Abbreviations: ACN, acyl carbon number; AN, acetonitrile; HPLC, high-performance liquid chromatography; MTBE, methyl-*tert*-butyl ether; PN, partition number; TLC, thin-layer chromatography; UV, ultraviolet.

was carried out on a Hewlett-Packard liquid chromatograph (Model HP-1050; Palo Alto, CA), equipped with an ultraviolet (UV) detector (220 nm) and a 60 mm × 10 mm Spherisorb 5 ODS 2 precolumn (Phenomenex, Torrance, CA) connected to a 250 mm × 10 mm Spherisorb 5 ODS 2 column, both maintained at 30°C. Data were acquired with a Hewlett-Packard ChemStation. Milk fat triacylglycerols were resolved with two consecutive linear gradient programs, followed by isocratic conditions, at a flow rate of 5 mL min^{-1}, using HPLC-grade solvents: (i) acetonitrile (AN)/MTBE from 90:10 to 80:20 (vol/vol) over a 42-min period; (ii) AN/MTBE from 80:20 to 70:30 (vol/vol), over the next 18 min; and (iii) AN/MTBE at 70:30 (vol/vol) for the remainder of the run. Samples (100 mg) were dissolved in chloroform (1.0 mL) and filtered. Aliquots of these solutions (100 µL) were injected, and the retention time of chloroform was taken as hold-up time (t_0). Fractions were collected using a Pharmacia fraction collector (Model 18-1010-19; Uppsala, Sweden), and the corresponding fractions from different runs were combined, dried over anhydrous sodium sulfate, filtered, concentrated at room temperature under a stream of dry nitrogen, and kept at −30°C until analysis. Collection times of the different fractions were based on retention times of a series of standards that were eluted under the same conditions, and were adjusted to collect triacylglycerols by PN. Mass of the recovered fractions allowed a precise determination of their relative proportions. Thus, calibration of the UV detector was not necessary.

Reference compounds. The following mono- and diacid triacylglycerols (99%, unless otherwise stated) were obtained from Sigma Chemical (St. Louis, MO) and used as standards: tributyrin, tricaprin, tricaproin, tricaprylin, trielaidin, trilaurin, trilinolein, trilinolenin, trimyristin, trimyristolein (98%), triolein, tripalmitelaidin, tripalmitin, tripalmitolein, tristearin, 1,2-dioleoyl-3-stearoyl-*rac*-glycerol, 1,2-distearoyl-3-myristoyl-*rac*-glycerol, 1,2-distearoyl-3-oleoyl-*rac*-glycerol, 1,3-distearoyl-2-oleoylglycerol, and 1,2-distearoyl-3-palmitoyl-*rac*-glycerol. Additional racemic diacid triacylglycerols were prepared for identification purposes, by chemical interesterification (13) of the following pairs of monoacid triacylglycerols, in ratios of 3 to 2: tributyrin and tricaprin; tributyrin and trimyristin; tributyrin and tripalmitin; tricaproin and tripalmitin; tricaprylin and tricaprin; tricaprylin and tripalmitin; tricaprylin and trilinolein; tricaprylin and trilinolenin; tricaprin and trimyristin; tricaprin and tripalmitin; tricaprin and triolein; trilaurin and trimyristin; trilaurin and tripalmitin; trilaurin and trilinolein; trimyristin and tripalmitin; trimyristin and triolein; tripalmitin and triolein; tripalmitin and trilinolein; triolein and trilinolein.

Degradation of milk fat triacylglycerols and thin-layer chromatography (TLC) separation. Grignard degradation was adapted from literature procedures (10–12) based on the use of a Grignard reagent. It was carried out on fractions that amounted to at least 4 mg (fractions 5 to 14), with ethylmagnesium bromide in slight excess (125%) of the required amount, to optimize the ratio of monoacylglycerols in the products, compared to large excesses usually utilized (11,12, 14,15). *sn*-2-Monoacylglycerols were isolated, and values for *sn*-1,3 positions were estimated from analysis of triacylglycerols and *sn*-2-monoacylglycerols (12). The procedure employed was as follows: to a solution of milk fat triacylglycerols (10 mg) in anhydrous diethyl ether (0.5 mL), contained in a flame-dried flask under an inert atmosphere, a solution of ethylmagnesium bromide in the same solvent (3.0 M; 42 µL, 5 eq) was added. After stirring 30 s at room temperature, glacial acetic acid (10 µL) was added slowly, followed by 300 µL of a 10% boric acid solution. The mixture was extracted with diethyl ether saturated with boric acid (4 × 2 mL), and the organic extracts were combined, washed sequentially with sodium bicarbonate (2%), water, and brine (saturated aqueous solution of sodium chloride), dried over anhydrous sodium sulfate, filtered, and concentrated under a stream of dry nitrogen. The residue was dissolved in a 1:1 mixture of chloroform/methanol (50 µL), and the solution was applied on a plate of silica gel (20 cm × 20 cm, 0.2 mm thickness). Standards of mono-, di-, and triacylglycerols were also applied as references. Migration was carried out with a mixture of chloroform/methanol (98:2 vol/vol), and the references were sprayed with dichlorofluorescin (0.1% in ethanol) and visualized under UV light. The band corresponding to monoacylglycerols was scraped off and extracted with diethyl ether (4 × 2 mL) aided by sonication. The solvent was evaporated and the residue was solubilized as before and applied on a homemade plate of silica gel containing 10% (w/w) boric acid (20 cm × 20 cm, 0.5 mm thickness). Migration was achieved with a mixture of chloroform/methanol (96:4 vol/vol). Usual treatment was carried out for isolation of *sn*-2 monoacylglycerols. Fractions 1 to 4 and 15, which were isolated in very small amounts (≤2.0 mg), were not analyzed for regiospecific distribution of fatty acids.

Gas chromatography of fatty acids. Methylation of fatty acids was carried out in a sealed tube with 0.4 N sodium methoxide in methanol (16). Analyses of the methyl esters were performed with a gas chromatograph (Model 5890 Series II; Hewlett-Packard) equipped with a flame-ionization detector and connected to a computer with a Hewlett-Packard ChemStation. Sample volumes (1.0 µL) in hexane were injected on a DB-225 capillary column (J&W, Folsom, CA; 30 m × 0.25 mm i.d., 0.25 µm film thickness), with a split ratio of 50:1. Hydrogen was the carrier gas, with a linear velocity of 35.5 cm s^{-1} at 50°C. The injector and detector temperatures were maintained, respectively, at 215 and 230°C, while the oven temperature was programmed from 35 to 180°C at 20°C min^{-1}, then up to 200°C at 3°C min^{-1}, and held for 13 min at this temperature, for a total time of 30 min. Fatty acid methyl ester standards (GLC Reference Standards, Catalogue No. GLC-60 and GLC-76; Nu-Chek-Prep, Elysian, MN) were used for identification and calibration of the detector response. The standard mixture was composed of saturated fatty acid esters C_4–C_{20}, including odd carbon number fatty acid esters C_{13}, C_{15}, and C_{17}; and unsaturated fatty acid esters, $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$.

RESULTS AND DISCUSSION

Fractionation of milk fat by HPLC. Reversed-phase HPLC fractionation of bovine milk fat was based on the utilization of standard triacylglycerols for collection times of the fractions. Although triglycerides were not as well resolved in preparative conditions as they were in analytical conditions (17–20), a total of 67 peaks, exclusive of shoulders, was divided into 15 differentiated peak groups (Fig. 1). As a result, starting with fraction 2, the HPLC fractions corresponded to PN of triacylglycerols (Table 1). Values reported in Table 1 show that fractions 7, 8, 12, and 13 constituted the major fractions, since they accounted for 54.0% of milk fat, which displayed a bimodal distribution for triacylglycerols.

Grignard degradation and TLC separation. Partial deacylation of triacylglycerols was carried out with ethylmagnesium bromide. To ensure there was no isomerization of the monoacylglycerols during degradation, workup, and TLC separation, the method was performed with synthetic *sn*-1,3-dipalmitoyl-2-oleoylglycerol. Fatty acid analysis of the *sn*-1,3 and *sn*-2 monoacylglycerols showed very little or no detectable isomerization with the procedure used. A two-step

TABLE 1
Partition Number and Composition of Milk Fat Fractions by High-Performance Liquid Chromatography

Fraction	Composition		
	Partition number	(wt%)	(mol%)[a]
1	≤24	0.7	1.0
2	26	0.6	0.8
3	28	0.8	1.0
4	30	1.8	2.2
5	32	3.9	4.8
6	34	8.6	10.0
7	36	14.1	15.4
8	38	11.2	11.8
9	40	6.3	6.6
10	42	7.8	7.5
11	44	8.1	7.5
12	46	13.2	11.9
13	48	15.5	13.4
14	50	5.2	4.4
15	52	2.0	1.7
Total	—	99.8[b]	100.0

[a]Mol% composition was calculated from wt% composition, based on the fatty acid composition of the individual fractions. Data not reported for fractions 1 to 4 and 15.
[b]Value does not total 100.0 because of rounding of figures.

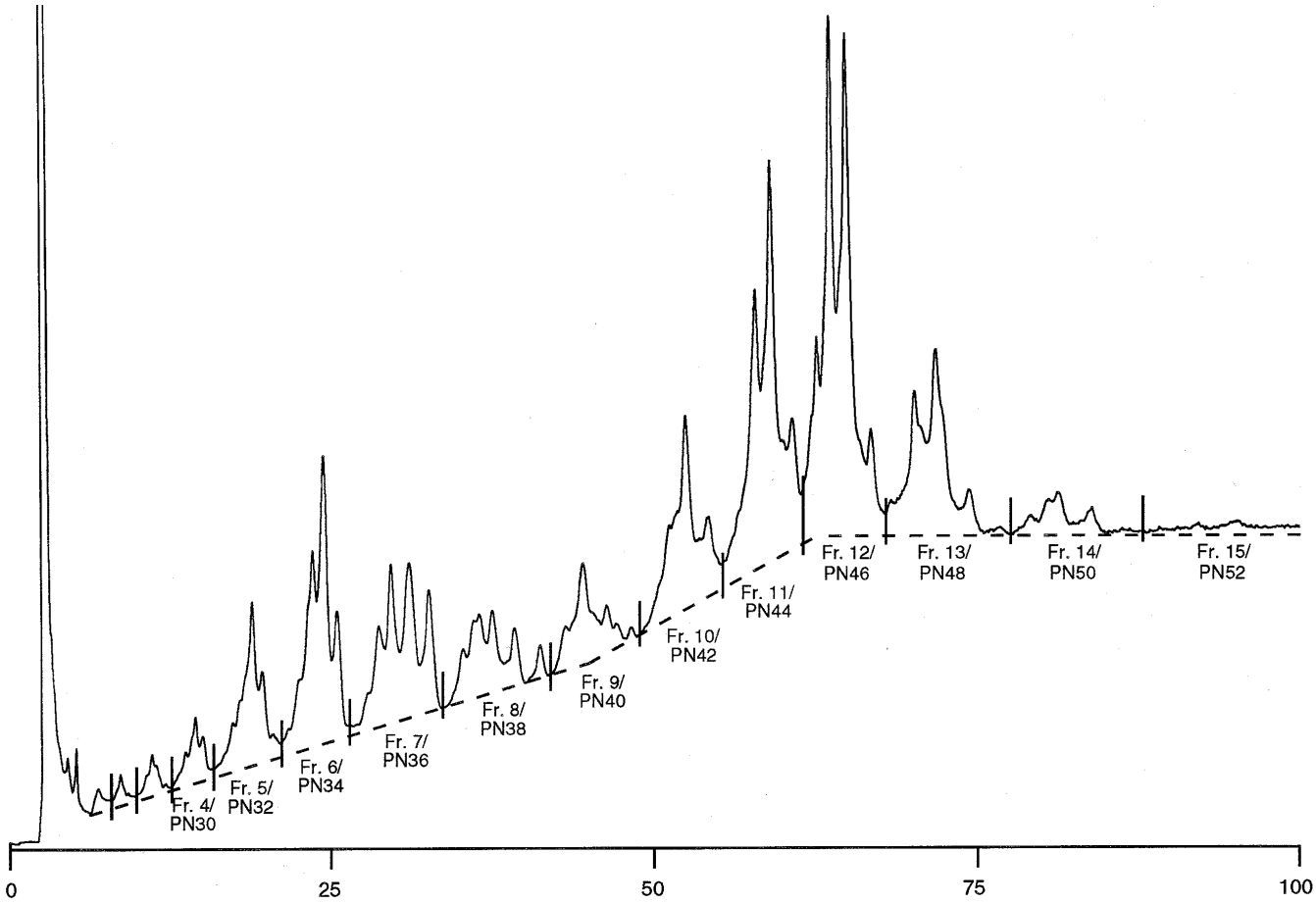

FIG. 1. High-performance liquid chromatogram of bovine milk fat, on a C-18 semipreparative column, using eluent mixtures of acetonitrile (AN)/methyl-*tert*-butyl ether (MTBE) from 90:10 to 80:20 (vol/vol) over a period of 42 min, followed by a second gradient of AN/MTBE from 80:20 to 70:30 (vol/vol), over the next 18 min, and an isocratic mixture of AN/MTBE 70:30 for the remainder of the run. The ultraviolet detector was set at 220 nm. The vertical lines indicate the border of the fractions (Fr.) corresponding to partition number (PN).

TLC separation was selected because it allowed a cleaner separation of the bands corresponding to *sn*-1,3 and *sn*-2 monoacylglycerols, compared to only one TLC migration (21), for which separation between *sn*-1,3 and *sn*-2 monoacylglycerols was never very clear with milk fat samples, unlike simple standards.

Fatty acid analysis. Fatty acid analyses of triacylglycerols and of monoacylglycerols resulting from their Grignard degradation were carried out for fractions 5 to 14 (Table 2). Fatty acids were analyzed as methyl esters, and only the 17 most common were quantified. The saturated fatty acids analyzed included arachidic, butyric, capric, caproic, caprylic, lauric, margaric, myristic, palmitic, pentadecanoic, stearic and tridecanoic, whereas unsaturated fatty acids were entirely of *cis*-configuration and of methylene-interrupted unsaturation (linoleic, linolenic, myristoleic, oleic, and palmitoleic acids). Neither branched-chain fatty acids, acids with conjugated unsaturation, nor unsaturated acids of *trans*-configuration were identified in the analysis.

Saturated fatty acids. C_4 to C_8 fatty acids were present only in the fractions corresponding to triacylglycerols with PN of 32 to 42 (Table 1). As expected, butyric acid was found exclusively in *sn*-1,3 position, with values of around 40% in triacylglycerols with PN32 and 34, in agreement with previous results by pancreatic lipase deacylation (2), by ^1H and ^{13}C nuclear magnetic resonance (22,23), and by direct measure-

TABLE 2
Composition of Fatty Acids (mol%) Esterified at the *sn*-1,3 and *sn*-2 Positions of Triacylglycerols in the High-Performance Liquid Chromatographic Fractions 5 to 14 of Bovine Milk Fat[a]

Fatty acid	Position	Fraction/(PN)[b] (mol%)									
		5 (32)	6 (34)	7 (36)	8 (38)	9 (40)	10 (42)	11 (44)	12 (46)	13 (48)	14 (50)
4:0	1,3	40.8 (100)[b]	39.5 (100)	31.4 (100)	13.8 (100)	6.7 (100)	1.3 (100)	—[c] (0)	— (0)	— (0)	— (0)
	2	—	—	—	—	—	—	— (0)	— (0)	— (0)	— (0)
6:0	1,3	6.7 (87)	5.7 (80)	8.3 (92)	18.5 (98)	7.8 (94)	0.9 (86)	— (0)	— (0)	— (0)	— (0)
	2	2.0 (13)	2.8 (20)	1.5 (8)	0.7 (2)	1.0 (6)	0.3 (14)	— (0)	— (0)	— (0)	— (0)
8:0	1,3	2.7 (71)	1.7 (74)	1.8 (78)	5.5 (90)	11.4 (88)	2.6 (76)	0.3 (60)	— (0)	— (0)	— 0)
	2	2.2 (29)	1.2 (26)	1.0 (22)	1.2 (10)	3.1 (12)	1.6 (24)	0.4 (40)	— (0)	— (0)	0.4 (100)
10:0	1,3	4.1 (46)	2.0 (57)	1.9 (63)	4.0 (70)	12.5 (77)	14.1 (75)	4.5 (76)	— (0)	— (0)	— (0)
	2	9.5 (54)	3.0 (43)	2.2 (37)	3.4 (30)	7.4 (23)	9.5 (25)	2.9 (24)	1.8 (100)	— (0)	0.8 (100)
12:0	1,3	6.4 (38)	2.4 (41)	1.54 (53)	2.2 (52)	4.8 (59)	6.8 (60)	11.0 (71)	2.4 (58)	0.6 (100)	— (0)
	2	21.0 (62)	6.8 (59)	2.7 (47)	4.0 (48)	6.7 (41)	9.0 (40)	9.2 (29)	3.5 (42)	— (0)	1.1 (100)
13:0	1,3	— (0)	0.4 (90)	0.2 (80)	— (0)	— (0)	0.2 (80)	0.3 (75)	— (0)	— (0)	— (0)
	2	— (0)	0.1 (11)	0.1 (20)	— (0)	— (0)	0.1 (20)	0.2 (25)	0.3 (100)	— (0)	— (0)
14:0	1,3	8.5 (40)	10.1 (35)	4.3 (33)	3.5 (36)	9.1 (49)	8.9 (48)	15.6 (58)	18.8 (63)	4.3 (42)	0.6 (16)
	2	25.8 (60)	37.2 (65)	17.1 (67)	12.6 (64)	18.9 (51)	19.4 (52)	22.2 (42)	21.7 (37)	11.9 (58)	6.2 (84)
14:1	1,3	0.9 (26)	0.4 (33)	0.4 (62)	0.6 (60)	1.4 (65)	2.4 (67)	3.8 (75)	0.6 (52)	— (0)	— (0)
	2	5.9 (74)	1.6 (67)	0.5 (38)	0.8 (40)	1.5 (35)	2.4 (33)	2.5 (25)	1.1 (48)	— (0)	— (0)
15:0	1,3	1.1 (65)	0.7 (50)	1.2 (53)	0.8 (47)	0.8 (55)	1.0 (57)	1.1 (55)	1.6 (62)	1.0 (49)	0.5 (42)
	2	1 .2 (35)	1.4 (50)	2.1 (47)	1.8 (53)	1.3 (45)	1.5 (43)	1.8 (45)	2.0 (38)	2.1 (51)	1.4 (58)
16:0	1,3	14.5 (61)	20.9 (61)	28.6 (54)	22.5 (50)	25.2 (59)	29.4 (65)	27.7 (62)	28.8 (60)	31.9 (54)	22.0 (47)
	2	18.9 (39)	26.6 (39)	48.4 (46)	45.2 (50)	35.4 (41)	31.6 (35)	33.4 (38)	38.9 (40)	54.6 (46)	50.4 (53)
16:1	1,3	1.1 (45)	1.4 (44)	0.5 (42)	0.5 (48)	1.0 (56)	1.2 (55)	2.3 (66)	2.9 (72)	1.1 (100)	0.1 (29)
	2	2.7 (55)	3.6 (56)	1.4 (58)	1.1 (58)	1.6 (44)	2.0 (45)	2.4 (34)	2.2 (28)	— (0)	0.5 (71)
17:0	1,3	— (0)	— (0)	0.4 (73)	0.9 (64)	0.7 (61)	0.6 (75)	0.6 (71)	0.8 (100)	1.4 (100)	0.9 (95)
	2	— (0)	— (0)	0.3 (27)	1.0 (36)	0.9 (39)	0.4 (25)	0.5 (29)	— (0)	— (0)	0.1 (5)
18:0	1,3	3.0 (80)	2.4 (69)	5.2 (79)	15.2 (76)	14.0 (79)	11.8 (79)	9.6 (78)	7.4 (66)	13.8 (76)	45.3 (85)
	2	1.4 (20)	2.2 (31)	2.9 (21)	9.4 (24)	7.7 (21)	6.5 (21)	5.4 (22)	7.7 (34)	8.9 (24)	16.3 (15)
18:1	1,3	6.6 (61)	10.3 (66)	13.6 (59)	10.6 (54)	3.6 (35)	17.4 (71)	21.0 (70)	33.3 (76)	44.6 (80)	30.4 (74)
	2	8.4 (39)	10.7 (34)	18.8 (41)	17.9 (46)	13.3 (65)	14.5 (29)	18.0 (30)	21.0 (24)	22.6 (20)	21.8 (26)
18:2	1,3	2.5 (95)	2.5 (69)	1.0 (67)	1.1 (73)	1.4 (72)	0.9 (64)	1.8 (77)	3.4 (100)	1.1 (100)	0.4 (100)
	2	0.9 (15)	2.2 (31)	1.0 (33)	0.8 (27)	1.1 (28)	1.0 (36)	1.1 (23)	— (0)	— (0)	— (0)
18:3	1,3	2.2 (94)	— (0)	— (0)	— (0)	— (0)	0.3 (100)	0.5 (100)	— (0)	— (0)	— (0)
	2	0.3 (6)	0.2 (100)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0	— (0))
20:0	1,3	— (0)	— (0)	— (0)	— (0)	— (0)	0.3 (100)	— (0)	— (0)	0.2 (100)	0.8 (100)
	2	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)	— (0)

[a]Values are means of duplicate analyses.
[b]Values in parentheses represent the percentage of fatty acids located between the *sn*-1,3 and *sn*-2 positions in any given fraction.
[c]Fatty acid not detected or value was less than 0.1%. PN, partition number, defined as acyl carbon number – 2 × DB, where DB is the number of double bonds.

ment by gas chromatography (24), which assigned this acid almost exclusively at either *sn*-3 or *sn*-1,3 position. Since butyric acid is found only in the *sn*-3 position of milk fat triacylglycerols (2), its level at this position of the glycerol is 80% in these two fractions. Caproic acid was found only in fractions containing triacylglycerols with PN32 to 42, and mostly in *sn*-1,3 positions, with the highest ratio in the fractions with PN36 to 40, which compares to the reported values (2,24). As only one butyric or caproic acid may be present in triacylglycerols with PN of 32 and 34, data in Table 1 suggest that more than 90% of those triacylglycerols contain either butyric or caproic acid. The distribution of caprylic acid was also nonrandom and was preferentially located at *sn*-1,3-positions with increasing frequency from PN32 to PN40. Above PN40, the proportion of C_8 in the *sn*-1,3 positions decreased. The distribution of capric acid (C_{10}) also varied with the triacylglycerol PN. It was preferentially located at the *sn*-2 position of triacylglycerols with PN32 and 34 and nearly random in triacylglycerols with PN of 36 and 38, but above PN40, it was predominantly located at *sn*-1,3 positions.

Generally, C_{12}–C_{15} fatty acids were preferentially located in the *sn*-2 position, but their frequency at the *sn*-2 position varied with the PN of triacylglycerol. The frequency of lauric acid (C_{12}) at the *sn*-2 position decreased gradually with increase in the PN of triacylglycerols until PN of 44 where its distribution was nearly random, but above PN44, its frequency at the *sn*-2 position increased again. The distribution pattern for myristic acid (C_{14}) was quite similar to lauric acid except that a nearly random distribution occurred at PN46.

Of the C_{16} to C_{20} saturated fatty acids, which included palmitic, margaric, stearic and arachidic acids, palmitic and stearic acids were the most abundant. The distribution of the latter two acids between *sn*-1,3 and *sn*-2 positions was essentially opposite and was greatly affected by PN, but with some exceptions. Palmitic acid (C_{16}) was nearly random in triacylglycerols with PN42 and was distributed predominantly at the *sn*-2 position in triacylglycerols with PN36, 38, 48, and 50. The distribution of stearic acid (C_{18}) was predominantly at the *sn*-1,3 position with two exceptions in triacylglycerols with PN of 34 and 46, where the distribution was nearly random. Concentration of palmitic acid at the *sn*-2 position was the highest of all the fatty acids in most of the HPLC fractions (fractions 7 to 14 or PN36 to PN50), with values ranging from 18.9% in fraction 5 (PN32) up to 54.6% in fraction 13 (PN48). Distributions of myristic and palmitic acids are in good agreement with reported values for distributions of fatty acids in triacylglycerols with PN of 48 (ACN50:1 and 52:2) (25).

Unsaturated fatty acids. Among the unsaturated fatty acids analyzed, oleic acid was by far the most abundant. The distribution pattern of oleic acid ($C_{18:1}$) was different from other major fatty acids. It was nearly random in triacylglycerols with PN32 and 34. Above PN34, it was predominantly located at the *sn*-2 position until PN40, where 65% of the oleic acid was at the *sn*-2 position. Above PN40, oleic acid was predominantly located at the *sn*-1,3 positions, somewhat similar to the stearic acid distribution in the PN range of 42 to 50. This distribution pattern was also true for myristoleic and palmitoleic acids. Myristoleic acid was the second-most abundant unsaturated fatty acid (5.0%) at the *sn*-2 position in fraction 5 (PN32), second only to oleic acid (8.4%). Linoleic and linolenic acids were mostly found in the *sn*-1,3 position, but their levels were low. Moreover, linolenic acid was found only in a limited number of fractions and was highest in fraction 5 (PN32).

The fatty acid distribution in bovine milk fat between *sn*-1,3 and *sn*-2 positions followed the general trends observed by Kuksis and co-workers for fractions enriched either in short- or long-chain triacylglycerols (2–4), but we report fatty acid distribution of milk fat on a number of fractions enriched in triacylglycerols varying in size. Our results also show the changes in the distribution of various fatty acids between the *sn*-2 and *sn*-1,3 positions with size of triacylglycerol. Fatty acids C_4, C_6, and C_8 are predominantly located at the *sn*-1,3 positions irrespective of the triacylglycerol size. The distribution pattern of the fatty acid C_{10} was mixed. It was predominantly located at the *sn*-2 position only in the short-chain triacylglycerols (PN32 and 34); above PN36, it was preferentially distributed at the *sn*-1,3 positions like the short-chain fatty acids and its frequency at the *sn*-2 position decreased with triacylglycerol size, similar to fatty acids C_{12} and C_{14}. Fatty acids C_{12} and C_{14} present a different pattern in that they are preferentially located at the *sn*-2 position, but their frequency at this position decreases with triacylglycerol size. Although the distributions of C_{16} and C_{18} are not greatly affected by size, the distribution of palmitic acid is predominantly at the *sn*-2 position and that of stearic acid in the *sn*-1,3 positions. However, the distribution of oleic acid presents a different pattern. It is distributed predominantly at the *sn*-2 position, somewhat like palmitic acid below PN40; but in longer-chain triacylglycerols it is predominantly located at the *sn*-1,3 positions, somewhat similar to stearic acid. Considering only the distribution of saturated vs. unsaturated fatty acids (Table 3), we observed that in fractions 5 to 9 (PN32–40), the *sn*-1,3 positions were more saturated than the *sn*-2 position. This trend was the opposite in fractions 10 to 14 (PN42 to 50). For all fractions except 12 and 13 (PN46 and 48), the difference between values for saturated and unsaturated fatty acids was not very high (3 to 10%). But for fractions 12 and 13, the difference was 16 to 24%. In addition, the *sn*-1,3 positions were about twice more unsaturated than the *sn*-2 position in these two fractions (Table 3).

Fractions of milk fat triacylglycerols corresponding to PN 32 to 50 were isolated and analyzed for their fatty acid distribution between the *sn*-1,3 and *sn*-2 positions. First, our results confirm that fatty acid distribution is nonrandom in milk fat triacylglycerols. Second, our results show that the distribution pattern of various fatty acids between the *sn*-2 and *sn*-1,3 positions varies with triacylglycerol size. Short-chain fatty acids (C_4–C_8) and stearic acid are located at the *sn*-1,3 position at high proportions irrespective of size. The frequency of the medium-chain fatty acid, C_{10}, at the *sn*-2 posi-

TABLE 3
Carbon Number, Saturated and Unsaturated Fatty Acid Composition Relative to Position of Milk Fat Fractions by High-Performance Liquid Chromatography

			Fatty acid (%mol)	
Fraction[a]	Position	CN[b]	Saturated	Unsaturated
5	1,3	10.0	87.4	13.4
	2	13.7	82.8	17.2
6	1,3	10.5	85.4	14.6
	2	14.6	81.9	18.1
7	1,3	11.4	84.5	15.5
	2	15.6	78.3	21.7
8	1,3	12.1	86.9	12.7
	2	15.7	79.5	20.6
9	1,3	12.8	92.9	7.5
	2	15.0	82.6	17.4
10	1,3	14.8	77.8	22.2
	2	14.9	80.2	19.9
11	1,3	15.5	70.5	29.5
	2	15.4	76.1	23.9
12	1,3	16.4	59.8	40.2
	2	15.9	75.7	24.3
13	1,3	17.1	53.2	46.8
	2	16.4	77.5	22.6
14	1,3	17.7	70.2	30.9
	2	16.5	77.6	22.4

[a]Fractions were obtained by reversed-phase high-performance liquid chromatography of milk fat, and fractions 5 to 14 accounted for 93.3% of milk fat.
[b]Carbon number (CN) was calculated from the fatty acid composition.

tion decreased with triacylglycerol size and it was preferentially located only in short-chain triacylglycerols. Other medium-chain fatty acids C_{12} and C_{14} are located at the *sn*-2 position in high proportions, but their proportion at the *sn*-2 position decreases with triacylglycerol size. Palmitic acid is predominantly located at the *sn*-2 position irrespective of the size of triacylglycerol. Interestingly, oleic acid distribution is unique in that it is located in high proportions at the *sn*-2 position in triacylglycerols of lower size, but at *sn*-1,3 positions in longer triacylglycerols. Our results also showed the *sn*-2 position was more unsaturated than the *sn*-1,3 position in the PN range of 32–40, but it was more saturated in triacylglycerols of higher PN owing to the distribution pattern of oleic acid.

ACKNOWLEDGMENTS

We acknowledge financial support of the Natural Sciences and Engineering Council of Canada, Dairy Farmers of Canada, and Agropur Coopérative Agro-alimentaire, Granby, Québec, Canada.

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[Received May 8, 1998, and in final revised form November 16, 1998; revision accepted 1998]

Calcium-Independent Phospholipase A_2 in Isolated Rabbit Ventricular Myocytes

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ABSTRACT: We characterized phospholipase A_2 (PLA_2) activity in isolated rabbit ventricular myocytes with respect to subcellular distribution, substrate specificity, and Ca^{2+} dependency. Membrane-associated PLA_2 was found to be an order of magnitude greater than cytosolic PLA_2. Ventricular myocyte PLA_2 activity was enhanced following protease-activated receptor stimulation with thrombin and was found to be largely Ca^{2+}-independent and selective for phospholipid substrates containing arachidonic acid at the *sn*-2 position. Immunoblot analysis using an antibody to cytosolic Ca^{2+}-independent PLA_2 from Chinese hamster ovary cells recognized a membrane-associated protein with a molecular mass of approximately 80 kDa; however, differences in pH optima, response to inhibitors, and substrate selectivity of membrane-associated and cytosolic PLA_2 activity suggest the presence of multiple Ca^{2+}-independent PLA_2. Pretreatment with bromoenol lactone, a specific inhibitor of Ca^{2+}-independent PLA_2, significantly attenuated membrane-associated and cytosolic PLA_2 in unstimulated and thrombin-stimulated myocytes. Pretreatment with methyl arachidonyl fluorophosphonate, mepacrine, or dibucaine had no significant effect on PLA_2 activity under all conditions tested. Ventricular myocyte PLA_2 activity was significantly inhibited by ATP, GTP, and their nonhydrolyzable analogs and was regulated by protein kinase C activity. These studies demonstrate the presence of one or more unique membrane-associated Ca^{2+}-independent PLA_2 in isolated ventricular myocytes that exhibit a preference for phospholipids with arachidonate at the *sn*-2 position and that are activated by thrombin stimulation.

Lipids 33, 1203–1212 (1998).

The present study was undertaken to characterize phospholipase A_2 (PLA_2) activity in isolated rabbit ventricular myocytes. Although detailed characterization of PLA_2 was described in whole myocardium from several species (1–4) and in vascular smooth muscle cells from the dog (5), little is known regarding detailed characterization of PLA_2 isoforms in ventricular myocytes. It is important to provide a detailed characterization of PLA_2 in different myocardial cell populations because of cell-specific differences in phospholipid composition and in the mechanisms of enzyme activation. For example, we showed that increased production of lysophospholipids and free fatty acids resulting from PLA_2 activation during ischemia or following thrombin stimulation in the heart plays an important role in the subsequent development of electrophysiologic alterations (6,7). Because there are likely to be significant differences in the nature of the electrophysiologic alterations produced by monoacyl lysophospholipids (derived from PLA_2-catalyzed hydrolysis of diacyl phospholipids in endothelial cells and smooth muscle cells) as opposed to lysoplasmalogens (derived from PLA_2-catalyzed hydrolysis of plasmalogens in ventricular myocytes), it is important to determine the substrate selectivity of the PLA_2 activity present in isolated cardiac myocytes and to distinguish this activity from that present in other nonmyocytic myocardial cell populations.

Previous studies of PLA_2 in whole myocardium demonstrate that Ca^{2+} is not required for PLA_2 activity (i.e., myocardial PLA_2 is Ca^{2+}-independent) and that PLA_2 is selective for arachidonylated ether-linked phospholipid substrates. Studies of purified cytosolic myocardial PLA_2 activity demonstrated that this isoform is tightly associated with phosphofructokinase (PFK) (8), is stimulated by ATP (9), and specifically binds to calmodulin in a Ca^{2+}-dependent fashion (10).

The goal of the present study was to characterize the subcellular distribution, Ca^{2+}-dependency, and substrate selectivity of PLA_2 activity in isolated ventricular myocytes. Since the phospholipid composition of isolated ventricular myocytes is unique because of the predominance of phospholipids containing an unsaturated ether covalent linkage at the *sn*-1 position (i.e., plasmalogen phospholipids) (11), our characterization of PLA_2 activity in isolated ventricular myocytes was performed in the absence (<1 nM) and presence (10 mM) of Ca^{2+} using phospholipid substrates containing *O*-acyl ester, saturated ether, or unsaturated (vinyl) ether-linked aliphatic groups at the *sn*-1 position and oleate or arachidonate at the *sn*-2 position. Alterations in ventricular myocyte PLA_2 activity in the presence of high-energy phosphates, thrombin, and modulated protein kinase C (PKC) activity are also described.

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Abbreviations: BEL, (*E*)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one or bromoenol lactone; $cPLA_2$, cytosolic PLA_2; CHO, Chinese hamster ovary; DAG, diacylglycerol; DTT, dithiothreitol; $iPLA_2$, calcium-independent PLA_2; LPC, lysophosphatidylcholine (this term is also used to refer collectively to 1-*O*-alkyl, 1-*O*-alk-1′enyl, and 1-*O*-acyl forms of monoradyl choline glycerophospholipids); MAFP, methyl arachidonyl fluorophosphonate; PAP, phosphatidic acid phosphohydrolase; PFK, phosphofructokinase; PKC, protein kinase C; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate.

MATERIALS AND METHODS

Isolation of ventricular myocytes. Adult female rabbits weighing 2–3 kg were anesthetized with intravenous pentobarbitone sodium (50 mg/kg), and the heart was rapidly removed. The heart was mounted on a Langendorff perfusion apparatus and perfused for 5 min with a Tyrode solution containing (in mmol/L) NaCl 118, KCl 4.8, $CaCl_2$ 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.2, and glucose 11 (all from Sigma Chemical Co., St. Louis, MO); the Tyrode solution was saturated with 95%O_2:5%CO_2 to yield a pH of 7.4. This was followed by a 4-min perfusion with a Ca^{2+}-free Tyrode solution containing EGTA (100 μM) and a final perfusion for 20 min with the Tyrode solution containing 100 μM Ca^{2+} and 0.033% collagenase (type II; Worthington Biochemical, Lakewood, NJ). The left and right ventricles were cut into small pieces which were then shaken in fresh enzyme solution. The first harvest of myocytes was discarded. Cells from the next three harvests were combined and washed with a HEPES buffer containing (in mmol/L): NaCl 133.5, KCl 4.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, HEPES 10, and glucose 10, plus 300 M $CaCl_2$, pH adjusted to 7.4 with 10 N NaOH. Extracellular Ca^{2+} concentration was increased to 1.2 mM in three stages at intervals of 20 min. Elongated myocytes were separated from rounded nonviable cells by repeated differential sedimentation.

PLA_2 assays. Myocytes were suspended in 1 mL of buffer containing (mmol/L): sucrose 250, KCl 10, imidazole 10, EDTA 5, dithiothreitol (DTT) 2 with 10% glycerol, pH = 7.8 with 10 N KOH (all from Sigma Chemical Co.). The suspension was sonicated on ice three times for 10 s, and the sonicate was centrifuged at 14,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 60 min to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant). PLA_2 activity in subcellular fractions was assessed by incubating enzyme (typically 8 μg membrane protein or 200 μg cytosolic protein) with 100 μM *sn*-2 radiolabeled plasmenylcholine, alkylacyl glycerophosphocholine, or phosphatidylcholine in assay buffer containing 10 mM Tris, 10% glycerol, pH = 7.0 with either 4 mM EGTA or 10 mM Ca^{2+} at 37°C for 5 min in a total volume of 200 μL. Synthesis of radiolabeled phospholipid substrates was described previously (3,6,8). The reaction was initiated by adding the substrate as a concentrated stock solution in ethanol (5 μL total volume) which was injected into a total volume of 200 μL of aqueous buffer to achieve a final substrate concentration of 100 μM. Reactions were terminated by the addition of 100 μL of butanol, and released radiolabeled fatty acid was isolated by application of 25 μL of the butanol phase to channeled Silica Gel G plates, development in petroleum ether/diethyl ether/acetic acid (70:30:1, by vol) and subsequent quantification by liquid scintillation spectrometry. The reaction conditions we selected resulted in linear reaction velocities with respect to both time and enzyme concentration for each substrate examined. Protein content of each sample was determined by the Lowry method utilizing freeze-dried bovine serum albumin (Bio-Rad Laboratories, Richmond, CA) as the protein standard as described previously (12).

Immunoblot analysis of PLA_2. Myocytes were suspended in lysis buffer containing (in mmol/L) HEPES 20 (pH 7.6), sucrose 250, DTT 2, EDTA 2, EGTA 2, β-glycerophosphate 10, sodium orthovanadate 1, phenylmethylsulfonyl fluoride 2, leupeptin 20 μg/mL, aprotinin 10 μg/mL, and pepstatin A 5 μg/mL. Cells were sonicated on ice for three bursts of 10 s and centrifuged at 14,000 × *g* at 4°C for 10 min to remove cellular debris and nuclei. Cytosolic and membrane fractions were separated by centrifuging the supernatant at 100,000 × *g* for 60 min. The pellet was resuspended in lysis buffer, and the suspension was centrifuged at 100,000 × *g* for 60 min twice to minimize contamination of the membrane fraction with cytosolic protein. The final pellet was resuspended in lysis buffer containing 0.1% Triton X-100. Protein (total protein, cytosol, or membrane) was mixed with an equal volume of SDS sample buffer and heated at 95°C for 5 min prior to loading onto a 12% polyacrylamide gel. Protein was separated by SDS/polyacrylamide gel electrophoresis at 200 V for 35 min and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) at 100 V for 1 h. Nonspecific sites were blocked by incubating the membranes with Tris-Buffered saline containing 0.05% (vol/vol) Tween-20 (TBST) and 5% (wt/vol) nonfat milk for 1 h at room temperature. The blocked polyvinylidene difluoride membrane was incubated with antibodies to cytosolic PLA_2 ($cPLA_2$; Santa Cruz) or Ca^{2+}-independent PLA_2 (iPLA, 1:2000 dilution; Cayman Chemical, Ann Arbor, MI) for 1 h at room temperature. Unbound antibodies were removed with three washes with TBST solution, and membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution). Following six washes with TBST, regions of antibody binding were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and exposure to film (Hyperfilm; Amersham).

RESULTS

In initial studies, examination of the metabolic pathways potentially responsible for the production of fatty acid from diradyl choline glycerophospholipid substrates and the stability of PLA_2 activity in sonicated cell preparations derived from freshly isolated cells was examined. These initial studies of ventricular myocyte PLA_2 used 1-*O*-hexadec-1′-enyl-2-[^{3}H]oleoyl-*sn*-glycero-3-phosphocholine (16:0, [^{3}H]18:1 plasmenylcholine) as substrate and unfractionated sonicates of isolated myocytes as the enzyme source in the absence (<1 nM) and presence (10 mM) of added Ca^{2+}. Following incubation of the substrate with sonicated cell preparations, the reaction products were subjected to thin-layer chromatographic analysis using both one- and two-dimensional thin-layer chromatographic techniques which permitted the separation of fatty acids from other neutral lipids and which clearly resolved choline lysophospholipids from the diradyl

TABLE 1
Quantitation of Radiolabeled Products Generated Following Incubation of 100 μM (16:0,[^{3}H]18:1) Plasmenylcholine (PlasCho) or Phosphatidylcholine (PC) Substrate with 8 μg Membrane Protein (for 5 min at 37°C)[a]

Substrate	Product	dpm	Substrate	Product	dpm
(16:0,[^{3}H]18:1)	Oleic acid	20934 ± 306	(16:0,[^{3}H]18:1)	Oleic acid	14952 ± 227
Plasmenylcholine	LPlasC	216 ± 17	Phosphatidylcholine	LPC	176 ± 27
	AAG	220 ± 23		DAG	132 ± 16
	PA	145 ± 21		PA	128 ± 21

[a]The individual radiolabeled products were isolated by thin-layer chromatography and the radioactivity determined by liquid scintillation counting. The majority of radioactivity released from the phospholipid substrate was observed in the free fatty acid fraction, with minimal production of lysoplasmenylcholine (LPlasC), lysophosphatidylcholine (LPC) (indicating plasmalogenase or phospholipase A_1 activity), alkylenyl acyl glycerol (AAG) or diacylglaycerol (DAG) (indicating phospholipase C activity), or the phosphatidic acid (PA) (indicating phospholipase D activity). Results are mean ± SEM for three separate determinations.

choline glycerophospholipid substrate. These studies demonstrated that radiolabeled free fatty acid production accounted for >94% of the total reduction in diradyl choline glycerophospholipid radioactivity under all reaction conditions tested. There was no evidence for significant production of radioactive diradylglycerol, phosphatidic acid, or lysophospholipid (Table 1). Thus, we could not find evidence for measurable phospholipase C (PLC), phospholipase D (PLD), plasmalogenase, or phospholipase A_1 (PLA_1) activity under these assay conditions during the short incubation period used. To rule out the possibility that radioactive free fatty acid production was the result of sequential PLA_1 (or plasmalogenase)-lysophospholipase activity, PLC-diglyceride lipase activity or PLD-phosphatidate phosphohydrolase-diglyceride lipase activity, unlabeled lysophosphatidylcholine (LPC) or diacylglycerol (DAG) was added to the incubation mixture. The presence of these unlabeled lipids would result in an apparent decrease in the rate of radiolabeled fatty acid production and cause the accumulation of radioactively labeled LPC or DAG as a result of an isotope dilution effect if there was substantial flux of radiolabeled substrate through the sequential enzymatic pathways described above. Following addition of unlabeled LPC or DAG, we did not detect any significant reduction in radiolabeled fatty acid production or accumulation of radiolabeled LPC or DAG. Accordingly, based on these results and the stoichiometric relationship between production of radioactive fatty acid and decrease in radioactivity of diradyl choline phospholipid substrate, we are confident that under the incubation conditions employed in our study, the rate of production of radiolabeled fatty acid represents the rate of regiospecific hydrolysis of the *sn*-2 fatty acyl moiety from the diradyl choline glycerophospholipid substrate molecule catalyzed by PLA_2.

The addition of protease inhibitors including leupeptin, pepstatin, and aprotinin had no significant effect on enzyme stability during the course of cell sonication and preparation of cytosol and membrane fractions. The addition of glycerol and DTT was essential to maintain enzyme activity in sonicated cell suspensions, suggesting the requirement for reduced sulfhydryl groups for expression of intracellular PLA_2 activity.

PLA_2 activity in cytosol and membrane fractions isolated from ventricular myocytes was examined for thermal stability. When membrane fractions were incubated at 37°C, an apparent half-life of 1 h for PLA_2 activity was observed. The loss of PLA_2 activity was greatly attenuated following storage at lower temperatures, with less than 10% activity lost after 4 h when membranes were stored on ice. Isolated membrane fractions frozen for up to 24 h maintained PLA_2 activity levels comparable to initial values.

Following isolation of ventricular myocytes, less than 10% loss of PLA_2 activity occurred over 4 h when cells were placed on ice in the presence of 2 mM DTT and 10% glycerol. For our studies, considerable effort was expended to ensure that the time which elapsed between removal of the heart, isolation of the cells, and assay of enzyme activity was consistent between cell preparations.

In cytosol and membrane fractions isolated from ventricular myocytes, we found linear reaction velocities with respect to protein concentration for 2–20 g membrane protein and 100–500 μg cytosolic protein. Using 8 μg membrane protein and 200 μg cytosolic protein, the rate of liberation of radiolabeled free fatty acid from 100 M (16:0[^{3}H]18:1) plasmenylcholine or (16:0[^{3}H]18:1) phosphatidylcholine was found to be linear up to 10 min of incubation. We decided to use an incubation time of 5 min for determination of PLA_2 activity to ensure that linear reaction rates were obtained. Longer incubation times were associated with a loss in linearity, and shorter incubation times were associated with less reproducibility of activity measurements. Maximal reaction velocities were consistently achieved with substrate concentrations >50 μM. We used 100 μM as the substrate concentration for our assays to ensure that maximal rate measurements were being made and to minimize any effects of isotope dilution by unlabeled endogenous substrates (in all assays, we calculated that exogenous radiolabeled substrate was present in >5–10-fold molar excess over all potential endogenous substrates in both cytosolic and membrane fractions).

The cytosolic and membrane subcellular distribution, Ca^{2+} requirements, and substrate selectivity of PLA_2 activity in isolated rabbit ventricular myocytes are presented in Figure 1. Basal ventricular myocyte PLA_2 activity exhibited the following characteristics: (i) The highest specific PLA_2 activity is in a membrane-associated form (Fig. 1A). After adjustment for the total protein content of each fraction and normalizing

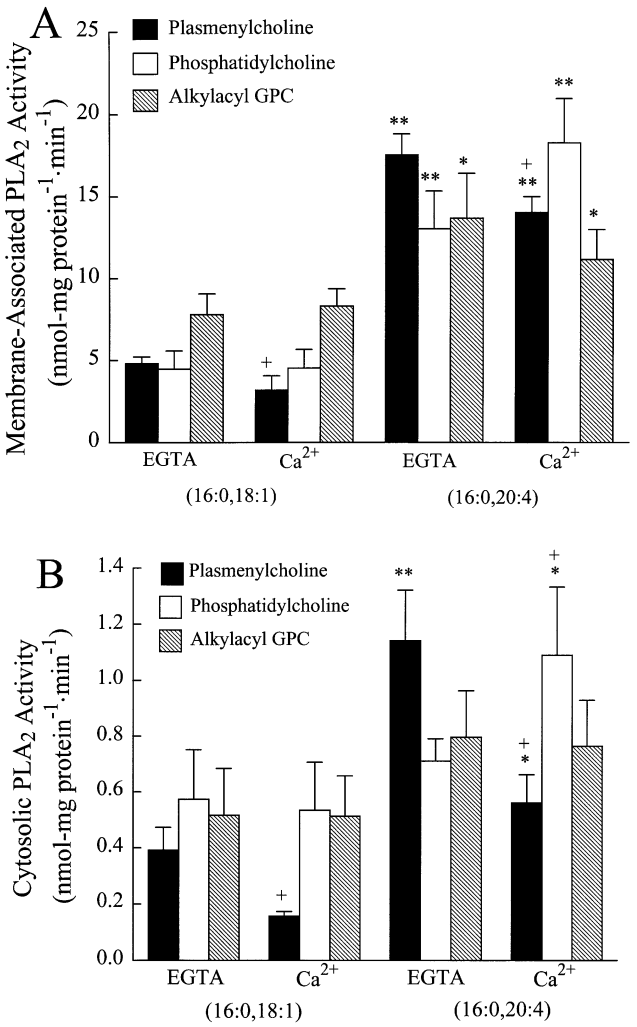

FIG. 1. Phospholipase A_2 (PLA_2) activity measured in the membrane (A) or cytosolic (B) fractions isolated from cardiac myocytes using plasmenylcholine, phosphatidylcholine, or alkylacyl glycerophosphocholine (GPC) substrates radiolabeled at the *sn*-2 position with oleic (16:0,[^{3}H]18:1) or arachidonic (16:0,[^{3}H]20:4) acid. Activity measurements were made in the absence (4 mM EGTA) or presence of (10 mM) Ca^{2+}. Values shown represent the mean ± SEM of independent results derived from four separate animals. $^*P < 0.05$, $^{**}P < 0.01$ for comparisons between (16:0,[^{3}H]18:1) and (16:0,[^{3}H]20:4) substrates; $^+P < 0.05$ for comparisons between the absence and presence of Ca^{2+} using the same substrate.

to 1×10^6 myocytes, total membrane-associated PLA_2 activity was 10- to 20-fold greater than total soluble (cytosolic) PLA_2 with all substrates tested. The PLA_2 activity in the membrane fraction could not be recovered in soluble form following repeated sonication, exposure to high- or low-ionic strength solutions, or exposure to EGTA and thus, on this basis, appears to represent a membrane-anchored or integral membrane protein. (ii) PLA_2 activity is influenced by the nature of the covalent linkage of the *sn*-1 aliphatic group and the composition of the *sn*-2 aliphatic group of the substrate molecule (Fig. 1). (iii) The majority of ventricular myocyte PLA_2 does not demonstrate a catalytic requirement for Ca^{2+} (i.e., ventricular myocyte PLA_2 is Ca^{2+}-independent).

Increasing the Ca^{2+} concentration from $<10^{-9}$ M (estimated concentration of Ca^{2+} in nominally Ca^{2+}-free solutions in the presence of 4 mM EGTA assay buffer) to concentrations greater than 10^{-6} M significantly decreased both membrane-associated and cytosolic PLA_2 activity using (16:0,[^{3}H]18:1) plasmenylcholine substrate. In contrast, increasing the Ca^{2+} concentration had no significant effect on cytosolic or membrane-associated PLA_2 activity measured using (16:0,[^{3}H]18:1) phosphatidylcholine substrate.

Comparisons of the pH profiles of PLA_2 in cytosolic and membrane fractions from isolated myocytes demonstrate a difference in pH for optimal activity in each fraction, further supporting the hypothesis of the presence of different PLA_2 isoforms in these subcellular fractions (Fig. 2). Membrane-associated PLA_2 activity measured using (16:0,[^{3}H]18:1) plasmenylcholine or (16:0, [^{3}H]18:1) phosphatidylcholine exhibited a pH optimum of 8.5 (Fig. 2A), whereas cytosolic PLA_2 activity was maximal at pH 7.0 using (16:0, [^{3}H]18:1) plasmenylcholine or (16:0, [^{3}H]18:1) phosphatidylcholine (Fig. 2B).

The marked differences in substrate selectivity, differential responses of PLA_2 activity to alterations in Ca^{2+} concentration, and differences in optimal pH for activity in cytosolic and membrane-associated PLA_2 activity suggest the presence of multiple PLA_2 isoforms in isolated ventricular myocytes. However, we cannot rule out the possibility that these observations are the result of Ca^{2+}-mediated alterations in the interaction of a single enzyme species with different aggregated phospholipid substrates.

By using our protocol for the preparation of cytosol and membrane fractions, unbroken myocytes, nuclei, and mitochondria are collectively sedimented following centrifugation at $14{,}000 \times g$ for 10 min. The total activity recovered in this fraction represents approximately 25% of the total enzyme activity recovered from the isolated cells. This enzyme activity was not further characterized. The $14{,}000 \times g$ supernatant contained both cytosolic and membrane-associated enzyme activities which were further fractionated by centrifugation at $100{,}000 \times g$ for 60 min to prepare cytosol (supernatant) and membrane (pellet) functions. Previous studies of cytosolic (e.g., Creatine Kinase, lactate dehydrogenase) and membrane (e.g., Na,K-ATPase, Ca-ATPase) marker enzyme activities showed that the cytosolic fraction from this final step contains no detectable membrane marker enzyme activity (13). The membrane fraction is composed predominantly of microsomes, including vesicles of sarcolemma and sarcoplasmic reticulum with >70% recovery of sarcolemma and sarcoplasmic reticulum marker enzyme activities in this fraction and minimal contamination by cytosolic proteins.

To determine whether previously characterized, intracellular PLA_2 isoforms could be identified in subcellular fractions isolated from ventricular myocytes, we used antibodies to known cytosolic, $cPLA_2$, and $iPLA_2$ enzymes for immunoblot

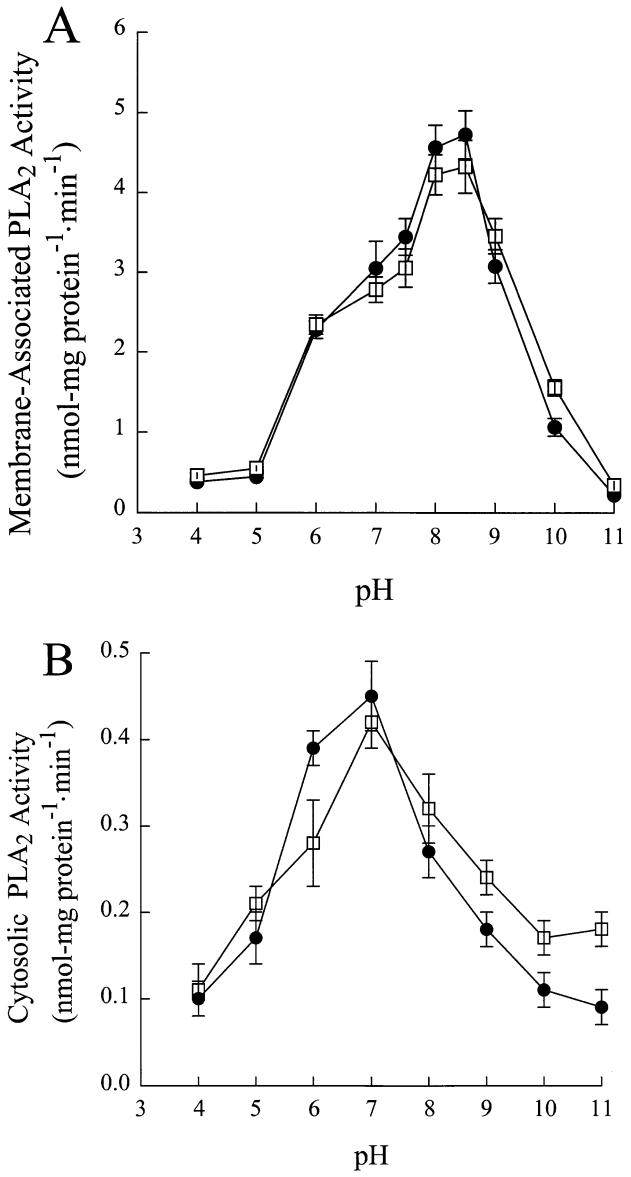

FIG. 2. The pH profile for PLA$_2$ activity at increasing pH values in membrane (A) and cytosolic (B) fractions from isolated cardiac myocytes using (16:0,[^{3}H]18:1) plasmenylcholine (●) and (16:0,[^{3}H]18:1) phosphatidylcholine (□). Values shown represent the mean ± SEM of independent results derived from three separate animals. See Figure 1 for abbreviation.

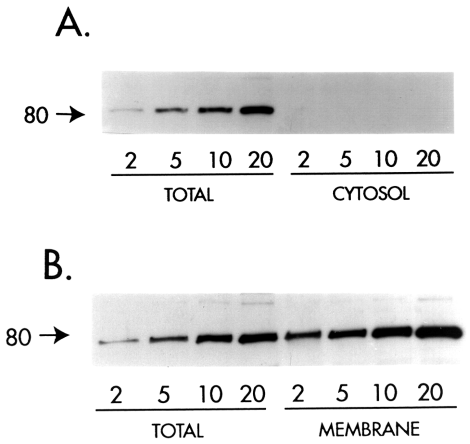

FIG. 3. Immunoblot analysis of calcium-independent PLA$_2$ (iPLA$_2$) in adult rabbit ventricular myocytes. Total (cytosol + membrane) and cytosolic protein (2–20 μg, A) and total and membrane protein (B) were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were probed with iPLA$_2$ antibody (1 in 2,000 dilution; Cayman Chemical, Ann Arbor, MI) for 60 min and incubated with horseradish peroxidase-linked secondary antibody (antirabbit, 1 in 5,000 dilution; Amersham, Arlington Heights, IL) for 45 min. Immunoblots were detected with enhanced chemiluminescence and exposure to film for 5 min. Specific iPLA$_2$ protein in the total and membrane protein migrated at approximately 80 kDa compared with molecular weight markers. No iPLA$_2$ was detectable in the cytosolic protein at comparable amounts of protein. For abbreviation see Figure 1.

analysis. By using a specific antibody to iPLA$_2$, a distinct band at approximately 80 kDa was observed in the total cellular protein and membrane protein samples. The intensity of the iPLA$_2$ band detected by immunoblot analysis was concentration-dependent over 2–20 μg protein (Fig. 3B). No band was observed in the cytosolic fraction (Fig. 3A). Thus, iPLA$_2$ appears to be selectively enriched in the membrane fraction of rabbit ventricular myocytes. Immunoblots probed with anti-cPLA$_2$ antibody did not reveal detectable cPLA$_2$ in either the cytosolic or membrane fractions from ventricular myocytes.

Ventricular myocyte membrane-associated PLA$_2$ activity was increased following stimulation of surface membrane protease-activated receptors with thrombin. Thrombin increased membrane-associated PLA$_2$ activity measured using (16:0,[^{3}H]18:1) plasmenylcholine (4.47 ± 0.87 to 9.65 ± 1.12 nmol-mg protein^{-1}·min^{-1}) phosphatidylcholine (4.37 ± 0.92 to 8.77 ± 1.37 nmol-mg protein^{-1}·min^{-1}) and alkylacyl GPC (7.04 ± 1.10 to 11.12 ± 1.63 nmol-mg protein^{-1}·min^{-1}) substrates in the absence of Ca^{2+}. To confirm that the majority of basal and thrombin-stimulated PLA$_2$ was Ca^{2+}-independent, myocytes were incubated with bromoenol lactone (BEL), a potent, mechanism-based inhibitor specific for myocardial iPLA$_2$ (14), and PLA$_2$ activity was measured in thrombin-stimulated and unstimulated cytosolic and membrane fractions using all phospholipid substrates in the absence of Ca^{2+}. Incubation of rabbit ventricular myocytes with BEL concentrations greater than 2 μM for 30 min resulted in a significant decrease in basal membrane-associated PLA$_2$ activity measured using all phospholipid substrates (Fig. 4A). Thrombin-stimulated PLA$_2$ activity was significantly inhibited at BEL concentrations greater than 5 μM and blocked completely by BEL concentrations greater than 10 μM (Fig. 4A). Cytosolic activity was also decreased significantly in BEL-pretreated myocytes (data not shown). Pretreatment with methyl arachidonyl fluorophosphonate, an irreversible inhibitor of both cPLA$_2$ and iPLA$_2$ (15) had no significant effect on basal or thrombin-stimulated PLA$_2$ activity in either the membrane fraction (Fig. 4B) or the cytosol (data not shown) at concentrations up to 20 μM. Pretreatment of isolated myocytes with the cPLA$_2$ inhibitors, 10 μM mepacrine, or 50 μM dibucaine

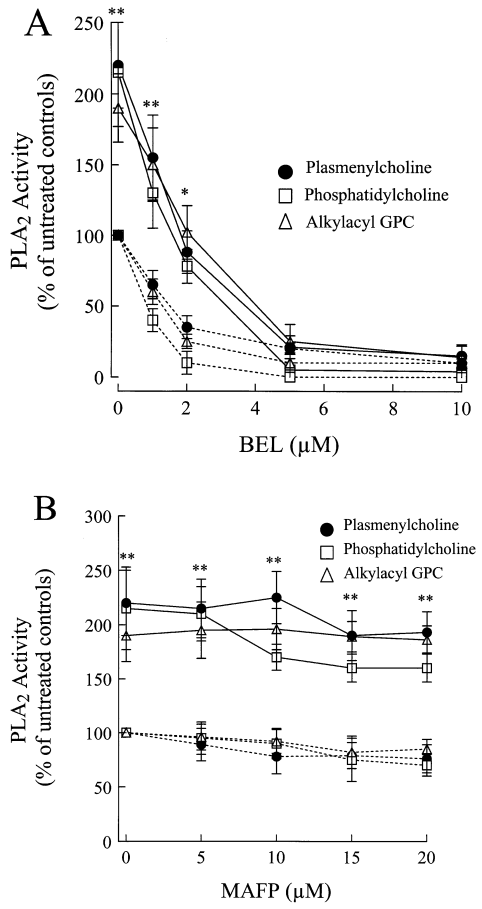

FIG. 4. Influence of preincubation of isolated ventricular myocytes with bromoenol lactone (BEL; 1 to 10 μM, 30 min, panel A), or methyl arachidonyl fluorophosphonate (MAFP; 5 to 20 μM, 15 min, B) on membrane-associated PLA_2 activity under control (dotted lines) or thrombin-stimulated (0.05 U/mL, 1 min, solid lines) conditions. Measurement of PLA_2 activity used (16:0,[^{3}H]18:1) plasmenylcholine, (16:0,[^{3}H]18:1) phosphatidylcholine, and (16:0,[^{3}H]18:1) alkylacyl GPC substrates in the absence of Ca^{2+}. Values are means ± SEM of independent results derived from six separate animals. $^*P < 0.05$, $^{**}P < 0.01$ compared to unstimulated values in the presence of the same concentration of inhibitor. Significant decreases in basal PLA_2 activity were observed in unstimulated cells at concentrations of BEL greater than 1 μM. See Figure 1 for other abbreviations.

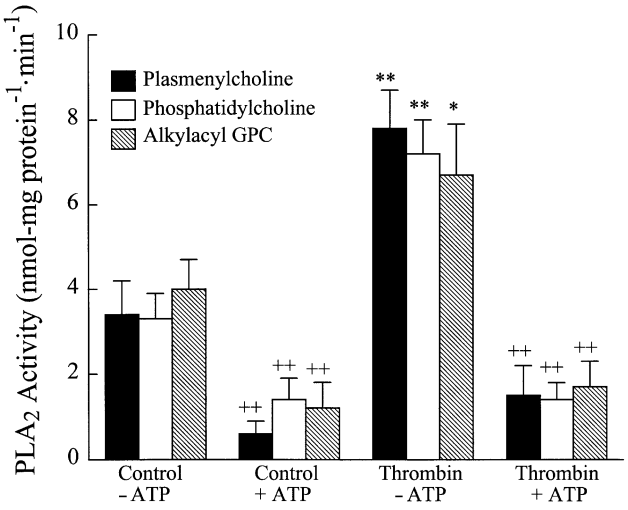

FIG. 5. Effect of including 10 mM ATP in the assay buffer on membrane-associated PLA_2 activity in cardiac myocytes under control and thrombin-stimulated (0.05 U/mL, 1 min) conditions. PLA_2 activity was measured using (16:0,[^{3}H]18:1) plasmenylcholine (filled bars), (16:0,[^{3}H]18:1) phosphatidylcholine (open bars), and alkylacyl GPC (hatched bars) substrates in the absence of Ca^{2+}. Values are means ± SEM of independent results derived from four separate animals. $^*P < 0.05$ compared to control values. $^{**}P < 0.01$ compared to control values; $^{++}P < 0.01$ compared to activity in the absence of ATP between unstimulated and thrombin-stimulated groups. See Figure 1 for abbreviations.

for 30 min had no significant effect on cytosolic or membrane-associated PLA_2 activity measured using all substrates under basal or thrombin-stimulated conditions (data not shown). These results further support our hypothesis that thrombin-stimulated, membrane-associated ventricular myocytes PLA_2 is an $iPLA_2$ isoform that is selectively inhibited by BEL.

Since previous studies of cytosolic $iPLA_2$ purified from whole myocardium indicate that the presence of ATP enhances activity, we measured ventricular myocyte PLA_2 activity in the presence and absence of 10 mM ATP. In contrast to previous findings (9), we found that the presence of ATP inhibits PLA_2 activity in both the membrane (Fig. 5) and cytosolic (data not shown) fractions isolated from rabbit myocytes that were either unstimulated or thrombin-stimulated. For example, by using (16:0,[^{3}H]18:1) plasmenylcholine, the presence of ATP reduced membrane-associated PLA_2 activity in unstimulated and thrombin-stimulated cells by 80% (Fig. 5; solid bars). To eliminate the possibility of ATP hydrolysis occurring during the PLA_2 assay procedure, we used nonhydrolyzable analogs of ATP in the assay buffer. The ATP-induced decrease in membrane-associated PLA_2 activity using (16:0,[^{3}H]18:1) plasmenylcholine in the absence of Ca^{2+} was also observed with the nonhydrolyzable analogs adenosine 5′-*O*-(3-thiotriphosphate) and adenosine 5′-(β,γ-methylene)triphosphate (Fig. 6). The addition of the nonhydrolyzable analogs of GTP, guanosine 5′-*O*-(3-thiotriphosphate) and guanyl-5′yl-imidodiphosphate, also reduced PLA_2 activity, but not as much as with the ATP analogs (Fig. 6).

Since stimulation of protease-activated receptors by thrombin is accompanied by activation of PKC, additional experiments were performed to determine whether ventricular myocyte PLA_2 activity is regulated by PKC. For these experiments, we treated cells with 100 nM phorbol 12-myristate 13-acetate (PMA) for 5 min (to activate PKC) or 24 h (to downregulate PKC). Incubation of myocytes with PMA for 5 min resulted in an approximate twofold increase in membrane-associated PLA_2 activity using all phospholipid substrates (Fig. 7). In contrast, 24-h incubation with PMA almost completely abolished PLA_2 activity (Fig. 7). Thrombin stimulation of ventricular myocytes following PMA pretreatment for 5 min or 24 h did not significantly alter PLA_2 activity from that following PMA pretreatment alone (data not shown).

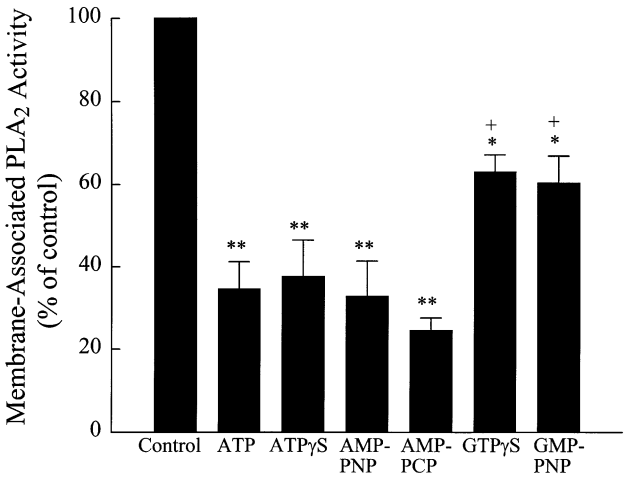

FIG. 6. Effect of ATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), adenyl-5′-yl-imidodiphosphate (AMP-PNP), adenosine 5′-(β,γ-methylene) triphosphate (AMP-PCP), guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), and guanyl-5′-yl-imidodiphosphate (GMP-PNP) inclusion in the assay buffer on PLA$_2$ activity in the membrane fraction isolated from cardiac myocytes. PLA$_2$ activity was measured using (16:0,[^{3}H]18:1) plasmenylcholine in the absence of Ca^{2+}. Values are means ± SEM of independent results derived from three separate animals. $^*P < 0.05$, $^{**}P < 0.01$ compared to activity in the absence of any analog. $^+P < 0.05$ for comparisons between corresponding ATP and GTP analogs. See Figure 1 for other abbreviation.

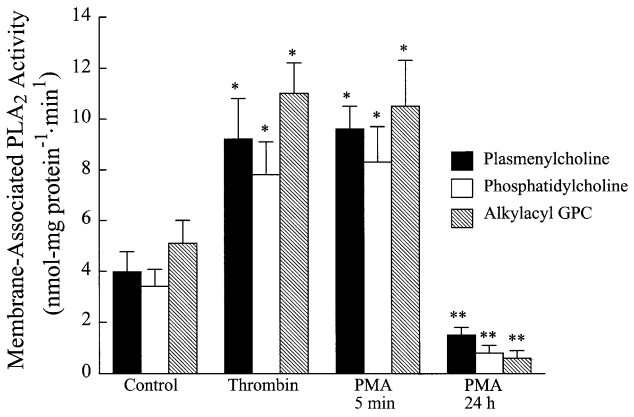

FIG. 7. Influence of incubation of isolated ventricular myocytes with phorbol 12-myristate 13-acetate (PMA) (100 nM) for 5 min or 24 h and thrombin (0.05 U/mL, 1 min) on membrane-associated PLA$_2$ activity. Measurement of PLA$_2$ activity used (16:0,[^{3}H]18:1) plasmenylcholine (filled bars), (16:0,[^{3}H]18:1) phosphatidylcholine (open bars), and (16:0,[^{3}H]18:1) alkylacyl GPC (hatched bars) substrates in the absence of Ca^{2+}. Values are means ± SEM of independent results derived from six separate animals. $^*P < 0.05$, $^{**}P < 0.01$ compared to control unstimulated values. See Figure 1 for other abbreviations.

Thus, PLA$_2$ activation in ventricular myocytes in response to thrombin stimulation is regulated by PKC activity. PKC activity also appears to play a role in the regulation of basal membrane-associated ventricular myocyte iPLA$_2$ since down-regulation of PKC activity significantly reduced basal iPLA$_2$ activity (Fig. 7).

DISCUSSION

This study demonstrates that ventricular myocyte PLA$_2$ displays a preference for phospholipid substrates with arachidonate esterified at the *sn*-2 position and does not exhibit a catalytic requirement for Ca^{2+}. The enzyme is thermolabile and requires reduced sulfhydryl groups for activity. These characteristics of PLA$_2$ in isolated ventricular myocytes clearly distinguish this enzyme activity from that catalyzed by the low-molecular weight secretory group I, II, and III PLA$_2$ since the low-molecular weight PLA$_2$ require mM concentrations of Ca^{2+} ion for maximal activity, are heat-stable, do not exhibit a distinct preference for highly unsaturated fatty acyl residues at the *sn*-2 position of phospholipid substrate molecules, and are inactivated following exposure to reduced sulfhydryl reagents (16–18).

The recently cloned 85 kDa cPLA$_2$ effectively utilizes ether-linked phospholipid substrates and displays a distinct preference for those phospholipid substrate molecular species containing esterified arachidonate (19,20). These features of substrate selectivity are virtually identical to that exhibited by ventricular myocyte PLA$_2$. Ventricular myocyte PLA$_2$ and the cloned 85 kDa cPLA$_2$ also do not require Ca^{2+} ion at the active site in order to catalyze phospholipid hydrolysis; however, cPLA$_2$ contains an amino terminal Ca^{2+}-dependent lipid binding domain that functions to mediate attachment of the enzyme to aggregated phospholipid substrate interfaces (19,20). This process greatly increases the dwell time for the enzyme at the interface, resulting in markedly enhanced rates of phospholipid hydrolysis in response to alterations in ionized Ca^{2+} concentration in the physiologic (0.1–10 M) range. Thus, the proposed mechanism for activation of cPLA$_2$ is Ca^{2+}-dependent translocation of the enzyme from the cytosolic fraction to the membrane interface mediated by the Ca^{2+}-dependent lipid binding domain. Cytosolic PLA$_2$ activity is also inhibited by methyl arachidonyl fluorophosphonate (MAFP) (16). In contrast, ventricular myocyte PLA$_2$ is maximally active at Ca^{2+} concentrations below 1 nM (i.e., nominally Ca^{2+}-free media at 4 mM EGTA concentration) and is not inhibited by MAFP; activity measured with ether-linked phospholipid substrates is significantly attenuated when the Ca^{2+} concentration is greater than 10^{-6} M. The differential responses to Ca^{2+} and MAFP and the membrane localization of ventricular myocyte PLA$_2$ illustrate substantial differences between ventricular myocyte PLA$_2$ and the 85 kDa cPLA$_2$. The membrane localization and differential Ca^{2+} responses of the ventricular myocyte PLA$_2$ also distinguish this enzyme activity from the cloned 30 kDa dimeric cytosolic PLA$_2$ isolated from platelets which exhibits considerable sequence similarity to the 14-3-3 family of signal transduction proteins (21).

A cytostolic iPLA$_2$ isoform was purified or cloned from Chinese hamster ovary (CHO) cells and P388D$_1$ macrophages. This iPLA$_2$ protein has a molecular weight of 80–85

kDa. Immunoblot analysis using antiserum against this $iPLA_2$ protein demonstrated that $iPLA_2$ immunoreactivity could be detected in the membrane fraction of rabbit ventricular myocytes but was not detected in the cytosol. The membrane-associated form of $iPLA_2$ in ventricular myocytes has a molecular mass of approximately 80 kDa, similar to that reported in $P388D_1$ macrophages and CHO cells. Thus, membrane-associated $iPLA_2$ in isolated rabbit ventricular myocytes has sufficient homology with the previously cloned $iPLA_2$ from CHO cells for the antibody to recognize it. However, the $iPLA_2$ present in isolated ventricular myocytes possesses several characteristics that distinguish it from $iPLA_2$ in CHO or $P388D_1$ cells. Previously described isoforms of $iPLA_2$ were demonstrated to be cytosolic enzymes (22,23), whereas the overwhelming amount of $iPLA_2$ in ventricular myocytes assessed by immunoblot or activity measurements is membrane-associated and not removed from the membrane by sonication, removal of Ca^{2+}, or variations in salt concentration. In addition, we demonstrated that $iPLA_2$ in ventricular myocytes is the major isoform responsible for arachidonic acid release and lysophospholipid production in response to stimulation, whereas studies in $P388D_1$ cells indicate that $iPLA_2$ plays a minor role in signal transduction (24). Further studies are necessary to determine whether ventricular myocyte and $P388D_1$ macrophage $iPLA_2$ represent alternative splicing products or post-translational modifications of the same gene product with different localization and intracellular function in different cell types.

Previous studies of PLA_2 activity prepared by homogenization of whole myocardium demonstrated the presence of both cytosolic and membrane-associated enzymes which also exhibit maximal activities in the absence of Ca^{2+} (i.e., nominally Ca^{2+}-free with mM concentrations of EGTA or EDTA) and demonstrate a preference for arachidonylated ether-linked phospholipid substrates, particularly plasmalogens (1–4). Cytosolic $iPLA_2$ and plasmalogen-selective PLA_2 were isolated from homogenates of dog, rabbit, and human myocardium (1–4). These enzymes exhibit catalytic features that are virtually identical to those described for ventricular myocyte PLA_2, but differ from the latter by the fact that these enzymes are present in soluble form, whereas the majority of ventricular myocyte PLA_2 appears to be firmly anchored to the membrane. In homogenates of whole myocardium, it was demonstrated that ischemia results in the selective activation of membrane-associated $iPLA_2$ (1,4). The increase in $iPLA_2$ activity during ischemia appears to be the result of the activation of a latent, membrane-associated enzyme and not the result of translocation of $cPLA_2$ to the membrane as described previously for $cPLA_2$ (21,25,26). Thus, it is reasonable to conclude that separate and distinct cytosolic and membrane-associated forms of $iPLA_2$ exist.

Since during the course of isolation, ventricular myocytes may undergo some degree of metabolic compromise, it is possible that the predominance of membrane-associated $iPLA_2$ activity we describe in isolated ventricular myocytes may reflect, in part, the increase in membrane-associated $iPLA_2$ activity described previously during myocardial ischemia (1,4). However, ischemia-induced increases in myocardial membrane-associated $iPLA_2$ are reversible upon reperfusion (1), and our isolated myocytes are maintained in oxygenated buffer and do not exhibit creatine kinase or lactate dehydrogenase release, electrophysiologic abnormalities, or morphologic changes suggestive of ischemic cell injury.

Several recent studies provided insight into the regulation of purified cytosolic PLA_2 from whole myocardium, suggesting that multiple mechanisms may regulate PLA_2 activity in the myocardium. Coordination of anaerobic glycolysis and phospholipolysis in myocardial ischemia appears to be regulated by the highly specific association of a PFK isoform with cytosolic $iPLA_2$ (8). This association between PFK and $iPLA_2$ allows increased production of glycolytically derived ATP during ischemia to result in allosteric alterations in the conformation of PFK which lead to an increase in cytosolic $iPLA_2$ activity, and increased phospholipolysis perhaps even under conditions of global ATP depletion. In addition, there is evidence that depletion of Ca^{2+} from intracellular stores, such as could occur during myocardial ischemia, may also activate cytosolic $iPLA_2$ by interactions between $iPLA_2$, calmodulin, and Ca^{2+} (10). Our studies demonstrating that ATP and its nonhydrolyzable analogs inhibit membrane-associated $iPLA_2$ in both basal and thrombin-stimulated conditions suggest that activation of membrane-associated ventricular myocyte PLA_2 could result from a decrease in global ATP concentration during myocardial ischemia. This effect could be additive and independent of the activation of cytosolic $iPLA_2$. In any case, our data demonstrating a distinct difference in pH optima for cytosolic and membrane-associated $iPLA_2$ and the differential ATP response of isolated ventricular myocyte membrane-associated $iPLA_2$ and purified cytosolic $iPLA_2$ from whole myocardium suggest that these enzyme activities may be mediated by distinct catalytic entities.

Previous studies suggested that PLA_2 activity is regulated by PKC (27–29), however, this was not demonstrated previously in isolated cardiac myocytes. We demonstrated that activation of PKC by brief exposure to PMA results in increased ventricular myocyte PLA_2 activity, whereas downregulation of PKC by prolonged PMA exposure almost completely abolishes PLA_2 . Recently, it was suggested that BEL may decrease arachidonic acid release by inhibiting phosphatidic acid phosphohydrolase (PAP) which would inhibit the production of DAG from phosphatidic acid (30). Since DAG activates PKC, and since we have demonstrated that PLA_2 activity is regulated by PKC, it is possible that attenuation of PLA_2 activity by BEL may be mediated through this alternative pathway. However, we were able to demonstrate inhibition of PLA_2 activity in ventricular myocytes with BEL concentrations as low as 2 μM. Complete inhibition of PAP with BEL was not observed with concentrations as high as 50 μM; thus, we believe that the dramatic inhibition of PLA_2 activity observed using BEL in this study is a result of direct inhibition of PLA_2, although we cannot rule out the possibility of the involvement of an alternative inhibitory pathway operating through inhibition of PAP by BEL.

In conclusion, we demonstrated the presence of PLA_2 activity in isolated mammalian ventricular myocytes, the majority of which is membrane-associated, Ca^{2+}- independent with maximal activity measured with phospholipid substrates with an ether linkage at the *sn*-1 position and arachidonic acid at the *sn*-2 position. Important differences between this enzyme(s) and PLA_2 enzymes reported previously suggest that this is a novel PLA_2 enzyme that has not been characterized to date.

ACKNOWLEDGMENTS

Research from the authors' laboratory was supported in part by the National Institutes of Health Grant HL54907-01 (JM), Veterans Administration Research Career Development Award Program (MHC), Veterans Administration Merit Review Grant Program (MHC), and the American Heart Association (JM and MHC). The authors gratefully acknowledge Hofmann La-Roche for the gift of BEL and the technical assistance of Jan Jones and RaeTreal McCrory.

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[Received March 10, 1998, and in final revised form September 25, 1998; revision accepted November 11, 1998]

Phytanic Acid α-Hydroxylation by Bacterial Cytochrome P450

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ABSTRACT: Fatty acid α-hydroxylase, a cytochrome P450 enzyme, from *Sphingomonas paucimobilis,* utilizes various straight-chain fatty acids as substrates. We investigated whether a recombinant fatty acid α-hydroxylase is able to metabolize phytanic acid, a methyl-branched fatty acid. When phytanic acid was incubated with the recombinant enzyme in the presence of H_2O_2, a reaction product was detected by gas chromatography, whereas a reaction product was not detected in the absence of H_2O_2. When a heat-inactivated enzyme was used, a reaction product was not detected with any concentration of H_2O_2. Analysis of the methylated product by gas chromatography–mass spectrometry revealed a fragmentation pattern of 2-hydroxyphytanic acid methyl ester. By single-ion monitoring, the mass ion and the characteristic fragmentation ions of 2-hydroxyphytanic acid methyl ester were detected at the retention time corresponding to the time of the product observed on the gas chromatogram. The K_m value for phytanic acid was approximately 50 μM, which was similar to that for myristic acid, although the calculated V_{max} for phytanic acid was about 15-fold lower than that for myristic acid. These results indicate that a bacterial cytochrome P450 is able to oxidize phytanic acid to form 2-hydroxyphytanic acid.

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Fatty acid α-hydroxylase (FAAH) activity has been found in various organisms, but this enzyme has not been fully characterized. We have studied FAAH isolated from *Sphingomonas paucimobilis* since this organism contains a large amount of 2-hydroxymyristic acid, a product of α-hydroxylation (1). FAAH purified from *S. paucimobilis* oxidized straight-chain saturated and unsaturated fatty acids in the presence of H_2O_2, with the oxygen atom from H_2O_2 being incorporated into the fatty acid to form the corresponding 2-hydroxy fatty acid (2,3). Recently, we have cloned the FAAH gene (4). Sequence analysis of the FAAH gene and spectral analysis of recombinant FAAH revealed that FAAH from *S. paucimobilis* is a novel member of the cytochrome P450 (P450) superfamily. This recombinant form of FAAH avidly α-hydroxylated myristic acid. Bacterial FAAH, a P450 enzyme, is a soluble enzyme similar to other bacterial P450s, but belongs to the E-class (5) like $P450_{BM-3}$. FAAH is saturated with very small amounts of H_2O_2 (approximately 60 μM), but not other hydroperoxides, to catalyze α-hydroxylation of fatty acids. In this regard, bacterial FAAH is a unique P450 enzyme.

In general, P450s catalyze hydroxylation of a variety of endogenous substances (e.g., fatty acids and steroids) and xenobiotics (e.g., drugs and environmental pollutants). Among bacterial P450s, $P450_{BM-3}$, isolated from *Bacillus megaterium,* is also involved in the metabolism of nonesterified fatty acids (6). Unlike FAAH, $P450_{BM-3}$ catalyzes ω-*n* (*n* = 1–3) hydroxylation of saturated and monounsaturated fatty acids. Recently, English *et al.* (7) demonstrated that phytanic acid (3,7,11,15-tetramethyl-hexadecanoic acid) derived from the phytyl chain of chlorophyll was a good substrate for $P450_{BM-3}$, as were straight-chain fatty acids.

On the other hand, in mammals, phytanic acid initially undergoes α-oxidation, by which the carboxyl carbon is removed, and then is degraded *via* β-oxidation (8). Refsum disease in humans is caused by a deficiency in phytanic acid α-hydroxylation, the rate-limiting step in the α-oxidation pathway (9).

These observations led us to investigate whether bacterial FAAH can utilize phytanic acid as a substrate. We wished to clarify the involvement of FAAH in phytanic acid α-oxidation of this bacterium and to compare bacterial FAAH to mammalian FAAH. The present study was undertaken to define the metabolism of phytanic acid by the use of the bacterial recombinant FAAH.

MATERIALS AND METHODS

Materials. Phytanic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was purchased from Wako Pure Chemicals (Osaka, Japan). The expression plasmid pGEX-4T-3 was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). Diazomethane was synthesized from *N*-nitroso-*N*-methylurea (Sigma Chemical Co., St. Louis, MO). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Preparation of recombinant FAAH and assay of FAAH activity. AgeI and EcoR52I sites were found 7 bp upstream from the translation initiation codon and 294 bp downstream of the termination codon of the FAAH gene, respectively. A blunt-ended AgeI-EcoR52I fragment including the FAAH gene was

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Abbreviations: FAAH, fatty acid α-hydroxylase; GC–MS, gas chromatography–mass spectrometry; P450, cytochrome P450.

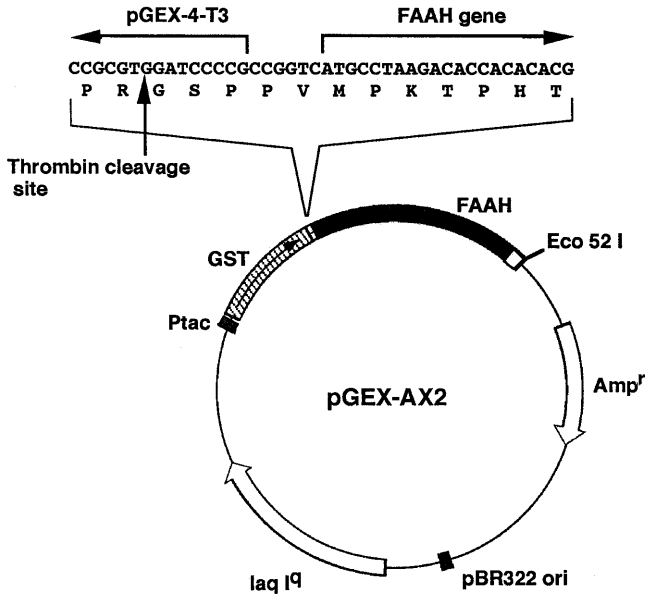

FIG. 1. Schematic representation of the expression plasmid including the full-length fatty acid α-hydroxylase (FAAH) gene.

ligated into blunt-ended EcoRI-NcoI-cleaved pGEX-4T-3 (Fig. 1). Expression and purification of the recombinant FAAH were performed as previously described (4). FAAH was purified to homogeneity on sodium dodecylsulfate-polyacrylamide gel. Specific content of the purified FAAH was 10.2 nmol/mg protein. In this procedure, the purified enzyme protein contained the entire FAAH enzyme with an additional five amino acids at its N-terminus. The standard reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 0.2 mM H_2O_2, 20 nmol of phytanic acid dissolved in 1 μL of ethanol, and 0.2 μg of the purified recombinant FAAH in a total volume of 0.2 mL. The reaction was performed at 37°C and terminated by the addition of 20 μL of 2 N HCl. Phytanic acid and the reaction product were extracted with ethyl acetate, and the ethyl acetate layer was collected. After washing with distilled water, the ethyl acetate layer was evaporated under vacuum. The resultant residue was then treated with diazomethane. Quantitation of phytanic acid and the reaction product was determined by gas chromatography (GC).

Identification of the reaction product by gas chromatography–mass spectrometry (GC–MS). GC–MS was performed on the 5971A mass spectrometer (Hewlett-Packard, Palo Alto, CA) with a 5890 series II gas chromatograph (Hewlett-Packard) using a DB-5MS capillary column (30 m × 0.25 mm, film thickness 0.1 mm, J&W Scientific, Folsom, CA). For GC, samples (1 μL) were introduced onto the column. Injector temperature was 280°C. A GC oven temperature program was then applied: an initial temperature of 80°C was held for 1 min, and then the temperature was raised to 280°C in 8°C/min increments and held at 280°C for 4 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC–MS interface temperature was 280°C. Electron-impact MS was performed with an ion source temperature of 280°C, emission current of 0.3 mA, and electron energy of 70 eV.

RESULTS AND DISCUSSION

When phytanic acid was incubated as a substrate with recombinant FAAH in the presence of H_2O_2, a reaction product was detected by GC at a retention time of 19.7 min (Fig. 2). To determine whether this reaction product was formed enzymatically, we investigated the effects of incubation period, the amount of H_2O_2 added to the reaction mixture, and the effects of heat treatment of FAAH on the amount of product formed. In the presence of H_2O_2, the amount of product increased in a time-dependent manner and reached a plateau at 20 min, whereas in the absence of H_2O_2, a product was not detected with any incubation time (Fig. 3A). When the FAAH enzyme was incubated with the substrate, the amount of product increased as the concentration of H_2O_2 in the reaction mixture increased and approached a plateau at a concentration of 0.2 mM (Fig. 3B). Conversely, when heat-treated enzyme was used, no product was formed with any concentration of H_2O_2. These results indicated that formation of the product was due to an enzymatic reaction of the recombinant FAAH.

To identify the reaction product, analysis by GC–MS was performed. The mass spectrum of the methylated product displayed a pattern identical to that of 2-hydroxyphytanic acid methyl ester (Fig. 4), which has been previously reported (10). The molecular ion is *m/z* 342. The characteristic fragment ion of 2-hydroxy fatty acid, *m/z* 283, has lost the car-

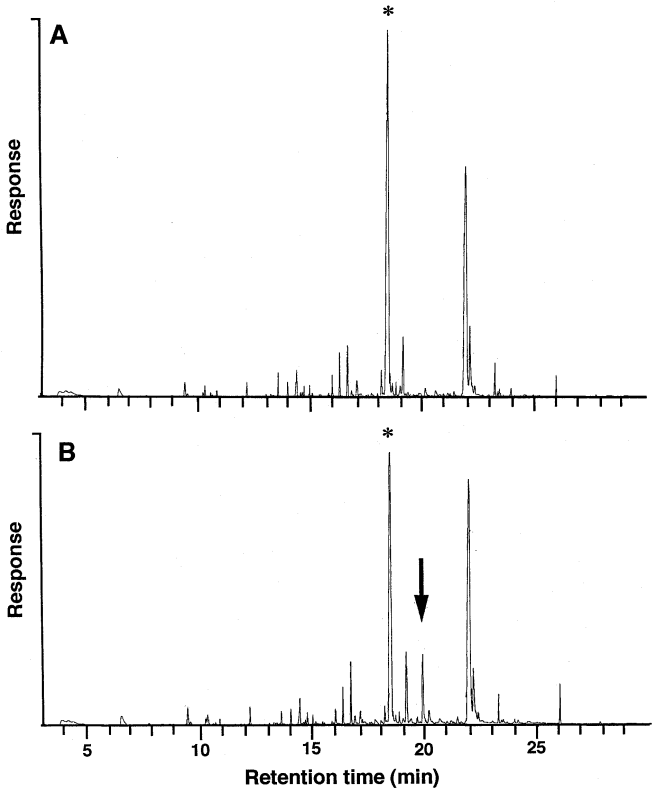

FIG. 2. Gas chromatograms of phytanic acid methyl ester and the methylated product at time 0 (A) and after a 10 min incubation with FAAH (B). The asterisks and arrows show phytanic acid methyl ester and the methylated product, respectively. For abbreviation see Figure 1.

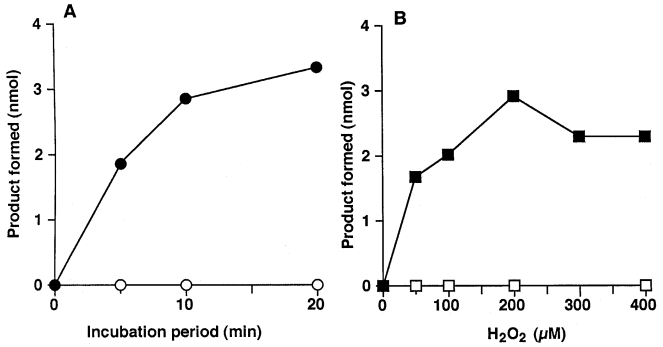

FIG. 3. Changes in the amount of product formed as a result of (A) varying the incubation period in the presence (●) and absence (○) of H_2O_2 and (B) varying the amount of H_2O_2 used with heat-treated (□) and non-treated enzyme (■). Heat treatment 100°C for 10 min.

boxylmethyl group of *m/z* 342. Cleavage of the C_2-C_3 bond and the McLafferty rearrangement yield *m/z* 90. The fragments *m/z* 117 and 159 are formed by cleavages between C_3 and C_4 and between C_6 and C_7, respectively. These ions (*m/z* 342, 283, 159, 117, 90) were observed at a retention time of 19.7 min (Fig. 5). Only negligible amounts of these ions were detected at other retention times. Therefore, we concluded that the reaction product was 2-hydroxyphytanic acid.

We compared K_m and V_{max} for phytanic acid to those for myristic acid. As shown in Figure 6, the K_m value for phytanic acid (50 μM) was similar to that for myristic acid (43 μM). However, the calculated V_{max} for myristic acid (44 μmol/min/mg protein) was about 15-fold higher than that for phytanic acid (2.9 μmol/min/mg protein).

In *S. paucimobilis*, 2-hydroxymyristic acid, a product of α-hydroxylation of myristic acid, was found as a component of sphingoglycolipids which are characteristic of this bacterium (1). The majority of the fatty acid of sphingolipid in this bacterium is 2-hydroxymyristic acid. Therefore, FAAH was speculated to be normally involved in sphingolipid metabolism where myristic acid is α-hydroxylated and incorporated into ceramide. Interestingly, phytanic acid was also α-hydroxylated by this bacterial FAAH. The bacterial FAAH metabolized phytanic acid at a slower rate than myristic acid, although the metabolic rate of phytanic acid by FAAH was still comparable to those of straight-chain fatty acids by mammalian ω-hydroxylase of CYP4A subfamily (11). However, judging from K_m values for these fatty acids, the affinity of

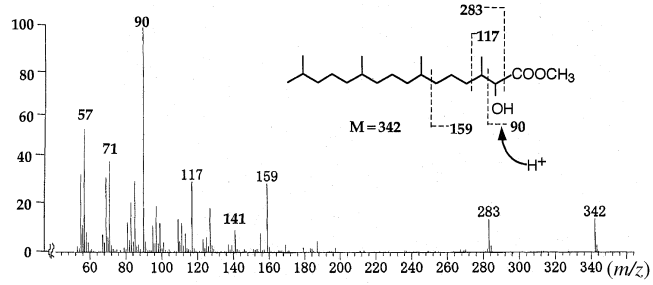

FIG. 4. Mass spectrum of the methylated product, having *m/z* of 342 for the mass ion (M).

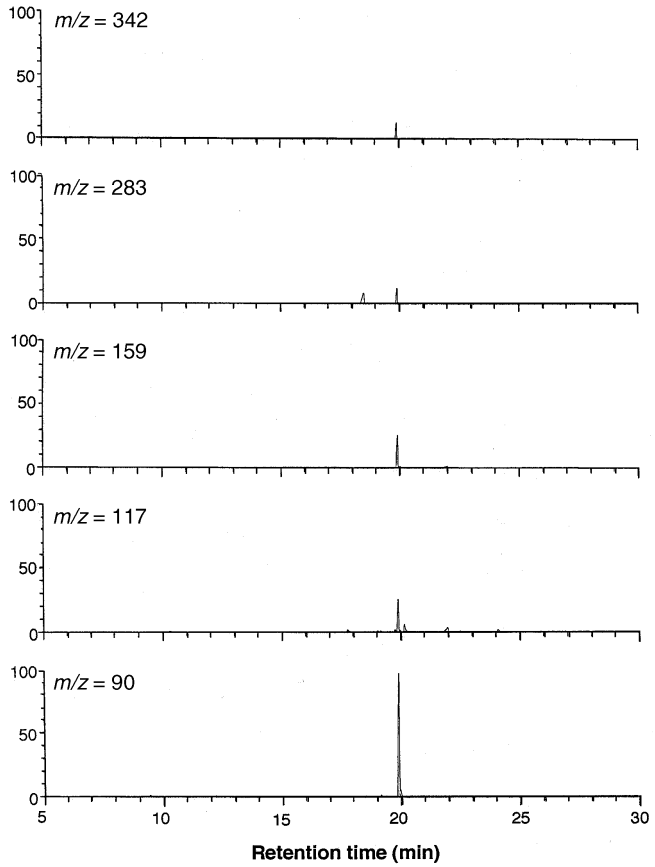

FIG. 5. Single-ion monitoring of mass ion (*m/z* 342) and fragmentation ions (*m/z* 283, 159, 117, and 90) of the methylated product.

phytanic acid for FAAH is almost the same as that of myristic acid, suggesting significant involvement of FAAH in catabolism of phytanic acid in this bacterium. Furthermore, *S. paucimobilis* is a soil bacterium, and thus it is possible that it will

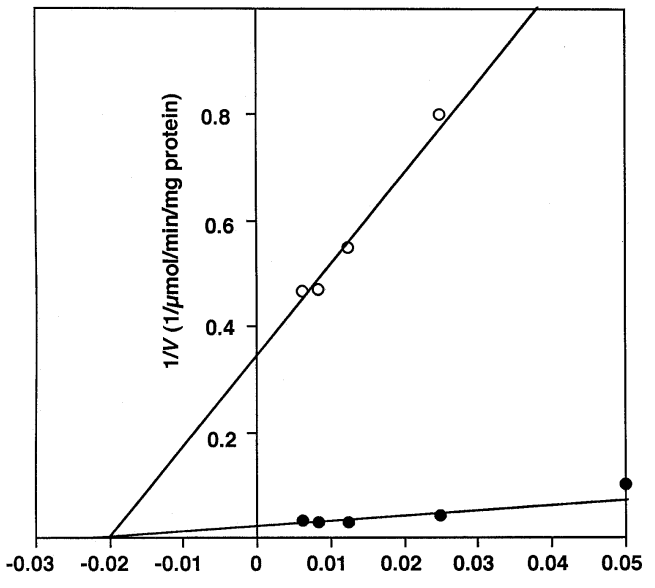

FIG. 6. Lineweaver-Burk plot of FAAH with phytanic acid (○) and myristic acid (●) as substrates. Incubation period was 5 min. For abbreviation see Figure 1.

be exposed to phytanic acid as a breakdown product of plant matter. Therefore, a metabolic pathway for phytanic acid would be useful for this bacterium. However, the exact significance of phytanic acid oxidation to 2-hydroxyphytanic acid by bacterial FAAH remains to be elucidated.

English *et al.* (7) demonstrated that phytanic acid is hydroxylated by $P450_{BM-3}$ to form (ω-1)-hydroxyphytanic acid. They also reported that phytanic acid and (ω-1)-hydroxyphytanic acid as xenobiotic compounds regulated transcription of the $P450_{BM-3}$ gene. We have not yet investigated the occurrence of regulation of FAAH gene expression by such fatty acids. FAAH significantly metabolized phytanic acid as a substrate. Thus, we are now interested in studying the regulation of the FAAH gene by phytanic acid.

In mammals, the α-oxidation pathway is indispensable in catabolizing 3-methyl-branched fatty acids, including phytanic acid, because the 3-methyl branch of phytanic acid prevents its degradation *via* the β-oxidation pathway. Refsum disease is an inherited human disorder caused by a deficiency of phytanic acid α-hydroxylation (9). Recently, Mihalik *et al.* (12) and Jansen *et al.* (13) identified the gene responsible for Refsum disease. They reported that this gene encoded phytanoyl-CoA α-hydroxylase, which is a 2-oxoglutarate-dependent dioxygenase. Bacterial FAAH does not metabolize acyl-CoA (2). Therefore, bacterial FAAH can apparently be distinguished from mammalian phytanoyl-CoA α-hydroxylase, due to differing properties of these enzymes such as substrate specificity. Thus, in bacteria, other catabolic pathways of phytanic acid may also exist to metabolize this exogenous fatty acid.

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[Received July 13, 1998, and in final revised form November 2, 1998; revision accepted November 11, 1998]

In Vivo Studies of the Biosynthesis of Vernolic Acid in the Seed of *Vernonia galamensis*

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ABSTRACT: *In vivo* radiotracer experiments using [1-^{14}C]acetate as the precursor were conducted to investigate the biosynthesis of vernolic acid (12, 13-epoxy-*cis*-9-octadecenoic acid) in the seeds of *Vernonia galamensis*. The acetate precursor radioactively labeled vernolate in phosphatidylcholine (PC), diacylglycerol, and triacylglycerol. Time-course kinetics of the incorporation of the radioactive tracer indicated that vernolate is synthesized while the acyl moiety is esterified to PC. Pulse-chase experiments provided additional supporting evidence that vernolate is synthesized while esterified to PC. These results are consistent with the hypothesis that linoleoyl PC is the precursor of vernoleoyl-PC. Subsequently, vernolate is quickly moved from the PC pool to the triacylglycerol pool, where it accumulates.

Lipids 33, 1217–1221 (1998).

Epoxidized oils such as epoxidized soybean and palm oils are widely used as plasticizers and stabilizers for plastics (1,2). The epoxy fatty acids can increase the flexibility and stability of plastics, particularly polyvinyl chloride. Currently, epoxidized oils are produced by the treatment of unsaturated oils with peracetic acid to introduce epoxy rings into the unsaturated positions of the fatty acids. Epoxidation of vegetable or animal oils and fats is a relatively expensive chemical process. Plant seed oils that contain vernolic (12,13-epoxy-*cis*-9-octadecenoic) acid, a natural epoxy fatty acid, could be substituted for chemically epoxidized oils. Only a few plants contain large percentages of vernolate in their seed triacylglycerol (TAG), and none of them are cultivated presently in the United States (1–4). *Vernonia galamensis*, whose seed contains 40% lipid of which 80% is vernolate, was successfully cultivated in Africa (3,5), but little has been done to improve the plant's agronomic properties.

Research on the epoxidation of fatty acids is receiving increasing attention (6–9). Bafor *et al*. (7) studied the formation of vernolate in the microsomes of *Euphorbia lagasca,* and they postulated that linoleoyl PC was the substrate for epoxidation, and the resulting vernoleoyl PC was hydrolyzed to give free vernolic acid, which was incorporated into TAG after activation as a CoA ester. More recently, the enzyme catalyzing the epoxidation of linoleate was identified from *Crepis palaestina* (9). We describe herein *in vivo* radiotracer studies of the biosynthesis of vernolic acid in the developing seeds of *V. galamensis*, using [1-^{14}C]acetate as the precursor.

MATERIALS AND METHODS

Materials. *Vernonia galamensis* was grown from seeds in a greenhouse under 14-h artificially supplemented illumination, at a temperature ranging between 22 and 25°C. To promote flowering, 90-d-old plants were grown for a period of 30 d in the same conditions, except illumination was 10 h per day. Under these conditions, seeds matured in about 35 d after flowering (DAF). Developing seeds rapidly accumulate lipids between 21 and 28 DAF and then start to desiccate. In our studies, seeds were collected between 21 and 28 DAF and expelled from their seedcoats. The decoated seeds were kept in ice-cold phosphate buffer (0.1 M, pH 7.2) and used immediately. Sodium [1-^{14}C]acetate (55 Ci/mol) solution was purchased from ICN (Irvine, CA). Chemicals and biochemicals were obtained from Sigma (St. Louis, MO) and Fisher Chemicals (Pittsburgh, PA).

In vivo *radioisotopic labeling*. In time-course experiments, approximately 6 g of decoated seeds were placed in 2 mL of 0.1 M phosphate buffer, pH 7.2, containing 10 μCi of sodium [1-^{14}C]acetate (55 Ci/mol) and incubated at room temperature (about 25°C) while shaking at 100 rpm. At various times after the start of the incubation, 15 seeds (about 1 g) were removed and washed three times with 1 mL of ice-cold water. The seeds were placed in 1 mL of water, heated for 15 min in a boiling-water bath to inactivate phospholipases, and stored at –30°C until the lipid was extracted.

For the pulse-chase experiments, about 7 g of decoated seeds were incubated as above in the presence of 20 μCi of [1-^{14}C]acetate (55 Ci/mol) for 1 h. After this pulse of label, the buffer containing the radioisotope was removed, and 15 seeds (about 1 g) were washed and heated in water as previously described. The remaining seeds were washed three times with 5 mL of phosphate buffer containing nonradioac-

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Abbreviations: DAF, days after flowering; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TAG, triacylglycerol; TLC, thin-layer chromatography.

tive 10 mM sodium acetate. Incubation was continued as before in fresh phosphate buffer in the absence of the radioisotope. At various times after the start of the chase incubation, samples of 15 seeds were removed, processed, and stored as previously described.

All *in vivo* radioactive labeling experiments were conducted three times, and very similar results were obtained each time. In each type of experiment, the data from one typical experiment were presented.

Lipid extraction. The seeds were homogenized with 1 mL of methanol in a 7-mL Broeck Tissue Grinder (Fisher Chemicals). The homogenates were centrifuged, the supernatant was removed and retained, and the residual solids were extracted twice with 2 mL of chloroform/methanol (2:1, vol/vol) and once with 2 mL of chloroform. All four extracts were combined, 0.9 mL of water was added, and the chloroform layer containing the lipids was recovered and concentrated by evaporation under reduced pressure at ambient temperature. The recovered lipids were dissolved in 1 mL of chloroform/methanol (2:1) and stored under nitrogen at –30°C.

Lipid separations and analyses. Lipid classes were fractionated by thin-layer chromatography (TLC) on silica gel H plates (200 × 200 × 0.5 mm) impregnated with 0.8 mM sodium carbonate (10). All lipids were identified by comparing their migration to authentic lipid standards. Polar lipids were fractionated with the solvent chloroform/methanol/acetic acid-water (25:15:8:4, by vol). Neutral lipids were fractionated with hexane/diethyl ether/acetic acid (60:40:1, by vol). These fractionations were undertaken either on separate plates or by double development. In the latter, the polar lipids were first fractionated by developing the plate half way to the top; the solvent was evaporated under a stream of nitrogen or carbon dioxide, and the neutral lipids, which were in the solvent front, were separated by developing the plate all the way to the top with the second solvent. After chromatography, the developing solvents were removed under a stream of nitrogen or carbon dioxide, and the neutral lipid bands were located by exposing the vertical edges of the plates to iodine vapor.

For fatty acid analysis, the portion of the silica gel containing each lipid that had not been exposed to iodine was scraped from the TLC plates, and the fatty acids were directly converted to methyl esters without removal of the lipids from the silica gel (11). To accomplish this, the silica was treated with 0.5 mL of 1 M sodium methoxide in methanol and 1 mL of diethyl ether and incubated with shaking at 30–40°C for 1 h. Next, 20 µL of glacial acetic acid and 1 mL of water were added, and the fatty acyl methyl esters were recovered by extraction with diethyl ether. The ether was evaporated under a stream of nitrogen or carbon dioxide and the residue was dissolved in 0.1–0.3 mL of methanol. The methyl esters were analyzed immediately after derivatization on a Beckman System Gold HPLC fitted with a 4 mm × 25 cm Supelco C-18 column (State College, PA), using 5% water in methanol as the mobile phase. The eluate of fatty acyl methyl esters was detected by their absorbance at 202 nm with an in-line ultraviolet detector (Beckman Model 166). Radioactivity was detected with an in-line radioactivity detector (Beckman Model 171) equipped with a 1-mL cell, using Ready Flow III (Beckman) as the scintillation cocktail. The methyl ester peaks were identified by comparing their retention times with those of standards.

The fatty acid compositions of lipids from developing and mature seeds were analyzed as methyl esters by GC (Model 5890; Hewlett-Packard, Wilmington, DE) fitted with a flame-ionization detector and a 15-m × 0.244 mm DB-23 capillary column (J&W, Deerfield, IL) operated at 200°C with a helium flow of 1 mL/min. Peaks were identified by comparing retention times with those of standards.

Determination of radioactivity. An aliquot of lipid sample was mixed with 4 mL of ScintiVerse BD (Fisher Scientific) in 4-mL polypropylene vials, and radioactivity was detected on a scintillation analyzer (Model 2200CA; Packard Instruments, Downers Grove, IL). Lipid bands on TLC plates were revealed with iodine vapor, the lipid-containing silica was scraped into vials, and radioactivity was determined as described.

RESULTS AND DISCUSSION

Lipid accumulation during seed development. In the greenhouse conditions used in these experiments, *V. galamensis* seeds matured by 35 DAF. During this period, the lipid content of the seeds increased gradually to reach 5% by 21 DAF, and then more rapidly to reach 28% at 28 DAF. The seeds subsequently desiccated and had a lipid content of 34% at 35 DAF (Fig. 1). Thus, in the experiments described below, seeds between 21 and 28 DAF were used because this is the period when lipids rapidly accumulate.

Vernolic acid was found mainly in the neutral lipids, TAG and diacylglycerol (DAG), where it accounts for 61.9 and 54.6% of the total fatty acids, respectively (Table 1). Linoleate was the second most prevalent acyl component in TAG and DAG. In polar lipids, linoleate was the major fatty acid (data not shown). Of the three phospholipids examined,

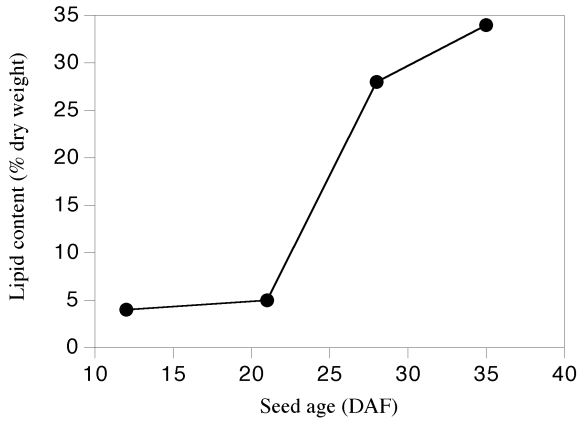

FIG. 1. Lipid accumulation in developing seeds of *Vernonia galamensis.* Average of two determinations. DAF, days after flowering.

TABLE 1
The Fatty Acid Compositions (wt%) of the Various Lipid Fractions in Developing Seeds 21 DAF[a]

Lipids	16:0	18:0	18:1	18:2	Vernolic
TAG	6.4	3.0	5.3	23.4	61.9
DAG	9.3	3.9	10.5	21.6	54.6
PC	15.5	5.9	13.7	59.4	5.3

[a]Average of two determinations. TAG, triacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; DAF, days after flowering.

phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylcholine (PC), only the latter contained detectable amounts of vernolic acid (5.3%).

In vivo *labeling of seed lipids with [1-^{14}C]acetate*. As shown in Figure 2A, [1-^{14}C]acetate was rapidly incorporated into lipids in the first 4 h of incubation, but in the subsequent 4 h little increase of radioactivity occurred either in total or individual lipids. PC was the most heavily radioactively labeled lipid throughout the incubation time. Initially DAG was more heavily radioactively labeled than TAG; however, after 2 h the radioactivity in DAG reached a plateau and by 4 h the radioactivity in TAG exceeded that in DAG. The only other lipids that incorporated radioactivity to any significant extent were PE and PI, which accounted for only 3 to 6% of the total radioactivity each and are not shown in the figures. No vernolate was detected in these lipids.

The distribution of radioactivity in each acyl group of the various lipids was determined by transesterification of each isolated lipid to form the methyl acyl esters, which were analyzed by radio-high-performance liquid chromatography. In the three lipids that were examined in detail (PC, DAG, and TAG), the oleoyl moieties were the most heavily labeled fatty acid in the initial stages of the incubation (Fig. 2B–D). With increasing time, the proportion of label in the oleoyl moieties declined as the proportion of label increased in the linoleoyl and vernoleoyl moieties of the three lipids. Vernolate first became radioactively labeled on PC (Fig. 2B and E). With increasing incubation time, radioactive label was incorporated into the vernoleoyl moieties of DAG and TAG. In all these lipids, saturated fatty acids (16:0 and 18:0, not shown in the figure) were the least labeled (~1%), and the percentage of label did not change with the incubation time.

The results of these experiments are consistent with the hypothesis that vernolate is initially synthesized while it is esterified to PC. Subsequently, the acyl moiety rapidly associated with the neutral lipids, DAG and TAG, TAG being the preferred lipid. It was not possible from these experiments to directly ascertain the immediate acyl precursor of vernolate.

In vivo *pulse-chase labeling of seed lipids with [1-^{14}C]acetate.* To further investigate the biosynthesis of vernolic acid, decoated developing *V. galamensis* seeds were exposed to [1-^{14}C]acetate, and after 1 h, the radioactive acetate was replaced with nonradioactive acetate, and the incubation was continued for an additional 8 h. Figure 3A shows the total incorporation of radioactivity into the lipids of the seeds at various times after the label was removed. The total amount of

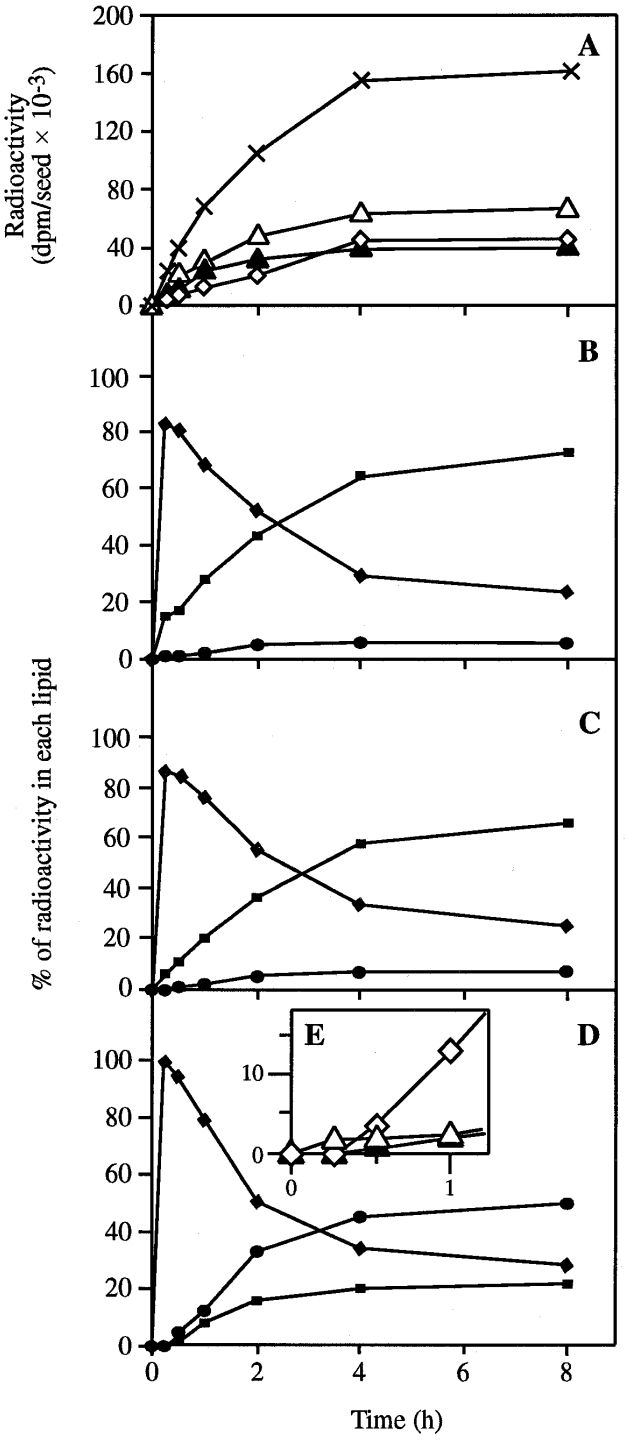

FIG. 2. Incorporation of radioactivity from [1-^{14}C]acetate into lipids and fatty acids of developing *Vernonia galamensis* seeds. Developing seeds were incubated in the presence of [1-^{14}C]acetate. (A) At the indicated times, the amount of radioactivity incorporated into total lipids (x), phosphatidylcholine (PC) (△), diacylglycerol (DAG) (▲), and triacylglycerol (TAG) (◇) was determined. The proportion of radioactivity in the oleoyl (◆), linoleoyl (■), and vernoleoyl (●) moieties of PC (B), DAG (C), and TAG (D) were determined. (E) Magnified view of the data in panels B, C, D to illustrate the proportion of radioactivity associated with the vernolate moiety of PC (△), DAG (▲), and TAG (◇). Vernolate is initially labeled on PC. Each data point is an average of two determinations.

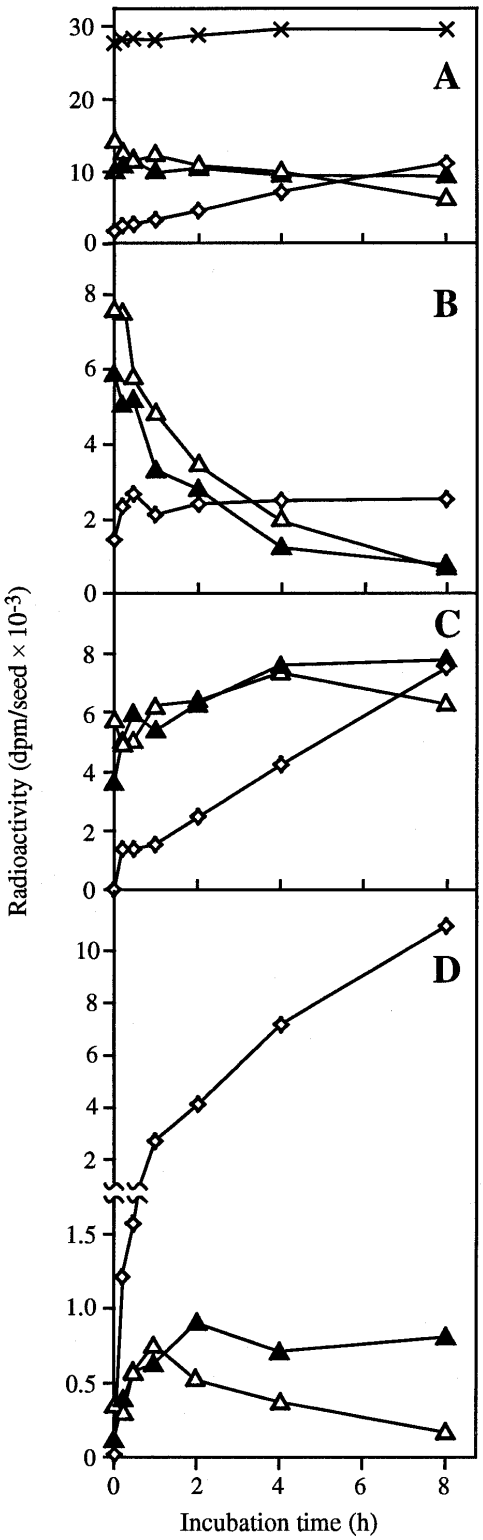

FIG. 3. Pulse-chase analysis of the incorporation of radioactivity from [1-^{14}C]-acetate into lipids and fatty acids of developing *Vernonia galamensis* seeds. Developing seeds were incubated in the presence of [1-^{14}C]-acetate for 1 h, after which, seeds were incubated for the indicated times in the presence of nonradioactive acetate. (A) The amount of radioactivity incorporated into total lipids (x), PC (△), DAG (▲), and TAG (◇). The amount of radioactivity in the oleoyl (B), linoleoyl (C), and vernoleoyl (D) moieties of PC (△), DAG (▲), and TAG (◇). Each data point is an average of two determinations. See Figure 2 for abbreviations.

radioactivity recovered in lipids was relatively constant during the chase phase of the experiment. PC was the most heavily labeled lipid at the end of the pulse phase and subsequently declined steadily during the chase phase of the experiment. Concomitant with the disappearance of radioactivity from PC, radioactivity associated with TAG increased, but radioactivity associated with DAG remained relatively stable. By the end of the chase, TAG was the most heavily labeled lipid. PE and PI contained a constant, low level (less than 5%) of radioactivity over the chase phase of the experiment (data not shown), and these lipids were not characterized further.

Figure 3B–D illustrates the distribution of radioactivity in oleate, linoleate, and vernolate, respectively, associated with PC, DAG, and TAG during the chase phase of the experiment. Palmitate was the only other acyl group labeled, but it contained only a small amount of radioactivity (data not shown). As expected from the experiment described in Figure 2, at the end of the pulse phase of the experiment, oleate esterified on PC and DAG was the most heavily labeled acyl moiety (Fig. 3B). During the chase, radioactivity in the oleoyl moiety of PC and DAG declined (Fig. 3B and C). This decline was associated with an increase in radioactivity in linoleate and vernolate (Fig. 3B and C). The most dramatic increases in radioactivity associated with these acyl moieties occurred in the TAG fraction. At the start of the chase phase of the experiment, the greatest amount of radioactivity associated with vernolate was associated with that esterified to PC (Fig. 3D). As the chase progressed, radioactivity in the vernoleoyl moiety of PC transiently increased, reached a peak at 1 h into the chase, and subsequently declined. This contrasts with the radioactivity associated with vernoleoyl-DAG, which although also at a low level reached a plateau at about 2 h into the chase. As with the previous experiments, these results indicate that vernolate is first synthesized while it is esterified to PC, and subsequently it rapidly becomes associated with the neutral lipids, particularly TAG.

In conclusion, epoxy fatty acids are found in a number of plant families (1–4,12,13), but only in Compositae and Eurphorbiaceae are high concentrations found. The reactive epoxide ring of these acids makes them ideal for use as stabilizers in vinyl plastics. Seeds of *V. galamensis* accumulate a large quantity of vernolic acid, most of which is esterified to TAG. Developing *V. galamensis* seeds accumulate seed lipids at maximal rates between 20 and 28 DAF. Such seeds proved highly amenable to radiotracer studies designed to elucidate the biosynthesis of this unusual fatty acid. The decoated seeds readily took up exogenously provided [1-^{14}C]acetate and incorporated into lipids, including vernolate.

The data gathered from *in vivo* radiotracer incubations and pulse-chase experiments are consistent with the hypothesis that vernolate is synthesized while it is esterified to PC. This conclusion is based on the observation that in developing *V. galamensis* seeds radioactivity from ^{14}C-acetate is first incorporated into vernolate while this acyl moiety is esterified to PC. In the pulse-chase experiments, the appearance of radioactive vernoleoyl-PC preceded the appearance of radioac-

tive vernoleoyl-TAG. However, these *in vivo* experiments did not clearly identify the acyl precursor of vernolate. Nevertheless our results are consistent with previous *in vitro* experiments with isolated microsomes from developing seeds of *Euphorbia lagascae*, which indicate that vernolate is synthesized by the epoxidation of linoleate while it is esterified to PC (6,7).

ACKNOWLEDGMENTS

Our thanks go to the Iowa Soybean Promotion Board and the Herman Frasch Foundation (Grant No. 322-HF92 to BJN) for partial financial support. Journal Paper No. J-17951 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011. Project No. 3414.

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[Received July 20, 1998, and in final revised form November 30, 1998; revision accepted December 1, 1998]

Inhibition of Lipoxygenase 1 by Phosphatidylcholine Micelles-Bound Curcumin

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ABSTRACT: Curcumin (diferuloyl methane) from rhizomes of *Curcuma longa* L. binds to phosphatidylcholine (PC) micelles. The binding of curcumin with PC micelles was followed by fluorescence measurements. Curcumin emits at 490 nm with an excitation wavelength of 451 nm after binding to PC-mixed micelles stabilized with deoxycholate. Curcumin in aqueous solution does not inhibit dioxygenation of fatty acids by Lipoxygenase 1 (LOX1). But, when bound to PC micelles, it inhibits the oxidation of fatty acids. The present study has shown that 8.6 μM of curcumin bound to the PC micelles is required for 50% inhibition of linoleic acid peroxidation. Lineweaver-Burk plot analysis has indicated that curcumin is a competitive inhibitor of LOX1 with K_i of 1.7 μM for linoleic and 4.3 μM for arachidonic acids, respectively. Based on spectroscopic measurements, we conclude that the inhibition of LOX1 activity by curcumin can be due to binding to active center iron and curcumin after binding to the PC micelles acts as an inhibitor of LOX1.
Lipids 33, 1223–1228 (1998).

Some natural phenolic antioxidants have potential preventive activity in the initial stages of antioxidant-related diseases (1). Recently, curcumin, the major constituent of the spice turmeric, has received much attention because of both its antioxidant and antiinflammatory activities (2–4). It has been shown to be highly effective in inhibiting the stimulation of HIV type 1 virus (5). It inhibits neutrophil activation, mixed lymphocyte reaction, and proliferation of smooth muscle cells, and suppresses nitrogen-induced proliferation of blood mononuclear cells (6). Further, it is a potent scavenger of reactive oxygen species (7), protects hemoglobin from nitrate-induced oxidation, and inhibits lipid peroxidation (8,9). Huang *et al.* (10) reported that the antitumor-promotion activity of curcumin in mouse epidermis is linked with the suppression of arachidonic acid metabolism. This metabolic pathway is one of the lipid peroxidation events in living organisms catalyzed by lipoxygenases.

Arachidonic acid lipoxygenases are involved in the biosynthesis of various bioregulators that are closely related to the pathogenesis of some diseases, such as allergy, atherosclerosis, and cancer (11). Lipoxygenase catalyzes the initial step in the production of leukotrienes and lipoxins, which mediate allergic and inflammatory responses (12). Since the discovery of the role of lipoxgenases in the biosynthesis of effectors critical in animal and plant physiology, extensive research has been carried out to study their effective inhibitory activities. Most studies have used soybean lipoxygenase (LOX1), whose three-dimensional structure and catalytic mechanism have been characterized in detail (13,14). Earlier reports showed that curcumin is a potent inhibitor for nonenzymatic lipid peroxidation (15,16).

The results presented in this paper suggest that curcumin inhibits LOX1-catalyzed oxidation of linoleic and arachidonic acids and demonstrate that this natural antioxidant inhibits the enzyme by binding to the active center iron. The inhibitory activity of curcumin is potentiated in the presence of hydrophobic environment, which can be provided by phosphatidylcholine (PC) micelles.

MATERIALS AND METHODS

Materials. Soybean LOX1 was isolated according to the method of Axelrod *et al.* (17) with some modifications as described earlier (18). The specific activity was 200–240 μmoles/min/mg of protein. Linoleic and arachidonic acids were from Nu-Chek-Prep (Elysian, MN). Egg PC, curcumin, and sodium deoxycholate (DOC) were purchased from Sigma (St. Louis, MO). Stock solutions of fatty acids were prepared with a concentration of 10 mM in 10% ethyl alcohol containing 50 mM borate buffer pH 9.0

Preparation of the mixed micelles. Mixed micelles were prepared using the mixture of PC and DOC. After solubilizing the PC and DOC in chloroform/methanol (2:1) mixture, the solvent was evaporated by flash evaporator and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris HCl, pH 7.4, and then sonicated for 5 min using a bath-type sonicator.

Fluorescence microscopy. PC micelles (10 mM), after mixing with curcumin (60 mM) solubilized in ethyl alcohol (the final concentration of alcohol did not exceed 2 mM), were immediately examined under Leitz Diaplan (Wetzlar, Germany) Fluorescence microscope fitted with exciter filter BP 450-490 nm and suppression filter block of 520 nm.

Assay of lipoxygenase and inhibition by curcumin. The concentration of LOX1 was calculated from A_{280} by using the value E_{280} = 14.0 (18). Activities were determined by spec-

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Abbreviations: DOC, deoxycholic acid; LOX1, lipoxygenase 1; PC, phosphatidylcholine.

trophotometry. The formation of hydroperoxide was followed at 234 nm (ε = 25,000 $M^{-1}cm$) in a solution of 50 mM Tris HCl (pH 7.4) containing 100 μM PC micelles and 100 μM of fatty acid solubilized in ethyl alcohol (the final concentration of alcohol did not exceed 1 mM). In the inhibition experiments, curcumin was added to the assay mixture as an ethanolic solution (the final concentration of alcohol was 2 mM). The residual activity was independent of the sequence of reagents addition, when related to the activity in the absence of the inhibitor. Reversibility of inhibition was assessed by assaying residual activity after dilution of the enzyme/inhibitor complexes with 50 mM Tris HCl, pH 7.4 at 4°C.

Curcumin fluorescence studies. Fluorescence measurements were performed with a Shimadzu (Kyoto, Japan) spectrofluorophotometer RF 5000. Temperature was maintained at 25°C by circulating the water through the thermostated cuvette holder. Fluorescence titrations of curcumin and PC micelles were made according to the method of Azzi (19). Samples were excited at 451 nm, and emission was recorded at 490 nm. The dissociation constant (K_{cur}) and the number of binding sites on PC micelles were determined using the Scatchard plot method. The interaction of LOX1 and metal ion, Fe^{3+} ($FeCl_3$), was made by following the fluorescence quenching. The PC micelles were saturated with curcumin as reflected in maximum relative fluorescence intensity, F_{max}, at 490 nm. To this PC–curcumin complex, LOX1 or $FeCl_3$ was added gradually and the decrease in fluorescence intensity, F, was recorded. The dissociation constant (K_D) for the fluorescence quenching ligand (L) was determined using equation $F_{max}/F = (1 + K_{cur}/[cur])(1 + [L]/K_D)$ (20), where K_{cur} is the dissociation constant for PC–curcumin determined by Scatchard plot, [cur] is the concentration of curcumin, and [L] is the concentration of LOX1 or $FeCl_3$ added during the titration. The reciprocal of the dissociation constant was used as the equilibrium constant (K_{eq}).

The affinity of curcumin for PC micelles in the presence of fatty acid was calculated using the fluorometric titration of curcumin with PC micelles and fatty acid, and K_{cur} was calculated using the Scatchard plot method. The concentration of bound curcumin used in the inhibition studies was determined using the fluorometric titration of curcumin against 100 μM of PC micelles and 100 μM fatty acid (the concentration similar to activity measurements); the following equation was used to calculate the bound curcumin concentration: $C_B = C_T - (K_D/n)$ (RFI/RFI_{max} – RFI), (21) where C_T was the concentration of curcumin used for the inhibition, K_D was the dissociation constant of LOX1 or Fe^{3+} with PC micelles in the presence of fatty acid, RFI_{max} was maximum fluorescence intensity which was the intercept of the reciprocal plot of curcumin against PC micelles in the presence of fatty acid, and RFI was obtained from the reciprocal plot for the corresponding C_T of curcumin.

RESULTS

Figure 1 shows that curcumin in the presence of PC micelles has a characteristic fluorescence emission maximum of 490 nm at an excitation wavelength of 451 nm. This result indicates that the binding of curcumin with PC micelles could be due to the interaction between the hydrophobic group of curcumin and the hydrophobic regions of PC micelles. This observation was confirmed by fluorescence microscopy measurements which clearly show the binding of curcumin to the PC micelles and the influence of curcumin fluorescence in visualizing the size and shape of PC-micelles (Fig. 2). The luminous disks had diameters of 0.1 to 0.3 μm. Curcumin in aqueous solution and in a solution containing only DOC has a weak fluorescence, but its fluorescence intensity is very high in organic solvents, and the quantum yield increases with increase in the hydrophobicity of the solvent (22). It has been

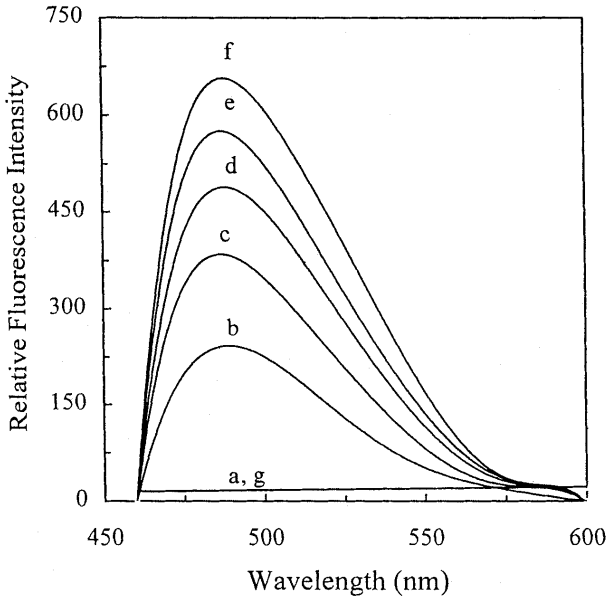

FIG. 1. Fluorescence emission spectra of curcumin with phosphatidylcholine (PC) micelles. To 50 μM PC micelles, various concentrations of curcumin were added in 50 mM Tris HCl buffer, pH 7.4 (a, 0; b, 1; c, 2; d, 3; e, 4; and f, 5 μM of curcumin; g, 5 μM curcumin in 100 μM dioxycholic acid).

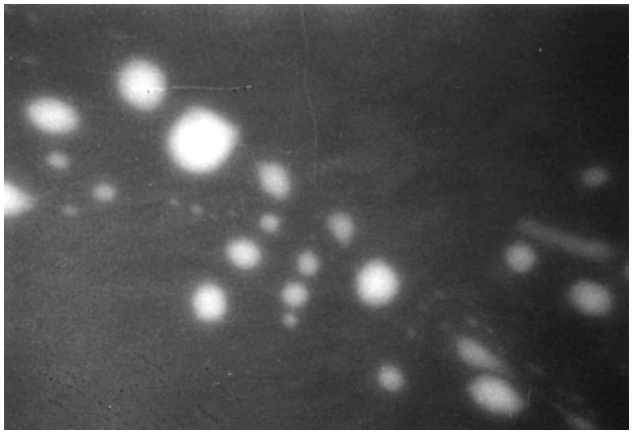

FIG. 2. Fluorescence photomicrograph of PC micelles–curcumin complex in 50 mm Tris HCl buffer, pH 7.4 (PC to curcumin molar ratio was 6.0). Magnification factor 40×. For abbreviation see Figure 1.

reported that curcumin in aqueous solvent is not stable in the alkaline pH and undergoes degradation at high alkaline pH. Therefore, all measurements were made at pH 7.4 in the present study.

LOX1 utilizes linoleic acid bound to the PC micelles as substrate, with a pH optimum of 7.4 (23). The LOX1-catalyzed rate of oxidation of linoleic and arachidonic acids inserted into PC micelles at pH 7.4 is given in Figure 3A. Only the fatty acids bound to the PC micelles are the substrates for LOX1, and the LOX 1 activity is maximum with equal concentrations of fatty acid and PC micelles. This method is very useful for LOX activity determination in the presence of curcumin, because measurements can be made at neutral pH. Although activity of LOX1 with linoleic and arachidonic acids solubilized in equal amounts of Tween 20 had a pH optimum around 9.0, the enzyme had around 50% of its activity at pH 7.4. The effect of different concentrations of curcumin in aqueous system on the LOX1 activity for the Tween 20-solubilized fatty acid substrate suggested that curcumin did not inhibit LOX1 activity (Fig. 3B). However, the inhibitory effect of curcumin on LOX1 activity was observed only with PC-bound fatty acid as substrate. These results clearly show that curcumin inhibits LOX1 in the presence of PC micelles, presumably by binding to the PC micelles, but not in aqueous solution.

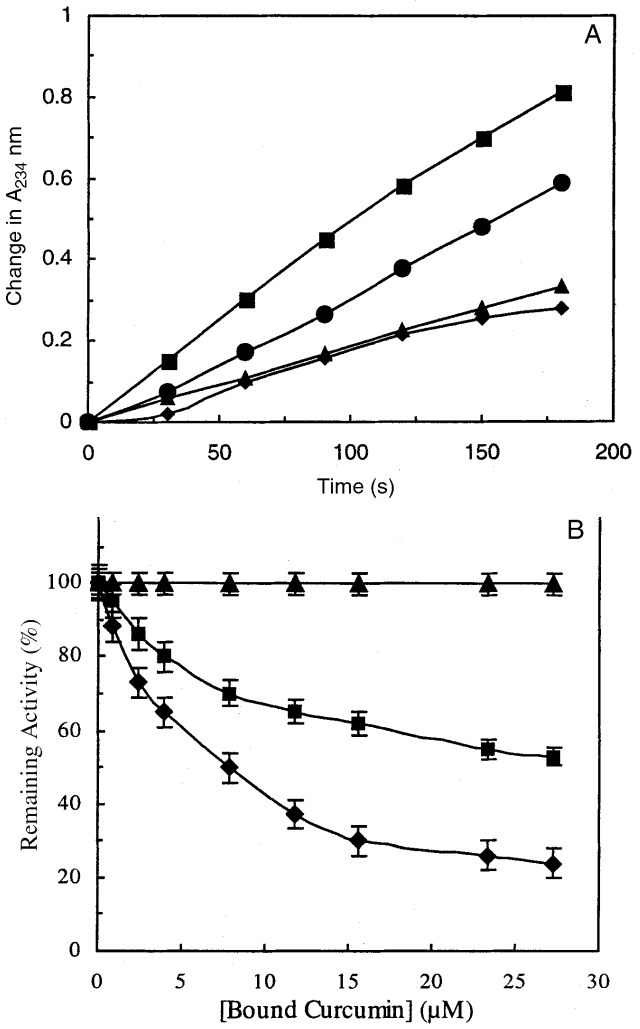

FIG. 3. (A) Soybean lipoxygenase 1 (LOX1)-catalyzed oxidation of fatty acids. Rates were measured in the presence of (i) 100 μM of fatty acid with 100 μM of micellar PC (arachidonic acid, ■; linoleic acid, ●) and (ii) 100 μM of fatty acid dispersed in Tween 20 (arachidonic acid, ▲; linoleic acid, ◆) at pH 7.4 (50 mM Tris HCl). (B) The concentration-dependence inhibition of curcumin on LOX1-dependent dioxygenation. The reaction mixture consisted of 100 μM fatty acid with (i) 100 μM PC micelles (linoleic acid, ◆; arachidonic acid, ■) and (ii) Tween 20 (arachidonic and linoleic acids, ▲) in 50 mM Tris HCl, pH 7.4, and various concentrations of ethanolic solution of curcumin. The reaction was initiated by adding LOX1. For other abbreviation see Figure 1. In Part B the error bars signify the percentage error in measurements.

LOX1 has optimal activity at 100 μM of fatty acid and 100 μM of micellar PC. Since only bound fatty acid is the substrate and the inhibitory effect by curcumin is also due to binding to PC micelles, the concentration of PC micelles is the limiting factor in the LOX1 activity and its inhibition by curcumin. As shown in Figure 3B, the incorporation of increasing amounts of curcumin into the PC micelles led to a successive inhibition of oxidation of fatty acid bound to the PC micelles. Curcumin inhibited dioxygenation of linoleic acid more effectively than arachidonic acid. The 50% inhibitory activity toward linoleic acid oxidation was observed with 11 μM curcumin, which corresponded to 8.6 μM bound curcumin.

When the effects of linoleic and arachidonic acids on lipoxygenase inhibition by curcumin were examined, the rate of oxidation became greater, as the concentration of substrate increased. The Lineweaver-Burk plots at a fixed curcumin concentration showed that K_m increased without changing the V_{max} of the reaction (Fig. 4), suggesting that curcumin is a competitive inhibitor of LOX1, K_i values being 1.7 μM and 4.3 μM for linoleic and arachidonic acid oxidation, respectively (Fig. 5).

Earlier reports on the antioxidant activity of curcumin suggested that curcumin can chelate the metal ion (24,25). Further, LOX1 contains iron as the cofactor. Therefore, studies were made to check whether the competitive inhibition of curcumin is due to binding to the active site iron. Fluorimetric titrations of curcumin with PC micelles with increasing concentrations of LOX1 and $FeCl_3$ suggest that curcumin inhibits LOX1 activity competitively by binding to the active site iron, as the LOX1 quenches the curcumin fluorescence in PC micelles. The equilibrium constant of curcumin bound to the PC micelles with LOX1 is 0.33×10^6 M^{-1}; this value is comparable with the affinity of curcumin with PC micelles, which is 0.37×10^6 M^{-1}. These data further confirm that curcumin can elicit its inhibitory activity only after binding to the PC micelles. The reversible inhibition of LOX1 by curcumin bound to the PC micelles was further confirmed by complete recovery of catalytic activity after dilution of enzyme/inhibitor complex with the buffer. Fe^{3+} also quenched curcumin fluorescence, which was used for determining the affinity of curcumin (PC bound) with Fe^{3+}. This had equilibrium constant of 0.23×10^6 M^{-1}. However, Fe^{2+} was more ef-

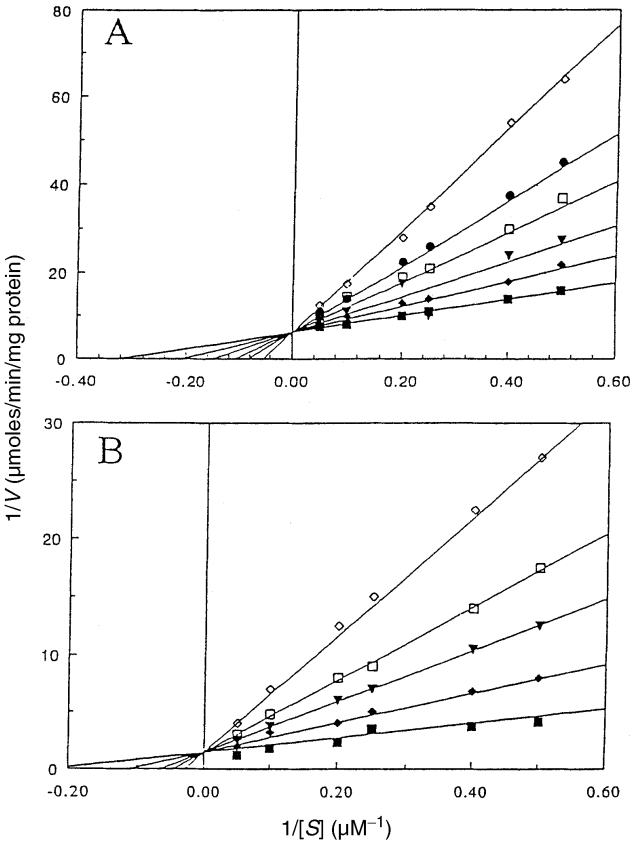

FIG. 4. Lineweaver-Burk plot analysis of the inhibitor of LOX1 by curcumin for the dioxygenation of (A) arachidonic acid and (B) linoleic acid. The concentrations of bound curcumin for arachidonic acid were 0 (■), 3.9 (◆), 7.8 (▼), 11.73 (□), 15.6 (●), and 24 μM (◇); and for linoleic acid 0 (■), 3.13 (◆), 6.25 (▼), 9.4 (□), and 15.6 μM (◇). The micellar PC concentration was 100 μM, and the fatty acid concentration was 100 μM. For abbreviations see Figures 1 and 3.

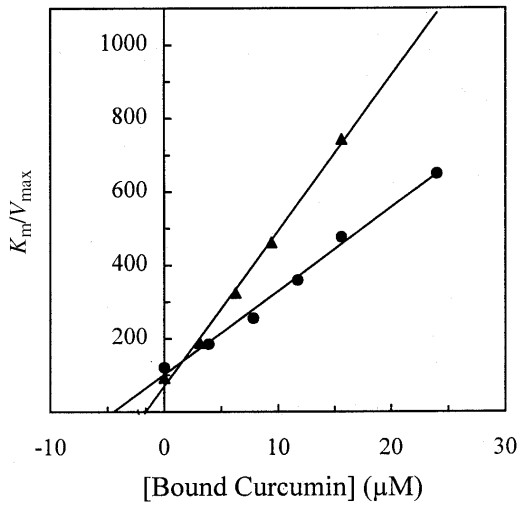

FIG. 5. The slope (K_m/V_{max}) of the lines described from the double reciprocal plot are plotted against the curcumin concentration (bound to PC micelles) in order to derive the K_i value for curcumin (linoleic acid, ◆; arachidonic acid, ●). For abbreviation see Figure 1.

Our earlier study demonstrated that LOX1 can oxidize fatty acids bound to the PC micelles at neutral pH (23). Therefore, this reaction system to monitor the LOX1 activity was used in the present study. Curcumin binds to PC micelles with a high affinity, exhibiting a characteristic fluorescence spectrum that could be attributed to the amphilic nature of curcumin. The binding of curcumin with PC micelles enhances its hydrophobicity. Further, from the equilibrium constants for curcumin–PC micelles and the LOX1–curcumin (bound to PC micelles), it can be inferred that enhancing the hydrophobicity of the curcumin makes it more effective in reaching the active site iron of LOX1. Similar observations

fective in quenching the curcumin fluorescence (data not shown). Comparison of equilibrium constant of PC micelles-bound curcumin with LOX1 (0.33×10^6 M^{-1}) and Fe^{3+} (0.23×10^6 M^{-1}) suggests that curcumin incorporated in PC micelles inhibits LOX 1 activity by binding to the active site iron (Fig. 6).

DISCUSSION

Several studies on the inhibition of LOX1 have shown that inhibitors can act through a number of mechanisms, for example, by reducing the catalytically active ferric enzyme to its inactive ferrous form through the formation of free-radical metabolites (26), or by preventing the formation of the activated Fe(III) form of LOX (27), or by binding to sites other than the active site of the enzyme molecule (28). In general, the inhibition of lipoxygenase reaction seems to be derived from inactivation of the active site of the enzyme or scavenging of free radical at the active site. Therefore, most antioxidants are inhibitors of LOX1. The results of the present study suggest that curcumin inhibits LOX1 by binding with the active site iron.

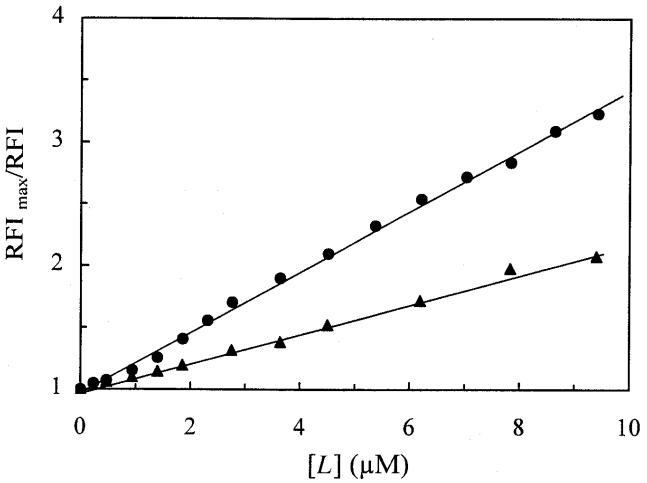

FIG. 6. Fluorescence titration of LOX1 (●) and $FeCl_3$ (▲) against curcumin bound to PC micelles. To 100 μM PC micelles, 100 μM fatty acid and 5 μM curcumin in 50 mM Tris HCl, pH 7.4, various concentrations of LOX1/$FeCl_3$ were added, and the fluorescence quenching was recorded. The plot was made as described in the Materials and Methods section. RFI_{max}, maximum fluorescence intensity, defined as the intercept of the reciprocal plot of curcumin against PC micelles in the presence of fatty acid. For other abbreviations see Figures 1 and 3.

have been made in the earlier study on the competitive inhibition of LOX1 by *n*-alcohols, where longer alkyl chains were shown to increase the affinity of these compounds for the enzyme (29). Similarly, 6-palmitoyl ascorbic acid inhibits more effectively than ascorbic acid (30). It has also been shown that some of the membrane-bound enzymes like protein kinase C and pp $60^{c\text{-}src}$, tyrosine kinase are inhibited by curcumin (31). Hence, it is conceivable that the inhibitory effect of curcumin could be potentiated after binding to the membranes.

The results of the present studies demonstrate that curcumin after binding to the PC micelles acts as competitive and reversible inhibitor of LOX1. Unlike other antioxidant inhibitors of LOX1, curcumin does not require radical formation during LOX inhibition. The dissociation constant of curcumin (bound to PC micelles) with LOX (3.03 μM) determined by fluorometric titrations is comparable with the K_i (1.72 μM) value of LOX inhibition by curcumin for linoleic acid oxidation, indicating clearly that curcumin inhibits LOX1 by binding to the active site iron, the binding is reversible, and the inhibition can be overcome by excess of fatty acid. On the other hand, the K_{eq} ($0.33 \times 10^6 M^{-1}$) for Fe^{3+} binding with PC-bound curcumin is comparable with the K_{eq} (0.23×10^6 M^{-1}) constant of LOX1 interaction with PC micelles-bound curcumin, suggesting that curcumin can bind the active Fe^{3+} form of LOX1. The present observations that PC micelle-bound curcumin binds to the Fe^{3+} can be explained using earlier reports, which suggested that the β-diketone moiety of curcumin could chelate the metal ion, and chelation affinity increased with the increase in hydrophobicity of the curcumin molecule. Hence, curcumin became more potent for inhibiting the LOX1 after its association with PC micelles.

The low K_i value of curcumin for linoleic acid oxidation (K_i = 1.72 μM) compared to arachidonic acid (K_i = 4.3 μM) implies that curcumin is more specific for inhibiting the LOX1 for linoleic acid peroxidation, although LOX 1 shows almost similar values of K_{cat} (4.53×10^7 $M^{-1}s^{-1}$) and K_m (9 μM) for arachidonic acid oxidation compared to linoleic acid oxidation ($K_{cat} = 3.6 \times 10^7$ $M^{-1}s^{-1}$ and K_m = 10 μM). However, some of the reported anti-inflammatory effects of curcumin could be due to inhibition of arachidonic acid peroxidation (10). It is also known that curcumin inhibits mammalian 5-lipoxygenase and cyclooxygenase (32).

These studies imply that curcumin, after binding to PC micelles, acts as a potent inhibitor of LOX1-dependent fatty acid peroxidation, although the micellar system in these experiments may not reflect directly the event of lipid peroxidation occurring *in vivo*. However, observations made in this study may have physiological significance, involving lipoxygenase in biomembrane alteration, both in animals and plants.

ACKNOWLEDGMENTS

We thank Dr. V. Prakash, Director CFTRI, for his keen interest; Dr. K. Udaya Sankar, for constant encouragement; and Dr. Umesh Kumar, for his help in fluorescence microscopy measurements. Senior Research Fellowships to G.B and E.S from CSIR, New Delhi, India, are greatfully acknowledged.

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[Received August 3, 1998, and in final revised form November 18, 1998; revision accepted November 23, 1998]

Lysophosphatidylcholine Acyltransferase Activity in *Saccharomyces cerevisiae*: Regulation by a High-Affinity Zn^{2+} Binding Site

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ABSTRACT: *Saccharomyces cerevisiae* cells were demonstrated to contain lysophosphatidylcholine (lysoPtdCho) acyltransferase (E.C. 2.3.1.23) activity. The enzyme displayed $K_{m(app)}$ of 69 μM for lysoPtdCho and 152 μM for oleoyl CoA. Enzyme activity was not affected by the addition of 1 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, or 200 mM EDTA. However, Zn^{2+} inhibited lysoPtdCho acyltransferase activity to 33% control values at 0.1 mM and to 7% at 1.0 mM Zn^{2+}. To further explore the possibility that lysoPtdCho acyltransferase may contain a high-affinity Zn^{2+} binding site, we tested the strong Zn^{2+} chelator *o*-phenanthroline for its ability to inhibit enzyme activity. LysoPtdCho acyltransferase activity was inhibited to 18 and 27%, respectively, those of control values in the presence of 2 and 1 mM *o*-phenanthroline, implying that a high-affinity Zn^{2+} binding site exists in lysoPtdCho acyltransferase or in an accessory protein that is essential for protein stability and/or activity. *Saccharomyces cerevisiae* lysoPtdCho acyltransferase activity displayed a broad lysoPtdCho fatty acyl chain substrate specificity utilizing lysoPtdCho molecules ranging in length from C_{10}–C_{20} (the entire range tested). In addition, the enzyme was capable of using the ether-linked analog of lysoPtdCho, 1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine, as a substrate. The ability of *S. cerevisiae* to incorporate radiolabeled 1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine into phosphatidylcholine *in vitro* was exploited to demonstrate a direct precursor–product relationship between lysoPtdCho molecules and their incorporation into phosphatidylcholine *in vivo*. Identical labeling results were obtained in *S. cerevisiae* cells disrupted for their major transacylase activity, *PLB1,* demonstrating that the incorporation of lysolipid was *via* acyltransferase, and not transacylase, activity.

Lipids 33, 1229–1234

Phosphatidylcholine (PtdCho) is a major constituent of eukaryotic membranes constituting approximately half of cellular phospholipid mass (1). In eukaryotic cells, PtdCho is synthesized *de novo via* the Kennedy (CDP-choline) pathway in all cell types except yeast and mammalian liver cells, which are also capable of synthesizing PtdCho through the methylation of phosphatidylethanolamine (2,3). The final step within the Kennedy pathway is catalyzed by cholinephosphotransferase *via* transfer of phosphocholine from CDP-choline to diacylglycerol to form PtdCho and CMP (4). The molecular species of diacylglycerol utilized by cholinephosphotransferase determines the fatty acyl composition of *de novo* synthesized PtdCho. *De novo* synthesized PtdCho undergoes fatty acyl chain remodeling through a phospholipase A_2-mediated deacylation followed by reacylation *via* lysophosphatidylcholine (lysoPtdCho) acyltransferase (5,6). This deacylation–reacylation mechanism is believed to be responsible for maintaining polyunsaturated fatty acid pools (e.g., arachidonic acid) at the *sn*-2 position of PtdCho (7,8). PtdCho-derived polyunsaturated fatty acid stores are released from PtdCho in an agonist-dependent manner during most inflammatory responses for subsequent conversion to prostaglandins, leukotrienes, and other inflammation-mediating lipid molecules (9). However, the deacylation–reacylation of PtdCho occurs in many cell types that do not mediate immune responses (10), implying a biological role beyond storage of fatty acyl pools for agonist-stimulated release.

A lysoPtdCho acyltransferase cDNA or gene has not been cloned from any source, and the technical difficulties associated with purifying intrinsic membrane proteins have prevented the purification of the enzyme to homogeneity (10). In this study we report the biochemical and enzymatic characterization of a microsomal lysoPtdCho acyltransferase activity in the yeast *Saccharomyces cerevisiae*. This activity displayed properties consistent with regulation *via* a high-affinity Zn^{2+} binding site either within the enzyme or an accessory protein. The ability to use the observed characteristics to isolate an *S. cerevisia*e gene coding for lysoPtdCho acyltransferase is also discussed.

MATERIALS AND METHODS

Materials. The *S. cerevisiae* strain w303a (*MAT* **a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*) (the kind gift of Robert M. Bell, Glaxo Wellcome, Inc.) was used for all experiments

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Abbreviations: GTE, buffer made from 20% glycerol, 50 mM Tris-HCl pH 7.4, 1 mM EDTA; lysoPAF, 1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine; lysoPtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; YPD, growth medium containing 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% glucose.

except the *PLB1* experiment in which DL882 MATa/MATα 1788 *plb1Δ*:::URA3/*plb1Δ*:::URA3, and its parental strain DL1788 MATa/MATα *leu2-3, 112 ura3-52, trp1-1, his4 can1*^{r} (the kind gifts of Susan A. Henry, Carnegie Mellon University), were used. Lipids were supplied by Avanti Polar Lipids (Alabaster, AL). Oleoyl CoA was supplied by Sigma Chemical Co. (St. Louis, MO), and [oleoyl-1-^{14}C]oleoyl CoA was supplied by Amersham Life Sciences (Arlington Heights, IL). Fatty acyl CoA stocks were stored in aliquots at −20°C. [Alkyl-1,2-^{3}H]1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine was obtained from Du Pont NEN (Boston, MA). Media components were obtained from Difco (Detroit, MI), and all other chemicals were obtained from Sigma.

Enzyme assay. Saccharomyces cerevisiae w303a cells were grown to mid-log phase in YPD medium (1% Bacto-Yeast Extract, 2% Bacto-Peptone, 2% glucose), pelletized by centrifugation at 3,000 rpm for 5 min, washed once with ice-cold GTE buffer (20% glycerol, 50 mM Tris-HCl pH 7.4, 1 mM EDTA), and resuspended in 0.7 mL GTE buffer. Cells were disrupted by vortexing in the presence of 0.5-mm glass beads for five bursts of 30 s separated by 30-s incubations on ice. The supernatant was transferred to a new tube, and the beads were rinsed twice with 0.5 mL GTE. The pooled sample was centrifuged at 16,000 × *g* for 15 min at 4°C, and the resulting supernatant was centrifuged at 450,000 × *g* for 15 min. The pellet was resuspended in 250 µL GTE by Dounce homogenization (11). Membranes were stored at −70°C. LysoPtdCho acyltransferase activity was stable for at least 2 mon at −70°C. Yeast lysoPtdCho acyltransferase assays were adapted from previously published assays for the mammalian enzyme (12,13). For a standard assay, 18:1 lysoPtdCho was dried under N_2 gas, resuspended in 5 µL H_2O by water-bath sonication for 5 min with occasional vortexing, and added to a freshly prepared reaction mixture containing 100 mM Tris-HCl (pH 7.4) and 20 µg microsomal protein. The reaction mixture was preincubated at 25°C for 5 min, and catalysis was initiated by the addition of [^{14}C]oleoyl CoA (2500 dpm/nmol). Final substrate concentrations were 150 µM for oleoyl CoA and 125 µM for lysoPtdCho, unless otherwise indicated, in a reaction volume of 100 µL. The reaction was allowed to proceed for 20 min at 25°C. Catalysis was terminated by the addition of 3 mL $CHCl_3/CH_3OH$ (2:1, vol/vol) and 1.4 mL 0.9% KCl. The mixture was centrifuged for 10 min at 1,400 × *g* to facilitate phase separation. An aliquot of the organic phase was dried under N_2, resuspended in 40 µL chloroform spiked with PtdCho, and applied to Whatman (Haverhill, MA) silica gel 60A thin-layer chromatography plates alongside a PtdCho standard. Lipids were separated in a solvent system containing $CHCl_3/CH_3OH/NH_4OH/H_2O$ (70:30:4:2, by vol) and analyzed for radiolabel with a BIOSCAN imaging scanner. PtdCho was identified by iodine staining, and the appropriate region of the silica gel plate was scraped into a scintillation vial for radioactivity determination.

*[^{3}H]1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine ([^{3}H]lysoPAF) metabolism.* [^{3}H]lysoPAF (1.0 µCi, 36 mCi/µmol) was added to mid-log phase *S. cerevisiae* cells grown in YPD broth, and 1-mL aliquots were removed at the indicated times. Cells were washed twice with 1 mL H_2O and resuspended in 1 mL $CHCl_3/CH_3OH$ (1:1, vol/vol). A modified Folch *et al.* (14) extraction procedure was used to extract lipids as described (11). Organic and aqueous phases were assessed for the accumulation of radiolabel by scintillation counting. In addition, the organic phase was dried under N_2 gas for thin-layer chromatography analysis (as described above) and phospholipid phosphorus determination.

Standard assays. Lipid phosphorus was assessed using the method of Ames and Dubin (15). Protein was determined by the method of Lowry *et al.* (16).

RESULTS

LysoPtdCho acyltransferase activity in S. cerevisiae. LysoPtdCho acyltransferase activity was present in *S. cerevisiae* microsomal membranes. Enzyme activity was linear at 25°C for 30 min in the presence of up to 50 µg microsomal protein (data not shown). To ensure linearity with both time and protein, standard assay conditions allowed catalysis to proceed for 20 min using 20 µg of microsomal membrane protein as enzyme source.

The effect of oleoyl CoA concentration on lysoPtdCho acyltransferase activity was investigated in the presence of 125 µM lysoPtdCho (Fig. 1). LysoPtdCho acyltransferase activity increased linearly up to 100 µM oleoyl CoA whereupon enzyme activity plateaued; activity was inhibited by oleoyl CoA concentrations beyond 300 µM, most likely owing to the detergent properties of the fatty acyl CoA solubilizing the membrane. Lineweaver-Burk double-reciprocal plot analysis (17) yielded a $K_{m(app)}$ of 152 µM for oleoyl CoA and a $V_{max(app)}$ of 10.9 nmol min^{-1} mg protein^{-1}. Varying the concentration of the second substrate, lysoPtdCho, in the presence of 150 µM oleoyl CoA resulted in lysoPtdCho activity that was linear up to 60 µM (Fig. 2). Enzyme activity was inhibited by lysoPtdCho concentrations of 150 µM and beyond, again most likely owing to the detergent properties of this substrate solubilizing the membrane used as enzyme source. Lineweaver-Burk plot-derived values for lysoPtdCho were $K_{m(app)}$ of 69 µM and $V_{max(app)}$ of 9.1 nmol min^{-1} mg protein^{-1}.

The specificity of the *S. cerevisiae* microsomal lysoPtdCho acyltransferase activity for *sn*-1-lysoPtdCho containing different acyl chains was determined (Table 1). LysoPtdCho acyltransferase activity was detectable using substrates with acyl chains ranging in length from 10 to 20 carbons, and the highest activity was measured using 18:1 lysoPtdCho as substrate. With 14:0 lysoPtdCho as substrate, the activity observed was 64% of that using 18:1 lysoPtdCho, followed by 12:0 (56%), 18:0 (49%), 16:0 (44%), 10:0 (34%), and 20:0 (30%). *Saccharomyces cerevisiae* was also capable of catalyzing the lysoPtdCho acyltransferase reaction utilizing the ether-linked lysoPAF as substrate. This was unexpected as *S. cerevisiae* does not contain ether-linked phospholipids (18).

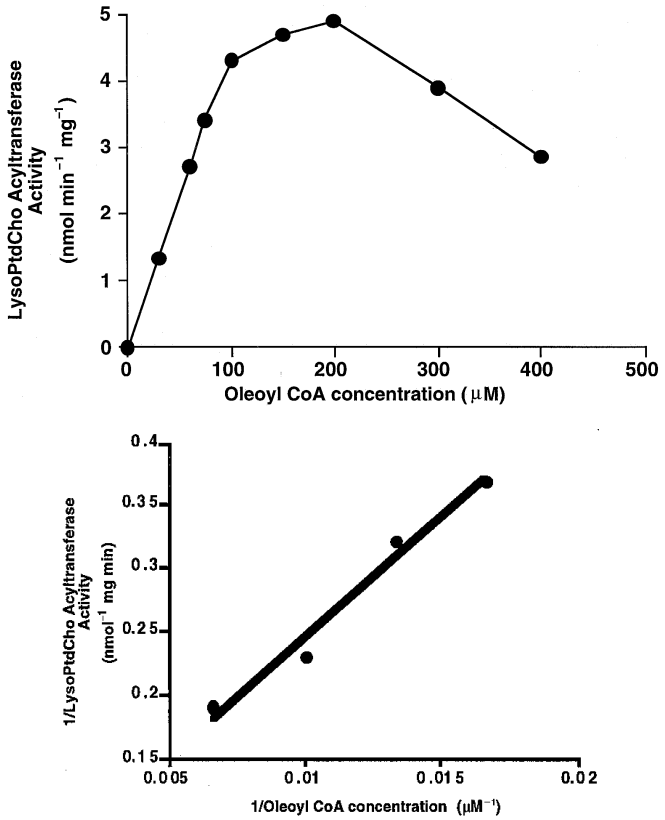

FIG. 1. Effect of oleoyl CoA concentration on *Saccharomyces cerevisiae* lysophosphatidylcholine (lysoPtdCho) acyltransferase activity. The activity of lysoPtdCho acyltransferase was determined in the presence of various concentrations of oleoyl CoA at a constant concentration of 125 μM lysoPtdCho. Each point represents the mean of four individual experiments performed in duplicate. All standard errors were less than 15% of the mean for all points.

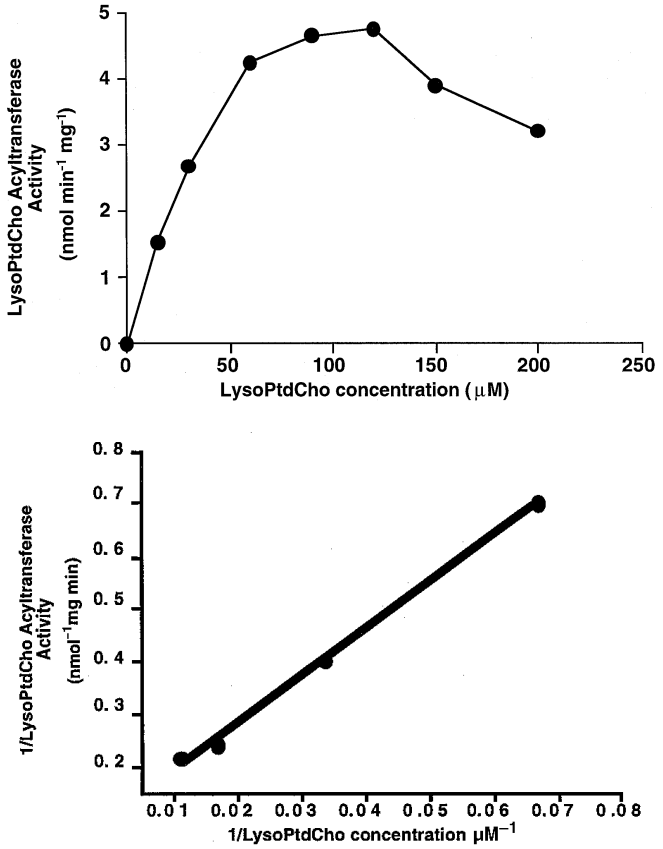

FIG. 2. Effect of lysoPtdCho concentration on *S. cerevisiae* lysoPtdCho acyltransferase activity. The activity of lysoPtdCho acyltransferase was determined in the presence of various concentrations of lysoPtdCho at a constant oleoyl CoA concentration of 150 μM. Each point represents the average of four individual experiments performed in duplicate. All standard errors were less than 15% of the mean for all points. For abbreviatins see Figure 1.

Effect of lipids and cations on lysoPtdCho acyltransferase activity. Many integral membrane-bound phospholipid-metabolizing enzyme activities are affected by their lipid environment, although the biological role of particular lipids affecting specific enzymes is just now being elucidated (19–21). The effect of lipids on lysoPtdCho acyltransferase activity was determined (Table 2). None of the lipids tested resulted in an inhibition of lysoPtdCho acyltransferase. However, several acidic phospholipids including phosphatidylinositol, phosphatidic acid, and cardiolipin increased lysoPtdCho activity to 212, 182, and 236% of control values, respectively.

TABLE 1
LysoPtdCho Fatty Acyl Specificity of LysoPtdCho Acyltransferase Activity[a]

LysoPtdCho fatty acyl chain species	Enzyme activity (nmol min^{-1} mg^{-1})
10.0	1.45 ± 0.21
12:0	2.41 ± 0.13
14:0	2.75 ± 0.38
16:0	1.92 ± 0.37
18:0	2.11 ± 0.32
18:1	4.32 ± 0.38
20:0	1.29 ± 0.17
LysoPAF	0.90 ± 0.13

[a]Results are expressed as mean ± standard error (n = 4). LysoPtdCho, lysophosphatidycholine; lysoPAF, 1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine.

LysoPtdCho acyltransferase activity was determined in the presence of divalent cations (Table 2). Neither Mg^{2+}, Mn^{2+}, Ca^{2+}, nor EDTA had an appreciable effect on lysoPtdCho acyltransferase activity. However, Zn^{2+} inhibited lysoPtdCho acyltransferase activity. Since many Zn^{2+} metalloenzymes are inhibited by Zn^{2+} *in vitro* (22–24), the effect of various concentrations of Zn^{2+} on lysoPtdCho acyltransferase activity was determined (Table 3). LysoPtdCho acyltransferase activity was reduced to 93% control values at 0.01 mM Zn^{2+}, 33% at 0.1 mM Zn^{2+}, and 7% at 1 mM Zn^{2+}. The strong Zn^{2+} chelator, *o*-phenanthroline, is capable of chelating (and thus removing) Zn^{2+} from high-affinity binding sites within proteins (EDTA does not chelate Zn^{2+} in a manner capable of physically removing Zn^{2+} from high-affinity sites). The addition of 1 or 2 mM *o*-phenanthroline to the assay mixture inhibited activity to 27 and 18% of control levels, respectively (Table 3). Since *o*-phenanthroline is delivered in an ethanol

TABLE 2
The Effect of Lipids and Cations on LysoPtdCho Acyltransferase Activity[a]

Treatment	Enzyme activity (% control)
None	100 ± 6
PtdCho	97 ± 7
PtdEtn	127 ± 13
PtdSer	91 ± 9
PtdIns	212 ± 22
PtdOH	182 ± 17
PtdGro	94 ± 14
CL	236 ± 28
Oleic acid	91 ± 5
DAT	135 ± 11
CDP-DAG	116 ± 18
SM	112 ± 15
200 mM EDTA	96 ± 9
1 mM $MgCl_2$	100 ± 9
1 mM $MnCl_2$	102 ± 12
1 mM $CaCl_2$	95 ± 8
1 mM $ZnCl_2$	7 ± 6

[a]The lipid mixture contained 10 mol% of the indicated mixture (relative to the lysoPtdCho concentration). Other assay conditions were as described in the Materials and Methods section. The control (100% activity) was 4.3 nmol min^{-1} mg^{-1}. Results are expressed as mean ± standard error (n = 4). Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdGro, phosphatidylglycerol; CL, cardiolipin; DAG, diacylglycerol; CDP-DAG, cytidine diphosphate-diacylglycerol; SM, sphingomyelin. For other abbreviation See Table 1.

suspension, the effect of 1% ethanol on lysoPtdCho acyltransferase activity was also tested to ensure that this organic solvent was not inhibiting activity. Ethanol did not inhibit lysoPtdCho acyltransferase activity (Table 3). The concentrations of Zn^{2+} and *o*-phenanthroline required for inhibition of lysoPtdCho acyltransferase activity are in the range observed for inhibition of activity of known Zn^{2+} metalloenzymes that contain high-affinity Zn^{2+} binding sites (22,23). In an attempt to gain insight into whether the Zn^{2+} bound directly to the lysoPtdCho acyltransferase enzyme itself or to an essential activator protein, heat denaturation experiments were performed. However, a time course of heat denaturation of lysoPtdCho activity at 55°C in the absence or presence of Zn^{2+} did not reveal significant differences in enzyme activity stability, preventing any conclusions with respect to a more precise role for the Zn^{2+} requirement to be drawn.

TABLE 3
Effect of Zn^{2+} on LysoPtdCho Acyltransferase Activity[a]

Cation/chelator	Activity (% control)
None	100 ± 15
None (1% EtOH)	90 ± 15
$ZnCl_2$, 1 mM	7 ± 6
$ZnCl_2$, 0.1 mM	33 ± 19
$ZnCl_2$, 0.01 mM	93 ± 14
o-Phenanthroline, 2 mM	18 ± 15
o-Phenanthroline, 1 mM	27 ± 18

[a]The prereaction mixture contained either $ZnCl_2$ or *o*-phenanthroline at the indicated concentration. The prereaction mixture was incubated for 30 min at room temperature before the addition of radiolabeled oleoyl CoA (to allow for efficient chelation of Zn^{2+} by *o*-phenanthroline). The 30-min preincubation (in the absence of assay modifiers) did not significantly reduce enzyme activity. The control (100% activity) was 4.3 nmol min^{-1} mg^{-1}. Results are expressed as mean ± standard error (n = 6).

[^{3}H]lysoPAF metabolism in S. cerevisiae. To conclusively prove that the observed lysoPtdCho acyltransferase activity was due to direct acylation of lysoPtdCho by fatty acyl CoA and not *via* indirect mechanisms including lipid biosynthetic and/or transacylation pathways, the observed incorporation of lysoPAF into PtdCho *in vitro* (Table 1) was exploited. Ether bonds are poorly metabolized in all eukaryotic cells (25), and the absence of ether-linked glycerophospholipids in *S. cerevisiae* (17) implies that the ability of *S. cerevisiae* cells to hydrolyze this linkage is highly unlikely. The capacity of mid-log phase *S. cerevisiae* cells to metabolize [^{3}H]lysoPAF was examined temporally (Fig. 3). Radiolabel was recovered exclusively in the organic (lipid) fraction (data not shown), and thin-layer chromatographic analysis of the label within the organic phase resulted in its accumulation exclusively in PtdCho (Fig. 3). These results indicate that the ether linkage was not metabolized and also demonstrate a direct precursor–product relationship between lysoPAF and PtdCho formation. To ensure that the acylation of [^{3}H]lysoPAF was not due to transacylase activity, the labeling protocol was repeated in a yeast strain carrying a disruption of the *PLB1* gene (devoid of measurable transacylase activity) (26). The *PLB1*-disrupted yeast cells acylated lysoPAF *in vivo* in a manner indistinguishable from the wild-type parental strain. That is, strain DL882 (*plb1Δ*URA3/*plb1Δ*::URA3), incorporated 1207 ± 39 dpm/nmol lipid P_i [mean ± standard error (n = 8)] 45 min after the addition of [^{3}H]lysoPAF to mid-log phase organisms grown in YPD medium, whereas the wild-type parental strain DL1788 incorporated 1344 ± 33 dpm/nmol lipid P_i under the same experimental conditions.

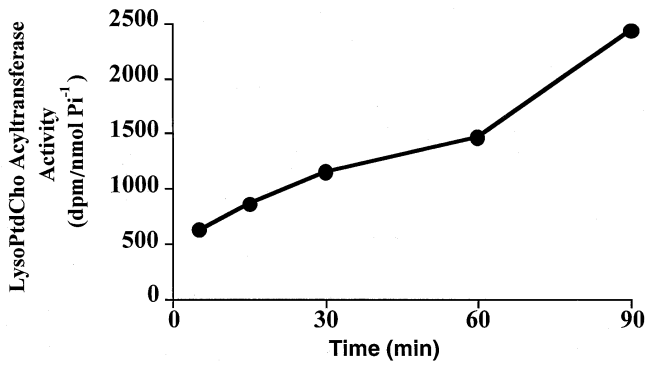

FIG. 3. Metabolism of [^{3}H]1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine by *S. cerevisiae*. [^{3}H]1-*O*-alkyl-2-hydroxy-*sn*-3-glycerophosphocholine was added to mid log-phase w303a yeast cells grown in YPD medium (1% Bacto-Yeast Extract, 2% Bacto-Peptone, 2% glucose). Aliquots were removed at the indicated times and analyzed as described in the Materials and Methods section. Each point represents the mean of two separate experiments performed in duplicate. Pi refers to inorganic phosphorus. See Figure 1 for abbreviations.

DISCUSSION

The distribution of fatty acyl species along the backbone of PtdCho molecules determines the availability of a particular PtdCho molecule for its use in many biological processes including the production of lung surfactant (27) and the release of arachidonic acid from PtdCho stores for conversion to leukotrienes and prostaglandins during agonist-induced inflammatory responses (7,9). Furthermore, unsaturated PtdCho fatty acyl species may undergo oxidative cleavage for the production of platelet-activating factor mimetic, and this process has been linked to the formation of atherosclerotic plaques (28,29).

The interrelationships between the activities and substrate specificities of cholinephosphotransferase and lysoPtdCho acyltransferase are the major dictators of the fatty acyl profile of PtdCho synthesized *de novo*, or *via* the Land's remodeling pathway, respectively. The isolation of lysoPtdCho acyltransferase protein or cDNA has met with limited success owing to the difficulties associated with purifying integral membrane proteins, as well as an absence of genetic tools.

Saccharomyces cerevisiae glycerophospholipids have limited diversity in acyl chain composition (18). Yeasts possess only Δ9 desaturase activity and hence do not contain polyunsaturated fatty acyl chains within their lipids, unless these are supplied from an exogenous source. Despite this limit in diversity, lysoPtdCho acyltransferase activity has been detected in yeast (30). Furthermore, phospholipid remodeling occurs in yeast (31), implying that fatty acyl chain remodeling plays a biological role other than the specific physiological roles listed above. Our study demonstrated that *S. cerevisiae* lysoPtdCho acyltransferase activity has many characteristics similar to those of lysoPtdCho acyltransferase activities present in other eukaryotic cell types. Most notable of these similarities was the inability of Mg^{2+}, Mn^{2+}, Ca^{2+}, and EDTA to affect activity (32). However, Zn^{2+} was found to inhibit lysoPtdCho acyltransferase activity dramatically in *S. cerevisiae* cell membranes. Many Zn^{2+} metalloenzymes are inhibited by high concentrations of Zn^{2+} (22–24). In addition, the strong Zn^{2+} chelator, *o*-phenanthroline, inhibited lysoPtdCho acyltransferase at concentrations similar to its inhibition of known Zn^{2+}-binding protein activities (22,23). Collectively, these results provide strong evidence that lysoPtdCho acyltransferase activity is dependent upon a high-affinity Zn^{2+} binding site on the enzyme itself or an essential accessory protein.

Our *in vitro* characterization of the lysoPtdCho fatty acyl species preferences of *S. cerevisiae* lysoPtdCho acyltransferase indicated that the enzyme was capable of utilizing a broad range of chain lengths, covering the entire range tested (C10–C20). Since each lysoPtdCho substrate was prepared as a micellar suspension, a direct comparison of the efficacy of substrate utilization is not practical because substrate presentation will vary from micelle to micelle. However, it was noted that lysoPAF was utilized as a substrate *in vitro*. LysoPAF is identical in structure to lysoPtdCho except that the fatty acyl group at the *sn*-1 position is linked through an ether bond as opposed to an ester bond. Ether-linked fatty acyl chains are metabolized at a much lower rate than ester-linked fatty acids in all eukaryotic cells (18,25). The addition of labeled lysoPAF to log-phase yeast cells resulted in its conversion to PtdCho in a time-dependent manner. PtdCho was the only radioactive metabolite detected, indicating that the ether bond was not metabolized and that PtdCho was formed directly from lysoPAF, and demonstrating that lysoPtdCho acyltransferase activity is operative *in vivo*.

Our characterization of *S. cerevisiae* lysoPtdCho acyltransferase activity has revealed several important characteristics: (i) the *S. cerevisiae* lysoPtdCho acyltransferase activity shares biochemical and enzymatic properties similar to those in other eukaryotic cell types (8,32); (ii) enzyme activity is inhibited by Zn^{2+} and the Zn^{2+} chelator, *o*-phenanthroline (22,23,33); this is the first demonstration of this effect on this enzyme activity from any source; and (iii) it can utilize a broad range of lysoPtdCho molecules as substrates. The ability of Zn^{2+} to inhibit lysoPtdCho acyltransferase activity coupled with the observation that a broad range of lysoPtdCho molecules (with varying degrees of organic and aqueous solubilities) can be utilized as substrates should aid in the isolation of a *S. cerevisia*e lysoPtdCho acyltransferase gene *via* the development of biochemical screens for mass colony-based assays (34,35).

ACKNOWLEDGMENTS

This work was supported by a Killam Foundation graduate scholarship and a Medical Research Council of Canada graduate studentship (to MGR), and Medical Research Council of Canada scholarship and operating awards (to CRM).

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[Received August 28, 1998, and in final revised form December 1, 1998; revision accepted December 4, 1998]

Chemiluminescent Determination of Cholesterol Hydroperoxides in Human Erythrocyte Membrane

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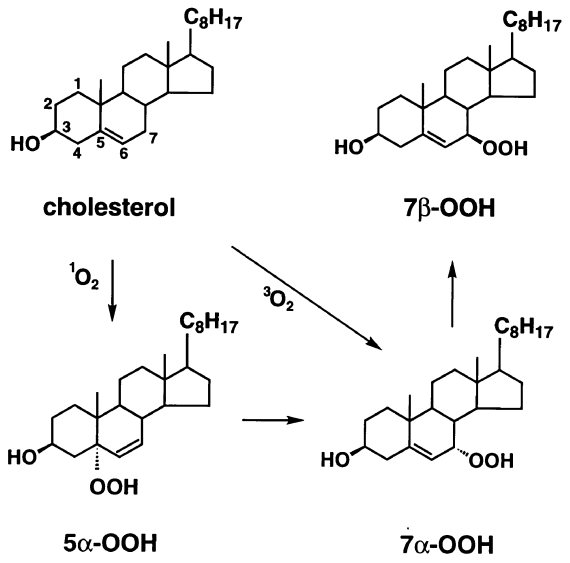
ABSTRACT: A method for separating, detecting, and quantifying cholesterol hydroperoxide (Ch-OOH) based on extraction, purification by solid-phase extraction cartridge, high-performance liquid chromatography with chemiluminescent detection (HPLC-CL), and liquid chromatography–mass spectrometry has been developed for human erythrocyte membrane. We prepared standard compounds of the cholesterol 5α-, 7α-, and 7β-hydroperoxides (Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH). An octyl silica column with methanol/water/acetonitrile 89:9:2 (by vol) as eluent was used to determine Ch-OOH. HPLC-CL that incorporated cytochrome c and luminol as the post-column luminescent reagent was used. We also investigated the optimal assay conditions and how to prevent formation of artifact Ch-OOH. Analysis of erythrocyte membranes from seven healthy volunteers identified Ch 7α-OOH and Ch 7β-OOH, but not Ch 5α-OOH, as commonly occurring components. The respective mean concentrations of Ch 7α-OOH and Ch 7β-OOH were 2.5 ± 1.6 and 5.4 ± 3.5 pmol/mL blood.
Lipids 33, 1235–1240 (1998).

Lipid peroxidation has been linked to a number of pathological conditions and diseases, including ischemia–reperfusion injury, inflammation, and atherosclerosis. It has been monitored on the basis of the formation of thiobarbituric acid-reacting substances (1) and conjugated diene determination (2), but these lack specificity.

Highly sensitive, specific methods for direct measurement of lipid peroxides have now been developed. Phospholipid hydroperoxide levels have been determined in human blood plasma (3) and human red blood cells (4) by high-performance liquid chromatography with chemiluminescent detection (HPLC-CL). Cholesterol ester hydroperoxide in human blood plasma has been analyzed by HPLC-CL (5) and by HPLC with coulometric detection (6).

The possible production of cholesterol hydroperoxide from cholesterol is outlined in Scheme 1. Peroxidation of cholesterol can be induced by such active oxygen species as singlet oxygen, producing cholesterol 5α-hydroperoxide (Ch 5α-OOH) as the first step, then rearrangement of the hydroperoxide giving cholesterol 7α-hydroperoxide (Ch 7α-OOH), and finally epimerization of Ch 7α-OOH giving cholesterol 7β-hydroperoxide (Ch 7β-OOH) (7). In contrast, cholesterol may be autoxidized, producing cholesterol 7-hydroperoxide Ch 7-OOH (8). Ch 7α-OOH and Ch 7β-OOH were detected in the rat by HPLC-CL (9, 10), and in humans were found by HPLC combined with electrochemical detection from photooxidized erythrocyte ghosts (11), but not from the human erythrocyte ghost itself.

We report a sensitive, simple method for determining cholesterol hydroperoxides (Ch-OOH) in erythrocyte membranes



SCHEME 1

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Abbreviations: 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; BHT, 3, 5-di-*tert*-butyl-4-hydroxytoluene; Ch-OOH, cholesterol hydroperoxides; Ch 5α-OOH, cholesterol 5α-hydroperoxide; Ch 7α-OOH, cholesterol 7α-hydroperoxide; Ch 7β-OOH, cholesterol 7β-hydroperoxide; CL, chemiluminescent, chemiluminescence; DMF, dimethylfuran; HPLC-CL, high-performance liquid chromatography with chemiluminescent detection; IS, internal standard; LC–MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; PCOOH, phosphatidylcholine hydroperoxide; UV, ultraviolet.

of healthy volunteers using HPLC-CL and liquid chromatography–mass spectrometry (LC–MS) with an atmospheric pressure chemical ionization interface to identify Ch-OOH. Furthermore, to prevent formation of artifact Ch-OOH during extraction, we examined purification using a solid-phase cartridge, and the influences of light and antioxidants.

MATERIALS AND METHODS

Materials. Cholesterol was obtained from Sigma (St. Louis, MO). 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), luminol (3-aminophthaloylhydrazine), and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH were synthesized as follows: A solution of cholesterol (960 mg) and rose bengal (10 mg) in pyridine (200 mL) was irradiated with a halogen lamp at 10°C with oxygen bubbling for 8 h. Subsequent evaporation of pyridine gave a crystalline residue, of which half was purified by medium-pressure column chromatography (silica gel) and recrystallized from benzene to give Ch 5α-OOH, m.p. 145–148°C. The product had an ^{1}H nuclear magnetic resonance (NMR) spectrum identical to that of an authentic sample (7). The remaining half of the crystals was dissolved in chloroform, and the resultant solution was stirred at room temperature for 3 d. Evaporation of the solvent gave a residue which was purified by medium-pressure column chromatography (silica gel), then recrystallized from ethyl acetate, affording Ch 7α-OOH, m.p. 144–147°C, and Ch 7β-OOH. Although Ch 7β-OOH was obtained as a 6:1 mixture with the 7α-isomer, both compounds had ^{1}H NMR spectra identical to those of authentic samples (12).

β-Sitosterol 5α-hydroperoxide was prepared by irradiating a solution of β-sitosterol (400 mg) and hematoporphyrin (7 mg) in pyridine (208 mL) with a high-pressure mercury lamp through a Pyrex filter at 10°C with oxygen bubbling for 9 h. The crude products were purified and recrystallized from ether to give β-sitosterol 5α-hydroperoxide. The product had a characteristic ^{1}H NMR spectrum as in the case of Ch 5α-OOH. β-Sitosterol 5α-hydroperoxide was the internal standard (IS).

HPLC-CL analysis. Ch-OOH were determined by reverse-phase HPLC with post-column chemiluminescent (CL) detection: HPLC was done in a column of TSK gel Octyl-80Ts (150 × 4.6 mm, i.d.) using an LC-10AD vp pump (Shimadzu, Kyoto, Japan). The column was kept at 40°C and flushed with methanol/water/acetonitrile (89:9:2), the mobile phase, at the flow rate of 0.7 mL/min. The premixed mobile phase was degassed by 5 min of sonication before use. A Rheodyne 7125 injector (100 μL, Cotati, CA) was used to inject the sample solution into the column. After passage through a SPD-6A spectrophotometric detector (Shimadzu) set at 210 nm, the eluate was mixed with a luminescent reagent in the post-column mixing joint, at the controlled temperature of 37°C, of a CLD-10A CL detector (Shimadzu). The luminescent reagent, prepared by dissolving cytochrome c and luminol in alkaline borate buffer (pH 10), was loaded with an LC-10AD vp pump at the flow rate of 0.5 mL/min. The CL generated by the reaction of the hydroperoxide with the luminescent reagent was measured with a CL detector.

The concentrations of cytochrome c and the luminol prepared, respectively, 10 and 2 μg/mL, were the same as those employed by Miyazawa *et al.* (13,14). The luminescent solution was 20 mM $H_3BO_3 \cdot Na_2CO_3$ buffer at pH 10. The effect of the flow rate of this reagent was examined between 0.2 and 0.8 mL/min. The injection volume and supply voltage also were examined in terms of the hydroperoxide-dependent CL intensities.

Standard curves were prepared by the analyses of 1, 2, 4, and 10 ng (2.39, 4.78, 9.58, and 23.9 pmol) of Ch 5α-OOH and of Ch 7β-OOH, and 0.5, 1, 2, and 5 ng (1.20, 2.39, 4.78, and 12 pmol) of Ch 7α-OOH with 2.5 ng of the IS. Individual peak areas were calculated with an integrator (Chromatopac C-R4A; Shimadzu). The ratios of the hydroperoxides to the IS also were calculated for the standard compounds and lipid extracts of the specimens. The recoveries from the sample extracts were determined by comparison of the peak areas obtained after injection of a sample extract spiked with a known concentration. The recoveries of Ch 7α-OOH and IS varied from 55 to 65%.

HPLC–MS analysis. An L-7000 series (Hitachi, Tokyo, Japan) liquid chromatography system fitted with spherisorb ODS-2-5 (250 × 4.6 mm, i.d.) and a model M-1200AP LC–MS system that incorporated an atmospheric chemical ionization system (Hitachi) were used. The mobile phase, methanol containing 0.1 M ammonium acetate or methanol alone, was delivered at the flow rate of 0.7 mL/min. By adding ammonium acetate to the methanol as the mobile phase, an ion appeared at *m/z* 401 so that Ch-OOH were distinguishable from the 7-hydroxycholesterols as shown in Figure 1, although most had similar retention times. Application parameters for the mass spectrometer were positive-ion measurement mode, a nebulizer temperature of 170°C, a desolvator temperature of 400°C, and a needle-electrode voltage of 3000 V.

Artifact formation. Two milligrams of cholesterol was extracted twice with 9 mL chloroform/methanol (2:1) containing 0.005% (50 ppm) BHT by the modified method of Folch *et al.* (15). The combined chloroform layer was concentrated in a rotary evaporator, then dried under a nitrogen stream. The residue was dissolved in 1 mL of methanol and a 10 μL portion injected into the HPLC column (Method A). A silica column (Bond Elut® Varian, Harbor City, CA) of 3-mL capacity, containing aminopropyl- derivatized silica (-NH_2) as packing material, was conditioned by washing it with 5 mL of acetone and 10 mL of *n*-hexane, after which 2 mg of cholesterol in a small amount of chloroform was passed through, followed by elution with a mixture of 2 mL chloroform and 1 mL isopropanol. The eluate was concentrated, and the residue subjected to HPLC (Method B), or after concentration it was passed through another Bond Elut® column with elution using 3 mL of 10% of ethyl acetate/*n*-hexane. The eluate was concentrated and the residue subjected to HPLC (Method C). When two cartridges of Bond Elut® were conditioned, 0.1%

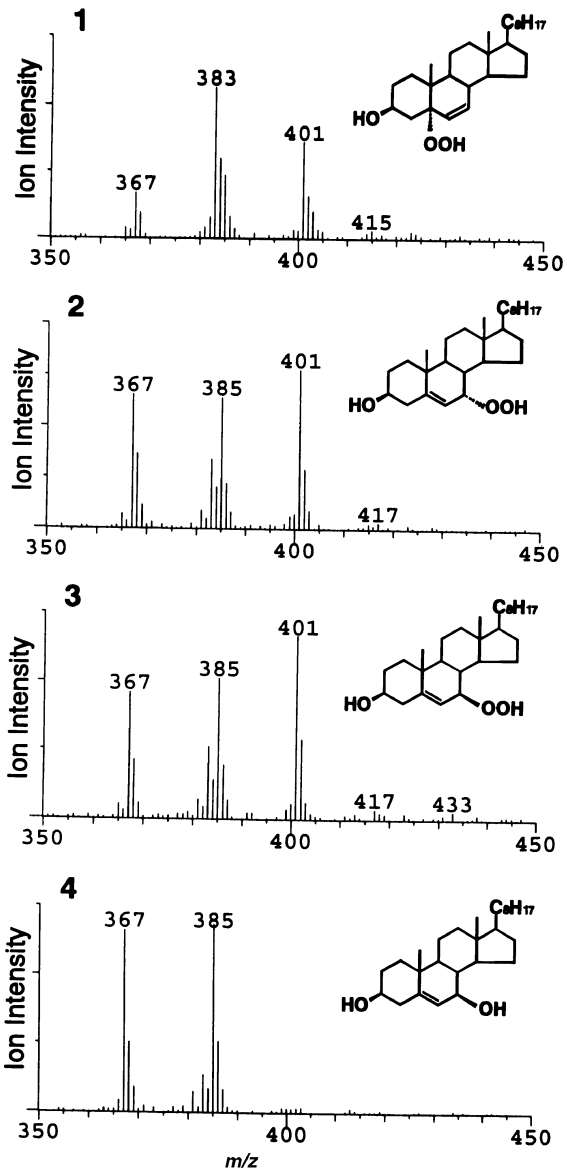

FIG. 1. Mass spectra of the cholesterol 5α-, 7α-, and 7β-hydroperoxides (M = 418) and 7β-hydroxycholesterol (M = 402) were detected by liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface. Methanol containing 0.1 M ammonium acetate was used as a mobile phase.

EDTA · 2Na was used before water and acetone (Method D). Dark test tubes were used during solid extraction with Bond Elut® (Method E). Two milligrams of cholesterol was extracted twice with 9 mL chloroform and methanol containing BHT and dimethylfuran (DMF) to give final concentrations of 10, 20, 50, 100, 400, and 1000 ppm BHT. The concentrations of DMF were 0, 15, 60, and 150 ppm. The chloroform layer was evaporated, and the residue was applied to a Sep-Pak® (Waters, Milford, MA) of 3-mL capacity containing aminopropyl-derivatized silica (-NH_2) packing material (Method F).

Extraction. Seven healthy male volunteers (39.3 ± 12.2 yr) participated in the study. After obtaining their informed consent, venous blood samples were drawn. Five milliliters of blood was collected in a glass test tube containing 0.5 mg EDTA · 2Na, then centrifuged at 4°C and 800 × *g* for 10 min; the erythrocytes were then fractionated. A method for erythrocyte ghost preparation was used (16). IS (200 pmol in 100 μL chloroform) was added to lipids from the white ghosts and extracted twice with 9 mL chloroform and methanol containing 0.005% BHT at room temperature, essentially by the method of Folch *et al.* (15). The combined chloroform layer was concentrated, and the residue applied to Sep-Pak®. Each column was conditioned by washing it with 5 mL of acetone and 10 mL of *n*-hexane. Before the column became completely desiccated, the crude membrane lipids dissolved in a small amount of chloroform were applied and drawn through, followed by elution with a mixture of 2 mL chloroform and 1 mL isopropanol. The eluate was concentrated. The residue was dissolved in 200 μL of methanol, and a 10-μL portion injected to an HPLC column.

Statistical analysis. Mann-Whitney's U-test was used to determine the statistical significance of the difference between group means. *P*-values of <.05 were considered statistically significant.

RESULTS

Figure 2 shows typical HPLC chromatograms obtained with a mixture of standard Ch-OOH and hydroxycholesterols. The 3 Ch-OOH (7β-, 7α, and 5α-OOH) separated when methanol/water/acetonitrile was the mobile phase, as shown by CL, and the two hydroxycholesterols also were separated as seen by ultraviolet (UV). The retention times were Ch 7β-OOH, 6.8 min; Ch 7α-OOH, 7.3 min; Ch 5α-OOH, 7.8 min; IS, 9.5 min; 7β-hydroxycholesterol (7β-OH), 6.6 min; and 7α-hydroxycholesterol (7α-OH), 7.1 min. Because CL detection is specific, the Ch-OOH were distinguishable from peaks 7α-OH and 7β-OH despite the similar retention times. Although Ch-OOH theoretically are detectable by CL and UV, no Ch-OOH peaks appeared on UV because of the small amount used. The CL detector response was about 300 times greater for Ch 7α-OOH than the UV detector response.

The injection volume of Ch 7α-OOH (20 pmol) vs. peak areas (CL intensity) was linear. We usually injected 10 μL of the sample to the HPLC. Standard curves (ratio of the IS vs. the amount of Ch-OOH injected) for Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH were linear. The detection limit for Ch 5α-OOH was 0.7 pmol, that for Ch 7α-OOH 0.3 pmol, and that for Ch 7β-OOH 0.3 pmol at the signal-to-noise ratio of six.

We investigated the effects of the supply of the chemiluminescent detection voltage (–0.7 to –0.9 kV) and the flow rate of the chemiluminescent reagent (0.2 to 0.8 mL/min) on the chemiluminescence response. At the supply voltage of –0.9 kV, Ch 7α-OOH (20 pmol) gave its maximum response. We used –0.7 kV as the supply voltage. At the flow rate of 0.5 mL/min, Ch 7α-OOH (20 pmol) gave its maximum CL response.

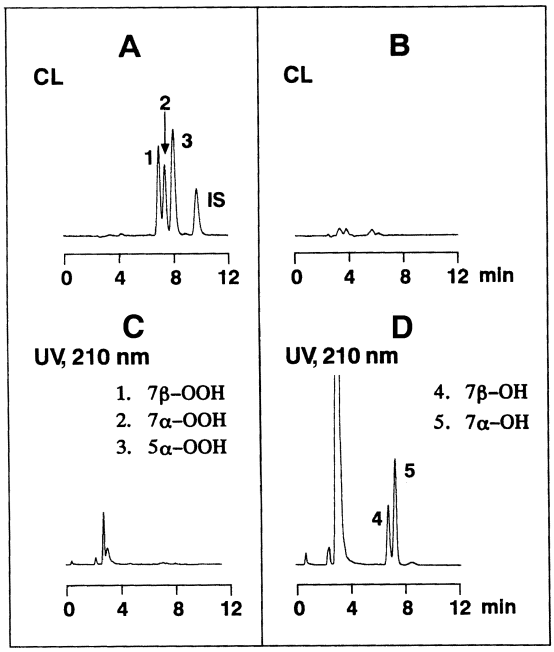

FIG. 2. Chromatographic separation of cholesterol hydroperoxides (Ch-OOH) and corresponding diols in standard mixtures. (A,C) A 10-μL sample containing known Ch-OOH in methanol [2 pmol cholesterol 7α-hydroperoxide (Ch 7α-OOH), 4 pmol cholesterol 5α-hydroperoxide (Ch 5α-OOH), and cholesterol 7β-hydroperoxide (Ch 7β-OOH)]. (B,D) A 10-μL sample containing known hydroxycholesterol in methanol [50 nmol 7α-hydroxycholesterol (7α-OH) and 25 nmol 7β-hydroxycholesterol (7β-OH)]. CL, chemiluminescent detection; UV, ultraviolet detection.

Table 1 compares artifact formation by Methods A to F. Method A, extraction of cholesterol itself by the method of Folch *et al.* (15), did not cause artifact formation, nor did passing cholesterol through one cartridge of Bond Elut® result in artifact formation (Methods B). Passing cholesterol through two cartridges (Method C), however, sometimes caused artifact formation, and formation was prevented neither by washing the cartridge with EDTA · 2Na (Method D) nor by shading it with a dark test tube (Method E). When we used DMF as well as BHT as the antioxidants in the extraction solvent (Method F), to prevent artifact formation 0.1% (1000 ppm) BHT and 0.015% (150 ppm) DMF were necessary. A large negative peak appeared, which disturbed the Ch-OOH peak. When BHT alone was the antioxidant in the extraction solvent (Method F), artifact formation did not occur at any concentration (10 to 100 ppm). We therefore did the extraction with chloroform and methanol containing 0.005% (50 ppm) BHT as the antioxidant, and followed it by purification with Sep-Pak® (Method F).

Figure 3 shows a typical HPLC chromatogram of artifact formation. Peak 1 seemed to be Ch 7β-OOH, peak 2 Ch 7α-OOH, and peak 3 Ch 5α-OOH because the retention times corresponded to these standard compounds. Peaks A, B, and C could not be determined.

Figure 4 shows chromatograms for the erythrocyte sample from a healthy volunteer, a mixture of standard Ch-OOH, and IS. Standard Ch 7β-OOH, Ch 7α-OOH, Ch 5α-OOH, and IS appeared, respectively, at 6.8, 7.3, 7.8, and 9.5 min. The extracts from human erythrocyte membranes contained Ch 7β-OOH and Ch 7α-OOH, but not Ch 5α-OOH. The addition of the standards Ch 7β-OOH and Ch 7α-OOH independently confirmed the peak identities.

The mean levels of Ch 7β-OOH and Ch 7α-OOH in erythrocyte membranes of seven healthy volunteers were 5.4 ± 3.5 (range 1.9–9.2) and 2.5 ± 1.6 (range 1.1–5.3) pmol/mL blood (means ± standard deviations). The concentration of Ch 7β-OOH was not significantly higher than that of Ch 7α-OOH.

DISCUSSION

There are many recent reports of analytical methods for determining phospholipid hydroperoxides and cholesteryl ester hydroperoxides in biological samples, but only a few researchers have given an analytical method for Ch-OOH and showed the presence of Ch-OOH. To develop an analytical

TABLE 1
Artifact formation by Methods A to F[a]

Method	BHT (ppm)	DMF (ppm)	Formation/sample
A: Extraction	50	0	0/2
B: Bond Elut®[b]	0	0	0/3
C: Bond Elut® + Bond Elut®	0	0	5/7
D: EDTA · 2Na + (Bond Elut® + Bond Elut®	0	0	2/4
E: Method D with dark test tube	0	0	2/3
F: Extraction + Sep-Pak®[c]	1000	150	0/6
Extraction + Sep-Pak®	400	60	2/3
Extraction + Sep-Pak®	100	150	2/2
Extraction + Sep-Pak®	100	15	2/3
Extraction + Sep-Pak®	100	0	0/3
Extraction + Sep-Pak®	50	0	0/3
Extraction + Sep-Pak®	20	0	0/2
Extraction + Sep-Pak®	10	0	0/2

[a]Abbreviations: BHT, 3,5-di-tert-butyl-4-hydroxytoluene; DMF, dimethylfuran.
[b]Varian Harbor City, CA.
[c]Waters, Milford, MA.

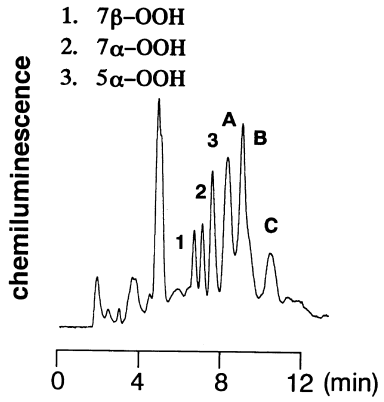

FIG. 3. Typical high-performance liquid chromatograms of artifact formation produced by passing cholesterol in chloroform through two cartridges of Bond Elut®-NH_2. A, B, C, unknown; for abbreviations see Figure 2.

method for Ch-OOH, we first prepared the standard compounds Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH. The standard Ch-OOH were subjected to LC–MS using an atmospheric pressure chemical ionization interface. It is important to confirm the chemical structure of the Ch-OOH, and this has not been previously achieved.

We then investigated the optimal assay conditions for HPLC-CL. We separated Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH from one another in a C8 column with methanol/water/acetonitrile, under similar conditions to those of Korytowski *et al.* (11). They, however, used HPLC combined with electrochemical detection, establishing the detection limit for Ch-OOH as ~25 pmol, so that they could analyze the Ch-OOH generated by photodynamic action, not from erythrocyte ghosts. Because of the report of Zhang *et al.* (17) that the HPLC-CL system employed by Miyazawa *et al.* (13,18,19) and them (20) combined with a cytochrome c–luminol cocktail was 16- to 100-fold more sensitive to phosphatidylcholine hydroperoxide (PCOOH) than the microperoxidase–isoluminol cocktail

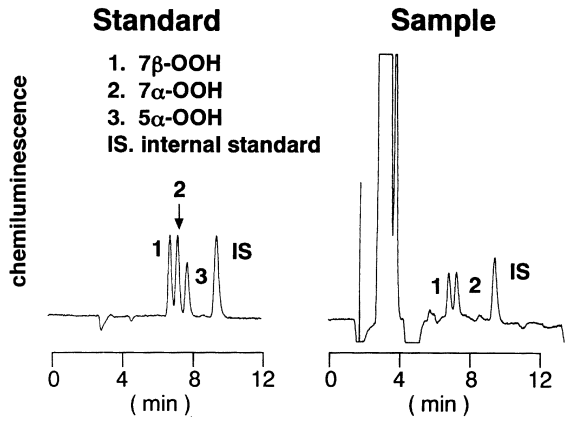

FIG. 4. Chromatograms from high-performance liquid chromatography with chemiluminesent detection for the erythrocyte sample from a healthy volunteer, a mixture of standard Ch-OOH, and the IS.

used by Yamamoto *et al.* (21), we used the cytochrome c–luminol cocktail. Brown *et al.* (22) reported a normal-phase HPLC method with UV detection that could resolve all the cholesterol products oxygenated at the 7-position: 7-ketocholesterol, Ch 7α-OOH, Ch 7β-OOH, 7α-OH, and 7β-OH, but they detected Ch-OOH from the lipid extract of atherosclerotic plaque only in trace amounts because of the low detection limit.

Lastly, we examined the extraction procedure and some antioxidants to avoid artifact formation. Cholesterol in low density lipoprotein was easily oxidized with the help of the metal ion Cu^{2+} (22,23) or a metal-independent peroxy-radical generated system (AAPH) (22) and yielded Ch 7α-OOH and Ch 7β-OOH. BHT (0.1%) and DMF (0.015%) are reported to be absolutely necessary as antioxidants to prevent artifact formation during the assay process (9,10), but both antioxidants at a high level produced a negative peak, thereby disturbing the Ch-OOH peak. In contrast, we found that the presence of BHT alone was sufficient to prevent artifact formation. Ozawa *et al.* (9,10) purified the lipid extract from rat skin using two Bond Elut® cartridges, but artifact formation might occur on passage through the two cartridges due to metal contaminants. Unknown peak A in the chromatograms of artifact formation (Fig. 3) was assumed to be cholesterol 6β-hydroperoxide based on a comparison with the chromatograms of Korytowski *et al.* (11).

In conclusion, for the first time we detected Ch 7α-OOH and Ch 7β-OOH in the erythrocyte membranes of healthy volunteers. The respective mean concentrations of Ch 7β-OOH and Ch 7α-OOH were 5.4 and 2.5 pmol/mL blood. Healthy human plasma is reported to contain 0.5 μM of PCOOH (18), 3 nM (5,24) and 4.2 nM (25) of cholesterol ester hydroperoxide, and less than 20 nM hydroperoxide of eicosatetraenoic acid (26). Plasma glutathione peroxidase was reactive with linoleic acid hydroperoxide but had a low reducing activity toward Ch 7α-OOH and no detectable activity with the Ch 5α-OOH (27); therefore, PCOOH might be absent but Ch-OOH might be present in human plasma. The concentration of total Ch 7-OOH in rat skin was 20–150 μmol/g skin (7), which was higher than the values we found, probably because lipid peroxides easily accumulate in the skin of the rat.

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[Received August 27, 1998, and in final revised form November 9, 1998; revision accepted November 18, 1998]